Prognostic markers and the individualized management of endometrial carcinomas
PROGNOSTIC MARKERS AND THE INDIVIDUALIZED MANAGEMENT OF ENDOMETRIAL CARCINOMAS

Proefschrift

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Voor mijn vader en moeder
Voor Karin
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CHAPTER 1

INTRODUCTION AND THESIS OUTLINE
Endometrial carcinoma

Endometrial carcinoma is the most common gynecological malignancy in the western world, and with a current incidence of 16/100,000 in the Netherlands it affects around 2000 women each year, most of them postmenopausal. Known risk factors for developing an endometrial carcinoma are exo- and endogenous estrogen exposure, diabetes, early menarche, nulliparity, late menopause, old age and tamoxifen use. The incidence of endometrial carcinomas is increasing, predominantly as a result of increases in both life expectancy and the prevalence of a high Body Mass Index. As a result, the prevalence of endometrial carcinomas in the United States is already as high as 24/100,000. Endometrial carcinomas are traditionally divided into endometrioid and non-endometrioid carcinomas, based on histology. Endometrioid carcinomas are assumed to arise from hyperplastic endometrium under the influence of unopposed estrogen stimulation, and generally have a favorable prognosis. Non-endometrioid carcinomas on the other hand, which have serous or clear cell histology, are assumed to arise from atrophic endometrium, and have a worse outcome. The majority of the endometrial carcinomas has endometrioid histology, and presents at an early stage with postmenopausal blood loss. Overall, the prognosis of endometrial carcinomas is therefore favorable, yet annually around 500 women still die of this disease in the Netherlands.

When endometrial pathology is suspected, the endometrium is visualized using a transvaginal ultrasound, and a subsequent endometrial biopsy is advised when the endometrium is measured to be ≥ 4mm thick. The primary treatment is based on the clinical stage, the histological type, and the tumor grade, and is most often surgical. In patients with a clinical stage I, low or intermediate grade endometrioid carcinoma a hysterectomy and a bilateral salpingo-oophorectomy are recommended. Studies have shown that in these patients there is no benefit from a routine systematic lymphadenectomy. In contrast, because high grade endometrioid, and non-endometrioid carcinomas have a substantial risk of lymph node metastasis, surgical staging is indicated for these carcinomas. Based on the FIGO stage (Table 1), the presence of non-endometrioid or high grade endometrioid histology, the depth of myometrial invasion, the presence of lymphovascular space invasion, and the age of the patient adjuvant radiotherapy to prevent a locoregional recurrence is recommended in the Netherlands.

Vaginal brachytherapy is preferred for low and low-intermediate risk endometrial carcinomas, whereas external beam radiotherapy is required for high-intermediate and high risk carcinomas. Although some studies have shown that chemotherapy might reduce the risk of distant spread in selected cases, there is no international consensus about the use of chemotherapy yet. FIGO stage IV disease is rare, and management of these cases is not yet standardized.

Endometrioid carcinoma types

Endometrioid and non-endometrioid carcinomas were shown to be different on immunohistochemical and genetic levels. Endometrioid carcinomas are characterized by expression of the estrogen receptor (ER) and progesterone receptor (PR), microsatellite instability, and PTEN, KRAS, PIK3CA, and CTNNB1 mutations. In contrast, non-
endometrioid carcinomas are characterized by $TP53$ mutations, reduced E-cadherin expression, and $ERBB2$ amplifications.$^{45, 47-49}$ However, there is a growing evidence that the traditional dualistic model is too rigid.$^{50, 51}$ On a histological level, classification of endometrial carcinomas in one of the two subgroups can be challenging, as some present with a mix of endometrioid and non-endometrioid histology, and a mixed molecular profile.$^{52, 53}$ Additionally, based on the current concept of endometrial carcinogenesis, the endometrium next to endometrioid carcinomas is expected to be hyperplastic. Interestingly, in a recent study atrophic endometrium was found next to 20% of the endometrioid carcinomas.$^{54}$ These carcinomas were shown to have a worse prognosis than endometrioid carcinomas with adjacent hyperplastic endometrium, and might represent a third type, but the molecular profile of these carcinomas has not yet been studied. More recently, studies have shown that genetic profiling can be used to identify non-endometrioid carcinomas which were misdiagnosed as endometrioid carcinomas, and to identify aggressive endometrioid carcinomas wrongly identified as low risk endometrioid carcinomas.$^{55, 56}$ One study has classified endometrial carcinomas purely based on their genomic profiles, and identified four subgroups: a hypermutated and an ultramutated group, and a copy-number low and a copy-number high group.$^{57}$ The ultramutated and copy-number low groups contained mainly endometrioid carcinomas, the copy-number high group contained non-endometrioid and high grade endometrioid carcinomas, and the hypermutated group contained high grade endometrioid carcinomas as well. Currently, little is known about the combined value of histological and molecular markers in improving the classification of endometrial carcinomas. Development of an endometrial carcinoma classification model which includes both histological and molecular characteristics of the carcinoma could improve endometrial carcinoma management in daily clinical practice.$^{50, 51, 58, 59}$

Carcinogenesis of endometrial carcinomas

Based on histological and molecular differences between the two types, endometrioid and non-endometrioid carcinomas are assumed to follow different carcinogenic pathways. Endometrioid carcinomas are assumed to arise from hyperplastic endometrium. Normal postmenopausal endometrium is supposed to be inactive and atrophic, but can become hyperplastic under the influence of unopposed estrogen stimulation.$^{60, 61}$ Hyperplasia can have either a simple or a complex architecture, and is considered to be atypical if nuclear enlargement with dispersed or clumped chromatin is present.$^{62}$ Studies have shown that there is a substantial risk of either a coexistent endometrial carcinoma or progression to an invasive carcinoma if endometrial hyperplasia with nuclear atypia is present.$^{62, 65}$
On a molecular level, endometrioid carcinogenesis is assumed to be driven predominantly by the PI3K/Akt, MAPK/ERK and Wnt pathways, which have been summarized in Figure 1. Mutations of the PTEN gene, a negative regulator of the PI3K/Akt pathway, and activation of the Wnt pathway, associated with unopposed estrogen stimulation and loss of PR, are believed to be early events, already present in atypical hyperplasia, whereas KRAS and PIK3CA mutations play a role in malignant transformation of endometrial hyperplasia. The carcinogenesis of non-endometrioid carcinomas is believed to be independent of estrogen stimulation and these carcinomas are assumed to originate from atrophic endometrium under the influence of TP53 mutations, loss of E-cadherin, and ERBB2 amplification, although a minority might in fact be dedifferentiated endometrioid carcinomas. In addition, it has not been clarified yet whether the components of endometrial carcinomas with mixed histology and mixed molecular profiles follow separate carcinogenic pathways, or whether one component originates from the other component. Our knowledge about the carcinogenesis is based on the traditional dualistic model and the molecular differences between the two subtypes, and is therefore challenged by recent studies showing that there are probably more subtypes. Unfortunately, these studies only analyzed endometrial hyperplasia, and it is unknown whether these additional subgroups follow different carcinogenic pathways. It is also uncertain whether atypical hyperplasia is the true premalignancy of endometrioid carcinomas, as studies analyzing the risk of a coexistent endometrial carcinoma or progression to an invasive carcinoma show conflicting results. Moreover, the prevalence of atypical hyperplasia was recently shown to be higher than expected, which implies that the risk of a coexistent carcinoma or progression is lower than currently assumed. Molecular analysis of benign, assumed premalignant, and malignant endometrium, as well as endometrium next to endometrial carcinomas would help us further understand endometrial carcinogenesis.

Progression of endometrial carcinomas

Although most endometrial carcinomas have a favorable prognosis, a minority presents in an advanced stage or with recurrent disease. These carcinomas cause the majority of the disease related mortality, and it is therefore essential to identify them as soon as possible. Endometrial carcinoma can spread hematogenous, lymphogenous, and intra-abdominal, and the symptoms can vary, depending on the location of the metastases. In ovarian carcinomas, different molecular profiles have been associated with the type of spread, but so far this has not been studied in endometrial carcinomas. It has been shown that abdominopelvic metastases generally have the same histological profiles as the primary tumor. This would suggest that it could indeed be possible to identify molecular markers which predict the presence of metastases. However, a specific mutation as a cause of endometrial carcinoma metastasis was not identified, and it has been suggested that either a great diversity of genetic events, or a combination of intergenic, epigenetic, and environmental effects contribute to the metastatic process. Endothelial-mesenchymal transition (EMT) is assumed to play a pivotal role in endometrial carcinoma progression and metastasis. During EMT, the carcinoma cells lose basal apical polarity and become spindle shaped instead, with weakening of cell-cell junctions; as a result the cells become more mobile and have an increased infiltrative capacity. On a molecular level, the cells show decreased expression of ER, PR, and loss of epithelial markers like E-cadherin, and increased expression of mesenchymal markers and β-catenin. The PI3K/Akt, MAPK/ERK, and Wnt pathways, which play a role in endometrial carcinogenesis, are involved in EMT as well. Transforming growth factor beta (TGF-β) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling play a role in EMT. The process is mainly controlled by the transcription factors TWIST, and members of the SNAIL, SLUG, and ZEB1 protein families. However, knowledge about the molecular characteristics of endometrial carcinoma progression have not yet led to markers which can be used to identify endometrial carcinomas that are prone to metastasize, and to predict the pattern of spread. Studies that compared the molecular profiles of primary carcinomas and recurrences reported differences in the expression of ER, PR, stathmin and p-mTOR, and a relationship between loss of ER and PR, and the presence of lymph node metastases has also been described. Additional research is needed to improve the identification of endometrial carcinoma progression.

Prognostic markers

At the moment, most prognostic models include the tumor histology, differentiation grade, percentage of myometrial invasion, presence of lymphovascular space invasion, and the age of the patient. However, a substantial number of the carcinomas which are considered to be low risk will recur, whereas a substantial number of the carcinomas which are considered high risk will not. To improve prognostic models we need to reevaluate existing markers, discover and validate new molecular markers, and study the combined value of existing and new markers. Several improvements to the currently used histological markers have already been proposed. In the currently used guidelines, lymphovascular space invasion plays a role in advising adjuvant radiotherapy to prevent locoregional recurrences, whereas it has shown to a strong marker of distant spread. In advising adjuvant radiotherapy to prevent locoregional recurrences, whereas it has shown to a strong marker of distant spread. Moreover, several alternatives to the currently used method of measuring myometrial invasion have been described. Further studies on the prognostic value and therapeutic consequences of myometrial invasion and lymphovascular space invasion could improve the individualization of endometrial carcinoma management. In addition to histological risk factors, several immunohistochemical and genetic markers have been described. The most extensively studied markers of poor prognosis are immunohistochemical loss of ER and PR, which was associated with lymph node metastases and disease recurrences. Especially loss of PR, a strong tumor suppressor, seems to be a valuable prognostic marker. More recently the L1 cell adhesion molecule (L1CAM) might be a strong prognostic marker in endometrial carcinomas as well. Other promising immunohistochemical markers of poor prognosis are increased expression of p53, MIB1, β-catenin, p21, p16, and stathmin, and reduced expression of E-cadherin. Studies focusing on the prognostic value of genetic markers found that activity of the PI3K/Akt pathway predicted aggressive behavior in low risk cases, and that genetic profiles could be used to identify non-endometrioid carcinomas which were falsely classified as endometrioid.
carcinomas.\textsuperscript{55, 56} Based on the genetic profiles, The Cancer Genome Atlas Research network described four distinct genetic subgroups, which had a different prognosis as well.\textsuperscript{57} However, the value of these new markers in addition to the currently used prognostic models is not well known, and as a result these markers are currently only used to support the histological diagnosis.\textsuperscript{56, 115, 116} Further analysis of the combined value of molecular and histological markers is necessary before these new findings can be translated to daily clinical practice.

Aims of the thesis

Currently, the management of endometrial carcinomas is completely based on histological identification of high risk carcinomas. There is a growing amount of evidence that this is too rigid, and as a result, a substantial number of assumed low risk carcinomas will recur, and of the assumed high risk cases will not.\textsuperscript{16, 50}

In order to improve endometrial carcinoma survival the identification of high risk carcinomas should be improved, both by improving prognostic markers already in use, and by integrating new molecular markers in the prognostic models. These new markers can be identified by studying the molecular characteristics of the carcinogenesis and progression of endometrial carcinomas. Subsequently, the value of these markers in a prognostic model, and their relation to other prognostic markers, should be tested and validated in large clinical cohorts, to translate these findings to daily clinical practice. The aims of this thesis therefore were to study:
- The molecular characteristics of endometrial carcinoma carcinogenesis and progression
- The value of and improvements to the currently used markers
- The prognostic value of molecular markers in the management of endometrial carcinomas

Outline of the thesis

In Chapter 2, the immunohistochemical and genetic profiles of endometrioid carcinomas next to atrophic endometrium, endometrioid carcinomas next to hyperplastic endometrium, and non-endometrioid carcinomas are analyzed.

In Chapter 3, the genetic characteristics of complex and simple hyperplasia with and without atypia, endometrioid carcinomas with adjacent hyperplastic or atrophic endometrium, the adjacent endometrium itself, and non-endometrioid carcinomas.

In Chapter 4, the immunohistochemical profiles of endometrial carcinomas without metastases, with intra-abdominal, and with distant metastases, as well as the corresponding metastases are analyzed.

In Chapter 5, the disease outcome of early stage endometrioid carcinomas with lymphovascular space invasion is analyzed.

In Chapter 6, the interobserver variability of measuring myometrial invasion using three different methods is analyzed.

In Chapter 7, the prognostic value of L1CAM expression in a large cohort of endometrial carcinomas from the European Network for Individualized Treatment of Endometrial Cancer is analyzed.
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CHAPTER 2

IMMUNOHISTOCHEMICAL AND GENETIC PROFILES OF ENDOMETRIOID ENDOMETRIAL CARCINOMA ARISING FROM ATROPHIC ENDOMETRIUM


*Both authors contributed equally to this work

Gynecol Oncol. 2015 May;137(2):245-51
Abstract

Background
Endometrial carcinomas are divided into type I endometrioid endometrial carcinomas (EECs), thought to arise from hyperplastic endometrium, and type II nonendometrioid endometrial carcinomas, thought to arise from atrophic endometrium. However, a minority (20%) of EECs have atrophic background endometrium, which was shown to be a marker of a worse prognosis. This study compares the immunohistochemical and genetic profiles of this possible third type to that of the known two types.

Materials and Methods
43 patients with grade 1 EEC and hyperplastic background endometrium (type I), 43 patients with grade 1 EEC and atrophic background endometrium (type II) were included (n=107). Tissue microarrays of tumor samples were immunohistochemically stained for PTEN, L1CAM, ER, PR, p53, MLH1, PMS2, β-catenin, E-cadherin and MIB1. The BRAF, KRAS, and PIK3CA genes were analyzed for mutations.

Results
A significantly higher expression of ER and PR, and a lower expression of L1CAM, p53 and MLH1 were found in type I and III compared to type II carcinomas. Expression of E-cadherin was significantly reduced in type III compared to type I carcinomas. Mutation analysis showed significantly less mutations of KRAS in type III compared to type I and II carcinomas (p<0.01).

Conclusion
There appear to be slight immunohistochemical and genetic differences between EEC with hyperplastic and atrophic background endometrium. Carcinogenesis of EEC in atrophic endometrium seems to be characterized by loss of E-cadherin and a lack of KRAS mutations. As expected, endometrioid and serous carcinomas were immunohistoschemically different.

Introduction
Cancer of the uterine corpus is the most common gynecologic malignancy among women in the developed world. In 2012, it affected 47,130 women and caused the death of 8,010 women in the US. It is generally accepted that endometrial carcinomas (ECs) can be divided into two subtypes. Type I endometrial carcinoma is the most common subtype. It affects women at a median age of 60 years and has a good prognosis. These tumors are usually related to unopposed estrogen stimulation and show endometrioid histology, arising from hyperplastic endometrium. In contrast, the less common type II carcinomas affect older women and have a poor prognosis. These tumors are not related to unopposed estrogen stimulation and are characterized by clear cell or serous histology, arising from atrophic endometrium.

Distinct carcinogenic pathways have been described in each subtype. Type I carcinomas are characterized by microsatellite instability and alterations of the PTEN, KRAS, PIK3CA and CTNNB1 genes, whereas type II carcinomas are often aneuploid and show over expression of p53 and Her2/neu.

However, some tumors do not fit within this dualistic model. In a recent study we reviewed slides from 527 patients with grade 1 endometrioid endometrial carcinomas and found that 17% of these carcinomas had atrophic background endometrium. Furthermore, the presence of atrophic background endometrium adjacent to endometrioid carcinomas was associated with several predictors of poor survival, and an independent predictor of reduced progression free survival in endometrioid endometrial carcinomas. Moreover, recent studies looking at the molecular basis of endometrial carcinomas also show that this dualistic model is too simplistic and propose to categorize them based on their molecular profile. These studies show that within the two groups as defined by the dualistic model, it is able to distinguish certain groups solely based on their molecular pattern. It might be possible that endometrioid endometrial carcinomas with atrophic background endometrium have a different molecular and therefore immunohistochemical pattern when compared to endometrioid endometrial carcinomas with hyperplastic background endometrium. The aim of the present study was to analyze the hypothesis that endometrioid endometrial carcinomas with a background of atrophic endometrium arise through different carcinogenic pathways than type I and II endometrial carcinomas. Therefore, the expression of several immunohistochemical markers and the presence of distinct genetic mutations in endometrioid endometrial carcinoma with a background of atrophic endometrium was compared to those of type I and II carcinomas.

Materials and Methods

Patients
For this study, patients with endometrial carcinoma from two cohorts, who were at least treated with a hysterectomy and bilateral salpingo-oophorectomy and who did not have a personal history of malignancy, were evaluated for inclusion. The first cohort is comprised of patients treated for grade 1 endometrioid endometrial carcinoma at the Radboud university medical center (Radboudumc) and the Canisius-Wilhelmina Hospital in Nijmegen, the Netherlands, between January 1999 and December 2009, and at the Mayo Clinic in Rochester, Minnesota, US, between January 2002 and December 2008. The second cohort
is comprised of patients with uterine serous carcinoma treated at the Radboud university medical center and the Canisius-Wilhelmina Hospital, Nijmegen between January 1999 and December 2009.14, 15 Slides of the primary carcinoma and background endometrium from the cohort of patients with grade 1 endometrioid endometrial carcinoma were reviewed with special attention to the nature of the background endometrium by experienced pathologists (JB, SB, or DV) who were unaware of the original pathology results and clinical outcome. In the case of doubt or discrepancy with the original pathology report a second review was performed by another pathologist and consensus was reached. Background endometrium was categorized as simple hyperplasia, simple atypical hyperplasia, complex hyperplasia, complex atypical hyperplasia, disordered proliferative, atrophic, and normal proliferative as previously described.1, 10, 16 Some cases had to be excluded because the tumor covered the entire cavity of the uterus and there was no background endometrium to be evaluated.

All patients with grade I endometrioid endometrial carcinoma and a background of pure (100%) atrophic endometrium (abbreviated to type III) as well as a similar amount of patients with grade I endometrioid endometrial carcinoma and a background of hyperplastic endometrium (type I) were included. Subsequently, all patients from the uterine serous carcinoma (type II) cohort of whom uterine tissue could be retrieved from the archive were included. This cohort consisted of carcinomas with both pure and mixed serous histology. It has been previously described that only about half of the serous carcinomas have pure serous histology.17

Tissue microarray and immunohistochemistry

Tissue microarrays were created from the primary carcinoma.18 Two representative areas of the carcinoma were selected on hematoxylin and eosin-stained slides. For the type II cases, areas with pure serous histology were selected. Two cylinders with a diameter of 2 mm were punched out of every donor block from the selected areas, and mounted into a recipient paraffin block using the Tissue-Tek Quick-Ray (Sakura Finetek, Torrance, CA, US) manual tissue microarrayer. The tissue microarrays were cut in 4 µm slides and immunohistochemically stained. Several markers were selected to be stained, based on the difference in their expression in type I and type II endometrial carcinoma.6, 6, 19, 20 An overview of the antibodies and dilutions used as well as the area of the cell was which was evaluated when scoring is shown in Table 1. Immunohistochemical analysis of PTEN, L1CAM, ER, PR, p53, MLH1, PMS2, β-catenin, E-cadherin and MIB1 expression was performed according to the local protocols. These markers were chosen because previous literature has shown that their expression is different in type I and II EC.6, 6, 20 In short, formalin fixed paraffin sections were stained with the primary antibody following EDTA antigen retrieval, blocking of endogenous background with Peroxidase Blocking Reagent and protein blocking using horse serum. Subsequently, a secondary antibody was added and visualization was performed with Vectastain and 3,3’-Diaminobenzidine (Zymed lab. California, US) as a substrate. Staining was enhanced in CuSO4 and slides were counterstained with Mayer’s hamatoxylin. Finally, slides were dehydrated and mounted.

