Endometriosis and Regional Lymph Node Involvement in Animal and Clinical Studies

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Abbreviations

CK           cytokeratin
DIE           deeply infiltrating endometriosis
ER           estrogen receptor
IELCs           isolated endometriotic-like cells
LN           lymph node
PR           progesterone receptor
PSLN           pelvic sentinel lymph node
rAFS           revised American Fertility Society
SLN           sentinel lymph node
Abstract

Animal Study - English

Objectives: To establish an experimental bowel endometriosis model in the rat and to investigate regional lymph node (LN) involvement.

Materials and Methods: Twenty Sprague-Dawley rats were subjected to autologous transplantation of endometrial tissue to the ileocecum by laparotomy. After two months, endometriotic lesions and mesenteric LNs were harvested by repeat laparotomy subjected to histological assessment regarding the histologically verified presence of endometriosis in the ileocecum and in mesenteric LNs.

Results: A total of 20 rats were subjected to autologous transplantation of endometrial tissue to the ileocecum. Ileocelecal endometriosis was successfully induced in 19/20 (95%) rats. In 18/20 animals the mesenteric LN was identified and resected. Regional LN involvement by endometriosis was identified in 0/18 mesenteric LNs.

Conclusions: Endometriosis can be successfully induced in a rat model by autologous transplantation of endometrial tissue. In this experimental rodent endometriosis model, regional mesenteric LN involvement by endometriosis was not identified.
Zielsetzungen: Zielsetzung dieser Arbeit war die Etablierung eines experimentellen Tiermodells für Darmendometriose in der Ratte mit spezieller Berücksichtigung der Ausbreitung von Endometriose in regionale Lymphknoten.


Clinical Study - English

Objective: To assess the prevalence of LN involvement in pelvic sentinel lymph nodes (PSLN) in women with ovarian and/or peritoneal endometriosis.

Materials and Methods: Women with clinically suspected endometriosis underwent diagnostic laparoscopy. After the confirmation of ovarian and/or peritoneal endometriosis by intraoperative frozen section analysis, PSLN sampling was conducted. The resected LNs were sent for histological evaluation for the presence of endometriotic lesions and immunohistochemical analysis of estrogen receptor (ER), progesterone receptor (PR), cytokeratin (CK) and CD10 expression. The intra- and postoperative complications were recorded.

Results: 26 women with suspected endometriosis were enrolled. Endometriosis was confirmed in 23 women and a PSLN was identified in 19 women. A total of 37 (right side: 20; left side: 17) PSLNs were removed. The prevalence of LN involvement in PSLN was 11% (2/19). Both lesions were ER-, PR-, CK-, and CD10-positive. Isolated endometriotic-like cells (IELCs) staining positive for ER and PR were identified in 16/19 (84%) and 14/19 (74%) of patients, respectively. All IELCs lacked CK staining, whereas CD10 staining was present in 16/19 (84%) cases, indicating a stromal origin. Intra- and/or postoperative complications were observed in 1/26 woman.

Conclusions: Endometriotic lesions in PSLN are present in 11% of women with ovarian and/or peritoneal endometriosis. IELCs in PSLN are common in endometriosis. Further studies to evaluate the prognostic and predictive value of LN involvement in endometriosis are warranted.
Clinical Study - German

Zielsetzungen: Ziel dieser Studie war es die Prävalenz von Endometrioseläsionen in pelvinen Sentinellymphknoten (PSLN) bei Frauen mit ovarieller und/oder peritonealer Endometriose zu erheben.


Schlussfolgerungen: Endometrioseläsionen in PSLN finden sich bei 11% der Frauen mit ovarieller und/oder peritonealer Endometriose. Disseminierte ELCs
in PSLN sind häufig und wahrscheinlich stromalen Ursprungs. Weitere Studien zum prognostischen und prädiktiven Wert der lymphknoteninfiltration bei Frauen mit Endometriose sind gerechtfertigt.
1 Introduction

Endometriosis is a common, benign, estrogen-dependent, chronic disorder in women of reproductive age, which is defined by the presence of endometrial glands and stroma outside the uterine cavity and musculature.\textsuperscript{1, 2}

