Lymphovascular space invasion in endometrial carcinoma

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LYMPHOVASCULAR SPACE INVASION IN ENDOMETRIAL CARCINOMA

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CHAPTER 1

GENERAL INTRODUCTION

General introduction

Although treatment has improved in recent years, a diagnosis of cancer is still too often fatal, an outcome mainly due to its capacity for invasive growth and metastatic spread throughout the body. Relapses and metastases lead to fatality when i) a cancer becomes insensitive to treatment, ii) it can no longer be surgically removed, or iii) the cancer has caused so much physical harm that the patient can no longer withstand further treatment.

Although not every cancer will acquire the ability to metastasize, the main route of tumor cell dissemination is via blood and lymphatic vessels, a phenomenon known as 'lymphovascular space invasion' (LVSI), which may occur long before lymph node and distant metastases become apparent. Vascular invasion is therefore an early indicator of metastatic potential. The role of LVSI in endometrial carcinoma will be discussed in detail in the following chapters. However, we will first briefly discuss general concepts of cancer development, invasion and metastasis, as well as tumor classification and staging.

A general concept in cancer development

Cancer cells often show uninhibited growth, a characteristic attributable to wide-ranging changes in cell homeostasis, perhaps the most important of which involve DNA and the failure of DNA repair mechanisms. The development and progression of cancer has been conceptualized in the "Hallmarks of Cancer" model [1, 2]. This model integrates diverse biological processes in order to categorize the events leading to malignant behavior, as well as providing a framework for understanding similarities and differences between the different types of cancer. This model was recently updated and now encompasses 14 hallmarks and enabling characteristics (figure 1). The hallmarks most relevant for invasive growth and metastasis are briefly discussed below.

Alterations in cancer cell metabolism lead to a high demand for the oxygen and nutrients transported by blood vessels. By ***inducing vascular growth (angiogenesis) or by improving access to vasculature,*** cancer cells ensure an adequate supply of oxygen and nutrients. Angiogenesis, an early event in tumorigenesis, is promoted by high levels of VEGF and is further enhanced by bone marrow-derived cells such as macrophages, neutrophils, mast cells and myeloid progenitors [1].

Activating invasion and metastasis requires cancer cells to invade surrounding tissue, to enter, travel through and extravasate from blood and lymph vessels, and to finally colonize distant tissues (figure 2) [3]. Invasion is promoted by the epithelial-mesenchymal transition (EMT) via the action of transcription factors involved in processes such as migration, some of which are also active during embryogenesis. Cancer cells signal to surrounding mesenchymal stem cells, which in turn signal cancer cells to promote invasion. In another form of crosstalk, cancer cells promote invasion inducing inflammatory cells to release enzymes that break down the extracellular matrix [1].

An anti-cancer immune response aimed at eradicating cancer cells can simultaneously and paradoxically enhance tumor growth via ***tumor-promoting inflammation***. Inflammatory responses can subsequently trigger angiogenesis, the release of growth factors and stimulate the modification of the extracellular matrix [1].

Senescent cells are characterized by an inability to undergo cell division, as well as morphologic and metabolic changes. This state is referred to as the 'senescence-associated secretory phenotype' and involves the release of bioactive proteins that act on biological processes considered cancer hallmarks. Senescent cancer cells can also reverse the senescent state, a capability that is thought to contribute to therapy resistance, progression and metastasis [2, 4].

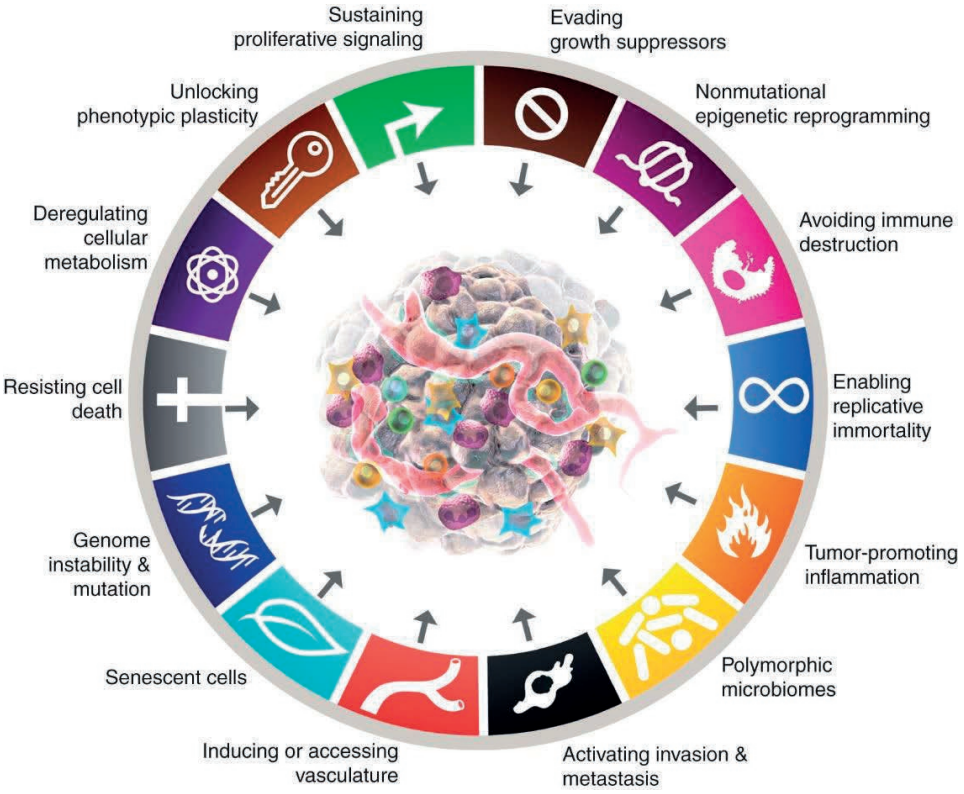

Figure 1. The 'Hallmarks of Cancer' model provides a framework for cancer conceptualization. The hallmarks and enabling characteristics play a role in cancer development, progression and maintenance (adopted from Hanahan, 2022 [2]).

The biology of invasion and metastasis

The transformation of a single tumor into metastatic disease can be modeled as a sequential process in which a metastasis is the successful outgrowth of disseminated cancer cells into a new tumor in distant tissue (figure 2 and table 1) [3].

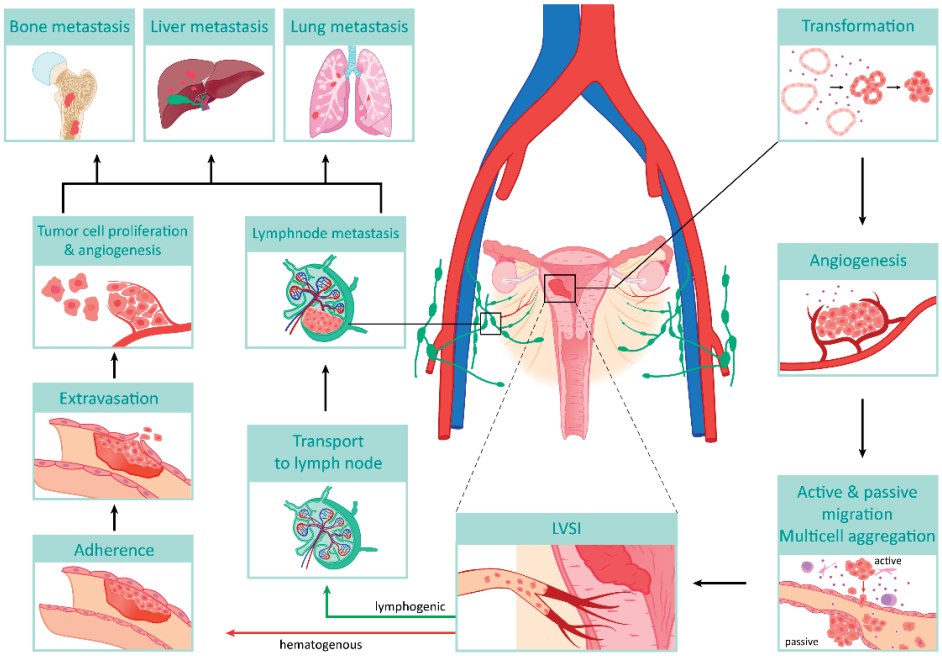

Figure 2. The sequential process resulting in metastatic disease. The progression model begins with cancer initialization by induction of cancer cells, followed by angiogenesis, invasion, migration, intravasation, circulation, extravasation, finally leading to colonization (adopted from Talmadge, 2010 [3]) Included are the two types of migration (below right): the active route is chemokine- and mitosis-driven, while the passive route is the result of competition for space and nutrients in a growing tumor (adopted from Bockhorn, 2007 [5]).

Table 1. Steps in the sequential process of metastases (adopted from Talmadge [3]).

1	After the initial transforming event, the growth of neoplastic cells is progressive and frequently slow.
2	For a tumor mass to exceed a 1- to 2-mm diameter vascularization is required. The synthesis and secretion of angiogenesis-promoting factors plays a critical role in establishing a vascular network within the surrounding host tissue.
3	Local invasion of the host stroma by tumor cells can occur via multiple routes, including, but not limited to, thin-walled venules and lymphatic channels, both of which offer little resistance to tumor cell invasion.
4	Detachment and embolization of tumor cell aggregates, which may increase in size via interaction with hematopoietic cells within the circulation.
5	Circulation of these emboli within both hematologic and lymphatic vessels.
6	Survival of tumor cells that trafficked through the circulation and arrested in a capillary bed.
7	Extravasation of the tumor embolus by mechanisms similar to those involved in initial tissue invasion.
8	Proliferation of tumor cells within the organ parenchyma, resulting in a metastatic focus.
9	Establish vascularization and defenses against host immune responses.
10	Reinitiate these processes for the development of metastases from metastases.

For the majority of solid tumors, the primary path of metastasis is vascular spread through lymphatic or blood vessels. The lymphatic system differs from blood vasculature in terms of function, anatomy and metastatic pattern. The smallest, proximal lymphatic vessels drain

extracellular fluids and can be differentiated from capillary vessels by endothelial cell shape and the type of tight junctions, which allow one-way fluid flow and the ingress of immune cells. Multiple minor vessels converge into larger collecting lymphatic vessels that transport immune cells, waste and antigens (lymph) to the draining lymph node. Eventually the lymph enters the blood stream [6]. Tumor cells in lymphatic vessels arrive in the tumor-draining lymph node where they need to adapt in order to survive in an organ rich with immune cells. These adaptations include metabolic alterations to suit a fatty acid-rich nutrient supply. Colonized lymph nodes are a potential source of subsequent hematogenous metastases, which involve invasion of afferent lymph node blood vessels by tumor cells [7].Thus, cancer cells can enter the blood stream either via lymph nodes or directly by invading the capillary vessels surrounding a tumor, from where circulating tumor cells give rise to distant organ metastases.

An alternative metastatic route is transcoelomic spread in which tumor cells disseminate through a body cavity such as the peritoneal cavity. A combination of peritoneal and vascular spreading patterns has been noted in endometrial, pancreatic, gallbladder and colorectal carcinoma. In ovarian cancer, peritoneal spread is the primary metastatic pathway. Peritoneal metastases grow from spontaneously detached single cells derived from the primary tumor and form multicellular aggregates (spheroids) which attach to the mesothelial surface and finally infiltrate the submesothelial extracellular matrix (figure 3) [8].

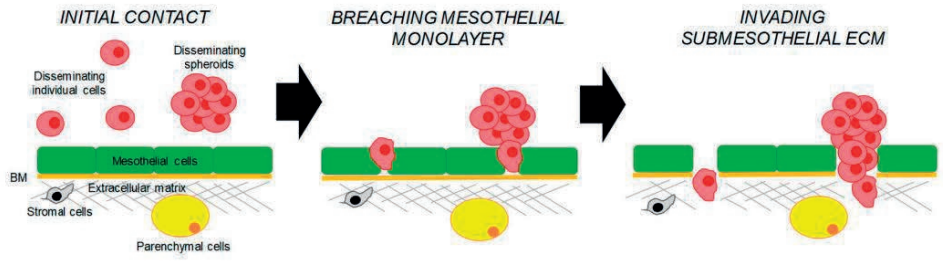

Figure 3. Modellation of transcoelomic spread.

In both lymphatic and hematogenous metastases, cancer cells enter the circulation early on in the course of disease, but are usually rapidly eliminated [9, 10]. Colonization of distant tissue often requires the radical adaptation of cancer cells to a new micro-environment and is therefore frequently unsuccessful [1]. This means that the presence of cancer cells or emboli in the circulation does not reliably indicate that metastasis has occurred. Metastatic processes involve much more than just the shedding of intact cancer cells into the circulation. Exosomes loaded with mRNA, microRNAs and ligands often precede cancer cells to induce 'terra-forming' processes at a host site that promote colonization. Thus, even before metastasis is initiated, the host site may have been primed and a pre-metastatic niche created [11].

The dissemination of cancer cells through vessel walls is called intravasation and has been most thoroughly studied in breast cancer models. Tumor-associated macrophages enhance invasion through vessels by secretion of epidermal growth factor (EGF) and colony-stimulating factor 1 (CSF1). Intravasation is also promoted when endothelial junctions are down-regulated by the local expression of VEGF [12]. The endothelial cell barrier is passed when single cells actively disrupt the endothelium through a mitosis-dependent mechanism in which cancer cells align along a vessel [13]. Another mechanism (figure 2, adopted from Bockhorn [5]) is the passive shedding of tumor cells into the circulation. As the majority of these cells appear to be dead or apoptotic, it is hypothesized that this passive shedding may be the result of competition for space and nutrients in a growing tumor [5].

Circulating tumor cells may be detected at an early phase of tumorigenesis, well before the primary tumor has been located [14, 15]. Some cancers even present as metastases without a known primary site [16]. Results from expression analyses of tumor samples aimed at predicting the risk of metastasis support the hypothesis that metastatic potential is already present early in tumorigenesis rather than being a solely evolutionary process [17]. Expression analysis of breast carcinomas has identified a number of signatures associated with prognosis, recurrence or metastases to specific sites. However, these studies did not address underlying mechanisms [18-21]. Nevertheless, several genes associated with intravasation have been identified and, together with a mechanistic hypothesis, have contributed to our understanding of the molecular events underlying intravasation. Sometimes these mechanisms appear to conflict, perhaps characteristic of the complexity of multifactorial processes, as illustrated by the role of E-cadherin in breast cancer. The two major types of breast cancer, 'no special type' (formerly ductal) and 'lobular', differ in terms of cell-surface E-cadherin expression (in the former but not the latter case). E-cadherin is both a cell-cell adhesion molecule and a tumor suppressor protein. While the two types of breast cancer show equal frequencies of lymph node involvement, the number of nodal metastases is higher in lobular breast cancer but LVSI is lower [22, 23]. One explanation is that circulating lobular cancer cells may be unable to form cell clusters due to the lack of E-cadherin. In most carcinomas, individual circulating tumor cells form clusters to increase chances of survival, and may also interact with platelets and neutrophils to evade immune cells [11]. Loss of E-cadherin promotes invasion, but also reduces proliferation, survival, number of circulating cancer cells, seeding and outgrowth in distant organs [24]. At the cellular level, loss of E-cadherin impacts gene expression involved in apoptosis regulation, with subsequent effects on invasion, dissemination and colony formation. When E-cadherin loss activates TNFα, TGFβ and p53, apoptosis is induced via reactive oxygen species and oxidative stress [24]. While loss of E-cadherin does not appear to promote tumor progression, and lobular carcinoma has a similar prognosis to ductal carcinoma when matched for clinicopathological characteristics, the prognosis of lobular carcinoma is worse when patients have additional high-risk features [25].

In the sequential cascade of metastasis, both migration and invasion precede intravasation. Genes involved in EMT and motility such as *SNAIL*, *SLUG* and *ZEB1* are upregulated, stimulating invasion and migration by repressing cell-cell adhesion molecules like E-cadherin [26]. Migrating cancer cells need to find vessels for intravasation, a process facilitated by VEGFC and CCR7, a lymphatic-homing chemokine receptor. Both are expressed by cancer cells and secreted VEGFC stimulates expression of CCL21 (CCR7 ligand) by endothelial cells. Expression of CCL21 by endothelial cells subsequently attracts CCR7-expressing cancer cells [27]. Compared to capillary vessels, intravasation of lymphatic vessels is easier due to the fenestrated junctions of endothelial cells, occasional pericytes and the lack of a basement membrane in lymphatic vessels. However, it is unclear to what extent vascular anatomy is decisive in predominantly lymphatic invasion compared to capillary intravasation, especially with respect to paracrine interactions between cancer cells and capillary endothelial cells [28]. Besides interactions with endothelial cells, cancer cells interact with stromal and immune cells. These include cancer-associated fibroblasts, which are modified stromal cells that stimulate growth, migration and invasion due to the proximity of and interaction with cancer cells. These processes are facilitated by Wnt/β-catenin signaling, expression of podoplanin and N-cadherin, and by the loss of interleukin 6 [28]. The lymphocytes that surround tumors mainly consist of T-lymphocytes and natural killer (NK) cells. NK cells, as well as a subset of T-cells (CD8+ cytotoxic and regulatory T-cells), are tumor suppressing, whereas CD4+ and FOXP3+ T-cells are tumor promoting via EGFR signaling [28]. The same pathway of tumor promotion is evident in the case of tumor-associated macrophages, in addition to enhancement of angiogenesis via IL-1, MMP2 and VEGF [29].

Signs of invasion and the metastatic process can be seen by pathologists during routine microscopic assessment. These include LVSI, stromal modifications (desmoplasia), tumor budding (dissociation of single cells or small clusters from the invasive front), inflammatory infiltrates in or around tumor cells, and an increased density of small vessels. All of these characteristics have been associated with (lymph node) metastases [30-33].

Tumor classification, tumor staging and the significance of LVSI

As described above, tumorigenesis and the invasive sequence leading to metastases are complex and multifactorial processes that have been elucidated to only a certain level. This represents one end of the spectrum. At the other end stands the patient in need of a diagnosis and answers to crucial prognostic questions such as “What are my chances of survival? and “What kind of treatment can you offer?”

The pathologist’s first task is to arrive at a diagnosis based on the World Health Organization (WHO) classification of tumors. In this system, tumors are classified according to the organ the tumor arises in. The diagnosis is made by the pathologist and is primarily based on tumor morphology. In recent decades tumor classification has shifted from morphology-based to molecular-based classifications. This shift gained momentum once it became clear that

molecular classification was much better at predicting biological behavior than morphology-based classification.

The next step is to determine the stage of the disease, which together with tumor classification is crucial for determining treatment options and prognosis. In the case of solid tumors, the TNM (and FIGO for gynecological tumors) staging system is usually applied. This system is based on three items: size and extent of the primary tumor (T), presence and extent of lymph node metastases (N), and presence of distant metastases (M).

Although LVSI is not incorporated in most of the staging systems, reporting the presence of LVSI provides valuable prognostic information in many types of cancer, especially in early-stage disease [34-39]. The association of LVSI with an early-stage tumor that has been completely removed without detectable lymph node metastases may explain why recurrence or metastases develop later in the course of disease. However, LVSI is not a perfect indicator, as detection can be complicated by artifacts or may simply be absent despite later metastases. The dynamics involved in LVSI are still poorly understood and important questions remain unanswered, such as 'What is the window of opportunity for LVSI detection?' or 'How long do tumor cells remain in vessels surrounding the tumor before they leave the organ?' (and can therefore no longer be detected as LVSI).

Endometrial carcinoma

Endometrial carcinoma (EC) arises in the epithelial lining of the uterus. EC typically affects post-menopausal women and is usually diagnosed at an early disease stage due to timely symptoms of post-menopausal bleeding. The standard treatment of early-stage EC is hysterectomy with bilateral salpingo-oophorectomy, with or without lymphadenectomy [40]. The need for and type of adjuvant treatment is dependent on the presence of risk factors such as substantial LVSI, high tumor grade, deep myometrial invasion and lymph node metastases [40].

Histopathological assessment after surgery is necessary for final staging and tumor classification. For gynecological tumors a staging system similar to TNM, designed by the Federation of Gynecology and Obstetrics (FIGO) [41], integrates factors including extent of tumor, lymph node involvement and spread or metastases to other organs (table 2). The WHO classification of EC identifies endometrioid and non-endometrioid carcinomas, including serous, clear cell, un/-dedifferentiated, mesonephric-like, mixed carcinomas, and carcinosarcoma [42]. Endometrioid carcinomas are graded based on the percentage of solid growth and degree of nuclear atypia (grade 1, 2 or 3), whereas non-endometrioid carcinomas are not graded but regarded as grade 3 by definition. The most recent trend is to move towards to a two-tiered grading system, consisting solely of low grade (grades 1 and 2 combined) and high grade (grade 3).

Table 2. International Federation of Gynecology and Obstetrics 2009 staging system for endometrial cancer

Stage			*Description*
Stage I			Tumor confined to the corpus uteri
	IA		<50% myometrial invasion
	IB		≥50% myometrial invasion
Stage II			Tumor invades the cervical stroma but does not extend beyond the uterus
Stage III			Localand/or regional spread of the tumor
	IIIA		Tumor invades the serosa of the uterus and/or adnexa
	IIIB		Vaginal and/or parametrial involvement
	IIIC		Metastases to pelvic and/or para-aortic lymph nodes
		IIIC1	Metastases to pelvic lymph nodes
		IIIC2	Metastases to para-aortic lymph nodes with or without pelvic lymph node involvement
Stage IV			Tumor invades bladder and/or bowel mucosa, and/or distant metastases
	IVA		Tumor invades the bladder and/or bowel mucosa
	IVB		Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes

In addition to morphological classification, a molecular classification has been introduced that has a strong prognostic value and superior reproducibility, reducing the value of the FIGO grading system. In the traditional classification system, high-grade tumors are associated with especially low reproducibility [43-45]. The molecular classification was inspired by The Cancer Genome Atlas (TCGA) initiative, which identified four prognostically significant subgroups of EC based on tumor molecular burden and somatic copy number alterations. The ultra-mutated (>100 mutations per megabase (mut/mb)) subgroup was characterized by mutations in the exonuclease domain of *POLE* and was associated with an excellent prognosis. The hypermutated (10-100 mut/mb) subgroup was mismatch repair-deficient (MMRd) and associated with an intermediate prognosis. By contrast, the subgroup characterized by high levels of copy-number alterations had frequent *TP53* mutations (p53abn) and was associated with a poor prognosis. The final subgroup, characterized by low levels of copy-number alterations, microsatellite stability and a non-specific molecular profile (NSMP), generally showed an intermediate prognosis.[46]. Subsequent work found that pragmatic use of *POLE* mutation analysis and simple surrogate markers (immunohistochemistry) can identify similar groups with prognostic relevance [47].

LVSI in endometrial cancer

Lymphovascular invasion (LVSI) is defined as the presence of (clusters of) tumor cells within an endothelial-lined vascular space beyond the invasive front. During routine light microscopic histological examination, LVSI can be seen in the myometrium surrounding the tumor. Although LVSI in EC is relatively uncommon, it is associated with high histological grade, deep myometrial invasion and advanced stages of disease [48-52]. LVSI is a significant prognostic factor and, like grade 3 histology and deep myometrial invasion, is an independent risk factor for recurrent disease [53-56], including lymph node recurrence [56-63], although this does not apply for vaginal relapse [64, 65]. LVSI is also a predictor of pelvic and para-aortic lymph node metastases [51, 66-69], distant metastases [49, 55, 58-60, 63, 70, 71] and reduced recurrence free survival [53, 68, 70, 72, 73], as well as reduced overall survival [52, 61, 68, 74, 75].

With growing evidence pointing to LVSI as an important prognostic factor, LVSI has been included in the most recent update of European clinical guidelines for the management of EC and, when present, has implications for treatment recommendations in stage I EC [40]. This shift was initiated by the pooled analyses of the PORTEC-1 and PORTEC-2 trials. The first PORTEC (Post-Operative RadioTherapy in Endometrial Carcinoma) trial showed that adjuvant external beam radiotherapy (EBRT) reduces locoregional recurrence in stage I EC [76]. The subsequent PORTEC-2 trial proved that vaginal brachytherapy is as effective as EBRT in reducing vaginal vault relapses, but with fewer toxic side effects [77]. LVSI was not a relevant prognostic factor in either study, but pooled analyses of the two studies showed that substantial LVSI is a strong prognostic factor, and that EBRT reduces pelvic recurrence risk when substantial LVSI is present [78].

Thesis outline

The traditional strength of pathology is that it can capture key cancer characteristics through simple microscopic assessment, something that can be performed across the world without a need for expensive ancillary tests. While recent molecular advances in EC are impressive and have clearly advanced the field, key H&E characteristics nevertheless remain at the center of patient management. Especially in the case of patients with EC confined to the uterus (stage I disease), accurate risk of recurrence assessment is critical to directing adequate adjuvant treatment decisions. By assessing morphological tumor (and micro-environmental) features known to predict behavior, pathologists are key players when it comes to predicting chances of recurrence. One feature in particular is relevant: the presence of LVSI. Debatably, LVSI is more important than any other variable in predicting disease outcome. It is therefore critical that assessment of LVSI is reproducible and is interpreted in a way that translates to clinical relevance. The chapters in this thesis underline the continuing relevance of LVSI as an important prognostic factor in EC, and hopefully contribute to the applicability, reproducibility and acceptance of this simple light microscopic assessment tool.

Chapter 2 reviews clinicopathological aspects of LVSI and provides tools for the assessment of LVSI in EC. In chapter 3 the prognostic value of several (semi)-quantitative assessment systems for LVSI in stage I endometrioid EC was analyzed based on the combined PORTEC-1 and -2 randomized clinical trials. In light of the variability in histological subtype diagnosis of high-grade EC, the value of a pathology review by experienced gynecologic pathologists was correlated to prognosis and is described in chapter 4. The value of substantial LVSI as a prognostic factor in high-risk EC is described in Chapter 5. The reproducibility of LVSI assessment (recognition and extent) was studied with the cooperation of an expert panel of European gynecologic pathologists and is presented in chapter 6. Chapter 7 describes the development of a threshold for clinically-relevant LVSI. Chapter 8 reports a pilot study of gene expression analysis among mismatch repair-deficient ECs. This study aimed to find a gene expression profile associated with LVSI. Additionally, we provide an overview of LVSI-associated

gene expression profiles in the literature. Chapter 9 summarizes the main results of this thesis and includes a general discussion with the focus on clinical practice and future research.

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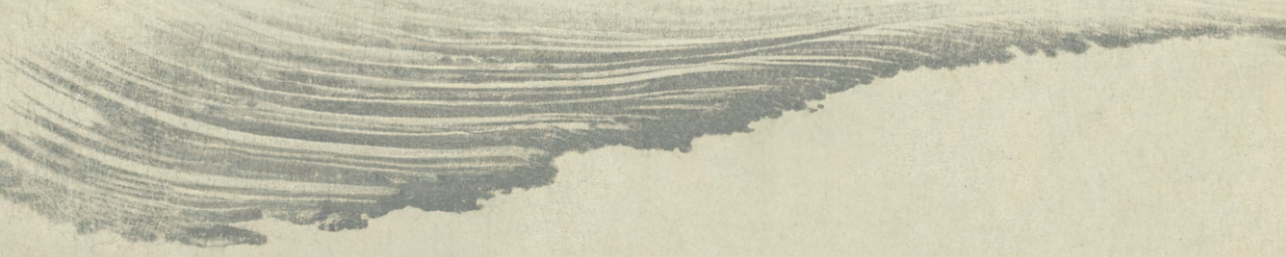

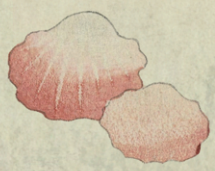

CHAPTER 2

EXPERT OPINION: PRACTICAL GUIDANCE FOR ASSESSMENT OF LYMPHOVASCULAR SPACE INVASION IN ENDOMETRIAL CANCER

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Submitted

Abstract

Lymphovascular space invasion (LVSI) is an important prognostic histological parameter in endometrial cancer (EC) and has gained increasing attention in the literature in recent years due to the expanding body of evidence on its independent prognostic value, especially when the presence of LVSI is quantified. The key strength of LVSI as a biomarker is that it can be detected on by routine microscopic examination, without additional ancillary tests, and so it can be assessed even in under-resourced regions. The weakness however is the lack of uniformly applied rules for assessment of LVSI, resulting in significant diagnostic interobserver variation. This is confounded by artefacts and other morphological features which may mimic LVSI (commonly referred to as pseudo-LVSI). Despite these factors, LVSI has been shown in multiple studies to be strongly associated with lymph node (LN) metastases and is an independent risk factor for LN recurrences and distant metastases. Consequently, the presence of substantial/ extensive LVSI has is an important factor in influencing adjuvant treatment recommendations. Herein, we review the current literature on LVSI in EC, discussing its role as a prognostic marker, the reproducibility of LVSI assessment and provide guidance on what represents true-LVSI as opposed to mimics and pseudo-LVSI. We provide illustrations and also discuss the current widely used three-tiered system of LVSI classification. This work is intended to provide guidance to practicing pathologists and unify the approach towards LVSI assessment in EC.

Introduction

Lymphovascular space invasion (LVSI) is an important histological prognostic parameter in many tumors, including endometrial cancer (EC), and can be visualized on routinely stained H&E- slides. LVSI is defined as the presence of tumor cells in a vascular space lined by endothelial cells [1]. Although this definition seems straightforward, LVSI assessment can be challenging due to a variety of factors, including scenarios that can mimic true LVSI. The incidence of LVSI in stage I ECs reported in the literature varies widely (3.2% to 35%) [2,3] suggesting there are local differences in LVSI assessment, for example overdiagnosis due to misinterpretation of "pseudoinvasion", or underrecognition through failure to recognize the sometimes focal nature or subtle features.

The presence of LVSI confirms that the tumor has gained critical capacities for tumor progression[4]. Consistent with this, the presence of LVSI is strongly associated with lymph node (LN) metastases in EC, and several studies have shown LVSI to be an important risk factor for LN recurrence and distant metastasis in early-stage disease. Since LVSI is an independent risk-factor it has earned a prominent position in the current risk stratification systems used for the clinical management of early-stage EC [5].

In recent years, a considerable body of evidence has shown that LVSI becomes an even more powerful prognostic factor in EC when it is quantified. This has resulted in the implementation of a semi-quantitative methodology, in which the presence of LVSI is categorized as either focal or substantial/ extensive (hereafter referred to as substantial). This approach strengthens the position of LVSI as an independent prognostic factor that should be acted upon in the adjuvant setting of early-stage disease [5]. Following on these observations, it has recently been proposed to integrate the presence of substantial LVSI into the new FIGO staging system for EC, resulting in upstaging of some cases of early-stage disease. Consequently, accurate and uniform identification of LVSI and quantification of this has become critical in clinical management of EC patients.

A relatively recent survey of members of The International Society of Gynecological Pathologists (ISGyP) showed that LVSI is reported on hysterectomy specimens by 90% of respondents, but is quantified by only 50%. We have noted significant practice differences in the interpretation of LVSI and pseudo-LVSI in our routine in-house and consultation practices and to provide guidance and attempt to unify the the assessment of LVSI in EC we herein provide guidance and outline a set of practical rules that we apply when assessing LVSI. We also provide illustrations of what represents true LVSI and of its histological mimics.

Definition of LVSI

Several terms (and abbreviations) to denote vascular invasion are used in the literature, depending on the organ and field of research. The term "intravasation" is common in basic science, whereas a multitude of terms is used in clinically orientated literature. The preferred

terms and abbreviations vary between organ systems and the lack of Medical Subject Headings (MeSH terms) does not contribute to harmonization.

LVSI is the most widely used and preferred terminology in EC and is defined as the presence of tumor cells in an endothelial-lined vascular space; in EC, it is recommended that this should be within the myometrium beyond the advancing front of the tumor since most recent publications looking at the prognostic impact of LVSI in EC have used this definition (figure 1). Several clues are helpful to recognize a lymphatic vessel. A lymphatic vessel is lined by a single layer of endothelial cells and the presence of venous and/or arterial vessels in close proximity is supportive. As is the case with most tumors, there is little evidence to support the distinction between lymphatic and blood vessel invasion and this distinction is not routinely performed. Herein we also discuss pseudo-LVSI, which is a collective term for artefacts mimickng LVSI (see *Clues to pseudo-LVSI, below*).

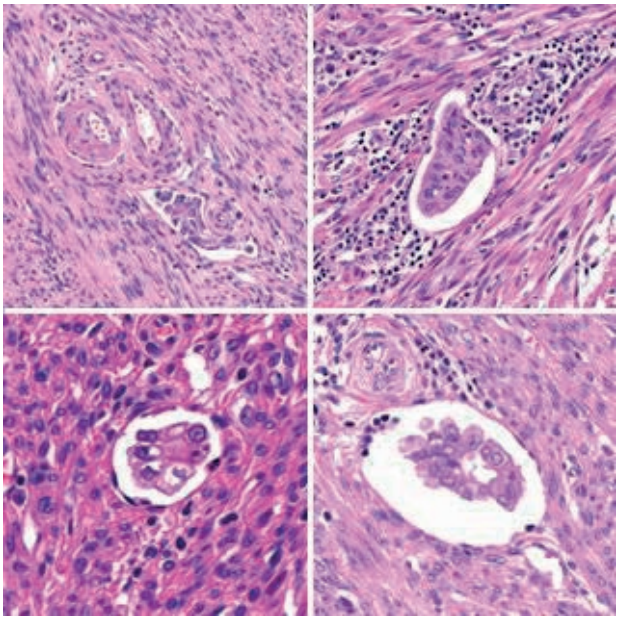

Figure 1. Examples of LVSI in EC. Cohesive clusters of tumor cells in a vascular space lined by endothelial cells. The vessels involved are situated in the myometrium beyond the invasive tumor border. Often perivascular lymphoid aggregates are found, or arterial and/or venous branches are close to the lymphatic vessel.