### Table 1. Antibodies and dilutions used in this study, as well as the areas of the cell used for scoring

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Company</th>
<th>Area scored</th>
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<tr>
<td>PTEN inactivation</td>
<td>6H2.1</td>
<td>Dako*</td>
<td>Cytoplasm/nucleus</td>
</tr>
<tr>
<td>L1CAM</td>
<td>U127</td>
<td>Thermo Scientific*</td>
<td>Membrane</td>
</tr>
<tr>
<td>ER expression</td>
<td>SP1</td>
<td>Thermo Scientific</td>
<td>Nucleus</td>
</tr>
<tr>
<td>PR expression</td>
<td>PgR 636</td>
<td>Dako</td>
<td>Nucleus</td>
</tr>
<tr>
<td>PS3 mutations</td>
<td>DO-7</td>
<td>Thermo Scientific</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Loss of MLH1</td>
<td>GI6B-15</td>
<td>BD'</td>
<td>Nucleus</td>
</tr>
<tr>
<td>Loss of PMS2</td>
<td>A16-4</td>
<td>BD</td>
<td>Nucleus</td>
</tr>
<tr>
<td>β-catenin alteration</td>
<td>14/Beta-Catenin</td>
<td>1:100</td>
<td>BD</td>
</tr>
<tr>
<td>E-cadherin alteration</td>
<td>UJ127</td>
<td>Thermo Scientific</td>
<td>Membrane</td>
</tr>
<tr>
<td>High proliferation rate</td>
<td>MIB1</td>
<td>Thermo Scientific</td>
<td>Nucleus</td>
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Tumor samples were given a score ranging from 0 to 9 by two independent evaluators (YG, and AT.) which was the product of the percentage of cells stained (0 = 0%, 1 = 1-10%, 2 = 11-50% and 3 = 51-100%) and intensity of staining (0 = none, 1 = weak, 2 = moderate and 3 = strong).13 The evaluators were unaware whether the tissue cylinders were from type I, type II or type III carcinomas. Samples with too little tissue to assess or samples not containing any malignant tissue were not included in the calculations. In case of a large discrepancy between the scores of the two evaluators (i.e. a difference in percentage or intensity score > 2 or disagreement on the presence of malignant tissue) a third independent reviewer (JB), who was unaware of the score given by the first evaluators, scored the sample as well. The final score per case (range 0 to 9) was calculated by adding all scores given to the two tissue samples and dividing them by the number of scores in the sum (which varied depending on the presence of tumor tissue in the sample and the need for a third review). The final semiquantitative score was used for analysis of PTEN, ER and PR according to the literature as well as for β-catenin and E-cadherin, because there is no consensus as to which scoring system should be used.21, 22 L1CAM was considered positive when there was staining in at least 10% of the malignant cells.19 Cases with a final score ≥ 4 concerning p53 were considered to be positive while those with a score < 4 were negative.20 MLH1 and PMS2 were considered lost when there was no staining at all, according to the international guidelines. Finally, MIB1 staining was considered positive at every intensity and categorized as 1-10%, 11-50% and 51-100% cells positive.

### Mutation analysis

Slides with at least 10% representative tumor tissue were selected for DNA isolation. For the cases from the Mayo Clinic, the two TMA tumor biopsies were used instead. DNA was isolated with TET-lysis buffer (10 mmol/L Tris-HCl, pH 8.5; 1 mmol/L EDTA, pH 8; 0.1% Tween-20) containing 5% Chelex-100 (Bio-Rad, Hercules, CA). Protein digestion was
performed by adding proteinase K to each sample and incubation at 56°C for 48 h. Next, Protein K was inactivated at 95°C for 10 min. The samples were centrifuged for 10 min at 14,000 rpm (RT) and the DNA concentration of the supernatant was measured using the Quant-iT picogreen dsDNA assay kit (Invitrogen, Carlsbad, CA, U.S.) before storage at 4°C. For the detection of mutations, DNA was amplified for exons of the KRAS, BRAF and PIK3CA genes using earlier published PCR primers.26 The amplified exons were assessed for mutations at 22 nucleotide positions by single nucleotide probe extension assays using a SNAPShot Multiplex kit (Applied Biosystems, Foster City, CA) as described previously.26, 27

Statistical analysis
The differences between the immunohistochemical marker scores of the three endometrial carcinoma subtypes were calculated using the Mann Whitney test, the χ2 test and the Fisher exact test. Differences between the amount of cases with genetic mutations per subtype were calculated using the χ2 test and Fisher exact test. As a measure for tumor heterogeneity the correlation between the scores given to the two biopsies per tumor was calculated using the Pearson correlation coefficient. This test was also used to calculate the correlation between different markers. Differences were considered to be statistically significant at a p-value ≤ 0.05. SPSS version 20 (SPSS IBM, New York, NY, U.S.) statistical software was used for analysis of the data.

Ethical approval
No ethical approval was needed for this study, which was performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies, www.federa.org).

Results
Patients
Of the 527 patients with grade 1 endometrioid endometrial carcinoma evaluated, background endometrium was hyperplastic in 387 (73%) and atrophic in 88 (17%). The background endometrium was normal premenopausal, proliferative in 27 (5%) patients and could not be assessed due to the size of the tumor in 25 (5%) patients. Pure atrophy was found in 43 (48.9%) of the 88 patients with atrophic background endometrium. Out of the 387 patients with hyperplastic background endometrium, 43 (11.1%) were randomly selected as controls. From the cohort of patients with uterine serous carcinoma, all patient data as well as tissue was present in 21 cases (out of 47 meeting the inclusion criteria), 14 (66.7%) of which had pure serous histology, which is slightly higher than that in the general population of patients with uterine serous carcinoma.17 In total, 107 cases were included for immunohistochemical and mutation analyses.

Immunohistochemistry
After initial evaluation of 2140 tissue samples, additional review because of discrepancy between the evaluators’ scores was necessary for 107 samples (44 type I, 19 type II and 44 type III). This was equally divided among all markers. Three type III cases were excluded from the analysis because both tumor samples contained too little representative tissue or because they did not contain malignant tissue (one ER case, one MLH1 case and one MIB1 case). The final marker scores per type of endometrial carcinoma and the differences between the scores are shown in Figure 1.

Figure 1. The different marker scores per type of endometrial carcinoma. P-values of the Mann Whitney test (for the boxplots) and χ2 test (for the other graphs) comparing the individual types are shown.
The correlation between the scores given to the two biopsies from the same tumor as a measurement of tumor heterogeneity is shown per marker in Table 2, categorized as high ($r$ between 0.7 and 0.9), moderate ($r$ between 0.4 and 0.7) and weak ($r$ between 0.1 and 0.4), according to Dancey and Reidy.28 It was moderate to high for most markers, only the scores for β-catenin were not correlated ($r = -0.01$, $p=0.92$).

Table 2. Marker heterogeneity, depicted as the strength of the correlation (according to Dancey and Reidy) between the score of the two biopsies per marker

<table>
<thead>
<tr>
<th>Strong</th>
<th>Moderate</th>
<th>Weak</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN ($r^*=0.72$, $p^†&lt;0.01$)</td>
<td>L1CAM ($r=0.44$, $p=0.01$)</td>
<td>β-catenin ($r=-0.01$, $p=0.92$)</td>
</tr>
<tr>
<td>ER ($r=0.80$, $p=0.01$)</td>
<td>p53 ($r=0.55$, $p=0.01$)</td>
<td></td>
</tr>
<tr>
<td>PR ($r=0.78$, $p=0.01$)</td>
<td>PMS2 ($r=0.48$, $p=0.01$)</td>
<td></td>
</tr>
<tr>
<td>MLH1 ($r=0.78$, $p=0.01$)</td>
<td>E-cadherin ($r=0.56$, $p=0.01$)</td>
<td></td>
</tr>
<tr>
<td>MIB1 ($r=0.60$, $p=0.01$)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Pearson’s correlation coefficient; † P-value for Pearson’s correlation coefficient

There was a strong positive correlation between the presence of PMS2 and MLH1 ($r = 0.78$, $p < 0.01$) as well as between the presence of ER and PR ($r = 0.70$, $p < 0.01$). L1CAM positivity had a slightly negative correlation with ER ($r = -0.33$, $p < 0.01$) and PR ($r = -0.42$, $p < 0.01$). When looking at the differences between the subtypes it can be seen that in both type I and III EC expression of ER and PR was significantly higher than that in type II EC, while expression of L1CAM, p53 and MLH1 was significantly lower in type I and III EC than that in type II EC. Expression of E-cadherin and MIB1 was significantly lower in type III EC compared to type I and II EC. Examples of E-cadherin and MIB1 staining in type I and III carcinomas are shown in Figure 2.

Figure 2. Examples of E-cadherin (A) and MIB1 (B) expression in type I (left) and type III (right) endometrial carcinomas.

The only significant difference was found with the KRAS gene, which was mutated in 15/43 (37.2%) of the type I and 6/21 (23.8%) of the type II carcinomas, compared to only 1/43 (2.3%) of the type III carcinomas. In the type I carcinomas eight of the mutations were located on codon 12, five on codon 13, one on codon 19 and one on codon 61 and in the type II carcinomas three mutations were located on codon 12 and two on codon 13. The mutation in the type III carcinoma was located on codon 19.

The BRAF gene was not mutated in any of the cases, while the PIK3CA was mutated more often in type II EC (23.8%) than in type I (14%) and III (11.6%) EC, but these differences were not significant.

Discussion

This study compared the immunohistochemical and genetic profiles of endometrioid endometrial carcinomas with hyperplastic background endometrium (type I) to those with atrophic background endometrium (hypothetical type III). While they were quite comparable, endometrioid carcinomas with atrophic background endometrium showed reduced expression of E-cadherin and less KRAS mutations compared to endometrioid carcinomas with hyperplastic background endometrium. The endometrioid carcinomas
were also compared to those with serous histology (type II), which had a different immunohistochemical profile as expected.

**Immunohistochemical profile**
The immunohistochemical differences between type I and II endometrial carcinoma in this study are in line with previous literature. As expected, ER and PR expression were significantly higher in type I carcinomas, while L1CAM, p53 and MLH1 were significantly higher in type II carcinomas. Moreover, while the differences in PMS2, E-cadherin, β-catenin and MIB1 expression were not significant, the observed trends were according to the findings in previous literature on these markers. Interestingly, PTEN expression was found to be lowest in serous carcinomas, while previous studies have shown loss of PTEN in endometrioid carcinomas.9, 29, 30

It was hypothesized that type III carcinomas would have a distinct immunohistochemical profile. For most markers, there was no difference in expression between type I and III. However, expression of E-cadherin and MIB1 were significantly reduced in type III compared to type I carcinomas.

Loss of E-cadherin has been described in both endometrioid and non-endometrioid carcinomas, while expression levels were found to be normal in hyperplastic endometrium.11-13 In epithelial cells, E-cadherin is the major molecule of the cadherin family, which is essential for tight cell-cell connections.14 Reduced expression of E-cadherin was associated with lymph node metastasis, deep myometrial invasion, tumor dedifferentiation and advanced disease stage.15-17 Loss of E-cadherin is likely to be responsible, at least partially, for the fact that type III carcinomas are associated with lymph node metastasis, deep myometrial invasion and advanced disease stage as well.20 Moreover, while increased expression of MIB1, a proliferation marker associated with aggressive tumors, might seem more likely in type III carcinomas, we found the opposite to be true. However, this finding is in line with the conclusions from a recent publication showing the prognostic value of E-cadherin over proliferation markers.14

As expected, staining was slightly heterogeneous for most markers. Only β-catenin staining showed very strong heterogeneity, the reason for which is unknown. Finally, a strong correlation between the presence of ER and PR as well as MLH1 and PMS2 was shown, which is in line with the literature.18, 19 A negative correlation between the presence of L1CAM and the loss of ER and PR was also shown, though weaker than previously described.20

**Genetic profile**
In line with previously published data, BRF mutations were found in none of the cases.26 PIK3CA mutations are associated with invasive growth and poor prognosis and were most frequently found in type II carcinomas, in accordance with previous findings.27-30

Mutation analysis of KRAS, which have been described in 30% of the type I, but only 10% of the type II carcinomas showed several interesting results.9 In type I carcinomas we observed a slightly higher amount of KRAS mutations than previously reported, which might be explained by the fact that most studies only look at codon 12 mutations, while we looked at mutations at codons 12, 13, 61 and 19. Most of the KRAS mutations that we found were located on codons 12, 13 and 61, which are the most likely locations of KRAS mutations according to the literature.41 Only in one type II and in the only mutated type III case was the mutation on codon 19, an infrequent location of mutations in some tumors that has not been described in endometrial carcinomas before.42 Codon 19 mutations of KRAS have been suggested to alter the treatment response of colorectal carcinomas.43 However, this does not explain the high amount of KRAS mutations that we observed in type II carcinomas. This finding might be explained by the fact that we chose to include representative type II cases, some of which had a minor component of non-serous histology.27 The KRAS mutations in three of the six type II cases might have been present in minor areas with endometrioid histology instead of those with serous histology. The discrepancy between previously published results and the amount of mutations that we observed in type II carcinomas was much larger. Finally, the amount of mutations in the KRAS gene in this study was significantly lower in type III compared to type I and II carcinomas.

KRAS mutations have been found to be an early event in the carcinogenesis of endometrioid endometrial carcinomas, present not only in endometrioid carcinoma, but endometrial hyperplasia as well.44, 45 These findings support the lack of KRAS mutations in endometrioid carcinomas arising from atrophic endometrium.

Our findings in type III carcinomas of loss of E-cadherin with a lack of KRAS mutations are reported in several other studies. Up-regulation of E-cadherin expression by KRAS mutations was demonstrated in one study, while another describes expression of Zeb1, a transcription factor repressing E-cadherin expression, in tumor cell lines that grow independent of KRAS mutations.46-48 These findings might suggest that loss of E-cadherin is an early event of carcinogenesis of type III carcinomas because there is a lack of KRAS mutations.

**Implications for the classifications of endometrial carcinomas**
In 1983 endometrial carcinomas were subdivided into two distinct morphological subgroups and this division has played a distinct role in the management of endometrial carcinomas ever since.2 This morphological subdivision has proven to be very useful and in more recent years was also shown to be present on the immunohistochemical and genetic levels.8, 9 However, recent immunohistochemical and genetic studies have also shown that a subdivision into two groups is too simplistic.11-13 Moreover, even on a morphological level there is reason to believe that this subdivision into type I carcinomas arising from hyperplastic endometrium and type II carcinomas arising from atrophic endometrium is incomplete. Studies from our group have not only shown that 17% of the endometrioid carcinomas have in fact atrophic background endometrium – and a worse prognosis – but that the background endometrium of 9.3% of the pure and 34.7% of the mixed serous carcinomas was in fact hyperplastic.44, 45 The results of the current study, show additional value of immunohistochemistry and genetics next to morphology, which is in line with other recent studies. While studies by McConkey et al. and The Cancer Genome Atlas have shown that type I and II carcinomas both have a distinct genetic profile, they also show the existence of more subgroups based on the genetic profile.11, 12 McConkey et al. describe the use of genetics to aid in distinguishing cases with a worse prognosis which are hard to distinguish morphologically, for example grade 3 endometrioid carcinomas and serous carcinomas.12 Interestingly, while the TCGA publication only looked at the overlap between their four genetic subtypes and the two morphological subtypes, they do describe two groups with a low number of KRAS mutations, which have a bad to intermediate prognosis.31 Based on our findings as well as the growing amount of literature describing alternative subdivisions of endometrial carcinomas, we believe that it is important to take a closer look at overlap between newly found immunohistochemical and genetic profiles. It is, for
be used to identify patients with a worse prognosis more accurately, as proposed by Chiang et al.67

Strengths and weaknesses of the study
This study is the first to analyze the immunohistochemical and genetic profiles of endometrioid endometrial carcinomas with atrophic background endometrium. A large set of immunohistochemical markers and genes was analyzed to give a clear view of the similarities and differences between the different endometrial tumor types. While many recent publications have shown that there are more than two types of endometrial carcinoma based on their molecular profile, this study shows that this is also true on an immunohistochemical level. It also shows that there is a significant heterogeneity within tumors, making a clear subdivision difficult.

A weakness of the study may be the fact that DNA was extracted for mutation analysis from whole slides instead of selected tumor tissue. However, while this might be responsible for the high mutation rate we found in type II carcinomas, it does not interfere with answering the question whether there is a difference between type I and III carcinomas. If anything, it highlights more clearly that very few KRAS mutations are found in type III carcinomas as well as the surrounding tissue. Lastly, it might have been better to match the patient characteristics of the patients with Type I and III carcinomas, because it is possible that the latter have a higher age and a lower BMI compared to the former, possibly influencing the carcinogenesis.

Conclusion
In conclusion, on the immunohistochemical and genetic levels, endometrioid carcinomas arising from atrophic background endometrium were shown to be quite comparable to endometrioid carcinomas arising from hyperplastic background endometrium. However, while KRAS mutations are an early event in carcinogenesis of endometrioid endometrial carcinoma in hyperplastic endometrium, these mutations were rarely seen in endometrioid carcinomas with atrophic background endometrium. Instead, carcinogenesis of these carcinomas seems to be characterized by loss of E-cadherin, which is uncommon in endometrioid carcinomas arising from hyperplastic endometrium. Immunohistochemical and genetic profiling might aid in distinguishing these cases which were previously shown to have a worse prognosis.

References


38. Catasus L, Gallardo A, Cuatrecasas M et al. PIK3CA mutations in the kinase domain (exon 20) of uterine endometrial adenocarcinomas are associated with adverse prognostic parameters. Mod Pathol 2008;21:131-139.


CHAPTER 3

MOLECULAR PROFILES OF BENIGN AND (PRE)MALIGNANT ENDOMETRIAL LESIONS


Carcinogenesis. 2017 Jan. Accepted
Abstract

Background
Endometrial carcinomas are histologically classified as endometrioid, assumed to originate from hyperplastic endometrium, or non-endometrioid carcinomas, assumed to originate from atrophic endometrium. However, both on a histological and a molecular level there are indications that there are more carcinoma types and carcinogenetic pathways. This study aims to analyze endometrial carcinogenesis on a molecular level.

Materials and Methods
The presence of known KRAS, PIK3CA, AKT1, CTNNB1, BRAF, EGFR and NRAS mutations was studied in proliferative, atrophic and hyperplastic endometrium, endometrioid and serous carcinomas, and the endometrium next to these carcinomas, using single molecule Molecular Inversion Probes.

Results
Mutations were found in 9 (15%) of the 62 non atypical, and in 6 (18%) of the 34 atypical hyperplasia cases. In comparison, mutations were found in 1 (3%) of the simple, and 8 (30%) of the 27 complex hyperplasia cases. In 12/22 (55%) endometrioid carcinomas a mutation was found. The KRAS gene was most often mutated in carcinomas next to hyperplastic endometrium, whereas PIK3CA and CTNNB1 mutations were found in endometrioid carcinomas with adjacent atrophic endometrium.

Conclusion
Complex hyperplasia rather than atypical hyperplasia appears to be the most important lesion in the carcinogenesis of endometrioid carcinomas, and KRAS, PIK3CA, and CTNNB1 mutations appear to play an important role in this process. Carcinogenesis of endometrioid carcinomas next to hyperplasia seems to be different to that of those next to atrophy. The value of these findings in managing endometrial hyperplasia and carcinoma should be studied.

Introduction
Endometrial cancer is the most common gynecological malignancy in the developed world, and its incidence is rising. Traditionally, endometrial carcinomas are divided into those with endometrioid and those with non-endometrioid histology, which were shown to have different molecular profiles as well. Endometrioid carcinomas are characterized by a heterogeneous molecular profile of mutations in PTEN, KRAS, CTNNB1, and PIK3CA, whereas TP53 mutations are most common in non-endometrioid carcinomas.