1.1 Pelvic and extrapelvic endometriosis

Endometriosis has been reported in nearly every organ system of the human body and mostly found in the pelvis – the ovaries, the cul-de-sac, the uterosacral ligaments, the posterior uterus and the posterior broad ligaments.\textsuperscript{3} The prevalence of pelvic endometriosis approaches 6–10\% in the general female population.\textsuperscript{2} Extrapelvic endometriosis is a rarity and the true prevalence is unknown, because of the absence of epidemiologically well-defined studies.\textsuperscript{4} Extrapelvic endometriosis can be found in many gynecologic (vulva, vagina, cervix) and nongynecologic sites. The latter include bowel, lungs and pleural cavity, brain, skin (episiotomy or surgical scars after cesarean section), lymph nodes (LN) and nerves.\textsuperscript{5} Endometriosis involving the heart and the spleen has not been reported.\textsuperscript{4} Regarding the pathogenesis of endometriosis, implantation and metaplasia theories are the most accepted explanations for pelvic endometriosis while extrapelvic endometriosis may result from vascular or lymphatic dissemination of endometrial cells.\textsuperscript{6} Extrapelvic endometriosis is diagnosed in an older population than pelvic endometriosis. The median age at the first diagnosis of extrapelvic endometriosis is 34–40 years, whereas in pelvic endometriosis it is 25–30 years of age.\textsuperscript{4, 7}
1.2 Subtypes of endometriosis

There are three subtypes of endometriosis, i.e. gross and microscopic inspection consisting of endometriomas (e.g. ovarian cysts), superficial endometriotic implants (e.g. peritoneal endometriosis), and deeply infiltrating endometriosis (DIE, e.g. rectovaginal nodules). It is still controversial whether these subtypes are different in pathogenesis or share the same origin. According to Nissole et al., ovarian, peritoneal and rectovaginal endometriotic lesions should be considered three separate entities because of the different pathogeneses. In detail, the occurrence of peritoneal endometriosis is due to the inflow and implantation of endometrial glands and stroma while DIE results from metaplasia of remaining tissues of the Müllerian duct. The formation of ovarian endometriotic lesions may involve both implantation theory and metaplastic histogenesis.

1.3 Red, black and white lesions

Laparoscopy is considered the standard technique for visual inspection of the pelvis and establishment of a definitive diagnosis. Characteristic findings under laparoscopy include typical (“powder–burn” or “gunshot”) lesions on the surfaces of the peritoneum. According to the revised American Fertility Society (rAFS) classification system issued in 1996, the morphology of peritoneal and ovarian implants are categorized as red (red, red–pink, and clear lesions), white (white, yellow–brown, and peritoneal defects), and black (black and blue lesions). Red lesions are characterized by numerous proliferative glands and epithelium, which are the most active and considered the first stage of peritoneal endometriosis. Black lesions are advanced endometriosis caused by the menstrual shedding and intraluminal debris while the subsequent fibrosis forms the inactive white lesions.
1.4 Clinical characteristics and classification

Endometriosis is an established cause of infertility and chronic pelvic pain: 21-47% of women presenting with infertility and 71-87% of those with chronic pelvic pain.\textsuperscript{16, 17} The associated symptoms include dysmenorrhea (in 79% cases), pelvic pain (69%), dyspareunia (45%), bowel symptoms (36%), infertility (26%), and dysuria (10%).\textsuperscript{18}

The current classification system of endometriosis by the American Society of Reproductive Medicine is the rAFS system issued in 1996 (Figure 1.1).\textsuperscript{15} The system is based on the appearance, size and depth of peritoneal and ovarian implants; the presence, extent and type of adnexal adhesions and the degree of cul–de–sac obliteration.\textsuperscript{19} The rAFS system is to date the best available tool to describe objectively the extent of endometriosis and relate it to spontaneous evolution and to therapeutic outcomes, such as relief of pain and improvement of fertility.\textsuperscript{15} However, it has also been criticized due to the poor correlation with endometriosis-related pain or infertility.\textsuperscript{20, 21}
Fig. 1.1 Revised American Fertility Society classification for endometriosis: scoring system and patient information. (American Society for Reproductive Medicine. Fertil Steril 1997).
1.5 Endometriosis and ovarian carcinoma

Endometriosis is considered a benign disorder because of the absence of criteria typical for malignancy, i.e. nuclear atypia, abnormal mitotic activity or increased nuclear to cytoplasmatic ratio.\textsuperscript{22, 23} However, endometriosis shows some distinct characteristics of malignant diseases, such as abnormal morphology, deregulated cell growth, cellular invasion, and neoangiogenesis.\textsuperscript{24} Endometriosis can be both locally and distantly metastatic and attach to other tissues, invade, and damage them.\textsuperscript{25} Wu et al. found that endometriotic tissue is monoclonal in origin in 100\% of the investigated samples.\textsuperscript{26} In addition, endometriotic tissue can be a precursor of some ovarian cancers.\textsuperscript{27, 28} Studies showed that endometriosis is associated with an approximately 3-fold statistically significant risk of endometrioid and clear cell ovarian cancer.\textsuperscript{29-31} In a recent pooled analysis which included 13 case-control studies using data from more than 23 000 women, the authors found that the association of a history of endometriosis with increased risk of ovarian cancer is only apparent for endometrioid, clear-cell and low-grade serous subtypes, while no association is noted between endometriosis and risk of mucinous or high-grade serous invasive ovarian cancer.\textsuperscript{32}

1.6 Endometriosis and LN involvement

Another characteristic feature shared by malignant diseases and endometriosis is regional LN involvement, which refers to the presence of endometriotic glands with typical epithelium and stroma in LN. The notion of regional LN involvement in women with endometriosis dates back to 1897, when Ries et al. reported ‘Drüseneinschlüsse’ in pelvic LNs of women with cervical carcinoma.\textsuperscript{33, 34} These lesions were described as ‘glandular inclusions’\textsuperscript{33} and ‘endometriosis-like formations’\textsuperscript{35}, similar to endometriosis and hence the
lesions were considered endometriosis. Regional LN involvement by endometriosis was subsequently also noted in endometriosis patients.