Features supportive for true LVSI

LVSI in EC is most commonly seen involving small vascular channels close to the invasive border of the tumor, just beyond the interface of tumor stroma and unaffected myometrium. LVSI in large blood vessels is usually not found in isolation, but in tumors with substantial LVSI involving small vascular channels, and the identification of tumor in large vessels only should result in consideration of pseudo-LVSI (see below). Tumors with broad pushing borders within the myoinvasive component are less likely to be positive for LVSI [6, 7], and with multiple patterns of myometrial invasion, LVSI is most likely to be seen where the advancing front is associated with a desmoplastic stromal response. Some clustering of LVSI is sometimes

observed, with a “spray-like” pattern (figure 2A). Clustering of LVSI makes it easier to recognize the intravascular component at low power. However, clustering may also be a result of a tortuous vessel which is cut at multiple different planes (figure 2B). Clues to this are that the vessels are of the same caliber lying in line with each other and this needs to be considered when defining the extent of LVSI (see section on *Extent of LVSI*). Because LVSI can be limited to a certain part of the tumor, extensive sampling of the tumor and surrounding myometrium may be necessary to avoid underestimating LVSI status. In accordance with most current guidelines, one section of tumor (some with adjacent myometrium) per centimeter of largest tumor diameter is required, and a minimum of four sections should be taken if possible. Submitting the entire tumor can be considered when it measures ≤ 3 cm [8].

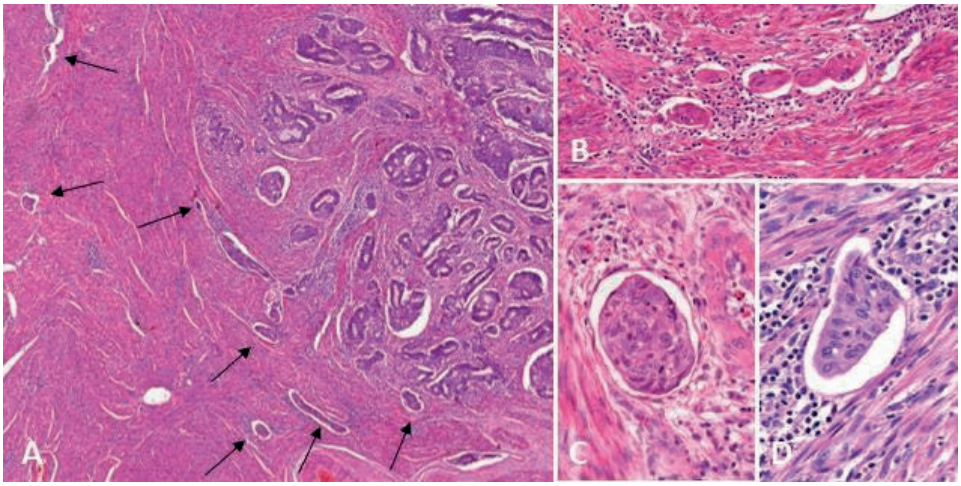

Figure 2. *2A:* LVSI is most likely to be seen near the advancing tumour front associated with a desmoplastic stromal response. Clustering of multiple vessels is often seen, appearing as a spray-like distribution of LVSI. *2B:* Example of a tortuous vessel with LVSI; due to a tortuous course it appears multiple times during sectioning. Note the same calibre and shape of both the embolus as well as the vessel. *2C:* a typical embolus has somewhat rounded shape, similar to the vessel. Tumor cells form a cohesive embolus with centrally located, rounded nuclei. *2D:* a perivascular infiltrate is often seen around involved vessels. Perivascular lymphoid infiltrates should trigger the pathologist to look for LVSI is this area.

The morphology of the tumor cells themselves may also help to distinguish between true LVSI and pseudo-LVSI. The tumor embolus in true-LVSI tends to have a smooth and rounded shape, which resembles the vessel it lies in and may sometimes be attached to the vessel wall. A true tumor embolus usually lacks any stromal elements and the tumor cells are cohesive (figure 2C). The tumor cells are “vital” with a clearly recognizable cell nucleus that is intact, surrounded by cytoplasms and shows no signs of karyorrhexis. The cells have a rounded shape and generally centrally located nucleus, which may resemble cell clusters in tissue culture. Lastly, vessels containing a true embolus are sometimes have a surrounding perivascular lymphocytic infiltrate (figure 2D). The presence of inflammatory cells (mainly lymphocytes) cuffing vessels with tumor emboli can often be appreciated at low power and can direct the attention of the

pathologist to higher power examination of foci suspicious for LVSI [9-11]; it is important to note that perivascular inflammatory cell infiltrates in the absence of LVSI are not of clinical significance [9].

Clues in support of a diagnosis of pseudo-LVSI

Awareness of the many pitfalls in LVSI assessment helps to enhance the quality and reliability of LVSI assessment. In EC, there are several frequently encountered artefacts and other factors which can mimic LVSI Artefacts can result from poor fixation and tissue preservation. To reduce factors related to poor fixation, the specimen should be transferred to the pathology laboratory as soon as possible and should be opened in the coronal plane and placed in ample formalin [8]. This reduces the likelihood of retraction artefacts and tumor displacement at grossing (figure 3A). Like necrotic tumor, autolytic tumor fragments get "transported" easily during grossing, commonly being displaced to cut surfaces, onto the serosa, and into large vessels and slit-like spaces in the myometrium. If the pathologist is aware of these artefacts, they are usually not mistaken for LVSI since the "lytic" cell changes, disorganized and fragmented clusters look like they have been wiped together.

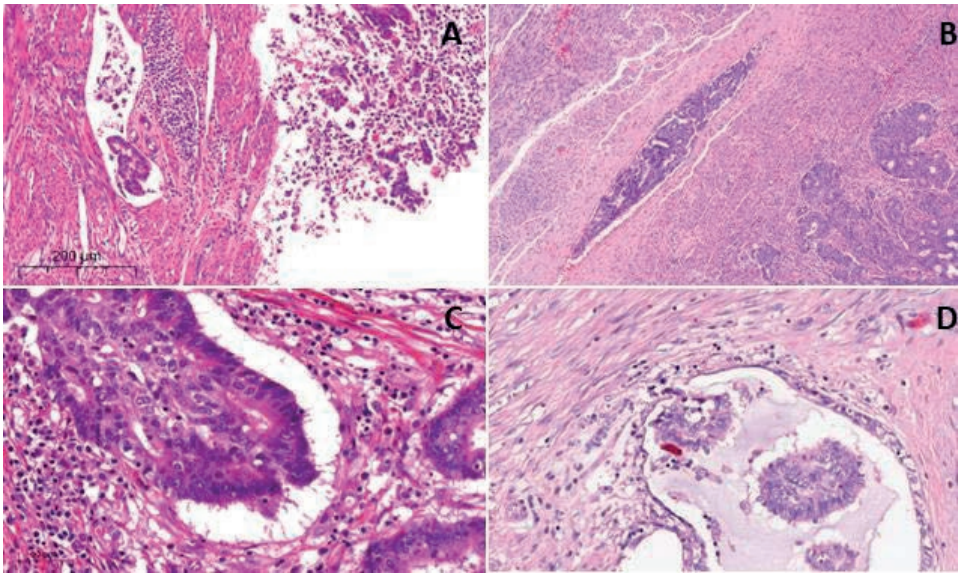

Figure 3. *3A:* lytic tumor fragments often become displaced to cut surfaces during grossing, and may also be displaced into vascular spaces. *3B:* tumor fragments pushed into vessels and in slit-like myometrial spaces. *3C:* retraction artefact shows little strands of cytoplasm between the embolus and the putative vessel wall. Also note the preserved glandular shape. **3D**: MELF type gland with partially flattened epithelial lining simulating endothelial cells. Note the delicate desmoplastic stromal response around the gland.

Another reason for tumor displacement is the use of an intrauterine manipulator during surgery. These devices are transvaginally inserted in the uterine cavity and they allow the surgeon to manipulate the uterus to improve access in the pelvis and identify anatomic structures.

However, when using the manipulator, tumor cells can be displaced into small and large vascular channels, slit-like artefactual spaces within the myometrium (which are caused by the manipulator), the lumina of the fallopian tubes or beyond (figure 3B) [12, 13]. Clues to the presence of pseudo-LVSI are that the degree of vascular involvement is often greater than that expected from the grade and stage of the tumor (for example, in a low-grade, stage IA endometrioid carcinoma, substantial LVSI would not be expected) and that the tumor emboli often preferentially involve large caliber blood vessels.

Retraction artefacts can be seen in the myometrium, forming slit-like spaces around tumor nests. These slit-like spaces can resemble a vessel and this can be challenging to distinguish from LVSI, especially since flattened myocytes can resemble endothelial cells. The following features should be considered in distinguishing between LVSI and retraction: first, be cautious about diagnosing LVSI if there are widespread obvious retraction artifacts in the tumor and the suspected focus looks similar. Second, view the embolus at high magnification and assess the border of the embolus; little strands of cytoplasm attaching the embolus to the putative vessel wall are a feature of retraction while a smooth surface suggests LVSI (figure 3C). In addition, assess the cytological features and architecture of the tumor cells; a well-retained glandular configuration with cylindrical cells and basally located nuclei support retraction.

The microcystic elongated and fragmented (MELF) pattern of myometrial invasion is associated with LVSI but can also result in mimicry of true LVSI. In MELF-pattern myometrial invasion, tumor glands may focally form microcysts lined by tumor cells which can resemble endothelial cells when they are extremely flattened and therefore become mistaken for a small vessel. Remaining glandular tumor fragments in the lumen may subsequently be interpreted as LVSI. The lining of these spaces, at least focally, typically shows obvious epithelial differentiation in the form of cuboidal cells and an epithelial, rather than endothelial lining, can be confirmed by positive staining with cytokeratin markers (figure 3D).

Use of immunohistochemistry in LVSI assessment

Routine examination of H&E-stained sections is usually sufficient to make a diagnosis of LVSI but when there is genuine doubt as to whether tumor is present within a vascular channel or not, immunohistochemistry may assist. However, as just stated this is typically not needed and will not distinguish between true LVSI and artefactual displacement of tumor emboli into vascular channels. A true vascular lining can be identified with the use of podoplanin (D2-40) or CD31 immunohistochemistry, although interpretation of these stains can be problematic, as discussed below. In one study, the detection rate of LVSI was increased threefold using a dual stain (cytokeratin AE1/AE3 and CD31) compared to evaluation of conventional H&E slides [14]. However, in another study, conventional detection of LVSI proved to be superior to immunohistochemical detection when the results were correlated with clinical outcome [15]. In addition, cancer associated fibroblasts surrounding tumors with a brisk stromal reaction often express podoplanin [16, 17] thereby making lymph vessel detection problematic. Taking

everything in consideration, the usefulness of podoplanin is limited to uncommon situations in which a diagnosis cannot be made based upon the features described above and in the absence of an intense stromal reaction. It is also important to stress that most studies that have shown LVSI to be an independent prognostic factor in EC did not use immunohistochemistry. This is important since LVSI can be adequately assessed even in under-resourced regions where immunohistochemistry is not widely available.

When the differential diagnosis is between MELF and LVSI, cytokeratin staining can confirm the presence of epithelial cells lining the space and support a diagnosis of MELF. Finally, cytokeratin immunohistochemical staining can help to discriminate between tumor cells and macrophages in rare case where there is doubt regarding the nature of the cells within a vascular space.

Reproducibility of LVSI assessment

Given the significance of substantial LVSI for individual adjuvant treatment recommendations (discussed in detail below), the diagnosis of LVSI must be reliable and reproducible. Implementation of the semi-quantitative LVSI reporting system in a series of EC cases meeting PORTEC-1 inclusion criteria [18], found substantial LVSI in 16.0% of tumors [19], around three times as many as reported in the combined PORTEC-1-2 cohorts (4.8%)[9]. Given the marked difference in the proportion of cases exhibiting substantial LVSI in the two cohorts despite similar composition, it is fair to conclude the definitions for extensive/ substantial LVSI should be improved to lead to reproducible results. The same criteria for LVSI were applied in two other studies and less divergent results were obtained for substantial LVSI (11.1% among 941 FIGO stage I EC (including endometrioid and non-endometrioid types)[20] and 4.2% among 524 FIGO stage I grade 1 and 2 endometrioid carcinomas [21]).

Reproducibility of LVSI assessment in EC was assessed in an interobserver variation study among a team of expert gynecological pathologists. The level of agreement in discriminating true LVSI from pseudo-LVSI and in assessing extent of LVSI assessment were studied. Although cases were enriched for difficult LVSI mimics, there was substantial agreement among participants in discriminating true LVSI from LVSI mimics. Assessment of the extent of LVSI resulted in substantial agreement, illustrating the definitions can be applied in daily practice for reliable semi-quantitative LVSI assessment [22].

Extent and quantification of LVSI

Review of PORTEC-1 and -2 prospective study cohorts for LVSI showed that a two-tier assessment of LVSI had the strongest prognostic value and for the first time, extent of LVSI was shown to be associated with prognosis [9]. This tow-tier system tool was adopted from a study by Fujimoto et al. and categorizes LVSI as none, focal or substantial LVSI [23]. Focal LVSI was originally defined as a single focus of LVSI beyond the invasive front while substantial LVSI was defined as diffuse or multifocal (figure 4). However, clearly this classification did not cover all

possible scenarios. The prognostic value of the two-tier system was subsequently confirmed in several other studies [20, 21, 24]. Previous retrospective studies had also shown that the risk of LN and distant metastases increased with the extent of LVSI [23, 25].

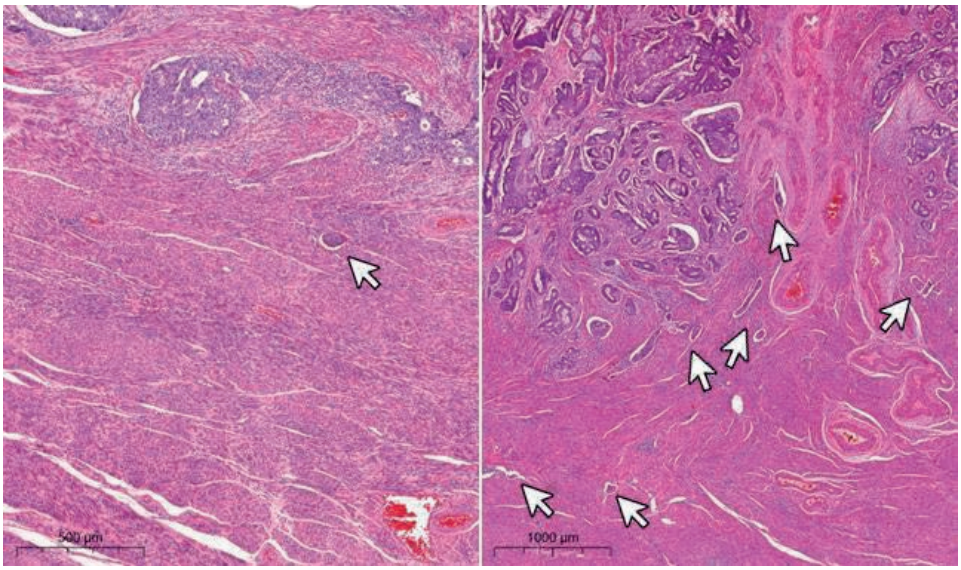

Figure 4. *Left:* in focal LVSI there are up to three foci of LVSI in a single H&E beyond the invasive front of a tumor. *Right:* substantial LVSI is charactarized by multiple foci of LVSI, usually easy to recognize and is diagnosed then at least four foci are present in a single H&E.

The definitions of focal and substantial LVSI used in the initial studies were useful but the lack of an explicit threshold for distinction between focal and substantial LVSI left room for confusion and differing interpretations in borderline cases. Both Tortorella and Winer et al. defined extensive LVSI as ≥3 vessels containing tumor emboli [21, 26] but the definitions used in those studies still lacked a sufficient level of detail. For instance, should substantial LVSI be diagnosed if the threshold of 3 vessels is met in at least one histological section, in every section with tumor, as an average per section, or cumulative of all sections? In an attempt to determine a threshold for substantial LVSI, additional analyses were done in the PORTEC-1-2 cohort and an independent validation cohort. The risk of pelvic LN recurrence was correlated with the number of emboli per histological section. Toxicity of adjuvant treatment was also taken into consideration to determine a threshold for "clinically-relevant" LVSI; the threshold should be set low enough to prevent patients at risk for regional recurrence being denied effective treatment. At the same time, the threshold should be high enough to prevent patients from being exposed to the toxicity of adjuvant treatment with limited reduction of risk of disease recurrence. In that study, it was concluded that substantial LVSI should be diagnosed when ≥4 positive vessels were present in at least one histological section [27]. To further complicate this issue, the latest World Health Organization (WHO) chapter on EC sets the threshold for substantial LVSI at ≥5 positive vessels [28]. Meanwhile, the College of American Pathologists (CAP) uses a threshold of

≥3 positive vessels in the cancer protocol template for examination of hysterectomies with EC. The International Society of Gynecological Pathologists recommends a threshold of 3 vessels while ESGO/ESTRO/ESP recommends 5, as per the WHO (5th edition, p.255 [29]). In these guidelines, it is not clear whether these figures are based on the number of vessels involved in all slides examined or in a single slide. In addition, the International Collaboration on Cancer Reporting (ICCR) recommends the semi-quantitative approach (no/focal/substantial) for LVSI reporting [1].

It is important to note that relatively few cases present any difficulty in classification as "none", "focal", or "substantial" LVSI. After publication of the paper by Bosse et al. [9] the Vancouver group adopted the semi-quantitative approach for LVSI assessment and data of LVSI scores were prospectively collected. LVSI scores of 3200 EC's were collected between 2016 and 2022 and show LVSI status was obvious (present or not) in 98% but it was inconclusive in 2% (figure 5A). Extent of LVSI was determined in 722 EC's (23%) and 71% had substantial and 29% had focal LVSI (figure 5B). The number of LVSI-positive vessels was counted in 58 tumors (figure 6), the number of cases with 3, 4 and 5 vessels containing tumor emboli, was 1, 2 and 1, respectively. This presents a challenge, however, as it means it will be difficult or impossible to generate data critically comparing a cut-off of 3 versus 4 versus 5 vessels, as the most clinically appropriate threshold. In contrast to the pragmatically choses thresholds of 3 and 5 vessels, the threshold of ≥4 positive vessels in at least one single H&E was based on an optimal balance between risk of disease recurrence versus toxicity of adjuvant treatment [27]. Given the small range these thresholds are in, none of them can be wrong, however the ≥4 vessel threshold also supports pathologists in the application of the threshold and therefore contributes to reproducibility. Therefore, substantial LVSI should be diagnosed when ≥4 positive vessels are present in at least one histological section from the uterus. and we recommend that the various protocols should be harmonized to reflect this.

Summary and future directions

The presence of and extent of LVSI has prognostic and therapeutic impact in EC depending on the stage of disease and can consequently affect patient management recommendations. We acknowledge LVSI assessment is not always straightforward, and provide an "LVSI checklist" (table 1) which we hope will be helpful in problematic cases. In some cases, deeper levelling or additional sampling may be of value and in general, we recommend in cases of uncertainty as to whether an embolus is present within a vessel to not count these foci.

The criteria we propose will reduce confusion and allow for uniform criteria to be used by all pathologists. While ongoing and future studies may refine the cut-off for substantial LVSI, there are practical considerations that will make such studies difficult, specifically the identification of sufficient numbers of cases near the diagnostic threshold between focal and substantial LVSI.

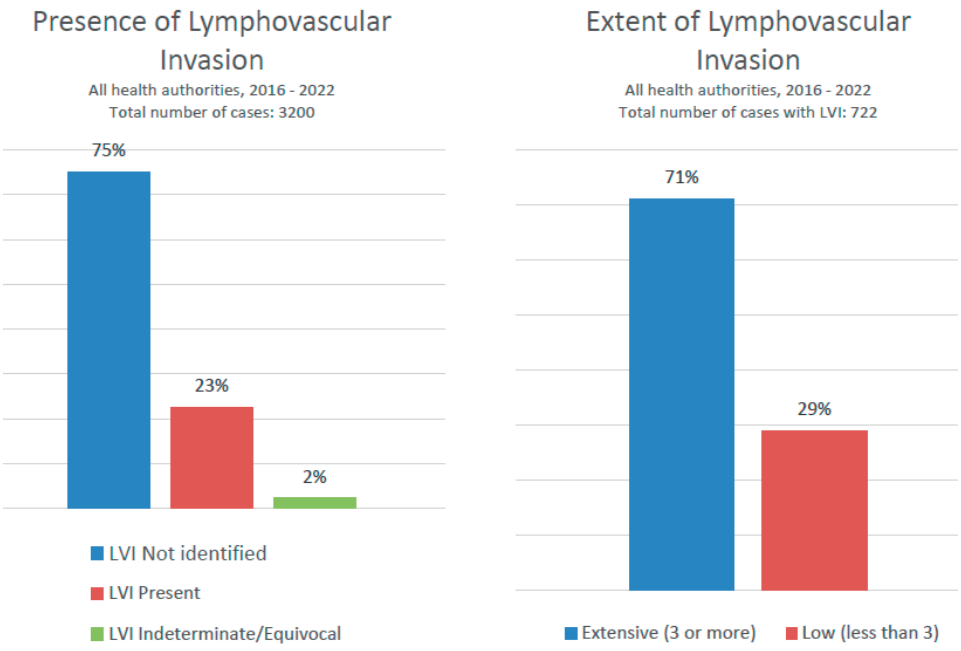

Figure 5. *5A* LVSI assessment of 3200 EC's according to LVSI status (not present, present or indeterminate). *5B*: semiquantitative LVSI assessment of 722 LVSI positive EC's.

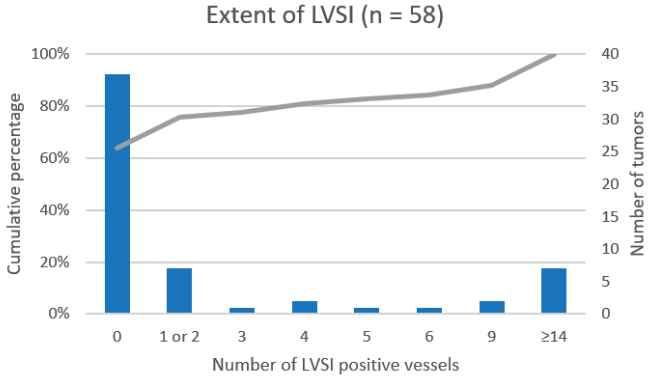

Figure 6. Quantitative LVSI assessment of 58 EC's. The number on emboli counted is on the X-axis. The right Y-axis shows the absolute count and the left Y-axis shows the cumulative percentage.

Table 1. LVSI Checklist

Clues for true LVSI	Features favoring true LVSI
Clues for artefactual LVSI	Located near invasive front
Consider IHC	Perivascular infiltrate
CD31/D2-40	Close to venous and/or arterial vessel
Pancytokeratin	Cohesive tumor embolus
Representative sampling	Molding of tumor embolus
One sample per cm tumor	Altered cell cytology
Focal LVSI	Close to infiltrative or MELF growth pattern
1, 2 or 3 emboli	More than one vessel involved
1 or 2 H&E's	
Small emboli	Features favoring pseudo-invasion
Substantial LVSI	Signs of poor fixation (autolysis) and/or spill
≥4 vessels in 1 H&E	Embolus with admixed inflammatory cells and debris
Often multiple H&E's	Close to retraction artefacts
Larger emboli	Surrounded by stromal reaction
Downgrade if in doubt	Preserved glandular structure and stroma

Summary of features in favor of LVSI and characteristics of pseudo-LVSI.

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CHAPTER 3

SUBSTANTIAL LYMPH-VASCULAR SPACE INVASION (LVSI) IS A SIGNIFICANT RISK FACTOR FOR RECURRENCE IN ENDOMETRIAL CANCER – A POOLED ANALYSIS OF PORTEC 1 AND 2 TRIALS

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Abstract

Lymph-vascular space invasion (LVSI) is an important adverse prognostic factor in endometrial cancer (EC). However, its role in relation to type of recurrence and adjuvant treatment is not well defined, and there is significant interobserver variation in diagnosing LVSI. This study aimed to quantify LVSI and correlate this to risk and type of recurrence.

In the post operative radiation therapy in endometrial carcinoma (PORTEC)-trials stage I EC patients were randomized to receive external beam radiotherapy (EBRT) versus no additional treatment after surgery (PORTEC-1, n=714), or to EBRT versus vaginal brachytherapy (PORTEC-2, n=427). In tumor samples of 926 (81.2%) patients with endometrioid tumors LVSI was quantified using 2-, 3- and 4-tiered scoring systems. Cox proportional hazards model was used for time-to-event analysis.

Any degree of LVSI was identified in 129 cases (13.9%). Substantial LVSI (N=44, 4.8%) using the 3-tiered approach had strongest impact on the risk of distant metastasis (hazard ration (HR) 4.5 confidence interval (CI) 2.4-8.5). In multivariate analysis (including: age, depth of myometrial invasion, grade, and treatment) substantial LVSI remained the strongest independent prognostic factor for pelvic regional recurrence (HR 6.2 CI 2.4-16), distant metastasis (HR 3.6 CI 1.9-6.8) and overall survival (HR 2.0 CI 1.3-3.1). Only EBRT (HR 0.3 CI 0.1-0.8) reduced the risk of pelvic regional recurrence.

Substantial LVSI, in contrast to focal or no LVSI, was the strongest independent prognostic factor for pelvic regional recurrence, distant metastasis and overall survival. Therapeutic decisions should be based on the presence of substantial, not ‘any’ LVSI. Adjuvant EBRT and/or chemotherapy should be considered for stage I EC with substantial LVSI.

Introduction

Lymph-vascular space invasion (LVSI) is found in about 8-10% of patients with International Federation of Gynaecology and Obstetrics (FIGO) stage I endometrial carcinoma (EC), and is increasingly found with higher tumor grade, deeper invasion and older age [1-3]. LVSI has been reported as a risk factor for recurrence and for both lymph node and distant metastasis [4-10]. Presence of LVSI has been related with a 5-fold risk of microscopic pelvic lymph node metastases [11], but LVSI is also an important risk factor for distant metastases in the absence of nodal involvement [5]. This has led to the question if LVSI can be used as a surrogate marker of nodal involvement in absence of surgical nodal staging [4].

A clinical dilemma arises when LVSI is found in a patient with otherwise intermediate risk features with regard to the recommendation for adjuvant radiotherapy. While LVSI was included as a risk factor in the definition of high-intermediate risk in the GOG#99 trial [12], it was not included in the PORTEC-1 definition [13]. In the PORTEC-1 trial LVSI was mainly found in the registered group with grade 3 and >50% myometrial invasion [1]. Apart from retrospective studies in which treatment was not controlled, the randomized trials of radiotherapy did not report separately on the outcome of patients with LVSI, making it difficult to draw firm conclusions [12-16].

Lack of uniform histological criteria to establish LVSI in EC specimens; the possibility that a quantification factor is important and the considerable interobserver variability in the assessment of LVSI might explain part of these conflicting findings. In most studies no definition for assessment of LVSI has been reported. Often a comment is made that there should be clear presence of LVSI, in contrast to cases presenting with focal or questionable LVSI that can be difficult to distinguish from retraction artifacts or so-called 'microcystic, elongated and fragmented' (MELF-like) growth pattern of invasion[17]. Two-, three- and four-tiered definitions of LVSI have been proposed, with increasing degrees of LVSI and the question is whether or not this semi-quantification is clinically relevant (Figure 1)[18, 19].

The hypothesis of the current study was that more prominent LVSI would result in higher risk of disease recurrence and stronger prognostic significance. The aim of this study was to analyze the prognostic value of two-, three- and four-tiered definitions in relation to adjuvant radiotherapy within the PORTEC trials.

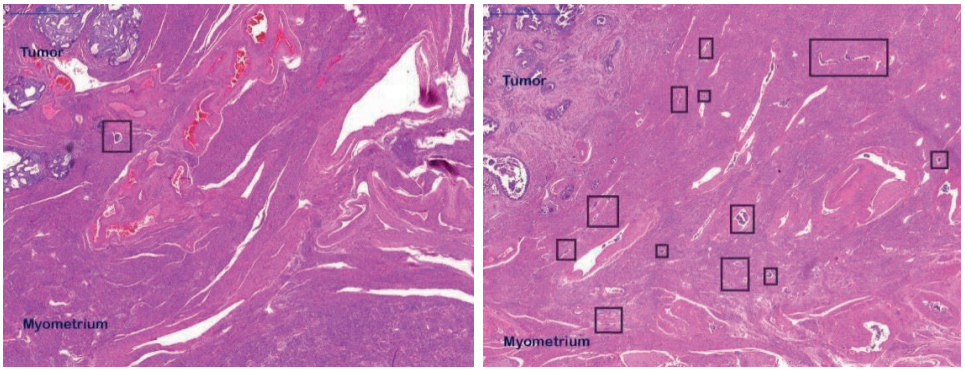
Methods

Study population

For this study patients and follow-up data from the PORTEC-1 and -2 studies were used. PORTEC-1 included 714 patients with FIGO (1988) stage IB grade 2 or 3 and stage IC grade 1 or 2 EC between 1990 and 1997 [13]. The PORTEC-2 study included 427 patients between 2002 and 2006 who had stage I EC with high-intermediate risk features (FIGO 1988 stage 1B grade 3, IC grade 1 or 2 or stage 2A) [15]. All patients underwent total hysterectomy and bilateral salpingo-

oophorectomy without lymphadenectomy and were randomly allocated to receive external beam radiation therapy (EBRT) versus no additional treatment (NAT, PORTEC-1) or EBRT versus vaginal brachytherapy (VBT, PORTEC-2). In both studies central pathology review was performed to assess histological type, stage, grade and LVSI. Representative histological slides and/or tumor samples were available from 926 (81,2%) of the 1141 patients.

Figure 1. Representative pictures of haematoxylin & eosin (H&E) stained slides (magnification 2.5x) illustrating how the



3-tiered scoring was applied. Representative examples of focal left) and substantial (right) Lymph-vascular space invasion (LVSI). Black boxes indicate foci of LVSI.

LVSI definition

LVSI was defined as the presence of tumor cells in a space lined by endothelial cells outside the immediate invasive border. In case of possible mimics such as retraction/shear artefacts, smear artefacts and MELF-type invasion there was restraint to seignate involved foci as LVSI. Intra tumoral LVSI foci were not considered. Supportive criteria used to define LVSI presence were: foci near other vessels and presence of a lymphocytic infiltrate around the involved vessel.

Scoring systems for LVSI in endometrial cancer

In order to semi quantify the above-described LVSI definition; we searched the endometrial cancer literature for LVSI scoring methods. The majority of publications describe LVSI as present or not present mostly without any further detail (*'two-tiered system'*). Two publications were identified with a more detailed description of LVSI and a semi-quantitative scoring method, including a *three-* and *four-tiered* scoring system [18, 19]. These scoring systems are outined in Table 1.

All available H&E slides were systematically screened at 10x10 magnification and scored by the first observer (EP) for the presence of LVSI. Addtionally, to further substantiate the semi-quantitative scoring systems, the number of involved vessels was counted. Finally, the presence of a perivascular infiltrate was noted, which has been described to be indicative for the presence of LVSI. To make our findings comparable to previous publications, a perivascular infiltrate was present if there were aggregates of >20 lymphocytes around a vessel per section [20].

All cases in which LVSI was reported at least once (original pathology report, central pathology review and/or first observer) or in which the presence of LVSI was uncertain, were scored by two additional observers (TB, VS). All reviews were performed blinded from previous reports and scores. Consensus was reached if the first and second observer agreed. If there was no consensus the case was discussed at a multiheaded microscope with all observers present until consensus was reached.

Table 1. Definitions of lymph vascular space invasion (LVSI)*

A	LVSI absent	Definition not met
	LVSI present	Definition met
B [18]	No LVSI	Definition not met
	Focal	A single focus of LVSI was recognized around a tumor
	Substantial	Diffuse or multifocal LVSI was recognized around the tumor
C [19]	No LVSI	Definition not met
	Minimal	Only a few lymph vascular vessels were involved on the border of the invasive front of the tumor
	Moderate	More vessels were involved in a wider area surrounding the tumor
	Prominent	Many vessels were diffusely involved in the deeper part of the myometrium

*See methods for definition of LVSI.

Statistical analyses

Patient, tumor and treatment characteristics were analyzed using Chi-square statistics or Fishers exact test in case of categorical and *t* test or analysis of variance (ANOVA) for continuous variables.

Time to event analysis were calculated from the date of randomization as starting point and patients who were alive and without recurrence were censored at the date of last follow-up. Data for survival curves were calculated using Kaplan-Meier method with log-rank test. For the following endpoints events between brackets were considered as event: vaginal recurrence rate (all vaginal recurrences); pelvic regional recurrence (all pelvic nodal or non-vaginal recurrences); distant metastasis (all distant metastasis); overall survival (all deaths). Cox proportional hazards models included established prognostic factors age, grade, depth of myometrial invasion and treatment received. All statistical analyses were done with IBM SPSS (version 20.0).

Results

Study population

Patient characteristics are detailed in Table 2 and supplementary Tables S1A-C. Since the PORTEC-2 trial include high-intermediate risk patients while the PORTEC-1 trial also included (low-)intermediate risk cases, patients in the VBT group were on average older and had more grade 3 tumors. Median follow-up for patients alive was 160 months for PORTEC-1 and 89 months for PORTEC-2.

Table 2. Patient characteristics by treatment received and after central review of pathology.

	Total (n = 926)	NAT (n = 287)		EBRT (n = 450)		VBT (n = 189)		*p*-Value
	N	N	%	N	%	N	%	
Age								
Mean (range)	67.8 (41–90)	66.3 (46–90)		67.7 (41–88)		70.2 (52–86)		<0.001
<60 years	158	77	48.7	74	46.8	7	4.4	
>60 years	768	210	27.3	376	49.0	182	23.7	
Myometrial invasion								
<50%	278	125	45.0	120	43.2	33	11.9	<0.001
>50%	648	162	25.0	330	50.9	156	24.1	
Differentiation grade								
1	673	186	27.6	337	50.1	150	22.3	0.001
2	137	48	35.0	67	48.9	22	16.1	
3	116	53	45.7	46	39.7	17	14.7	
LVSI								
Absent	856	274	32.0	410	47.9	172	20.1	0.065
Present	70	13	18.6	40	57.1	17	24.3	

LVSI: lymph vascular space invasion; NAT: no additional treatment; EBRT: external beam radiotherapy; VBT: vaginal brachytherapy.

Lymph-vascular space invasion

In the original pathology reports, LVSI had been found in 64 (6.9%) tumors. While in the current analysis any degree of LVSI was found in 129 (13.9%) tumors, LVSI was more frequently observed in tumors with deep (>50%) myometrial invasion (15.9%) than in those with superficial invasion (9.4%, p=0.008, Table S1C). The agreement between the original reports and the current analysis was low (Kappa 0.30). Results using the different LVSI definitions are shown in Table 3. Both the three- and four-tiered approaches showed an increase in the number of involved vessels.

