Lymphovascular space invasion in endometrial carcinoma

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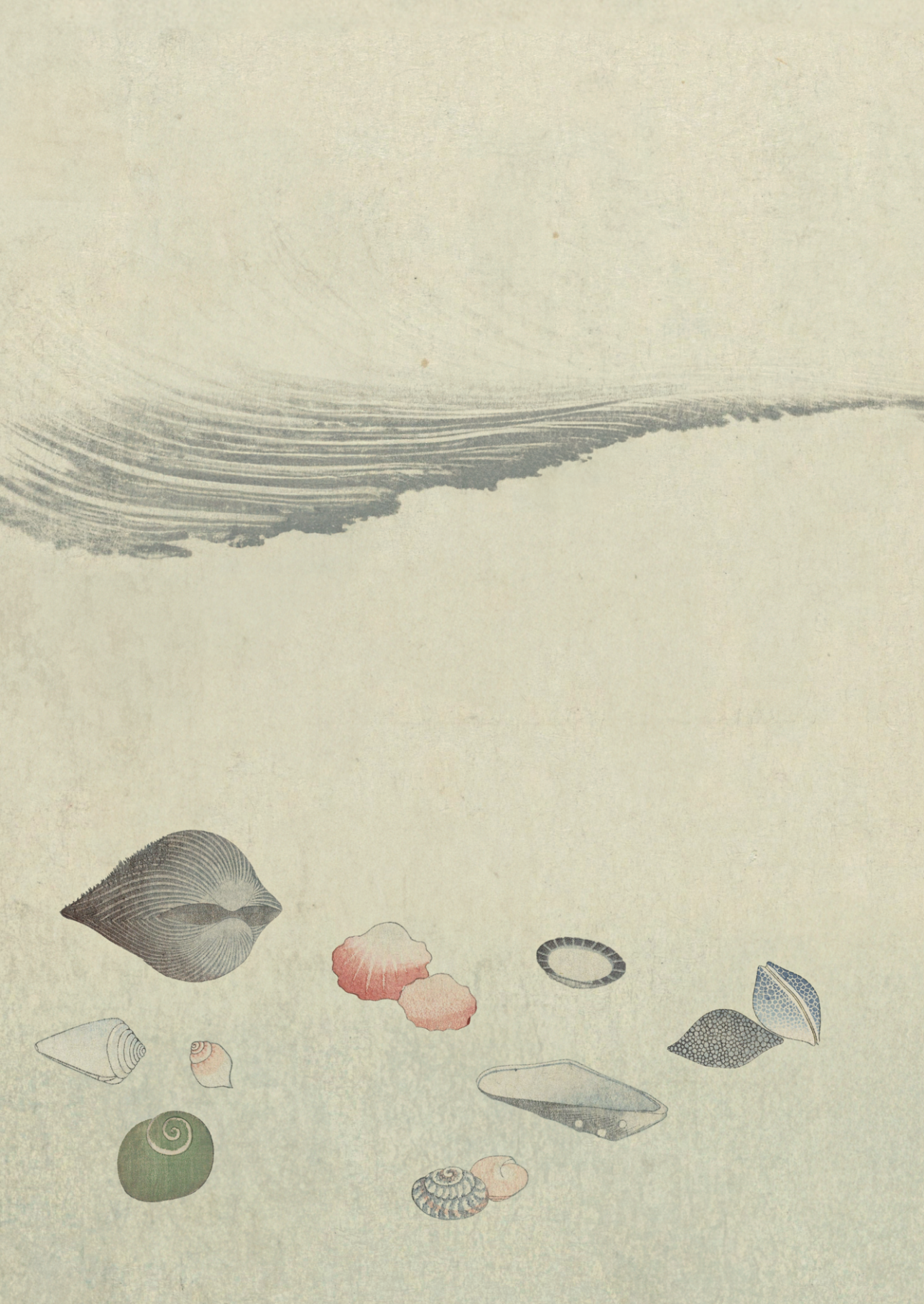

CHAPTER 9

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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