Table 1.1 and 1.2 list the case reports as well as clinical studies of LN involvement in women with endometriosis (PubMed search with keywords: endometriosis, endometrioma, lymph node; search date: 11-November-2011). At first, LN involvement was indentified incidentally in patients with bowel endometriosis. Insabato and Pettinato reported three cases of bowel endometriosis with LN involvement. In a series of 35 women with rectosigmoid endometriosis, Abrao et al. described LN involvement in 26% of pericolic LNs. Lorente Poyatos et al. reported mesenteric LN involvement in one woman with rectosigmoid endometriosis. Pelvic LN involvement was also described by other groups in women with deeply infiltrating bowel and/or rectovaginal endometriosis and the prevalence reached up to 33%. A recent study comprising 26 patients with rectosigmoid endometriosis found LN involvement in 11/26 cases (42%). Regarding rectovaginal endometriosis, Thomakos et al. described pelvic LN endometriosis in a case of rectovaginal endometriosis and Mechsner et al. reported endometriotic lesions in 3/12 (25%) pelvic sentinel LNs in women with deeply infiltrating rectovaginal endometriosis. These data demonstrate that regional LN involvement is a common event in DIE and that endometriosis metastasizes into regional LNs in a significant number of cases.
### Case report

<table>
<thead>
<tr>
<th>Case report</th>
<th>No. of cases</th>
<th>Type of endometriosis</th>
<th>Type of LNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Javert 1949</td>
<td>5</td>
<td>Bowel</td>
<td>Obturator, iliac and ureteral</td>
</tr>
<tr>
<td>Lange 1955</td>
<td>1</td>
<td>Bowel</td>
<td>Obturator and iliac</td>
</tr>
<tr>
<td>Kniepkamp 1959</td>
<td>1</td>
<td>Adenomyosis</td>
<td>Iliac</td>
</tr>
<tr>
<td>Regidor-Brandau 1994</td>
<td>1</td>
<td>None*</td>
<td>Obturator and iliac</td>
</tr>
<tr>
<td>Insabato et al. 1996</td>
<td>3</td>
<td>Rectosigmoid</td>
<td>Pericolic</td>
</tr>
<tr>
<td>Lorente Poyatos 2003</td>
<td>1</td>
<td>Rectosigmoid</td>
<td>Mesenteric</td>
</tr>
<tr>
<td>Thomakos et al. 2006</td>
<td>1</td>
<td>Rectovaginal</td>
<td>Obturator</td>
</tr>
<tr>
<td>Barrier et al. 2007</td>
<td>1</td>
<td>Ileocaecal</td>
<td>Pericolic</td>
</tr>
<tr>
<td>Rafailidis et al. 2010</td>
<td>1</td>
<td>Rectosigmoid</td>
<td>Pericolic</td>
</tr>
<tr>
<td>Beavis et al. 2011</td>
<td>1</td>
<td>Endometrioma</td>
<td>Para-aortic</td>
</tr>
<tr>
<td>Namkung et al. 2011</td>
<td>1</td>
<td>Rectal</td>
<td>Pelvic</td>
</tr>
</tbody>
</table>

Table 1.1 Case reports of LN involvement in women with endometriosis.

- **LN**: lymph node.
- *The patients had no anamnesis or clinical symptoms or surgical evidence of endometriosis.

### Clinical study

<table>
<thead>
<tr>
<th>Clinical study</th>
<th>No. of pts/no. pts with LN</th>
<th>Type of endometriosis</th>
<th>Type of LNs</th>
<th>LN involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abrao et al. 2006</td>
<td>35/19</td>
<td>Bowel</td>
<td>Pericolic</td>
<td>5/19 (26.3%)</td>
</tr>
<tr>
<td>Noel et al. 2008</td>
<td>26/26</td>
<td>Rectosigmoid</td>
<td>Pericolic</td>
<td>11/26 (42.3%)</td>
</tr>
<tr>
<td>Mechsner et al. 2008</td>
<td>14/12</td>
<td>Rectovaginal</td>
<td>PSLN</td>
<td>3/12 (25.0%)</td>
</tr>
<tr>
<td>Mechsner et al. 2010</td>
<td>24/24</td>
<td>DIE</td>
<td>Mixed</td>
<td>8/24 (33.3%)</td>
</tr>
<tr>
<td>Tempfer et al.** 2011</td>
<td>26/19</td>
<td>Ovarian/peritoneal</td>
<td>PSLN</td>
<td>2/19 (10.5%)</td>
</tr>
</tbody>
</table>

Table 1.2 Clinical studies of LN involvement in women with endometriosis.

