Semi-invasive Diagnosis of Endometriosis

Doctoral Thesis

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Budapest

2010

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2. Abbreviations

AFS: American Fertility Society
AHSG: Alpha-2 Heremans-Schmidt glycoprotein
Bcl-2: B-cell lymphoma/ leukemia-2
CA-125: Cancer antigen-125
CA-19-9: Cancer antigen-19-9
CCR1: Cognate chemokine receptor 1
CD44: Cluster of differentiation 44
CD68: Cluster of differentiation 68
CGRP: Calcitonine gene–related polypeptide
CK 7: Cytokeratin
CK 8/18: Cytokeratin 8/18
COX-2: Cyclooxygenase-2
CRP: C-reactive protein
HGF: Hepatocyte growth factor
ICAM-1: Intercellular cell adhesion molecule-1
IFN-gamma: Interferon-gamma
IGF-BP 3: Insulin-like growth factor binding protein-3
IL-1β, 2, 4, 6, 8, 10, 12: Interleukin
IL-1β: Interleukin-1β
LOO–CV: Leave–One–Out cross validation
LSSVMs: Least Squares Support Vector Machines
MCP-1: Monocyte chemotactic protein-1
M-CSF: Macrophage colony stimulating factor
MMP-1: Matrix metalloproteinase-1
MMP-2: Matrix metalloproteinase-2
MMP-3: Matrix metalloproteinase-3
MMP-7: Matrix metalloproteinase-7
MMP-9: Matrix metalloproteinase-9
NF: Neurofilament protein
NPY: Neuropeptide Y
PGP9.5: Protein gene product 9.5
RANTES: Regulated on activation normal T-cell expressed and secreted
sICAM-1: Soluble intercellular cell adhesion molecule-1
SP: Substance P
sVCAM-1: Soluble vascular cell adhesion molecule-1
TGF-β: Transforming growth factor-β
TNF alpha: Tumor necrosis factor alpha
TNFR2: Tumor necrosis factor receptor 2
T-plastin: T isoform plastin
VIP: Vasoactive intestinal peptide

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5. Introduction

5.1. Pathogenesis

Endometriosis is a common, benign, oestrogen-dependent, gynaecological disorder associated with pelvic pain and infertility. While endometriosis has been described for more than one hundred years, our current knowledge of its pathogenesis remains unclear.

There are a number of theories which have been proposed to explain the pathogenesis of endometriosis:

Retrograde menstruation/transplantation
Coelomic metaplasia
The induction theory
Genetic background
Altered cellular immunity
Environmental basis

The ectopic transplantation of endometrial tissue, originally proposed by Sampson in 1924, is the most widely accepted theory on the pathogenesis of endometriosis. It claims that the disorder originates from retrograde menstruation of endometrial tissue sloughed through patent fallopian tubes into the peritoneal cavity [1]. Retrograde menstruation occurs in 70% to 90% of women [2, 3], and may be more common in women with endometriosis than in those without the disease [4]. The presence of endometrial cells in the peritoneal fluid, indicating retrograde menstruation, has been reported in 59% to 79% of women during menses or in the early follicular phase [5-7].

The development of endometriosis in the first few years after menarche has been associated with a high rate of obstructing genital-tract anomalies. These include non-communicating rudimentary uterine horns, cervical stenosis, cervical atresia, vaginal or transverse septum agenesis, or an imperforate hymen [8, 9]. As a general rule, women with a stricture at the level of the cervix have a higher incidence of endometriosis than women with a stricture lower in the genital tract. Furthermore, women with shorter intervals between menstruation and longer duration of menses are more likely to have retrograde menstruation and are at higher risk for endometriosis [10, 11].

The transformation (metaplasia) of coelomic epithelium into endometrial tissue has been proposed as a mechanism for the ontogenesis of endometriosis. This theory is best

supported by the fact that genetic induction of ovarian endometriosis is possible in mice suggesting that ovarian endometriotic lesions may arise directly from the ovarian surface epithelium through a metaplastic differentiation process induced by activation of an onco-genic K-ras allele [12].

The induction theory is, in principle, an extension of the coelomic metaplasia theory. It proposes that an endogenous biochemical factor in menstrual fluid present in the peritoneum during menstruation can induce undifferentiated peritoneal cells to develop into endometrial tissue. This theory has been somehow supported by experiments in rabbits [13, 14] but has not been substantiated in women and primates.

Genetic basis

The risk or endometriosis is 7 times greater if a first-degree relative has been affected by endometriosis [15, 16]. No specific Mendelian inheritance pattern has been identified, therefore multifactorial inheritance has been postulated. A relative risk for endometriosis of 7.2 has been found in mothers and sisters, and a 75% incidence has been noted in homozygotic twins of patients with endometriosis [17]. Epidemiological research in the Icelandic population has shown that endometriosis occurs concordantly in monozygotic twins, that pain symptoms start at a similar age in affected nontwin sisters, and that the prevalence of endometriosis is 6- to 9 times increased among first-degree relatives of women with endometriosis when compared to the general population[17].

The induction of humanlike endometriosis in mice by genetic activation of an oncogenic K-ras allele lends further support to the genetic basis of this disorder [12]. More recent studies showed aneuploidy for chromosomes 11, 16, and 17 [18], increased heterogeneity of chromosome 17 aneuploidy [19], and losses of lp and 22q (50%), 5p (33%), 6q (27%), 70 (22%), 9q (22%), and 16 (22%) of 18 selected endometriotic tissues [20].

Recent study shows relationship between the genetic basis of the reumatoid arthritis and endometriosis [21].

The relationship between endometriosis and single gene polymorphisms is controversial [22]. Positive correlations have been shown for single nucleotide polymorphisms linked to cytokines and inflammation, steroid-synthesizing enzymes and detoxifying enzymes and receptors, estradiol metabolism, other enzymes and metabolic systems, and adhesion molecules and matrix enzymes. Apoptosis, cell-cycle regulation, and oncogenes seems to be negatively correlated with the disease, whereas the group of hormone receptors,

growth factor systems, and especially groups of the HLA-system components show a relatively strong correlation.

An ongoing web project (http://www.well.ox.ac.uk/krinaz/) describes allele and genotype frequencies of SNPs in association studies and provides a useful tool for the study of genetic variants and the pathogenesis of endometriosis[22].

Immunologic mechanisms are believed to be involved in the pathogenesis of endometriosis. Several theories have been proposed to explain enhanced implantation and defective clearing of endometrial cells from the pelvic cavity, facilitating the development of endometriosis.

Although retrograde menstruation appears to be a common event in women, not all women who have retrograde menstruation develop endometriosis. The immune system may be altered in women with endometriosis, and it has been hypothesized that the disease may develop as a result of reduced immunologic clearance of viable endometrial cells from the pelvic cavity [23]. According to this hypothesis, endometriosis can be caused by decreased clearance of peritoneal fluid endometrial cells resulting from reduced natural killer (NK) cell and macrophage activity[24]. In contrast, endometriosis can also be considered a condition of immunologic tolerance for ectopic endometrium, which essentially is self-tissue.

Substantial evidence suggests that endometriosis is associated with a state of subclinical peritoneal inflammation, marked by an increased peritoneal fluid volume, increased peritoneal fluid white blood cell concentration (especially macrophages with increased activation status), and increased inflammatory cytokines, growth factors, and angiogenesis-promoting substances. It has been reported in baboons that subclinical peritoneal inflammation occurs both during menstruation and after intrapelvic injection of endometrium [25, 26]. Furthermore, there are data supporting that a higher basal activation status of peritoneal macrophages in women with endometriosis may impair fertility by reducing sperm motility, increasing sperm phagocytosis, or interfering with fertilization [27, 28] possibly by increased secretion of cytokines such as tumor necrosis factor-α(TNF-α) [11, 29].

Macrophages or other cells may promote the growth of endometrial cells by secretion of growth and angiogenetic factors such as epidermal growth factor (EGF) [30], macrophage-derived growth factor (MDGF) [31], fibronectin, and adhesion molecules

such as integrins [32]. After attachment of endometrial cells to the peritoneum, subsequent invasion and growth appear to be regulated by matrix metalloproteinases (MMP) and their tissue inhibitors [33, 34].

Aromatase activity is absent in normal endometrium. Contrarily, aromatase is expressed aberrantly in endometriosis, which gives rise to very high levels of aromatase activity in the endometriotic tissue. Both aromatase expression and activity are stimulated by PGE2. This results in local production of estrogen, which induces PGE2 formation and establishes a positive feedback cycle [35, 36].

The subclinical pelvic inflammatory status associated with endometriosis is also reflected in the systemic circulation. Increased concentrations of C-reactive protein, serum amyloid A (SAA), TNF-a, membrane cofactor protein-1, interleukin-6, interleukin-8 and chemokine (C-C motif) receptor 1 (CCR1) have been observed in peripheral blood samples of patients with endometriosis when compared with controls [37].

Environmental factors

The links between reproductive health, infertility, and environmental pollution are controversial. Attention has been directed to the potential role of dioxins in the pathogenesis of endometriosis, but the issue remains controversial. A recent meta-analysis concluded that currently there is insufficient evidence in women or in nonhuman primates that endometriosis is caused by dioxin exposure [38].

5.2. Future research

Future research on the pathogenesis of endometriosis, should focus on the early interactions between endometrial and peritoneal cells in the pelvic cavity at the time of menstruation. Proteomic and genomic approaches can detect potential differences between eutopic endometrium and myometrium in women with and without endometriosis [39].

5.3. Prevalence

Based on the few reliable data, the prevalence of the condition can reasonably be assumed to be around 10% [40]. Endometriosis is predominantly found in women of reproductive age but can also be found in adolescents and in postmenopausal women

receiving hormonal replacement [40]. It is found in women of all ethnic and social groups. In women with pelvic pain or infertility, a high prevalence of endometriosis (20 % - 90%) has been reported [41, 42]. In asymptomatic women undergoing tubal ligation, the prevalence of endometriosis ranges from 3% to 43% [43].

5.4. Diagnosis

The current clinical opinion is that laparoscopy is required for definitive diagnosis of endometriosis [44].

History and physical examination can yield a number of significant findings suggestive for endometriosis including affected first degree relatives, chronic pelvic pain and dysmenorrhea, retroverted uterus, adnexal masses, cul de sac nodularity and uterosacral ligament thickening and tenderness, but none are diagnostic for endometriosis.

Well known risk factors for endometriosis include short cycle length [45], heavier menstruation, and longer flow duration [46], probably related to a higher incidence of retrograde menstruation. Patient height and weight are positively and negatively, respectively, associated with the risk of endometriosis [47]. Although a higher prevalence of endometriosis has been found among patients with coitus during menses [48], more research is needed to address the role of sexual habits in the development of endometriosis.

Endometriosis can be associated with significant gastrointestinal symptoms (pain, nausea, vomiting, early satiety, bloating and distention, altered bowel habits) as well.

A characteristic motility change, along with bacterial overgrowth, has been documented the bowel system of most women with endometriosis [49].

The average delay between the onset of pain symptoms and surgically confirmed endometriosis is quite long: mean 8 years in the United Kingdom and 9 to 12 years in the United States [50]. However over the past 20 years, there has been a steady decrease in the delay in diagnosis and a decline in the prevalence of advanced endometriosis at first diagnosis [51].

5.5. Pain

In adult women, dysmenorrhea is especially suggestive of endometriosis if it begins after

years of pain-free menses. Dysmenorrhea often starts before the onset of menstrual bleeding and continues throughout the menstrual period. In adolescents, dysmenorrhee may be present after menarche without an interval of pain-free menses. The distribution of pain is variable but most often is bilateral [11].

Local symptoms can arise from rectal, ureteral, and bladder involvement, and lower back pain can occur. Most studies have failed to detect a correlation between the degree of pelvic pain and the severity of[52] endometriosis [42]. Some women with extensive disease have no pain, whereas others with only minimal disease may experience severe pelvic pain. Severe pelvic pain and dyspareunia may be associated with deep infiltrating subperitoneal endometriosis [53].

Possible mechanisms causing pain in patients with endometriosis include local peritoneal inflammation, deep infiltration with tissue damage, adhesion formation, fibrotic thickening, and collection of shed menstrual blood in endometriotic implants, resulting in painful traction with the physiologic movement of tissues [52, 53]. In rectovaginal endometriotic nodules, a close histologic relationship has been observed between nerves and endometriotic foci and between nerves and the fibrotic component of the nodule [54].

5.6. Subfertility and Infertility

An association between endometriosis and subfertility is generally accepted, based on epidemiological, retrospective or cross-sectional studies in women and on nonhuman primate research[55]. In women with moderate or severe endometriosis, major pelvic adhesions can impair oocyte release from the ovary or inhibit ovum pickup or transport[56]. In women with mild endometriosis, the monthly fecundity rate (MFR) is lower (5%-11%) than observed in a normally fertile population (25%,) [57]. The association between minimal endometriosis and infertility has been explained by numerous mechanisms (ovulatory dysfunction, luteal insufficiency, luteinized unruptured follicle syndrome, recurrent abortion, altered immunity, and intraperitoneal inflammation)[57], but remains controversial[55, 58]. It is possible that functional disorders of the endometrium may both predispose to the development of endometriosis and impair implantation mechanisms in affected women[11]. Both the prevalence of

endometriosis and the proportion of moderate to severe endometrisos are higher in infertile women (27% and 71%) than in women of proven fertility women (3% and 43%)[55].