Endometrioid and non-endometrioid carcinomas are assumed to follow different carcinogenic pathways as well. Normal postmenopausal endometrium is atrophic, but can become hyperplastic, mainly as a result of unopposed estrogen stimulation. Endometrial hyperplasia can be categorized based on both the presence of simple or complex architecture, and the absence or presence of atypical nuclei. It is assumed that endometrioid carcinomas originate mainly from hyperplasia with atypia, and the World Health Organisation (WHO) therefore advises to categorize hyperplasia as either non-atypical or atypical, and to perform a hysterectomy when atypia is present. Non-endometrioid carcinomas on the other hand are assumed to originate from atrophic endometrium. On a molecular level, PTEN and KRAS mutations are assumed to be early events in endometrioid carcinogenesis, already present in endometrial hyperplasia, whereas PIK3CA mutations appear related to invasive transformation. In serous carcinomas, TP53 mutations were shown play an important role, but other non-endometrioid carcinomas have more heterogeneous molecular profiles. Moreover, it has been suggested that some non-endometrioid carcinomas are in fact dedifferentiated endometrioid carcinomas, as they have both non-endometrioid and endometrioid molecular characteristics.

In a recent study analyzing the endometrium of asymptomatic postmenopausal women, who were expected to have atrophic endometrium, a high prevalence of endometrial hyperplasia, including hyperplasia with atypical nuclei was found. In addition, although endometrioid carcinomas are assumed to originate in endometrial hyperplasia, the endometrium next to these carcinomas is atrophic in around 20% of the cases, and these cases were shown to have a worse prognosis. Moreover, studies categorizing endometrial carcinomas based on their molecular profiles have concluded that there are most likely more than two subgroups. These recent findings challenge the dualistic nature of endometrial carcinogenesis, and the fact that a large proportion of the atypical endometrial hyperplasia will progress into a carcinoma. It is therefore important to analyze the molecular characteristics of endometrial carcinogenesis in relation to these recent findings. This information is not only valuable for the clinical management of endometrial carcinomas, but for the management of endometrial hyperplasia as well.

Materials and Methods

Cases
This study included a benign (proliferative and atrophic endometrium), a premalignant (hyperplastic endometrium), and a malignant (endometrioid carcinomas and serous carcinomas) cohort. The latter cohort also included the endometrium adjacent to the carcinomas.
- **Benign:** Patients who underwent a hysterectomy for uterovaginal prolapse at the Radboud University Medical Center Nijmegen between 1999 and 2009. This cohort included a large number of patients with benign endometrial lesions.

- **Premalignant:** Patients who underwent a hysterectomy for endometrial hyperplasia, at the Radboud University Medical Center Nijmegen between 1999 and 2009. This cohort included a large number of patients with premalignant endometrial lesions.

- **Malignant and adjacent endometrium:** Patients treated for endometrioid carcinomas or serous carcinomas at the Radboud University Medical Center or Canisius-Wilhelmina Hospital in Nijmegen from 1999 to 2009. This cohort included a large number of patients with malignant endometrial lesions.

Hematoxylin and eosin stained endometrial slides were reviewed by a gynecological pathologist (JB) with respect to the diagnosis, and representative areas were marked. Atrophic endometrium was defined as shallow endometrium with a thin basal layer and with a few tubular glands lined by inactive endometrium. Proliferative endometrium was defined as glandular proliferation, but no hyperplasia based on the gland:stroma ratio in postmenopausal women, and as widely spread, sometimes tortuous, tubular glands showing mitotic activity and abundant stroma in premenopausal women. Hyperplasia was defined as glandular proliferation with an increased gland:stroma ratio of 3:1, and was classified as simple or complex according to the [old] World Health Organization criteria. Atrioya was defined as enlarged, rounded, polymorphic nuclei with loss of polarity, prominent nucleoli, chromatin clumping and an increased nucleus to cytoplasm ratio. Both the carcinoma and adjacent tissue were marked in the carcinoma groups. Cases with no or insufficient tissue were excluded.

**DNA isolation**

The previously marked representative areas were separated by either macro- or laser microdissection from respectively 20µm or 10µm thick section from formalin-fixed, paraffin embedded tissue. For laser micro dissection, slides were mounted on poly ethylene naphthalate (PEN) membrane slides (Leica Microsystems, Buffalo Grove, IL, US), pretreated with ultraviolet light, and visualized with hematoxylin.

The separated tissue was digested overnight at 56°C in TET-lysis buffer (10mM Tris/HCl pH 8.5, 1mM EDTA pH 8.0, 0.01% Tween-20) with 5% Chelex-100 (Bio-Rad, Hercules, CA, US) and 0.2% proteinase K. Subsequently, proteinase K was inactivated at 95°C for ten minutes, and the supernatant was transferred after centrifugation into a clean tube. DNA concentration was determined using the Qubit Broad Range Kit (Thermo Fisher Scientific, Waltham, MA, US).

**smMIP design and library preparation**

The samples were analyzed using single molecule Molecular Inversion Probes (smMIPs). Both the design of the smMIPs and the preparation of the library were performed as previously described (Eijkelenboom A. et al. (in press)). Reliable Next Generation Sequencing of FFPE tissue using single molecule tags. J Mol Diagn). In short, a panel of smMIPs, targeting both strands of hotspots listed in Supplementary Table 1 in a tiled manner, was designed. The smMIP probes contained extension and ligation probe arms (together 40pb long), and these arms were separated by an 112bp gap. These genes were chosen because they have been previously described to play a role in the PI3K/AKT, MAP/ERK and Wnt pathways, which play a role in the development of endometrial carcinomas, and because mutations of all of these genes have been previously described in endometrial carcinomas. A common backbone sequence was inserted between the targeting arms and eight nucleotides were inserted between the backbone and ligation probe. The smMIP probes were produced by Integrated DNA Technologies (Leuven, Belgium) were mixed and phosphorylated with 1ul of T4 polynucleotide kinase (M0201, New England Biolabs, Ipswich, MA, US) per 25ul of 100U/mL smMIPs and ATP-containing T4 DNA ligase buffer (B0202, New England Biolabs, Ipswich, MA, US). The molecular ratio between genomic DNA and smMIPs was set to 1:3200 for every individual smMIP, and genomic DNA input was expected to be 100 ng.

A capture mix was made by adding the phosphorylated smMIP-pool, 1 unit of Ampligase DNA ligase (A0110K, Epic Bio, Madison, WA, US) with Ampligase Buffer (A1905B, Epic Bio, Madison, WA, US), 3.2 units of Hemo Klentaq (M0332, New England Biolabs, Ipswich, MA, US) and 8 ul of dNTPs (28:4065-20/-12/-22/-32, GE Healthcare, Hoevelaken, the Netherlands) to 20 µl of sample containing genomic DNA. After denaturation at 95°C for 10 minutes, the mix was incubated for probe hybridization, extension and ligation at 60°C for 18 hours and cooled prior to exonuclease treatment. Exonuclease I (10 units M0293, New England Biolabs, Ipswich, MA, US) and III (50 units, M0206, New England Biolabs, Ipswich, MA, US) and Ampligase Buffer were added to the capture volume, adding up to a total of 27 µl, and incubated for 45 minutes at 37°C followed by inactivation at 95°C for 2 minutes. A total of 20 µl of the exonuclease treated capture was used for PCR in a total volume of 50ul with a common forward primer, bar-coded reverse primers, and iProof high fidelity master mix (1725310, Bio-Rad. Veenendaal, the Netherlands). The resulting PCR products were pooled prior to purification with 0.8x volume of Agencourt Ampure XP Beads (A63881, Beckman Coulter, Woerden, the Netherlands).

**Sequencing and analysis**

Sequencing of libraries diluted to a concentration of 1.2 pM was performed using a NextSeq500 device (Illumina, San Diego, CA, US) and the 300 cycles Mid Output sequencing kit, resulting in 2x150bp paired-end reads. The Bcl files were converted to fastq files, and bar-coded reads were demultiplexed. Duplicate reads were used to assemble consensus reads using Sequence Pilot software (JSI Medical Systems, Costa Mesa, CA, US) with the following settings: Tags active: yes; R1 tag length: 8; R2 tag length: 0; Min abs. cov. cons.: 1; Min per. cov. cons.: 50%; Ignore cons. read thresh.: 30%; Ignore N tags: yes; Ignore low Q tags: yes. The following settings were used for variant calling using Sequence Pilot: Required Coverage / Min abs. cov.: 20 combined; Mutations / Min abs. cov.: 5 combined; Min % cov.: 1% per dir. Mutations in well sequenced parts of the DNA, which had an allele frequency of at least >5% in benign and >10% in malignant samples, and which were previously described in the Catalogue of Somatic Mutations in Cancer (COSMIC, cancer.sanger.ac.uk/cosmic) were considered to be clinically relevant.

**Ethical approval**

No ethical approval was needed for this study, which was performed according to the Code for Proper Secondary Use of Human Tissue (Dutch Federation of Biomedical Scientific Societies, www.federa.org).
CHAPTER 3

Molecular profiles of benign and (pre)malignant endometrial lesions

Results

Cases

DNA isolation, PCR and sequencing were performed for a total of 179 samples, and were successful in 137 (77%), as shown in Table 1 per subgroup. The number of cases with mutations per subgroup, and the number of successfully analyzed genes and mutations per gene are shown in Table 2 and 3. Of the 41 pairs of endometrial carcinoma and adjacent endometrium, analysis was unsuccessful for seven complete pairs, eight endometrial carcinomas, and four adjacent endometrium carcinoma samples, leaving 22 pairs to be analyzed. Hyperplastic and atrophic endometrium next to the endometrioid carcinomas, both eleven cases, are shown separately. The endometrium next to the serous carcinomas was heterogeneous, with four cases with pure atrophy, two with pure endometrial hyperplasia, and two with a mix of atrophic and hyperplastic endometrium. Due to the small numbers these cases are not shown separately in the tables. The location and allele frequency of all mutations found are shown in Supplementary Table 2.

Table 1. Percentage of the included samples which was successfully analyzed (number of samples successfully analyzed/total number of samples analyzed)

<table>
<thead>
<tr>
<th>Study group</th>
<th>% Successful</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferative endometrium</td>
<td>64% (7/11)</td>
</tr>
<tr>
<td>Atrophia</td>
<td>60% (12/20)</td>
</tr>
<tr>
<td>Simple hyperplasia</td>
<td>100% (19/19)</td>
</tr>
<tr>
<td>Simple atypical hyperplasia</td>
<td>94% (16/17)</td>
</tr>
<tr>
<td>Complex hyperplasia</td>
<td>90% (9/10)</td>
</tr>
<tr>
<td>Complex atypical hyperplasia</td>
<td>90% (18/20)</td>
</tr>
<tr>
<td>Atrophia next to EEC*</td>
<td>73% (11/15)</td>
</tr>
<tr>
<td>Hyperplasia next to EEC</td>
<td>60% (9/15)</td>
</tr>
<tr>
<td>EEC next to atrophia</td>
<td>73% (11/15)</td>
</tr>
<tr>
<td>EEC next to hyperplasia</td>
<td>73% (11/15)</td>
</tr>
<tr>
<td>Endometrium next to USC</td>
<td>55% (6/11)</td>
</tr>
<tr>
<td>Serous carcinoma</td>
<td>73% (8/11)</td>
</tr>
<tr>
<td>Total</td>
<td>77% (130/168)</td>
</tr>
</tbody>
</table>

Table 2. The percentage of endometrial hyperplasia cases and subgroups with mutations, as well as the prevalence of specific mutations in these groups. The absolute number of cases with (specific) mutations relative to the number of cases successfully analyzed are shown between brackets.

<table>
<thead>
<tr>
<th>Mutated</th>
<th>KRAS</th>
<th>PIK3CA</th>
<th>AKT</th>
<th>CTNNB1</th>
<th>BRAF</th>
<th>NRAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>15% (9/62)</td>
<td>5% (3/49)</td>
<td>7% (4/58)</td>
<td>3% (2/62)</td>
<td>2% (1/60)</td>
<td></td>
</tr>
<tr>
<td>Not atypical</td>
<td>11% (3/28)</td>
<td>0% (-/21)</td>
<td>8% (2/25)</td>
<td>0% (-/28)</td>
<td>4% (1/27)</td>
<td></td>
</tr>
<tr>
<td>Atypical</td>
<td>18% (6/34)</td>
<td>11% (3/28)</td>
<td>6% (2/33)</td>
<td>6% (2/34)</td>
<td>0% (-/33)</td>
<td></td>
</tr>
<tr>
<td>Simple</td>
<td>3% (1/35)</td>
<td>0% (-/22)</td>
<td>0% (-/32)</td>
<td>0% (-/35)</td>
<td>3% (1/33)</td>
<td></td>
</tr>
<tr>
<td>Complex</td>
<td>30% (8/27)</td>
<td>11% (3/27)</td>
<td>15% (4/26)</td>
<td>7% (2/27)</td>
<td>0% (-/27)</td>
<td></td>
</tr>
<tr>
<td>SHa</td>
<td>5% (1/19)</td>
<td>0% (-/12)</td>
<td>0% (-/17)</td>
<td>0% (-/19)</td>
<td>6% (1/18)</td>
<td></td>
</tr>
<tr>
<td>SAHa</td>
<td>0% (-/16)</td>
<td>0% (-/10)</td>
<td>0% (-/15)</td>
<td>0% (-/16)</td>
<td>0% (-/15)</td>
<td></td>
</tr>
<tr>
<td>CHc</td>
<td>22% (2/9)</td>
<td>0% (-/9)</td>
<td>25% (2/8)</td>
<td>0% (-/9)</td>
<td>0% (-/9)</td>
<td></td>
</tr>
<tr>
<td>CAHc</td>
<td>33% (6/18)</td>
<td>17% (3/18)</td>
<td>11% (2/18)</td>
<td>11% (2/18)</td>
<td>0% (-/18)</td>
<td></td>
</tr>
</tbody>
</table>

*Simple hyperplasia without atypia, *Simple atypical hyperplasia, *Complex hyperplasia without atypia, *Complex atypical hyperplasia

Table 3. The percentage of carcinoma cases and adjacent tissue cases with mutations, as well as the prevalence of specific mutations in these groups. The absolute number of cases with (specific) mutations relative to the number of cases successfully analyzed are shown between brackets.

<table>
<thead>
<tr>
<th>Mutated</th>
<th>KRAS</th>
<th>PIK3CA</th>
<th>AKT</th>
<th>CTNNB1</th>
<th>BRAF</th>
<th>NRAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Next to EEC*</td>
<td>10% (2/20)</td>
<td>7% (1/15)</td>
<td>6% (1/18)</td>
<td>0% (-/20)</td>
<td>0% (-/15)</td>
<td>0% (-/20)</td>
</tr>
<tr>
<td>Atrophia</td>
<td>9% (1/11)</td>
<td>0% (-/8)</td>
<td>30% (1/10)</td>
<td>0% (-/13)</td>
<td>0% (-/7)</td>
<td>0% (-/11)</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>11% (1/9)</td>
<td>14% (1/7)</td>
<td>0% (-/9)</td>
<td>0% (-/8)</td>
<td>0% (-/9)</td>
<td>0% (-/8)</td>
</tr>
<tr>
<td>EEC next to Atrophia</td>
<td>55% (12/22)</td>
<td>27% (6/21)</td>
<td>18% (4/22)</td>
<td>1 (5%)</td>
<td>9% (2/19)</td>
<td>0% (-/22)</td>
</tr>
<tr>
<td>Atrophia</td>
<td>55% (6/11)</td>
<td>9% (1/11)</td>
<td>27% (3/11)</td>
<td>1/11 (9%)</td>
<td>20% (2/10)</td>
<td>0% (-/11)</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>55% (6/11)</td>
<td>50% (5/10)</td>
<td>9% (1/11)</td>
<td>0% (-/13)</td>
<td>0% (-/9)</td>
<td>0% (-/11)</td>
</tr>
<tr>
<td>Next to serous</td>
<td>17% (1/6)</td>
<td>25% (1/4)</td>
<td>0% (-/5)</td>
<td>0% (-/6)</td>
<td>0% (-/4)</td>
<td>0% (-/5)</td>
</tr>
<tr>
<td>Serous</td>
<td>13% (1/8)</td>
<td>0% (-/6)</td>
<td>17% (1/6)</td>
<td>0% (-/8)</td>
<td>0% (-/5)</td>
<td>17% (1/6)</td>
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*Endometrioid endometrial carcinoma
Mutations in benign and premalignant endometrium

The number of mutated benign and premalignant cases, as well as the genes mutated in these cases, are shown in Table 2. No mutations were found in the proliferative and atrophic endometrium cases. Of the 62 analyzed cases with endometrial hyperplasia, but no adjacent carcinoma, nine (15%) contained mutations, eight of which contained one mutation, and one contained two mutations. A mutation of the EGFR gene was found in one case with simple hyperplasia without atypia. Two PIK3CA mutations were found in the cases with complex hyperplasia without atypia. All other mutations were found in the cases with complex atypical hyperplasia: three KRAS mutations, two PIK3CA mutations and two AKT1 mutations. In comparison, mutations were slightly more common in cases with atypia than in cases without atypia (18% vs. 11%), and more common in complex than in simple hyperplasia (30% vs. 3%). When comparing the subgroups, cases with complex hyperplasia without atypia and complex atypical hyperplasia were mutated in 22% and 33%, respectively, compared to only one (5%) mutation found in simple hyperplasia without atypia, and none in the cases with simple atypical hyperplasia.

Mutations in carcinomas and adjacent endometrium

The number of mutated carcinoma cases and of the adjacent endometrium, as well as the genes mutated in these cases, are shown in Table 3. Of the 30 analyzed carcinomas (both endometrioid and non-endometrioid), 14 (43%) contained mutations, 11 of which contained one mutation, and three contained two mutations. Of the 22 included endometrioid carcinomas, twelve cases had a total of 14 mutations, most of which in the KRAS and PIK3CA genes. Five of the six mutations in endometrioid carcinomas next to hyperplastic endometrium were in the KRAS gene and one in the PIK3CA gene. Endometrioid carcinomas next to atrophic endometrium were more heterogeneous: the six cases with mutations contained three PIK3CA mutations, two CTNNB1 mutations, and a KRAS, an AKT1, and an N-RAS mutation. One of the three cases with PIK3CA mutations contained two mutations. Two of the cases contained two mutations: one case had a PIK3CA and a CTNNB1 mutation, and the other a PIK3CA and an N-RAS. There was only one mutated serous carcinoma, which contained both a PIK3CA and a BRAF mutation.

In the 26 endometrium next to a carcinoma samples, we identified only three (12%) mutations: a KRAS mutation in hyperplastic endometrium next to an endometrioid carcinoma, a PIK3CA mutation in atrophic endometrium next to an endometrioid carcinoma, and a KRAS mutation in, interestingly, atrophic endometrium next to a serous carcinoma. Only the KRAS mutation in the hyperplastic endometrium next to an endometrioid carcinoma was identified in the corresponding carcinoma.

Discussion

This study analyzed the presence of mutations in the endometrium, and found no mutations in the included cases with benign endometrium, and only one mutation in the cases with simple hyperplasia. Complex hyperplasia without atypia and complex atypical hyperplasia appear to be important steps in endometrial carcinogenesis, as most mutations were found in these cases. Interestingly, KRAS mutations were very common in endometrioid carcinomas next to hyperplastic endometrium, but not in endometrioid carcinomas next to atrophic endometrium. There were only a few mutations in the endometrium adjacent to the carcinomas.

Mutations

AKT1. Although the PI3K/AKT pathway is frequently affected in endometrial carcinomas, more often than in any other cancer type analyzed by The Cancer Genome Atlas, AKT1 mutations do not seem to play a major role.\(^21\)\(^,\)\(^22\) Shoji et al. analyzed 89 endometrial carcinomas, and found AKT1 mutations in only two cases, both at location c.49, and in both cases there were no mutations in the KRAS, PIK3CA, and ERBB2 genes.\(^23\) This is comparable to our findings in endometrial carcinomas: we found only one AKT1 mutation, which was at the same position. Interestingly, we found two AKT1 mutations in complex hyperplasia as well, which could imply that these do play a role in endometrial carcinogenesis. However, the allele frequency of these mutations was low, and it is quite possible that they do not play a tumor driving role. This is supported by the fact that we found a much more prevalent KRAS mutation in one of the cases with an AKT1 mutation.