- **pts**: patients; **LN**: lymph node; **PSLN**: pelvic sentinel lymph node; **DIE**: deeply infiltrating endometriosis.
- **The present study.**
1.7 Research questions

The clinical problem of LN involvement in endometriosis, however, has only recently attracted attention. The significance of this phenomenon for the pathogenesis and prognosis of endometriosis remains unclear. Most reported cases are associated with one subtype of endometriosis, namely DIE.\textsuperscript{36, 37, 40, 46} However, regarding the other subtypes, for example, the most common form of endometriosis - ovarian and/or peritoneal endometriosis - the prevalence of LN involvement has not yet been investigated. To date, there are only a few clinical studies to elucidate this phenomenon (table 1.2) and many questions remain. That may be mainly because of the difficulty to obtain LN samples from women with endometriosis. For benign diseases like endometriosis, there is at present no indication for regional LN dissection. Therefore, the clinical part of my thesis will focus on this issue. In addition, we have established an animal model of endometriosis with LN involvement, which may contribute to the understanding of this phenomenon.
2 Animal study

2.1 Objectives

The aim of the animal study was to establish a bowel endometriosis model in the rat using autologous transplantation of uterine tissue to the ileocecum and to investigate regional LN involvement in successfully induced endometriosis.

2.2 Materials and methods

Approval for this study was obtained by the Institutional Review Board and the Animal Care and Use Committee of Tongji University School of Medicine, Shanghai, China. Twenty female Sprague-Dawley rats weighing 250g to 300g were used in this experiment. The study was performed at the Animal Experiment Center, Tongji Hospital, Shanghai, China. All animals were raised in a restricted-access room with a stable temperature (22°C) and a 12-hour light-dark cycle. Standard laboratory chow and drinking water were provided ad libitum.

Bowel endometriosis was surgically induced by midline laparotomy under phenobarbital anesthesia. After ligation of the uterus, the distal 1cm of the right uterine horn was resected and immersed immediately in warm (37°C) sterile physiologic saline. The endometrium was exposed by opening the horn with a pair of sterile scissors, and 2 pieces of uterine tissue measuring 5mm × 5mm were obtained, containing endometrium, stroma, and myometrium. Each of the four corners of the tissue specimens was sutured to the surface of ileocecum next to the mesenteric vessels with 4-0 sterile silk with the two pieces situated next to each other. Postoperative infection was prevented by muscular injection of cidomycin (HFQ Animal Pharmaceutical, Shanghai, China) for 5 days.
Two months after the first surgery, animals were sacrificed by phenobarbital anesthesia and a second laparotomy was conducted. The mesenteric LN, located at the radix of the mesentery next to the ileocaecal junction, was defined as the target LN for this experiment. The size of the mesenteric LN was calculated as $4\pi ab^2/3$ ($\pi=3.14$, $a=$length of the LN, $b=$width of the LN) and recorded pre- and post-operatively. Visible endometriotic lesions and the mesenteric LNs were harvested and histologically examined using 5 to 10 serial sections of formalin-fixed, paraffin-embedded sections of 2 mm thickness. After hematoxylin and eosin (H&E) staining, endometriosis lesions and LNs were evaluated under a light microscope. Lesions were counted as endometriosis, if they showed growth of at least one implant in vesicle form with a major diameter of $\geq 2$mm.\textsuperscript{48}

2.3 Results

Twenty rats underwent laparotomy and the autologous transplantation procedure was successfully performed in all 20 animals. After two months, all 20 animals underwent re-laparotomy and endometriosis lesions were visually identified in 19/20 animals. Among them, 17 animals had two lesions and 2 animals had one lesion. One animal had no endometriosis lesion on visual inspection. A total of 36 endometriosis lesions were resected. In 19/20 (95.0\%) animals, bowel endometriosis was identified by histological examination. All 36 resected lesions had endometriosis on histological examination. All endometriotic lesions showed typical structures with the three layers of glandular epithelial cells, stromal cells, and myometrium (Figure 2.1).

A total of 18 mesenteric LNs were harvested in 18/20 animals. In one animal, no mesenteric LN was found and in one animal with no visible endometriotic lesion, the mesenteric LN was not resected. The average size of the LNs was 72.1 (minimum 28.3, maximum 140.8) mm\textsuperscript{3}. No abnormal appearance of the
LNs was noted. The average distance between the endometriotic lesion and the mesenteric LN was 1.4 (minimum 1.1, maximum 1.8) cm. On histologic examination, no endometriotic lesion was found in any of the resected LNs.