Table 3. Different approaches for scoring of LVSI by number of involved vessels and the prognostic efficacy for distant metastasis.

	Total		Involved vessels		Distant metastasis			
	N	%	Mean (95% CI)	*p*-Value	HR (95% CI) unadjusted	*p*-Value	HR (95% CI) adjusted*	*p*-Value
Original reports								
No LVSI	863	93.1						
LVSI present	64	6.9			3.3 (1.9–5.9)	<0.001	3.1 (1.8–5.7)	<0.001
Central review								
No LVSI	856	92.4			1		1	
LVSI present	70	7.6			2.6 (1.4–4.8)	0.001	2.2 (1.2–4.1)	0.012
Two-tiered								
No LVSI	797	86.1	0	<0.001	1		1	
LVSI present	129	13.9	2.5 (2.1–2.9)		3.1 (2.0–5.0)	<0.001	2.9 (1.8–4.6)	<0.001
Three-tiered								
No LVSI	797	86.1	0	<0.001	1		1	
Focal	85	9.2	1.8 (1.5–2.1)		2.4 (1.3–4.9)	0.004	2.4 (1.3–4.5)	0.005
Substantial	44	4.8	3.9 (3.1–4.7)		4.5 (2.4–8.5)	<0.001	3.6 (1.9–6.8)	<0.001
Four-tiered								
No LVSI	797	86.1	0	<0.001	1		1	
Minimal	46	5.0	1.2 (1.0–1.3)		2.8 (1.3–5.8)	0.007	3.0 (1.4–6.3)	0.004
Moderate	55	5.9	2.4 (2.0–2.9)		2.6 (1.3–5.3)	0.008	2.3 (1.1–4.7)	0.023
Prominent	28	3.0	4.9 (3.9 – 6.0)		4.9 (2.3–10.3)	<0.001	3.8 (1.8–8.1)	0.001

CI: confidence interval; HR: hazard ratio; LVSI: lymph vascular space invasion.

* Adjusted for age, review grade, review depth of myometrial invasion and treatment.

Perivascular lymphocytic infiltrates were found in 305 (32.7%) tumors. Although these changes were found more frequently tumors with LVSI, only 26.4% of patients with perivascular lymphocytic infiltrates had LVSI (Table S1C).

Prognostic value

Hazard ratios (HR) for the risk of distant metastases in relation to LVSI using the different definitions, both unadjusted and adjusted for age, depth of myometrial invasion, grade and treatment received are shown in Table 3. There was no prognostic difference between minimal and moderate LVSI in the four-tiered definition, and therefore this definition had no added value over the three-tiered approach (Table 3, Figure 2A and 2B). In the three-tiered scoring system there was a stepwise increase in the prognostic impact of focal LVSI and substantial LVSI, with a markedly increased HR of substantial LVSI compared to LVSI in the two-tiered definition (4.5 vs. 3.1). For these reasons, the three-tiered definition was included in a multivariate Cox regression analysis (Table 4).

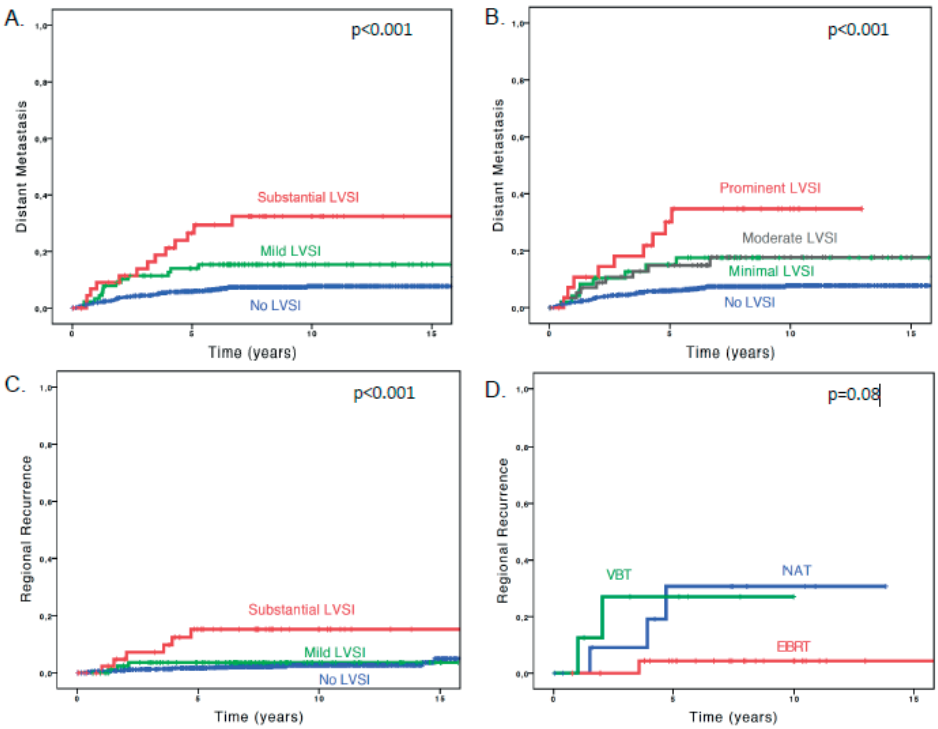


Figure 2. Kaplan Meier curves for the risk of distant metastasis for the three-tiered (A) and four-tiered definition (B) of LVSI. Kaplan Meier curves of the risk of pelvic regional recurrence using a three-tiered definition of LVSI (C) and for treatment received in the subgroup of 46 patients with substantial LVSI (D).

Substantial LVSI was an independent prognostic factor for pelvic regional recurrence, distant metastasis (DM) and overall survival (OS). Substantial LVSI was the strongest independent prognostic factor for an increased risk of pelvic regional recurrence (at 5 years, the risk for no

LVSI was 1.7%, for focal LVSI 2.5% and for substantial LVSI 15.3%), while EBRT (but not VBT) independently decreased the risk of pelvic regional recurrence (Table 4 and Figure 2C). In the subgroup of patients with substantial LVSI, the risk of pelvic regional recurrence at 5 years after EBRT was 4.3% compared to VBT 27.1% and NAT 30.7% (Figure 2D). In addition to substantial LVSI, grade 3 was an independent risk factor for pelvic regional recurrence. Both focal and substantial LVSI and grade 3 were independent prognostic factors for DM. Age >60 years, grade 3 and substantial LVSI were independent prognostic factors for a decreased OS. For the risk of vaginal recurrence, both EBRT and VBT were the strongest independent predictive factors for a decreased risk, both age >60 years and grade 3 increased the risk while presence of LVSI was no independent prognostic factor. Finally, the presence of a perivascular lymphocytic infiltrate was not associated with endometrioid EC recurrence (HR 1.0, CI 0.74-1.44).

Discussion

In this large cohort of 926 intermediate to high-intermediate risk Stage I EC patients randomized in the PORTEC-1 and -2 trials, 4.8% were found to have substantial LVSI in a three-tiered semi-quantitative scoring system, which was the strongest independent prognostic factor for pelvic regional recurrence, distant metastasis and overall survival. LVSI was not predictive for the risk of local vaginal recurrence when adjusted for treatment received, showing the large risk reduction with both EBRT and VBT. Importantly, EBRT was associated with a decrease in the risk of pelvic regional recurrence, in contrast to VBT. EBRT and VBT did not impact on the risk of distant metastasis and overall survival.

The assessment of LVSI in hysterectomy specimens is not easy due to frequently found artifacts such as tumor spill due to bad fixation or retraction artifacts. Also, a MELF like growth pattern can mimic LVSI [17]. Additionally, there is no uniformity in the definitions used to describe LVSI. This is possibly one of the explanations for the broad variation in reported prevalence of LVSI in stage I EC, and for the low interobserver agreement. In this study all available H&E slides were systematically screened for the presence of any degree of LVSI. This was done at high magnification, adequate to identify tumor cells along with sufficient view of its surroundings, and doubled the amount of LVSI positive cases compared to initial pathology reports. However, most cases had focal LVSI and the number of cases with more clinical relevant substantial LVSI was reduced compared to the initial pathology reports. Low magnification was sufficient to recognise cases with substantial LVSI. Despite the stepwise increase of number of involved vessels in the largest embolus within both the three- and four-tiered scoring system, the four-tiered approach had no stronger prognostic significance than the three-tiered approach, due to the lack of difference between minimal and moderate LVSI. Identification of perivascular infiltrates did not contribute to the prognostic significance of LVSI.

Table 4. Multivariate Cox proportional Hazard regression models for the three-tiered scoring system for LVSI

	Vaginal recurrence			Pelvic Regional Recurrence			Distant Recurrence			Overall survival		
	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*	HR	95% CI	*p*
Age												
<60	1			1			1			1		
>60	3,15	1,10–9,01	0,032	2,00	0,58–6,89	0,275	1,29	0,68–2,45	0,437	3,19	2,15–4,74	<0,001
Differentiation grade												
1	1			1			1			1		
2	1,68	0,75–3,76	0,212	2,13	0,82–5,55	0,120	1,89	1,05–3,42	0,035	1,19	0,87–1,62	0,285
3	2,31	1,01–5,26	0,046	2,75	1,02–7,42	0,045	3,72	2,12–6,53	<0,001	1,79	1,30–2,48	<0,001
Myometrial invasion												
<50%	1			1			1			1	1	
>50%	1,47	0,74–3,03	0,301	1,89	0,73–4,97	0,195	1,25	0,74–2,12	0,409	1,08	0,83–1,41	0,546
LVSI												
No LVSI	1			1			1			1		
Focal	1,86	0,65–5,35	0,251	1,10	0,26–4,74	0,900	2,42	1,31–4,45	0,005	1,36	0,93–2,00	0,111
Substantial	1,68	0,51–5,66	0,393	6,19	2,35–16,3	<0,001	3,61	1,90–6,84	<0,001	2,02	1,30–3,12	0,002
Treatment received												
NAT	1			1			1			1		
EBRT	0,17	0,08–0,37	<0,001	0,30	0,11–0,80	0,016	1,14	0,67–1,93	0,640	1,04	0,81–1,34	0,734
VBT	0,13	0,04–0,43	0,001	1,16	0,47–2,87	0,745	1,21	0,63–2,33	0,568	0,82	0,56–1,21	0,319

HR: hazard ratio; CI: confidence interval; LVSI: lymph vascular space invasion; NAT: no additional treatment; EBRT: external beam radiotherapy; VBT: vaginal brachytherapy.

The three-tiered definition confirmed our hypothesis that more LVSI would result in higher risk of disease recurrence. Substantial LVSI in the three-tiered definition had a markedly increased HR compared to the two-tiered approach and to the original pathology reports, and its prognostic significance was strongest and most clinically relevant in the multivariate Cox regression analysis. In this scoring system focal LVSI was defined as a single focus of LVSI. However, analyses of number of involved vessels shows that on average two involved vessels were found, indicating that the interpretation of this definition is not absolute. An interobserver study regarding identification of LVSI has been initiated to determine if the use of the three-tiered system will lead to more reproducible reporting of substantial LVSI with clinical consequences.

While the obvious strengths of this analysis are the inclusion of a large cohort of randomized, uniformly treated patients with complete follow-up data, and the central review of pathology, there are limitations. Although an effort was made to include as many H&E slides per case as possible, for a proportion of the patients there was only one tumor-containing slide available, which might have led to underreporting of LVSI. However, based on the prevalence of LVSI in the original pathology reports and during initial central pathology review and the low agreement with the current analysis including more of the mild LVSI cases, this is most likely minor underreporting. In addition, despite the inclusion of more than 900 cases, the proportion of patients with substantial LVSI (N=44) was small, with corresponding wide confidence intervals.

Well-known risk factors in endometrial cancer are age, FIGO stage, histological subtype, tumor grade and depth of myometrial invasion. In stage I-II disease, most studies reported LVSI (and grade 3) as a significant risk factor for distant metastasis, and showed that the presence of LVSI was associated with microscopic lymph node metastases in lymphadenectomy specimens [4, 7, 8, 10, 11]. Most studies that investigated prognostic factors in EC patients were cohort studies in which adjuvant treatment was not controlled, hampering conclusions with regard to pelvic recurrence. The randomized trials reporting on the role of radiotherapy in EC have not specifically reported on the outcomes of patient with and without LVSI [12-16] Based on previous results in GOG studies LVSI was included in GOG#99 as a risk factor for defining high-intermediate risk[12], while in PORTEC-1 the high-intermediate risk factors (age >60 years, grade 3, >50% myometrial invasion) were based on multivariate regression analysis of prognostic factors within the trial population. LVSI was found in 5% of 714 randomized patients, but was mainly found in 17% of the cohort of 99 patients with deep invasive grade 3 tumors that were registered but not randomized [1, 13]. For these reasons LVSI was not included in the PORTEC definition of high-intermediate risk. Currently VBT is preferred in high-intermediate risk patients based on its capability of ensuring vaginal control with only minimal toxicity and without any negative impact on quality of life [15, 21]. Vaginal brachytherapy is a local treatment of the vaginal vault region (where 75% of the local recurrences in the NAT arm of the PORTEC-1 trial were located), leaving regional pelvic nodes untreated. Clinical pelvic regional recurrence only

occurred in 3.4% of the NAT patients in PORTEC-1 and in 3.8% of the VBT patients in PORTEC-2 at 5 years and most had synchronous distant metastases for which systemic therapy was needed. However, the optimal adjuvant treatment of patients whose tumors have substantial LVSI can be debated.

In both PORTEC trials routine staging lymphadenectomy was not perfomed, in contrast to GOG#99. However, even after routine lymphadenectomy in GOG#99 recurrence was reduced with pelvic radiotherapy [12]. With two large randomized showing no survival benefit but increased morbidity, it is currently accepted that a staging lymphadenectomy is not indicated in low- and intermediate-risk EC [22, 23]. Available evidence points in the direction that (substantial) LVSI in the primary tumor serves as a surrogate marker for both (microscopically) involved lymph nodes and more distant disease spread. Pelvic EBRT offers a significant reduction in the risk of both pelvic nodal recurrence and vaginal recurrence in patients with risk factors, both with and without lymphadectomy. Patients with substantial LVSI who received NAT or VBT had a 5-year risk of pelvic regional recurrence of 25-30% that was reduced to 5% with EBRT. These patients were only 5% of all PORTEC-1 and -2 trial patients, and these may well be the small subgroup of patients with increased risk of pelvic and distant relapse justifying the use of EBRT as for them the benefits outweigh the risks [24, 25]. Given the increased risk of distant metastasis in cases with substantial LVSI, it seems logical to explore adjuvant systemic treatment in these patients. However, despite that adjuvant chemotherapy is increasingly employed in high-risk EC, there is no data showing a benefit of chemotherapy specifically for patients with (substantial) LVSI. Recently the results of the GOG#249 trial in stage I-II, high-intermediate and high-risk EC patients have been presented and showed no benefit of the combination of VBT and 3 adjuvant cycles of carboplatin/paclitaxel compared to EBRT alone [26] The results of the PORTEC-3 and GOG#258 trials comparing EBRT plus chemotherapy vs. EBRT alone and vs. chemotherapy alone, respectively, are therefore eagerly awaited.
It will be essential to determine which specific patients benefit from adjuvant therapy. In the near future, molecular factors may be used for selecting specific tumors that are sensitive for systemic therapies. In conclusion, substantial LVSI using a three-tiered scoring system (see Table 1 for detailed description) is the strongest independent prognostic factor for pelvic regional recurrence, distant metastasis and overall survival. Adjuvant EBRT should be considered for the small subgroup of stage I EC patients who have substantial LVSI, especially those with grade 3 tumors, and the role of systemic therapy should be determined.

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Supplementary table 1A. Patient characteristics by trial and after central review of pathology.

	Total (n=954)		PORTEC-1 (n=563)		PORTEC-2 (n=391)	
	N	%	N	%	N	%
Age						
Mean (range)	67,9 (41 – 90)		66,5 (41 – 90)		69,9 (46 – 88)	
<60	163	17,1	147	26,1	16	4,1
>60	791	82,9	416	73,9	375	95,9
Myometrial invasion						
<50%	293	30,7	228	40,5	65	16,6
>50%	661	69,3	335	59,5	326	83,4
Differentiation grade						
1	681	71,4	372	66,1	309	79,0
2	143	15,0	104	18,5	39	10,0
3	130	13,6	87	15,4	43	11,0
LVSI						
absent	882	92,5	535	95,0	347	88,7
present	72	7,5	28	5,0	44	11,3
Treatment received						
NAT	294	30,8	292	51,9	2	0,5
EBRT	466	48,8	271	48,1	195	49,9
VBT	194	20,3			194	49,6

LVSI: lymph vascular space invasion; NAT: no additional treatment; EBRT: external beam radiotherapy; VBT: vaginal brachytherapy.

CHAPTER 4

PROGNOSTIC IMPACT OF HISTOLOGICAL REVIEW OF HIGH-GRADE ENDOMETRIAL CARCINOMAS IN A LARGE DANISH COHORT

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Abstract

The aim of this study was to investigate the outcome of histological subtype review of high-grade EC and its prognostic impact in a large well-documented Danish nationwide cohort. From the Danish Gynecological Cancer Database (DGCD) 2005-2012 cohort we included 425 patients with an original diagnosis of high-grade EC, independent of histologic subtype. Of these, at least one haematoxylin and eosin (H&E) stained slide from 396 cases (93.2%) was available for review. The histologic subtype was reviewed by specialized gynecopathologists blinded to the original diagnosis and clinical outcome. Interobserver variability between original and revised histologic subtype was analysed using simple Kappa statistics. Hazard ratios (HR), recurrence free survival (RFS) and overall survival were calculated for original and revised subtypes, respectively.

Overall histologic subtype agreement was moderate (kappa=0.42) with the highest agreement for endometrioid-type EC (EEC; 75.5%) and serous-type EC (SEC; 63.8%). For clear cell carcinoma and un-/dedifferentiated EC, agreement was significantly lower; 30.1% and 33.3% respectively. Of the 396 reviewed cases, only two (0.5%) were re-classified as low-grade EEC upon revision. Interestingly, GR3 EEC had better RFS than SEC with stronger significance after revision, HR 2.36 (95% CI 1.43-3.89), p=0.001, compared to original diagnosis, HR 1.74 (95% CI 1.07-2.81), p=0.024.

In conclusion, this study confirmed that pathology review results in substantial shift in histological subtype in high-grade EC. After review, a stronger prognostic benefit for GR3 EEC as compared to other histological subtypes was observed. This work supports maintaining a low threshold for pathology revision of high-grade EC in clinical practice.

Introduction

The prognostic relevance of histologic subtype within high-grade endometrial carcinomas (EC) is poorly defined. It is however generally accepted that high-grade endometrioid-type (EEC, GR3) have a slightly better prognosis than the high-grade non-endometrioid ECs. For adjuvant treatment decisions, a risk stratification (e.g., low/intermediate/high-intermediate/high risk) is made, which relies on a combination of clinicopathological risk factors including The International Federation of Gynecology and Obstetrics (FIGO) stage, grade, age, lymphovascular space invasion (LVSI) and histologic subtype. FIGO stage III/IV disease is considered high-risk per definition, independent of any of the other factors. In stage I/II disease the risk assignment is stratified depending, among other factors, on grade and histotype [1]. For risk-assignment of a patient with stage I/II disease with a high-grade EC, histologic subtype is considered relevant: patients with FIGO stage IA myoinvasive grade 3 endometrioid-type EC (GR3 EEC) without substantial LVSI are considered intermediate risk, whereas myoinvasive stage IA non-endometrioid-type (non-EEC) are considered high risk. Similarly, FIGO stage IB GR3 EEC are high-intermediate risk, whereas FIGO stage IB non-EEC would be considered high-risk [1]. Therefore, in the context of stage I/II disease, distinguishing histologic subtype of a high-grade EC may have consequences for clinical management.

High-grade EC is an heterogenous group of tumours consisting of GR3 EEC and non-EECs including serous carcinoma (SEC), clear cell carcinoma (CCC), mixed epithelial carcinomas, de-/undifferentiated endometrial carcinomas (DEC) and uterine carcinosarcoma (UCS). Despite the apparently clear histological description of high-grade histologic subtypes in the WHO classification [2], it has now been well documented that significant interobserver variability exists, even among experts [3–7]. This is likely due of the morphologic heterogeneity of this disease, in which a significant number of cases are difficult to classify. Although in these ambiguous high-grade EC immunohistochemical markers may help (e.g., Napsin A for the diagnosis of CCC), these markers are frequently not conclusive [8, 9]. This is causing a problem for the clinical management of those stage I/II patients for which the risk assignment relies on histologic subtype.

Research groups aware of this problem invest significant amounts of time reviewing retrospective cohorts by specialized gynecopathologists to ensure uniformity in the research setting [7, 10–12]. In addition, this interobserver variability issue has resulted in the recommendation to apply a low threshold for pathology revision of high-grade EC in clinical practice, suggesting that experienced and specialized pathologists maybe in a better position to assign histologic subtype. The obvious downside of this practice is the time and costs involved, both in clinical and research setting. The consequences of this general practice are only poorly studied; hence it is worth to clarify the impact of possible changes on clinical outcome in relation to the revised diagnosis. Therefore, the aim of this study was to investigate

the effects of histological review of high-grade EC and its prognostic impact in a large national Danish cohort.

Materials and methods

The Danish Gynecological Cancer Database (DGCD) includes 4707 EC patients diagnosed between January 1, 2005 to December 31, 2012 [13]. The DGCD holds prospectively registered information about initial surgical and adjuvant treatment, pathology diagnosis and follow-up data [14]. From the DGCD 2005-2012 cohort we included 425 patients with an original diagnosis of high-grade EC (all histologic subtypes except uterine carcinosarcomas). Of these, at least one haematoxylin and eosin (H&E) stained slide from 396 cases (93.2%) could be retrieved for review (Figure 1).

These cases were originally diagnosed at 19 different pathology institutes distributed throughout Denmark. Distribution in age, original histologic subtype, stage, lymphovascular space invasion (LVSI) status and risk group according to ESMO-ESGO-ESTRO 2016 [15] are shown in Table 1.Follow-up data for Cox analyses and Kaplan-Meier curves were retrieved from the database, from the national patients file registry and patient's medical records. Missing data regarding recurrences were retrieved from the pathology reports in the Danish pathology database. Deaths were retrieved from the Danish Person Register and Cause of Death Register.

Pathology revision

The review was performed by four gynecopathologists (EEMP, ALC, VTHBMS and TB). Even though in some instances immunohistochemistry was used for the original diagnosis, the histology review for this study was performed with H&E slides only. The vast majority of cases included H&E slides from the hysterectomy specimen (394/396; 99.5%), but in two cases it was limited to an H&E of the endometrial biopsy (2/396; 0.5%). The average and median number of slides reviewed per case was 10.9 (range 1-70, median 10), and cases were equally and randomly distributed among the members of the reviewing group. Prior to final histologic subtype assignment, all cases with ambiguous morphology (68/396; 17.2%) were discussed by the review group together to reach consensus diagnosis. The review group was blinded to the original diagnosis and any of the other clinicopathological variables listed in Table 1. The pathology review focused on histologic subtype and did not include re-assessment of grade or FIGO stage. The review group also assessed LVSI extent in this study cohort, results of which will be published separately.

The cases included were originally diagnosed as high-grade carcinomas including GR3 EEC, SEC, CCC or un-/dedifferentiated carcinoma (DEC). For histologic subtype assignment the review group used the terminology of the WHO 2014 [2]. In a minority of cases, histology could not be assessed due to poor tissue fixation, too small tumour, or no remaining tumour in the available slides from the hysterectomy.

Table 1. Distribution in age, original histological type, FIGO stage, lymphovascular space invasion (LVSI) status and risk group according to ESMO-ESGO-ESTRO 2016 [15]

All patients	n	396
	Median	Range
Age at diagnosis (years)	69	43-94
Lower quartile (years)	63	
Upper quartile (years)	76	
	Median	Range
Follow-up time	8.5	5.1-13.0
Histological type	n	%
GR3 EEC	163	41.2
SEC	141	35.6
CCC	83	21.0
DEC	9	2.3
FIGO stage	n	%
Stage I	292	73.7
Stage II	31	7.8
Stage IIIc1	46	11.6
Stage IIIc2	19	4.8
Stage IV	8	2.0
Risk group	n	%
High risk	324	81.8
High-intermediate risk	72	18.2
LVSI	n	%
No	210	53.0
Yes	98	24.7
Unknown	88	22.2

SEC: Serous EC; CCC: Clear cell carcinoma; DEC: De-/undifferentiated EC; GR3 EEC: Grade 3 endometrioid-type EC

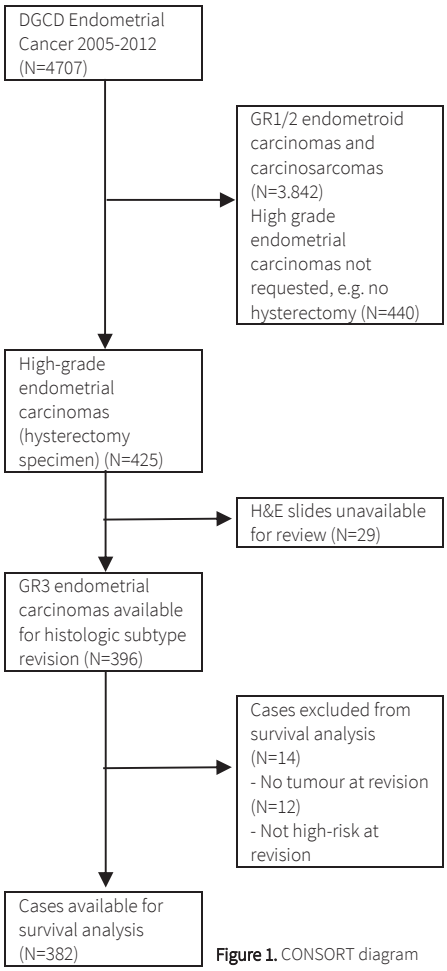

Figure 1. CONSORT diagram

Statistics

For statistical analysis regarding interobserver variability between original diagnosis and reviewed diagnosis we used eight categories as shown in Table 2, similar to a categorization made in two other studies, that were based on histological cell type or major/minor disagreement, respectively [3, 5]. Mixed cell carcinomas were categorized according to their high-grade component or to the major component in case of two high-grade components. Interobserver variability was analysed using simple Kappa statistics and calculated with 95% confidence limits. Furthermore, interobserver variability was stratified by original diagnosis from subspecialized or general institute and stage, respectively, and tested for differences with hypothesis of equal means. Calculations were done using SAS v.9.4 (SAS Institute, Cary, NC, USA).

For statistical analyses regarding clinical outcome, a predefined categorisation into four groups was used. This allowed for a comparison between GR3 EEC, SEC, CCC and other high-grade EC. The other group contained all other histological subtypes of high-grade EC, such as DEC and UCS. Recurrence free survival (RFS) was calculated from time of surgery to first recurrence, omitting patients dying from other causes than EC. Overall survival (OS) was calculated from time of surgery to death. The Kaplan-Meier method was used to calculate survival rates, p-values for Kaplan-Meier curves being based on log rank test. Hazard ratios were calculated with Cox regression analyses, where adjustments were made for age, comorbidity using ASA score, FIGO stage, lymph node resection and/or adjuvant treatment. GR3 EEC was used as reference. Cases that were not high-grade carcinoma at revision were omitted from calculations of RFS and OS. P-values for RFS and OS were calculated using adjusted Cox proportional hazards model. Calculations were done using STATA 11 (StataCorp, College Station, TX, USA).

Table 2. Categories for histological types

Histological type categories for interobserver variability	Original histological type	Revised histological type
SEC	SEC	SEC; Mixed SEC/EEC; Mixed SEC/CCC
CCC	CCC	CCC; Mixed CCC/EEC; Mixed CCC/SEC
DEC	DEC	DEC
GR3 EEC	GR3 EEC	GR3 EEC
EIN	N.A.	EIN
UCS	N.A.	UCS
MC	N.A.	MC
Cannot assess	N.A.	Poor tissue fixation; tumour too small or no tumour in available slides

SEC: serous EC; CCC: clear cell carcinoma; EEC: endometrioid type EC; DEC: de-/undifferentiated EC; GR3 EEC: grade 3 EEC; EIN: endometrioid intraepithelial neoplasia; UCS: uterine carcinosarcoma; MC: mucinous carcinoma

Results

The distribution of the original histologic subtypes and the revised histologic subtypes are shown in Table 3. Of a total of 396 high-grade EC, histology review could be performed on 384 (97%). These 384 cases were originally diagnosed as GR3 EEC (n=163; 41.2%), SEC (n=141;

35.6%), CCC (n=83; 21.0%) and un-/dedifferentiated carcinomas (n=9; 2.3%). This distribution changed substantially after review, including one additional category: GR3 EEC (n=181; 45.7%), SEC (n=133; 33.6%), CCC (n=38; 9.6%), DEC (n=17; 4.3%) and UCS (n=13; 3.3%). Only two cases were not considered to be high-grade EC on review (0.5%), but EIN (0.25%, n=1) and mucinous carcinoma (0.25%, n=1), respectively. In both these outlier cases, the available H&E slides were from representative tumour from the hysterectomy specimen. The original diagnosis of these two cases were GR3 EEC and CCC, respectively. Furthermore, 12 cases (3.0%) could not be revised; 10 due to lack of tumour in the available H&E slides and 2 due to insufficient fixation quality for assessment. The distribution of these cases is presented in Table 4.

Overall kappa value was 0,42. The highest concordance was obtained for GR3 EEC and SEC with 75.5% and 63.8%, respectively. For CCC and undifferentiated carcinoma, the concordance was considerably lower with 30.1% and 33.3%, respectively. The main histologic subtype shift was from SEC to GR3 EEC (26/43; 60.5%), followed by GR3 EEC to SEC (19/39; 48.7%). Interestingly, review of the 83 original CCC resulted in 29 GR3 EEC and 23 SEC, while only 25 remained CCC. Examples of CCC that were reclassified are shown in Figure 2.

Table 3. Original and revised histological types

Original histologic al type		Revised histological type									Total discrepant cases
		SEC	CCC	DEC	GR3 EEC	EIN	Cannot assess	UCS	MC	Total	
SEC	n	90	11	2	26	0	8	4	0	141	43
	%	63.8	7.8	1.4	18.4	0.0	5.7	2.8	0.0	100	
CCC	n	23	25	1	29	0	3	1	1	83	55
	%	27.7	30.1	1.2	34.9	0.0	3.6	1.2	1.2	100	
DEC	n	1	0	3	3	0	0	2	0	9	6
	%	11.1	0.0	33.3	33.3	0.0	0.0	22.2	0.0	100	
GR3 EEC	n	19	2	11	123	1	1	6	0	163	39
	%	11.7	1.2	6.8	75.5	0.6	0.6	3.7	0.0	100	
Total		133	38	17	181	1	12	13	1	396	

SEC: serous EC; CCC: clear cell carcinoma; EEC: endometrioid type EC; DEC: de-/undifferentiated EC; GR3 EEC: grade 3 EEC; EIN: endometrioid intraepithelial neoplasia; UCS: uterine carcinosarcoma; MC: mucinous carcinoma

Table 4. Distribution of cases that could not be revised

Original histological type	Reason not revised		
	No tumour (n)	Cannot assess (n)	Total (n)
SEC	7	1	8
CCC	3	0	3
DEC	0	0	0
GR3 EEC	0	1	1
Total	1	2	12

SEC: serous EC; CCC: clear cell carcinoma; EEC: endometrioid type EC; DEC: de-/undifferentiated EC; GR3 EEC: grade 3 EEC

Looking at concordance per stage, there were no statistically significant differences. Most of the patients were stage I (n = 292), and the distribution and type of discrepancies of stage I were completely in line with the overall results. For stage II–IV, numbers of patients were too small to

draw any conclusions, but we saw no obviously different tendencies. Also, there were no significant differences in concordance whether the original diagnosis was made at a general or subspecialized institute.

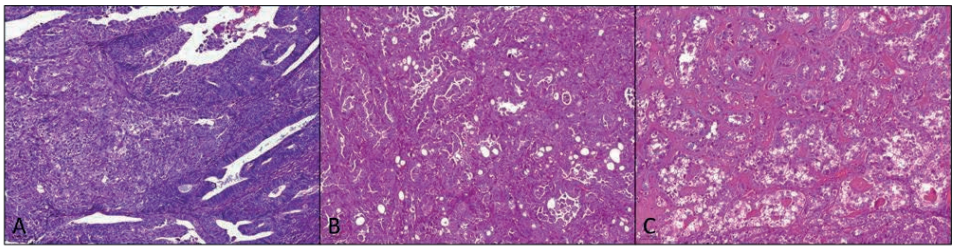

Figure 2. Original CCC that were re-classified as either GR3 EEC (A), SEC (B) or remained CCC (C)

Table 5. Five year overall survival and recurrence free survival, HR with 95% CI and *p*-values based on Cox proportional hazards model. GR3 EEC serves as reference

Overall five-year survival

	Original				Revision			
	%	*p*	HR	95% CI	%	*p*	HR	95% CI
GR3 EEC	66		1		71		1	
SEC	59	0,676	1,09	0,74 – 1,61	56	0,138	1,34	0,91 – 1,98
CCC	65	0,452	0,83	0,52 – 1,32	61	0,759	1,10	0,60 – 1,20
Other	22	0,078	2,10	0,92 – 4,78	40	0,002	2,41	1,39 – 4,16

Recurrence free five-year survival

	Original				Revision			
	%	*p*	HR	95% CI	%	*p*	HR	95% CI
GR3 EEC	79		1		83		1	
SEC	65	0,024	1,74	1,07 – 2,81	63	0,001	2,36	1,43 – 3,89
CCC	76	0,625	1,16	0,64 – 2,12	72	0,134	1,79	0,84 – 3,82
Other	60	0,174	2,35	0,69 – 8,06	55	<0,001	3,65	1,81 – 7,35

GR3 EEC serves as reference. SEC: Serous EC; CCC: Clear cell carcinoma; DEC: De-/undifferentiated EC; GR3 EEC: Grade 3 endometrioid-type EC; Other: Other types of high-grade EC.