In a recent study authors have published a fundamental article about the disturbances of implantation in endometriosis. The endometrium is receptive to embryonic implantation during a well-defined ‘window of implantation’ in the midsecretory phase, when pinopods appear on the surface of the endometrium. The genes and gene products aberrantly expressed in the endometrium from women with endometriosis include aromatase, endometrial bleeding factor, hepatocyte growth factor, 17β-hydroxysteroid dehydrogenase, homeobox genes A-10 and A-11, leukaemia inhibitory factor, matrix metalloproteinase-7 and -11 and progesterone receptors [59].

Aromatase is only expressed in the eutopic and ectopic endometrium of women with endometriosis, but not in the endometrium of healthy women. Endometrial bleeding factor is downregulated during the window of implantation. It is a marker of uterine non-receptivity and is abundantly expressed during the window of implantation in women with endometriosis and infertility [60].

Matrix metalloproteinases degrade extracellular matrix components, and are normally expressed in the endometrium during menstrual breakdown and subsequent oestrogen-mediated endometrial growth, and are suppressed by progesterone during the secretory phase. It has been shown that there is a persistent expression of MMP-7 and -11 during the secretory phase, probably caused by complex interactions between progesterone and local cytokines in endometriosis [61].

Lessey et al. [62] reported that the αVβ3 integrin chain is co-expressed with other specific integrins only during the implantation window, and is a biomarker for endometrial receptivity. In women with endometriosis, it appears that αVβ3 integrin expression is reduced. It was suggested that a direct effect is exerted on the endometrium by inflammatory factors contained in the peritoneal fluid of women with endometriosis.

Giudice et al.[63] have screened 588 genes, and observed a marked up-regulation for cytokines, growth factors and other gene products in endometriosis in decidualized stromal cells, which supports a major role for paracrine interactions between the stroma and other endogenous and transient cell populations within the endometrium and during early pregnancy.

Kao et al. have applied paralleled gene expression profiling using high-density oligonucleotide microarrays to investigate differentially regulated genes in endometrium (obtained during the implantation window) from women with versus women without endometriosis. By analysis of these data, they propose novel candidate genes for implantation failure and infertility in endometriosis, such as GlcNAc6ST, olfactomedin, C4BP, IL-15, Dickkopf-1, purinenucleoside phosphorylase, neuronal pentraxin II, glycodelin S100E and BSEP. The other candidate genes may promote an inhospitable endometrial milieu for embryonic implantation, due to embryo toxicity, immune dysfunction, inflammatory or apoptotic responses [64].

Recently, Montgomery et al. reviewed gene mapping studies in endometriosis and the prospects of finding gene pathways contributing to disease using the latest genome-wide strategies. Genetic variants in 76 genes have been examined for association, but none shows convincing evidence of replication in multiple studies. However, there is evidence for genetic linkage to chromosomes 7 and 10, but the genes (or variants) in these regions contributing to disease risk have yet to be identified [65].

The above mentioned data could suggest that the main reason for reproductive failure in women with endometriosis lies in the endometrium and its altered characteristics during the crucial implantation window. However, it has been shown [66] that patients with endometriosis undergoing IVF with oocyte donation have the same chances of implantation and pregnancy as other recipients when oocytes came from donors without known endometriosis. At the same time, when the results of oocyte donation were classified according to origin of the oocytes donated, patients who received embryos derived from endometriotic ovaries showed a significantly reduced implantation rate as compared with the remaining groups.

These observations may suggest that infertility in endometriosis patients may be related to alterations within the oocyte, which in turn result in embryos with decreased ability to implant. Some think that a good quality embryo may overcome the slight decrease observed in endometrial receptivity [66].

Although much work has been done in identifying of cytokines, growth factors and gene products, there is not yet a clear understanding of their importance in (recurrent) pregnancy loss and implantation failure.

Based on controlled prospective studies, there is no evidence that endometriosis is associated with pregnancy loss [67], or that medical or surgical treatment of endometriosis reduces the spontaneous abortion rate [68, 69].

5.7. Endocrinologic disorders

Endometriosis has been associated with anovulation, abnormal follicular development, reduced circulating E2 levels during the preovulatory phase, disturbed luteinizing hormone (LH) surge patterns, premenstrual spotting, the luteinized unruptured follicle syndrome, galactorrhea and hyperprolactinemia [11]. However, there are not sufficient convincing data indicating that the prevalence of these endocrinologic abnormalities is significantly increased in women with endometriosis [55].

5.8. Clinical Examination

In many women with endometriosis, no abnormality is detected during the clinical examination. The vulva, vagina, and cervix should be inspected for any signs of endometriosis, although the occurrence of endometriosis in these areas is rare (2). Other possible signs of endometriosis include uterosacral or cul-de-sac nodularity, lateral or cervical displacement caused by uterosacral scarring[70], painful swelling of the recto-vaginal septum, and unilateral ovarian enlargement. In more advanced disease, the uterus is often in fixed retroversion, and the mobility of the ovaries and fallopian tubes is reduced. Evidence of deeply infiltrative endometriosis (deeper than 5 mm under the peritoneum) in the rectovaginal septum with cul-de-sac obliteration or cystic ovarian endometriosis should be suspected by clinical documentation of uterosacral nodularities during menses, especially if CA125 serum levels are higher than 35 IU/mL [71, 72].

Extrapelvic endometriosis is rare (0,5 -2%) and may result from vascular or lymphatic dissemination of endometrial cells to many gynecologic (vulva, vagina, cervix) and non-gynecologic sites [11]. Although extrapelvic endometriosis is often asymptomatic, it should be suspected when symptoms of pain or a palpable mass occur outside the pelvis in a cyclic pattern. Endometriosis involving the intestinal tract, especially colon and rectum, is the most common site of extrapelvic disease, and is associated with cyclic or chronic abdominal and back pain, abdominal distention, cyclic rectal bleeding,

constipation, and obstruction. Ureteral involvement can lead to obstruction and result in cyclic higher back pain, dysuria, and hematuria. Pulmonary endometriosis can manifest as pneumothorax, hemothorax, or hemoptysis during menses. Umbilical endometriosis should be suspected when a patient has a palpable mass and cyclic pain in the umbilical area[11] .

Although clinical examination is a useful tool in the detection of endometriosis, the diagnosis of endometriosis should be confirmed by biopsy of suspicious lesions that are obtained laparoscopically.

5.9. Imaging and Endometriosis

Gynecologic transvaginal [73] or transrectal ultrasonography [74] is an important diagnostic tool [75] in the assessment of ovarian endometriotic cysts and of rectovaginal endometriosis (sensitivity, 97%; specificity, 96%). The presence of filling defects detected by hysterosalpingography has also a significant positive correlation with endometriosis. The positive predictive value of this finding is 84% and negatíve predictive value is 75% [76]. Other imaging techniques, including computed tomography (CT) and MRI, can be used to provide additional and confirmatory information, but they cannot be used for primary diagnosis [77].

5.10. Laboratory tests

There is no blood test available for the diagnosis of endometriosis. Levels of CA125, a coelomic epithelium marker common to most nonmucinous epithelial ovarian carcinomas, have been found to be significantly higher in women with moderate or severe endometriosis, but not in women with minimal or mild disease, when compared to women with a normal pelvis [78]. Serum CA 125 levels are reported to increase during menstruation according to some [79, 80] but not all [81] investigators, and this phenomenon many be limited to patients with moderate to severe endometriosis [82]. Compared with laparoscopy, measurement of serum CA125 levels has no value as a diagnostic tool [77]), but serial CA125 determinations may be useful to predict the recurrence of endometriosis after therapy [83].

5.11. Laparoscopic findings

Unless disease is visible in the vagina or elsewhere, most guidelines state that laparoscopic visualization of suspicious lesions is the gold standard for the definitive diagnosis of endometriosis [77]. During diagnostic laparoscopy, the pelvic and abdominal cavity should be systematically investigated for the presence of endometriosis. The type, location, and extent of all lesions and adhesions should be documented in the operative notes ideally, should be recorded [77, 84]. Characteristic findings include typical ("powder-burn" or "gunshot") lesions on the serosal surfaces of the peritoneum. These lesions are black, dark brown, or bluish nodules or small cysts containing old hemorrhage surrounded by a variable degree of fibrosis (Fig.1.)

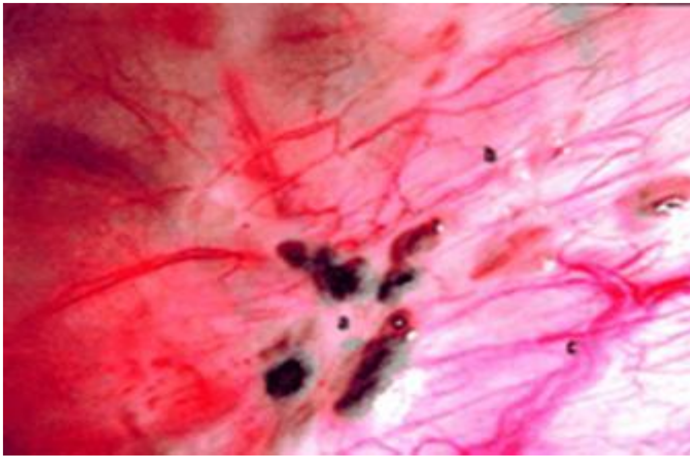

Figure 1. Peritoneal endometriotic laesions

Endometriosis can appear as subtle lesions also called 'atypical', including red implants (petechial, vesicular, polypoid, hemorrhagic, red flamelike), serous or clear vesicles and sometimes white plaques or scarring, yellow-brown discoloration of the peritoneum, and tubo-ovarian adhesions (Fig.2.)

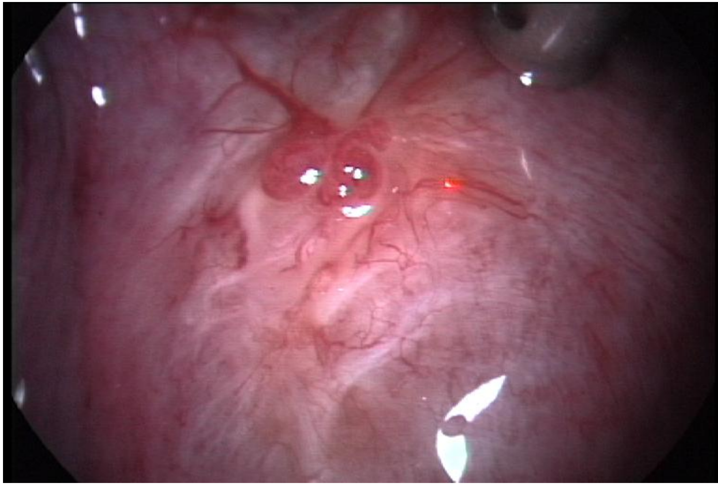

Figure 2. Subtle lesions serous and clear vesicles

[85] Histologic confirmation of the laparoscopic impression is essential is important for the diagnosis of endometriosis, not only for subtle lesions but also for typical lesions reported to be histologically negative in 24% of cases [86], and should be considered as ideal practice [77]. In the presence of ovarian endometrioma (greater than 3 cm in diameter) and deeply infiltrating disease, histology should be obtained to identify endometriosis and to exclude rare instances of malignancy [85].

Deeply invasive endometriosis is associated with reduced depth and volume of the pouch of Douglas (Fig 3.) suggesting that this phenotype of endometriosis does not develop in the recto-vaginal septum but is the consequence of intraperitoneally buried anterior rectal wall adhesions [87].

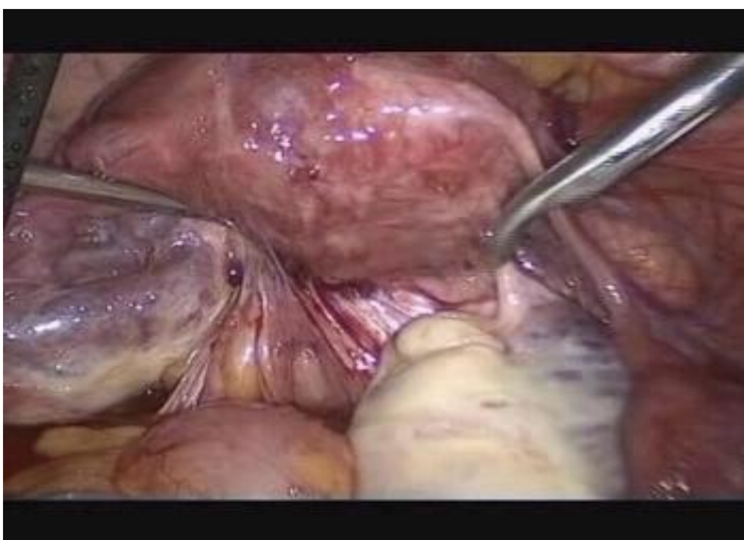

Figure 3. Deeply infiltrating endometriosis of the rectum

Superficial ovarian endometriosis can present as both typical and subtle lesions (Fig.4.).

