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].

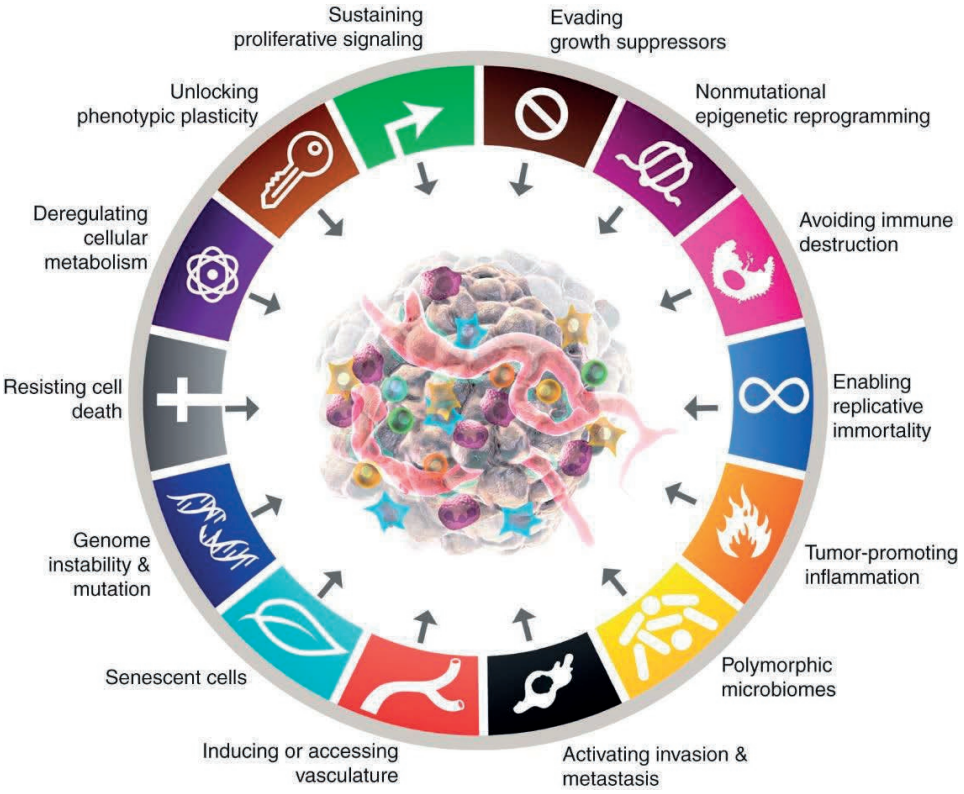

Figure 1. The 'Hallmarks of Cancer' model provides a framework for cancer conceptualization. The hallmarks and enabling characteristics play a role in cancer development, progression and maintenance (adopted from Hanahan, 2022 [2]).

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].

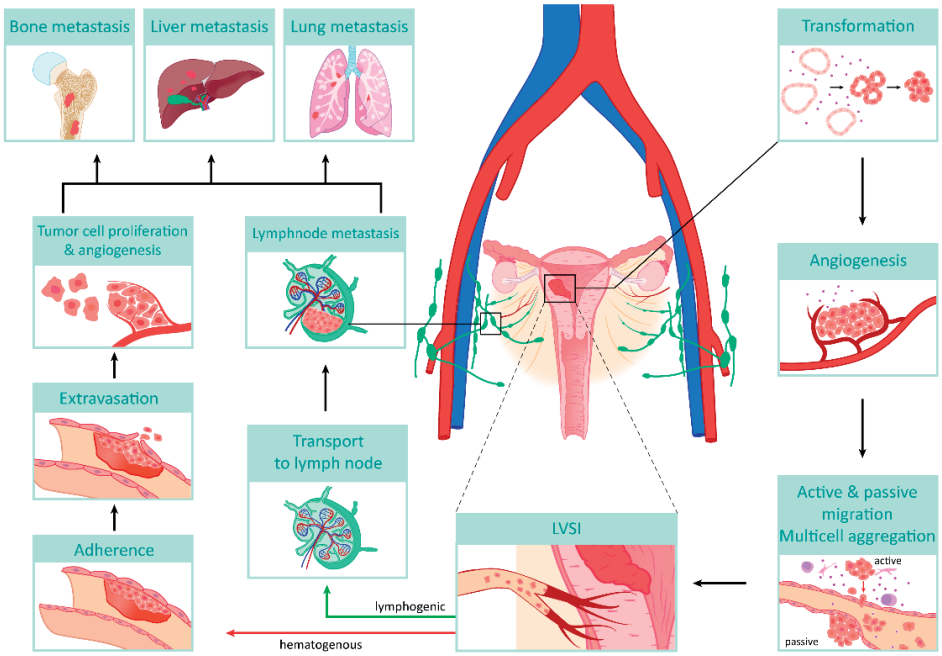

Figure 2. The sequential process resulting in metastatic disease. The progression model begins with cancer initialization by induction of cancer cells, followed by angiogenesis, invasion, migration, intravasation, circulation, extravasation, finally leading to colonization (adopted from Talmadge, 2010 [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].

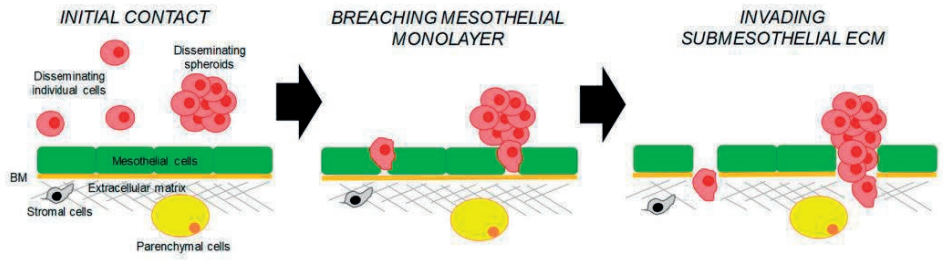

Figure 3. Modellation of transcoelomic spread.

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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