Five-year survival, hazard rates and p-values based on Cox proportional hazards model for OS and RFS are shown in Table 5 and Kaplan-Meier curves for OS and RFS in Figure 3. The OS of patients originally diagnosed with GR3 EEC, SEC and CCC were not significantly different, and despite the shift in histologic subtypes after revision, there were no significant differences. However, patients with SEC had a poorer RFS than GR3 EEC with stronger significance after revision, HR 2.36 (95% CI 1.43-3.89), p=0.001, compared to original diagnosis, HR 1.74 (95% CI 1.07-2.81), p=0.024. Finally, patients with an EC falling under the "other" category, consisting of un-/dedifferentiated carcinoma and UCS after review, had significantly worse OS and RFS than those with GR3 EEC for revised diagnoses with HR 2.41 (95% CI 1.39-4.16; p=0.002) and HR 3.65 (95% CI 1.81-7.35; p<0.001), respectively, while there was no statistically significant difference for original diagnoses with HR 2.10 (95% CI 0.92-4.78; p=0.078) and HR 2.35 (95% CI 0.69-8.06; p=0.174), respectively.

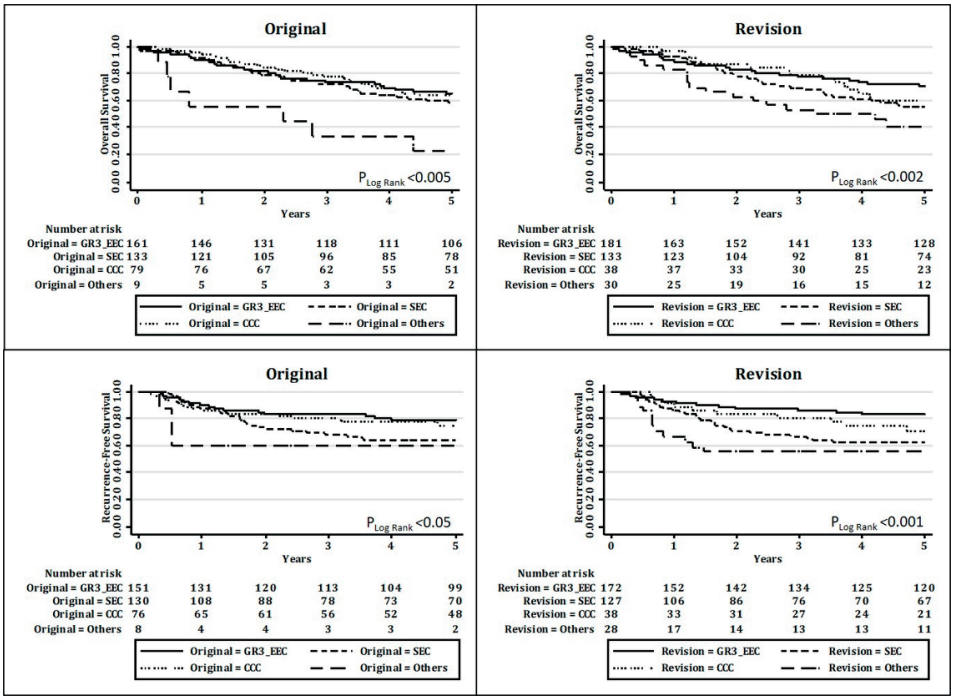

Figure 3. Kaplan-Meier curves for five-year overall and survival recurrence-free survival, original and revised diagnosis.
SEC: Serous EC; CCC: Clear cell carcinoma; DEC: De-/undifferentiated EC; GR3 EEC: Grade 3 endometrioid-type EC; Other: Other types of high-grade EC.

Discussion

We present an interobserver pathology study of a large nationwide high-grade EC cohort including well documented clinical outcome data. We were able to retrieve 90% of all high-grade EC cases and thereby the data presented are a good reflection of the true distribution of high-grade EC in Denmark.

It was re-assuring to find that after revision as much as 99.5% of cases were consistently diagnosed high-grade EC by specialized gynecopathologists, despite the fact that the original diagnosis was made by 19 different pathology institutes, subspecialized as well as general. However, this study showed once again that histological subtyping of high-grade EC is poorly reproducible. From a clinical management perspective, one may argue that this inconsistency in histological type assignment has limited consequences, as adjuvant treatment recommendations according to international guidelines [1] would be altered for a minority of patients. This mainly involves reallocation from GR3 EEC to non-EEC and vice versa in FIGO stage I/II. In Denmark, currently the only exception would be the indication for omentectomy in SEC and DEC, which is not considered to be relevant for patients with GR3 EEC. In other countries, other choices are made, why the impact of the observed diagnostic shift may vary per country.

The overall agreement of histologic subtype assignment in our high-grade EC cohort was just moderate with a kappa value of 0.42. This is in agreement with other studies with kappa values of 0.30-0.68 for high-grade EC [4, 5, 7, 10], illustrating the limited reproducibility of histological subtyping of high-grade EC. The highest reproducibility was obtained for GR3 EEC (75.5%) and serous EC (63.8%), respectively. In addition, 13 cases were reclassified as uterine carcinosarcomas upon revision. The higher number of revised histological types is likely a reflection of the lack of reproducible histologic subtype specific features. This appeared particularly problematic for the diagnosis of CCC, as CCC was the subtype with the worst reproducibility.

CCC often includes a mixture of architectural patterns and can be difficult to distinguish from variants of EEC and SEC. In the new WHO classification published in 2020 [16] it was stressed that strict adherence to architectural and cytological diagnostic criteria is required to optimize the diagnostic reproducibility of CCC. Adding an immunohistochemical panel of ER/PR, p53, NapsinA and HNF1Beta likely improves the correct diagnosis of CCC, but is not always helpful [8, 9]. Consequently, the WHO 2014 histology-based classification of EC is an insufficient basis for histotype-directed clinical treatment decisions and forms a poor basis for clinical trial inclusion. The WHO 2020 [16] introduced the molecular classification, which relies on the analysis of surrogate markers in order to identify the four subgroups analogous to the ones described by The Cancer Genome Atlas (TCGA) [17]. This novel classification has a strong prognostic value and higher reproducibility than the histology based classification [17–20] and therefore may be a better basis for future clinical trials [19]. Most of the data on the molecular EC classification is derived from analysis of EEC and SEC, however small series of CCC indicate that the molecular classification may also be applicable to CCC [21, 22].

Although the interobserver variability of high-grade EC diagnosis has been addressed in previous works, this is the first study to include an assessment of the impact of revision on RFS or OS. This is of obvious importance, as histologic classification systems are meant to serve as an important prognostic variable and guide treatment. The shift between the high-grade subtypes GR3 EEC, SEC and CCC at revision had no significant impact on overall survival. However, the group of GR3 EEC had better RFS with much stronger significance after revision compared to the original diagnosis. Furthermore, there were significantly poorer RFS and OS of the revised DEC and UCS. These findings support the most recent European guidelines which differentiates between GR3 EEC and non-endometrioid subtypes to assign risk groups and consequently different adjuvant treatment recommendations [1]. Therefore, our study builds on previous work and argues in favour of central pathology review for all high-grade EC in routine clinical practice.

This study is not without limitations. Due to the study design (selection of high-grade EC), there is an over-representation of serous carcinomas compared to the general EC population in Denmark where 70-80% are EEC and 10% are SEC according to the Danish national guideline

group [23], and therefore we cannot generalise our findings to low-grade EC. We note that previous studies analysing the interobserver reproducibility of histological diagnosis had a lower proportion of SEC [3, 4, 11, 12], however, their results did not differ substantially from the present work. Furthermore, due to our approach we did not adjust for stage in COX regression analysis, and therefore the role of stage in this context could not be addressed. Finally, for some cases only selected slides were available for review, possibly omitting the part of the tumour with the most representative morphology. This limitation is counterbalanced by our ability to retrospectively review cases with an average number of 10.9 H&E slides/case. Additionally, review diagnoses were solely based on H&E without any immunohistochemistry (IHC), although this would likely improve agreement, particularly in cases with ambiguous morphology [8, 9]. Therefore, an interesting future study would be to look at the value of a standard IHC marker panel on the interobserver variability of high-grade EC.

In conclusion, we confirmed the substantial interobserver variability in histologic subtyping high-grade EC in a large Danish population cohort. All but two cases remained high-grade, however a major shift in histologic subtype was observed, most significant for CCC. After revision, endometrioid-type high-grade carcinomas had strongly significant better RFS than SEC, and better RFS and OS than the group of DEC and UCS, but otherwise the shift between the different subtypes of high-grade EC did not change the outcome in terms of RFS or OS. We suggest keeping a low threshold for pathology revision of high-grade EC in clinical practice and foresee that molecular classification of high-grade EC will be a better fundament for future clinical management as it is built upon more objective parameters.

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CHAPTER 5

SUBSTANTIAL LYMPHOVASCULAR SPACE INVASION IS AN ADVERSE PROGNOSTIC FACTOR IN HIGH-RISK ENDOMETRIAL CANCER

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Abstract

Approximately 15% of patients with endometrial cancer present with high-risk disease (HREC). Moreover, assessing the extent of lymphovascular space invasion (LVSI) may provide prognostic insight among patients with HREC. The aim of this study was to determine whether the extent of LVSI can serve as a prognostic factor in HREC.

All cases of ESMO-ESGO-ESTRO 2016 classified HREC in the Danish Gynecological Cancer Database (DGCD) diagnosed from 2005-2012 were reviewed for the presence and extent of LVSI (categorized using a three-tiered definition). We used Kaplan-Meier analysis to calculate actuarial survival rates, both adjusted and unadjusted Cox regression analyses were used to calculate the proportional hazard ratio (HR).

A total of 376 patients were included in our analysis. Among 305 patients with stage I/II HREC, 8.2% and 6.2% had focal or substantial LVSI, respectively, compared to 12.7% and 38.0% of 71 patients with stage III/IV HREC, respectively. Moreover, the estimated 5-year recurrence-free survival rate was significantly lower among patients with substantial LVSI compared to patients with no LVSI for both stage I/II (HR: 2.8; p=0.011) and stage III/IV (HR: 2.9; p=0.003) patients. Similarly, overall survival was significantly lower among patients with substantial LVSI for both stage I/II (HR: 3.1; p<0.001) and stage III/IV (HR: 3.2; p=0.020) patients.

In patients with HREC, substantial LVSI is an independent adverse prognostic factor for lymph node and distant metastases, leading to reduced survival. Thus, the extent of LVSI should be incorporated into routine pathology reports in order to guide the appropriate choice of adjuvant treatment.

Introduction

The majority of women with endometrial cancer present with either low-risk or intermediate-risk disease according to ESMO-ESGO-ESTRO 2016 risk classification. However, approximately 15% of patients present with high-risk disease (HREC), including stage IB grade 3 endometrioid endometrial cancer (EEC), stage II-III EEC, and stage I-IVA non-endometrioid endometrial cancer tumor types[1, 2], and these patients generally have a higher risk of disease recurrence and reduced overall survival [3-6].

Lymphovascular space invasion (LVSI) is a well-known adverse prognostic factor in endometrial cancer and is associated with an increased risk of lymph node (LN) involvement, as well as distant metastases [7-11]. In patients with high-intermediate-risk EEC, assessing the extent of LVSI using a three-tiered scoring system revealed that substantial LVSI was the strongest independent factor for predicting pelvic LN recurrence [12]. In addition, retrospective and case-control studies have confirmed the prognostic effect of assessing the extent of LVSI on both recurrence and survival [13, 14]; thus, LVSI assessment is currently being incorporated into international guidelines for reporting diagnostic pathology and is used in risk stratification models [1]. Nevertheless, whether substantial LVSI is an independent risk factor in HREC is currently unknown, particularly among stage IIIC patients with LN metastases at diagnosis, as these patients are already at a higher risk of developing recurrent disease [6].

The aim of this study was to determine the prognostic value of assessing the extent of LVSI in a large population-based cohort of patients with HREC.

Materials and Methods

The Danish Gynecological Cancer Database (DGCD) includes nearly all patients with endometrial cancer diagnosed in Denmark from January 1, 2005 through December 31, 2012. The DGCD contains prospectively registered information regarding the initial surgical and adjuvant treatment, pathology-based diagnosis, and follow-up data. Any missing data and histology-confirmed recurrences were retrieved from the pathology reports in Patobank, the Danish National Pathology Database. Non-histology verified recurrences were retrieved from medical records based on gynecological or radiological examination. The Danish National Patient Register (*Landspatientregistret*) and the patients' individual medical records were used to retrieve information regarding adjuvant treatment, recurrence, and missing data. Information regarding patient death was retrieved from The (Danish) Central Person Registry.

A total of 376 hysterectomy specimens of ESMO-ESGO-ESTRO 2016 classified HREC [1] were available for assessment of LVSI (Supplementary Figure S1). The patients' clinical data were originally collected by Ørtoft *et al.* [3, 15] in order to evaluate the introduction of systematic lymphadenectomy in Denmark. All pathology slides were obtained from local hospitals for a central pathology review with respect to tumor type, tumor grade (EEC only), and disease stage [3]. Carcinosarcomas were excluded.

LVSI was assessed by four trained pathologists (authors EEMP, ALC, VTHBMS and TB) using hematoxylin and eosin (H&E)–stained slides, without immunohistochemistry. Ethical approval to perform revision, denied access of the study pathologists to the original pathology reports. The presence of LVSI was defined as the unequivocal presence of non-necrotic tumor cells or tumor cell clusters in an endothelial lined space within the uninvolved myometrium (i.e., beyond the invasive border of the tumor). In tumors that were determined to be positive for LVSI, the extent of LVSI was further specified as either focal or substantial using a qualitative approach as described previously [12]. In brief, focal LVSI was defined as a "single focus" of LVSI, and substantial LVSI was defined as the "multifocal/diffuse" presence of LVSI [12]. Intra-tumoral LVSI was not considered. In case of artefacts like smear, retraction or mimics like MELF-type invasion, there was reticence to diagnose LVSI. Consensus among the four pathologists was required in order to classify a case as having substantial LVSI. In addition, the following quantitative features were also recorded for each case: 1) the total number of H&E-stained slides from the uterus available for review, 2) the number of tumor-containing H&E-stained slides, 3) the number for H&E-stained slides showing the presence of LVSI, and 4) the number of involved vessels in each H&E-stained slide showing LVSI. In case of any doubt about (artificial) LVSI, tumor type, tumor grade or disease stage, cases were discussed with all study pathologists present at a multiheaded microscope where also all cases with substantial LVSI were discussed until consensus was reached.

Calculations on the number of slides with done using *t* test. Recurrences were defined as local, pelvic/para-aortic LN, distant LN, and/or distant metastases. Recurrence-free survival (RFS) was calculated as either the interval between the date of surgery and the date of first recurrence or—in event-free patients—until death or the date of last follow-up, censoring patients who died from a cause other than endometrial cancer. Cancer-specific survival (CSS) was calculated as the interval between the date of surgery and death due to endometrial cancer, censoring patients who died from a cause other than endometrial cancer. Overall survival (OS) was calculated as the interval between the date of surgery and the date of death due to any cause. The Kaplan-Meier method was used to calculate the actuarial survival rate. Adjusted and unadjusted Cox regression analyses were used to calculate the proportional hazard ratio; adjustments were made for the following clinically relevant but potentially confounding prognostic factors: age, comorbidity using the American Society of Anesthesiologists (ASA) score, lymphadenectomy, and/or adjuvant treatment (radiotherapy and/or chemotherapy). Since only HREC were included, no correction for grade and depth of myometrial invasion was performed. Data were analyzed using Stata 11 (StataCorp LLC, College Station, TX), and differences with a two-sided p-value ≤ 0.05 were considered statistically significant.

Results

Study population

A total of 4707 patients with endometrial cancer were diagnosed from 2005 through 2012 and

registered in the DGCD. After central pathology review of 623 cases and excluding patients with low- or intermediate-risk endometrial cancer, a total of 376 HREC cases were included in our LVSI analysis (Supplementary Figure S1). During the initial central review, 68 of the 376 HREC cases (18%) were downgraded from high-risk to high-intermediate risk due to changes in the histological type; however, these cases were still included in our analysis. The median number of H&E-stained slides available for assessing LVSI was 9 (range: 1-70) and the mean was 6 (SD 3,1) respectively. For 15 (4,0%) cases only 1 H&E was available for LVSI assessment.

The patient characteristics are summarized in Table 1. Systematic pelvic lymphadenectomy was performed in 266 patients (71%), including 195 stage I/II patients. In 172 of the 195 stage I/II patients (88%), lymphadenectomy was limited to pelvic LN regions; the remaining 23 patients also underwent a para-aortic lymphadenectomy. A total of 267 patients did not receive adjuvant treatment; 255 of these patients (96%) had stage I/II disease. Of 71 LN-positive patients, 59 (83%) received adjuvant treatment; 47 of these 59 patients (80%) received chemotherapy alone, 7 (12%) received external beam radiotherapy (EBRT) alone, and 5 (8.5%) received both chemotherapy and EBRT.

LVSI

Among the 376 patients who were evaluated for LVSI, 80 (21%) had evidence of LVSI visible in at least one H&E-stained slide. Among the 305 patients with stage I/II disease, 44 (14%) had either focal (25 cases; 57%) or substantial (19 cases; 43%) LVSI. Among the 71 patients with stage III/IV disease, 36 (51%) had either focal (9 cases; 25%) or substantial (27 cases; 75%) LVSI.

Recurrence

An analysis of the five-year recurrence rate revealed that substantial LVSI was a significant risk factor for overall recurrence, pelvic/para-aortic LN recurrence, and distant LN recurrence—but not local recurrence and distant metastases—among patients with stage I/II disease (Table 2). The adjusted hazard ratio (HR) between substantial LVSI compared to no LVSI was the strongest for pelvic and para-aortic LN recurrence (HR: 4.8; 95% CI: 1.76-13.3). In contrast, focal LVSI was not a significant risk factor for any site of recurrence, and neither focal nor substantial LVSI was a significant risk factor for any site of recurrence among patients with stage III/IV disease.

Prognosis

The estimated five-year OS rate for all patients with stage I/II disease was 68% (95% CI: 62-72%). When these patients were stratified based on LVSI status, 5-year OS was 42% among patients with substantial LVSI compared to 70% among patients with no LVSI (HR: 3.1, 95% CI: 1.6-5.9, $p<0.001$). Among the patients with stage III/IV disease, the estimated 5-year OS was 44% (95% CI: 32-55%); when stratified for LVSI status, OS was 22% among patients with LVSI compared to 57% among patients with no LVSI (HR: 3.2; 95% CI: 1.5-6.5, $p<0.05$). Similarly, substantial LVSI was a major risk factor for reduced 5-year CSS and RFS among both patients with stage I/II disease and patients with stage III/IV disease.

Table 1: Summary of patient characteristics, stratified by disease stage and LVSI status.

		All patients		Stage I and II								Lymph node metastases							
				All		No LVSI		Focal		Substantial		All		No LVSI		Focal		Substantial	
		N=376		N=305		N=261 (85,6%)		N=25 (8,2%)		N=19 (6,2%)		N=71		N=35 (42,3%)		N=9 (12,7)		N=27 (38%)	
			SD		SD		SD		SD		SD		SD		SD		SD		SD
	Follow-up (years)	8,55	2,1	8,6	2,2	8,6	2,1	8,3	2,5	9,0	2,4	8,5	1,9	8,5	1,9	8,7	2,1	8,4	2,0
	Age at diagnosis	69	9,7	70	9,8	70	9,8	70	11,4	69	8,5	67	9,0	66	10,1	68	4,7	67	8,6
	ASA	1,8	0,6	1,8	0,6	1,8	0,6	1,7	0,7	1,9	0,6	1,8	0,5	1,8	0,5	1,8	0,7	1,7	0,5
		n	%	n	%	n	%	n	%	n	%	n	%						
Stage	I-II		81,1	305		261	85,6	25	8,2	19	6,2								
	IIIc-IV		18,9									71		35		9		27	
Histo-logy	EEC GR3		38,0	120	39,3	104	39,9	6	24,0	10	52,6	23	32,4	11	31,4	4	44,4	8	29,6
	Non-EEC		62,0	185	60,7	157	60,1	19	76,0	9	47,4	48	67,6	24	68,6	5	55,6	19	70,4
Risk group	High-Intermediate	68	18,1	68	22,3	60	22,9	4	16,0	4	21,1								
	Stage I	208	55,5	208	68,2	176	67,4	19	76,0	13	68,4								
	Stage II	29	7,7	29	9,5	25	8,2	2	8,0	2	10,5								
	Stage IIC and IV	71	18,9									71		35	49,3	9	12,7	27	38
Lymph-adenectomy	No	110	29,3	110	36,9	95	36,4	10	40,0	5	26,3	0							
	Pelvic	216	57,5	172	54,6	146	55,9	13	52,0	13	68,4	44	62,0	24	68,6	5	55,6	15	55,6
	Para-aortic	1	0,3									1	1,4	1	2,9				
	Pelvic & para-aortic	49	13,0	23	7,5	20	7,7	2	8,0	1	5,3	26	36,6	10	28,6	4	44,4	12	44,4
Adjuvant therapy	No	267	71,0	225	83,6	218	83,5	23	92,0	14	73,7	12	16,9	5	14,3	2	22,2	5	18,5
	EBRT	33	8,8	21	6,9	17	6,5	1	4,0	3	15,8	12	16,9	8	22,9	2	22,2	2	7,4
	Chemo	82	21,8	30	9,8	27	10,3	1	4,0	2	10,5	52	73,2	25	71,4	5	66,6	21	77,8

SD, standard deviation; ASA, American Society of Anesthesiologists physical status classification system; EEC, endometrioid endometrial cancer; LN, lymph node; EBRT, external beam radio therapy.

Table 2. Summary of the 5-year recurrence rate relative to date time of surgery, stratified by disease stage and LVSI status.

		Stage I/II (n=305)			Stage III/IV, pN1 (n=54)		
		No LVSI (n=261)	Focal LVSI (n=25)	Substantial LVSI (n=19)	No LVSI (n=32)	Focal LVSI (n=6)	Substantial LVSI (n=16)
Overall recurrence rate	5-YRR	21,6	23,0	44,3	40,8	33,3	65,1
	HR		1,1	2,8		0,5	2,1
	95% CI		0,43-2,78	1,26-6,24		0,10-3,10	0,81-5,31
	P		0,849	0,011		0,496	0,130
Local	5-YRR	12,0	23,0	25,5	17,1	0,0	37,0
	HR		2,0	2,7			3,5
	95% CI		0,74-5,22	0,94-7,96			0,76-16,20
	P		0,177	0,065		1,000	0,109
Pelvic / para-aortic LN	5-YRR	8,0	15,1	34,3	17,1	20,0	46,7
	HR		2,1	4,8		1,2	3,7
	95% CI		0,60-7,45	1,76-13,3		0,09-14,28	0,92-14,68
	P		0,242	0,002		0,911	0,065
Distant LN	5-YRR	4,0	5,3	15,2	17,1	0,0	26,7
	HR		1,7	4,6			2,0
	95% CI		0,21-13,47	0,95-21,92			0,40-10,32
	P		0,632	0,058		1,000	0,395
Distant metastases	5-YRR	18,5	19,9	40,6	40,8	33,3	58,1
	HR		1,1	2,8		0,7	1,7
	95% CI		0,39-3,13	1,18-6,64		0,12-4,17	0,62-4,39
	p		0,848	0,019		0,692	0,314

5-YRR: 5 year recurrence rate, HR: hazard ratio, 95% CI: 95% confidence interval, LN: lymph node
Notes: Stage I/II includes patients with and without lymphadenectomy at time of primary surgery. Stage III/IV, lymph node–positive patients with progressive disease early after primary surgery were excluded from the analysis (n=17 patients). Overall recurrence refers to any recurrence regardless of site. Local refers to non-nodal recurrence in the vagina, bladder, and/or rectum. Distant LN refers to lymph node recurrence in a site other than the pelvic or para-aortic lymph nodes. Distant metastasis refers to non-nodal recurrence in the abdomen or extra-abdominal site. HR adjusted for significant and/or potential confounders by multivariate analysis, including age, ASA score, lymph node resection, adjuvant radiotherapy, and/or chemotherapy

In contrast, focal LVSI was not a significant risk factor for OS, CSS, or RFS. The 5-year OS, CSS, and RFS rates stratified by LVSI status and disease stage are summarized in Table 3 and Kaplan-Meier curves are shown in Figure 1 (for OS) and Figure 2 (for RFS).

Subgroup analyses

Among the 305 patients with stage I/II disease, 195 underwent a lymphadenectomy and were histologically confirmed to have no LN metastases (pN0), and the remaining 110 patients were determined to have no LN metastases based on clinical findings. Patients who had stage I/II disease and substantial LVSI had significantly higher rates of overall recurrence, pelvic/para-aortic LN, and distant metastasis. However, similar 5-year recurrence rates were obtained from a subgroup analysis of the 195 patients with stage I/II pN0 disease.

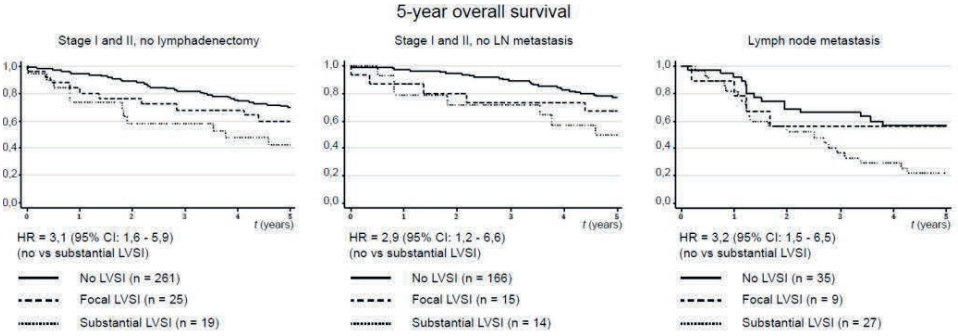

Figure 1. Kaplan-Meier curves of 5-year overall survival for patients in the indicated patient groups.

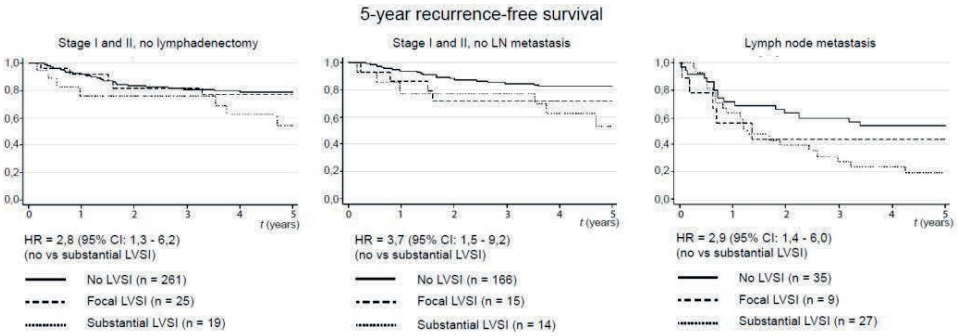

Figure 2. Kaplan-Meier curves of 5-year recurrence-free survival for patients in the indicated patient groups.

Patients who had stage I/II disease and focal LVSI did not have higher rates of recurrence; in the subgroup of stage I/II pN0, these patients had a significantly higher rate of local recurrence rate in cases with focal LVSI compared to cases with no LVSI (HR: 3.7, 95% CI: 1.18-11.50). These results are summarized in Supplementary Table S1.

We also performed a subgroup analysis of patients with stage I/II disease stratified by histological tumor type (EEC versus NEEC) and found that substantial LVSI was a significant risk factor for local recurrence only among patients with EEC; however, it should be noted that this analysis was based on only 9 patients with focal LVSI and 10 patients with substantial LVSI. In contrast, substantial LVSI was a significant risk factor for pelvic/para-aortic LN recurrence (HR: 4.1), distant LN recurrence (HR: 9.7), distant metastases (HR: 3.2), reduced OS (HR: 5.0) and CSS (HR: 3.7), among patients with NEEC (Supplementary Table S2 and supplementary figure S1).

Discussion

By analyzing a relatively large, well-documented patient cohort, we found that the presence of substantial LVSI is an independent adverse prognostic factor in patients with high-risk endometrial cancer. Specifically, we found that substantial LVSI was more prevalent among patients with stage III/IV disease. Moreover, substantial LVSI was associated with significantly

Table 3. Summary of the 5-year survival rate expressed relative to the date of surgery, stratified by disease stage and LVSI status.

		All (n=305)		No LVSI (n=261)		Focal LVSI (n=25)		Substantial LVSI (n=19)	
		% 5Y survival (95% CI)		% 5Y survival (95% CI)					
Stage I/II	OS	68	62-72	70	64-75	60	38-76	42	20-62
	CSS	81	76-86	82	77-87	86	63-95	61	33-80
	RFS	77	72-81	78	73-83	77	53-90	54	27-75
		% 5-year survival		% 5-year survival					
Stage III/IV, pN1	OS	44	32-55	57	39-72	56	20-80	22	9-39
	CSS	46	34-57	60	42-74	56	20-80	23	10-41
	RFS	40	29-51	54	37-69	44	14-72	20	7-37

		Focal vs no LVSI			Substantial vs no LVSI		
		HR	95% CI	*p*	HR	95% CI	*P*
Stage I/II	OS	1,7	0,9-3,3	0,128	3,1	1,6-5,9	<0,001
	CSS	0,9	0,3-3,1	0,907	2,8	1,2-6,8	0,020
	RFS	1,1	0,4-2,8	0,849	2,8	1,3-6,2	0,011
Stage III/IV, pN1	OS	1,0	0,3-3,7	0,070	3,2	1,5-6,5	0,020
	CSS	1,2	0,3-4,2	0,817	3,2	1,6-7,0	0,002
	RFS	1,3	0,4-4,1	0,649	2,9	1,4-6,0	0,003

OS, overall survival; CSS, cancer-specific survival; RFS, recurrence-free survival; HR, hazard ratio adjusted for significant and/or potential confounders by multivariate analysis, including age, ASA score, lymph node resection, adjuvant radiotherapy, and/or chemotherapy; 95% CI, 95% confidence interval.

lower rates of recurrence-free survival, cancer-specific survival, and overall survival compared to patients with no LVSI; in contrast, focal LVSI was not associated with the risk profile among these patients, confirming that the extent of LVSI plays an important role in determining outcome. Together with our novel finding that substantial LVSI was an independent adverse prognostic factor for survival in patients with stage III/IV disease, our results indicate that substantial LVSI is a strong predictor of recurrence and survival among patients with HREC regardless of disease stage, even in patients with documented lymph node metastases.

The presence of LVSI in endometrial cancer is a well-known risk factor repeatedly reported to be associated with poor outcome, including reduced OS, CSS, and RFS [8-10, 16-18]. Moreover, a recent nationwide Swedish population–based study by Stålberg *et al.* [18] found that LVSI was the strongest prognostic factor for LN metastases and was independently associated with decreased survival among patients with EEC. Nevertheless, assessing the extent of LVSI, rather than simply reporting the presence or absence of LVSI, is a relatively new concept. Using a three-tiered qualitative approach (i.e., no LVSI, focal LVSI, or substantial LVSI), we previously reported that substantial LVSI was the strongest independent predictor of locoregional recurrence, distant metastases, and OS in patients with intermediate-risk endometrial cancer [12]. We also recently reported that this three-tiered classification is reproducible in an interobserver study addressing diagnosis and assessment of LVSI extent in patients with endometrial cancer, reaching a considerable level of agreement [19]. Using this same method in the current study confirms that the presence of substantial LVSI is an independent risk factor

among women with HREC, who otherwise already have a relatively poor prognosis. Importantly, the hazard ratios associated with substantial LVSI among patients with high-risk endometrial cancer are consistent with our previous results on intermediate-risk patients [12]. Moreover, our results add to the results obtained in a retrospective study by Winer *et al.* [20], who found that extensive LVSI (defined by the authors as the involvement of ≥3 vessels) was an independent prognostic factor for overall survival among patients with stage I/II serous type endometrial cancer.

The latest update of the guideline for EC management by ESGO-ESTRO-ESP [21] has made changes to the prognostic risk groups by incorporation molecular classification and extent of LVSI. As a consequence, a part of the cases in the current study would be assigned to the high-intermediate risk group according to the new classification. Nevertheless, substantial LVSI is acknowledged as a risk factor for pelvic and para-aortal LN recurrence and as a consequence EBRT should be considered. The benefit of added chemotherapy was established for patients with serous endometrial cancer or stage III endometrial cancer to increase both failure-free survival and overall survival [22]. However, little is known how this relates to LVSI status, let alone extent of LVSI. Although the guidelines are based on clinical trials in which LVSI status is often known, the extent of LVSI has not been reported, and the effect of adjuvant chemotherapy on the risk of distant metastases among patients with endometrial cancer and substantial LVSI remains unknown. By incorporating substantial LVSI in the latest ESGO-ESTRO-ESP guideline, the reporting of the extent of LVSI is being encouraged which offers possibilities to study the effect of adjuvant chemotherapy on recurrence in patients with substantial LVSI in the future.

Between 2005 and 2012, systematic lymphadenectomy was introduced as part of the standard management and serves primarily as a diagnostic procedure. Here, we found that both LVSI status and recurrence rate were largely similar among patients with stage I/II disease regardless of whether surgical lymph node staging was performed. The similarity in recurrence rates suggests that clinical staging is relatively accurate, consistent with the results of two large randomized trials involving patients who underwent systematic pelvic lymphadenectomy [23, 24]. We did find a significant risk for local recurrence for patients with focal LVSI in stage I/II pN0. However, this finding is based on small numbers of events and patients and therefore deserves caution.

This study has several strengths. First, the data were retrieved from a national population-based registry, resulting in a relatively large, well-documented cohort for which extensive follow-up data were available, thus ensuring reliable data regarding recurrence and survival, as well as detailed information regarding LN status. Second, LVSI status was reviewed independently by four trained pathologists using a large number of slides prepared from the hysterectomy specimens. Finally, the majority of patients (71%) with stage I/II disease did not receive adjuvant therapy, allowing us examine the putative effects of LVSI independent of adjuvant treatment.

Despite these strengths, this study also has several limitations that warrant discussion. First, the patients with HREC were originally selected (using local pathology reports) from the DGCD in order to evaluate the introduction of systematic lymphadenectomy in Denmark, which may have led to a relative underrepresentation of patients with stage II, IIIa, and/or IIIb disease. Second, the central pathology review resulted in the re-classification of 18% of patients with stage I disease from high-risk to high-intermediate risk due to changes in the histological type. Finally, although the majority of cases had a relatively high number of H&E-stained hysterectomy slides, only one slide was available for 15 patients which may have resulted in an underestimation of LVSI positive cases. Although there is no evidence providing guidance on the minimum number of H&Es to be assessed to avoid under-reporting, we recommend to include at least one block per cm of tumor.