Figure 2.1 Endometriotic bowel lesion in the ileocecum two months after autologous transplantation of endometrial tissue. Hematoxylin & eosin staining, magnification x100 (Yao GONG et al. Wien Klin Wochenschr 2011).

2.4 Discussion

In the present study, we found that bowel endometriosis can be successfully induced in a rodent model by autologous transplantation of uterine tissue. In this experimental rodent endometriosis model, regional mesenteric LN involvement by endometriosis was not identified.

In the experiment, we chose the mesenteric LN as the target LN, because most of the reported cases of LN involvement were found in mesenteric LNs in women with bowel endometriosis. Second, the mesenteric LN is bigger than pelvic LNs and harbors more lymphatic ducts, thus potentially increasing the chance of a positive finding. Our data demonstrate that this rat model of
endometriosis is successful, but it may not be suitable for studying the phenomenon of regional lymphatic spread in endometriosis. If accepted as a valid model, our data argue against lymphatic spread as a regular phenomenon in bowel endometriosis. However, the validity of our study may be limited due to the small sample size, the time between induction of endometriosis and harvesting of the LN, and the choice of the mesenteric LN as target. A higher number of animals, a longer latency period between induction of endometriosis and harvesting of lymphatic material, and a more thorough LN dissection may yield different results. In addition, we cannot rule out that single cells have migrated into the lymphatic system.

Also, the observed absence of regional LN involvement in this model may be due to the fact that endometrium, but not endometriotic tissue, has been transplanted. Endometriotic cells differ from eutopic endometrium\(^1,49\) and this may affect the ability to spread into the regional lymphatic system. Therefore, nude mice with endometriotic human xenografts may be a more successful model for lymphatic spread of endometriosis. These limitations have to be acknowledged when interpreting the results of our work, awaiting confirmation by other investigators.

In summary, we present an experimental bowel endometriosis model, which fails to demonstrate regional mesenteric LN involvement by endometriosis.
3 Clinical study

3.1 Objectives

In a prospective clinical study, we aimed to assess the prevalence of LN involvement in pelvic sentinel lymph nodes (PSLN) in women with ovarian and/or peritoneal endometriosis.

3.2 Materials and methods

3.2.1 Patients

Women with clinical suspicion of endometriosis between 18 and 50 years were included at the department of Obstetrics and Gynecology, Medical University of Vienna. Suspected diagnosis of endometriosis was based on the presence of secondary dysmenorrhea and/or dyspareunia and/or chronic pelvic pain and/or cycle-independent chronic pelvic pain. All women included have signed informed consent. Exclusion criteria were cervicitis and/or vaginitis, known disease of the lymphatic system, and known allergy to blue dye. This study was approved by the Ethics Committee of the Medical University of Vienna (protocol number 473/2009).

3.2.2 Surgical treatment and PSLN procedure

All women underwent diagnostic laparoscopy following a standard protocol established at our institution. If a diagnosis of endometriosis of the ovarian and/or peritoneum was established by visual inspection, a frozen section analysis of excised endometriotic tissue was performed. The manifestation of visual inspection included ovarian cysts, i.e. chocolate cysts, red, white or gun-powder lesions. After the histological confirmation of the diagnosis, we
conducted the PLSN procedure as follows. First, the pelvic region was visually inspected to note any presence of abnormal LN, such as enlarged LNs or LN with gun-powder lesions. The results were recorded. Then 1.5 mL of blue dye (Guerbet, Villepinte, France) was injected into the four quadrants of the cervix uteri under the epithelium. After a minimum of 10 minutes, the peritoneum was incised parallel to the external iliac vessels. The area of iliac vessels and the obturator fossa were screened for the presence of a PSLN marked with blue dye. The blue-stained PSLN was then removed. If no SLN was identified, no lymphadenectomy was performed. No specific drainage of the retroperitoneum was applied. The surgery was finished after the complete haemostasis. All surgical procedures were performed by the study team members experienced in laparoscopic PSLN dissection. Intra- and postoperative complications were recorded. All patients were contacted 3 months after surgery and telephone interviewed regarding the occurrence of late complications of surgery. Figure 3.1 shows the flow diagram of the surgical procedure in our study.