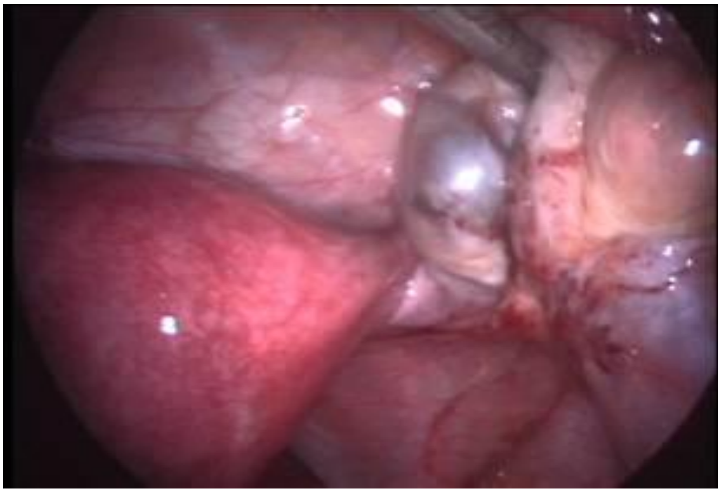

Figure 4. Superficial ovarian endometriosis

Ovarian endometriotic cysts are usually located on the anterior surface of the ovary and are associated with retraction, pigmentation, and adhesions to the posterior peritoneum (Fig.5.).

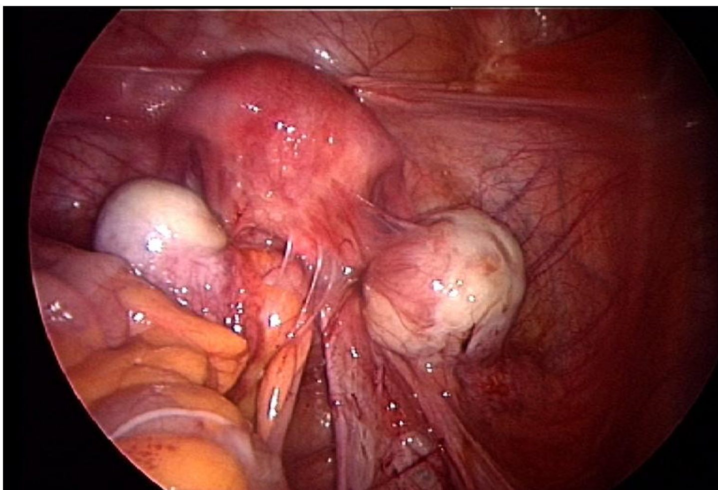

Figure 5. Ovarian endometriotic cysts

These ovarian endometriotic cysts often contain a thick, viscous dark brown fluid (chocolate fluid) composed of hemosiderin derived from previous intraovarian hemorrhage. Since such fluid may also be found in other conditions, such as in

hemorrhagic corpus luteum cysts or neoplastic cysts, biopsy and preferably removal of the ovarian cyst for histologic examination is desirable [88].

5.12. Histologic Confirmation

Histologic confirmation is useful tool but not essential in the diagnosis of endometriosis. In a study of 44 patients with chronic pelvic pain, endometriosis was laparoscopically diagnosed in 36%, but histologic confirmation was obtained in only 18%. This approach resulted in a low diagnostic accuracy of laparoscopic inspection with a positive predictive value of only 45%, explained by a specificity of only 77% [11]. Microscopically, endometriotic implants consist of endometrial glands and stroma, with or without hemosiderin-laden macrophages (Fig.6.).

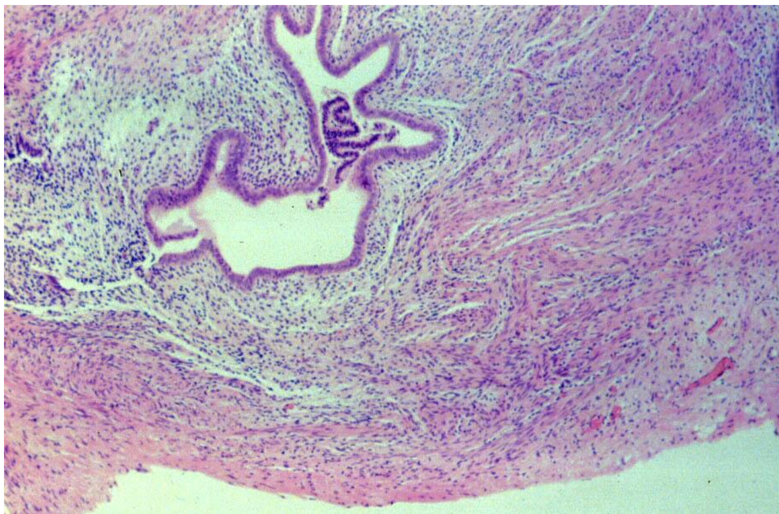

Figure 6. Histologic aspect of endometriosis

Endometrioid stroma may be more characteristic of endometriosis than endometrioid glands [89]. Vascularization, mitotic activity, and the three-dimensional structure of endometriosis lesions are key factors.

The current classification system of endometriosis is Revised American Fertility Society Classification which has been revised without major changes [90]. The classification is based on the appearance, size, and depth of peritoneal and ovarian implants; the degree of cul-de-sac obliteration and the presence, extent, and type of adnexal adhesions.

In the new classification system, the morphologic aspect of the lesions is additionally categorized as red (red, red-pink, and clear lesions), white (white, yellow-brown, and peritoneal defects), and black (black and blue lesions) [90].
Despite of the criticism of several authors, the revised classification of endometriosis is the only internationally accepted system, it appears to be the best available tool to describe objectively the extent of endometriosis.

5.13. Spontaneous evolution during pregnancy

The characteristics of endometriosis are variable during pregnancy, and lesions tend to enlarge during the first trimester but regress thereafter [91]. Studies in baboons have revealed no change in the number or surface area of endometriosis lesions during the first two trimesters of pregnancy [92]. These results do not exclude a beneficial effect that potentially may occur during the third trimester or in the immediate postpartum period.

5.14. Prevention

There are no successful strategies to prevent endometriosis. Although a reduced incidence of endometriosis has been reported in women who engaged in aerobic activity from an early age the possible protective effect of exercise has not been investigated thoroughly [11]. There is insufficient evidence that oral contraceptive offers protection against the development of endometriosis [93] .

5.15. Therapy

Treatment must be individualized, taking into consideration the clinical problem in its entirety, including the impact of the disease and the effect of its treatment on quality of life [44]. Complaints may persist despite adequate medical or surgical treatment of the disease. In such circumstances, a multidisciplinary approach involving a pain clinic and counseling should be considered early in the treatment plan [44].

5.16. Endometrial sensory nerve fibres in endometriosis

5.16.1. Small diameter sensory nerve fibres in endometrium

It has been known for some time that the myometrium, the endometrial–myometrial interface and the deeper portion of the basal endometrium can be innervated by nerve fibres, but that nerve fibres are absent from the superficial two-thirds of the endometrium (the functional layer) in the normal human uterus [94]. The function of these nerve fibres in normal basal endometrium is not well understood. However, some acetylcholinesterase (AChE)-immunoreactive nerve fibres were detected in the basal layer of normal human endometrium [94]. Neuropeptide - (NPY), substance P- (SP), vasoactive intestinal peptide- (VIP) and neurotensin (NT)- immunoreactive nerve fibres were also present in normal human endometrium [95]. Yet little is known about the functions of these nerve fibres in human endometrium. Some researchers have studied nerve fibres in normal endometrium in animals. Calcitonin gene-related peptide-(CGRP) [96], AChE- and NPY-immunoreactive nerve fibres were detected in rat endometrium [97] and many CGRP-immunoreactive nerve fibres were near the surface epithelium [96]. SP and CGRP were also localized in myometrial nerves in a rat model [96].

A variety of clinical conditions including endometriosis, pelvic inflammatory disease, uterine fibroids, adhesions and uterine contractions can cause pelvic pain. Various investigators have demonstrated nerve fibres in endometriotic plaques, abdominal adhesions and myometrium [54, 98-101]. Endometriotic plaques in a rat model were innervated by SP, CGRP (sensory C and Aδ fibres) and vesicular monoamine transporter (VMAT) fibres [101].

Nerve fibres were present in human peritoneal endometriotic plaques, and transforming growth factor (TGF-β1) was expressed in the nerve fibres only in red and white lesions [100]. Rectovaginal endometriotic nodules from women with severe dysmenorrhoea and deep dyspareunia showed the presence of nerve fibres staining with the nonspecific antibody, S-100 protein [54]. Human peritoneal adhesions contained synaptophysin- CGRP-, SP-, vasoactive intestinal peptide- (VIP)- and tyrosine hydroxylase (TH)-immunoreactive nerve fibres [99] and 78% of endometriosis-related adhesions showed

NF-immunoreactive nerve fibres [98]. SP- and CGRP-immunoreactive nerve fibres were present in human myometrium [102] and SP-induced contractions were inhibited by CGRP. It has been reported that women with endometriosis had uterine contractions with higher frequency, amplitude and basal pressure tone during menses [103]; therefore, SP- and CGRP-immunoreactive nerve fibres may play a role in the pain of women with endometriosis.

5.16.2. Nerve growth factors

If the implantation theory is true, the nerve fibres in endometriotic plaques may originate from nerve fibre progenitors in the functional layer of the endometrium or, more probably, from ingrowth of local nerve fibres due to the secretion of nerve growth factors (NGFs) and increased expression of two NGF receptors, namely Trk-A and p75, by the implanting endometrium. It is not yet clear what neural stimuli may initiate the severe pain sensations which some women experience with endometriosis. However, a number of molecules that are capable of stimulating nerve fibres, such as tumour necrosis factor-α (TNF-α) [104], prostaglandin I2 [105], prostaglandin E2 and F2α [106], and NGF [107] can be released from endometriotic plaques. It seems highly probable that the endometrium of women destined to develop endometriosis is functionally abnormal [108-110]. It may also be programmed to secrete large amounts of nerve trophic factors, resulting in the remarkable ingrowth of unmyelinated small nerve fibres demonstrated in this study. This may also be translated into ingrowth of nerve fibres into new endometriotic plaques on the peritoneum or ovary. These nerve fibres may have an important role in the mediation of pain symptoms. No other satisfactory theory has yet been advanced for the genesis of pain symptoms in women with endometriosis. This demonstration of small nerve fibres in the functional layer of eutopic endometrium of women with endometriosis is so striking in the present study that we believe it could become a relatively simple surrogate marker of this condition using endometrial biopsies [111]. It is even possible that an endometrial biopsy may demonstrate nerve fibres in women destined to develop the condition later in life.

5.17. Need for non-invasive diagnosis

Non invasive diagnosis of endometriosis by detection of biomarkers in plasma/serum, urine, saliva, peritoneal fluid.

In a recent meta-analysis [112] authors conducted a systematic review of the literature from the last 25 years to assess critically the clinical value of all proposed biomarkers for endometriosis in serum, plasma and urine.

The majority of the latter studies have focused on single markers, and many results have been inconsistent and, at times, contradictory. Several narrative reviews have been published, but most authors have failed to use methods that are now recommended for systematic reviews of diagnostic tests [113].

The biomarker that has been used in clinical practice over the last 20 years is CA125. However, in a meta-analysis published in 1998, [114] authors showed convincingly that the biomarker's performance in diagnosing endometriosis was low, even though it showed some promise in detecting more severe disease. Since their meta-analysis was published, we identified 15 further studies reporting a correlation between endometriosis and CA125. More recent studies tend to assess the use of CA125 in monitoring treatment [115, 116]. Although these studies suggest CA125 levels fall during treatment, they have not shown a correlation with disease response. Owing to the ethical constraints on performing second-look laparoscopies in these studies (and the impracticalities in a modern clinical setting), it may be useful to include some form of health-related questionnaire such as the Endometriosis Health Profile-30 [117], to try to gain more information about disease activity.

In our analysis, we took into consideration the strong possibility of reporting bias. There is statistical evidence to suggest that the data available are heavily skewed as a result of investigators' failure to present negative data for publication and the disinclination of journals to publish negative data [118]. It is common knowledge that negative data are often either not submitted to scientific journals or not accepted by the reviewers or editors. However, if a study is well designed then negative data can be extremely informative and should, in our opinion, be published. In fact, as patients consent to collect and use their samples to help increase knowledge of the disease and/or find new diagnostic tools, it is ethically highly questionable when negative data are not published.

It is worth noting that surgery often plays a vital role in the treatment of endometriosis. Furthermore, it may also be of importance in the management of other conditions which present in a similar manner (e.g. tubal infertility). The use of a biomarker may well be tempered in these circumstances, but it may still help to reduce the need for diagnostic surgery in some women, enable monitoring of the disease progression by non-surgical methods, and potentially allow for better pre-operative assessment of women with endometriosis. Finally, the majority of the studies included in our review focused on assessing the diagnostic performance of single biomarkers. Realistically, however, a reliable diagnostic tool for endometriosis is likely to consist of a panel of biomarkers, not a single molecule. Table 1. shows the summary of representative proteins mainly cytokines, angiogenic, adhesion and growth factors aberrantly expressed in women with endometriosis.

Table 1. Representative proteins aberrantly expressed in women with endometriosis.