One caveat to the qualitative nature in which LVSI assessment was performed in our study is that this to some extent limits clinical implementation due to interobserver variability. This interobserver variability may partly explain the differences in the reported prevalence of substantial LVSI [12, 25, 26], although some of these differences can also be explained by study cohort composition. However, we acknowledge that borderline cases exist in which the qualitative nature of this approach will result in variability in the interpretation of focal versus substantial LVSI. Furthermore, correct interpretation of true LVSI versus artifactual displacement remains another challenge and may also impact reported prevalence. Intriguingly, despite all of this, the prognostic power of substantial LVSI is generally found across many independent cohorts [12, 18, 20, 25]. We foresee that the increased attention on this topic will eventually result into international consensus, which through training will lead to improved agreement among pathologists. To further support progress in this field a quantitative definition for what is clinically relevant LVSI extent is much needed. In our companion paper by Peters *et al*. (27), we used the cohorts available to us to address this specific point.

In conclusion, we report that substantial LVSI, but not focal LVSI, is an independent significant risk factor for survival in patients with high-risk endometrial cancer. These results both support and validate previous findings and underscore the clinical value of assessing the extent of LVSI in routine pathological testing. Finally, since extent of LVSI has recently been incorporated in (European) clinical guidelines for EC management, we recommend reporting of extent of LVSI in EC pathology reports [21].

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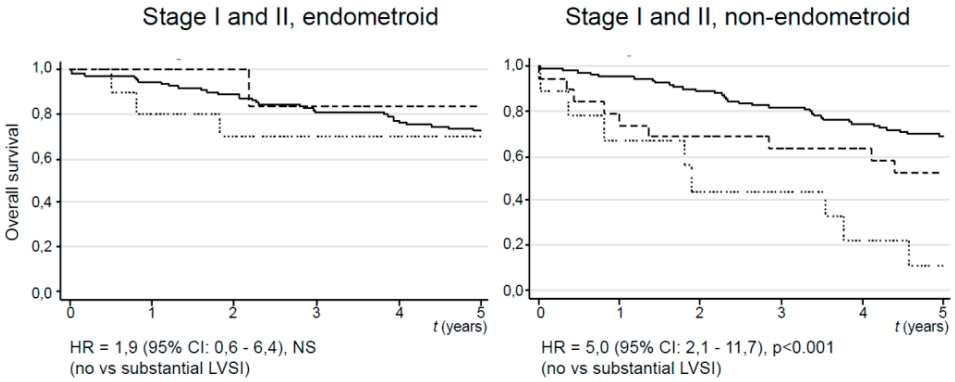

Supplementary Figure S1: 5-year recurrence and 5-year survival rates for patients with stage I/II disease, stratified by histological subtype (EEC vs. NEEC) and LVSI status. Notes: 5-year recurrence and 5-year survival are relative to the date of surgery. Overall refers to any recurrence regardless of site.

Supplementary Table S1. Summary of recurrences for patients with stage I/II disease and patients with stage I/II pN0 disease, stratified by LVSI status.

		Overall recurrence rate				Local				Pelvic/para-aortic LN			
All stage I/II (n=305)	N	5YRR (%)	HR	95% CI	p	5YRR (%)	HR	95% CI	p	5YRR (%)	HR	95% CI	p
No LVSI	261	21.6				12.0				8.2			
Focal LVSI	25	23.0	1.1	0.43-2.78	0.849	23.0	2.0	0.74-5.22	0.177	15.1	2.1	0.60-7.45	0.242
Substantial LVSI	19	44.3	2.8	1.26-6.24	0.011	25.5	2.7	0.94-7.96	0.065	34.3	4.8	1.76-13.3	0.002
Stage I/II, **pN0** (n=195)		5YRR (%)	HR	95% CI	p	5YRR (%)	HR	95% CI	p	5YRR (%)	HR	95% CI	p
No LVSI	166	17.8				9.0				7.9			
Focal LVSI	15	28.2	1.9	0.65-5.45	0.240	28.0	3.7	1.18-11.50	0.025	15.2	2.4	0.51-10.83	0.271
Substantial LVSI	14	46.1	3.7	1.47-9.17	0.005	24.0	3.1	0.87-11.19	0,081	39.7	7.8	2.55-23.84	<0.001

		Distant LN				Distant metastases			
All stage I/II (n=305)	N	5YRR (%)	HR	95% CI	p	5YRR (%)	HR	95% CI	p
No LVSI	261	4.0				18.5			
Focal LVSI	25	5.3	1.7	0.21-13.5	0.632	17.9	1.1	0.39-3.13	0.848
Substantial LVSI	19	15.2	4.6	0.95-21.9	0.058	40.6	2.8	1.18-6.64	0.019
Stage I/II, **pN0** (n=195)		5YRR (%)	HR	95% CI	p	5YRR (%)	HR	95% CI	p
No LVSI	166	3.4				15.6			
Focal LVSI	15	8.7	4.3	0.47-38.7	0.199	28.2	2.3	0.79-6.73	0.129
Substantial LVSI	14	17.7	6.0	0.99-36.2	0.051	46.1	4.5	1.77-11.47	0,002

HR, hazard ratio adjusted for significant and/or potential confounders by multivariate analysis, including age, ASA score, lymph node resection, adjuvant radiotherapy, and/or chemotherapy; 95% CI, 95% confidence interval; 5YRR, five year recurrence rate.

Notes: Overall recurrence refers to any recurrence regardless of site. Local refers to non-nodal recurrence in the vagina, bladder, and/or rectum. Distant LN refers to lymph node recurrence in a site other than the pelvic or para-aortic lymph nodes. Distant metastasis refers to non-nodal recurrence in the abdomen or extra-abdominal site.

Supplementary Table S2: Subgroup analyses of 5-year recurrence and 5-year survival rates for patients with stage I/II (EEC vs. NEEC)

	EEC stage I/II (n=120)			*Focal vs no LVSI*			*Substantial vs no LVSI*		
	No LVSI (n =104)	Focal (n=6)	Substantial (n=10)	HR	95% CI	*p*	HR	95%CI	*p*
Overall (%)	15,3	16,6	32,3	1,0	0,1-8,2	0,998	3,4	0,95-	0,059
Local (%)	6,1	16,7	21,1	3,5	0,3-36,3	0,298	6,5	1,2-34,0	0,028
Pelvic/para-aortic LN (%)	1,0		23,8						
Distant LN (%)	4,2								
Distant metastasis (%)	10,8	16,7	23,8	1,4	0,2-11,8	0,746	2,8	0,6-13,5	0,192
5-yr OS rate (%)	72	83	70	0,9	0,1-6,6	0,882	1,9	0,6-6,4	0,302
5-yr CSS rate (%)	89	83	78	1,9	0,2-16,6	0,574	2,7	0,6-12,8	0,220
5-yr RFS rate (%)	85	83	69	1,0	0,1-8,2	0,998	3,4	1,0-12,4	0,059

	NEEC stage I/II (n=185)			*Focal vs no LVSI*			*Substantial vs no LVSI*		
	N0 LVSI (n=157)	Focal (n=19)	Substantial (n=9)	HR	95% CI	*p*	HR	95% CI	*p*
Overall (%)	26,0	25,4	62,5	1,1	0,4-3,1	0,867	2,8	0,9-8,5	0,066
Local (%)	16,0	25,5	38,1	1,7	0,6-5,3	0,327	1,8	0,4-8,4	0,434
Pelvic/para-aortal LN (%)	13,0	20,8	46,4	2,1	0,6-7,5	0,277	4,1	1,0-16,2	0,044
Distant LN (%)	3,8	7,4	38,1	2,9	0,3-28,1	0,364	9,7	1,4-68,3	0,023
Distant metastasis (%)	23,4	18,0	62,5	0,9	0,3-3,1	0,902	3,2	1,0-9,9	0,043
5-yr OS rate (%)	69	53	11	2,0	0,9-4,2	0,072	5,0	2,1-11,7	<0,001
5-yr CSS rate (%)	78	88	36	0,7	0,2-3,0	0,641	3,7	1,1-12,2	0,030
5-yr RFS rate (%)	74	74	36	1,1	0,4-3,1	0,588	2,8	0,9-8,5	0,066

HR, hazard ratio adjusted for significant and/or potential confounders by multivariate analysis, including age, ASA score, lymph node resection, adjuvant radiotherapy, and/or chemotherapy; 95% CI, 95% confidence interval; OS, overall survival; CSS, cancer-specific survival; RFS, recurrence-free survival Notes: 5-year recurrence and 5-year survival are relative to the date of surgery. Overall refers to any recurrence regardless of site. Local refers to non-nodal recurrence in the vagina, bladder, and/or rectum. Distant LN refers to lymph node recurrence in a site other than the pelvic or para-aortic lymph nodes. Distant metastasis refers to non-nodal recurrence in the abdomen or extra-abdominal site.

CHAPTER 6

REPRODUCIBILITY OF LYMPHOVASCULAR SPACE INVASION (LVSI) ASSESSMENT IN ENDOMETRIAL CANCER

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Abstract

Aims: Lymphovascular space invasion (LVSI) in endometrial cancer (EC) is an important prognostic variable impacting on a patient's individual recurrence risk and adjuvant treatment recommendations. Recent work has shown that grading the extent of LVSI further improves its prognostic strength in patients with stage I endometrioid EC. Despite this, there is little information on the reproducibility of LVSI assessment in EC. Therefore, we designed a study to evaluate interobserver agreement in discriminating true LVSI from LVSI-mimics (phase 1) and reproducibility of grading extent of LVSI (phase 2).

Materials and results: Scanned haematoxylin and eosin (H&E) slides of endometrioid EC (EEC) with a predefined possible LVSI-focus were hosted on a website and assessed by a panel of six European gynaecological pathologists. In phase 1, 48 H&E slides were included for LVSI assessment and in phase 2, 42 H&E slides for LVSI grading. Each observer was instructed to apply the criteria for LVSI used in daily practice. The degree of agreement was measured using the two-way absolute agreement average-measures intra-class correlation coefficient (ICC). Reproducibility of LSVI assessment (ICC: 0.64 ($p < 0.001$)) and LVSI grading (ICC of 0.62 ($p < 0.001$)) in EEC was substantial among the observers.

Conclusions: Given the good reproducibility of LVSI, this study further supports the important role of LVSI in decision algorithms for adjuvant treatment.

Introduction

Classic histopathological parameters are the cornerstone of the current risk-assessment and guide adjuvant treatment for patients with early stage (stage I/II) endometrial carcinoma (EC). Tumour factors included in the risk assessment of early-stage disease are histological type, tumour grade, cervical stromal involvement, depth of myometrial invasion and lymphovascular space invasion (LVSI). Combinations of these factors stratify early-stage EC patient into low-risk (LR), high-intermediate risk (HIR) and high-risk (HR) for recurrence with differential adjuvant treatment choices [1-3].

Currently, significant advances in our understanding of molecular alterations in EC are reshaping the risk-assessment by incorporating molecular features. Novel models in which molecular factors are integrated to further refine the risk assessment are being developed [4, 5]. These integrated approaches still rely on the most relevant histological variables mentioned above. The Achilles heel of those histological variables, however, is the reproducibility among pathologists. One of the strongest prognostic variables in this context is the presence (or absence) of LVSI.

LVSI has gained a prominent position in most of the risk stratification systems for EC [5-7]. Adjuvant radiation treatment for patients with grade 1 or 2 stage I EEC is recommended in the presence of LVSI, independent of the depth of myometrial invasion [7]. It is interesting that the adjective "*unequivocal*" is used for LVSI in the most recent ESMO-ESTRO-ESGO clinical guidelines[7], as it advises to report LVSI only when there is no other interpretation possible. This immediately evokes the question "how reproducible among pathologists is unequivocal LVSI". In addition, recent work shows that substantial LVSI in EC may have a stronger prognostic significance than focal LVSI [52, 78]; similar effects are reported for LVSI grading in breast cancer [10].

A diversity of LVSI definitions can be found in the EC literature, reflecting different ways to approach its assessment. Irrespective of the exact formulation, all these refinements are aimed to help distinguish LVSI from LVSI-mimics. The most frequently encountered LVSI-mimic is artefactual displacement of tumour within myometrial clefts or large endothelial lined vessels. These displacements are likely the result of manipulation of the uterus by an intrauterine balloon during surgery [11] or an artefact induced by inappropriate grossing of a friable tumour [12]. Artefactual displacement is more likely to occur in cases with poor fixation or in EC with abundant necrosis. Another frequent artefact that mimics LVSI, is stromal retraction around invading tumour glands. Furthermore, "emboli" in vascular spaces are not always clearly composed of viable tumour cells. There may be degenerative changes and infiltration of inflammatory cells may obscure the presence of tumour cells in these emboli. A specific type of myometrial invasion referred to as microcystic elongated and fragmented (MELF)-type invasion [13], may also be confused with LVSI, but importantly is also associated with true LVSI.

Additional histological criteria, such as proximity to a venous and arterial vessel [10] or perivascular lymphocytes, have been proposed to favour true LVSI [14].

The reported prevalence of LVSI in stage I EC varies widely (3.2-35%), indicating there may be local differences in how LVSI assessment is conducted and reported [15, 16]; however, interobserver variability studies focusing on LVSI in EC are sparse. Given the significance of LVSI evaluation in risk allocation of EC, and the widely accepted difficulties in LVSI assessment, this study was initiated to examine interobserver agreement on the presence of LVSI and LVSI grading. To our knowledge, this is the first study to assess the reproducibility of the recently proposed grading system for LVSI.

Materials and methods

In a previous study [8] haematoxylin and eosin (H&E) slides of EEC from 926 patients derived from the PORTEC 1 and 2 trials [2, 17] were locally re-reviewed for the presence of LVSI by the study-pathologists (EEMP, TB and VTHBMS). At review, the presence of LVSI-mimics was also noted.

In phase 1, to determine agreement of LVSI assessment, 48 cases were selected by the study pathologists, composed of challenging LVSI-mimics (n = 29) and cases with convincing true LVSI (n = 19). The LVSI-mimics were composed of MELF (n = 8); retraction artefact (no endothelial lining) (n = 10); artefactual tumour displacement (n = 5) and LVSI-mimics of emboli without tumour cells (n = 6). H&E slides were scanned and hosted on a website designed for this purpose. To ensure all observers evaluated the same focus, they were guided to the predefined, digitally annotated putative LVSI focus. It remained possible for the observers to view the whole section and not just the preselected focus, by scrolling through the complete scanned slide. In this phase observers were asked to indicate if the selected focus was true LVSI, using the LVSI definition they used in daily practice. When observers did not consider the marked focus as true LVSI, they were asked to specify what type of LVSI-mimic was present (supplementary table 1A). In this phase we also asked the observers to explain their choice. We also asked the observers for the definition of LVSI, they used in everyday practice.

In phase 2, we set out to determine agreement of LVSI grading. For this, a new selection of 42 cases was put together by the study pathologists. All 42 cases were considered positive for true-LVSI on re-review and were graded as either focal (n = 20) or substantial LVSI (n = 22). Cases were presented to the same group of observers on the same website, asking them first to confirm LVSI and next to grade LVSI positive cases as either focal LVSI or substantial LVSI. Focal LVSI was defined semi quantitatively as “the presence of a single focus of LVSI around the tumour”. Substantial LVSI was defined as “diffuse or multifocal LVSI around the tumour“ (supplementary table 1B) [18]. Free text comments were optional.

Six experienced gynaecologic pathologists (observers) were recruited via the ENITEC network. We aimed to include pathologists of different nationalities and from different European institutes, in order to assure differing training backgrounds.

Statistics

Raw data was stored on the website, downloaded and processed prior to analysis. Agreement among observers was measured using the two-way absolute agreement average-measures intra-class correlation coefficient (ICC). Due to the lack of a gold standard for true-LVSI, this method results in a measure of intra-observer and inter-observer variability[19]. SPSS 23.0 package was used for statistical analyses. An ICC value reflects slight (0 – 0.19); fair (0.2 – 0.39); moderate (0.4 – 0.59; substantial (0.6 – 0.79) or almost perfect (>0.8) agreement. Additionally, agreement was qualitatively expressed as: "full agreement" when all observers agreed; "partial agreement" when 4 or 5 observers agreed and "no agreement" when 3 or less observers agreed [20].

Results

Table 1 lists the LVSI definitions provided by the gynaecologic pathologists (observers). These definitions all capture the key element of the consensus definition of LVSI, namely the presence of tumour cells in a vessel lined by endothelial cells. Some observers also include exclusion criteria or components such as: adherence to the vessel wall, and the presence of erythrocytes.

Table 1. Definitions of LVSI as used by the observers.

Observer	What definition of LVSI do you use in daily practice?
A	Cohesive aggregates of tumour cells located inside a vascular space (defined by the presence of an endothelial lining) and preferentially juxtaposed to the vessel wall.
B	Carcinoma cells adherent to vessel wall (with endothelial cells).
C	Definite tumour cells within an endothelial lined channel and no features to suggest artefactual vascular invasion.
D	Presence of tumour cells in lymphatics or vessels, which is not caused by artefacts (such as smears, retraction).
E	Tumour cells usually as a group or nest within a space that is covered by endothelial cells and does not contain a significant number of erythrocytes.
F	The presence of a tumour embolus within a vessel (capillary or lymphatic), usually well defined, rounding up to the contour of the vessel, may or may not be attached to the inner surface, may include red cells or fibrin; absence of marked autolysis.

Phase 1: Reproducibility of LVSI assessment

Full agreement about the presence or absence of LVSI was found in 10 out of 48 cases (21%); partial agreement in 23 cases (48%) and no agreement in 15 cases (31%) (table 2). Individual scores are presented in supplementary table 2. One observer was a noted outlier and appeared to have a low threshold for diagnosing true-LVSI. Overall, these outcomes resulted in substantial agreement (ICC of 0,64 ($p < 0,001$)) in LVSI assessment.

Table 2. Qualitative level of agreement in LVSI assessment (phase 1) and LVSI grading (phase 2), according to initial central review.

Level of agreement	Phase 1 Initial review LVSI positive (n = 19)	Initial review LVSI negative (n = 29)	Phase 2 Initial review focal LVSI (n = 20)	Initial review substantial LVSI (n = 22)
Full	5	5	3	3
Partial	10	13	11	13
No	4	11	6	6

Some representative examples of LVSI mimics from the study are illustrated in figure 1. Interestingly, there was little agreement upon the various reasons to score the focus as negative for LVSI. There were 26 cases in which at least two observers stated there was no LVSI. In just eight of these cases (31%) the same explanation was given. In the remaining 18 cases (69%) at least two different reasons for "no LVSI" were given. This is illustrated in figure 2, a case in which mimics co-exist resulting in more than one reason to reject true LVSI.

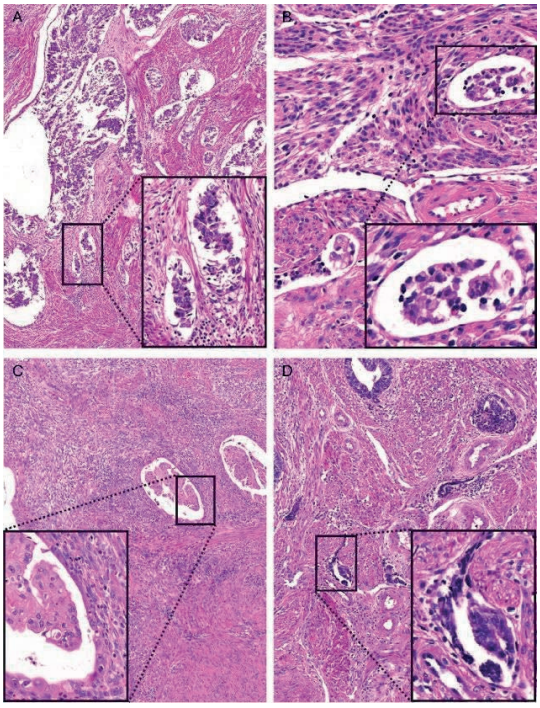

Figure 1. Representative examples of LVSI mimics presented in phase I. **A**: Retraction artefact around poorly preserved invading tumour. **B**: A cluster of inflammatory cells within a vessel, mimicking tumour cells. **C**: A microcyst aligned by flattened epithelial cells with a cluster of tumour cells in the centre, mimicking true LVSI. **D**: A cluster of tumour cells trapped within a myometrial cleft without an endothelial lining. Note the lack of perivascular infiltrate in all LVSI-mimics.

Phase 2: Reproducibility of LVSI grading

Full agreement was achieved in six cases (14%); partial agreement in 23 cases (55%) and no agreement in 13 cases (31%). Figure 3 is an example of a case with full agreement on focal LVSI. Figure 4 illustrates a case with partial agreement on substantial LVSI. The overall reproducibility in this phase was moderate (ICC 0,54 ($p < 0.001$)). However, one pathologist consistently scored

cases as negative for LVSI, whereas two pathologists had a noted tendency to diagnose substantial LVSI. Individual scores are presented in supplementary table 3. LVSI grading in cases recognized by the observers as true-LVSI resulted in substantial agreement (ICC 0.62 ($p < 0.001$) using the predefined semi quantitative definitions for grading LVSI.

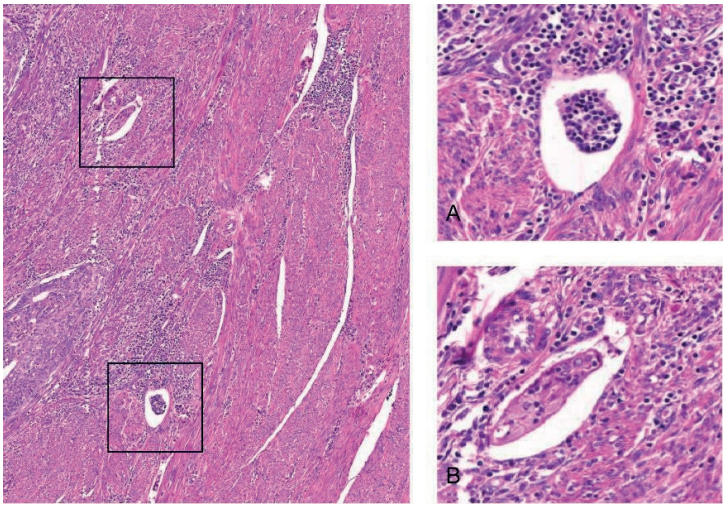

Figure 2. A representative example of a case with no consensus on LVSI assessment. This case shows two suspected foci of LVSI close to each other. The lower focus (detail A) shows the presence of endothelial cells indicating that this is a vessel, however the cell cluster within this vessel does not unequivocally contain tumour cells. The upper focus (detail B) shows a vessel with a cluster of epithelioid cells infiltrated by a few lymphocytes. Three observers scored this case as LVSI positive, two scored negative arguing the lack of tumour cells, one scores negative because of lack of endothelial cells. In this case subsequent IHC would likely result in higher level of agreement.

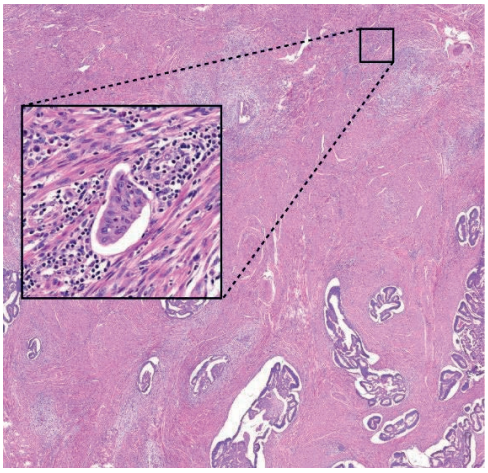

Figure 3. A case derived from phase II with full agreement on focal LVSI. The overview shows infiltrating tumour glands surrounded by an extensive stromal reaction. Some glands are surrounded by retraction artefacts. There is a focus top right (detail shown left) suspected for LVSI. The focus contains a perivascular lymphocytic infiltrate and is adjacent to a venule. This was the only LVSI focus on this H&E. All observers graded this as focal LVSI.

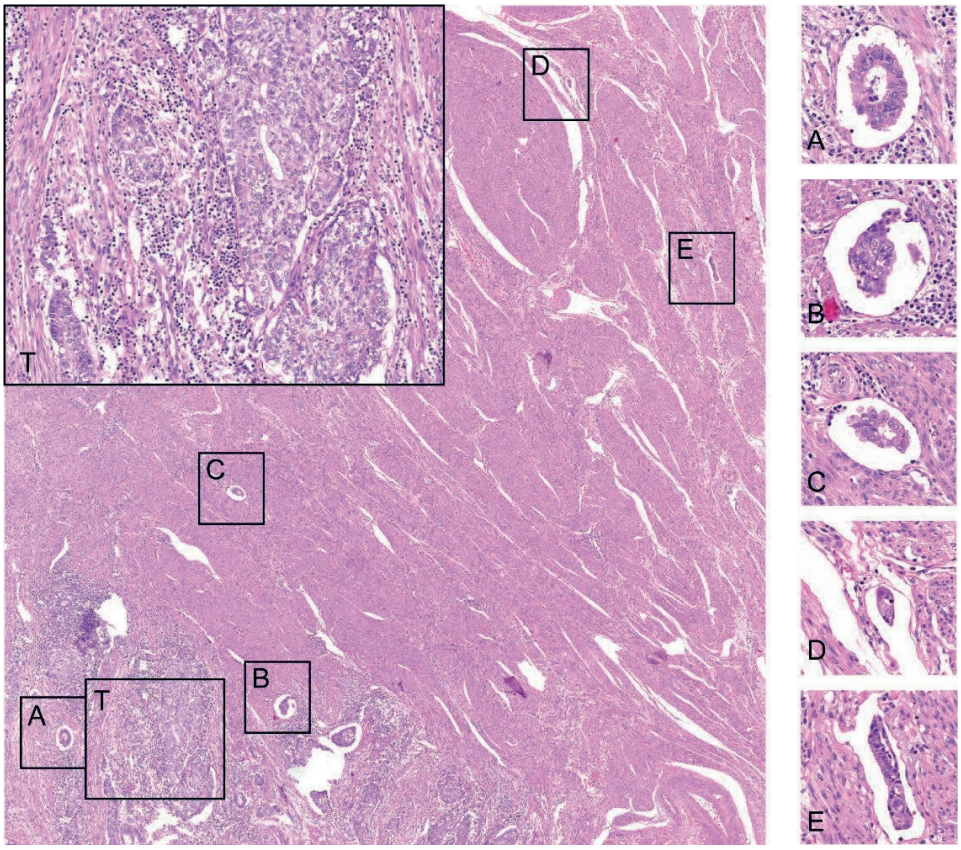

Figure 4. A case derived from phase II with partial agreement on substantial LVSI. Box T shows a detail of the EEC with a prominent peritumoral infiltrate. Inset A-E show details of putative LVSI foci that were annotated for this case that was called substantial LVSI by the study pathologist. Five observers diagnosed this case as positive for LVSI and four agreed to grade this as substantial LVSI.

Discussion

In this study we explored the interobserver reproducibility in both diagnosing LVSI and in the application of a recently introduced LVSI grading system[8]. As presence of LVSI is considered one of the strongest predictors of recurrence in early-stage EC, it is critical to assess reproducibility and identify problematic areas to further improve LVSI assessment. Here, we show that gynaecological pathologists reach substantial agreement in LVSI assessment.

We did not provide the observers with a LVSI definition, because in literature a consensus definition for LVSI is lacking. A variety of elements in the definition of LVSI can be found in the literature, such as: the presence of an endothelial lining [21], use of ancillary studies [22-24], position of the LVSI focus relative to the tumour [25], attachment of the embolus to the vessel wall or not [26, 27], nature of the vessel (lymphatic, vascular, “capillary-like”) [25, 28, 29], vitality and shape of the embolus [30] and presence of surrounding erythrocytes [31] or perivascular

infiltrates [14]. We did however ask our observers to provide the LVSI definition they use in their daily practice. Theses definitions showed significant overlap, and all LVSI to be defined as "tumour cells" located in a "vessel". The minor differences in refinements to this definition are unlikely a source of varying interpretations.

With this study we add to previous studies regarding reproducibility of pathological reporting of other EC specific characteristics like histological typing, tumour grading assessment of cervical involvement and assessment of myometrial invasion [32-35]. Levels of reproducibility of these tumour characteristics are similar to our results for LVSI assessment. None of the previous studies specifically focused on LVSI assessment, but there are two studies that report on reproducibility of LVSI in EC[33, 36]. LVSI and other tumour characteristics were reviewed as part of upfront pathology review before randomisation in the PORTEC-3 trial [36]. A high rate of inter-observer agreement between the original pathology report and central pathology review was found for LVSI ($\kappa = 0{,}72$). In the study by Guan et al., LVSI assessment was part of an alternative binary grading system in EC [33]. Here, LVSI was defined as clusters of malignant epithelial cells within vascular spaces located outside the main tumour. Assessment was performed on H&E slides and CD31 was used to identify the endothelial lining in indeterminate or suspicious cases. Assessment of 254 EC by four pathologists resulted in a disappointing κ-value of 0.23 for LVSI. Several explanations may be considered as to why our study resulted in much higher κ-values. First, LVSI was one parameter among three others, making observers less focussed on one particular parameter. Second, in our study observers were guided to a predefined focus, ensuring that all observers looked at the same area of interest. Last, the observers in our study were selected based on their special interest in gynaecological pathology with an assumption that they are familiar with common LVSI-mimics in EC.

Some of the observers in our study commented that they would have used immunohistochemistry (IHC) to prove the presence of endothelial cells in a subset of the presented cases. Although, the role of adding IHC to LVSI assessment was not part of the study design, it seems obvious that difficult cases may benefit from the use of IHC. Appropriate IHC to help demonstrate LVSI are pan endothelial (CD31) or lymph vessel specific (podoplanin/D2-40) antibodies. Weber et al. found D2-40 IHC increases the proportion of LVSI positive cases in EC compared to H&E evaluation alone. Interestingly, all D2-40 positive cases could be retrospectively identified on H&E [37]. Alexandre-Sefre et al. compared routine H&E LVSI detection with dual pancytokeratin and CD31 staining and found a threefold increase in the LVSI detection rate from 18% with H&E to 54% using IHC in stage I EC[25]. However, both studies fail to illustrate how the increased detection with IHC would affect the clinical relevance/prognostic strength of LVSI detection. There may also be reasons to be reluctant to apply IHC universally. Cancer-associated fibroblasts surrounding adenocarcinoma of the lung [38], and breast [39] have been shown to express podoplanin. Although nonspecific fibroblastic reactivity was not described in the studies of Weber et al. and Alexandre-Sefre et al., it is

possible that an extensive fibroblastic reaction in EC (e.g., in MELF-infiltrative growth pattern) could exhibit podoplanin positivity and results in an incorrect diagnosis of LVSI. Furthermore, Harris et al, showed that the assessment of both small and large vessel involvement in colorectal carcinoma could not be not improved by application of D2-40 and CD31[40]. We acknowledge however that the use of IHC can be useful in selected difficult cases (e.g., cases with extensive retraction artefact), and when used in the correct context will likely further improve interobserver agreement.

Reproducibility of LVSI assessment has also been studied in the context of other tumours like hepatocellular carcinoma (HCC)[41], colorectal cancer [40] and squamous cell carcinoma of the floor of the mouth [42]. In the HCC study [41] inter- and intraobserver reproducibility of six pathologists were analysed. LVSI definitions were not provided and 126 slides and 26 images circulated among the observers twice. There was moderate overall agreement in both attempts (first round $\kappa = 0.50$, second round $\kappa = 0.43$), with slightly lower agreement among non-hepatopathologists compared to hepatopathologists. A study in colorectal cancer [40] included 50 cases from which one H&E slide circulated among six gastrointestinal pathologists assessing small and large vessel invasion using the individual pathologists own criteria. The agreement for small vessel invasion on H&E slides was fair ($\kappa = 0.28$). Agreement was not improved with the use of CD31 ($\kappa = 0.26$) or D2-40 ($\kappa = 0.32$). LVSI assessment in squamous carcinoma of the floor of the mouth [42] was performed on H&E slides from 58 cases by three pathologists using their own criteria. This resulted in substantial agreement for LVSI ($\kappa = 0.64$), comparable to our findings. The variation in levels of agreement between these studies shows that reproducibility of LVSI assessment is likely tumour type specific.

A three-tiered LVSI grading system for EEC (no, focal, substantial) has only recently been proposed [8]. Despite its novelty, this study showed that the observers were able to apply the semiquantitative system with good agreement. Focal LVSI was defined as “a single focus of LVSI around a tumour” and substantial LVSI was defined as “diffuse or multifocal LVSI around a tumour”. Given the considerable reproducibility of this system, this seems a very reasonable approach in daily practice. We do, however, recognize that problematic cases exist, in which this semiquantitative approach may not suffice. For example, cases with 2-5 involved vessels, clustered in a small focus, may be regarded as “focal” by some (if assumed that all the foci of LVSI involve a single vessel) and “substantial” by others. Although this scenario is rare and therefore will be a minor problem in practice, the grading system may benefit from more precise cut-off values. One would anticipate that this would result in further improvement of the reproducibility. At the time of this study, no evidence based cut-off values were available.

Like all interobserver studies, this study is not without its limitations. Importantly, given the lack of a gold standard, we had to rely on the assessment of the study-pathologists for case selection. The study cohort was enriched for cases with potential LVSI, including a selection of LVSI artefacts and mimics, and therefore represents a selected and diagnostically difficult

cohort. The level of interobserver agreement in this study, therefore, likely represents an underestimation of the true agreement for LVSI assessment in EC. A more realistic unselected routine cohort would include many LVSI-negative cases without artefacts or mimics, which would likely result in a much higher agreement. Furthermore, we did not provide serial sections or additional stains to the observers, which in selected cases may have improved agreement.

In summary, this study shows that gynaecological pathologists are able to adequately discriminate unequivocal LVSI from LVSI-mimics. LVSI grading using a recently proposed 3-tiered system (no, focal, substantial) was reproducible. Given the prognostic relevance [8], this study further supports the implementation of this LVSI grading system to routine clinical practice.

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Supplementary table 1A. Questions and response options in phase 1.

Take a look at the indicated focus. Do you think this is a focus of LVSI?	
	Yes
	No, this is a shrinkage artifact
	No, there is no endothelial lining
	No, there are no tumour cells
	No, this is a focus with MELF pattern invasion (MELF = micro cystic, elongated and fragmented)
	No, this is tumour spill
	No, because of other reasons
	Unsure (explain below)
We would like to learn from your answer. You can elucidate your answer in the box below. (optional)	

Supplementary table 1B. Questions and response options in phase 2.