BRAF. Although BRAF is part of the PI3K/AKT pathway as well, it does not seem to play a major role in endometrial carcinomas. There is one publication which analyzed BRAF exon 11 and 15, and found mutations in 21% of the endometrial carcinomas, and in 11% of the complex atypical hyperplasia cases, but not in benign endometrium.\(^24\) However, all of these were novel mutations, and other studies have contested these findings.\(^25\)\(^,\)\(^26\) BRAF mutations do not seem to play a major role in the samples we analyzed, but we did find one previously described BRAF mutation with a high allele frequency in one of the serous carcinomas.

CTNNB1. Previous studies analyzing the CTNNB1 gene in endometrial carcinomas have found CTNNB1 mutations in 15-25% of the endometrioid carcinomas.\(^25\)\(^,\)\(^26\) We found less CTNNB1 mutations than previously described, but it has to be noted that analysis of CTNNB1 was not possible in a substantial number of cases, most likely because FFPE tissue was used for this study. We did not find any CTNNB1 mutations in the other subgroups, but previous studies have only analyzed carcinomas, and we are therefore not able to compare this finding.

EGFR and ERBB2. Immunohistochemical expression of EGFR and Her2/Neu, activated by the PI3K/AKT and MAP/ERK pathways, is frequently seen in endometrial carcinomas, especially in serous carcinomas. EGFR mutations in endometrial carcinomas have not been described, whereas ERBB2 mutations are common in serous carcinomas and uncommon in endometrioid carcinomas.\(^27\)\(^,\)\(^28\) In accordance with these findings, we found only one EGFR mutation in the parts of the gene we sequenced, in a case with simple hyperplasia without atypia. Given the lack of these mutations in complex hyperplasia and endometrial carcinomas, it is unlikely that this gene plays a role in endometrial carcinogenesis. We included only a small number of serous carcinomas, which might explain why we were unable to find any ERBB2 mutations.

KRAS. The presence of KRAS mutations in endometrioid carcinomas has been extensively studied, and these were found in 9-19%.\(^21\)\(^,\)\(^32\)\(^,\)\(^34\) There is one previous study analyzing the presence of KRAS mutation in both endometrioid carcinomas (n=58) and atypical hyperplasia (n=22) next to the carcinomas.\(^23\) This study found KRAS mutations in 19% of the carcinoma, and in 5% of the hyperplasia cases. In comparison, we found KRAS mutations in 27% of the endometrioid carcinomas, almost all of which in endometrioid carcinomas next to hyperplastic endometrium. We also found one KRAS mutation in the hyperplasia next to the carcinoma, and it was present in the adjacent carcinoma as well. In addition, we found...
K Ras mutations in 5% of the endometrial hyperplasia cases with no adjacent carcinoma, all of which were complex atypical hyperplasia cases.

H Ras and N Ras. Both H Ras and N Ras play a role in the PI3K/AKT pathway as well, and although they have been described in other cancer types which are driven by this pathway, H Ras mutations have never been described in endometrial carcinomas, and N Ras mutations were only found in only 1.8% of the endometrial carcinomas (both endometrioid and non-endometrioid).36-38 This is in line with our findings: we found no H Ras mutations and N Ras mutations in only 5% of the endometrioid carcinomas.

PI3KCA. Oda et al. found PI3KCA mutations in 36% of the 66 endometrial carcinomas studied, and in 26% of the cases PTEN was mutated as well.39 In addition, Hayes et al. analyzed both these genes in 29 cases with complex atypical hyperplasia and 44 cases with an endometrial carcinoma.40 They found PTEN mutations in 48% of the complex atypical hyperplasia cases and 57% of the endometrial carcinoma cases. In contrast, they found PI3KCA mutations in only 7% of the complex atypical hyperplasia cases, and in 39% of the endometrial carcinoma cases. They hypothesized that PTEN mutations are a very early event, whereas PI3KCA mutations, amongst others, are important for the invasive potential. This is supported by numerous studies showing either PTEN mutations or loss of immunohistochemical PTEN expression in endometrial hyperplasia.40-42

Premalignancies

Endometrioid carcinomas are assumed to originate from hyperplastic endometrium under the influence of unopposed estrogen expression, and many different mutations have been associated with this process. Especially PTEN mutations are assumed to be an early event, already present in a substantial number of cases with atypical hyperplasia, whereas other mutations, especially of the K Ras and PI3KCA genes, may play a role in the progression from hyperplasia into a carcinoma.41, 42, 43, 44 Based on several recent studies describing a high risk of up to 30% of progression from atypical hyperplasia into an endometrioid carcinoma, the WHO advises a subdivision of endometrial hyperplasia into non-atypical and atypical hyperplasia.45, 46, 47 In contrast, the previous subdivision had four categories, and was based on both the architecture (simple or complex) and the absence or presence of atypical nuclei.48 This was based on a study by Kurman et al., which shows a progression risk of 1% in simple hyperplasia without atypia, 3% in complex hyperplasia without atypia, 8% in simple atypical hyperplasia, and 29% in complex atypical hyperplasia.49 Nevertheless, most recent studies concluding that there is a risk of progression when atypical nuclei are present have pooled simple and complex atypical hyperplasia.50, 51, 52 However, combining these cases, and subsequently advising a hysterectomy for both diagnoses, is challenged by our findings that almost all mutations are found in complex hyperplasia without atypia and complex atypical hyperplasia, and not in simple atypical hyperplasia. The new WHO subdivision was also based on the fact that many of the mutations present in endometrioid carcinomas were already present in atypical hyperplasia.53 As previous studies have shown that PTEN mutations are a very early event, followed by other mutations which lead to malignant progression, it might very well be possible that PTEN mutations are already present in simple atypical hyperplasia, but progression mainly occurs as a result of other mutations, which we found predominantly in complex and complex atypical hyperplasia.54-56 The traditional morphological characterization has also been challenged by studies suggesting that the identification of endometrioid premalignancies, which were called endometrial intraepithelial neoplasia (EIN), should include assessment of glandular volume, architectural complexity, and nuclear abnormality.57 Coexistent carcinomas and progression to a carcinoma were found in a substantial number of the EIN cases.41, 42 Interestingly, EIN was predominantly diagnosed in cases with complex hyperplasia without atypia and complex atypical hyperplasia.43, 44 Moreover, progression to a carcinoma was most likely to occur if both EIN and complex atypical hyperplasia were present.41, 42 Little is known about molecular characteristics of EIN, besides the fact that the combined presence of EIN and loss of PTEN predicts the progression risk better than the presence of EIN alone.43 All together, these findings suggest that it may be too simplistic to consider all atypical hyperplasia to be the endometrioid premalignancy, and future studies should investigate possible improvements to the histological classification, as well as the combined value of histology and molecular markers in the identification of cases at risk of a coexistent carcinoma or progression to a carcinoma.

Carcinogenesis

It has previously been shown that endometrial hyperplasia can be very focal, and it has been hypothesized that only a few hyperplastic glands are required for endometrial carcinogenesis.44 This is further supported by the fact that next to around 20% of the endometrioid carcinomas the assumed precursor lesion, endometrial hyperplasia, is not present.45 Interestingly, in the current study we failed to find mutations present in the carcinomas in most of the corresponding endometrial samples, which would support that endometrial carcinogenesis may be a focal process. Furthermore, previous studies found prognostic and molecular differences between endometrioid carcinomas next to hyperplastic and atrophic endometrium.46, 47 In the current study almost all mutations found in the endometrioid carcinomas next to hyperplastic endometrium were in the K Ras gene, whereas endometrioid carcinomas next to atrophic endometrium were in several other genes other than K Ras. It is of course unknown if these latter carcinomas originated from focal hyperplastic endometrium or directly from atrophic endometrium, but it seems likely that their carcinogenic pathway is different than that of endometrioid carcinomas next to hyperplastic endometrium. This is further supported by a recent study by Berg et al., which shows that the carcinogenesis of endometrial carcinomas is dependent on the body mass index (BMI), and is characterized by PTEN mutations and PI3KCA mutations in non-obese women, and by K Ras mutations in obese women.46 Interestingly, a previous study has shown that the BMI of patients with an endometrioid carcinoma with adjacent atrophic endometrium is significantly lower than that of patients with an endometrioid carcinoma with adjacent hyperplastic endometrium. Even when taking into account the fact that we did not include PTEN mutations in our analyses, the fact that we found mutations in only 55% of the endometrioid carcinomas underlines the fact that carcinogenesis of these carcinomas appears to be a very heterogeneous process. Moreover, in light of recent studies classifying endometrial carcinomas based on their molecular profiles, it would be interesting to study the carcinogenesis in relation to this new classification into four subgroups.47, 58 The carcinogenesis of non-endometrioid carcinomas is believed to be independent of estrogen stimulation and these carcinomas are assumed to originate directly from atrophic endometrium, although a minority might in fact be dedifferentiated endometrioid carcinomas.49 Interestingly, there was one case which had both a BRAF and a PI3KCA mutation,
and was adjacent to hyperplastic endometrium. This might in fact be a dedifferentiated endometrioid carcinoma.1, 2

Strengths and weaknesses
This is the first study analyzing endometrial tissue with smMIPs, which has several advantages over other Next Generation Sequencing techniques (Eijkelenboom A. et al. (in press). Reliable Next Generation Sequencing of FFPE tissue using single molecule tags. J Mol Diagn). 46 The number of false positive variants with an allele frequency >5% is significantly reduced in the smMIP-NextSeq500 approach, while the detection of clinically relevant mutations is comparable. Additionally, overestimation of the actual number of analyzed molecules as a result of sequence analysis of PCR duplicates is a problem when poor quality FFPE-derived gDNA is used. Because smMIPs have a unique molecular tag the exact number of analyzed input DNA molecules can be calculated, preventing overestimation. Moreover, this is the first study analyzing a substantial number of genes in an extensive and well described, and revised cohort containing benign, premalignant and malignant lesions, as well as endometrium next to the malignant lesions. Unfortunately, the smMIP analysis was not successful in all cases, most likely as a result of DNA fragmentation caused by the use of FFPE tissue which has been archived for years. In addition, a smMIP probe targeting PTEN was not included in the panel. Although PTEN alterations undeniably play an important role in the development of endometrial carcinomas, this has already been extensively described in other publications highlighting its role in both premalignant and malignant endometrial tissue. The genes described in this study have been described to play a role in a later stage of carcinogenesis, but there is little data on the presence of these mutations in endometrial tissue other than endometrial carcinomas.

Conclusion
This study reinforces the heterogeneous genetic origin of endometrial carcinogenesis. On a molecular level, complex endometrial hyperplasia appears to be the most important step in this process. Endometrioid carcinogenesis seems to follow different pathways in the presence of hyperplastic and atrophic background endometrium. More research into these distinct carcinogenic changes and the role of these findings in the management of endometrial carcinomas is needed. Moreover, it should be studied whether non invasive follow-up of patients with simple atypical hyperplasia might be sufficient, with possibly repeated mutation analyses of the endometrial biopsies.

References
8. Lacey Jv, Jr., Chia VM. Endometrial hyperplasia and the risk of progression to carcinoma. Maturitas 2009;63:39-44.


**Supplementary Table 1.** The hotspots targeted by the smMIPs

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<tr>
<th>Gene</th>
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<th>Ensembl ID</th>
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<td>L858-L861</td>
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<td>08</td>
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<td>c.161 to c.245</td>
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**Supplementary Table 2.** The locations, allele frequency and study groups of the mutations found in this study.

<table>
<thead>
<tr>
<th>Location (allele frequency)</th>
<th>Study group</th>
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<td>KRAS c.35G&gt;T (14%)</td>
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</tr>
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<tr>
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CHAPTER 4

IMMUNOHISTOCHEMICAL PROFILES OF ENDOMETRIOID ENDOMETRIAL CARCINOMAS WITH AND WITHOUT METASTATIC DISEASE


*Both authors contributed equally to this work

**Abstract**

**Background**
A minority of endometrial carcinomas present at an advanced stage with a poor prognosis, and should be identified to individualize treatment. Immunohistochemical markers have been studied, but most have not been directly linked to metastasis. This study analyzes the immunohistochemical profile of endometrioid endometrial carcinomas (EECs) with and without metastases, and corresponding metastases.

**Materials and Methods**
Tissue Micro Array slides from stage I EECs, and stage III-IV EECs and corresponding metastases, and corresponding metastases. EECs without metastasizing.14 Endometrial carcinomas can be divided into two subtypes with different clinical and pathologic characteristics.1 The most common endometrioid endometrial carcinoma (EEC) is believed to arise from hyperplastic endometrium under the influence of unopposed estrogen stimulation. They often present at an early stage and have a good prognosis. The less common non-endometrioid carcinomas, most with serous or clear cell histology, arise from atrophic endometrium and have a worse prognosis. These two subtypes were shown to differ on a molecular level as well. EECs often have microsatellite instability and mutations in PTEN, PIK3CA, KRAS and CTNNB1, whereas TP53, STK15, CDKN2A (p16), CDH1 and ERBB2 are often mutated in non-endometrioid carcinomas.2 Recent studies have shown that there are more than two subgroups, based on the molecular profile, but these new findings have not yet been incorporated in clinical practice.3-5 Although most EECs present at an early stage with a favorable prognosis, some may already be advanced stage at the time of diagnosis. It is important to identify these patients in order to further individualize treatment, and although several immunohistochemical prognostic markers have been studied, only a few have been directly linked to the presence of metastatic lesions.6-8 Expression of the estrogen receptor (ER), progesterone receptor (PR), stathmin and p-mTOR was found to be different in endometrial carcinoma recurrences when compared to the primary tumor, and loss of ER and PR in preoperative endometrial biopsies was associated with the presence of lymph node metastases.9, 10 In addition, E-cadherin is assumed to play a role in epithelial to mesenchymal transition, a process important in tumor progression and metastasis.11 Although EECs can spread intra-abdominal and to a distant site (lymphogenous or hematogenous), it is unknown whether there is a molecular difference between these two.12 In ovarian carcinomas distinct molecular differences have been found in cells spreading to an intra-abdominal site, and lymphogenously or hematogenously to a distant site.13 Interestingly, it has been shown that endometrial cancer cells can be intra-abdominal without metastasizing.14 Understanding the mechanisms underlying metastasis of EECs is of great value in individualizing the treatment. This study therefore aims to compare the immunohistochemical profile of EECs without metastases to that of EECs with metastases, of the primary tumor to that of corresponding metastases, and of intra-abdominal metastases to that of distant metastases.

**Results**
Primary tumors with distant metastases had a significantly lower ER expression than those without metastases or with intra-abdominal metastases. Distant metastases had a significantly lower PR expression than the corresponding primary tumor and intra-abdominal metastases. In contrast, p16 and PTEN expression was significantly higher in intra-abdominal metastases compared to corresponding primary tumors.

**Conclusion**
Immunohistochemistry predicts both presence and location of EEC metastases. Loss of ER and PR was related to distant spread, and increased expression of PTEN and p16 was related to intra-abdominal spread. Additional research should assess the use of these markers in the diagnostic workup as well as the possibility to target metastases through these markers.

**Introduction**
Endometrial carcinoma is the most common gynecologic malignancy in the developed world and its incidence is increasing.1 Endometrial carcinomas can be divided into two subtypes with different clinical and pathologic characteristics. The most common endometrioid endometrial carcinoma (EEC) is believed to arise from hyperplastic endometrium under the influence of unopposed estrogen stimulation. They often present at an early stage and have a good prognosis. The less common non-endometrioid carcinomas, most with serous or clear cell histology, arise from atrophic endometrium and have a worse prognosis. These two subtypes were shown to differ on a molecular level as well. EECs often have microsatellite instability and mutations in PTEN, PIK3CA, KRAS and CTNNB1, whereas TP53, STK15, CDKN2A (p16), CDH1 and ERBB2 are often mutated in non-endometrioid carcinomas. Recent studies have shown that there are more than two subgroups, based on the molecular profile, but these new findings have not yet been incorporated in clinical practice.

Although most EECs present at an early stage with a favorable prognosis, some may already be advanced stage at the time of diagnosis. It is important to identify these patients in order to further individualize treatment, and although several immunohistochemical prognostic markers have been studied, only a few have been directly linked to the presence of metastatic lesions. Expression of the estrogen receptor (ER), progesterone receptor (PR), stathmin and p-mTOR was found to be different in endometrial carcinoma recurrences when compared to the primary tumor, and loss of ER and PR in preoperative endometrial biopsies was associated with the presence of lymph node metastases. In addition, E-cadherin is assumed to play a role in epithelial to mesenchymal transition, a process important in tumor progression and metastasis. Although EECs can spread intra-abdominal and to a distant site (lymphogenous or hematogenous), it is unknown whether there is a molecular difference between these two. In ovarian carcinomas distinct molecular differences have been found in cells spreading to an intra-abdominal site, and lymphogenously or hematogenously to a distant site. Interestingly, it has been shown that endometrial cancer cells can be intra-abdominal without metastasizing. Understanding the mechanisms underlying metastasis of EECs is of great value in individualizing the treatment. This study therefore aims to compare the immunohistochemical profile of EECs without metastases to that of EECs with metastases, of the primary tumor to that of corresponding metastases, and of intra-abdominal metastases to that of distant metastases.

**Materials and Methods**

**Patient selection**
For this case-control study the nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA) was searched for all patients surgically treated between January 1999 and January 2010 for International Federation of Gynaecology and Obstetrics (FIGO) stage III or IV EEC at the Radboud university medical center and the Canisius-Wilhelmina Hospital in Nijmegen, and the TweeSteden and St. Elisabeth Hospital in Tilburg, the Netherlands. Subsequently, a control group of patients with stage I EEC, matched for tumor grade, was collected from a well-defined cohort of patients treated for endometrial cancer between January 1999 and January 2010 at the Radboud university medical center and the Canisius-Wilhelmina Hospital.
All histological slides of the primary carcinoma and the metastatic site were retrieved from the pathology archives and reviewed systematically with respect to the histological type and tumor grade of both the primary tumor and the metastasis. Moreover, primary tumor slides were reviewed with respect to depth of myometrial invasion, presence of lymphovascular space invasion, and the nature of the background endometrium as well. Review was performed in every hospital separately by an independent pathologist (JB, SB, or AW) who was unaware of the original pathology reports and the clinical outcome. When there was doubt about the diagnosis or discrepancy between the review and the original pathology report, the three reviewing pathologists reached consensus.

Clinical data were recorded from patient records: age, menopausal state, body mass index (BMI), parity, personal medical history, treatment, stage of disease, date of recurrence of disease, date of death, and cause of death.

### Tissue microarray and immunohistochemistry

Representative areas of the primary carcinomas and the metastatic lesions were selected on haematoxylin and eosi-stained slides. These areas were combined into tissue microarrays (TMAs) by punching a cylinder with a diameter of 3 mm from the corresponding formalin-fixed paraffin-embedded (FFPE) block and mounting it into a recipient paraffin block using a manual tissue microarray (Tissue-Tek, Quick-Ray, Sakura Finetek, USA).

Slides (4 μm) cut from the TMA blocks were immunohistochemically stained. In short, following antigen retrieval of deparaffinized and rehydrated slides, endogenous background was blocked using Peroxidase Blocking Reagent (3% H2O2 in PBS) and protein was blocked using horse serum. After incubating with the primary antibody, a secondary antibody was added and visualization was performed with Vectastain and 3,3’-Diaminobenzidine (Zymed lab, California, USA) as substrate, and slides were counterstained with Mayer’s haematoxylin. Finally, slides were dehydrated and mounted. Table 1 contains an overview of the antibodies used in this study.