Proteins	**Molecular Weight(kDa)**	**Eutopic endometrium**	**Peritoneal Fluid**	**Endo-lesions**
IL-6	23-30 kDa	↑		↑
IL-8	8kDa		↑	↑
VEGF	32-42kDa	↑		↑
sICAM-1	90-95kDa	↑		
TNF alpha	17.5kDa	↑		↑
IGF-BP 3	17.7kDa		↑	
IL-4	10.8kDa		↑	
IL-10	39kDa			↑
alpha-2Heremans-Schmidt glycoprotein (AHSG)	64kDa	↑		
T-plastin	74kDa		↑	
Annexin II	38 kDa		↑	
Annexin V	36 kDa		↑	

MMP-1	54 kDa		↑	
MMP-3	54 kDa.		↑	
MMP-7	30 kDa		↑	
MMP-9	92 kDa		↑	
Transgelin	22-23kDa		↑	
TGF-β	Dimeric(25kDa)			↑
sVCAM-1	110 kDa	↑		
ICAM-1	85-110 kDa.		↑	↑
IL-1β	17kDa			↑
IL-12	75kDa			↑
CA-125	200 kDa	↑		↑
CA-19-9	200-1000kDa			
Aromatase	58 kDa.		↑	
RANTES	8kDa			↑
COX-2	72 kDa			
MCP-1	8.7 kDa	↑	↑	↑
IFN-gamma	20-25 kDa.	↑		
HGF	85kDa			↑
CD44	85 - 90 kDa			↑
CCR1	41 kDa			↑
IL-2	15.5 kDa		↑	
TNFR2	75kDa	↑		
Bcl-2	26kDa			↑
M-CSF	45 - 100 kDa			↑
CRP	251.1kDa	↑		

Ultimately, with more studies investigating the use of technologies such as genomics, proteomics and metabolomics, it can be expected that a panel of molecules or a typical profile of gene or protein expression will in the future help to distinguish between patients with and without disease. In combination with imaging techniques, such a panel

of biomarkers may indicate which women need a laparoscopy and eliminate countless unnecessary operations.

5.18. Cells of peritoneal cavity

The pathogenesis of endometriosis can to a certain extent be explained by retrograde menstruation of endometrial tissue sloughed through patent fallopian tubes into the peritoneal cavity [1]. However, it has never been shown that the prevalence of endometrial (EM) cells in peritoneal fluid (PF) is higher in women with endometriosis than in controls during menstruation. In fact, the cytology of retrograde menstruation has never been studied in depth.

Based on epidemiological and experimental data, it can be hypothesized that the quantity of retrograde menstruation and the consecutively flushed endometrial cells play an important role in the development of endometriosis [123, 179] In previous research, retrograde menstruation, defined as red stained PF [172], has been observed during culdoscopy in 50% [136], and during laparoscopy in 70-90% of patients at the time of menstruation [3]. However, the presence of red blood cells in PF is not a proof of the presence of viable EM cells at the time of menstruation. Furthermore, in most studies the identification of PF EM cells has been limited to classical histological analsyis of cell clumps present in PF [139, 180]. Not surprisingly, the presence of endometrial cells in PF has been reported to vary between 0 - 59% [6]. Using more objective immunocytochemical methods, some investigators [9] reported that PF contains single epithelial cells, rather than endometrial tissue fragments, in women with patent tubes and that these cells might be of endometrial origin. However, there is no evidence that the EM cell concentration in PF is higher during menstruation than in other phases of the menstrual cycle.

Erythrocytes represent a part of the cell population in PF which also contains other free floating cells like macrophages, mesothelial cells, lymphocytes, eosinophil and mast cells. Several studies show that there is an increase in erythrocyte count and consecutively in hemoglobin content in the peritoneal fluid of women with peritoneal endometriosis when compared with controls with a normal pelvis [10]. Hemoglobin overload might have numerous cytotoxic effects in the peritoneal environment [181, 182]. Its nonprotein moeity, heme, and its ferrous iron core are known as pro-oxidant and

proinflammatory molecules [183] and might be involved in the pathogenesis of endometriosis through several mechanisms including induction of oxidative stress, stimulation of cell adhesion, and cytokine production by macrophages[10]. However, it is not known if the PF concentration of red blood cells and hemoglobin is higher during menstruation than during nonmenstrual phases of the cycle. Endometriosis is associated with a state of subclinical peritoneal inflammation, marked by an increased PF volume, increased PF white blood cell concentration (especially macrophages with increased activation status), and increased inflammatory cytokines, growth factors, and angiogenesis-promoting substances [3, 26, 119][5, 14-16]. Moreover, it has been reported in baboons that subclinical peritoneal inflammation occurs both during menstruation and after intrapelvic injection of endometrium and both the incidence and recurrence of retrograde menstruation is increased in baboons with spontaneous endometriosis when compared to healthy controls [25, 120, 184]. However, it is not known if the PF concentration of white blood cells is higher during menstruation than during nonmenstrual phases of the cycle.

The lack of knowledge regarding potential differences in the presence and distribution of PF cell populations during menstruation between women with and without endometriosis is a major obstacle with respect to the validity of the Sampson hypothesis. The aim of our study was to test the hypothesis that menstruation is associated with a higher PF concentration of RBCs, WBCs and EM cells when compared to nonmenstrual phases of the cycle.

Women with early stages of endometriosis have the most pronounced increases of total leukocytes, macrophages, helper T lymphocytes and natural killer cells compared to fertile controls. There is compelling evidence for increased *white blood cell* populations in the PF of women with endometriosis as compared to women without endometriosis [119].

It has been proven that, the white blood cell concentration in peritoneal fluid is elevated in primates in the subgroup with recent endometriosis when compared with the other subgroups with a normal pelvis, long-term endometriosis or induced disease [120]. Moreover, it has been reported in baboons that subclinical peritoneal inflammation occurs both during menstruation and after intrapelvic injection of endometrium [7].*Granulocytes*, normally present in small numbers, are greatly increased with pelvic inflammation [121].

These findings support an active immunological process.

Macrophages are attracted to the peritoneal environment more abundantly than any other cell type [121-127]. These cells originate in the bone marrow, circulate as monocytes, and then migrate to various body cavities where they function primarily as phagocytes when activated. Macrophage-directed host defence mechanisms resulting in the recognition, phagocytosis, and destruction of micro-organisms are well known. Macrophages digest and process peritoneal debris such as spermatozoa and endometrial tissue and present antigens to the T cells.

Recent studies [119, 128] suggests that activated macrophages are increased in the peritoneal fluid of women with endometriosis. These activated macrophages secrete numerous macromolecules that may contribute to the implantation of endometrial cells and the progression of endometriosis [129-131]. Macrophages are capable of secreting various substances, such as growth factors, cytokines, prostanoids, complement components and hydrolytic enzymes [119, 132-134].

Macrophages also promote cellular growth and viability through secretion of growth factors and cytokines. Furthermore, macrophages release low amounts of reactive oxygen metabolites, such as superoxide anion, hydrogen peroxide and singlet oxygen.

Previous studies suggest that menstrual effluent contains factors inducing local destruction of the peritoneal mesothelium, thereby creating adhesion sites for *endometrial cells*. Experiments by Witz et al. [135] have given further support to this theory, specifically demonstrating that endometrium—both stroma and epithelium—can easily and rapidly adhere to an intact mesothelium.The experimental model involved plating explants of peritoneum and culturing them in the presence of endometrium in form of cellular aggregates or isolated epithelial and stromal cells or menstruated fragments. The attachment process was evaluated by transmission electron microscopy and a confocal laser-scanning microscope. The results indicate that endometrial attachment to an intact mesothelium occurs within 1 hours and that transmesothelial invasion occurs between 1 and 18–24 hours. Thus, and in contrast to previous observations the intact mesothelium does not seem to constitute a defence barrier to the adhesion of endometrial fragments and traumas to the mesothelial lining are not a prerequisite for endometrial cell adhesion.

Several studies have shown that the attachment of endometrial cells is enhanced by the induction of adhesion molecules and their receptors in case of endometriosis. After

adhesion, endometrial cells proliferate and gradually invade the peritoneal tissue and some factors induce vascularization of endometriotic implants, allowing their further development.
Cytokines and growth factors such as TGFβ, IL-8, IL-1, TNFα, IFNγ, vascular endothelial cell growth factor (VEGF) and hepatocyte growth factor (HGF) and have been implicated as inducers of these attachment, proliferation, and neovascularization.
The adhaesion phenomenon of the epithelial cells with possible endomterial origin may affect the detection of the endometrial cells in PF.

5.19. Retrograde menstruation

Several investigations have shown that retrograde menstruation is quite common. Polishuk et al. [136], performed culdoscopy during the menstrual period, found blood-stained PF in 50% of the patients. Others have found that 90% of normal women experience retrograde menstruation, with 70'% exhibiting grossly bloody PF during menstruation [3]. However, the occurrence of red blood cells in PF during menstruation is not proving retrograde transport of viable endomelrial cells. Studies analysing the incidence of endometrial cells in PF are conflicting; the incidence varying from 0 to 59% [137]. Several investigators have found endometrial tissue in the PF of women with or without endometriosis with equal frequency [138, 139]. Only one study has identified endometrial tissue more frequently in the PF of women with endometriosis [140].

The hypothesis that the quantity of endometrial cells deposited into the peritoneal cavity during menstruation is higher among women who develop endometriosis is supported by epidemiological evidence [3]. It is well known that women with short cycles and long duration of menstrual flow are more likely to develop endometriosis [141]. Furthermore, outflow obstruction of the menstrual effluent, resulting in excessive retrograde menstruation, has been associated with endometriosis both in humans and in animal experiments[142]. The development of endometriosis in the first few years after menarche has been associated with a high rate of obstructing genital-tract anomalies. These include non-communicating rudimentary uterine horns, cervical stenosis, cervical atresia, vaginal or transverse septum agenesis, or an imperforate hymen. As a general rule, women with a stricture at the level of the cervix have a higher incidence of

endometriosis than women with a stricture lower in the genital tract. This explains the observation that in women with Mullerian anomalies, those with outflow obstruction were more likely to have endometriosis than those without (77 vs. 37%)[11] . Furthermore, studies in baboons have demonstrated that supracervical ligation can cause obstructed uterine outflow, resulting in decreased duration of antegrade menstruation and increased retrograde menstruation. Endometriosis has been observed in animals as early as 3 months after supracervical ligation suggesting that even modest degrees of uterine outflow obstruction may play a role in the development of endometriosis [10].

These data suggest that retrograde transport of viable endometrial cells during menstruation occurs in most women with patent tubes, suggesting that another factor(s) aside from the presence of endometrial cell reflux is critical for the pathogenesis of endometriosis.

The mesothelium is a simple squamous epithelium that lines the peritoneal cavity. It has been shown that human *peritoneal cells* (mesothelial cells) are capable of producing haematopoietic growth factors, either constitutively (IL- 1. IL-6. IL-8. MCP-I, granulocyte colony-stimulating factor, macrophage colony-stimulating factor) or in response to a variety of stimuli, including TNFα, IL- 1 and epidermal growth factor (EGF) [143]. In addition, these cells secrete CA- 125 from their apical surfaces [144].
These findings suggest that mesothelial cells play an important role in the regulation of peritoneal inflammation and tissue regeneration.
There is increasing evidence that local inflammation and secretion of prostaglandins (PG) is related to differences in endometrial aromatase activity between women with and without endometriosis [36]. The subclinical pelvic inflammatory status associated with endometriosis is also reflected in the systemic circulation. Increased concentrations of C-reactive protein, serum amyloid A (SAA), TNF-a, membrane cofactor protein-1, interleukin-6, interleukin-8 and chemokine (C-C motif) receptor 1 (CCR1) have been observed in peripheral blood samples of patients with endometriosis when compared with controls [37].

5.19.1. Quality of viable endometrial cells

It has been hypothesised that the quality of endometrial cells in PF of women with endometriosis is different from women with normal pelvis. Viable endometrial cells from human endometriotic biopsies but not from human endometrial biopsies are invasive in an in vitro collagen invasion assay, probably because they have a higher proportion of potentially invasive E-cadherin-negative epithelial cells [145]. Inflammatory cytokines (TNF-α, IL- 8 and IL-6) produced by endometrial cells probably contribute to this adhesion process [146-148]. IL-8 has been shown to stimulate the adhesion of endometrial cells to fibronectin [146]. TNF-α has been reported to also promote endometrial stromal cell proliferation in vitro [147] and endometrial stromal cell adhesion to extracellular matrix components [148]. TNF-α may induce IL-8 gene and IL-8 protein expression in a dose-dependent manner, and the stimulating effect of TNF-α on endometrial stromal cell proliferation can be reversed by adding anti-IL8 antibodies[145] [30]. Does endometriosis then only occur among women with a high degree of endometrial-peritoneal adhesion? This is unknown at present, since it is impossible to study this process in women in vivo.

Debrock and colleagues [149] reported a 80–100% success rate of endometrial-peritoneal adhesion in cultured explants after 48 hours, regardless of the presence or absence of endometriosis. Witz et al [135] showed endometrial adhesion occurs within 1 hour and transmesothelial invasion occurs within 18 hours. However, all these assays are merely descriptive and there is a need to develop a quantitative in vitro assay to measure endometrial-peritoneal adhesion. Endometrial quality can also be affected by local estrogen production in eutopic/ectopic endometrium. Indeed, the expression of uncontrolled aromatase mRNA in endometriotic lesions [150] suggests that a local estrogenic milieu is important in the development of endometriosis. It is possible that persistent expression of aromatase and 17β-hydroxysteroid dehydrogenase in endometriotic lesions may also be driven by a T-like autoantibody response. Indeed, autoantibodies recognising T-like antigens have been reported to be upregulated in endometriosis and may trigger the synthesis of cytokines such as IL- 1, TNF-α and IL-6,

which in turn may induce the expression of aromatase and 17β-hydroxysteroid dehydrogenase in endometriotic lesions [151].