Take a look at the indicated focus and its surroundings. Do you think this case shows LVSI?	
	No, there is no LVSI
	Yes, this case shows mild LVSI
	Definition: mild LVSI = a single focus of LVSI around a tumour.
	Yes, this case shows substantial LVSI
	Definition: diffuse or multifocal LVSI around a tumour.
We would like to learn from your answer. You can elucidate your answer in the box below. (optional)	

Supplementary table 2. Raw data phase 1.

	OBSERVER					
CASE	A	B	C	D	E	F
1	1	1	1	1	1	1
2	6	1	6	9	1	6
3	7	3	1	1	1	1
4	6	1	6	1	7	6
5	6	1	6	6	1	1
6	1	1	1	1	1	1
7	6	1	1	1	1	1
8	1	4	4	1	1	3
9	1	1	1	1	1	1
10	1	1	3	1	1	1
11	1	6	9	9	1	1
12	1	1	1	1	1	9
13	1	1	1	1	1	1
14	3	3	9	2	3	2
15	6	1	1	1	1	1
16	1	3	5	1	1	1
17	2	1	1	1	1	3
18	6	6	7	6	6	6
19	6	1	9	1	1	1
20	6	2	1	1	1	1
21	1	1	1	1	1	1
22	1	1	9	1	1	1
23	9	1	1	2	1	2
24	6	5	1	6	1	1
25	5	1	1	5	1	5
26	6	1	1	1	1	9
27	1	1	1	1	1	1
28	5	3	5	5	5	5
29	6	2	6	1	1	1
30	1	6	6	1	1	7
31	7	4	1	4	1	1
32	1	1	1	1	1	1
33	2	2	1	2	1	2
34	5	3	1	5	1	5
35	1	1	5	5	1	5
36	2	2	1	1	1	2
37	1	1	6	1	1	1
38	1	1	9	1	1	1
39	1	1	1	5	1	2
40	6	1	1	9	1	9
41	1	1	1	1	1	2
42	1	2	1	1	1	1
43	9	5	1	6	1	2
44	1	2	1	1	1	1
45	4	4	4	4	1	6
46	3	3	1	3	1	2
47	4	4	9	1	1	1
48	1	1	1	1	1	1

Key: A to F: observers. 1: LVSI positive; 2: No LVSI – shrinkage; 3: No LVSI – no endothelial lining; 4: No LVSI – no tumour cells; 5: No LVSI – MELF; 6: No LVSI – spill; 7: No LVSI – other reasons; 9: Unsure.

Supplementary table 3. Raw data phase 2.

	OBSERVER					
CASE	A	B	C	D	E	F
1	2	2	2	2	2	2
2	1	1	2	0	2	2
3	1	2	1	2	2	2
4	1	2	2	2	2	2
5	0	1	2	1	2	2
6	2	1	2	2	1	2
7	2	1	2	2	1	2
8	1	0	2	1	1	0
9	0	2	2	2	2	2
10	2	2	2	2	2	2
11	2	2	0	2	2	2
12	0	0	2	2	1	1
13	1	1	1	1	1	1
14	0	1	2	1	2	2
15	0	1	1	0	1	1
16	0	1	2	1	2	2
17	1	1	2	2	2	2
18	2	2	2	1	2	2
19	1	1	2	2	2	2
20	1	1	0	0	1	1
21	2	2	2	2	2	2
22	0	2	2	2	2	2
23	0	0	2	1	1	1
24	2	1	2	2	2	2
25	2	2	1	2	2	2
26	2	0	2	1	2	2
27	1	1	2	2	1	2
28	2	1	2	2	2	2
29	0	1	2	2	2	2
30	0	2	2	2	2	2
31	2	1	2	0	1	2
32	1	2	2	2	2	2
33	1	1	2	1	2	2
34	0	2	2	2	1	2
35	2	2	2	2	2	2
36	2	2	2	2	2	2
37	2	1	2	1	1	2
38	1	0	2	0	1	2
39	1	1	2	0	2	2
40	0	1	2	1	1	2
41	0	2	2	2	2	2
42	0	2	2	0	2	2

Key: A to F: observers. 0: no LVSI; 1: focal LVSI; 2 substantial LVSI.

CHAPTER 7

DEFINING SUBSTANTIAL LYMPHOVASCULAR SPACE INVASION IN ENDOMETRIAL CANCER

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Abstract

Lymphovascular space invasion (LVSI) occurs in a minority of endometrial cancer (EC) cases, and the extent of LVSI is an important risk factor for recurrence and/or metastases. Our aim was to improve the reproducibility of measuring clinically meaningful LVSI by performing a quantitative analysis of the correlation between LVSI and the risk of pelvic lymph node recurrence (PLNR) in EC.

EC samples from the PORTEC-1 and PORTEC-2 trials were retrieved and used to collect quantitative data, including the number of LVSI-positive vessels per H&E-stained slide. Using a predefined threshold for clinical relevance, the risk of PLNR risk was calculated (Kaplan-Meier method, with Cox regression) using a stepwise adjustment for the number of LVSI-positive vessels. This analysis was then repeated in the DGCD (Danish Gynecological Cancer Database) cohort.

Among patients in the PORTEC-1 and PORTEC-2 trials who did not receive external beam radiotherapy, the 5-year PLNR risk was 3.3%, 6.7% (p=0.51), and 26.3% (p<0.001) when 0, 1-3, or ≥4 vessels had LVSI involvement; similar results were obtained for the DGCD cohort. Furthermore, both the average number of tumor cells in the largest embolus and the number of LVSI-positive H&E slides differed significantly between focal LVSI and substantial LVSI.

Based on these results, we propose a numeric threshold (≥4 LVSI-involved vessels in at least one H&E slide) for defining clinically relevant LVSI in EC, thereby adding supportive data to the semi-quantitative approach. This will help guide gynecologic pathologists to differentiate between focal- and substantial LVSI, especially in borderline cases.

Introduction

In endometrial cancer (EC), a combination of histopathological factors is typically used to assess clinical risk and forms the basis for recommendations regarding the use of adjuvant treatment. For example, lymphovascular space invasion (LVSI) is one of the most robust indicators of an increased risk of lymph node involvement, pelvic recurrence, and distant metastasis [1-5], and LVSI is commonly used in most risk-assessment algorithms, including the algorithm used in the ESGO-ESTRO-ESP guideline [6]. In addition, LVSI is often a critical factor in new risk models that incorporate molecular features [7], including the model used in the ongoing PORTEC-4a trial [8]. Moreover, recent studies have shown that the extent of LVSI—and not merely the presence or absence of LVSI—is the strongest predictor of recurrent regional and metastatic disease in patients with stage I disease [9, 10].

We previously reported that a three-tiered, semi-quantitative LVSI assessment based on no LVSI, focal LVSI, and substantial LVSI provided the strongest evidence with respect to recurrence among patients with intermediate-risk and high/intermediate-risk EC in the PORTEC-1 and PORTEC-2 trials [10]. Specifically, patients with substantial LVSI had a significantly higher risk of recurrent disease compared to patients with either no LVSI or focal LVSI. Moreover, substantial LVSI was the strongest independent prognostic factor for an increased risk of pelvic lymph node recurrence (PLNR), with a 5-year risk of 15.3% compared to 1.7% and 2.5% for no LVSI and focal LVSI, respectively. Importantly, among patients with substantial LVSI, external beam radiotherapy (EBRT) reduced the 5-year PLNR risk from 31% to 4.3% [10]. This finding suggests that the presence of substantial LVSI is clinically relevant with respect to recommending EBRT, as reflected by current guidelines.

For use in clinical practice, the definition and quantitative assessment of LVSI should be clear to practicing pathologists. In previous studies, however, “focal LVSI” was defined as a focus of LVSI, and “substantial LVSI” was defined as diffuse or multifocal LVSI present beyond the advancing tumor front [11]. Although the use of these semi-quantitative definitions provides a reasonable level of reproducibility among gynecologic pathologists—as we showed recently in an interobserver study [12]—using a numerical threshold to define clinically relevant LVSI may help distinguish between focal LVSI and substantial LVSI, particularly in borderline cases, thus providing improved standardization and reproducibility.

The aim of this study was to numerically define the threshold at which the extent of LVSI becomes clinically relevant with respect to the risk of PLNR, thereby establishing an objective threshold between focal LVSI and substantial LVSI. We therefore analyzed the number of LVSI-positive vessels, the number of tumor cells in the largest embolus, the distance between the deepest embolus and the serosal surface, and the number of H&E-stained slides containing LVSI-positive vessels in all previously annotated endometrioid EC (EEC) cases in the PORTEC-1 and PORTEC-2 trials [10]; in addition, we validated our findings using an independent Danish population-based EC cohort.

Materials and Methods

Study cohorts

For this study, we used the combined PORTEC-1 and PORTEC-2 [10] cohort and the Danish Gynecological Cancer Database (DGCD) cohort to quantitatively analyze the correlation between the extent of LVSI and the risk of PLNR. These two cohorts are described in detail below.

The PORTEC-1/PORTEC-2 cohort

The PORTEC-1 trial was a randomized controlled trial (RCT) involving 714 patients with intermediate-risk EC randomly assigned to receive either adjuvant EBRT or no adjuvant treatment following total abdominal hysterectomy and bilateral salpingo-oophorectomy without lymphadenectomy [13]. The PORTEC-2 trial was an RCT involving 427 patients with high/intermediate-risk EC randomly assigned to receive either adjuvant EBRT or adjuvant vaginal brachytherapy following total abdominal hysterectomy and bilateral salpingo-oophorectomy without lymphadenectomy [14]. All patients in PORTEC cohorts had endometrioid cancers. The risk classifications were based on ESMO-ESGO-ESTRO guidelines [15, 16].

The DGCD cohort

The second cohort in our study was derived from a Danish population-based study of patients with high-risk EC [6, 17] and is described in detail in our companion paper by Peters et al. [18]. Cases were collected from the DGCD, a Danish nationwide database that includes nearly all 4,707 patients diagnosed with EC from January 1, 2005 through December 31, 2012. All patients underwent a hysterectomy and 70.7% underwent systematic lymphadenectomy; 80% were FIGO stage I-II; 60% had non-endometrioid histology; and 70.3% of patients did not receive adjuvant therapy. Follow-up data were also registered in the DGCD and included the date and site of recurrence as well as the date and cause of death.

LVSI review

Four pathologists (authors EEMP, ALC, VTHBMS and TB) comprehensively reviewed the H&E–stained hysterectomy slides obtained from 926 cases in the PORTEC cohort and 401 cases in the DGCD cohort. The presence of LVSI was established when a tumor embolus was present in an endothelial-lined space within the myometrium beyond the invasive front of the tumor. The presence of LVSI was determined without the use of immunohistochemistry. For all cases, we noted the total number of H&E slides available, the number of tumor-positive H&E slides, the number of LVSI-positive H&E slides, and the number of LVSI-positive vessels per H&E slide. In addition, we noted the semi-quantitative assignment of LVSI (i.e., no LVSI, focal LVSI, or substantial LVSI). Focal LVSI was defined as a single focus of LVSI beyond the invasive border. Substantial LVSI was defined as diffuse or multifocal LVSI around the tumor [10]. For the PORTEC cohort only, we also measured the area of tumor-free myometrium per slide (in mm^2), the number of cells in the largest embolus, the distance between the deepest embolus and the

serosal surface (in mm), and the largest distance between the embolus and the invasive tumor front (in mm).

Statistics

Differences between two groups were analyzed using the Student's *t*-test. The time to event for PLNR was calculated from the date of randomization (for the PORTEC cohort) or the date of surgery (for the DGCD cohort) and analyzed using Cox regression and the Kaplan-Meier method with log-rank test, with stepwise adjustment of cut-off points by the highest number of tumor emboli counted in a H&E slide. If multiple slides were positive for LVSI, the highest number of emboli per slide was used for analyses. No calculations of the total number of emboli were made in case of multiple LVSI-positive slides. PLNR in PORTEC-1 and 2 was defined as any regional nodal recurrence and confirmed by a biopsy [13, 14]. For the DGCD cohort histology proven recurrences were located retrieving data from the Danish pathology database, non-histology verified recurrences were retrieved from medical records based on gynecological or radiological examination [17]. Because a small percentage of patients in the DGCD cohort received adjuvant treatment, the Cox regression analysis was adjusted for age, ASA classification score (in cases with comorbidity), lymph node resection, and adjuvant treatment. For the PORTEC cohort, the analyses were performed based on the patients in the PORTEC-1 and PORTEC-2 trials who were randomized to receive either vaginal brachytherapy or no additional treatment (n = 502, including 59 LVSI-positive patients). All patients (regardless of LVSI status) randomized to receive EBRT were excluded from this analysis, as EBRT has been shown to reduce the risk of pelvic failure [78]. The PORTEC data were analyzed using SPSS version 23.0 (IBM Corp., Armonk, NY), and the DGCD data were analyzed using Stata 11 (StataCorp LLC, College Station, TX). Differences with a p-value <0.05 were considered significant.

Criteria used to establish a numerical threshold for clinically relevant LVSI

Ideally, a clinically relevant threshold should indicate an increase in the risk of recurrence sufficient to justify providing adjuvant treatment despite the associated risk of side effects. In low-risk EC, the 5-year recurrence-free survival rate is 95% [19]; thus, the remaining 5% risk is not sufficient to justify adjuvant treatment. For our analysis, a PLNR risk of 10% was consider the upper range of acceptable risk for not recommending EBRT. Additionally, we took the Hazard Ratios (HRs) into consideration, seeking for a trade-off between the highest possible PLNR HR for substantial LVSI, versus the lowest possible PLNR HR for focal LVSI.

Results

We first analyzed a variety of quantitative factors measured in the H&E slides from the PORTEC cohort for any possible association with the number of LVSI-positive vessels. For this analysis, H&E-stained slides were available from 108 of the 129 (83.7%) LVSI-positive EC cases. For each LVSI-positive EC case, an average of 1.8 (range: 1-5) tumor-containing H&E slides were available, including 70 cases with focal LVSI and 38 cases with substantial LVSI. If LVSI was present, this

was not present in all samples taken from the tumor and myometrium. As shown in Table 1, the focal LVSI cases and the substantial LVSI cases differed significantly with respect to the number of H&E slides containing LVSI-positive vessels, the number of LVSI-positive vessels per H&E slide, and the number of cells contained in the largest embolus; in contrast, we found no significant difference with respect to the number of tumor-positive H&E slides, the myometrial area per H&E slide, the distance between the deepest tumor-positive vessel and the serosa, or the distance between the deepest tumor-positive vessel and the invasive border. The largest embolus in focal LVSI contains on average 31 cells (SD 32 cells), whereas for substantial LVSI the average size is 55 cells (SD 53) ($p = 0.002$). Focal LVSI was associated with less LVSI positive H&E's than substantial LVSI (1.19 (SD 0.46) H&E's and 1.5 (SD 0.73) H&E respectively; $p = 0.019$).

Table 1. Summary of pathological findings in LVSI positive cases in the combined PORTEC-1 and PORTEC-2 cohort, based on the extent of LVSI.

	Focal LVSI (n = 70)		Substantial LVSI (n = 38)		*p*
	Mean	(SD)	Mean	(SD)	
Number of H&E's with tumour	1.71	(0.94)	1.97	(1.1)	0.199
Number of H&E's with LVSI	1.19	(0.46)	1.5	(0.73)	0.019
Myometrium surface per H&E (mm^2)	157	(100)	159	(92.6)	0.851
Distance of deepest embolus to serosa (mm)	4.6	(2.9)	3.8	(2.4)	0.107
Distance of deepest embolus to advancing front (mm)	2.2	(2.2)	2.5	(1.9)	0.395
Number of LVSI-positive vessels	1.9	(2.5)	5.6	(6.4)	< 0.001
Number of tumour cells in the largest embolus	31	(32)	55	(53)	0.002

Establishing a clinically relevant threshold based on the number of LVSI-positive vessels
Repeated Cox regression and Kaplan-Meier analyses were used to calculate the risk of PLNR in order to establish a numerical threshold for defining clinically relevant LVSI. Table 2 shows the risk for PLNR of the study cohort with application of the semi-quantitative and several quantitative definitions We found that PLNR risk increased as the number of tumor-positive vessels per slide increased. In the study cohort, when LVSI was present in ≥3 vessels, the 5-year PLNR risk was 20.8%; in patients with <3 LVSI-positive vessels, this risk was 5.3%, which was slightly higher than the risk in LVSI-negative patients (3.3%). At a threshold of <6/≥6 LVSI-positive vessels, the 5-year PLNR risk in patients with <6 LVSI-positive vessels was 10.2%, which exceeds the predefined upper border of clinical acceptability. The lowest HR for PLNR was reached when focal LVSI was defined as <3 positive vessels per slide. The highest HR for PLNR was reached if substantial LVSI was defined as ≥6 positive vessels. The optimal trade-off was found when at least ≥4 LVSI-positive vessels were present in at least one H&E slide and is shown in a Kaplan-Meier curve in Figure 1.

Review of LVSI in 401 EC cases in the DGCD validation cohort resulted in 321 cases (80.0%) without LVSI, 37 cases (9.2%) with focal LVSI, and 43 cases (10.7%) with substantial LVSI. Over a five-year follow-up period, a total of 25 cases had PLNR, of which 12 cases were LVSI-positive. The results with respect to PLNR were analysed using a similar numerical approach as

Table 2. Repeated Kaplan-Meier analyses of the risk of pelvic lymph node recurrence (PLNR) based on stepwise increases in the total number of LVSI-positive vessels.

N emboli	patients	events	*p*	HR	95% CI			5-year PLNR risk
No LVSI	443	18	*NA*	*NA*		*NA*		3.3
focal	38	3	0.256	2.0	0.6	-	6.9	8.3
substantial	21	5	< 0.001	8.2	3.0	-	22.2	30.2
< 2	12	0	0.978	0.0	0.0			0.0
≥ 2	47	8	< 0.001	5.1	2.2	-	11.7	19.8
< 3	20	1	0.822	1.3	0.2	-	9.4	5.3
≥ 3	39	7	< 0.001	5.4	2.2	-	12.9	20.8
< 4	32	2	0.513	1.6	0.4	-	7.0	6.7
≥ 4	27	6	< 0.001	6.9	2.7	-	17.5	26.3
< 5	36	3	0.193	2.3	0.7	-	7.7	9.0
≥ 5	23	5	< 0.001	6.5	2.4	-	17.6	25.8
< 6	42	4	0.091	2.6	0.9	-	7.5	10.2
≥ 6	17	4	< 0.001	7.6	2.4	-	22.7	30.4
< 10	46	4	0.111	2.4	0.8	-	7.1	9.6
≥ 10	13	4	< 0.001	9.1	3.1	-	27.1	34.4
< 11	48	5	0.034	2.9	1.1	-	7.9	11.4
≥ 11	11	3	< 0.001	7.7	2.3	-	26.3	31.4
< 12	52	5	0,052	2,67	0,99	-	7,20	10,50
≥ 12	7	3	< 0,001	13,35	3,91	-	45,64	50,00
< 13	54	7	0,004	3,62	1,51	-	8,68	14,40
≥ 13	5	1	0,082	5,98	0,80	-	44,96	25,00
< 15	55	7	0,005	3,54	1,48	-	8,49	14,10
≥ 15	4	1	0,042	8,15	1,08	-	61,34	33,30
< 18	57	8	0,001	3,91	1,70	-	9,01	15,70
≥ 18	2	0	0,983	0,00	0,00			0,00

HR: hazard ratio, 95% CI: 95% confidence interval, PLNR: pelvic lymph node recurrence. The hazard ratio (HR) with 95% CI is based on the "no LVSI" reference group. This analysis included 502 patients in the combined PORTEC-1 and PORTEC-2 cohort who received either adjuvant vaginal brachytherapy or no adjuvant treatment but did not receive external beam radiotherapy.

described above and are summarized in Table 3. When we set the threshold to ≥4 LVSI-positive vessels, the 5-year PLNR risk was 26.1%, and 8.9% in cases with <4 LVSI-positive vessels. When we set the threshold to ≥5 LVSI-positive vessels, the 5-year PLNR risk for cases with <5 LVSI-positive vessels was 12.1%, which exceeded the predefined upper border of clinical acceptability. The lowest HR for PLNR in case of focal LVSI was 2,5 at the threshold of <4/≥4

vessels. With a HR of 7,4, the highest HR for PLNR was reached for substantial LVSI at the same threshold; i.e. at least 4 LVSI positive vessels in a H&E slide.

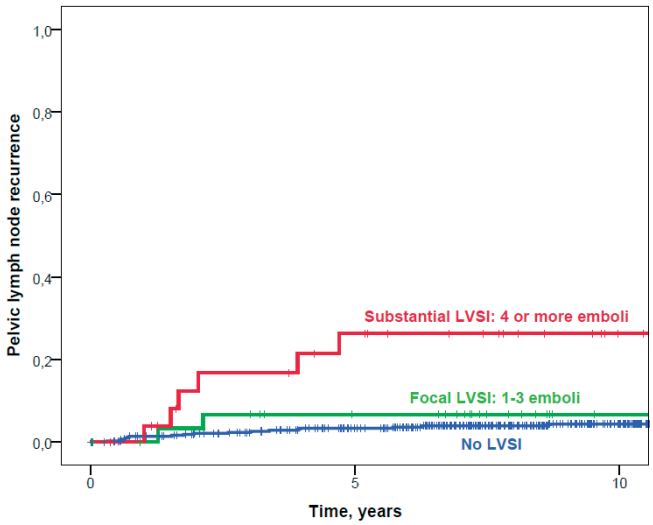

Figure 1. Kaplan-Meier curves for the 5-year risk of pelvic lymph node recurrence in the combined PORTEC-1 and PORTEC-2 cohort, for no LVSI, 1 to 3 LVSI positive vessels or 4 or more LVSI positive vessels.

Table 3. Pelvic lymph node recurrence (PLNR) based on stepwise increases in the total number of LVSI-positive vessels.

N emboli	patients	events	*p*	HR	95% CI		5-year PLNR risk
No LVSI	321	13	*NA*	*NA*	*NA*		4.7
focal	37	4	p = 0.015	4.1	1.3	12.8	14.5
substantial	43	8	p < 0.001	6.7	2.6	16.8	24.3
< 3	21	2	p = 0.173	2.8	0.6	12.5	11.6
≥ 3	59	10	p < 0.001	5.7	2.5	13.0	22.8
< 4	27	2	p = 0.242	2.5	0.6	11.1	8.9
≥ 4	53	10	p < 0.001	7.4	3.1	17.8	26.1
< 5	29	3	p = 0.050	3.6	1.0	12.9	12.1
≥ 5	51	9	p < 0.001	6.7	2.7	16.4	24.7
< 6	38	4	p = 0.017	4.0	1.3	12.4	14.0
≥ 6	42	8	p < 0.001	6.9	2.7	17.4	25.1

HR: hazard ratio, 95% CI: 95% confidence interval, PLNR: pelvic lymph node recurrence. Repeated analyses of the risk of PLNR based on stepwise increases in the total number of LVSI-positive vessels. The hazard ratio (HR) with 95% CI is based on the "no LVSI" reference group.

Discussion

Several studies have shown that the extent of LVSI is clinically relevant and can be used to improve the stratification of patients with intermediate/high-risk and high-risk EC [9, 10, 20]. Moreover, introducing the concept of measuring the extent of LVSI resulted in pathologists

reporting the extent of LVSI using a semi-quantitative approach (i.e., focal LVSI vs. extensive/substantial LVSI), with acceptable interobserver variation [12]. Despite the advantages associated with this semi-quantitative approach, the addition of a strictly quantitative (i.e., numerical) threshold is likely to increase reproducibility, particularly in cases in which no clear distinction can be made between focal LVSI and substantial LVSI. In this respect, our results show that the presence of LVSI is a biological variable, with the risk of PLNR increasing as the number of LVSI-positive vessels increases. In the PORTEC cohort, the threshold of ≥5 LVSI-vessels was sufficient to achieve the predefined upper border of a 10%increase in 5-year PLNR risk without the need for EBRT. In an independent cohort (the DGCD cohort), the upper border was reached when ≥4 vessels were involved, yielding a similar 5-year PLNR risk. This slight difference in threshold between the two cohorts (i.e., 5 vs. 4 vessels in the PORTEC and DGCD cohorts, respectively) may be explained—at least in part—by the inclusion of predominantly grade 3 and non-endometrioid tumors in the DGCD cohort and/or fewer H&E slides available in the PORTEC cohort. Nevertheless, in both cohorts the difference between choosing 4 or 5 vessels as the threshold would have affected the classification of only 4 patients in the PORTEC cohort and 2 patients in the DGCD cohort. Although the semi-quantitative (i.e., no LVSI, focal LVSI, or substantial LVSI) approach did not perform poorly in this study, we recommend to replace it with a numerical threshold of ≥4 LVSI-positive vessels in at least one H&E slide for the diagnosis of substantial LVSI, which will also improve acceptance and reproducibility.

For our analysis, we used a PLNR risk of 10% as the upper border of the acceptable risk associated with not recommending EBRT in these patients. This threshold was based on the following factors: 1) in low-risk EC the 5-year recurrence-free survival rate is 95% with general agreement that adjuvant treatment is not indicated, despite the remaining 5% risk; 2) the use of EBRT in patients with substantial LVSI reduces the risk of PLNR to <5%; and 3) there is general agreement among patients and clinicians that intensive adjuvant therapy should be recommended if it can reduce risk by 10% [21].

In this study, we extensively characterized LVSI, defined as a tumor embolus in an endothelial-lined space within the myometrium and beyond the invasive front of the tumor. In addition to counting the number of LVSI-positive vessels per H&E-stained slide, we also measured the area of tumor-free myometrium per slide, the number of cells in the largest embolus, the distance between the deepest embolus and the serosal surface, the largest distance between the embolus and the invasive tumor front, and the number of LVSI-positive H&E slides. Among these factors, we found that both the number of tumor cells in the largest embolus and the number of LVSI-positive H&E slides were associated with an increased risk of PLNR. This finding suggests that histological factors should be taken into account when assessing the extent of LVSI. The distribution of cell counts is skewed and there is great overlap in size between emboli found in focal and substantial LVSI. Therefore, cell counts on its own are not reliable to

distinguish focal from substantial LVSI. Additionally, cell counting is time consuming and prone to errors and therefore not applicable for every-day practice. Acknowledging LVSI is often present in multiple H&E slides as opposed to focal LVSI can aid to decide about the extent of LVSI, but is not an argument on its own.

Determining an evidence-based threshold in histopathology can be challenging, and such thresholds should ideally be established based on clinically annotated series, as the biological behavior of any given tumor is multifactorial. Therefore, we used well-documented cohorts of EC cases with high-quality pathology review data and robust data based on long-term clinical outcome [22]. Although these cohorts are relatively large, substantial LVSI is relatively uncommon and therefore challenging to study. Another aspect requiring vigilance is the risk of statistical noise that is easily introduced when studying phenomena with low incidence such as LVSI. A minor change in numbers of cases or events can have major effect on risk calculation. We excluded patients who received EBRT from our analyses, as EBRT can effectively prevent pelvic recurrence and would potentially have concealed the effect of LVSI on PLNR. Furthermore, fewer slides were available for review from the PORTEC cohort compared to what we would expect in routine practice, potentially causing an underestimation of the prevalence and/or extent of LVSI. The chance of underestimating the prevalence of LVSI was minimal because for the previous study a successful effort was made to collect as many H&E slides from cases that were LVSI positive [10]. For the current study less H&E slides from PORTEC were available for measurements and counting of emboli. Nevertheless, our review of LVSI in the DGCD cohort—in which the number of H&E slides was similar to routine practice—yielded similar outcome results. Moreover, approximately 70% of patients in the DGCD cohort did not receive adjuvant treatment and were therefore well suited to demonstrate the risk of PLNR associated with LVSI, adjusted for adjuvant treatment.

In conclusion, our results provide the first numeric threshold for clinically relevant LVSI in EC. This threshold—namely, ≥4 LVSI-positive vessels in at least one H&E slide—provides valuable supportive information that can help gynecologic pathologists classify the extent of LVSI, particularly in cases in which the distinction between focal LVSI and substantial LVSI is unclear. We therefore recommend pathologists to report the number of emboli in equivocal cases. The semi-quantitative approach has shown to be meaningful and reproducible in a population-based validation cohort and will generally be sufficient in most EC cases. By correlation of quantitative data, we were able to provide additional supportive factors such as the number of tumor cells in the largest LVSI embolus. We encourage other research groups to validate our proposed threshold in independent cohorts.

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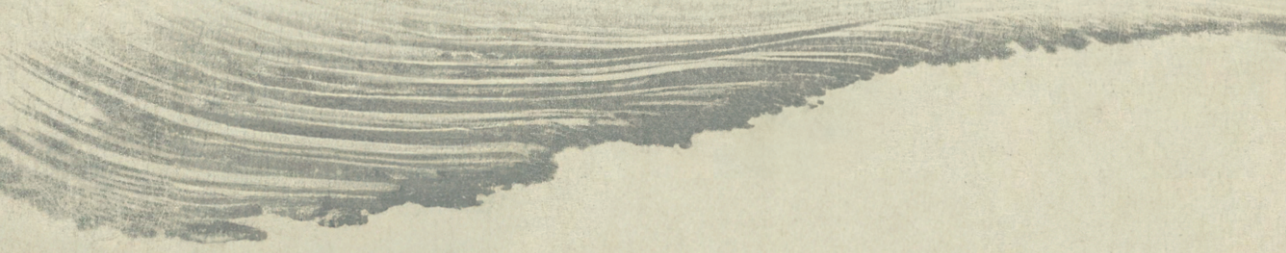

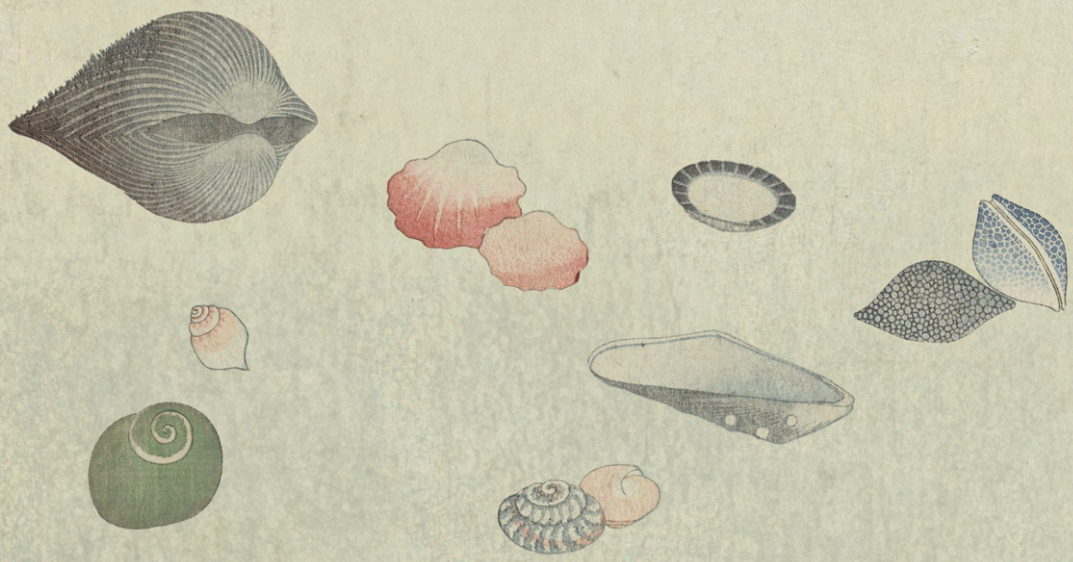

CHAPTER 8

GENE EXPRESSION ANALYSIS OF MISMATCH REPAIR DEFICIENT ENDOMETRIAL CANCERS WITH LYMPHOVASCULAR SPACE INVASION

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In preparation

Abstract

Background: the prevalence of lymphovascular space invasion (LVSI) in endometrial cancer (EC) is not evenly distributed among molecular subgroups and the molecular events leading to LVSI are largely undefined. The aim of this study was to find a vascular invasion profile (VIP) by comparing DNA expression of MMRd ECs with substantial LVSI and without LVSI.

Methods: first, differential DNA expression analysis was performed on TCGA data. Next, DNA expression of archived MMRd ECs with substantial LVSI was compared to MMRd ECs without LVSI using NanoString technologies. Lastly, literature was reviewed for VIPs among and beyond ECs (3) Results: TCGA analysis revealed 94 differentially expressed genes between ECs without LVSI and those with extensive LVSI. No differentially expressed genes were found in the NanoString analysis of MMRd ECs. Among 13 published VIPs containing 345 genes, 12 overlapping genes recurred in two or three VIPs.

Conclusions: although this study did not reveal a VIP for MMRd ECs, there may be indications LVSI is associated with differential gene expression. Further research using a wider panel and larger sample may be helpful.

Introduction

Lymphovascular space invasion (LVSI) in endometrial cancer (EC) is associated with an increased risk of recurrent disease, lymph node (LN) metastases, distant metastases and reduced survival [1-3]. Especially substantial LVSI, rather than focal LVSI, has proven to be the strongest prognostic factor in early-stage EC [4, 5]. LVSI is more prevalent in high grade EC and in higher stages of disease, compared to low grade and early-stage EC [6, 7]. While the clinical relevance of LVSI has been studied well, the underlying molecular biology leading to LVSI and potential markers to predict LVSI are largely undefined.

The TCGA molecular classification of EC describes four molecular subgroups, including 1) the polymerase epsilon (*POLE*) mutated (*POLE*-mt) tumors, characterized by an ultra-high mutated phenotype; 2) mismatch repair deficient (MMRd), characterized by a hypermutated phenotype; 3) *p53-abnormal* (p53abn), characterized by high copy number alterations and 4) tumors without a specific molecular profile (NSMP), lacking any of these characteristics [46]. The incidence of LVSI is highest in p53abn tumors, followed by MMRd tumors, but has a low incidence in *POLE* mt and NSMP tumors. In a pooled histopathological characterization of molecular groups, LVSI was present in 48,8%; 41,3%; 32,7% and 13,8% for p53abn, MMRd, *POLE*-mt and NSMP EC respectively [154]. In a combined early stage, high-intermediate risk EC study population, substantial LVSI was present in 5,4%; 8,9%, 0% and 2,4% in p53abn, MMRd, *POLE*-mt and NSMP EC respectively [10]. Potentially, these differences in the incidence of LVSI between the molecular subgroups may suggest molecular mechanisms resulting in LVSI.