26 Patients with suspected endometriosis for diagnostic laparoscopy

Resection of the lesions and frozen section analysis

23 Patients with histological confirmation of the diagnosis

Blue dye injection into the four quadrants of the cervix

19 Patients with pelvic sentinel lymph node

Lymphadenectomy

Figure 3.1 The flow diagram of the patients receiving lymphadenectomy for pelvic sentinel lymph node.
3.2.3 Immunohistochemistry

Immunohistochemistry studies were conducted as follows:

For estrogen receptor (ER) and progesterone receptor (PR) analysis we deparaffinized the slides using 4 μm paraffin sections in xylene for 5 min at room temperature (RT). This was done twice. Then, sections were rehydrated in decreasing concentrations of ethanol for 5 min at RT in 100%-100%-95%-80%-70% ethanol solutions. After rinsing the sections with tris-buffered saline (TBS), the slides were immersed in a 30% H$_2$O$_2$ solution (H$_2$O$_2$:methanol=3:7) for 15 min at RT for catalase removal. A heat induced epitope retrieval (HIER) procedure was then performed by microwave cooking of the slides using a target retrieval solution (Dako, Germany) at 700 W for 20 min. After rinsing with TBS, goat serum (GS) was applied to minimize non-specific staining. The so processed sections were incubated for 1 h at RT with an anti-ER and anti-PR rabbit monoclonal primary antibody (CONFIRM antibody; Ventana Medical Systems Inc., USA). After rinsing with TBS, an anti-rabbit immunoglobulin (IgG) in a 1:400 dilution was applied as a secondary antibody for 40 min. Finally, streptavidin (Roche, Germany) in a 1:400 dilution was applied for 25 min. Diaminobenzidine (DAB, Dako; Denmark) was used to visualize the specific immunoreactivity. The slides were then counterstained with hematoxylin and dehydrated. Lastly, the coverslips were mounted.

For cytokeratin (CK) analysis, we used deparaffinization, rehydration and the HIER procedures as described above. Then, the sections were incubated with proteinase K (Dako, Denmark). After rinsing with TBS, GS was applied. The sections were then incubated for 1 h at RT with a monoclonal mouse anti-cytokeratin antibody (clones AE1/AE3; DAKO, Denmark). After rinsing with TBS, an anti-mouse immunoglobulin (IgG) in a 1:400 dilution was applied for 40 min and the procedure was completed as described above.

For CD10 analysis, we applied the deparaffinization, rehydration, and HIER
procedures by microwave cooking of the probes in citrate buffer (0.1 M citric acid and 0.1 M sodiumcitrate, pH 6.0 at 700 W, 20 min). After rinsing with TBS, GS was applied. Sections were incubated for 1 h at RT with an anti-CD10 monoclonal mouse antibody (NCL-CD10-270; Novocastra Laboratories Ltd.) to recognize CD10.

As controls for nonspecific binding, we used both negative and positive controls in a simultaneous staining round. For negative controls, we used nonspecific IgG (dilution 1:50; DAKO, Denmark) omitted the primary antibody. For positive controls, we used samples of endometrium of non pregnant women.

3.2.4 Statistical analysis

Variables of interest are described by median and range and mean and standard deviation in case of skewed and normal distributions, respectively.

3.3 Results

3.3.1 Surgical observations and PSLN detection

A total of 26 patients (mean age: 33±6.1 years) with suspected endometriosis signed the inform consent and underwent diagnostic laparoscopy. Twenty three of them had confirmed endometriosis by histological examination of the frozen section. Among them, 8 had ovarian endometriosis, 7 had peritoneal endometriosis and 8 had both ovarian and peritoneal endometriosis. RAfS stages II was observed in 3 cases, rAFS stages III in10 cases and rAFS stages IV in 10 cases. Before the injection of the blue dye, we did not find any enlarged LNs in pelvic peritoneum.

After the injection of the blue dye, the blue stained LN, namely SLN was observed in 19 of the 23 patients (82.6%). In eighteen patients, the LNs
situated adjacent to the external iliac vein and in 1 patient in the obturator fossa. Histological examination of the PSLN samples revealed a total of 37 LNs (right side: 20, left side: 17). No intraoperative complications were noted in lymphadenectomy of the PSLN. One patient had blue stained face on the day of the surgery and it resolved in 48 hours. No other complications were observed in this patient. Three months after the surgery, no complications were reported by gynecological examination or telephone interview.

3.3.2 Prevalence of LN involvement in endometriosis

In all the 37 PSLNs from 19 patients, we found endometriotic lesions in 2 LNs from 2 patients. The prevalence of LN involvement in ovarian and/or peritoneal endometriosis was 10.5% in the present study. The size of the lesions in LN was 1.0 mm and 2.5 mm respectively. The lesions were endometrioid glands with epithelium and surrounding stroma and located in the peripheral sinus of the PSLN with no local reaction (Figure 3.2). The capsule of both LNs was intact. Regarding the stage of the endometriosis with LN involvement, one patient had rAFS stages III, the other rAFS stages IV. There was no significant difference of stage of endometriosis between patients with LN involvement and patients without LN involvement.