5.20. Diagnostic delay in the diagnosis of endometriosis

Endometriosis is a common, chronic gynaecological disease defined by the ectopic presence of endometrial glands and stroma, most commonly in the pelvis. It is symptomatically associated with infertility and pelvic pain including dysmenorrhoea, dyspareunia, dyschezia and chronic pelvic pain [50, 152]. Endometriosis–associated pain can be caused by peritoneal inflammation, adhesion formation, and specific innervation of endometriotic lesions and is correlated with the presence of deep infiltrating disease [107, 153-155]. However, there is a poor correlation between pain and the degree of endometriosis [53] (minimal–mild–moderate–severe), as determined according to the revised staging system of American Society for Reproductive Medicine. For a definitive diagnosis of endometriosis, visual inspection of the pelvis at laparoscopy is the 'gold standard' investigation, ideally combined with histological confirmation [44]. However, laparoscopy is a surgical procedure with rare but significant potential risks for the patients [156].

Due to the lack of a no–or semi–invasive diagnostic tool, the delay between onset of pain symptoms and surgically confirmed endometriosis is as long as 8 years in the United Kingdom and United States [50, 51]. The current delay in diagnosis and treatment contributes to years of suffering and potential infertility if the disease is left untreated. Clearly, a simple noninvasive diagnostic method may greatly help to reduce this delay, especially for minimal–mild endometriosis which cannot be diagnosed by clinical examination or ultrasound.

Attempts for noninvasive diagnosis of endometriosis based on analysis of biomarkers in peripheral blood have been limited by insufficient sensitivity and specificity [157-159]. Based on the fact that eutopic endometrium from women with endometriosis is biologically different from women with a normal pelvis [160-162], a semi–invasive diagnostic test for endometriosis can potentially be developed in endometrium obtained after transcervical endometrial biopsy. Whatever method is used, the most important property of any diagnostic test is high sensitivity in order to ensure that no women with

endometriosis or other significant pelvic pathology are missed who might benefit from surgery for infertility and/or pain [163].

In recent studies a higher density of small unmyelinated nerve fibres has been shown in the functional layer of endometrium from women with confirmed endometriosis when compared with women without endometriosis, especially in the secretory phase of the cycle [164, 165]. Indeed, sensory nerve fibers can be identified in functional layer endometrium by immunohistochemical analysis of various neural transmitters such as substance P (SP), vasoactive intestinal polypeptide (VIP), or neural proteins like protein gene product 9.5 (PGP9.5), neurofilament (NF), neuropeptide Y (NPY) and calcitonin gene–related protein (CGRP). The detection of endometrial nerve fibers has been proposed as a diagnostic tool for endometriosis in a recent pilot study [166]. However, this study was limited by the lack of uniform histological confirmation of endometriosis, inclusion of variable number of patients from all stages of the disease and by cycle phase related changes of endometrium.

In the present study we tested the hypothesis that women with minimal and mild endometriosis express a higher density of sensory small diameter nerve fibres in the functional layer of endometrium than women with a normal pelvis in order to develop a possible semi–invasive diagnostic tool for minimal to mild endometriosis.

6. Aims

In our experiments we aimed to develop a potential semi-invasive diagnostic test of endometriosis by using eutopic endometrial samples and to assess the biological changes in the peritoneal cavity during the menstrual cycle as a basis for biomarker development. We were also focused on the study of retrograde menstruation a crucial factor in understanding the pathogenesis of endometriosis.

Our specific aims were to:

1. Test the hypothesis that multiple sensory small diameter nerve fibers are present in a higher density in endometrium from patients with endometriosis when compared to women with a normal pelvis.
2. Prove that the assumed difference enables the development of a semi-invasive diagnostic test for minimal-mild endometriosis.
3. Investigate whether menstruation is associated with a higher concentration of endometrial cells in peritoneal fluid (PF).
4. Confirm that endometriosis is associated with an active immunologic process with increased white and red blood cell concentration in PF when compared to nonmenstrual phases of the cycle.

7. Material and Methods

7.1. Tissue collection

In this study 40 endometrial samples were selected from the biobank at the Leuven University Fertility Centre where tissues from women undergoing laparoscopies for infertility and/or pain have been stored since 1998. Endometrial biopsies were obtained after hysteroscopy and before laparoscopy using a Pipelle (Pipelle de Cornier, Paris, France), which is a sterile and disposable plastic cannula for sampling endometrium [167]. All patients had signed a written informed consent before recruitment and the study protocol had been approved by the Institutional Ethical and Review Board of University Hospital Gasthuisberg.

Endometrial samples were selected based on cycle phase, on the presence/absence of endometriosis, and on the absence of medical treatment for endometriosis within 3 months before sample collection. Menstrual cycle stage was reported as per the patient's report of last menstrual period and by histological evaluation of the endometrial tissues according to the criteria of Noyes [168].

Only samples collected during the secretory phase of the cycle were selected, since the density of multiple small nerve fibers is higher during this phase than during other phases of the cycle [164]. Twenty endometrial samples were selected from women with laparoscopically and histologically confirmed minimal (n=10) or mild (n=10) endometriosis (mean age 33 ±10 years), staged according to the revised staging system of American Society for Reproductive Medicine (American Society for Reproductive Medicine, 1996). Another 20 endometrial samples were selected from women with a laparoscopically confirmed normal pelvis (mean age 32 ± 5 years). Demographic data of our study population are shown in the Table 2.

Table 2. Demographic Characteristics of the Study Population (n=40)

	Endometriosis (n=20)	Controls (n=20)
Age (years, mean±SD)	33±10	32±5
Gravidity/parity (mean±SD)	0.1±0.3 /0.05±0.22	0.35±0.87/0.15±0.7
Primary/secondary infertility [n(%)]	18(90) /2(10)	17(85) /3(15)
Chronic pelvic pain [n(%)]	0(0)	0(0)
Dysmenorrhoea [n(%)]	3(15)	2(10)
Dyspareunia [n(%)]	0(0)	1(5)
Concurrent hormonal medication [n(%)]	0(0)	0(0)
Previous treatment for infertility [n(%)]	3(15)	4(20)
Ovulation induction	1(5)	0(0)
Laparoscopic surgery	2(10)	4(20)
Indication for surgery [n(%)]		
Infertility	2(10)	4(20)
Pelvic pain	0(0)	0(0)
Ethnicity [n(%)]		
Caucasian	20(100)	19(95)
Asian	0(0)	1(5)

7.2. Histology

All biopsies had been fixed in 10% neutral buffered formalin immediately after collection for at least 24 hours, processed, paraffin embedded, and stored at room temperature until further use. For this study, paraffin blocks were sectioned at 4µm thickness on a Leica microtome (type 2055 Autocut; Nussloch, Germany). One hundred serial sections were collected in sets of 4 subsequent sections on 25 silane-coated slides and were air dried at 37°C. Every tenth slide of this series was stained with hematoxylin-eosin for morphological evaluation. For immunohistochemical evaluation we selected sections which exhibited clear histological features consistent with a normal secretory phase.

7.3. Immunohistochemistry

Tissue sections were preheated for 2 hours at 55 ^{o}C, then deparaffinized and rehydrated. After rinsing in 0.01M Tris buffered saline (TBS), the tissue sections were heat retrieved in 0.01M TBS pH 9 with 0.001M EDTA. Serial sections were incubated overnight at 4°C with monoclonal mouse antihuman NF (ready to use; Dako, Glostrup, Denmark) polyclonal rabbit anti–PGP 9.5 (diluted 1:900; Dako), polyclonal rabbit anti–SP (diluted

1:2000; Serotec, Raleigh, NC, USA), monoclonal mouse anti–CGRP (diluted 1:2000; Sigma, St. Louis, MO, USA), polyclonal rabbit anti–VIP (diluted 1:1400; Chemicon, Temecula, CA, USA), and polyclonal rabbit anti–NPY (diluted 1:2000, Chemicon) respectively. The antibodies were detected with REAL Detection System, Alkaline Phosphatase/RED, Rabbit/Mouse (Dako) according to manufacturers instructions. Non–specific immunoglobulin binding was blocked with a mixture of BSA (2%), Tween–80 (0.1%) and non–fat–dried milk (1%) applied for 15 to 45 minutes before the first and the second antibody incubations. 0.01M TBS was used for all dilutions and rinsing steps throughout the staining procedure and all steps were carried out at room temperature except when state otherwise. Sections were counterstained lightly with Mayer's hematoxylin and mounted in glycerine jelly. We used normal human skin as a positive control as it reliably contains myelinated and unmyelinated nerve fibers expressing PGP9.5, VIP, SP, CGRP NPY, and NF. Rabbit and mouse immunoglobulin fractions were used as respective negative controls, the concentrations were matched with the concentrations of the antibodies.

Assessment of nerve fibre density was performed using image analysis software KS400 3.0 (Zeiss, Göttingen, Germany) linked to a Zeiss microscope (Axioskop 50) fitted with a Zeiss color camera (Axiocam MRc5). The evaluation of all immunohistochemical stainings was done blindly by the evaluation of the whole surface of each section on high power images (objective 40x, optovar 1, resolution 860x644 Px) of adjacent non overlapping fields from left to right and from top to down. Each high power field (HPF) covered a maximal area of 0.0789 mm2 from which all irrelevant zones (i.e. artefactual or not belonging to the actual tissue) were subtracted before measurement of the actually assessed field area. Within these HPFs, all nerve fibre profiles expressing neural markers were counted with exclusion of those crossing the right or the bottom side of the field frame respectively, thus avoiding to count these fibre profiles twice. After summation of the nerve fibre counts and the HPF area values for the whole section, the total number of nerve fibers was divided by the total surface area of the examined endometrium to obtain the nerve fiber density for the current section. The average duration of screening of one specimen was 30±10 minutes.

7.4. Statistical analysis

Data are presented as mean (SD) number of nerve fibres per mm2. Numerical data were analysed using Excel (version 5.0; Microsoft Corporation, Redmond, WA, USA). The K–S Lilliefors/Shapiro–Wilks test was used to test normality in order to determine whether parametric or non–parametric tests were to be used in further analyses.

The differences of nerve fibre density between eutopic endometrium from women with and without endometriosis were tested for significance by the Mann–Whitney U–test, using the statistical package Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

Multivariate analysis was done using stepwise logistic regression (SAS 9.1.3 for Windows, Cary, NC, USA) and Stepwise Logistic Regression and Least Squares Support Vector Machines (LS–SVM) (MATLAB scripts were downloaded from LS–SVMlab version 1.5 http://www.esat.kuleuven.ac.be/sista/lssvmlab/). For stepwise logistic regression only variables with significant odds ratios (p–value <0.05) were allowed in the model.

Both stepwise logistic regression and LS-SVM have been used frequently by our group in developing models for diagnostic testing for clinical applications [169-171]. Moreover, both models can be implemented as a formula in excel to be used for diagnostic testing.

For LS–SVM analysis feature selection was performed based on Leave–One–Out cross validation (LOO–CV) analysis. Briefly, in each LOO–CV the neural markers were ranked according to their p–value (Mann–Whitney U–test). Then the top “n” –features were selected where “n” ranged from 1, 2,… to 6 (corresponding to all neural markers). The “n” with the lowest LOO–CV error was selected to build a model on the full data set. The models were evaluated based on their Area Under the ROC curve (AUC) [172]. Additionally, an operating point on the ROC curve was chosen corresponding to the maximum of the sum of sensitivity and specificity. Then models were also evaluated by their sensitivity, specificity, accuracy, positive predictive value (PPV) and negative predictive value (NPV).

7.5. Patients and sample collection for the PF study

Our study protocol was approved by the Institutional Ethical and Review Board of Gasthuisberg University Hospital, KU Leuven for the protection of human subjects. Informed consent was obtained from all patients before entry into this study. A diagnostic laparoscopy for investigation of pelvic pain and/or infertility was performed in 107 reproductive age women (range 22-44 years, demographic data in Table 3).

Table 3. Demographic Characteristics of the Study Population (n=107)

	Endometriosis (n=59)	Controls (n=48)
Age (years, mean±SD)	31.76±3.71	28.27±3.18
Duration of infertility (years, mean±SD)	2.67±2.06	2.13±1.12
Primary/secondary infertility[n(%)]	39(66.1) /20(33.9)	30(62.5)/18(35)
Concurrent medication[n(%)]	2(3.38)	0
Phase of the menstrual cycle [n(%)]		
Menstrual	18(78.26)	5(21.74)
Nonmenstrual total	38(45.23)	46(54.77)
Follicular	22(26.19)	16(19.04)
Luteal)	16(19.04)	30(35.73)
Indication for surgery [n(%)]		
Infertility	58(98.3)	45(93.75)
Pelvic pain	1(1.71)	3(6.25)

A normal pelvis was observed in 48 women. Endometriosis was found in 59 women and was classified (American Society for Reproductive Medicine, 1996) into minimal (n=25), mild (n=20), moderate (n=6) and severe (n=8) disease. Endometriosis was confirmed histologically in all patients (n=59). All 107 patients had patent tubes. Samples of PF were collected during the luteal (n=46), follicular (n=38) or menstrual (n=23) phase of the cycle. The PF was aspirated from the pouch of Douglas before any surgical manipulation was started and immediatelly processed. Special precaution was taken to avoid blood or other fluid (saline, methylene blue dye) contamination. Peritoneal washing was not performed. In all PF samples the colour was noted and divided in the following categories: clear, light yellow, yellow, orange, pink, light red, red, dark red.