### Table 1. Antibodies used for staining

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Vendor</th>
<th>Retrieval</th>
<th>Dilution</th>
<th>Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-catenin</td>
<td>14/β-catenin</td>
<td>BD</td>
<td>EDTA 10'</td>
<td>1:100</td>
<td>Membranous</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>SPM471</td>
<td>Thermo Scientific</td>
<td>EDTA 10'</td>
<td>1:300</td>
<td>Membranous</td>
</tr>
<tr>
<td>ER</td>
<td>SP1</td>
<td>Thermo Scientific</td>
<td>EDTA 10'</td>
<td>1:80</td>
<td>Nuclear</td>
</tr>
<tr>
<td>PR</td>
<td>PgM636</td>
<td>Dako</td>
<td>citrate 10'</td>
<td>1:250</td>
<td>Nuclear</td>
</tr>
<tr>
<td>PTEN</td>
<td>6H2.1</td>
<td>Dako</td>
<td>EDTA 10'</td>
<td>1:100</td>
<td>Nuclear, cytoplasmatic</td>
</tr>
<tr>
<td>p16</td>
<td>G175-405</td>
<td>BD</td>
<td>citrate 10'</td>
<td>1:20</td>
<td>Nuclear, cytoplasmatic</td>
</tr>
<tr>
<td>MLH1</td>
<td>G168-15</td>
<td>BD</td>
<td>EDTA 10'</td>
<td>1:50</td>
<td>Nuclear</td>
</tr>
<tr>
<td>PMS2</td>
<td>A16-4</td>
<td>BD</td>
<td>EDTA 10'</td>
<td>1:100</td>
<td>Nuclear</td>
</tr>
<tr>
<td>L1CAM</td>
<td>UJ127</td>
<td>Thermo Scientific</td>
<td>EDTA 10'</td>
<td>1:100</td>
<td>Membranous</td>
</tr>
<tr>
<td>p53</td>
<td>DO-7</td>
<td>Thermo Scientific</td>
<td>EDTA 10'</td>
<td>1:400</td>
<td>Nuclear</td>
</tr>
<tr>
<td>p21</td>
<td>CD5-60.2</td>
<td>Thermo Scientific</td>
<td>citrate 10'</td>
<td>1:75</td>
<td>Nuclear</td>
</tr>
<tr>
<td>MIB</td>
<td>MIB-1</td>
<td>Dako</td>
<td>citrate 10'</td>
<td>1:100</td>
<td>Nuclear</td>
</tr>
</tbody>
</table>


### Evaluation of staining

Percentage (0 = 0%, 1 = 1-10%, 2 = 11-50% and 3 = 51-100% positive) and intensity of staining (0 = none, 1 = weak, 2 = moderate and 3 = strong) were scored per slide independently by two evaluators (YG and JB). The product of the percentage and intensity scores (range 0 to 9) was calculated and the average of these 2 scores per case was considered to be the final score. This semiquantitative score was used unaltered for ER and PR, in accordance with the literature, and for β-catenin, E-cadherin, PTEN and p16 because there is no consensus which scoring system should be used. MLH1 and PMS2 were considered lost when there was no scoring at all, according to the international guidelines. L1CAM was considered positive when staining was seen in at least 10% of the malignant cells. Cases with a final p53 score ≥ 4 were considered positive. All p21 scores below the first quartile (score 0 for the tumors and score 1.5 for the metastatic lesions) were considered negative. Finally, MIB1 staining was considered positive at every intensity and categorized as 1%-10%, 11%-50% and 51%-100% cells positive.

### Statistical analysis

For statistical analysis, metastases were classified according to the hypothesized route of spread: intra-abdominal (tubal, ovarian, peritoneal and omental), and lymphogenous or hematogenous (vaginal, nodal and other organs). Clinical and pathologic parameters of the case and the control groups were compared using the Pearson χ² or Fisher exact test for categorical variables, and the Mann-Whitney test for continuous variables. Differences in marker scores between the case and the control groups as well as between intra-abdominal and distant metastases were calculated using the Mann-Whitney test for β-catenin, E-cadherin, ER, PR, PTEN and p16, and Pearson χ² or Fisher exact test for MLH1, PMS2, L1CAM, p53, p21 and MIB1. Differences in marker scores between the primary tumors and the corresponding metastases were calculated in a paired fashion using the Wilcoxon test for β-catenin, E-cadherin, ER, PR, PTEN and p16, and McNemar’s test for MLH1, PMS2, L1CAM, p53, p21 and MIB1. P-values < 0.05 were considered statistically significant.

Statistical analyses were performed using SPSS version 20 (SPSS IBM, New York, NY, USA).

### Results

#### Patients

A total of 53 patients with FIGO stage III or IV EEC were retrieved from nationwide network and registry of histo- and cytopathology. After review 15 cases were excluded: 10 because non-endometrioid histology was seen, 4 because insufficient material was available and 1 because the metastatic site was found to be a second primary malignancy. Of the remaining 38 patients, 5 had FIGO stage IIIA disease, 6 stage IIIB, 9 stage IIIC, 5 stage IVA and 13 stage IVB. Three tumors were grade 1, 16 grade 2 and 19 grade 3. The matched control group consisted of 37 patients with FIGO stage I EEC. Four had a grade 1 tumor, 17 grade 2 tumor and 16 a grade 3 tumor.

Of the metastases, 23 were considered to be intra-abdominal: 7 peritoneal, 6 ovarian, 4 omental, 2 tubal, 2 parametrial, 1 of the mesovarium and 1 of the uterosacral ligament. Of these patients, 4 had another lesion at a distant site suspected to be a metastasis, but of
which no tissue was available. Fifteen metastases were considered to be distant: 11 lymph nodes, 3 vaginal, and 1 liver metastasis. Clinical and pathological characteristics of the included patients are shown in Table 2. There were no differences between patients with and without metastatic disease in terms of age at diagnosis, BMI and menopausal age. However, deep myometrial invasion, cervical involvement and lymphovascular space invasion were found significantly more often in the case group compared to the control group. As a result, patients in the case group were treated with adjuvant radio- and chemotherapy more often and were more likely to die as a consequence of the carcinoma, and the follow-up was therefore longer in the control group.

<table>
<thead>
<tr>
<th>Table 2. Comparison of the clinical and pathologic characteristics of the case and control groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of patients</strong></td>
</tr>
<tr>
<td><strong>Median age (years)</strong></td>
</tr>
<tr>
<td><strong>Median BMI (kg/m²)</strong></td>
</tr>
<tr>
<td><strong>Menopausal state</strong></td>
</tr>
<tr>
<td>Premenopausal</td>
</tr>
<tr>
<td>Postmenopausal</td>
</tr>
<tr>
<td><strong>FIGO stage</strong></td>
</tr>
<tr>
<td>IA</td>
</tr>
<tr>
<td>IB</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>III</td>
</tr>
<tr>
<td>IV</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td><strong>Myometrial invasion</strong></td>
</tr>
<tr>
<td>&lt;1/2</td>
</tr>
<tr>
<td>≥1/2</td>
</tr>
<tr>
<td><strong>Cervical involvement</strong></td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>Endocervical</td>
</tr>
<tr>
<td>Stromal</td>
</tr>
<tr>
<td><strong>LVI</strong></td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>Yes</td>
</tr>
</tbody>
</table>

*aP-value for the Mann-Whitney U test in case of continuous variables and the χ² test for categorical variables.*

**Evaluation of staining**

All immunohistochemical scores are shown in Figure 1. Primary tumors with a metastasis had a significantly lower ER expression compared to primary tumors without a metastasis (p = 0.04). This was attributable to a significantly lower ER expression (p = 0.02) in primary tumors with a distant metastasis compared to those without a metastasis, as there were no differences between primary tumors with an intra-abdominal metastasis and primary tumors without a metastasis. Moreover, distant metastases had a significantly lower PR expression than the corresponding primary tumor (p = 0.04) as well as than intra-abdominal metastases (p = 0.04).

Both PTEN (p = 0.02) and p16 (p < 0.01) expression was significantly increased in metastatic lesions compared to the corresponding primary tumor. This difference was caused by a significantly increased expression of PTEN (p = 0.05) and p16 (p = 0.01) in intra-abdominal metastases compared to the corresponding primary carcinoma. There were no significant differences between distant metastatic lesions and the corresponding primary tumor in PTEN and p16 expression, although there was a trend towards increased p16 expression in distant metastases compared to the corresponding primary tumor.

Other, non-significant observations were loss of β-catenin in distant metastases, loss of E-cadherin in all metastases, increased expression of L1CAM in distant metastases, increased expression of p53 in EECs with distant metastases and in all metastatic lesions, and finally loss of p21 in EECs with metastases, but not in the corresponding metastases.

**Discussion**

In this study primary EECs with distant metastases were shown to have a significantly reduced immunohistochemical expression of ER compared to EECs without metastases, and had a significantly lower expression of PR than corresponding primary tumors as well as intra-abdominal metastases. In contrast, intra-abdominal metastases were found to have and increased expression of PTEN and p16 compared to the corresponding primary EEC.

**ER and PR in distant metastasis**

In line with the previous literature, we found ER expression to be significantly lower in EECs with distant metastases compared to EECs confined to the uterus. Moreover, the ER expression was significantly lower in distant metastases than in intra-abdominal metastases. The carcinogenesis of EECs is thought to be directly related to estrogen and progesterone exposure, and binding of these hormones to their respective receptor leads to specific phenotypic effects.17 Estrogen promotes cell proliferation, inhibits apoptosis and modulates tumor suppressor function, and loss of ER and PR have been shown to be independent prognostic factors for survival and recurrence in many studies.2, 18 Moreover, loss of ER has been described in recurrences of endometrial carcinomas, and ER and PR expression in grade 2 and 3 EECs has been associated with an improved outcome.9, 19 Finally, it has been previously shown that loss of ER and PR in preoperative endometrial biopsies is related to lymphogenous spread.10
PTEN and p16 in intra-abdominal metastasis

We found that expression of PTEN is significantly higher in intra-abdominal metastatic lesions than in the corresponding EECs. Inactivation of PTEN frequently occurs in EECs, but seldom in aggressive, non-endometrioid endometrial carcinomas.\textsuperscript{20, 21} In addition, increased expression of p16 was found in intra-abdominal metastases compared to the corresponding primary carcinomas. Previous studies looking at p16 in EECs have shown conflicting results. Several studies have associated a decreased expression of p16 with a poor prognosis in endometrial carcinomas, whereas others found increased p16 expression to be associated with high FIGO stage in grade 3 EECs and to be expressed in the invasive front of endometrial carcinomas.\textsuperscript{22-27} A possible explanation for these conflicting data might be the fact that CDKN2A alterations can occur through homozygous deletion, promoter hypermethylation, or point mutations, all leading to different immunohistochemical expression patterns.\textsuperscript{24, 28, 29} Increased expression of p16 might for example reflect accumulation of dysfunctional proteins as is frequently observed in p53 expression.\textsuperscript{18}

The differences found in PTEN and p16 expression between intra-abdominal metastases and corresponding primary tumors might have been the result of tumor heterogeneity, or the presence of mixed histology.\textsuperscript{2} Expression of PTEN and p16 in endometrial carcinomas has been associated with serous histology, and expression of PTEN has been described in the serous component of mixed carcinomas.\textsuperscript{30, 31} Serous carcinomas have been shown to be prone to metastasize intra-abdominal.\textsuperscript{12} It is possible this component was missed either when sampling the primary tumor or when creating the TMA.

It is worth noting that many different are reported in studies assessing PTEN and p16 expression in endometrial carcinomas. A semiquantitative staining index developed for research purposes was used in the current study. For the possible future use of PTEN and p16 expression in the management of endometrial carcinomas consensus should be reached about standardized scoring systems.

Other molecular markers

Expression of E-cadherin, p53 and L1CAM, other known predictors of poor survival, was not significantly different between the groups.\textsuperscript{7, 8} There was a trend towards increased p53 expression in primary EECs with distant metastases. Moreover, metastatic lesions had an increased p53 expression and a decreased E-cadherin expression compared to the corresponding primary tumor. Complete absence of p53 staining has been associated with the presence of TP53 nonsense mutations and a poor prognosis.\textsuperscript{32} However, completely p53 negative cases did not have a high MIB1 staining index, and were distributed equally among all subgroups (data not shown)\textsuperscript{33, 34} They were therefore categorized as negative.

Finally, increased expression of L1CAM was found in distant metastases compared to the corresponding primary EECs, primary EECs without metastatic disease and intra-abdominal metastases. The number of lesions might not have been sufficient to find significant differences for these markers. L1CAM for example is known to be expressed only in a minority of the EECs, and only in a few in this study.\textsuperscript{9}

Implications for the management of endometrial carcinomas

Dividing endometrial carcinomas into two subgroups has proven to be a simple and effective way to individualize treatment. In addition to this, many immunohistochemical markers of poor prognosis have been identified, which might be used in the future to characterize and
treat endometrial carcinomas. More recently, molecular markers of poor prognosis have also been identified and a new subdivision completely based on the molecular profile of endometrial carcinomas has even been proposed by The Cancer Genome Atlas. The findings of the current study confirm that ER and PR are strong and useful pre- and postoperative markers. This study also shows that, like in ovarian carcinomas, intra-abdominal metastases of EECs have a distinct molecular profile. Based on both morphological and molecular comparison they conclude that there are only subtle differences between primary carcinomas and recurrences, which are possibly the result of either tumor progression or suboptimal sampling. The most notable immunohistochemical difference they describe is a reduced expression of PR in recurrences compared to the primary carcinoma. A substantial number of cases was found to have ambiguous or mixed histology, and peritoneal metastases in their study were related to the presence of serous histology. All cases with p16 overexpression had serous histology. The findings of both their study and the current study suggest that more extensive sampling and immunohistochemical analysis of the primary tumor is worthwhile, especially when the metastasis and primary tumor are immunohistochemically discordant.

Strengths and weaknesses
To our knowledge, this is the first study analyzing a large set of immunohistochemical markers in a cohort of EEC patients with histologically proven metastatic disease on both the primary tumors and the metastatic lesions. In addition, the control group was matched for tumor grade, avoiding possible bias in the immunohistochemical comparison between patients with and without metastatic disease. A limitation might be the fact that, in accordance with national guidelines, no routine lymphadenectomy was performed and only suspicious lesions were surgically removed. This means the absence of metastatic disease was not histologically proven for most patients in the control group. However, none of these patients developed recurrent disease during a median follow-up period of 44 months, which makes it highly unlikely there were metastatic lesions at the time of the primary treatment. Although the use of TMA slides has been validated for most markers in this study, they might not be an accurate reflection on heterogenic markers. For example, a heterogeneous staining pattern of MLH1 and PMS2 has previously been described. However, two representative areas were selected per case, the number of cases with no MLH1 or PMS2 expression was in accordance with the literature. Conclusion
This study confirms there is a strong association between loss of ER and PR from primary EECs and distant spread of the disease. However, it also shows expression of PTEN and p16 in intra-abdominal metastases is comparable to that of serous carcinomas, and not to the corresponding primary tumor. Immunohistochemistry of metastatic lesions in addition to primary EECs might be of value in managing endometrial cancer and future research should clarify the underlying processes of tumor progression and investigate the implications of these immunohistochemical findings.

References


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CHAPTER 5

Lymphovascular space invasion and the treatment of stage I endometrioid endometrial cancer

Abstract

Background
Treatment of clinical early-stage endometrioid endometrial cancer (EEC) in The Netherlands consists of primary hysterectomy and bilateral salpingo-oophorectomy. Adjuvant radiotherapy is given when 2 or more of the following risk factors are present: 60 years or older, grade 3 histology, and 50% or more of myometrial invasion. Lymphovascular space invasion (LVSI) is a predictor of poor prognosis and early distant spread. It is unclear whether adjuvant radiotherapy is sufficient in patients with LVSI-positive EEC.

Materials and Methods
Eighty-one patients treated from 1999 until 2011 for stage I LVSI positive EEC in 11 Dutch hospitals were included. The outcomes of patients with 0 to 1 risk factors were compared with those with 2 to 3 risk factors, and both were compared with the known literature.

Results
Eighteen patients presented with recurrent disease, and 12 of those recurrences had a distant component. Overall and distant recurrence rates were 19.2% and 11.5% in patients with 0 to 1 risk factors followed by observation and 25.5% and 17% in patients with 2 to 3 risk factors who received adjuvant radiotherapy. Only 1 patient with grade 1 disease had a recurrence.

Conclusion
In stage I LVSI-positive EEC with 0 to 1 risk factors, observation might not be adequate. Moreover, despite adjuvant radiotherapy, a high overall and distant recurrence rate was observed in patients with 2 to 3 risk factors. The use of systemic treatment in these patients, with the exception of patients with grade 1 disease, should be investigated.

Introduction
Endometrial cancer is the most common gynecologic malignancy in postmenopausal women in developed countries. Most patients present with endometrioid endometrial cancer (EEC) at an early stage and have a favorable prognosis. Primary treatment consists of hysterectomy and bilateral salpingo-oophorectomy. Risk factors for recurrent disease are myometrial invasion ≥50%, grade 3 histology, and an age of 60 years or older. External beam radiotherapy (EBRT) and vaginal brachytherapy (VBT) have been shown to reduce the risk of locoregional recurrence when at least two of these three risk factors are present. However, the morbidity of VBT was significantly less than that of EBRT. Therefore, adjuvant treatment of patients with stage I EEC and at least two of the three risk factors consists of VBT in the Netherlands.

Despite the good prognosis of stage I EEC, factors leading to an unexpected worse outcome are still under investigation. Lymphovascular space invasion (LVSI) has been reported as an important factor in poor outcome and is associated with a reduced progression-free and overall survival, as well as increased lymph node metastasis. Concerning the adjuvant treatment of patients with stage I endometrial cancer with LVSI, two studies demonstrated that EBRT is superior to both observation and VBT. In contrast, other studies showed that VBT is equally effective to EBRT in patients with endometrial cancer and LVSI. In the presence of LVSI, both lymphadenectomy and systemic treatment have been proposed. So far, the implications of LVSI in patients with stage I EEC on either primary or adjuvant treatment have not been well established. If there is a strong association between LVSI and distant spread, adjuvant radiotherapy is expected to be of limited value in LVSI-positive EEC. Moreover, all these studies look at centrally diagnosed LVSI, while this is commonly done by the primary pathologist. As the interobserver variability of LVSI is unknown, the exact relevance of these findings on the daily clinical practice is not known.

The aim of the current study was to examine the recurrence pattern in patients with stage I, LVSI-positive EEC, as diagnosed by the primary pathologist, who received adjuvant radiotherapy based on the presence of two or three risk factors. Furthermore, the aim was to evaluate whether or not the use of radiotherapy in these patients is sufficient, or if more aggressive therapy may be warranted.

Materials and Methods

Patients and treatment
Patients treated within the Comprehensive Cancer Center South (CCCS, a collaboration between nine hospitals in the Netherlands) between January 2005 and December 2011, and at the Radboud university medical center and the Canisius-Wilhelmina Hospital in Nijmegen, the Netherlands, between January 1999 and January 2009, were reviewed for inclusion. Only patients with surgical stage I EEC according to the 2009 criteria of the International Federation of Gynecology and Obstetrics (FIGO), and documented LVSI, as indicated in the primary pathologist’s report, were included. LVSI was defined by the presence of malignant cells in endothelial-lined channels and was assessed on hematoxylin and eosin stained slides as previously described. Patients were treated according to the Dutch guidelines. In cases of clinical stage I EEC, a hysterectomy and bilateral salpingo-oophorectomy with sampling of suspicious nodes is advised. Based on the Post-Operative Radiation Therapy in...
Endometrial Carcinoma (PORTEC) trials, adjuvant radiotherapy is advised in the presence of at least two out of three risk factors (myometrial invasion ≥50%, grade 3 histology, and age ≥60 years). Observation is recommended if <2 risk factors are present. Patients with histological types of endometrial cancer other than endometrioid, patients who did not undergo a hysterectomy and bilateral salpingo-oophorectomy, and patients who received either neoadjuvant treatment or adjuvant chemotherapy were excluded.

Patient data were extracted from files at the hospital where the patient received treatment. Age, date of diagnosis, date of surgery, type of surgery, lymphadenectomy, origin and number of dissected lymph nodes, tumor grade, myometrial invasion, adjuvant treatment, date of last follow-up, date of recurrence, location of recurrence, treatment for recurrence, date of death, and cause of death were recorded. Vaginal recurrences and pelvic masses were considered local recurrences, pelvic and iliac lymph node metastases were considered regional recurrences, and all others were considered distant recurrences. All histological data were retrieved from the primary pathologist’s report and were not reviewed centrally. No ethical approval was needed for this observational study according to the Code of Conduct for the use of data in Health Research (Dutch federation of Biomedical Scientific Societies, www.federa.org).

Outcome

Primary outcome was defined as the recurrence rate location related to primary and adjuvant therapy. Secondary outcome was defined as the demographic and tumor characteristics of patients with recurrence.