Few studies have described an EC-specific vascular invasion profile (VIP) for LVSI based on differential DNA expression analyses. Watanabe et al. designed a 55-gene profile to predict LVSI in EC [11]. In addition, Mannelqvist et al. found an 18-gene profile for vascular invasion in EC [12]. Although both gene panels were designed for EC, no overlapping genes are listed. A 55-gene expression profile to predict lymph node metastases in EC, which is strongly associated with vascular invasion, included one overlapping gene (*CLCN2*) with one of the previous panels [13]. Differential expression analyses seeking vascular invasion profiles in other tumor types like breast [14-17], ovarian [18], hepatocellular [19-21], non-small cell lung carcinoma (NSCLC) [22] as well as gastric adenocarcinoma [23, 24] have all resulted in signatures but show little overlap between, as well as within tumor types.

Previous studies exploring a vascular invasion expression profile for EC have not incorporated the molecular EC classification. Given differences in LVSI incidence among the four molecular subgroups, we hypothesized there are molecular alterations associated with LVSI which may not be similar between molecular subgroups. Since LVSI is associated with MMRd, gene expression analysis was performed on archived MMRd EC, stratified by LVSI to evaluate altered gene expression associated with LVSI.

Materials and Methods

TCGA

Publicly available whole genome expression data from the TCGA Uterine Corpus Endometrial Carcinomas (UCEC) cohort was used to explore differentially expressed genes (DEG) in LVSI positive EC [25]. LVSI status was extracted from the accompanying pathology report. LVSI was reported by local pathologists as: 'no', 'yes', 'focal', 'extensive' or 'indeterminate'. DEG analysis using the genes in the MsigDB hallmark gene sets [170] was carried out on the following subsets: all EC without LVSI versus EC with extensive LVSI; all EC without LVSI versus EC with any extent of LVSI; MMRd EC without LVSI versus MMRd EC with any extent of LVSI.

Patient and tissue selection

For this study 48 patients were selected from molecularly classified archived EC that were previously collected by our research group, including the international PORTEC-1/2/4a clinical trials [27-29]. Pathology review was performed to asses histological subtype, stage, grade and extent of LVSI. For the purpose of this study, all cases with documented (substantial) LVSI were re-reviewed by the study pathologists (E.E.M.P. and T.B.) to confirm LVSI status. Substantial LVSI was defined by the presence of at least four LVSI positive vessels in at least one hematoxylin and eosin (H&E) slide [30]. MMRd EC with substantial LVSI and MMRd EC without LVSI, matched for International Federation of Gynecology and Obstetrics (FIGO) stage and grade were selected for gene expression analysis. MMRd status was confirmed by immunohistochemistry [31].

RNA isolation

RNA was isolated from formalin-fixed paraffin-embedded (FFPE) tissue blocks using the automated Tissue Preparation System (Siemens Healthcare Diagnostics). RNA was extracted by taking four 0.8mm cores from the invasive border of the tumor, annotated by the study pathologists. The quality and quantity of the RNA was measured using the 2100 Bioanalyser (Agilent, Santa Clara, California USA). Samples were considered of sufficient quality for further analysis when >20% of RNA fragment length was at least 300 bases, and the corrected concentration remained >42 µg/µL.

Panel selection for NanoString

In order to select the best performing gene panel, we applied three available NanoString panels (PanCancer Progression Panel, PanCancer Pathways Panel (Human) and PanCancer IO 360 Panel) to TCGA cases and gene expression was performed, comparing tumors without LVSI to tumors with extensive LVSI. The panels contained 770 key genes of pathways involved in angiogenesis, extracellular matrix, epithelial-mesenchymal transition, signaling, and/or immune response and 30 to 40 housekeeping genes. Selection of the panel was based on the highest number of differentially expressed genes. The available nCounter gene panels allowed customization by adding up to 30 genes of choice. We choose to add the 15 most up-regulated and 15 most down-regulated genes from the TCGA comparison.

NanoString gene expression analysis

A total of 300 ng RNA was hybridized to the PanCancer IO 360 Panel for 17 hours, following the manufacturer's instruction (NanoString Technologies, Seattle, Washington, USA). The nCounter FLEX stsytem (Nanostring Technologies) was used to scan 490 Field of Views and count the number of copies per gene in each sample. Gene expression data was analyzed using the Advanced Analysis Module in the nSolver Analysis Software version 4.0 (NanoString Technologies, Washington, USA). The nSolver Analysis Software enables for quality control, normalization, cluster analysis and DEG analysis. Raw data was normalized by subtracting the mean plus one standard deviation of eight negative controls and technical variation was normalized through internal positive controls. Data was corrected for input volume via internal housekeeping genes.

Statistical analyses

Gene expression analyses on TCGA data were done using R version 4.0.2. MsigDB datasets were retrieved using the EGSEAdata package version 1.16 [173], and the genes from the NanoString panels were retrieved from the manufacturer documentation [33]. In order to limit the analysis to biologically relevant genes for the TCGA analysis, only genes belonging to the hallmark gene sets (category = "H") were selected. TCGA data of the selected gene sets and NanoString panels was retrieved using the cgdsr package version 1.3.0 [34]. Genes were filtered out when less than 50% of the samples had a valid data point. The statistical analysis was performed using voom from the limma package version 3.44.3 [35]. Genes were considered differentially expressed when the adjusted p-value according to Benjamini and Hochberg was below 0.05. Expression data was incomplete for one LVSI negative tumor and therefore excluded from further analyses.

Literature review for vascular invasion profiles

Literature on vascular invasion profiles in epithelial cancers was searched using the search terms listed in box 1.

Box 1. Entries used to search PubMed for vascular invasion profiles in cancer.

("Transcriptome"[Mesh] OR "gene expression profile"[all fields] OR "gene expression profiles"[all fields] OR "Transcriptome profile"[all fields] OR "Transcriptome profiles" OR "Gene expression signature"[all fields] OR "gene expression signatures"[all fields] OR "gene expression signature"[all fields] OR "gene expression signatures"[all fields]) AND ("lymphovascular space invasion"[all fields] OR "lymphvascular space invasion"[all fields] OR "lymphovascular space involvement"[all fields] OR "lymphvascular space involvement"[all fields] OR "vascular space invasion"[all fields] OR "vascular space involvement"[all fields] OR "vascular invasion"[all fields] OR "vascular involvement"[all fields] OR "LVI"[all fields] OR "LVSI"[all fields])

Results

TCGA

Gene expression data was available for 176/529 (33,3%) EC in the TCGA dataset. According to the accompanying pathology reports, 88/176 (50,0%) tumors had no LVSI; 78/176 (44,3%) were positive for LVSI and LVSI status was missing in 10/176 (5,6%) tumors. The extent of LVSI was specified in 28/78 (35,9%) of LVSI positive tumors and reported as extensive (n=19/78; 24,4%),

focal (n=8/78; 10,3%) and intermediate (n=1/78; 1,3%). Extensive LVSI was most prevalent in p53abn EC (n=14/19; 73,7%) as shown in table 1. Molecular subtype was unknown in 9 (9/176; 5,1%).

Table 1. LVSI status or extent according to molecular classifier for TCGA cases with gene expression data.

Molecular classifier	No LVSI	LVSI positive	Focal LVSI	Extensive LVSI	LVSI missing
POLE mutated	8 (62%)	3 (23%)	0 (0%)	2 (15%)	2
MMR deficient	19 (48%)	15 (38%)	3 (7.5%)	3 (7.5%)	2
TP53 mutated	38 (49%)	24 (31%)	2 (2,6%)	14 (18%)	4
NSMP	19 (70%)	5 (19%)	3 (11%)	0 (0%)	1

* Information about LVSI status of TCGA cases with gene expression data (n = 176) was retrieved from pathology reports. If LVSI was reported to be present, the extent of LVSI (focal or extensive) as stated in the pathology report was adopted. The extent of 'LVSI positive' cases is unknown. LVSI status in TCGA is not verified by pathology review, nor definitions are given of the extent of LVSI. LVSI status was reported 'indeterminate' in one NSMP case and left out from this table. Nine cases were not assigned to a molecular classifier, including four without LVSI, three were LVSI positive, LVSI was 'indeterminate' in one and LVSI status was missing in another one. Abbreviations: *POLE*: polymerase ε; MMR: mismatch repair, NSMP: no specific molecular profile.

Analysis of differential expression of genes between 19 tumors with extensive LVSI and 87 without LVSI revealed 94 DEGs (table 2, supplementary table 1 for details). In one of 88 LVSI negative tumors DNA expression data was incomplete and excluded from analysis. No DEGs were found in comparison between LVSI positive (any extent) versus no LVSI regardless of molecular group; any LVSI versus no LVSI among MMRd (table 3).

Table 2. Differentially expressed genes in alphabetical order

ABCD1	*CD207*	*DES*	*GLS*	*MAST4*	*NID2*	*PSMD3*	*SLC7A2*	*TOMM40*
AMOT	*CDC25B*	*DHRS2*	*HADH*	*MPPED2*	*NOP56*	*PVR*	*SLN*	*TRIB3*
AMPH	*CDC42*	*DUSP5*	*HSPB2*	*MSX1*	*NPHP1*	*RBP2*	*SMAD3*	*TUBA4A*
ATP1A3	*CDH16*	*EHD1*	*ICA1*	*MT1E*	*OASL*	*PHOF*	*SNX10*	*UPP1*
ATP2B4	*CLCF1*	*EI24*	*IFI30*	*MX2*	*OSTC*	*SCHIP1*	*SORBS2*	*WFS1*
AURKA	*CLCN2*	*EPHX1*	*IL6*	*MYL4*	*OVOL2*	*SGCB*	*SPHK1*	
BCL2L1	*CLDN19*	*ESR1*	*IRF6*	*NDP*	*PCDH7*	*SKP1*	*SSH2*	
CANT1	*CYB5A*	*FABP3*	*KCNQ2*	*NDRG2*	*PDCD4*	*SLC1A1*	*SST*	
CANX	*CYB5R1*	*FGFR1*	*KDM4B*	*NEFH*	*PERP*	*SLC27A1*	*TEAD4*	
CASP10	*CYP2C18*	*FUT1*	*L1CAM*	*NEXN*	*PEX2*	*SLC3A2*	*THY1*	
CASP6	*DDC*	*GAL*	*LRIG1*	*NFKB2*	*PLA2G12A*	*SLC6A12*	*TMED10*	

Panel selection for NanoString analysis

Selection of the NanoString gene panel was done in a two-way comparison. First, the genes listed in the PanCancer IO360, PanCancer Progression and PanCancer Pathways panels were

Table 3. Differential gene expression analysis in several comparisons.

Case selection	n	Reference	n	Number of DEG
Extensive LVSI*	19	No LVSI*	87	94
Extensive LVSI or LVSI positive*	68	No LVSI*	87	0
Any LVSI (MMRd)	21	No LVSI (MMRd)	19	0

*Any molecular classifier. Abbreviations: DEG: differentially expressed genes; MMRd: mismatch repair deficient.

compared to the list of 94 DEGs in extensive LVSI in the TCGA for overlapping genes and revealed 12, 10 and 9 overlapping genes, respectively. Additionally, a gene expression analysis

using the genes listed in these panels was performed for the TCGA selection of 19 tumors with extensive LVSI versus 87 without LVSI and resulted in 12, 44 and 31 DEGs respectively (table 4). Based on these results, the PanCancer Progression panel was selected for further analysis and enriched with the top 30 DEGs from the 94 DEGs from the TCGA analysis (table 5).

Table 4. Performance of different NanoString gene panels

NanoString panel	DEG in TCGA dataset[1]	Overlapping genes[2]
PanCancer IO 360	12	12
PanCancer Progression	44	10
PanCancer Pathways	31	9

[1] Differential expression analysis of TCGA data with the genes in the panels listed in the comparison of "no LVSI" versus "extensive LVSI". [2] Number of genes listed in the NanoString panel which were also among the 94 differentially expressed genes in the initial TCGA analysis ("no LVSI" versus "extensive LVSI")

Table 5. List of genes customized to the NanoString PanCancer progression panel.

AMOT	*CLDN19*	*ESR1*	*LRIG1*	*NDRG2*	*SLC6A12*
AMPH	*CYP2C18*	*FABP3*	*MPPED2*	*NEFH*	*SLC7A2*
ATP1A3	*DDC*	*GAL*	*MSX1*	*PCDH7*	*SLN*
CD207	*DES*	*KCNQ2*	*MT1E*	*RBP1*	*SORBS2*
CDH16	*DHRS2*	*L1CAM*	*MYL4*	*SLC1A1*	*SST*

NanoString gene expression analysis

Sufficient RNA for analyses was extracted from 32 MMRd EC without LVSI and 16 MMRd EC with extensive LVSI. None of the pathways or gene clusters involved in angiogenesis, extracellular matrix, epithelial-mesenchymal transition, signaling or immune response was associated with LVSI status. Analysis of differential expression of genes revealed no significant DEGs. In particular, the 30 genes customized to the panel which were differentially expressed in the TCGA analysis, showed no significant differential expression.

Literature review for vascular invasion profiles (VIPs)

Using the entries listed in Box 1, a PubMed search was performed which resulted in 13 articles with VIPs on eight epithelial tumor types, including endometrial, lung, breast, hepatocellular, serous ovarian, colon, urothelial cell and esophageal squamous cell carcinomas. A summary of these studies is listed in Table 6. DEGs per study are listed in supplementary table 2. There were three VIPs for EC, these included 17, 12 and 55 genes respectively with one overlapping gene (*CLCN2*) in two of the three VIPs. In the comparison of the three VIPs for EC with our TCGA analysis, three overlapping genes were found: *CLCN2*, *AURKA* and *NOP56*. In the comparison of the 13 published VIPs, 8 genes appeared in two VIPs (*AURKA, ATP2B4, CCNB2, HMGA1, MED1, MT1E, NDP* and *TM7SF2*) and 4 genes were listed in 3 VIPs *CLCN2, ESR1, NOP56* and *UBE2C*). 7 of these were also differentially expressed in our TCGA analysis (*AURKA, ATP2B4, CLCN2, ESR1, MT1E, NDP* and *NOP56,* figure 1). Overlap was found in VIPs for EC (*AURKA, CLCN2, ESR1* and *NOP56*) breast cancer (*MT1E, NDP* and *NOP56*), NSCLC (*ATP2B4*) and colon cancer (*ESR1*).

Table 6. Overview of reported vascular invasion profiles.

Author	Tumor	Input[1]	Cases (n)	DEG[2]
Mannelqvist[12]	Endometrial	FF	57	17
Kang[13]	Endometrial	FF	330	12
Watanabe[11]	Endometrial	FF	88	55
Regan[22]	NSCLC	Cell lines	9	17
Fidalgo[17]	Breast	FF	57	22
Kurozumi[14]	Breast	FF, data set	1565 + 854	99
Asaoka[15]	Breast	Data set	835	3
Minguez[19]	Hepatocellular	FF	214	35
Ho[21]	Hepatocellular	FF	53	14
Yue[18]	Serous ovarian	Data set	192	43
Jiang[36]	Colon	FF	47	20
Poyet[37]	Urothelial	FF	23	3
Sonohara[38]	Esophageal squamous	FF, data set	267 + 96	5

[1] FF: fresh frozen tissue. [2] DEG: differentially expressed genes; number of genes in the vascular invasion profile.

Discussion

This study aimed to identify differentially expressed genes associated with LVSI in EC. The initial analysis of TCGA data confirmed LVSI is more frequent in MMRd and p53abn tumors compared to NSMP and POLE-mutated tumors. Extensive LVSI was associated with differential expression of 94 genes. In the subsequent gene expression analysis of 48 MMRd EC on the NanoString platform we were not able to establish a gene expression profile associated with substantial LVSI. Literature review of differential expression of genes associated with vascular invasion among several tumor types resulted in a longlist of genes with limited overlap between and within tumor types.

The motivation for this study was the hypothesis that LVSI is associated with specific molecular differences between tumors which is manifested by altered gene expression. The analysis of TCGA data yielded 94 DEG supporting this hypothesis. The candidate genes are ideally confirmed in an independent study set. Due to the coordination of the various PORTEC RCTs, our research group has access to a large amount of data and FFPE EC from which this study set could be compiled.

NanoStrings direct hybridization technique without amplification work up, enables to perform expression analysis on FFPE tissue, instead of fresh frozen tissue only. The downside for choosing this platform is that expression analysis is limited to genes included in a fixed panel with limited options for customization. Despite of a well-considered selection and customization of the panel, no DEGs were found. The NanoString panel we choose was directed towards genes involved in angiogenic, tumor progression and metastatic processes. However, genes included in other VIPs are only involved in these processes to a limited extent.

Eight of the 94 differentially expressed genes associated with substantial LVSI in TCGA were also listed in other VIPs. These genes are involved in divergent cellular processes, like homeostatic processes involving *ATP2B4* (intracellular calcium homeostasis), *CLCN2* (voltage gated chloride channel) and *MTE1* (intracellular Acyl-CoA regulation), and not directly associated with vascular invasion. Other genes like *ESR1* encode for the estrogen receptor and like *MED1* contributes to transcription. *AURKA* contributes to the regulation of cell cycle progression and *NDP* activates the Wnt signaling pathway, associated with carcinogenic processes, but not directly linked to LVSI. Direct association with LVSI is even less evident for *NOP56,* which plays a role in pre-ribosomal RNA processing.

There are several explanations for the lack of concordance between our results and previous VIPs for EC. First, the latter were designed without knowledge of the molecular subtype. Second, stratification for LVSI was done using a binary system for LVSI (present or absent) without quantification [11, 12], presumably including focal LVSI we know have no clinical impact [4]. Third, the profile by Kang et al. was designed to predict nodal metastases which is strongly associated with LVSI, but is not the same [13]. Last, but not least, different methods were used.

Multiple studies have shown substantial LVSI is a relevant prognostic factor, in contrast to focal LVSI [4, 39-41]. Differences in prognosis and prevalence of LVSI are observed among the four molecular subtypes of EC and this study was designed to find a VIP within one molecular subtype. There were two reasons to limit this study to MMRd EC. First, we expected the mutations resulting from the MMRd nature, to appear randomly which would not impede the detection of specific, LVSI associated altered gene expression. Second, MMRd ECs have a relatively good prognosis, but substantial LVSI is the strongest prognostic factor for adverse prognosis for EC [4]. However, it is unknown how substantial LVSI affects the prognosis of patients with MMRd EC, a VIP for MMRd EC could be a predictive factor to identify patients with a high risk for recurrence among a group of patients with a relatively good prognosis. Third, by limiting the study to one molecular subtype, possible bias relating to the molecular subgroup was excluded.

Despite the well-considered study design, sample analysis revealed no VIP. This was in line with our TCGA subanalysis of MMRd ECs which did not result in DEGs associated with LVSI. A possible explanation is the sample size was inadequate to detect altered gene expression related to LVSI among hypermutated tumors in which genetic heterogeneity possibly overturns the specific molecular alterations associated with LVSI. Another possibility is the lack of relevant genes in the panels used.

There are some limitations to this study. First, TCGA analysis was used as input for panel selection and customization, however the majority of tumors with extensive LVSI in TCGA had a p53abn phenotype in contrast to the MMRd study set. TCGA analysis among MMRd only resulted

in a small sample size without DEGs and therefore could not be used for panel selection and customization. Second, although substantial LVSI occurs more often in MMRd and p53abn tumors [42], it is still a rather uncommon event, limiting the number of cases we could select for this study. Third, whole exome sequencing (WES) is the golden standard for gene expression studies. The archived tumor material was FFPE only and however the quality of extracted RNA from FFPE tissue was adequate for the NanoString platform, the quality was insufficient for WES.

Conclusions

In this study we were not able to establish a VIP for MMRd EC. Nevertheless, based on differences in the prevalence of LVSI between the molecular subgroups in EC, TCGA data analysis and literature search, there are sufficient indications there are molecular alterations underlying LVSI. The molecular mechanisms resulting in LVSI and the key genes involved are, however, still largely unknown. As a result, the search for a VIP is still unfocused, but advancing insights into tumor biology and adequate sample size will increase the likelihood of finding a VIP in future studies.

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Table S1: Differential expression analysis results of TGCA DNA expression data in a comparison of tumors with extensive LVSI (n=19) versus tumors without LVSI (n=87)

Gene	logFC	AveExpr	t	P value	adj P value	B
FABP3	-2,0299	4,1149	-0,5937	0,0000	0,0001	8,4812
MYL4	-2,0264	-1,5864	-0,5750	0,0000	0,0002	6,7377
MT1E	-2,2278	5,1918	-0,5330	0,0000	0,0006	5,9762
SCHIP1	-1,5680	4,3878	-0,5343	0,0000	0,0006	6,0127
CDC25B	-1,1029	7,5442	-0,5080	0,0000	0,0013	4,9084
CDH16	-3,6105	1,6080	-0,5001	0,0000	0,0015	4,5424
NDP	2,9624	2,6835	0,4824	0,0000	0,0027	3,5495
BCL2L1	-0,6438	8,4508	-0,4754	0,0000	0,0032	3,6023
IL6	-2,3317	1,4848	-0,4719	0,0000	0,0033	3,3896
SLC6A12	-2,2233	3,1225	-0,4664	0,0000	0,0037	3,3489
ESR1	2,6802	8,3879	0,4635	0,0000	0,0038	3,2320
KCNQ2	-3,4968	-1,4394	-0,4554	0,0000	0,0048	2,6266
SNX10	-1,3123	4,4351	-0,4501	0,0000	0,0055	2,7796
WFS1	1,0287	7,7131	0,4471	0,0000	0,0057	2,5860
NOP56	-0,6416	8,5424	-0,4378	0,0000	0,0077	2,1839
CANT1	0,6326	7,5680	0,4298	0,0000	0,0096	1,9460
CLCF1	-1,0251	5,1740	-0,4289	0,0000	0,0096	2,0048
KIFAP3	0,9217	6,3257	0,4266	0,0000	0,0099	1,9244
PCDH7	2,5788	6,3626	0,4229	0,0000	0,0108	1,8177
NEXN	-1,3893	2,9305	-0,4210	0,0001	0,0110	1,7247
SLC3A2	-0,5464	8,8881	-0,4191	0,0001	0,0113	1,5014
CASP10	-1,2263	4,6060	-0,4108	0,0001	0,0120	1,3971
CYB5R1	0,9276	7,1347	0,4119	0,0001	0,0120	1,3558
KDM4B	0,7460	7,3263	0,4126	0,0001	0,0120	1,3586
LRIG1	1,6564	8,7247	0,4115	0,0001	0,0120	1,2747
NDRG2	1,6820	6,8336	0,4124	0,0001	0,0120	1,4313
SST	-3,4973	1,9205	-0,4138	0,0001	0,0120	1,5022
SGCB	1,1724	6,7380	0,4081	0,0001	0,0129	1,2666
NEFH	-2,5620	2,2822	-0,4044	0,0001	0,0137	1,1867
NID2	-1,2089	4,1919	-0,4037	0,0001	0,0137	1,1711
SLN	-1,7418	-1,9876	-0,4043	0,0001	0,0137	0,9518
TMED10	0,5535	9,8296	0,4025	0,0001	0,0138	0,9190
RHOF	-1,4629	5,2830	-0,4010	0,0001	0,0142	1,0137
PSMD3	-0,8079	8,6224	-0,3857	0,0002	0,0215	0,3505
PVR	-0,6556	7,1363	-0,3869	0,0002	0,0215	0,4379
SPHK1	-1,0033	3,9545	-0,3862	0,0002	0,0215	0,6065
TOMM40	-0,6261	7,8381	-0,3862	0,0002	0,0215	0,3870
TRIB3	-1,1477	5,2422	-0,3888	0,0002	0,0215	0,6185
PERP	0,9467	9,1857	0,3844	0,0002	0,0220	0,3158
AURKA	-0,8133	5,9878	-0,3826	0,0002	0,0227	0,3667
NFKB2	-0,6685	7,3977	-0,3822	0,0002	0,0227	0,2665
L1CAM	-2,5584	3,9183	-0,3771	0,0003	0,0259	0,2816

Table S1. *Continued*

Gene	logFC	AveExpr	t	P value	adj P value	B
PLA2G12A	0,6663	6,5599	0,3776	0,0003	0,0259	0,2357
AMOT	1,7704	5,8448	0,3762	0,0003	0,0261	0,2871
SSH2	-0,7353	5,6436	-0,3756	0,0003	0,0261	0,1755
TEAD4	-0,7139	5,5822	-0,3747	0,0003	0,0263	0,1510
MPPED2	2,8872	4,4531	0,3715	0,0003	0,0288	0,1109
CANX	0,6814	10,6966	0,3696	0,0003	0,0291	-0,1886
DHRS2	-2,4084	0,7854	-0,3703	0,0003	0,0291	0,0682
RBP1	1,6826	8,3220	0,3695	0,0003	0,0291	-0,1129
NPHP1	1,1209	4,7290	0,3657	0,0004	0,0325	-0,0257
CYB5A	0,8665	6,6845	0,3637	0,0004	0,0340	-0,2086
DDC	-2,0983	-1,5586	-0,3604	0,0005	0,0340	-0,2889
EI24	0,4955	8,3705	0,3603	0,0005	0,0340	-0,4519
IFI30	-0,8229	9,0822	-0,3612	0,0005	0,0340	-0,4545
MAST4	1,5399	3,6844	0,3601	0,0005	0,0340	-0,2195
MX2	-1,2863	5,4121	-0,3610	0,0005	0,0340	-0,2993
SLC1A1	1,6747	3,5042	0,3629	0,0004	0,0340	-0,1484
SLC7A2	-1,8538	4,2930	-0,3615	0,0005	0,0340	-0,2084
SMAD3	-0,7800	6,2815	-0,3597	0,0005	0,0340	-0,3915
HADH	0,8239	7,4176	0,3591	0,0005	0,0341	-0,4219
CD207	1,7645	1,0437	0,3578	0,0005	0,0351	-0,3566
AMPH	2,0462	0,7544	0,3562	0,0005	0,0364	-0,4139
CDC42	0,4488	9,2736	0,3559	0,0006	0,0364	-0,6185
PDCD4	0,7863	7,8440	0,3548	0,0006	0,0372	-0,5914
ABCD1	-0,6411	5,7953	-0,3540	0,0006	0,0376	-0,5197
ATP1A3	-1,6121	-0,1840	-0,3525	0,0006	0,0390	-0,4837
IRF6	1,2689	5,8938	0,3519	0,0006	0,0392	-0,4794
GLS	-1,1404	6,3986	-0,3510	0,0007	0,0398	-0,6864
DES	-1,9365	4,4076	-0,3458	0,0008	0,0434	-0,7041
FGFR1	1,0630	8,3473	0,3453	0,0008	0,0434	-0,9019
HSPB2	-1,2239	2,1203	-0,3458	0,0008	0,0434	-0,6199
ICA1	0,8456	6,5131	0,3452	0,0008	0,0434	-0,7645
OASL	-1,2933	4,2848	-0,3465	0,0008	0,0434	-0,6402
OVOL2	0,9084	4,9861	0,3471	0,0007	0,0434	-0,5845
SKP1	0,4125	9,0654	0,3471	0,0007	0,0434	-0,8871
SLC27A1	0,9219	6,4060	0,3460	0,0008	0,0434	-0,7228
TUBA4A	-0,9864	6,5425	-0,3447	0,0008	0,0435	-0,8871
CLDN19	-2,2421	-1,8508	-0,3441	0,0008	0,0436	-0,7328
FUT4	-0,9036	4,2550	-0,3439	0,0008	0,0436	-0,7011
EHD1	-0,3938	7,4339	-0,3433	0,0008	0,0438	-0,9662
THY1	-0,9798	7,3973	-0,3430	0,0009	0,0438	-0,9869
DUSP5	-1,1731	5,2921	-0,3425	0,0009	0,0441	-0,8470
OSTC	0,5092	8,1118	0,3419	0,0009	0,0444	-1,0146
ATP2B4	0,8375	8,7907	0,3403	0,0009	0,0457	-1,0792

Table S1. *Continued*

Gene	logFC	AveExpr	t	P value	adj P value	B
EPHX1	1,0299	9,4952	0,3404	0,0009	0,0457	-1,0931
CASP6	0,6181	6,2815	0,3395	0,0010	0,0463	-0,9249
CLCN2	-0,8221	4,3454	-0,3388	0,0010	0,0463	-0,8522
GAL	-2,0534	0,8143	-0,3390	0,0010	0,0463	-0,8226
PEX2	0,5866	6,8017	0,3384	0,0010	0,0464	-1,0161
MSX1	2,8680	8,2560	0,3369	0,0010	0,0478	-1,0358
CYP2C18	-2,1826	-0,8609	-0,3361	0,0011	0,0480	-0,9296
SORBS2	2,0008	6,0159	0,3341	0,0011	0,0500	-0,9752
UPP1	-0,8969	5,4543	-0,3339	0,0011	0,0500	-1,1048

logFC: fold change of gene expression; AveExpr: average expression; t: P-value; adj P value: *p*-value after Benjamini Hochberg correction for multiple testing.

Table S2. Overview of differentially expressed genes in vascular invasion profiles available in literature.

Mannelqvist (2011) Endometrial carcinoma	*Upregulated:* ANGPTL4, COL8A1, FPR2, IL8, MMP, SERPINE1, TNFAIP6 *Downregulated:* ALDH1A2, ATCAY, C1orf114, COL4A6, FGFR2, ITIH5, KLHL13, MAMDC2, OGN, OSR2, SEMA5A
Kang (2018) Endometrial carcinoma	CLCN2, CPB1, ESR1, FMO2, GREM2, PKHDIL1, PRR9, RPTN, SLC9C2, TCHHL1, TMEM212
Watanabe (2019) Endometrial carcinoma	AUNIP, AURKA, BUB1, BUB1B, C5orf34, CAD, CCNA2, CCNB2, CDC20, CDCA8, CENPA, CHEK1, CKAP2, CKS1B, CLCN2, CSE1L, EIF2AK2, ESPL1, FAM38B, FANCA, FTL, GEN1, GINS1, HJURP, HMGA1, ISYNA1, KIF14, KIF20A, KIFC1, LAGE3, LSM5, LY6K, MCM4, MSH6, NDUFB9, NMU, NOP56, NT5DC2, PHGDH, PLCXD1, PLP2, PRR11, RACGAP1, RAD51AP1, RECQL4, SOX12, SRM, STON2, TACC3, TM7SF2, TNNT1, TONSL, TOP2A, TPX2, UBE2C
Regan (2016) NSCLC	*Upregulated:* VEGFC, LYPD6B, TSC22D1, ERGIC2, RPL7A, FTH1, PTPLAD2, ATP2B4, MTRNR2L1 *Downregulated:* ZNF280B, LYPD5, NF2, C10orf125, UNC5A
Fidalgo (2015) Breast carcinoma	*Upregulated:* C19orf33, LGALS7B, CPE, AGBL2, ARSG, UMOD, C13orf31, MYCBPAP, CXXC4, MED1, SHISA5 *Downregulated:* GFRA1, MF12, TBX21, KRT15, KFASC, FZD5, TCF7L1, MYBPC2, CRABP1, CIB2, DUSP3
Kurozumi (2019) Breast carcinoma	*Upregulated:* APOC1, APOE, CALML5, CCNB2, CDCA5, COX6C, DNAJA4, EEF1A2, ELF3, ERBB2, GNAS, HMGA1, HMGB2, HSPB1, IDH2, IFI27, ISG15, KRT18, KRT18P55, KRT19, KRT7, KRT8, LAPTM4B, LRRC26, LY6E, MMP11, MX1, NME1, NOP56, PGAP3, PITX1, PTTG1, S100P, SCD, SLC52A2, SLC9A3R1, SPDEF, TM7SF2, UBE2C, UBE2S, UCP2, YWHAZ *Downregulated:* ACTG2, ANG, ANXA1, C15, CDC42EP4, CEBPD, CFB, CFD, CLIC6, CXCL12, CXCL14, CYBRD1, CYP4X1, DCN, DKK3, DPYSL2, DUSP1, EEF1B2, FBLN1, FCER1A, FCGBP, FGD3, FOS, FST, GAS1, GSTP1, HBA2, HBB, HLA-DQA1, IL17RB, MAOA, MFAP4, MGP,MT1E, NDP, NINJ1, PDGFRL, PLGRKT, PYCARD, RPL3, S100A4, SELENOM, SERPINA3, SERPINE2, SGCE, SLC40A1, SLC44A1, SRPX, STC2, SUSD3, TNS3, TPM2, TXNIP, UBD, VIM, VTCN1, ZBTB20
Asaoka (2020) Breast carcinoma	*Upregulated:* STK26, CDH1, MKI67 *Downregulated:* -
Minguez (2011) Hepatocellular carcinoma	*Upregulated:* CD24, PGLS, HDLBP, GORASP2, TYMS, UBE2C, CPD, XPOT, YY1AP1, CDKN3, NARF, KDELR1, NOMO2, NDUFS8 *Downregulated:* PAH, PPARGC1A, MASP2, DEPDC7, GLYAT, UGT2B15, ZFAND5, KLF9, CYP3A4, SLC38A4, PIK3R1, PON1, DPYS, SLC38A2, GLYATL1, PCK1, MYLK, AASS, MAT1A, ADH4, RCL1

Table S2. *Continued*

Ho (2006) Hepatocellular carcinoma	AMDP3, TAF4B, SLC4A7, RAB38, RYR1, KIAA0010, DKFZP727M111, KIAA1441, TRIM8, THIN, OGG, MAFA, CSF3R
Yue (2019) Serous ovarian carcinoma	*Upregulated:* POSTN, LUM, THBS2, COL3A1, COL5A1, COL5A2, FAP, FBN1 *Downregulated:* -
Jiang (2019) Colon carcinoma	*DNA copy number alterations:* EP300, NOTCH1, ESR1, AKT1, DVL1, STAT5A, MED1, STAT3, STAT5B, SIRT1, PPARA, HDAC2, ARNT, CSNK1E, HDAc10, KAT2A, MYB, CITED2, IGF1R, MAPK1
Poyet (2017) Urothelial cell carcinoma	*Upregulated:* PDPN, LYVE1, SLP76 *Downregulated:* -
Sonohara (2019) Oesophageal squamous cell carcinoma	PLAC8, SLC12A8, CSPG4, TFPI, TNFSF10
Peters (2023) Endometrial carcinoma	*Upregulated:* CDH16, SST, KCNQ2, NEFH, L1CAM, DHRS2, IL6, CLDN19, MT1E, SLC6A12, CYP2C18, DDC, GAL, FABP3, MYL4, DES, SLC7A2, SLN, ATP1A3, SCHIP1, RHOF, NEXN, SNX10, OASL, MX2, CASP10, HSPB2, NID2, DUSP5, TRIB3, GLS, CDC25B, CLCF1, SPHK1, TUBA4A, THY1, FUT4, UPP1, IFI30, CLCN2, AURKA, PSMD3, SMAD3, SSH2, TEAD4, NFKB2, PVR, BCL2L1, NOP56, ABCD1, TOMM40, SLC3A2, EDH1 *Downregulated:* SKP1, CDC42, EI24, OSTC, TMED10, PEX2, CASP6, CANT1, PLA2G12A, CANX, KDM4B, PDCD4, HADH, ATP2B4, ICA1, CYB5A, OVOL2, KIFAP3, SLC27A1, CYB5R1, PERP, WFS1, EPHX1, FGFR1, NPHP1, SGCB, IRF6, MAST4, LRIG1, SLC1A1, NDRG2, RBP1, CD207, AMOT, SORBS2, AMPH, PCDH7, ESR1, MSX1, MPPED2, NDP

CHAPTER 9

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

General discussion and future perspectives

Although present in a minority of endometrial cancers (EC), lymphovascular space invasion (LVSI) is a risk factor for lymph node and distant metastases, as well as disease recurrence and poorer survival. When present, LVSI is usually found in the peritumoral myometrium and can be detected during routine light microscopic assessment of H&E slides derived from a uterine specimen. The aim of the studies included in this thesis was to improve our understanding of the extent to which the prognosis of EC is affected by LVSI quantity, to measure and improve reproducibility of LVSI assessment, and to initiate study of the molecular biology of LVSI in EC.