3.3.3 Immunohistochemical analysis of the PSLN

Immunohistochemical analysis of the PSLN showed both epithelial and stromal cells in both lesions expressed ER, PR, CK (Figure 3.3) and CD10. Interestingly, we also found isolated endometriotic-like cells (IELCs) stained by ER, PR and CD10 in other LNs (Figure 3.4 A and B). The three markers, ER, PR and CD10 were positive in 16/19 (84.2%), 14/19 (73.7%) and 16/19 (84.2%) of patients, respectively. These IELCs were located in peripheral sinus. Apart from the two LNs with endometriotic lesions, no CK positive cells were observed.
Figure 3.2 Endometriotic lesion in a pelvic sentinel lymph node in a woman with endometriosis, demonstrating endometrioid glands with epithelium (white arrow) and stroma (black arrow). Estrogen receptor staining is positive in both epithelial and stromal cells. Magnification×40 (Clemens B. TEMPFER et al. Fertil Steril 2011).

Figure 3.3 Endometriotic lesion in a pelvic sentinel lymph node in a woman with endometriosis, demonstrating CK-positive endometriotic epithelial cells. Magnification×100 (Clemens B. TEMPFER et al. Fertil Steril 2011).
Figure 3.4 Isolated endometriotic-like cells in peripheral sinus of a pelvic sentinel lymph node in women with endometriosis. A: positive estrogen receptor staining. B: positive progesterone receptor staining. Magnification×100 (Clemens B. TEMPFER et al. Fertil Steril 2011).
3.4 Discussion

In the present study, we identified LN involvement in 10.5% of the women with ovarian and/or peritoneal endometriosis. It was the first attempt to assess the prevalence of LN involvement in such subtype of endometriosis. We also found IELCs in 84.2% cases, which were positive for ER, PR and CD10, but negative for CK staining.

3.4.1 Limitations of the study

The results of this prospective study have to be interpreted with caution. First, the sample size is small. That is mainly due to the ethical issue of the surgical procedure. Endometriosis is considered a benign disorder and there is at present no indication for PSLN resection for the surgical treatment of endometriosis. The small sample size may result in the overestimation of the prevalence of LN involvement in endometriosis. Second, the patients recruited were from a tertiary hospital, which could not represent the general population of women with endometriosis. The results of the study may thus be influenced by selection bias. Third, we did not use a control group to detect the presence of LN involvement in patients without endometriosis, so we cannot rule out the possibility that SLN in non-endometriosis women also harbor IELCs or endometriotic lesions.

3.4.2 The source of IELCs and endometriotic lesions in LN

There are possibly four explanations regarding the source of IELCs and endometriotic lesions in LN.

First, IELCs and endometriotic lesions in LN may result from direct invasion by the adjacent endometriosis. In Abrao’s study, the LNs were isolated from the pericolic adipose tissue. The bowel endometriotic lesion may penetrate the LN membrane and infiltrate the LNs. The authors did not describe the exact
location of the lesions in LNs. However, in other studies,\textsuperscript{23, 46, 47} we can rule out this possibility because the surrounding capsule in all affected LNs was intact and endometriotic lesions were found in the peripheral sinus only.

Second, the IELCs may be immune cells. In normal LNs, there are some single CD10 and CK positive cells in the medulla.\textsuperscript{50, 51} However, after searching in the literature, we did not find immune cells with positive ER and PR staining in LNs.

A third explanation could be an occasional occurrence of IELCs in lymphatic tissue. This hypothesis could be verified by assessing LNs from women without endometriosis. In the prospective study by Mechsner et al.,\textsuperscript{23} they chose 9 LNs from patients without endometriosis as control tissue and failed to document the presence of IELCs or endometriotic lesions. It seems highly unlikely but cannot be excluded that endometriosis in LN would arise de novo. In a case report, endometriosis was found in two LNs in the retroperitoneum of a patient with cervical cancer, who had no clinical or surgical evidence of endometriosis.\textsuperscript{34}

The most likely source of the IELCs and endometriotic lesions in LN is from the drainage of the lymphatic vessels. There is evidence that fragments of endometriotic tissue can migrate to the lymphatic system.\textsuperscript{40, 41} Using immunohistochemical assessment of lymphovascular invasion by a D2-40 antibody, Noel et al. observed lymphovascular invasion consisting of cytogenic stroma or glands in 4/11 (36.3\%) patients with LN involvement in rectosigmoid endometriosis.\textsuperscript{40} In an animal study,\textsuperscript{52} Hey-Cunningham et al. induced endometriosis in baboons and found the number of endometrial stromal cells was significantly increased in LNs from endometriosis group compared to control group. In the present study, we considered the IELCs were stromal cells because of the CD10 positive staining.\textsuperscript{47} Mechsner et al. hypothesized that IELCs may have the potential to develop into a fully differentiated endometriotic lesion composed of epithelial glands and surrounding stromal
cells, which indicated the IELCs could be the precondition of the endometriosis lesions in LN.\textsuperscript{23}