Statistical analysis

Differences between patients who had a recurrence and those who did not were assessed using the Student’s t-test for parametric data; the Mann-Whitney U test for non-parametric data; and the χ², the Fisher exact, and the Freeman-Halton exact tests for categorical data. Differences were considered significant at a p-value ≤ 0.05. Patients were compared with respect to age, duration of follow-up, risk factors, primary treatment, and adjuvant treatment. The same was done when comparing patients who were followed by observation and those who received adjuvant treatment. Three year disease-free and overall survival was calculated using the Kaplan-Meier method. IBM SPSS 20.0 (SPSS IBM, New York, NY, US) was used for statistical analysis of the data.

Results

Patients and treatment

In total, 81 patients with stage I, LVSI-positive EEC were included in this study (51 from the CCCS and 30 from the two hospitals in Nijmegen). Clinical and tumor characteristics of all included patients, as well as patients stratified according to adjuvant therapy, are shown in Table 1. Out of the 81 included patients, 32 (39.5%) were followed by observation only and 49 (60.5%) received adjuvant radiotherapy. The median time of follow-up was 48 months (range 5–134) for all patients and 50 months (range 12-134) for those not deceased. All patients underwent a hysterectomy and bilateral salpingo-oophorectomy. Additional lymphadenectomy was performed in 16 patients (19.8%) and as only patients with surgical stage I EEC were included, all nodes were shown to be disease free.

### Table 1. Demographic and tumor characteristics of all patients combined and patients grouped by adjuvant treatment.

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>No adjuvant therapy</th>
<th>Adjuvant radiotherapy</th>
<th>p (*)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patients</strong></td>
<td>81</td>
<td>32</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Mean age in years</td>
<td>67 (sd 9.1)</td>
<td>66 (sd 10.5)</td>
<td>68 (sd 7.9)</td>
<td>0.25</td>
</tr>
<tr>
<td>Median months follow-up</td>
<td>48 (5–134)</td>
<td>47 (5–113)</td>
<td>49 (8–134)</td>
<td>0.34</td>
</tr>
<tr>
<td><strong>Surgery</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laparotomy</td>
<td>55 (67.9%)</td>
<td>21 (65.6%)</td>
<td>34 (69.4%)</td>
<td>0.72</td>
</tr>
<tr>
<td>Minimally invasive</td>
<td>26 (32.1%)</td>
<td>11 (34.4%)</td>
<td>15 (30.6%)</td>
<td></td>
</tr>
<tr>
<td><strong>Lymphadenectomy</strong></td>
<td>16 (19.8%)</td>
<td>9 (28.1%)</td>
<td>7 (14.3%)</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>21 (25.9%)</td>
<td>11 (34.4%)</td>
<td>10 (20.4%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>40 (49.4%)</td>
<td>17 (53.1%)</td>
<td>23 (46.9%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>20 (24.7%)</td>
<td>4 (12.5%)</td>
<td>16 (32.7%)</td>
<td></td>
</tr>
<tr>
<td><strong>Myometrial invasion</strong></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>22 (27.2%)</td>
<td>20 (62.5%)</td>
<td>2 (4.1%)</td>
<td></td>
</tr>
<tr>
<td>≥50%</td>
<td>59 (72.8%)</td>
<td>12 (37.5%)</td>
<td>47 (95.9%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>15 (18.5%)</td>
<td>11 (34.4%)</td>
<td>4 (8.2%)</td>
<td></td>
</tr>
<tr>
<td>≥60 years</td>
<td>66 (81.5%)</td>
<td>21 (65.6%)</td>
<td>45 (91.8%)</td>
<td></td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>0–1</td>
<td>28 (34.6%)</td>
<td>26 (81.3%)</td>
<td>2 (4.1%)</td>
<td></td>
</tr>
<tr>
<td>2–3</td>
<td>53 (65.4%)</td>
<td>6 (18.7%)</td>
<td>47 (95.9%)</td>
<td></td>
</tr>
<tr>
<td><strong>Adjuvant treatment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VBT a</td>
<td>22 (27.2%)</td>
<td>-</td>
<td>22 (44.9%)</td>
<td></td>
</tr>
<tr>
<td>EBRT b</td>
<td>23 (28.4%)</td>
<td>-</td>
<td>23 (46.9%)</td>
<td></td>
</tr>
<tr>
<td>VBT + EBRT</td>
<td>4 (4.9%)</td>
<td>-</td>
<td>4 (8.2%)</td>
<td></td>
</tr>
<tr>
<td><strong>Recurrence</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18 (22.2%)</td>
<td>5 (15.6%)</td>
<td>13 (26.5%)</td>
<td>0.29</td>
</tr>
<tr>
<td>Local</td>
<td>4 (4.9%)</td>
<td>2 (6.3%)</td>
<td>2 (4.1%)</td>
<td>0.65</td>
</tr>
<tr>
<td>Regional</td>
<td>5 (6.2%)</td>
<td>-</td>
<td>5 (10.2%)</td>
<td>0.15</td>
</tr>
<tr>
<td>Distant</td>
<td>12 (14.8%)</td>
<td>3 (9.4%)</td>
<td>9 (18.4%)</td>
<td>0.35</td>
</tr>
<tr>
<td><strong>Death of disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>9 (11.1%)</td>
<td>1 (6.3%)</td>
<td>7 (14.3%)</td>
<td>0.31</td>
</tr>
</tbody>
</table>

a Vaginal brachytherapy, b External beam radiotherapy, *p*-values for Student’s t-test for parametric data, the Mann-Whitney U test for non-parametric data, and the χ², Fisher exact, and Freeman-Halton exact tests for categorical data
Treatment was according to the recommended guidelines in 26/28 (92.9%) patients with 0–1 risk factors who were followed by observation and 47/53 (88.7%) patients with 2–3 risk factors who received adjuvant radiotherapy. In total, 73/81 (90.1%) patients were treated according to the recommended guidelines. The reasons for the discrepancy between the actual treatment and the guidelines varied from patients or clinicians preference to the fact that some patients were not eligible for radiotherapy. Other than the presence of the three risk factors, there were no significant differences between patients being observed and those receiving adjuvant radiotherapy. Adjuvant radiotherapy consisted of VBT in 22 (44.9%) patients and EBRT+/-VBT in 27 (55.1%) patients.

Outcome
A total of 18 (22.2%) patients were diagnosed with recurrent disease. The recurrences had a local component in four (22.2%) patients, a regional component in five (27.8%) patients, and a distant component in 12 (66.7%) patients. This results in local, regional, and distant recurrence rates of 4.9%, 6.2%, and 14.8%, respectively. Nine patients (11.1%) died as a consequence of endometrial cancer, eight of whom had a distant recurrence. Characteristics of patients with and without recurrent disease are shown in Table 2. With respect to the primary treatment, there was no significant difference in the recurrence rate among patients that underwent open surgery compared to those who underwent minimally invasive surgery (p = 0.92), nor between patients who underwent a lymphadenectomy (with negative nodes) and those who did not undergo a lymphadenectomy (p = 0.62). Only one of the patients with grade I EEC in this study recurred. Consequently, the number of patients with grade 2 or 3 disease was significantly higher in the population of patients who had a recurrence compared to the patients who did not.

All but one of the recurrences were found in patients who received adjuvant treatment according to the guidelines. The overall and the distant recurrence rate in patients with 0–1 risk factors followed by observation (n=26) were 19.2% and 11.5%, respectively. In patients with 2–3 risk factors who received adjuvant radiotherapy (n=47) they were 25.5% and 17%. There were no differences with respect to the recurrence rate when comparing VBT to EBRT +/- VBT (data not shown).

The four year disease-specific and disease-free survival rates were 87% and 77%, respectively. They were 91% and 77%, respectively, for patients with 0–1 risk factors who were followed by observation, and 85% and 75% for those with 2–3 risk factors who received adjuvant radiotherapy.

### Table 2. Demographic, tumor, and treatment characteristics of patients with or without recurrent disease.

<table>
<thead>
<tr>
<th></th>
<th>Recurrence</th>
<th>No recurrence</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients (% of total)</td>
<td>18 (22.2%)</td>
<td>63 (77.8%)</td>
<td>0.30</td>
</tr>
<tr>
<td>Mean age in years</td>
<td>69 (sd 9)</td>
<td>66 (sd 9.2)</td>
<td>0.30</td>
</tr>
<tr>
<td>Median months to recurrence</td>
<td>19 (4–63)</td>
<td>-</td>
<td>0.78</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td></td>
<td>0.78</td>
</tr>
<tr>
<td>Laparotomy</td>
<td>13 (72.2%)</td>
<td>42 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>Minimally invasive</td>
<td>5 (27.8%)</td>
<td>21 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>Lymphadenectomy</td>
<td>4 (22.2%)</td>
<td>12 (19%)</td>
<td>0.75</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>1</td>
<td>1 (5.6%)</td>
<td>20 (31.7%)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>12 (66.7%)</td>
<td>28 (44.4%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>5 (27.8%)</td>
<td>15 (23.8%)</td>
<td></td>
</tr>
<tr>
<td>Myometrial invasion</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>&lt;50%</td>
<td>5 (27.8%)</td>
<td>17 (27%)</td>
<td></td>
</tr>
<tr>
<td>≥50%</td>
<td>13 (72.2%)</td>
<td>46 (73%)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>3 (16.7%)</td>
<td>12 (19%)</td>
<td></td>
</tr>
<tr>
<td>≥60 years</td>
<td>15 (83.3%)</td>
<td>51 (81%)</td>
<td></td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
<tr>
<td>0–1</td>
<td>6 (33.3%)</td>
<td>22 (34.9%)</td>
<td></td>
</tr>
<tr>
<td>2–3</td>
<td>12 (66.7%)</td>
<td>41 (65.1%)</td>
<td></td>
</tr>
<tr>
<td>Adjuvant treatment</td>
<td></td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>Observation</td>
<td>5 (27.8%)</td>
<td>27 (42.9%)</td>
<td></td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>13 (72.2%)</td>
<td>36 (57.1%)</td>
<td></td>
</tr>
<tr>
<td>VBT*</td>
<td>9 (50%)</td>
<td>13 (20.6%)</td>
<td>0.71</td>
</tr>
<tr>
<td>EBRT*+/-VBT</td>
<td>4 (22.2%)</td>
<td>23 (36.5%)</td>
<td></td>
</tr>
</tbody>
</table>

*p-Values for Student’s t-test for parametric data, the Mann-Whitney U test for non-parametric data, and the χ², Fisher exact, and Freeman-Halton exact tests for categorical data.
Lymphovascular space invasion and the treatment of stage I endometrioid endometrial cancer

The strength of this study is the inclusion of a large number (n = 81) of a very specific group of patients with stage I, LVSI-positive EEC. Most other studies include either a small number of patients with stage I, LVSI-positive EEC or include other histological types and higher-stage disease. Only in the study by Simpkins et al. was a large group of patients with LVSI-positive, early-stage endometrial cancer included. Moreover, none of the studies looked specifically at the impact of LVSI on the treatment of stage I EEC based on the number of present risk factors. The statistical power of our study is limited by the low event rate and the lack of a LVSI-negative control group. Moreover, there has been a selection bias because only patients with 2–3 risk factors were to receive adjuvant radiotherapy and very few of these patients were followed by observation alone, which is no surprise given the proven value of adjuvant radiotherapy in preventing locoregional recurrence. However, this selection bias does not interfere with the research questions. Finally, the fact that there was no central pathology review might be seen as a shortcoming which reduces the relevance of this study. However, while all previous studies had central review and a very detailed description of the LVSI present, this is not in line with clinical practice. With this study we wanted to demonstrate that the presence of LVSI, when assessed in daily practice, is of prognostic relevance. Criteria for the presence of LVSI were used by the primary pathologist in accordance with the known literature. This means there was no systematic assessment of for example retraction artifacts, pseudoinvasion, artificial vascular involvement and immunohistochemically stained slides. The impact of these aspects on our findings is unknown. But as it was shown that even LVSI diagnosed by the primary pathologist has prognostic importance in stage I EEC, the need to include these aspects in the assessment of the primary pathologist should be studied.

In conclusion, when LVSI is present in stage I EEC patients with 0–1 risk factors, adjuvant radiotherapy might prove to be beneficial. Moreover, in patients with LVSI and 2–3 risk factors a substantial overall and distant recurrence rate is observed, despite adjuvant radiotherapy. The use of systemic treatment in these patients should be investigated, possibly with the exception of patients with two risk factors and grade 1 disease, who showed a favorable outcome.

Discussion

In this retrospective study of 81 patients with stage I, LVSI-positive EEC, a recurrence rate of 22.2% was observed. The majority of these recurrences had a distant component, especially in patients with 2–3 risk factors. In the presence of 0–1 risk factors and after observation the recurrence rate was 19.2%, while it was 25.5% in patients with 2–3 risk factors, despite adjuvant radiotherapy. Only one of the patients with grade 1 histology had recurrent disease. The current practice of treating patients with two or three risk factors with adjuvant radiotherapy is based on data from multiple large studies. In the PORTEC I trial, 714 patients with stage I endometrial cancer with 0–3 risk factors were randomly assigned to observation or EBRT. Keys et al. performed a comparable study in patients with FIGO 1988 stage Ib, Ic, and Ila endometrial cancer who underwent a lymphadenectomy. These studies showed that, in patients that did not receive adjuvant radiotherapy, the overall recurrence rate was 15.3–16.7%, and the distant recurrence rate was 5.6–6.4%. In patients that received EBRT, these rates were 6.8–9.9% and 5.3–6.8%, respectively. Both studies showed that adjuvant EBRT reduced locoregional recurrence in stage I EEC patients with two or three risk factors. Subsequently, EBRT was replaced by VBT in The Netherlands, since the PORTEC II trial showed that this was equally effective in preventing locoregional recurrence, but with a significantly reduced morbidity.

When comparing these results to the current study, it must be noted that in the current study patients were not randomized, but treated according to the presence of risk factors, which leads to a selection bias. Our data show that observation might not be sufficient when LVSI is present next to 0–1 risk factors, as they had a much higher recurrence rate than patients with comparable risk factors and treatment who were not selected based on the presence of LVSI, as assessed by the primary pathologist. Moreover, the overall (25.5%) and distant (12%) recurrence rate in patients with LVSI-positive EEC next to 2–3 risk factors who received adjuvant radiotherapy were much higher than those of patients with comparable risk factors and treatment who were not selected based on the presence of LVSI, as assessed by the primary pathologist. In our study, all patients who died of endometrial cancer had recurrent disease and all but one had a distant component. A group of patients that did exceptionally well were those with grade 1 EEC. Even in the presence of the other two risk factors, only one of these patients recurred.

It is hypothesized that patients with stage I, LVSI-positive EEC and two or three risk factors may have a high risk of distant recurrence, which would not be reduced by any form of adjuvant radiotherapy. Compared to recurrence rates in studies of patients with stage I endometrial cancer who were not selected based on the presence of LVSI, our results show that LVSI in stage I EEC appears to be a predictor of distant recurrence.

Several studies have shown LVSI to be an independent predictor of poor prognosis and an indication of early distant spread in patients with stage I EEC. While these studies all had central pathology review done, the current study included patients who had LVSI according to the primary pathologist. Interestingly, the findings of these studies are in line with our findings in the clinical practice setting. It is therefore worthwhile to investigate the role of adjuvant treatment in patients with LVSI and 0–1 risk factors and adjuvant systemic treatment in with LVSI and 2–3 risk factors, which has previously been proposed. An exception may be made for patients with grade 1 disease and two risk factors, who seem to have an excellent prognosis with radiotherapy alone.
Lymphovascular space invasion and the treatment of stage I endometrioid endometrial cancer


References

13. Cohn DE, Horowitz NS, Mutch DG et al. Should the presence of lymphvascular space involvement be used to assign patients to adjuvant therapy following hysterectomy for unstaged endometrial cancer? Gynecol Oncol 2002;87:243-246.

CHAPTER 5

Lymphovascular space invasion and the treatment of stage I endometrioid endometrial cancer
CHAPTER 6

REPRODUCIBILITY OF MEASUREMENT OF MYOMETRIAL INVASION IN ENDOMETRIAL CARCINOMA

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CHAPTER 6

Reproducibility of measurement of myometrial invasion in endometrial carcinoma

Abstract

Background
Myometrial invasion (MI) as a percentage (%MI), categorized into <50% or ≥50%, is an important predictor of prognosis in endometrial carcinoma. Recent studies suggest that tumor free distance (TFD) to serosa and the absolute depth of invasion (DOI) might be stronger predictors of prognosis. Although reproducibility is important in clinical practice for patient prognostication and treatment, reproducibility of these methods for the measurement of MI is largely unknown.

Materials and Methods
One or two slides from 50 patients with FIGO stage I endometrioid endometrial carcinoma were viewed by seven gynecological pathologists, who were requested to measure %MI, TFD and DOI. We categorized %MI as <50% (including no MI) or ≥50%, TFD as ≤1.75mm or >1.75mm (including no MI), ≤7mm or >7mm (including no MI), and ≤10mm or >10mm (including no MI) and DOI as <4mm (including no MI) or ≥4mm. Light’s kappa for multi-rater agreement was calculated.

Results
The %MI, TFD and DOI could be measured in 88%, 83% and 79% of cases, respectively. Kappa was 0.75 for %MI, 0.77, 0.73 and 0.69 respectively for TFD with cut-offs of 1.75mm, 7mm and 10mm, and 0.59 for DOI.

Conclusion
Pathologists reach substantial agreement when measuring %MI and TFD, and moderate agreement when measuring DOI. The %MI can be measured in more cases than TFD and DOI. This supports the use of %MI in daily clinical practice, but future studies should compare %MI and TFD more extensively, including inter-observer variability.

Introduction
Endometrial carcinoma is the most common gynecological malignancy in developed countries, and its incidence is increasing.1, 2 Primary treatment of endometrial carcinoma consists predominantly of hysterectomy and bilateral salpingo-oophorectomy. Additional staging is typically undertaken for non-endometrioid and high grade endometrioid carcinomas, and when tumor stage is advanced. Most patients are diagnosed with FIGO stage I disease and low grade (grade 1 or 2) endometrioid histology and have a good prognosis.3 After primary surgery, the decision to administer adjuvant radiotherapy to prevent locoregional recurrences relies on the presence of predictors of poor outcome, such as high tumor grade, lymphovascular space invasion, deep myometrial invasion (MI) and patient age >60 years.4 Traditionally, the percentage of myometrial invasion (%MI), categorized as <50% or ≥50%, is one of the parameters used in the determination of the need for adjuvant radiotherapy.3-5 However, more recently, two other methods of measuring MI been proposed: tumor-free distance to serosa (TFD, the distance in millimeters between the deepest point of invasion and the serosa) and absolute depth of invasion (DOI, the distance in millimeters between the endometrial/myometrial junction and the deepest point of MI). A study comparing TFD and DOI and another study comparing %MI, TFD and DOI concluded that TFD is superior in predicting disease extension as well as outcome.6, 7 Two comparable studies, on the other hand, have shown that DOI is superior in predicting nodal involvement, recurrent disease, and disease related mortality.8, 9 One study comparing TFD and DOI concluded that DOI is a stronger predictor of outcome, but TFD is easier to measure, but kappa statistics were not reported.10 If measurement of TFD or DOI is superior to that of %MI, it might improve identification of high risk patients and individualization of adjuvant treatment. However, reproducibility of these measurements is important to support their prognostic value in daily clinical practice. Because all previous studies were single-center studies and measurements were performed by a limited number of pathologists, reproducibility of TFD and DOI is currently unknown. Some studies have reported on reproducibility of %MI, but only one study included kappa statistics with a kappa value of 0.83.11 The aim of our study was to assess inter-pathologist reproducibility of %MI, TFD and DOI.

Materials and Methods

Included cases
Slides from patients treated for stage I endometrioid endometrial carcinoma at the Radboud university medical center (Radboudumc), Nijmegen, the Netherlands, between January 1999 and December 2009 were reviewed by a gynecological pathologist (JB). All pathologists collaborating in the European Network for Individualized Treatment of Endometrial Cancer (ENITEC) were invited to participate in this study, and seven expressed their interest. The sample size calculation was based on previous studies assessing reproducibility of the %MI measurement, as the kappa for TFD and DOI measurements is unknown.11, 13 Based on a kappa of 0.8 for %MI, we calculated that 50 cases should be included in order to have 90% assurance that the two-sided 95% confidence interval would be no more than 0.1.13, 15
Myometrial invasion measurement
All cases were assessed independently by seven expert gynecological pathologists who work in large referral centers (AW, KV, CB, SG, BD, WGM and GT), using the same set of slides. Scoring was performed according to the instructions shown in Figure 1.