Substantial LVSI is associated with an adverse prognosis

In the combined PORTEC-1 and PORTEC-2 cohorts involving high-intermediate risk EC patients, the extent of LVSI correlated with prognosis (chapter 3). LVSI assessment using a three-tiered scoring system (no, focal or substantial LVSI) proved to be the strongest independent prognostic factor for pelvic regional recurrence, distant metastasis and overall survival. The risk of pelvic recurrence declined strongly when patients with substantial LVSI received adjuvant external beam radiotherapy (EBRT), and as a result the ESGO/ESTRO/ESP multidisciplinary guideline now recommends adjuvant EBRT when substantial LVSI is present in stage I EC [1].

In chapter 5, the impact of LVSI extent on prognosis was studied in high-grade EC. The same three-tiered system was applied to assess LVSI and again substantial LVSI but not focal LVSI proved to be an independent adverse prognostic factor for lymph node and distant metastases, leading to reduced survival. Both studies showed that the extent of LVSI is important for prognosis, while focal LVSI has no significant prognostic impact.

For a wide range of cancers the association between LVSI and lymph node metastasis and/or prognosis is undisputed. One could hypothesize that the extent of LVSI may have a similarly high prognostic value in cancers arising in other organs. However, while the number of studies is limited, studies of cervical (CC) [2-4], breast (BC) [36] and ovarian (OC) [5] carcinoma have confirmed that extensive LVSI is associated with a poorer prognosis. The presence, but not the extent, of LVSI has been incorporated in guidelines for BC and CC [7,8], but not OC [9]. As in the case of EC, advice regarding early-stage BC and CC could become more targeted if the prognostic relevance of (semi) quantitative LVSI assessment could be confirmed in studies with a high level of evidence. This is less likely for OC however, because the primary route of dissemination is peritoneal [10] rather than lymphatic as in EC, BC and CC.

If extent of LVSI also proves relevant in colorectal cancer (CRC), this will have major therapeutic consequences for early-stage tumors. Since the introduction of national screening programs for CRC, the detection of early-stage CRC has increased [11]. The vast majority of patients with a T1 CRC (infiltration of only the submucosa) do not have lymph node metastases and can be curatively treated via endoscopic resection. Surgical resection with lymph node (LN) dissection

is indicated when LN metastases are suspected, but prediction of LN metastases using CT colonography has limited accuracy [12]. The risk of LN metastases is 3%-14% for T1 tumors and is strongly associated with LVSI [13-15]. For pedunculated T1 CRC, a risk calculator has been developed. This estimates the risk of LN metastases based on five histological parameters, including LVSI, and is used to determine the need for additional surgical treatment. LVSI in CRC is reported using a two-tiered system (present or not) and is applied accordingly in the risk calculator. If, in analogy with EC, the risk of LN metastases in CRC is strongly associated with the extent of LVSI, the prediction models for LN metastases could be improved, ultimately leading to a reduction of overtreatment. This would necessitate a study design similar to those we describe in chapters 3 and 5 and would require a sample of approximately 2,500 T1 CRCs, taking into account an estimated prevalence of LVSI of 3-5% [16-18]. The annual incidence of CRC is approximately 14,000 and half of patients are stage I (T1-T2), of whom 38% are treated via local excision [19]. This means that the proposed study population could be achieved using tissues and data collected over two years within the screening program.

Molecular characteristics associated with LVSI

In the field of molecular pathology, more advanced and ever faster techniques allow us to investigate the smallest molecular details of tumors. At the same time, these new techniques require increasingly advanced bioinformatic knowledge to interpret the wealth of data. In the 2020 WHO classification, molecular analyses of EC identified four subgroups [20]. The prognostic relevance of these subgroups is undisputed and the characteristics per subgroup are well described [21-23]. LVSI is more frequent in mismatch repair-deficient (MMRd) and *TP53*-mutated (p53abn) tumors compared to *POLE*-mutated (*POLE*-mt) and tumors with “no specific molecular profile” (NSMP). In an early-stage EC cohort, LVSI prevalence was 8.9%, 5.4%, 0% and 2.4% for MMRd, p53abn, *POLE*-mt and NSMP, respectively [24]. In a multivariate analysis including the molecular subgroups, this study also found that substantial LVSI is an independent prognostic factor [24]. However, the prognostic impact of substantial LVSI within each subgroup is unknown and might be relevant. For example, the reported prevalence of LVSI among *POLE*-mt EC varies widely (0% to almost 40% in stage I [125, 196]) and seems of little influence within a subgroup with an excellent prognosis (for whom adjuvant treatment can be omitted in stage I-II [1]). However, does this still apply when there is substantial LVSI?
The ongoing PORTEC-4a trial may shed some light on this question. In this trial, 500 high-intermediate risk endometrioid EC are prospectively allocated to adjuvant treatment schemes according to an integrated risk profile based on molecular subgroup, L1-CAM expression, *CTNNB1* mutational status and substantial LVSI. In the study arm all *POLE*-mt EC will allocate to the favorable group regardless of LVSI and observation is recommended [25]. The first results are expected in 2023 and will reveal if substantial LVSI and *POLE*-mt do co-occur [26]. Nevertheless, numbers will be too small for prognostic impact analyses, which would require the combination of multiple databases.

The uneven distribution of LVSI among molecular subgroups has led to the hypothesis that LVSI may be associated with specific molecular events. Chapter 8 describes a pilot study designed to detect differences in gene expression between tumors with substantial LVSI and those without LVSI. In this study RNA levels of MMRd EC's with and without substantial LVSI were compared, but did not reveal differentially-expressed genes. In a comparison of LVSI-associated expression profiles (both EC as well as non-EC), eight overlapping genes were identified, none of which were referenced as key regulators in carcinogenic cell biologic processes (table 1).

Table 1. List of recurrent genes in vascular invasion-associated gene expression profiles

Gene	*Full name*	*Synonyms*	*Hallmark*	*Action*
AURKA	Aurora kinase A	AIK, ARK1, AURA, BTAK, STK6, STK7, STK15, PPP1R47	a) Deregulating cellular metabolism; b) Resisting cell death; c) Inducing or accessing vasculature	a) Promoting glycolysis [63]; b) Avoiding autophagic cell death and dysregulation of DNA damage response [64, 65]; c) Inducing angiogenesis [66]
ATP2B4	ATPase plasma membrane Ca2+ transporting	ATP2B2, MXRA1, PMCA4, PMCA4b, PMCA4x	Activating invasion and metastasis	p38 MPAK pathway induced migration [67]
CLCN2	Chloride voltage-gated channel 2	CIC-2, CLC2, ECA2, ECA3, EGI11, EGI3, EGMA, EJM6, EJM8, HALD2, LKPAT, cIC-2	Sustaining proliferative signaling	Activation of β-catenin [68]
ESR1	Estrogen receptor 1	ER, ESR, ESRA, ESTRR, Era, NR3A1	Sustaining proliferative signaling	Multiple [69]
MT1E	Metallothionein 1E	MT-1E, MT-IE, MT1, MTD	Multiple	Multiple [70]
NDP	Norrin cystine knot growth factor NDP	EVR2, FEVR, ND	Sustaining proliferative signaling	Activation of β-catenin [RefSeq, Feb 2009]
NOP56	NOP56 ribonucleoprotein	NOL5A, SCA36	Enabling replicative immortality	Indirectly associated with telomerase activity [71]
UBE2C	Ubiquitin-conjugating enzyme E2 C	UBCH10, dJ447F3.2	Genomic instability and mutation	Contributes to genomic instability by avoiding mitotic checkpoints [72]

The table shows the action of the gene and the associated hallmarks.

Literature covering molecular drivers associated with LVSI is limited and mainly focuses on breast cancer. Despite differences in tumorigenic drivers between breast and endometrial cancer, a breast cancer model is useful when LVSI is viewed at as a crucial step in the metastatic pathway and is being studied in the context of cell migration leading to intravasation, cell survival in the circulation and colonization of lymph nodes or distant sites. However, processes involving the micro-environment, such as epithelial-mesenchymal transition and interactions with inflammatory and stromal cells, might differ due to divergent environments in the muscular uterus compared to fatty, collagenous breast tissue. The steps of the metastatic pathway and associated processes in breast cancer have been thoroughly reviewed by Fares et al. and Kariri et al. [27, 28], but the key genes involved in processes leading to LVSI did not

overlap with those recurrently present in LVSI-related expression profiles. It is unlikely that this is due to differences between the tumor's originating organs; this is more likely explained by differences in study design. For example, at one end of the spectrum, research in the field of cell migration, including LVSI, makes use of cell lines and/or organoids. Cell lines are often commercially available and genetic properties known. These cell lines are maintained in controlled, well-documented environments to reduce experimental bias and to ensure reproducible results. This type of model represents an optimal system in which to study cell biological processes, but there are also limitations. Advantages include reproducibility and a wide range of experimental options, whereas the obvious drawback is translational, i.e. moving from an *in vitro* controlled environment to *in vivo* complexity. At the other end of the spectrum, expression profiles of vascular invasion resulting from patient-derived tumor samples reflect a totally uncontrolled real-life setting. After stratification for the presence of LVSI, these profiles contain a selection of genes with predictive value for the probability of LVSI that does not necessarily correspond to the underlying mechanisms of LVSI. In addition, the lack of recurrent genes in LVSI-associated expression profiles (between and within tumor types) throws further doubt on the importance of these genes. Clearly, substantial gaps in knowledge concerning mechanisms of the metastatic pathway and LVSI-associated gene expression remain to be bridged. To bridge these gaps, future research should focus on integrating cell biological and clinical knowledge. The integration of morphological patterns with molecular data will help generate new mechanistic questions that will likely yield novel insights, but this will require a multidisciplinary approach. For example, when low grade EC lacking inflammatory infiltrates and desmoplastic reactions infiltrates with a pushing border, LVSI is usually absent. However, in areas of infiltrative growth with desmoplastic stroma and lymphocytic infiltrate, LVSI is more likely. Are immune cells involved in processes that contribute to LVSI, and if so, how? Both MMRd and *POLE*-mt tumors are associated with dense lymphocytic infiltrates, but LVSI is seen less frequently in *POLE*-mt tumors. Immune infiltrates surrounding tumors are composed of divergent cells with inhibitory as well as facilitating capabilities [28], so the exact composition might be crucial for LVSI. It is also possible that there is no direct association between LVSI and the composition of the infiltrate. One theory proposes that the infiltrate is triggered by hypermutation and consequent neoantigen formation, thus intravascular *POLE*-mt cells, relative to MMRd cells, may struggle to adapt to the intravascular micro-environment due to their widespread genomic aberrations.

LVSI definition and reproducibility

The wide variability in the prevalence of LVSI reported in stage I EC most likely results from the lack of uniformity in defining LVSI and the frequent artifacts that hamper diagnosis. While a study enriched for difficult cases showed good quantification and reproducibility of LVSI recognition (chapter 6), it may be advisable to incorporate a cut-off value in the definition of 'substantial LVSI'. To address this need we designed a study (chapter 7) that proposes a detailed and easy-to-apply definition of 'substantial LVSI'. With a threshold set at ≥4 LVSI-

positive vessels in at least one H&E slide, we anticipate that publication of the practice guideline will encourage implementation of LVSI assessment (chapter 2).

Immunohistochemical staining (IHC) was not used to diagnose LVSI in the studies included in this thesis. Firstly, as our aim was to investigate whether semi-quantification in everyday practice has prognostic value, the usual diagnosis of LVSI using standard H&E slides appeared the best option. Secondly, as relevant tissue blocks from the PORTEC-1, -2 and DCGD studies have limited availability, LVSI diagnosis supported by the use of IHC was never under serious consideration. Nonetheless, the use of IHC to assist LVSI diagnosis has been studied by others and has been shown to increase the detection rate in comparison to standard H&E assessment [29-31]. However, IHC was not superior in the detection of clinically-relevant LVSI [32-34] which, in light of our results (chapters 3 and 5), suggests that additional cases mainly included focal LVSI.
Following the incorporation of a definition of 'substantial LVSI' in the European guideline for EC management, reporting of LVSI has gained importance. This development may in turn lead to an increased use of IHC, which appears especially useful in difficult cases, for example those exhibiting artifacts such as retraction. To conclude, routine application of IHC is unlikely to be efficient, as H&E-based confirmation of both vitality and nature of the cells (tumor or macrophages) is still required. Additionally, the current threshold (≥4 LVSI positive vessels in at least one slide) was not designed for this purpose and might result in overcalling.

In an era of swift molecular and digital evolution, promoting a light microscopic assessment tool for assessment of a tumor characteristic may seem outdated. However, our results have shown that quantification of LVSI is robust and is a very strong prognostic factor, even in high grade EC and independent of molecular class [35]. Furthermore, it is simple and effective, even in low resource settings. Digital evolution in pathology is evolving rapidly and artificial intelligence (AI) is an emerging diagnostic aid especially suited to the detection and quantification of patterns and objects like LVSI. The transition in AI from engineering, which requires the definition of specific features, to deep learning (DL) by training allows development of algorithms that can detect patterns such as LVSI [36]. In the future AI will be used to identify or, more likely, will assist the pathologist with LVSI detection. One example is the Gleason score in prostate carcinoma, which is an important prognostic marker and as such reminiscent of LVSI [37]. Traditional grading of the Gleason score shows significant interobserver variability [38], but recent work by Bulten et al. showed that AI can support and improve Gleason grading of prostate biopsies [39]. The DL model used in the study was previously validated and had a grading performance similar to pathologists [40], while AI-assisted pathologists outperformed both unassisted as well as a standalone AI system. Moreover, AI-assisted Gleason grading resulted in reduced intra- and interobserver variation and therefore improved diagnostic quality [39]. Similarly, LVSI assessment is based on pattern recognition (but unlike Gleason, also object detection) that might be improved by similar AI systems. The development of this type of

system is challenging however. LVSI prediction models based on computed tomography (CT) and magnetic resonance imaging (MRI) have been developed for EC, gastric, hepatocellular and pancreatic cancer [41-44], but no AI-assisted LVSI detection system for H&E has been developed to date and development will require large numbers of digitalized whole slide EC images. At the moment, models able to predict the molecular subgroup of EC are being constructed [45], with the major advantage of models being that they can use molecular data. Ground-truth annotations for LVSI are currently lacking, however, and their development will require time-consuming manual annotation, and will also need to overcome challenges introduced by artifacts. Nonetheless, there are various models to choose from, with varying levels of supervision and types of learning [46]. Besides the large amount of training material required, any model will need time, expertise and sufficient resources with respect to computational facilities and data storage. Once a model has been designed, built and validated, implementation could potentially be hampered by even seemingly minor issues like interlaboratory variation in standard H&E staining [47]. Nevertheless, the development of a DL model for synergistic LVSI assessment should be encouraged and will likely improve diagnosis, thereby contributing to cost-effective treatment as well as a fast and efficient workflow. Moreover, the integration of further clinical data will help extend the frontiers of the pathologist beyond the microscopic slide.

Is substantial LVSI a surrogate marker for (sentinel) lymph node positivity?

Around 10% of women with EC, initially thought to have cancer limited to the uterus eventually prove to have LN metastases at the time of diagnosis [48]. Currently available pre-operative imaging for detection of LN metastases has a low sensitivity, leading to a risk of a false negative diagnosis [49, 50].

Surgical treatment with total hysterectomy and bilateral salpingo-oophorectomy is widely accepted, whereas systematic lymphadenectomy remains controversial. Systematic lymphadenectomy involves the removal of pelvic and/or para-aortic lymph nodes, and is regarded a diagnostic procedure only, since therapeutic benefit has not been demonstrated [51, 52]. The procedure is controversial because it is associated with complications including lower extremity lymphedema, affecting quality of life in these mainly elderly women [53]. While some consider the procedure to be therapeutic for prevention of LN recurrence, others claim it is unnecessary as regional (pelvic) disease control can also be achieved by selective adjuvant pelvic radiotherapy for women at risk of recurrence [54]. Sentinel node (SLN) mapping is increasingly being utilized for nodal staging purposes in EC. In a comparison with systematic lymphadenectomy it was shown to be superior for pelvic and non-inferior for para-aortic LN staging [55]. In addition, the procedure resulted in fewer perioperative and lymphatic complications [56-58]. LVSI (without quantifying the extent) is associated with SLN metastases [59-61], so future studies should determine the prognostic value of substantial LVSI in the context of SLN staging and subsequent adjuvant therapy strategies. It is currently unclear which method of estimating recurrence risk in stage I EC is superior, but as liquid biopsy (LB) is an

emerging technique in other tumors, it may also be relevant to LVSI. LB relies on the fact that growing tumors shed tumor cells, exosomes and cell-free, circulating tumor DNA (ctDNA) into blood, in which specific mutations may be detected using next generation sequencing (NGS). This approach provides a real-time impression of disease activity and therefore has broad clinical potential when monitoring residual disease, relapse and therapeutic efficacy [62]. Although experience with ctDNA analyses in EC is currently limited, as soon as issues concerning sensitivity and cost-effectiveness have been resolved new applications are expected.

Conclusion

The studies constituting this thesis have demonstrated that the presence of substantial LVSI is a strong and independent prognostic factor for recurrence, distant metastasis and overall survival among high-intermediate and high-risk EC patients. The method has proven to be robust and its implementation in everyday practice has gained momentum following its incorporation in the European clinical guideline for EC management. We are proud to note that the work described in this thesis played an important role in this implementation, and we hope it will inspire cancer researchers with other specialties to consider the role of LVSI. In a future characterized by algorithms and data integration, AI-assisted LVSI detection is expected to further boost reliability. Finally, indications of an association between LVSI and the genetic profile of ECs call for follow-up research, starting with the leads produced by the PORTEC 4a study in particular.

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APPENDICES

NEDERLANDSE SAMENVATTING
LIST OF PUBLICATIONS
CURRICULUM VITAE

Nederlandse samenvatting

Een ander woord voor endometriumcarcinoom in de Nederlandse taal is baarmoederkanker. Het ontstaat in het slijmvlies aan de binnenkant van de baarmoeder, vooral bij vrouwen na de menopauze en uit zich meestal in een vroege fase van de ziekte met vaginaal bloedverlies. Als de diagnose endometriumcarcinoom gesteld is, vindt in de meeste gevallen een chirurgische behandeling plaats waarbij de baarmoeder, eileiders, eierstokken en soms lymfklieren verwijderd worden. Het hangt af van risicofactoren of er hierna nog aanvullende behandeling nodig is.

Die risicofactoren worden vastgesteld door pathologisch onderzoek van de verwijderde organen. Zo worden het type en de graad van het endometriumcarcinoom vastgesteld, maar ook of, en hoe diep de tumor in de spierlaag van de baarmoeder groeit en of er sprake is van doorgroei in andere organen. Bij microscopisch onderzoek wordt ook beoordeeld of de tumor in de lymfbanen groeit. De groei van tumor in de lymfbanen en bloedvaten heet lymfangioinvasieve groei (LVSI) en dit is een risicofactor. Door de risicofactoren en tumoreigenschappen te combineren met de fase waarin de ziekte zich bevindt, wordt een risicoprofiel opgesteld. Dit risicoprofiel zegt iets over de kans dat de ziekte terugkomt en de kans om eraan te overlijden. Het risicoprofiel wordt bovendien gebruikt om vast te stellen of er aanvullende behandeling nodig is en welke behandeling dit dan zou moeten zijn.

Dit proefschrift gaat voornamelijk over pathologische aspecten van LVSI bij endometriumcarcinoom. In grote lijnen komen de volgende aspecten aan de orde: de wijze waarop de diagnose LVSI wordt gesteld, de betekenis van de diagnose voor de prognose van de patiënt en de onderliggende tumorbiologie van LVSI.

Het belang van LVSI in grote lijnen

LVSI wordt vastgesteld tijdens het microscopisch onderzoek van de tumor door de patholoog. LVSI wordt gedefinieerd als de aanwezigheid van tumorcellen in een lymfvat dat zich bevindt in het normale spierweefsel rondom de tumor. Het vaststellen van LVSI is soms moeilijk, bovendien wordt niet altijd gerapporteerd of het aanwezig is. In de meest recente Europese richtlijn heeft het vaststellen van LVSI en de mate ervan een belangrijke rol gekregen en daarom is het van belang dat pathologen dit structureler en beter gaan rapporteren. Met dit doel is hoofdstuk 2 geschreven. Het gaat in op belangrijke aspecten bij de pathologische beoordeling van LVSI. Zo wordt uitgelegd dat het vaststellen van de mate van LVSI van groot belang is voor de prognose. Ook wordt uiteengezet wat bekend is over LVSI in relatie tot tumorkenmerken als tumor type, groeipatroon, moleculaire kenmerken en de reactie van weefsel rondom de tumor. Nadat de context van LVSI geschetst is, wordt in het laatste deel van het hoofdstuk ingegaan op praktische aspecten waarmee een patholoog rekening moet houden bij de beoordeling van LVSI en die het makkelijker maken om LVSI te onderscheiden van pseudo-LVSI.

Betekenis van substantiële LVSI voor de prognose

In hoofdstuk 3 wordt de studie beschreven waarin onderzocht is of de mate van LVSI aanvullende prognostische waarde heeft. Histologische coupes die beschikbaar waren van patiënten die deelnamen aan twee gerandomiseerde klinische studies (PORTEC-1 en PORTEC-2) werden beoordeeld op de aanwezigheid van LVSI volgens verschillende definities. In totaal werden 926 tumoren onderzocht en werden de scores gecorreleerd met gegevens over terugkerende ziekte in lymfklieren of uitzaaiingen. Substantiële LVSI (LVSI in ernstige mate) kwam niet veel voor (4,8% van de patiënten), maar bleek een zeer goede voorspeller van terugkerende ziekte in lymfklieren. Deze voorspelling was onafhankelijk van andere risicofactoren en bovendien bleek dat het risico op terugkerende ziekte op deze plek voorkomen kon worden door uitwendige bestraling van dit gebied na de operatie. Ook bleek substantiële LVSI een verhoogde kans te geven op uitzaaiingen op afstand en afname van de overlevingsduur.

De patiënten die onderzocht werden binnen PORTEC-1 en PORTEC-2 hadden allemaal een hoog-intermediair risicoprofiel. In een vervolgstudie werd onderzocht of het vaststellen van de mate van LVSI volgens dezelfde systematiek ook prognostische waarde heeft voor patiënten met een hoog risicoprofiel. In hoofdstuk 5 wordt de studie beschreven die werd uitgevoerd op weefsel en klinische gegevens van patiënten met een hoog risicoprofiel afkomstig uit de Deense Gynaecologische Kanker Database (DGCD). Histologische coupes van 376 tumoren werden beoordeeld op aanwezigheid en mate van LVSI. Deze informatie werd gecombineerd met gegevens over terugkerende ziekte en overlevingsduur. Uit de analyses bleek dat substantiële LVSI ook bij patiënten met ongunstige tumorkenmerken leidt tot een verhoogd risico op terugkerende ziekte in de lymfklieren, uitzaaiingen op afstand en afname van zowel ziektevrije als totale overlevingsduur. Zelfs bij patiënten die op het moment dat de diagnose gesteld wordt al lymfklier uitzaaiingen hebben, beïnvloedt substantiële LVSI het ziektebeloop nadelig.

De typering van hooggradige endometriumcarcinomen is moeilijk en uit hoofdstuk 4 blijkt dat revisie van deze tumoren door gespecialiseerde pathologen zinvol is. Revisie leidt tot een nauwkeuriger diagnose en daarmee een betere inschatting van de prognose. In een enkel geval blijkt de tumor niet hooggradig te zijn en hoeft een patiënt waarschijnlijk minder zware nabehandeling te ondergaan.

Betrouwbaarheid van de diagnose LVSI en het aanscherpen van de definitie

Nu blijkt dat het vaststellen van de mate van LVSI belangrijke informatie verschaft over de prognose van de patiënt, is het van belang om te weten of de diagnose LVSI ook betrouwbaar gesteld kan worden en of onafhankelijke beoordelingen leiden tot dezelfde diagnose. Die vraag werd beantwoord door de interobserver studie in hoofdstuk 6 waarin enerzijds werd getoetst of pathologen in staat zijn LVSI te onderscheiden van pseudo-LVSI en waarin anderzijds werd getoetst of er in geval van LVSI, overeenstemming was over de mate waarin dit aanwezig was.

De studie werd verricht onder een internationaal panel van experts op het gebied van gynaecologische pathologie. Voor het eerste deel van de studie werden 48 ingescande coupes gepresenteerd waarbij gevraagd werd te beoordelen of er in een specifiek gebied sprake was van LVSI of niet. In het tweede deel van de studie werd gevraagd om de definities van focale en substantiële LVSI toe te passen op een nieuwe selectie van 42 endometriumcarcinomen. De mate van overeenstemming van het panel werd uitgedrukt in de intraclass correlatiecoëfficiënt en was 0,64 (op een schaal van 0 tot 1) in het eerste deel van de studie. De score was 0,62 in het tweede deel van de studie, waarbij de scores voor de mate van overeenstemming in woorden voor beide onderdelen kan worden omschreven als 'voldoende'.

Hoewel uit deze studie bleek dat de definitie voldoende toepasbaar was voor gebruik in de praktijk, werd tijdens presentaties en op congressen toch herhaaldelijk gevraagd om criteria voor substantiële LVSI die makkelijk toepasbaar zijn in de praktijk. Om aan die wens te beantwoorden is een studie verricht met het doel de definitie van substantiële LVSI scherp te stellen, deze wordt beschreven in hoofdstuk 7. Voor deze studie werden alle coupes van tumoren met LVSI uit PORTEC-1 en -2 opnieuw beoordeeld en werden details genoteerd over het aantal beoordeelde coupes met tumor, het aantal coupes met LVSI, het aantal lymfvaten met daarin een cluster tumorcellen, het aantal tumorcellen in het grootste cluster. Ook werd de oppervlakte van de spierlaag berekend waarin LVSI kon voorkomen en werden afstanden gemeten tussen het diepste lymfvat met LVSI en de buitenkant (serosa) van de baarmoeder en ten slotte tussen het diepste lymfvat met LVSI en het diepste punt waarin de tumor de spierlaag ingegroeid was. Vervolgens werden berekeningen gemaakt waarbij op verschillende afkappunten de kans op terugkerende ziekte in lymfklieren in het bekken werd berekend. Er werd vastgesteld dat het risico op terugkerende ziekte bij tenminste vier positieve lymfvaten in tenminste één coupe zo groot was dat het ook belastende aanvullende behandeling (uitwendige radiotherapie) zou rechtvaardigen. Vervolgens werd de systematiek herhaald in een onafhankelijke patiëntenpopulatie (DCGD) om te toetsen of de definitie adequaat was. In de onafhankelijke patiëntenpopulatie werden vergelijkbare resultaten behaald waarmee de definitie van substantiële LVSI werd vastgesteld als de betrokkenheid van ten minste vier lymfbanen in tenminste één histologische coupe.

Moleculaire biologie van LVSI

In hoofdstuk 8 wordt een aanzet gegeven om de biologie die ten grondslag ligt aan het ontstaan van LVSI bloot te leggen. Het DNA van tumorcellen heeft veranderingen ondergaan waardoor onder meer ongeremde celgroei, uitzaaiingen en ontsnappen aan mechanismen mogelijk worden die zouden moeten leiden tot celdood. Bij endometriumcarcinoom kunnen vier groepen worden onderscheiden op basis van veranderingen aan het DNA (moleculaire classificatie): polymerase epsilon (*POLE*)-gemuteerd (POLE-mt); mismatch repair deficiënt (MMRd); *TP53*-gemuteerd (p53abn) en tumoren zonder een van deze kenmerken, dus zonder specifiek moleculair profiel (NSMP). De mate waarin LVSI voorkomt verschilt tussen de groepen,

zo komt LVSI het meest voor bij p53abn en MMRd tumoren, nauwelijks bij NSMP en bijna nooit bij POLE-mt. Deze verschillen leiden tot de hypothese dat er ook verschillen zijn in tumor DNA tussen tumoren met en zonder LVSI die aangetoond kunnen worden door genexpressie analyse. Uit een publieke database (cbioportal.org) werd genexpressie data verkregen die vergeleken werd tussen tumoren zonder LVSI (n=88) en substantiële LVSI (n=19). Daarbij werden 94 genen gevonden die in belangrijke mate verschilden in expressie. In een daaropvolgende pilotstudie werd RNA geïsoleerd uit 32 MMRd tumoren zonder LVSI en dit werd vergeleken met RNA van 16 MMRd tumoren met substantiële LVSI waarbij de expressie van 800 genen bestudeerd werd. Hierbij werden geen genen gevonden die in deze vergelijking in belangrijke mate verschillend tot expressie komen.

Concluderende opmerkingen

Samenvattend hebben de studies in dit proefschrift bijgedragen aan de onderkenning van substantiële LVSI als belangrijke prognostische factor bij endometriumcarcinoom. Dit blijkt uit het feit dat substantiële LVSI opgenomen is in de Europese behandelrichtlijn die geldt als leidraad voor behandeling voor vrouwen met endometriumcarcinoom, en recentelijk ook in de nieuwste FIGO 2023 classificatie. Dit proefschrift heeft duidelijk gemaakt dat niet zozeer de aanwezigheid, maar vooral de mate van LVSI belangrijk is bij endometriumcarcinoom. Toekomstige klinische studies zullen laten zien of aanwezigheid van substantiële LVSI consequenties moet hebben voor de aanvullende behandeling. Het is ook denkbaar de mate van LVSI in andere orgaansystemen relevant is, zoals bij vroege dikke darmtumoren.

Daarnaast is de definitie van substantiële LVSI aangescherpt en daardoor beter toepasbaar voor pathologen. Het is te verwachten dat door het aanscherpen van de definitie van substantiële LVSI de betrouwbaarheid van de diagnose verbetert, maar toekomstig onderzoek zal moeten aantonen of dit zo is. De opmars van kunstmatige intelligentie in de pathologie biedt een andere veelbelovende mogelijkheid om de beoordeling van LVSI te verbeteren.

Dit proefschrift heeft niet kunnen bijdragen aan betere inzichten in de tumorbiologie die ten grondslag ligt aan LVSI. Dat het niet gelukt is om op DNA-niveau specifieke veranderingen vast te stellen die vaker voorkomen bij substantiële LVSI, wil niet zeggen dat deze er niet zijn. De wens om het ontstaan van LVSI te begrijpen motiveert om breder te zoeken met andere technieken, in meer en beter geselecteerde tumoren en in samenwerking met fundamenteel onderlegde experts.

List of publications

Peters EEM, León-Castillo A, Hogdall E, Boennelycke M, Smit VTHBM, Hogdall C, Creutzberg CL, Bosse T, Nout RA, Ørtoft G. *Substantial Lymphovascular Space Invasion Is an Adverse Prognostic Factor in High-risk Endometrial Cancer.* Int J Gynecol Pathol. 2022 May 1;**41**(3):227-234. PMID: 34392268.

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Curriculum vitae

Elke (Elizabeth Maria) Peters werd in Ottersum geboren op 5 augustus 1980. De middelbare schooltijd werd aangevangen op het Elzendaalcollege in Boxmeer en in 1999 afgerond met een VWO diploma behaald aan het ROC voor volwassenenonderwijs in Nijmegen. Hierna begon zij met de studie geneeskunde aan de Rijksuniversiteit Groningen en deze werd in 2006 afgerond aan Radboud Universiteit in Nijmegen. Als student kwam zij in de UMC Raad voor het eerst in contact met pathologen die door hun rustige uitstraling en scherpe analytische blik waarschijnlijk de eerste interesse voor het vak pathologie hebben gewekt. Zij maakte kennis met wetenschappelijk onderzoek in Christchurch, Nieuw-Zeeland waar zij dierexperimenteel onderzoek deed naar de invloed van peptiden op GnRH afgifte door de hypofyse van ratten.

Na het afronden van de studie geneeskunde begon zij in 2007 als arts-onderzoeker bij de afdeling pathologie van het RadboudUMC die zij verliet om in 2008 als beleidsadviseur eerstelijns zorg aan het werk te gaan bij het Ministerie van Volksgezondheid, Welzijn en Sport en vervolgens bij Zorgverzekeraars Nederland in de functie van medisch adviseur. Maar in 2011 solliciteerde zij naar de opleiding tot patholoog in het LUMC omdat het gemis aan diepgang en concreet bijdragen aan het geneeskundige proces te groot was. Tijdens de opleiding tot patholoog werd de eerste hand gelegd aan het onderzoek dat zou leiden tot dit proefschrift.

Eind 2016 werd de opleiding tot patholoog afgerond en na het verwerven van een positie binnen de staf van het Haaglanden Medisch Centrum in 2017 werd het volbrengen van dit onderzoek een van de prioriteiten. Elke is moeder van Nout (2011) en Fieke (2013).