Also, it may be speculated that stem cells enter the lymphatic and form endometriotic lesions in regional LN. Numerous studies showed that the basal layer of the endometrium contains endometrial stem/progenitor cells.\textsuperscript{53, 54} Extrauterine stem/progenitor cells, derived from the bone marrow or an alternative source, are likely to travel to distant ectopic sites via the lymphatic system.\textsuperscript{55} However, until now, no direct evidence for the role of endometrial stem/progenitor cells in the pathogenesis of human endometriosis has been reported.\textsuperscript{56}

Whether the cells are derived from the eutopic endometrium, the ectopic endometrium or both remain unclear. It is possible that cells from the endometriotic lesions outside the LN can spread through the lymphatic system. There is to date no robust evidence to support this hypothesis. It can explain that the presence of LN involvement is in proportion with the size of the frank lesions in DIE, which was observed in several studies.\textsuperscript{23, 37, 46} In addition, as shown in table 1.2, the prevalence of LN involvement in our study is lower than that reported by others. The distinction may be caused by the different subtypes of endometriosis investigated. Our study focused on ovarian/peritoneal endometriosis while the others studied mainly DIE, which is characterized by tissue infiltration and may increases the likelihood of cells entering the lymphatic vessels and subsequently colonizing regional LN.

**3.4.3 Characteristics of IELCs and endometriotic lesions in LN**

In the present study, we stained the LN with markers of ER, PR, CK and CD10, which was also done by Mechsner et.al.\textsuperscript{23} In both studies, we observed two manifestations in LN related to endometriosis: one is endometriotic lesions with positive staining of all four markers. The other is IELCs with positive ER and PR staining. Mechsner et al. found ER and PR positive staining cells in 58%
and 89% of PSLN, respectively, a finding consistent with our results. These IELCs were CD10 negative in the Mechsner’s study but positive in ours. However, the two set of cells were most likely the same. The discrepancy may be due to the different antibodies used in immunohistochemistry.

Until now, we do not know if the IELCs and endometriotic lesions in LN will be influenced by the hormones and experience cyclical changes like the frank lesions out of LN. There were no symptoms caused by the affected LN. Most of the LNs by visual inspection showed no signs of enlargement or gunpowder lesions. The lesions in LN have normal epithelium, unlike typical lesions out of LN where the presence of epithelium is rare. Ki67 expression was seen in 5-10% of epithelial and stromal cells in endometriotic lesions in LN but IELCs showed no proliferative activity by Ki67. Furthermore, the tissues around the lesions in LN showed no signs of inflammation. This raised the question whether we should take the LN involvement in endometriosis as a disease entity. To date, no studies have been conducted to compare IELCs and endometriotic lesions in LN with those in the primary endometriotic lesions. These questions may direct future studies.

3.4.4 The SLN procedure in endometriosis

In the present study, we have applied the PSLN procedure to women with established ovarian/peritoneal endometriosis and PSLNs were identified in 83% of cases. Mechsner et al. observed SLNs in the iliac region in 85% of cases of women with rectovaginal endometriosis. LN dissection in women with endometriosis is an experimental procedure. Since the prognostic, predictive, and therapeutic value of LN dissection in women with endometriosis has not been proven yet, SLN sampling was used in order to minimize the morbidity associated with LN sampling. Compared to systematic LN dissection, SLN sampling is safer and less invasive, which has been verified in women with breast cancer and other gynecological malignancies such as cervical and
vulvar cancer.\textsuperscript{57-60} No complications along with this procedure were observed in Mechsner’s and our study except for a transient blue colouring of the facial skin in one case. Therefore, SLN sampling is a safe and efficient procedure which may be used in future trials investigating endometriosis and LN involvement.

3.4.5 The clinical significance of LN involvement in endometriosis

In bowel endometriosis, the presence of endometriosis in LN was directly proportional to the extent of the bowel endometriotic lesion.\textsuperscript{37} This correlation was also found in rectovaginal endometriosis: the larger the size of the primary lesions, the greater the number of IELCs and endometriotic foci in the LN.\textsuperscript{23} In ovarian/peritoneal endometriosis investigated in our study, such correlation was not found. To date, no study found a correlation between LN involvement and the severity of clinical symptoms in women with endometriosis. In addition, there are no clinical studies testing whether or not regional LN involvement is of prognostic and/or predictive value regarding disease recurrence and hormone therapy efficiency. Does the LN involvement predispose to a worse prognosis and a higher recurrence rate of endometriosis? Will the resection of regional LNs decrease the recurrence rate of endometriosis and hence become part of the surgical treatment of this disease?\textsuperscript{61} Will the benefit of SLN procedure outweigh the risk of complications? All these issues can be elucidated only based on randomized intervention trials in sufficiently large patient populations.
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