Statistical analysis
For statistical analysis, %MI was categorized as <50% (including no invasion) or ≥50%. Reproducibility of TFD was calculated for all three previously reported cut-off values: ≤1.75mm or >1.75mm (including no invasion), ≤7mm or >7mm (including no invasion), and ≤10mm or >10mm (including no invasion). Only one earlier study described a cut-off for DOI, which was categorized as <4mm (including no invasion) or ≥4mm. Light’s Kappa for multi-rater agreement was calculated for categorized %MI, TFD and DOI scores, bootstrapped (1000 runs) and 95% confidence intervals were calculated. Missing scores were excluded in a pairwise fashion. Kappa was categorized into slight (0.01-0.20), moderate (0.41-0.60) substantial (0.61-0.80) or almost perfect (0.81-0.99) agreement. R statistical software was used to perform the calculations.

Results
Myometrial invasion measurement
The results of the measurements are shown in Table 1. As there were 50 cases, measured by seven pathologists, a total of 350 measurements were possible per method. In 95% of the 350 measurements the pathologists were able to assess whether or not there was MI, ranging from 82% to 100% of the 50 measurements per pathologist. For the %MI, TFD and DOI measurements this was 88% (64-98%), 83% (78-88%) and 79% (24-100%), respectively. For the presence of MI and the measurement of TFD the median number of measurements per case available to calculate Light’s multi-rater kappa, was 7, for the %MI measurement this was 6.5 and for the DOI measurement 6. Almost all cases had two or more measurements per method, allowing calculation of a kappa value. In four cases, it was impossible to calculate the kappa value for the TFD measurement, because no or only one measurement was performed.

The pathologists reported MI in 76% of the measurements with a kappa of 0.63, and ≥50% myometrial invasion in 28% of the measurements with a kappa of 0.75. The median TFD was 7mm (range 0.1mm to 25mm), and ≥4mm in 43% of the measurements, with a kappa of 0.77, 0.73, and 0.69, respectively. Median DOI was 5mm (range 0.1mm to 25mm), and ≥4mm in 43% of the measurements, with a kappa of 0.59. Examples of cases with good or poor reproducibility are shown in Figure 3.

Difficulties in measuring myometrial invasion
Table 2 shows how the pathologists rated the difficulty of the three measurements relative to the percentage of cases measured. For the %MI the number of measurements performed with a reported difficulty was 211, for TFD this was 201 and for DOI 189. The measurements were perceived to be easy in 54% for %MI, 72% for TFD and 44% for DOI; they were moderate in 30% for %MI, in 21% for TFD and in 43% for DOI and difficult in 16% for %MI, in 7% for TFD and in 33% for DOI. For all three measurements the kappa value of the perceived difficulty was smaller than 0.1.
Table 1. Characteristics and reproducibility of myometrial invasion measurements

<table>
<thead>
<tr>
<th></th>
<th>Number of cases</th>
<th>Measurements possible per method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
<td>350</td>
</tr>
</tbody>
</table>

Is there myometrial invasion?

<table>
<thead>
<tr>
<th></th>
<th>Measurable</th>
<th>Range (95% - 100%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>79 (24%)</td>
<td>82-100%</td>
</tr>
<tr>
<td>Yes</td>
<td>252 (76%)</td>
<td>331 (95%)</td>
</tr>
</tbody>
</table>

Kappa (95% confidence interval)

|                              | 0.63 (0.5-0.78) |

Table 1. Characteristics and reproducibility of myometrial invasion measurements (continued)

<table>
<thead>
<tr>
<th></th>
<th>Measurable</th>
<th>Range (64-98%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median measurements per case</td>
<td>7</td>
<td>3-7</td>
</tr>
</tbody>
</table>

<50%                          | 220 (72%)       | 87 (28%)            |
≥50%                          | 87 (28%)        | 87 (28%)            |

Kappa (95% confidence interval)

|                              | 0.75 (0.60-0.87) |

Table 2. Difficulty of the myometrial invasion measurements of cases with myometrial invasion

<table>
<thead>
<tr>
<th></th>
<th>MI</th>
<th>TFD</th>
<th>DOI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage measurable</td>
<td>88%</td>
<td>83%</td>
<td>79%</td>
</tr>
<tr>
<td>Number of measurements with MI and known difficulty</td>
<td>211</td>
<td>201</td>
<td>189</td>
</tr>
</tbody>
</table>

Per cent difficulty (range)

<table>
<thead>
<tr>
<th></th>
<th>Easy</th>
<th>Moderate</th>
<th>Hard</th>
</tr>
</thead>
<tbody>
<tr>
<td>54% (21-77)</td>
<td>72% (59-93)</td>
<td>24% (0-39)</td>
<td></td>
</tr>
<tr>
<td>30% (23-54)</td>
<td>43% (28-68)</td>
<td>33% (24-64)</td>
<td></td>
</tr>
<tr>
<td>16% (0-29)</td>
<td>7% (0-23)</td>
<td>33% (24-64)</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3. Several examples of slides scored in this study. Slides A and B were scored with little agreement concerning the DOI, and it was commented that it was hard to distinguish the endometrial/myometrial junction. Slides C and D on the other hand were scored with perfect agreement for all measurements.
Discussion

This study shows that gynecological pathologists reach substantial agreement when measuring %MI and TFD and moderate agreement when measuring DOI. Pathologists found measuring DOI more difficult than measuring %MI and TFD.

Myometrial invasion measurement

It is widely accepted that high tumor grade, non-endometrioid histology, lymphovascular space invasion, and deep myometrial invasion are predictors of poor prognosis in endometrial carcinoma and important parameters to decide on individualized treatment. Although many studies reported on reproducibility of tumor grading and histological typing, reports on reproducibility of assessment of MI and lymphovascular space invasion is limited. Jacques et al. and Ali et al. reported discrepancies between MI measured by the pathologist who reported the case and a reviewing pathologist, but without calculating the kappa value. This study reported a kappa value of 0.83 for two pathologists measuring myometrial invasion in 177 cases of endometrial cancer. Other studies determined the percentage of agreement between pathologists when measuring MI, but without calculating the kappa value. Ali et al. reported a discrepancy between the original %MI and the specialist reviewer %MI measurement in 12% of endometrial cancer cases. Jacques et al. reported discrepancies between MI measured by the pathologist who reported the case and a reviewing pathologist in 31.5% of cases. In that study, MI was categorized as not present, less than one third, and equal to or more than one third and discrepancies most commonly resulted in upstaging from no to less than one third MI. A comparable study by Chafe et al. described differences between the original pathology report and a review in 34% of 226 cases, but the percentage of cases with discrepancies in the categorization of %MI was not separately mentioned.

One study reported a kappa value of 0.83 for two pathologists measuring myometrial invasion in 177 cases of endometrial cancer. Other studies determined the percentage of agreement between pathologists when measuring MI, but without calculating the kappa value. Ali et al. reported a discrepancy between the original %MI and the specialist reviewer %MI measurement in 12% of endometrial cancer cases. Jacques et al. reported discrepancies between MI measured by the pathologist who reported the case and a reviewing pathologist in 31.5% of cases. In that study, MI was categorized as not present, less than one third, and equal to or more than one third and discrepancies most commonly resulted in upstaging from no to less than one third MI. A comparable study by Chafe et al. described differences between the original pathology report and a review in 34% of 226 cases, but the percentage of cases with discrepancies in the categorization of %MI was not separately mentioned.

Lindauer et al. assessed the prognostic value of the TFD measurement in 153 cases, but the reproducibility between two pathologists was only determined in five cases. We show that gynecological pathologists reach substantial agreement with respect to the presence of MI and the measurement of %MI and TFD, and moderate agreement with respect to the measurement of DOI. Interestingly, for both %MI and TFD reproducibility was better than for assessment of the presence of MI. This is in line with the studies of Jacques et al. and Ali et al., who found most discrepancies between cases with no MI and cases with superficial MI. Because the revised 2009 FIGO staging system does not differentiate between no MI and superficial MI, this finding does not affect staging and has been shown to be of no clinical significance.

In comparing agreement between pathologists with respect to %MI, TFD and DOI measurements, the best agreement was reached when measuring TFD with a cut-off of 1.75mm. This was closely followed by the %MI measurement, and TFD with cut-offs of 7 and 10mm. The most relevant cut-off for TFD needs to be determined, but the differences in reproducibility are small and probably without clinical importance, as are the differences between the reproducibility of the %MI and TFD measurements.

Measuring MI is more difficult in the presence of an irregular endometrial/myometrial junction, of a polypoid tumor or of adenomyosis, and also when the pattern of MI is unusual, such as diffusely infiltrative, or microcystic, elongated and fragmented (MELF). Because these are not yet regularly reported in daily clinical practice, this was beyond the scope of our study. However, it would be interesting to analyze the effect of different invasion patterns on the reproducibility of MI measurements.

Difficulties in measuring myometrial invasion

Pathologists found measurement of DOI more difficult than that of %MI and TFD, which is reflected in the lower average reproducibility of these measurements. However, perception of difficulty per case varied widely between pathologists, as reflected in a low kappa value. Comments of the participating pathologists indicated that sampling and sectioning of the endometrium and myometrium varies between institutions. Nonetheless, moderate to substantial agreement was obtained. However, further standardization of the guidelines might decrease inter-observer variability of these three measurements, which might improve their prognostic value. Possible improvements might be 1) standardization of the method to open the uterus as well as the location and direction in which the tissue samples are taken relative to the tumor, the myometrium, and the serosa, 2) photographic documentation of the specimen and 3) standardization of identification of the deepest point of invasion and the definition of the endometrial/myometrial junction.

Strengths and weaknesses of this study

This is the largest study assessing inter-pathologist reproducibility of MI measurement and the first assessing the reproducibility of the TFD and DOI measurement. Although the 95% confidence intervals of the kappa values were slightly wider than expected, in part due to the fact that not all measurements were performed, they remained acceptable. For a study on reproducibility in daily practice, our use of slides from daily practice rather than cases optimized for measurability makes the results relevant for daily practice. A limitation is that these slides were from one institution, while significant differences exist between institutions regarding sectioning and measuring procedures. Standardization of guidelines might further improve inter-observer reproducibility.

Conclusions

We show that gynecological pathologists reach substantial agreement when measuring %MI and TFD, but only moderate agreement when measuring DOI. Measurement of %MI and TFD was perceived to be easier than DOI measurement and %MI was the measure most often successful. This supports use of %MI but also of TFD. These two parameters merit further study, always by at least two pathologists as this will provide insight in inter-observer variability. Guidelines for gross examination, sectioning, and measuring of MI should be standardized to improve the inter-observer variability and improve on prognostic value.
CHAPTER 6

Reproducibility of measurement of myometrial invasion in endometrial carcinoma


References


CHAPTER 7

L1CAM EXPRESSION IN ENDOMETRIAL CARCINOMAS:
AN ENITEC COLLABORATION STUDY


CHAPTER 7

L1CAM expression in endometrial carcinomas: an ENITEC collaboration study

Abstract

Background
Identification of aggressive endometrioid endometrial carcinomas (EEC) and non-endometrioid carcinomas (NEEC) is essential to improve outcome. L1 cell adhesion molecule (L1CAM) expression is a strong prognostic marker in stage I EECs, but less is known about L1CAM expression in advanced-stage EECs, and NEECs. This study analyzes L1CAM expression in a clinically representative cohort of endometrial carcinomas.

Materials and Methods
The expression of L1CAM was immunohistochemically determined in 1199 endometrial carcinomas, treated at one of the European Network for Individualized Treatment of Endometrial Cancer (ENITEC) centers. Staining was considered positive when >10% of the tumor cells expressed L1CAM. The association between L1CAM expression and several clinicopathological characteristics and disease outcome was calculated.

Results
In all, L1CAM was expressed in 10% of the 935 stage I EECs, 18% of the 160 advanced-stage EECs, and 75% of the 104 NEECs. The expression of L1CAM was associated with advanced stage, nodal involvement, high tumor grade, non-endometrioid histology, lymphovascular space invasion, and distant recurrences in all cases, and with reduced survival in the EECs, but not in the NEECs.

Conclusion
The expression of L1CAM is a strong predictor of poor outcome in EECs, but not NEECs. It is strongly associated with non-endometrioid histology and distant spread, and could improve the postoperative selection of high-risk endometrial carcinomas. The value of L1CAM expression in the preoperative selection of high-risk endometrial carcinomas should be studied.

Introduction
Endometrial carcinoma is the most common gynecological malignancy in developed countries. These carcinomas can be histologically classified as either endometrioid endometrial carcinomas (EECs) or non-endometrioid endometrial carcinomas (NEECs). In general, EECs have a favorable prognosis, and are characterized by expression of the estrogen en progesterone receptors, microsatellite instability and PTEN, KRAS, PIK3CA, and CTNNB1 mutations. The most common NEECs have serous or clear cell histology, and a worse prognosis. They are characterized by TP53 mutations, and PTEN and PIK3CA mutations, respectively. Less common carcinomas with non-endometrioid histology are those with undifferentiated histology, characterized by microsatellite instability, those with mucinous histology, with a prognosis and molecular characterization similar to EECs, and the carcinosarcomas. However, a substantial number of endometrial carcinomas do not fit within this dualistic model, and have mixed histology or hybrid molecular and histological characteristics, which makes the diagnosis challenging.

More recently a new subdivision into four subgroups, based on the molecular profile, was proposed by The Cancer Genome Atlas Research network. However, these data have not been incorporated in clinical practice yet. In addition, several immunohistochemical markers have been shown to be associated with poor outcome. Expression of the transmembrane L1 cell adhesion molecule (L1CAM) seems to be one of the most powerful ones described to date. The L1CAM plays an important role in neurogenesis, but has been associated with poor outcome in various cancer types. Two large studies have shown a strong association between L1CAM expression in stage I EEC and poor disease outcome. However, there was a wide variation both in the percentage of cases expressing L1CAM, and in the strength of the association between L1CAM and disease outcome found by these studies. More recently, several smaller studies have highlighted the association between L1CAM expression and poor disease outcome in advanced-stage EECs, and NEECs, as well as a strong association between L1CAM expression and non-endometrioid histology. However, these studies have limited clinical applicability because neither of these studies analyzed the subgroups separately, and the number of included advanced-stage EECs and NEECs is still limited. In addition, Dellinger et al. used mRNA L1CAM expression with a different cut-off than the other studies, which limits the comparability.

The aim of the current study is therefore to analyze the value of immunohistochemical L1CAM expression in a large, clinically representative cohort of endometrial carcinomas, including substantial numbers of all histological types and FIGO stages.
CHAPTER 7

L1CAM expression in endometrial carcinomas: an ENITEC collaboration study

Materials and Methods

Patients
This study was performed within the European Network for Individualized Treatment of Endometrial Cancer (ENITEC), a European Society of Gynecological Oncology (ESGO) consortium aiming to improve and individualize treatment of women with uterine cancers by sharing expertise. All ENITEC members were invited to participate in this study and to include patients treated for stage I EEC (a maximum of 150 cases per center), stage II-IV EEC, or NEEC. Cases with any non-endometrioid component were included in the NEEC group, except for the mucinous carcinomas, that were included in the EEC groups as their characteristics and prognosis are similar to that of endometrioid carcinomas. Only cases diagnosed by an expert gynecological pathologist, with complete data on treatment and pathology, and at least 36 months of follow-up were included. Clinical and pathological data were recorded from the patient files into a database, including patient age, date of diagnosis, surgical treatment (including lymphadenectomy and omentectomy), tumor histology and grade, myometrial invasion, cervical invasion, LVSI, FIGO stage, adjuvant treatment (including radiotherapy, chemotherapy and chemoradiation), residual disease, recurrent disease, and death.

Tissue and staining
One representative slide was selected per case. Blank 4µm sections, cut from the corresponding formalin fixed, paraffin embedded tissue blocks, on Superfrost slides were sent to the Radboud university medical center. A hematoxylin and eosin and an immunohistochemically stained L1CAM slide were made for every case. Immunohistochemical staining was performed in semiautomatic staining devices using an optimized version of the previously described staining protocol. In short, after EDTA antigen retrieval and blocking of endogenous peroxidase with hydrogen peroxide, slides were incubated with 1:100 diluted L1CAM antibody (purified anti-CD171 (L1) antibody clone 14.10, Biolegend, San Diego, CA, US). They were subsequently incubated with PowerVision+ Poly-HRP and visualized with PowerVision DAB substrate solution (Leica Biosystems, Buffalo Grove, IL, US). Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted. Staining of the nerves was used as internal positive control.

Scoring
All slides were scored twice, by independent pathologists. First by N. Visser or K. van de Vijver, and subsequently by M. Santacana, P. Bronsert or J. Bulten. They were blinded for clinical and pathological data, as well as each other’s scores. They were asked to score the number of stained tumor cells as 0%, 1-10% 11-50% or 51-100%, and cases were considered to express L1CAM when one or both pathologists considered >10% of the tumor cells to be stained, in accordance with the previously described cutoff. In short, after EDTA antigen retrieval and blocking of endogenous peroxidase with hydrogen peroxide, slides were incubated with 1:100 diluted L1CAM antibody (purified anti-CD171 (L1) antibody clone 14.10, Biolegend, San Diego, CA, US). They were subsequently incubated with PowerVision+ Poly-HRP and visualized with PowerVision DAB substrate solution (Leica Biosystems, Buffalo Grove, IL, US). Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted. Staining of the nerves was used as internal positive control.

Statistical analysis
Clinicopathological differences between L1CAM positive and negative cases were compared using the χ² and Fisher exact tests for categorical and the Mann-Whitney U-test for continuous variables, and corresponding p-values are shown in Tables 1-4. The association between L1CAM expression and other known risk factors expressed as an odds ratio (OR) and corresponding 95% confidence interval (95% CI), was calculated using univariate logistic regression analysis. These risk factors were deep myometrial invasion, patient age over 60 years, LVSI, non-endometrioid (except mucinous) histology, advanced FIGO stage, and nodal involvement.

Kaplan-Meier curves of 10-year disease-free and overall survival were generated for the stage I EEC, stage II-IV EEC, and NEEC subgroups. The corresponding hazard ratio (HR) and 95% CI was calculated using Cox regression analysis. For stage I EECs a multivariate analysis was performed including covariates which were significantly associated with outcome in the univariate analysis. Sample size calculation accounted only for multivariate Cox regression analysis of the stage I EECs.

Statistical differences were considered significant at a two-sided p-value ≤0.05. SPSS version 22 (SPSS IBM, New York, NY, US) statistical software was used to perform the statistical analyses.

Ethical approval
The study was approved by the institutional review board (IRB) of all participating centers.

Results

L1CAM in endometrial carcinomas
There were 1199 cases included from ten European centers, including 935 stage I EECs, 160 stage II-IV EECs and 104 NEECs. L1CAM was expressed in 200 (17%) cases, and was scored with a κ of 0.82. Table 1 shows demographic and tumor characteristics of all cases and a comparison between the L1CAM-negative and -positive cases. These two groups were significantly different concerning demographics, treatment, tumor characteristics and disease outcome.