In the first 32 patients included in our study, PF cells were used for a detailed cytological analysis using Papanicolau staining and immunocytochemical analysis. These 32 patients included 13 controls and 19 patients with minimal (n=8), mild (n=5), moderate (n=2) or severe (n=4) endometriosis. In these 32 patients, PF samples were collected during the luteal (n=20), the follicular (n=5) or the menstrual (n=7) phase of the cycle.

7.6. Cell counts

Cell counts (leucocytes, erythrocytes) were determined on a Symex SE 9500 cell counter (Sysmex Co., Kobe, Japan). Leucocyte counts below 0.6 x 10^9 /L were redone by the counting chamber method (Nageotte counting chamber, Marienfeld Laboratory Glassware, Lauda-Königshofen, Germany).

7.7. Immunocytochemistry

PF specimens were centrifuged for 10 minutes at 3000 rpm. The pellet was processed and fixed with CytoRich (Becton Dickinson, Franklin Lakes, NJ. USA) then one thin-layer (Papanicolau stain) was prepared with PrepStain (Becton Dickinson, Franklin Lakes, NJ. USA).

All PF samples underwent both Papanicolau and immunocytochemical staining with monoclonal antibodies against CK 7 (1:100; Dako, Glostrup Denmark), CK 8/18 (1:20; Novocastra, Newcastle upon Tyne, UK), Ber-Ep4 (1:200; Dako, Glostrup Denmark), vimentin (1:100; Dako, Glostrup Denmark), calretinin (1:2000; Swant, Bellinzona, Switzerland) and CD68 (1:10; Kp1, Dako, Glostrup Denmark).

These antibodies were selected since they are commonly used in effusion cytology and they are relevant to identify endometrial epithelial or stromal cells, mesothelial cells and macrophages in PF. Ber-Ep4 is a marker for cells with epithelial (in some cases mesothelial) origin. CD68 is specific for cells from monocyte/macrophage lineage, CK7 and CK8/18 are markers for both endometrial epithelial and mesothelial cells, whereas calretinin and vimentin are markers for both endometrial stromal and mesothelial cells (Table 4).

Table 4. Immunocytological markers for different cell populations present in peritoneal fluid

Marker	Endometrial epithelial /glandular cells	Endometrial stromal cells	Mesothelial cells	Macrophages	NK cells	Lymphocytes
Ber Ep4	+	-	-	-	-	-
Vimentin	-	+	+	-	-	-
Calretinin	-	+	+	-	-	-
Cytokeratin7	+	-	+	-	-	-
Cytokeratin8	+	-	+	-	-	-
Cytokeratin18	+	-	+	-	-	-
CD68	-	-	-/+	+	-	-

Immunocytochemistry was done according to our routinely used protocol with respect to the dilution of the primary antibody, application of the heat-induced epitope retrieval and visualization of the antibody complexes through Envision-HRP with DAB (Dako, Glostrup, Denmark). Slides prepared by the PrepStain method were evaluated by an experienced cytopathologist.

7.8. Statistical analysis

Numerical data were analysed using MS Office Excel (version 6.0; Microsoft Corporation, Redmond, WA, USA). Kolomogorov-Smirnov/ Lilliefors and Shapiro–Wilks test was used to test normality to determine whether parametric or non-parametric tests were to be used in further analyses. For statistical analyses, Mann-Whitney and Fisher exact tests were performed. Statistical calculations were performed by using the GraphPad Prism version 5. 00 for Windows, (GraphPad Software, San Diego California USA), and $P \leq 0.05$ was considered significant.

8. Results

8.1. Results of the endometrial sensory nerve fibre study

In 90% (18/20) of women with endometriosis, nerve fibers were observed in the endometrium (Table 5.).

Table 5. Quantitative assessment of the endometrial nerve fibre density stained against different neural markers in patients with and without endometriosis

Marker	Em nerve fiber density: total number of nerve fibers/mm² em surface area screened median (range) mean±sd		Total number of nerve fibers present in total em surface area screened/ patient median (range) mean±sd		Total em surface area screened (mm²)/ Patient median (range) mean±sd
	Endo(n=20)	Control(n=20)	Endo(n=20)	Control(n=20)	
PGP9.5	2.30(0–9.23) 2.62±2.19‡	0.0(0–0.79) 0.21±0.28‡	9(0–32) 11.10±7.92‡	0(0–5) 1.20±1.73‡	5.74(2.13 –10.55) 5.65±2.02
NPY	1.73(0–18.05) 2.52± 3.91‡	0.0(0–0.62) 0.15±0.23‡	5(0–31) 7.7± 7.76‡	0(0–5) 1.05±1.70‡	5. 84(1.57 –9.82) 5.68±2.44
CGRP	1.58(0–4.9) 1.94±1.58‡	0.0(0–0.68) 0.08±0.19‡	5(0–31) 6.85±7.2‡	0(0–3) 0.45±0.99‡	5.54(1.89 –10.05) 5.53 ±2.70
SP	1.50(0–8.45) 2.29±2.2‡	0.0(0–0.56) 0.1±0.2‡	6(0–27) 7.95±7.04‡	0(0–3) 0.55±1.05‡	5.92(1.43 –10.07) 5.54±2.5
VIP	0.71(0–16.79) 2.37±3.77‡	0.0(0–0.43) 0.06±0.15‡	4.5(0–22) 7.75±6.9‡	0(0–3) 0.85±1.95‡	5.84(1.01–9.81) 5.63±2.12
NF	0.0 (0–0.45) 0.02±0.10¶	0.0(0–4.68) 0.25±1.04¶	0(0–1) 0.05±0.22¶	0(0–30) 1.60±6.70¶	6.19(1.73 9.99) 5.92±2.32

EM= endometrium

Endo = patients with endometriosis

Control = women with a normal pelvis

‡= P<0.0001

¶=NS

In this group, immunohistological staining was positive for PGP 9.5, SP, CGRP, VIP and NPY but not for NF, except in one patient, suggesting that almost all small nerve fibers were unmyelinated and represent a mixture of sensory C, adrenergic and (in smaller amount) sensory Aδ and cholinergic nerve fibers (Figure 7).

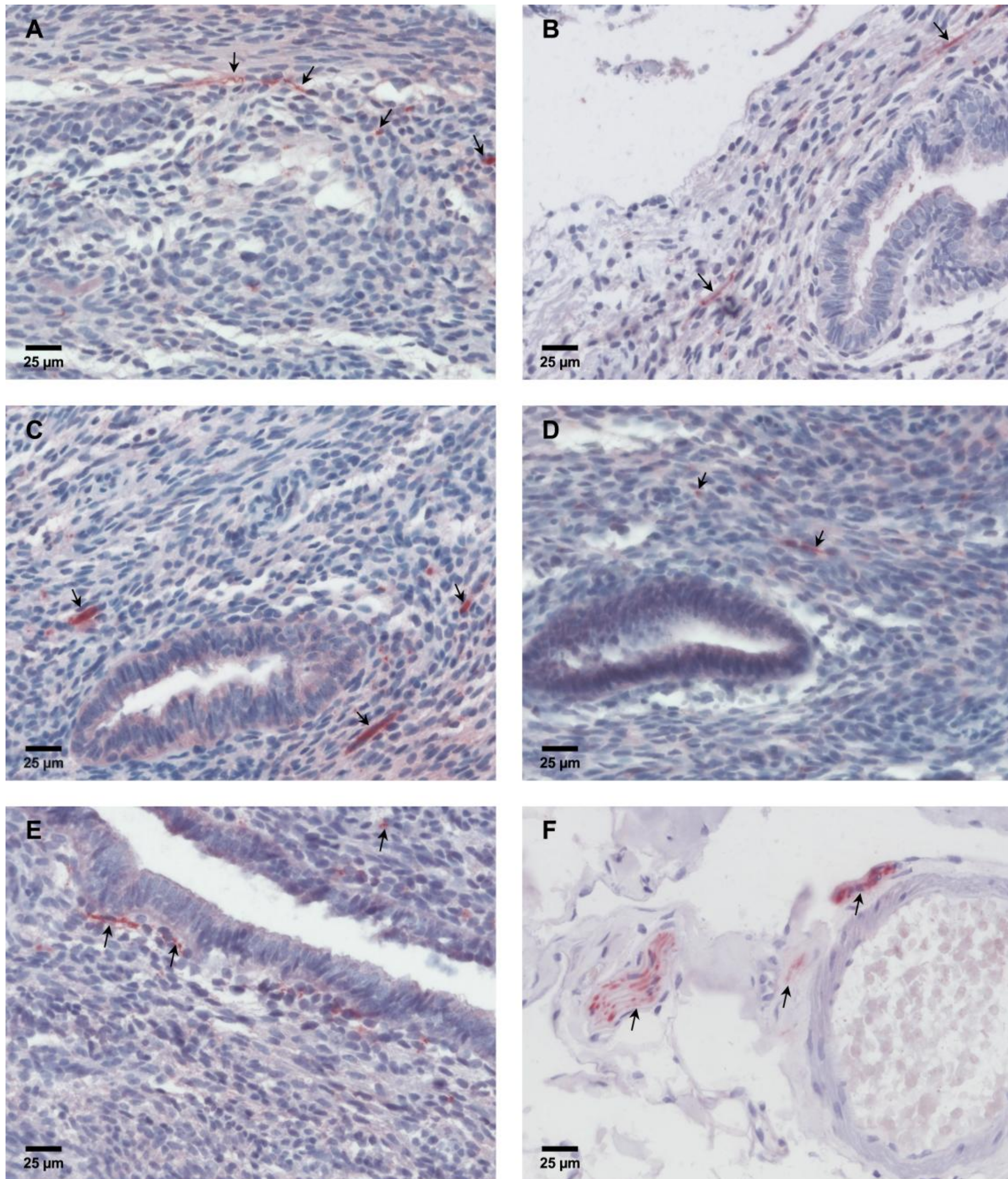

Figure 7**.** Small diameter nerve fibers in eutopic endometrium in women with minimal and mild endometriosis. Eutopic endometrium stained with PGP9.5(A), with VIP(B), with SP(C) with NPY(D) and with CGRP(E). Arrows denote tiny positive multiple nerve fibers. Eutopic endometrium from woman with endometriosis stained with NF(F). Arrows denote perivascular myelinated nerve fibres. Magnification, x400. Scale bars represent 25 μm.

However, these nerve fibers were not distributed homogeneously throughout the endometrium. The density of nerve fibers was markedly skewed, with few specimens showing counts above 30/mm2 and with most between 0–10 per mm2. There was no significant difference between the nerve fibre densities in women with confirmed minimal endometriosis (2.1±2.87) and those with mild endometriosis (1.84±2.59, p=0.46).

In only 40% (8/20) of women without endometriosis, only small numbers of PGP9.5–stained nerve fibers were present and were positive for SP, CGRP, VIP, and NPY but not for NF, except in two patients (Table 6.).

Table 6. Univariate analysis of different endometrial neural markers for the semi-invasive diagnosis of minimal and mild endometriosis

Marker	Sensitivity (95% CI)	Specificity (95% CI)	PPV	NPV	Cut off value for nerve fiber density	AUC (95% CI)
PGP9.5	95(75.13–99.87)	75(50.90–91.34)	79.19	93.75	0.49	0.94(0.86–1.02)
VIP	95+(75.13–99.87)	80(56.34–94.27)	82.6	84.21	0.08	0.94(0.87–1.00)
CGRP	90(68.30–98.77)	85(62.11–96.79)	85.71	89.47	0.23	0.92(0.83–1.01)
SP	95(75.13–99.87)	80(56.34–94.27)	82.6	84.21	0.20	0.90(0.85–1.01)
NPY	95(75.13–99.87)	65(40.78– 84.61)	72	86.66	0.13	0.90(0.80– 0.99)
NF	95(75.13–99.80)	10(1.23–31.70)	0.33	48.64	0.19	0.52 (0.34–0.70)

Data are presented as percentages. PPV= positive predictive value; NPV= negative predictive value, AUC=area under the curve for diagnostic accuracy

Univariate and multivariate analysis is shown in Table 6 and in Table 7 respectively. Table 7. Selecting the number of neural markers for LS–SVM modelling based on Leave One Out–Cross Validation (LOO–CV).

Number of neural markers	AUC (SE)
1	0.56 (0.10)
2	0.84 (0.07)
3	0.98 (0.02)
4	0.94 (0.05)
5	0.96 (0.03)
6(all)	0.94 (0.04)

AUC: Area under the ROC curve

SE: Standard error

No multivariate logistic regression model could be built that corresponded to our criteria (p–value <0.05 on odds ratios). Using Leave–One–Out cross validation (LOO–CV) analysis with LS–SVM modelling (Table 8.), the best result was obtained when selecting the top 3 neural markers based on their p–value (Mann–Whitney U–test). A LS–SVM model, built on the complete data set with the top 3 neural markers VIP, PGP9.5 and SP had an AUC of 0.99, (SE 0.01). (Figure 8).

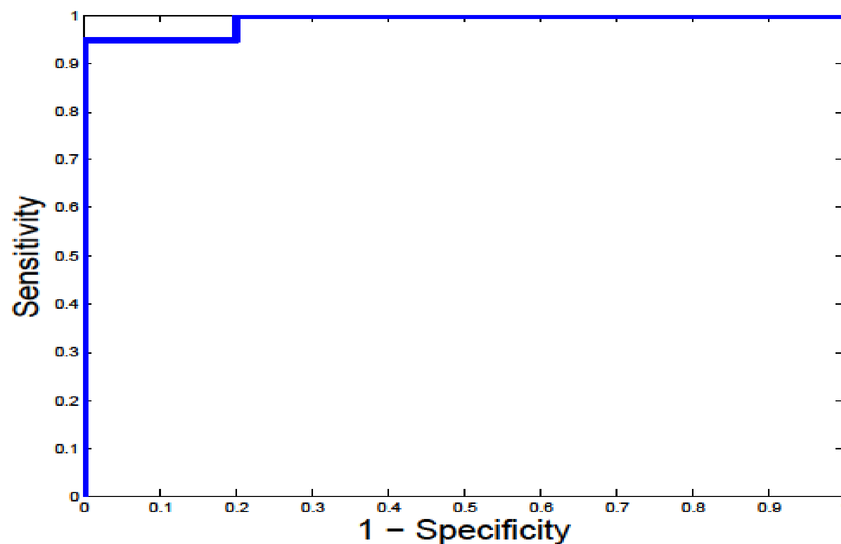

Figure 8. ROC curve of the LS–SVM model built using PGP9.5, VIP and SP

After choosing an operating point, this model allowed the diagnosis of endometriosis with a sensitivity of 95%, specificity of 100%, accuracy of 97.5%, PPV of 100%, and NPV of 95%, corresponding to one endometriosis patient classified as control by the model (i.e. false negative).

8.2. Results of the peritoneal fluid cytology study

In comparison with the nonmenstrual phase of the cycle (n=84), analysis of PF during menstruation (n=23) showed a 3 fold increased concentration of leucocytes, a 4 fold increase in the concentration of basophilic granulocytes, and a 13, 10 and 8 fold increase in the concentration of erythrocytes, hematocrit and hemoglobin, respectively (Table 8).

Table 8. Cell Counts in Menstrual vs. Nonmenstrual Phase of Cycle

	Menstrual phase (n=23) mean±SD	Nonmenstrual phase (n=84) mean±SD	P value
Leucocytes x109/L	3.3±1.1	0.8±0.5	0.03
Granulocytes			
Basophilic x109/L	0.2±0.08	0.04±0.02	0.002
Eosinophilic x109/L	0.1± 0.09	0.04±0.03	0.09
Erythrocytes x1012/L	0.3±0.2	0.02±0.01	0.006
Haematocrit L/L*	0.03±0.01	0.003±0.02	0.01
Haemoglobin g/dL	0.8±0.7	0.1±0.17	0.01

* calculated values

Morphological evaluation of the Pap-stained specimens showed that the prevalence in PF of single cells with an endometrial phenotype was low and comparable during menstruation (1/7), follicular phase (1/5), and luteal phase (2/20 including one case of single cells and one case of a group of cells). In contrast, mesothelial cells and histiocytes were present in all PF samples.

Prevalence of patients with PF cells stained positively by immunocytochemistry for 7 different cell markers among 32 patients with (endo, n=19) or without (control, n=13) endometriosis is shown in Table 9.

Table 9. Prevalence of patients with PF cells stained positively by immunocytochemistry

Marker	Endometrial epithelial /glandular cells		Endometrial stromal cells		Mesothelial cells		Macrophages/ Monocytes	
Ber Ep4	Endo	Contr	Endo	Contr	Endo	Contr	Endo	Contr
Total	2/19	0/13	0/19	0/13	0/19	0/13	0/19	0/13
Menstrual	2/5	0/2	0/5	0/2	0/5	0/2	0/5	0/2
Follicular	0/4	0/1	0/4	0/1	0/4	0/1	0/4	0/1
Luteal	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Vimentin								
Total	0/19	0/13	17/19	11/13	17/19	11/13	0/19	0/13
Menstrual	0/5	0/2	5/5	2/2	5/5	2/2	0/5	0/2
Follicular	0/4	0/1	4/4	1/1	4/4	1/1	0/4	0/1
Luteal	0/10	0/10	8/10	8/10	8/10	8/10	0/10	0/10
Calretinin								
Total	0/19	0/13	11/19	9/13	11/19	9/13	0/19	0/13
Menstrual	0/5	0/2	4/5	1/2	4/5	1/2	0/5	0/2
Follicular	0/4	0/1	2/4	1/1	2/4	1/1	0/4	0/1
Luteal	0/10	0/10	5/10	7/13	5/10	7/10	0/10	0/10
Cytokeratin7								
Total	10/19	6/13	0/19	0/13	10/19	6/13	0/19	0/13
Menstrual	3/5	2/2	0/5	0/2	3/5	2/2	0/5	0/2
Follicular	3/4	1/1	0/4	0/1	3/4	1/1	0/4	0/1
Luteal	4/10	3/10	0/10	0/10	4/10	3/10	0/10	0/10
Cytokeratin8								
Total	4/19	3/13	0/19	0/13	4/19	3/13	0/19	0/13
Menstrual	1/5	0/2	0/5	0/2	1/5	0/2	0/5	0/2
Follicular	1/4	0/1	0/4	0/1	1/4	0/1	0/4	0/1
Luteal	2/10	3/10	0/10	0/10	2/10	3/10	0/10	0/10
Cytokeratin18								
Total	4/19	3/13	0/19	0/13	4/19	3/13	0/19	0/13
Menstrual	1/5	0/2	0/5	0/2	1/5	0/2	0/5	0/2
Follicular	1/4	0/1	0/4	0/1	1/4	0/1	0/4	0/1
Luteal	2/10	3/10	0/10	0/10	2/10	3/10	0/10	0/10
CD68								
Total	0/19	0/13	0/19	0/13	0/19	0/13	17/19	11/13
Menstrual	0/5	0/2	0/5	0/2	0/5	0/2	5/5	2/2
Follicular	0/4	0/1	0/4	0/1	0/4	0/1	4/4	1/1
Luteal	0/10	0/10	0/10	0/10	0/10	0/10	8/10	8/10

Mesothelial cells stained positively with CK7, CK8/18, vimentin, calretinin and sometimes weakly with CD68. Cells positive for epithelial marker Ber-Ep4 were not observed, except in two patients investigated during menses who had a few positive cells. In 9/32 patients, the single cell population contained 10-50% cells with very weak, probably nonspecific staining for calretinin.

In all patients 50-98% of single cells were strongly positive for both vimentin and CD68. Most of the single cells present in PF were histiocytes or belonged to the monocyte/macrophage lineage.

The prevalence of mesothelial cells and macrophages staining positively for the antibodies tested (Table 9.) was comparable between menstrual and nonmenstrual phases of the cycle and between patients with and without endometriosis.

9. Discussion

To the best of our knowledge, our study provides the first evidence that assessment of nerve fiber density in eutopic secretory phase endometrium can be used as a diagnostic test for minimal to mild endometriosis [90] with high sensitivity (95%) and high specificity (100%). Only women with endometriosis who had not received any hormonal treatment of endometriosis within 3 months of endometrial biopsy were included, since it has been reported that hormonal medical treatment significantly decreases the multiple small nerve fibre density in the functional layer of endometrium [173].

Our study design is superior to that of previous studies [166] for several reasons. Firstly, only patients with the highest need for a non–invasive diagnostic test, i.e. patients with minimal–mild endometriosis were included, whereas in previous studies [166] a mixed population of women with minimal–severe endometriosis were studied. It is well accepted that women with moderate–severe endometriosis are less in need of a non–invasive diagnostic test since this degree of endometriosis can be diagnosed clinically and by imaging methods fairly accurately [44].

Secondly, all cases with endometriosis were confirmed by both laparoscopy and histology, whereas in the previous study histological confirmation was not available in all cases [166].

Thirdly, only secretory phase endometrium was selected since the highest density of nerve fibers is observed during this phase and since we wanted to rule out cycle phase dependent changes in endometrial nerve fiber density [164], whereas in previous studies endometrium from mixed unspecified phases of the cycle was studied [166].

Fourthly, taking into consideration our observation and previous reports showing that nerve fibers are not distributed homogeneously in the functional layer of endometrium [164-166], we examined the whole surface of all specimens to avoid inevitable bias resulting from randomly chosen fields, whereas previous investigators only assessed randomly chosen fields ([164-166].Using this meticulous approach, it is not surprising that the range of nerve fiber densities was considerably lower in our study (0–18.05 / mm2) than in previous studies (1.6–125 / mm2) [166], and that the mean nerve fibre density for PGP 9.5 positive nerve fibres in secretory phase endometrium was 6 times lower in our patients with minimal to mild endometriosis (2.62±2.19/ mm,2 mean±SD) compared to the patients with mixed stages of endometriosis investigated in a previous

study (13±6/ mm,2 mean±SD)[164]. This methodology may also explain why our diagnostic model could not confirm previously reported [166] results showing 100% sensitivity and 100% NPV for the diagnosis of minimal–severe endometriosis.

Fifthly, the multivariate statistical methods used in our study were superior to the univariate analyses described by previous investigators [166].

In our study, we selected a panel of neural biomarkers that are known [164-166], to identify and differentiate nerve fibres. We used PGP 9.5 which is a panneuronal marker [174, 175], whereas SP and CGRP are sensory nerve fiber markers which can be present in both Aδ and C fibres [95]. VIP is a specific marker for parasympathetic neurons and can be present in both sensory and cholinergic nerve fibres [176], while NPY is a specific marker for sympathetic neurons and can be present in both sensory and adrenergic nerve fibres [95]. NF is specific marker for myelinated nerve fibres [177]. Our data confirm that the endometrium of patients with minimal to mild endometriosis is predominantly innervated by multiple small diameter sensory (mostly C fibres), adrenergic and, in smaller amount, Aδ and cholinergic nerve fibres, as reported previously [164, 165, 178].

For the understanding of the pathomechanism of endometriosis, in order to develop a potential semi-invasive diagnostic tool, is essential to investigate the possible etiological factors of the disease such as the retrograde menstruation.

To the best of our knowledge, our study presents for the first time evidence that menstruation in women is associated with increased PF concentration of leucocytes and erythrocytes when compared to nonmenstrual phases of the cycle. Our data indicate that menstruation is associated with pelvic inflammation and are in agreement with our previous observation in nonhuman primates demonstrating higher levels of white blood cells in PF during menstruation [179].

Our data also show that menstruation is associated with a significant increase in erythrocyte count and consecutively in free hemoglobin content in PF, and provide a quantitative basis for the concept of retrograde menstruation. Hemoglobin accumulation in the peritoneal cavity may be a consequence of increased influx caused by red blood cell degradation, from retrograde menstrual reflux and/or a deficiency in the hemoglobin inactivating system [182] and may be involved in the pathogenesis of endometriosis [10].

In the context of the pathogenesis of endometriosis, retrograde menstruation has to be diagnosed not only as an increased presence of erythrocytes in PF but also as the presence of endometrial cells in PF. Nevertheless, many investigators [3, 4, 185, 186]

have interpreted the presence of red stained PF at the time of menstruation as sufficient evidence for retrograde menstruation of EM cells. However, it is well known that red-stained PF can be observed not only during menstruation but also during the first 5 days after ovulation [187] and during other phases of the cycle [3, 188] and that there is only a weak correlation between the presence of endometrial cells and the color of PF [189].

In our study, we could not confirm the hypothesis that the prevalence and/or quantity of EM cells in PF is increased in women with endometriosis (when compared to controls) and increased during menstruation (when compared to nonmenstrual phases of the cycle). Scientific evidence related to this hypothesis is controversial for several reasons.

Firstly, all studies addressing PF cytology are limited by small sample size, variable and sometimes unspecified phases of the cycle studied, subjective methods to define or detect EM cells or tissue in PF, and prior flushing of uterus and tubes via hysteroscopy before laparoscopic aspiration of PF cells.

Secondly, most investigators have used Papanicolaou or Giemsa staining to detect EM cells in PF and have considered clusters of cells in PF with positive staining to be EM cells [3, 189]. However, there is no proof that PF clusters of cells represent EM cells, since they may also include mesothelial cells or macrophages and hystiocytes. Indeed, recent evidence [188] suggests that EM cells rarely agglutinate in PF to become macroscopically visible tissue fragments containing endometrial glands and stroma during menstruation (in only 17% or 3/18 cases and similar in women with (2/9) and women without (1/9) endometriosis) or during all combined phases of the cycle (in only 16% or 16/99 cases and similar in women with (13/65 or 20%) and women without (3-34 or 9%) endometriosis).

Thirdly, immunocytological identification of EM cells in PF is not evident as reported in our study and in 3 papers published by 3 others groups of investigators [188, 190][9].

Comparison between these 4 studies is difficult since native PF cells were analyzed in our study and in only one other paper [9] whereas PF cell cultures were used in the 2 other reports [188, 190]. It is possible that immunocytological detection of EM cells is facilitated after cell culture when compared to analysis of EM cells in native PF. In our study the PrepStain method was used for the first time to study the cytology of native PF in women with endometriosis. This liquid-based preparation technique is adequate for the immunocytochemical study of numerous cell lines in effusion specimens [191] since cells are transferred directly into the liquid medium at the time of collection and therefore

the artefact of air-drying artifact, a culprit in many limited or inadequate conventional smears, is prevented. The results of our study are in line with a previously published immunocytological analysis of PF cells [9] obtained during the early follicular phase from 8 women with endometriosis and 8 with a normal pelvis. In that study [9] all PF samples except one contained cells stained positively with monoclonal antibodies against vimentin, cytokeratin 18 and 19, and 9 out of 16 PF samples contained cells that stained positively with monoclonal antibody BW495/36, an epithelial marker present in endometrium and absent in peritoneal epithelium. These data and our data are consistent with the interpretation that EM cells are present in PF from patients with patent fallopian tubes,and that their immunocytological staining profile is not different between women with and without endometriosis. However, among PF cells obtained during the late follicular phase prior to uterine flushing and cultured in vitro, other investigators could not identify EM cells that were immunocytologically positive for cytokeratins 5/7/8/14/19, cytokeratin 7/18, epithelial marker: BW495/36, HMFG 2, EM epithelial marker NEND-3 or ovarian carcinoma related markers OV-TL3, OV-TL10, OC-125 [190].

Fourthly, it is very difficult to identify with 100% certainty specific PF cell types by specific immunocytological markers, since endometrial epithelial, endometrial stromal, mesothelial cells and macrophages all stain positively for more than one marker (Table 3). For example, it was impossible to make a firm distinction in our study between endometrial stromal cells and mesothelial cells, since both cell types stained positively for vimentin and calretinin. It can be argued that our study was limited by the fact that endometrial stromal cell marker CD10 was not used, but the problem is that also other cell types stain positively for CD10, most importantly cervical stromal cells [192] and other cell types (normal renal tubular and glomerular cells, renal carcinoma, hepatocellular carcinoma lymphoid cells, mesonephric tumors, and acute lymphoblastic leukemia and lymphoma).

Taking together the evidence from our study and other studies, it is remarkable to conclude that even today there is no sound scientific evidence based on the analysis of PF fluid cells that retrograde menstruation is associated with the increased presence of endometrial cells in PF. Recent in vitro evidence demonstrating that attachment in vitro of endometrial cells on mesothelial cells occurs within 1 hour [193] supports the possible explanation that EM cells, refluxed via the Fallopian tube during menstruation into the

pelvic cavity, attach in a very short time to the peritoneal wall before they are detectable as free floating cells or clusters in PF. However, it is also possible that endometriosis does not (only) arise from retrograde menstruation, but may also be a consequence of mesothelial metaplasia induced by menstruation or other factors. In support of this metaplasia/induction theory, peritoneal endometriosis lesions appear to contain only stromal endometriosis in 45% of the cases, and stromal endometriosis can be found in about 7% of macroscopically normal peritoneum [194][31].

10. Conclusion

Our data have potentially high impact on the clinical diagnosis and management of endometriosis. Transcervical endometrial biopsy represents an acceptable semi–invasive technique, much less invasive than a laparoscopy, but still possibly associated with some degree of pelvic pain at the time of biopsy. An early semi–invasive diagnosis of minimal–mild endometriosis in women with or without pain who try to conceive should enable gynaecologists to select them for laparoscopic excision of endometriosis which improves pain and fertility and may prevent progression of endometriosis to a moderate to severe stage, since endometriosis is a progressive disease in at least 50% of the cases. We plan additional research to validate and confirm our results in a prospective controlled study.

The results of our second study demonstrate for the first time that menstruation in women is associated with an increased PF concentration of leucocytes, erythrocytes and hemoglobin when compared to nonmenstrual phases of the cycle, supporting the concept of retrograde menstruation. An increased PF concentration of PF endometrial cells was not observed during menstruation when compared to nonmenstrual phases of the cycle.

11. Summary

In our experiments we were focused on the development of a potential non/semi-invasive diagnostic test of endometriosis and on the assessment of retrograde menstruation, a crucial factor in understanding the pathogenesis of endometriosis. The aim of our first study was to test the hypothesis that multiple sensory small diameter nerve fibers are present in a higher density in endometrium from patients with endometriosis when compared to women with a normal pelvis, enabling the development of a semi-invasive diagnostic test for minimal-mild endometriosis. Secretory phase endometrium samples (n=40), obtained from women with laparoscopically/histologically confirmed minimal-mild endometriosis (n=20) and from women with a normal pelvis (n=20) were selected from the biobank at the Leuven University Fertility Centre. Immunohistochemistry was performed to localise neural markers for sensory C, Aδ, adrenergic and cholinergic nerve fibers in the functional layer of the endometrium. Sections were immunostained with antihuman protein gene product 9.5 (PGP9.5) anti-neurofilament protein (NF), anti-substance P (SP), anti-vasoactive intestinal peptide (VIP), anti-neuropeptide Y (NPY), and anti-calcitonine gene-related polypeptide (CGRP). Statistical analysis was done using Mann-Whitney test, Receiver Operator Characteristic (ROC) analysis, Stepwise Logistic Regression and Least Squares Support Vector Machines (LSSVMs). The density of small nerve fibres was about 14 times higher in endometrium from patients with minimal-mild endometriosis (1.96±2.73) when compared to women with a normal pelvis (0.14±0.46, $p < .0001$). The combined analysis of neural markers PGP9.5, VIP, SP could predict the presence of minimal-mild endometriosis with 95% sensitivity, 100% specificity, and 97.5 % accuracy. Since we believe that the detailed investigation of the pathogenesis of endometriosis will create a solid background for future research with clinical relevance, such as the development of a diagnostic method, we have investigated the biological changes during the mentsrual cycle in the peritoneal cavity.

In our second experiment we have tested the hypothesis that menstruation is associated with a higher concentration of endometrial cells in peritoneal fluid (PF) and with increased white and red blood cell concentration in PF when compared to nonmenstrual phases of the cycle. PF was obtained at laparoscopy from 107 women with endometriosis (n= 59) and controls with a normal pelvis (n= 48) during the luteal

(n=46), follicular (n=38) or menstrual (n=23) phase of the cycle. Endometriosis was classified according to the classification of the American Society for Reproductive Medicine (rAFS into minimal (n=25), mild (n=20), moderate (n=6) and severe(n=8) disease. Cell counts (leucocytes, erythrocytes, thrombocytes) were determined on a cell counter. In a subset of 32 patients (13 controls and 19 women with endometriosis), PF was fixed, processed and thinlayers were prepared and stained with Papanicolaou method and with immunocytochemistry using monoclonal antibodies against cytokeratin 7 (CK 7), CK 8/18, Ber-Ep4, vimentin, calretinin and CD68. Ber-Ep4 is a marker for cells with epithelial origin (in some cases for mesothelial cells as well). CD68 is specific for cells from monocyte/macrophage lineage; CK7 and CK8/18 are markers for both endometrial epithelial and mesothelial cells, whereas calretinin and vimentin are markers for both endometrial stromal and mesothelial cells. In comparison with the nonmenstrual phase of the cycle, analysis of PF during menstruation showed an increased concentration of leucocytes (3.3x109/L vs 0.8x109/L, P = 0.03), erythrocytes (0.3x1012/L vs 0.02x1012/L, P = 0.006), hematocrit (0.03 L/L vs 0.003 L/L, P = 0.01) and hemoglobin (0.8 g/dL vs 0.1 g/dL, P = 0.01). Mesothelial cells stained positively with CK7, CK8/18, vimentin, and calretinin. Cells positive for Ber-Ep4 were not observed, except in 2 patients with endometriosis we investigated during menstruation.

Összefoglalás

Az endometriosis gyakori, ösztrogén dependens, krónikus nőgyógyászati betegség, melynek lényege, az endometriumhoz hasonló szövet jelenléte a méh üregén kívül, legtöbbször a kismedencében. Etiológiája ismeretlen, azonban napjainkig számos elmélet született a betegség patomechanizmusának tisztázására. A legszélesebb körben elfogadott magyarázat a transzplantációs elmélet, mely szerint az endometriális sejtek retrográd menstruácio során jutnak a hasüregbe. Nincs azonban bizonyíték arra, hogy a peritoneális folyadékban található endometriális sejtek koncentrációja, vörös-és fehérvérsejt valamint hemoglobintartalma menstruáció során magasabb lenne, mint a menstruációs ciklus többi részében. Az endometriosis diagnózisa a kismedence laparoscopos áttekintésével lehetséges. Non-invazív diagnosztikai módszer hiányában, a tünetek kezdete és a definitív diagnózis között átlagosan 8 év telik el, a diagnosztikus késés, amennyiben a betegség kezelés nélkül marad, éveken át tartó szenvedéshez illetve meddőséghez vezethet. A perifériás vérből kimutatott biomarkereken alapuló non-invazív diagnosztikai eljárások hatékonysága, elégtelen szenzitivitásuk és specificitásuk miatt, korlátozott. Az endometriosisban szenvedők endometriumát túlnyomórészt kis átmérőjű szenzoros (főként C-tipusú), adrenerg és kisebb számban Aδ és cholinerg neuronok idegzik be. Transcervicálisan nyert endometriális szenzoros idegrostok immunhisztokémiai analízisét végeztük olyan neurális transmitterek és fehérjék kimutatásával mint a substance P (SP), a vasoactiv intestinalis polypeptid (VIP), a protein gene product 9.5 (PGP9.5), a neurofilamentum (NF), a neuropeptid Y (NPY) valamint a calcitonin gene–related protein (CGRP). A betegség pathomechanizmusának vizsgálata révén lehetőség nyílhat klinikai jelentőségű eljárások kifejlesztésére. Ezért második vizsgálat sorozatunkban a peritoneális folyadék ciklikus biológiai változásait tanulmányoztuk. A retrográd menstruáció vizsgálatára irányuló kísérletünkben laparoscopia során nyertünk peritonális folyadékmintát endometriózisban szenvedő páciens kismedencéjéből illetve egészséges kontrollokból, a menstruációs ciklus luteális, follikuláris és menstruációs fázisaiban. Citokeratin 7 (CK), CK 8/18, Ber-Ep4, vimentin, kalretinin és CD68-ellenes monoklonális antitesteket használtunk a periotnealis folyadék sejtjeinek immuncytokémiai identifikációja céljából. Eredményeink bizonyítják, hogy a női menstruáció során a peritoneális folyadék leukocita, eritrocita és hemoglobinkoncentrációja magasabb a menstruációs ciklus többi fázisával összevetve, alátámasztva a retrográd menstruáció elméletét. Azonban a peritoneális folyadék endometriális eredetű sejtkoncentrációjában nem emelkedett a ciklus menstruációs fázisában. A dolgozatban vizsgált vékony, velőhüvely nélküli, szenzoros idegrostok

előfordulási gyakorisága nagyobb az igazoltan endometriosisban szenvedő nők endometriumának funkcionális rétegében, mint az egészséges kontrollokéban. Eredményeink alapját képezik egy potenciális semi-invazív tesztnek mellyel lehetővé válhat az endometriosis korai klinikai diagnózisa.

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13. List of publications related to the thesis

Articles

Bokor A, Kyama CM, Vercruysse L, Fassbender A, Gevaert O, Vodolazkaia A, De Moor B, Fülöp V, D'Hooghe T. (2009) Density of small diameter sensory nerve fibres in endometrium: a semi-invasive diagnostic test for minimal to mild endometriosis. Hum Reprod, 24:827–834.

Bokor A, Debrock S, Drijkoningen M, Goossens W, Fulop V, D'Hooghe T. (2009) Quantity and quality of retrograde menstruation: a case control study. Reprod Biol Endocrinol, 7:123.

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Berkes E, Bokor A, Rigó J Jr. (2010) Current treatment of endometriosis with laparoscopic surgery. Orv Hetil, 151:1137-1144. (In Hungarian)

Bokor A, Kyama CM, Vercruysse L, Fassbender A, Gevaert O, Vodolazkaia A, Rigó J, De Moor B, Fülöp V, D'Hooghe T. The non-invasive diagnosis of endometriosis. Magy. Nőorv.Lapja In Press (In Hungarian)

Textbook chapters

Bokor A, Meuleman C, D'Hooghe T. The Role of the Fallopian Tube in the Development of Endometriosis and Associated Infertility In: Allahbadia G N, Saridogan E, Djahanbakhch O, (editors) The Fallopian Tube. Anshan Ltd., Kent, 2008: 449-457.

Bokor A, D'Hooghe T. Endometriosis and Miscarriage: Is there any Association?
In: Garcia-Velasco J A, Rizk B R M B (editors) Endometriosis:current therapy and future trends. Jaypee, New Delhi, 2009: 136-142.

Bokor A, Meuleman C, D'Hooghe T.The Clinical Aspects of Endometriosis
In: Carrell D, Peterson CM, (editors) Reproductive Endocrinology and Infertility: Integrating Modern Clinical and Laboratory Practice. Springer, New York, 2010: 191-207.

14. Acknowledgement

I would like to express my deep and sincere gratitude to my promoter, Professor Thomas D'Hooghe. His wide knowledge and his logical way of thinking have been of great value for me. His understanding, encouraging and personal guidance have provided a fundamental basis for the present thesis.

I wish to express my warm thanks to Professor János Rigó who introduced me to the field of clinical research in obstetrics and gynaecology and highlighted the importance of scientific thinking.

I am deeply grateful to my supervisor, Professor Vilmos Fülöp his detailed and constructive comments, and for his important support throughout this work.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project: Professor Sophie Debrock, Lisbeth Vercruysse, Cleophas Kyama, Amelie Fassbender, Alexandra Vodolazkaia and Steffi Mayer.