In univariate regression analysis, L1CAM expression was significantly associated with advanced stage (OR 5.1, 95% CI 3.5-7.3), nodal involvement (OR 5.0, 95% CI 3.2-7.7), and non-endometrioid histology (OR 24.0, 95% CI 14.8-38.8).
Table 1. Comparison of the clinical and pathologic characteristics and disease outcome of all included carcinomas with respect to the L1CAM expression.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>All (1199)</th>
<th>L1CAM- (999, 83%)</th>
<th>L1CAM+ (200, 17%)</th>
<th>p'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (years)</td>
<td>64 (range 31-93)</td>
<td>63 (range 31-93)</td>
<td>69 (range 39-93)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Median follow-up (months)</td>
<td>62 (range 0-229)</td>
<td>64 (range 0-229)</td>
<td>50 (range 0-185)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphadenectomy</td>
<td>795 (66%)</td>
<td>645 (65%)</td>
<td>150 (75%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>563 (47%)</td>
<td>467 (47%)</td>
<td>96 (48%)</td>
<td>0.86</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>123 (10%)</td>
<td>72 (7%)</td>
<td>51 (26%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>965 (80%)</td>
<td>849 (85%)</td>
<td>116 (58%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>II</td>
<td>74 (6%)</td>
<td>58 (6%)</td>
<td>16 (8%)</td>
<td>0.05</td>
</tr>
<tr>
<td>III</td>
<td>125 (10%)</td>
<td>76 (8%)</td>
<td>49 (25%)</td>
<td>0.05</td>
</tr>
<tr>
<td>IV</td>
<td>35 (3%)</td>
<td>16 (2%)</td>
<td>19 (10%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endometrioid</td>
<td>1095 (91%)</td>
<td>973 (97%)</td>
<td>122 (61%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Non-endometrioid</td>
<td>104 (9%)</td>
<td>26 (3%)</td>
<td>78 (39%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>467 (39%)</td>
<td>441 (44%)</td>
<td>26 (13%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2</td>
<td>474 (40%)</td>
<td>417 (42%)</td>
<td>57 (29%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3</td>
<td>258 (22%)</td>
<td>141 (14%)</td>
<td>117 (59%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Myometrial invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1/2</td>
<td>746 (62%)</td>
<td>656 (66%)</td>
<td>90 (45%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>≥1/2</td>
<td>453 (38%)</td>
<td>343 (34%)</td>
<td>110 (55%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LVSI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>813 (83%)</td>
<td>723 (88%)</td>
<td>90 (60%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Yes</td>
<td>162 (17%)</td>
<td>101 (12%)</td>
<td>61 (40%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Unknown</td>
<td>224</td>
<td>175</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual disease</td>
<td>40 (3%)</td>
<td>15 (2%)</td>
<td>25 (12.5%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Recurrence</td>
<td>154 (13%)</td>
<td>100 (10%)</td>
<td>58 (33%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Locoregional</td>
<td>76 (7%)</td>
<td>57 (6%)</td>
<td>19 (11%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Distant</td>
<td>98 (8%)</td>
<td>53 (5%)</td>
<td>45 (26%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Deceased</td>
<td>171 (14%)</td>
<td>104 (10%)</td>
<td>67 (34%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Endometrial carcinoma</td>
<td>99 (8%)</td>
<td>48 (5%)</td>
<td>51 (26%)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*Median follow-up including deceased patients

L1CAM expression in endometrial carcinomas

Patient and tumor characteristics of all stage I EEC cases (n = 935, including four mucinous carcinomas) with respect to L1CAM expression are shown in Table 2. L1CAM was expressed in 93 (10%) cases. These patients were older, had a higher tumor grade and LVSI, and more often presented with distant recurrence and disease-related mortality.

In univariate regression analysis, L1CAM expression was significantly associated with grade 3 histology (OR 4.1, 95% CI 2.5-6.8) and LVSI (OR 2.9, 95% CI 1.5-5.6), but not with deep myometrial invasion.

Table 2. Comparison of the clinical and pathologic characteristics as well as disease outcome of stage I endometrioid endometrial carcinomas with respect to L1CAM expression.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>All (935)</th>
<th>L1CAM- (842, 90%)</th>
<th>L1CAM+ (93, 10%)</th>
<th>p'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (years)</td>
<td>63 (range 32-93)</td>
<td>63 (range 32-91)</td>
<td>67 (range 39-93)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Median follow-up (months)</td>
<td>64 (range 1-210)</td>
<td>65 (range 1-210)</td>
<td>55 (range 6-185)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphadenectomy</td>
<td>586 (63%)</td>
<td>519 (62%)</td>
<td>67 (72%)</td>
<td>0.05</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>400 (43%)</td>
<td>359 (43%)</td>
<td>41 (44%)</td>
<td>0.79</td>
</tr>
<tr>
<td>VBT</td>
<td>206 (22%)</td>
<td>189 (23%)</td>
<td>17 (18%)</td>
<td></td>
</tr>
<tr>
<td>EBRT+/− VBT</td>
<td>190 (20%)</td>
<td>166 (20%)</td>
<td>24 (26%)</td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>36 (4%)</td>
<td>31 (4%)</td>
<td>5 (5%)</td>
<td></td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>442 (47%)</td>
<td>418 (50%)</td>
<td>24 (26%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>2</td>
<td>389 (42%)</td>
<td>348 (41%)</td>
<td>41 (44%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>3</td>
<td>104 (11%)</td>
<td>76 (9%)</td>
<td>28 (30%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Myometrial invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1/2</td>
<td>664 (71%)</td>
<td>604 (72%)</td>
<td>60 (65%)</td>
<td>0.15</td>
</tr>
<tr>
<td>≥1/2</td>
<td>271 (29%)</td>
<td>238 (28%)</td>
<td>33 (36%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LVSI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>703 (91%)</td>
<td>645 (92%)</td>
<td>58 (81%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Yes</td>
<td>67 (9%)</td>
<td>53 (8%)</td>
<td>14 (19%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Unknown</td>
<td>165</td>
<td>144</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recurrence</td>
<td>85 (9%)</td>
<td>66 (8%)</td>
<td>19 (20%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Locoregional</td>
<td>48 (5%)</td>
<td>41 (5%)</td>
<td>7 (8%)</td>
<td>0.27</td>
</tr>
<tr>
<td>Distant</td>
<td>42 (5%)</td>
<td>29 (3%)</td>
<td>13 (14%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Deceased</td>
<td>88 (9%)</td>
<td>69 (8%)</td>
<td>19 (20%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Endometrial carcinoma</td>
<td>37 (4%)</td>
<td>26 (3%)</td>
<td>11 (12%)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*Median follow-up including deceased patients

Lymphovascular space invasion, p-value for the Mann-Whitney U test for continuous variables, and Chi square test for categorical variables.
**L1CAM in advanced-stage endometrioid endometrial carcinomas**

Patient and tumor characteristics of all advanced-stage EEC cases (n = 160, including five mucinous carcinomas) with respect to L1CAM expression are shown in Table 3. L1CAM expression was present in 28 (18%) cases. These cases had a higher tumor grade, a more advanced FIGO stage, and more often presented with distant recurrence and disease-related mortality.

In univariate regression analysis, L1CAM expression was significantly associated with the presence of nodal disease (OR 4.1, 95% CI 1.5-11.5) and LVSI (OR 3.0, 95% CI 1.1-8.0).

**Table 3.** Comparison of the clinical and pathologic characteristics as well as disease outcome of advanced stage endometrioid endometrial carcinoma cases with respect to L1CAM expression.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>All</th>
<th>L1CAM-</th>
<th>L1CAM+</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>160</td>
<td>131 (82%)</td>
<td>29 (18%)</td>
<td></td>
</tr>
<tr>
<td>Median age (years)</td>
<td>64 (range 37-93)</td>
<td>64 (range 37-93)</td>
<td>68 (range 47-84)</td>
<td>0.40</td>
</tr>
<tr>
<td>Median follow-up (months)</td>
<td>55 (range 1-227)</td>
<td>58 (range 3-227)</td>
<td>37 (range 1-106)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphadenectomy</td>
<td>122 (76%)</td>
<td>103 (79%)</td>
<td>19 (65%)</td>
<td>0.13</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>112 (80%)</td>
<td>95 (73%)</td>
<td>17 (59%)</td>
<td>0.14</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>41 (26%)</td>
<td>31 (24%)</td>
<td>10 (36%)</td>
<td>0.23</td>
</tr>
<tr>
<td>Chemoradiotherapy</td>
<td>7 (5%)</td>
<td>6 (5%)</td>
<td>1 (4%)</td>
<td>1.00</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>59 (37%)</td>
<td>54 (41%)</td>
<td>5 (28%)</td>
<td>0.02</td>
</tr>
<tr>
<td>II</td>
<td>83 (52%)</td>
<td>62 (47%)</td>
<td>21 (72%)</td>
<td>0.02</td>
</tr>
<tr>
<td>IV</td>
<td>18 (12%)</td>
<td>15 (12%)</td>
<td>3 (11%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>25 (16%)</td>
<td>23 (18%)</td>
<td>2 (7%)</td>
<td>0.20</td>
</tr>
<tr>
<td>2</td>
<td>82 (51%)</td>
<td>68 (52%)</td>
<td>14 (48%)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>53 (33%)</td>
<td>40 (31%)</td>
<td>13 (45%)</td>
<td></td>
</tr>
<tr>
<td>Myometrial invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1/2</td>
<td>43 (27%)</td>
<td>39 (30%)</td>
<td>4 (14%)</td>
<td>0.11</td>
</tr>
<tr>
<td>≥1/2</td>
<td>117 (73%)</td>
<td>92 (70%)</td>
<td>25 (86%)</td>
<td></td>
</tr>
<tr>
<td>LVSI*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>67 (54%)</td>
<td>60 (58%)</td>
<td>7 (32%)</td>
<td>0.052</td>
</tr>
<tr>
<td>Yes</td>
<td>58 (46%)</td>
<td>43 (42%)</td>
<td>15 (68%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>35</td>
<td>28</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Residual disease</td>
<td>19 (12%)</td>
<td>13 (10%)</td>
<td>6 (21%)</td>
<td>0.12</td>
</tr>
<tr>
<td>Recurrence</td>
<td>41 (26%)</td>
<td>28 (24%)</td>
<td>13 (57%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Locoregional</td>
<td>15 (9%)</td>
<td>12 (10%)</td>
<td>3 (13%)</td>
<td>0.71</td>
</tr>
<tr>
<td>Distant</td>
<td>31 (19%)</td>
<td>20 (17%)</td>
<td>11 (48%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Deceased</td>
<td>38 (24%)</td>
<td>25 (19%)</td>
<td>13 (45%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Endometrical carcinoma</td>
<td>26 (16%)</td>
<td>16 (12%)</td>
<td>10 (35%)</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

*Median follow-up including deceased patients *Lymphovascular space invasion, p-value for the Mann-Whitney U test for continuous variables, and Chi square test for categorical variables
L1CAM in non-endometrioid carcinomas

Patient and tumor characteristics of all NEEC cases (n = 104) with respect to L1CAM expression are shown in Table 4. L1CAM expression was present in 78 (75%) cases, and varied between the subgroups: 77% of the serous carcinomas, 82% of the clear cell carcinomas, 64% of the carcinosarcomas, and 57% of the undifferentiated carcinomas expressed L1CAM. Patients with L1CAM expression were older, more often had LVSIs, and more often presented with distant recurrences and disease related mortality. In univariate regression analysis, L1CAM expression was significantly associated with the presence of LVSIs (OR 4.6, 95% CI 1.5-14.1).

L1CAM expression and survival

The 10-year disease-free and overall survival Kaplan-Meier plots are shown in Figure 1. Corresponding HRs are shown in Table 5. Multivariate analysis of the stage I EEC cases showed that expression of L1CAM is a strong and independent predictor of both reduced disease-free survival and overall survival, along with several known prognostic markers. Patient age >60 was the strongest predictor of reduced overall survival, but not of reduced disease-specific survival (data not shown). L1CAM expression and the presence of LVSIs in advanced-stage EEC cases were significantly associated with reduced disease-free, and overall survival in univariate analysis. Grade 3 histology and deep myometrial invasion predicted a reduced overall survival as well, but the myometrial invasion HR had a very wide CI.

Analysis of the NEEC cases showed that L1CAM expression is not associated with reduced disease-free and overall survival. Patient age >60 and advanced FIGO stage were associated with reduced disease-free survival, and the presence of LVSIs and advanced FIGO stage were associated with reduced overall survival.

![Figure 1. Kaplan-Meier plots of the 10-year disease-free and overall survival of the stage I endometrioid, advanced-stage endometrioid, and non-endometrioid cases, with respect to L1CAM expression.](image-url)

**Table 5.** Results of the Cox regression analysis, depicting the association between several risk factors and outcome. The table shows Hazard Ratio’s and corresponding 95% Confidence Intervals. Bold Hazard Ratio’s are significantly associated with the respective outcome variable. For the stage I EEC subgroup, additional multivariate Cox regression analysis was performed, including covariates which were significantly associated with outcome in the univariate analysis.

<table>
<thead>
<tr>
<th></th>
<th>Stage I EEC</th>
<th>Stage II-IV EEC</th>
<th>NEEC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Univariate</td>
<td>Multivariate</td>
<td>Univariate</td>
</tr>
<tr>
<td></td>
<td>HR (95%CI)</td>
<td>HR (95%CI)</td>
<td>HR (95%CI)</td>
</tr>
<tr>
<td>L1CAM+</td>
<td>3.1 (1.9-5.1)</td>
<td>2.3 (1.3-4.1)</td>
<td>3.9 (2.0-7.7)</td>
</tr>
<tr>
<td>Grade 3</td>
<td>2.7 (1.6-4.4)</td>
<td>2.0 (1.1-3.5)</td>
<td>1.3 (0.7-2.5)</td>
</tr>
<tr>
<td>MII&gt;50%</td>
<td>1.5 (1.0-2.3)</td>
<td>-</td>
<td>1.8 (0.8-4.0)</td>
</tr>
<tr>
<td>Age&gt;60</td>
<td>2.0 (1.2-3.3)</td>
<td>2.0 (1.1-3.4)</td>
<td>1.3 (0.7-2.6)</td>
</tr>
<tr>
<td>LVSIs</td>
<td>2.9 (1.6-5.1)</td>
<td>2.3 (1.3-4.1)</td>
<td>4.1 (1.5-6.7)</td>
</tr>
<tr>
<td>FIGO 3/4</td>
<td>-</td>
<td>-</td>
<td>1.6 (0.8-3.2)</td>
</tr>
</tbody>
</table>

*HR = Hazard Ratio, *95%* CI = 95% Confidence Interval, *Disease-free Survival, *Overall Survival*

Discussion

This large, well-documented series of 1199 endometrial carcinomas shows a strong association between L1CAM expression and poor outcome in stage I EECs and advanced-stage EECs, but not in NEECs. Moreover, L1CAM expression was shown to be associated with the presence of nodal disease, grade 3 histology, LVSIs, distant disease recurrences, but especially with non-endometrioid histology.

Prognostic value of L1CAM expression in stage I endometrioid carcinomas

Two large studies have described the prognostic value of L1CAM expression in stage I EECs. Zeimet et al. found L1CAM expression in 17% of the cases, with HRs of 16.33 for recurrence and 15.01 for death. The PORTEC group found L1CAM expression in 7%, with HRs of 2.55 for pelvic recurrence, 3.48 for distant recurrence, and 2.05 for death. In comparison, we found L1CAM expression in 10% of the stage I EECs, with HRs of 2.3 for recurrences, 2.8 for distant recurrences, and 2.4 for death.

The number of lymphadenectomies performed in our study was slightly higher than in the Zeimet study. The PORTEC study does not mention the exact number of lymphadenectomies, but it is described that routine lymphadenectomies were not performed, and only suspicious nodes were removed. Patients in our study received radiotherapy, especially external beam radiotherapy, more often than patients in the Zeimet study, but less often than those in the PORTEC study. Patients in the PORTEC study more often had a low grade tumor and
deep myometrial invasion, which was related to the inclusion criteria of the study. LVSI was present in a large number of carcinomas in the Zeimet study compared to both our study and the PORTEC study. It was hypothesized that a lower number of included grade 1 cases might explain the higher number of L1CAM positive cases in the Zeimet study. 10 Although we show a strong association between L1CAM expression and grade 3 histology, our study included even fewer grade 1 cases, which makes it unlikely this explains the difference in L1CAM expression. As we show a strong association between the presence of L1CAM expression and LVSI it is possible that the high prevalence of LVSI in the Zeimet study is responsible for the high number of L1CAM positive cases, and the exceptionally strong association between L1CAM expression and poor outcome they describe.

Several smaller studies included stage I EECs in addition to advanced-stage EECs and NEECs. Unfortunately, both Van Gool et al. and Geels et al. did not analyze the value of L1CAM expression specifically in the stage I EECs, making it impossible to compare our findings to these studies. 17, 18 Interestingly, Dellinger et al. included a sizeable amount of stage I EECs and analyzed the association between mRNA expression of L1CAM and prognosis, but were not able to validate the strong prognostic value of L1CAM expression.19 This might be explained by the fact that they considered all cases with an mRNA L1CAM expression above the median to be positive, which resulted in a much higher proportion of L1CAM positive cases in their study compared with other L1CAM studies.

Prognostic value of L1CAM expression in advanced-stage endometrioid carcinomas and non-endometrioid carcinomas

Several previous studies described the prognostic value of L1CAM expression in advanced-stage EECs and NEECs. Fogel et al. described L1CAM expression in all of the ten included FIGO stage III and IV cases, but the histology was not mentioned, and three previous studies described L1CAM expression in 73% of the 15, 58% of the 12, and 55% of the 20 included NEEC cases. 7, 15, 16 We found L1CAM expression in 75% of the 104 NEEC cases. Three studies included both advanced-stage EECs and NEECs and described the association between L1CAM expression and clinicopathological variables, as well as between L1CAM expression and outcome.7, 18-19 Geels et al. found an association between L1CAM expression and high tumor grade and LVSI, Dellinger et al. between L1CAM expression and advanced FIGO stage, non-endometrioid histology, high grade tumor, deep myometrial invasion, and nodal spread, and Van Gool et al. between L1CAM expression and non-endometrioid histology and high tumor grade, but not FIGO stage, depth of myometrial invasion, and LVSI. In comparison, we found an association between L1CAM expression and advanced FIGO stage, non-endometrioid histology, high tumor grade, LVSI, and nodal spread. We found these associations in all three subgroups, but unfortunately neither of these studies analyzed the association between L1CAM and clinicopathological variables within the subgroups. In addition, neither of these studies analyzed the disease-free and overall survival separately in the advanced-stage EECs and NEECs. Our subgroup analyses have shown that there is indeed a strong association between L1CAM expression and poor outcome in the advanced-stage EECs, but not in the NEECs, possibly because the presence of non-endometrioid histology itself is a strong marker of poor prognosis, and the majority were L1CAM positive. The fact that previous studies have combined the advanced-stage EECs and the NEECs in the high risk carcinoma group might therefore have influenced their results, and additional studies are required to validate the prognostic value of L1CAM expression separately in the advanced-stage EECs and the NEECs.

This study shows that there is a very strong association between L1CAM expression and the presence of non-endometrioid histology. Up until now, p53 expression has been used to identify NEECs, and whereas p53 is frequently expressed in serous carcinomas, it does not play an important role in other NEECs. 1 Previous studies reported p53 expression in 62-67% of all NEECs, whereas we found L1CAM expression in 75% of the NEECs, including 77% of the serous and 82% of the clear cell carcinomas. 17, 18 Based on these findings L1CAM expression appears to be the most powerful marker to identify NEECs described to date. Interestingly, Van Gool et al. did not find an association between L1CAM expression with a cut-off of 10% and poor outcome in their cohort of high-risk carcinomas, including high-risk stage I EECs, but did find this association when using a cut-off of 50%. 17 We however did find an association between L1CAM expression with a cut-off of 10% and poor outcome in the advanced-stage EECs and NEECs, and preliminary analyses did not show a major advantage of using a higher cut-off (data not shown). Using one cut-off in all subgroups would be preferable, because having to determine the tumor histology, tumor grade, and the depth of myometrial invasion before choosing the L1CAM cut-off would greatly limit the clinical applicability. It has to be noted that mixed carcinomas were included in the NEEC group of our study, and pure non-endometrioid histology was more common in the L1CAM positive cases. There is a possibility that mixed cases with a large endometrioid component were considered L1CAM negative in our study, even though L1CAM was expressed in over 10% of the non-endometrioid component. Although the presence of non-endometrioid histology itself is a marker of poor prognosis, it is interesting to see that those with L1CAM expression have an even worse prognosis, which is in agreement with previous findings in serous ovarian carcinomas.

Relationship between these findings and the function of L1CAM

The L1CAM was shown to have several extracellular and intracellular functions in cancer, both in an intact and cleaved form, as it has an influence on cell migration, cell survival, angiogenesis and tumor progression. 22-24 It is known that epithelial-mesenchymal transition (EMT) plays an important role in endometrial cancer invasion and metastasis. 25 Several studies suggest that L1CAM expression induces an EMT-like transition that increases the metastatic potential, without altering the invasive capabilities. Comparable to EMT, expression of L1CAM was shown to be TGFβ and Slug dependent. 26 Moreover, several studies have shown that intracellular L1CAM signaling activates NF-κB, which was shown to be essential for EMT and metastasis of breast cancer. 27-28 In colorectal cancer cell lines, L1CAM expression was shown to increase cell motility and liver metastasis, without changes in expression of epithelial or mesenchymal markers. Introduction of major EMT regulators changed expression of epithelial and mesenchymal markers, but did not increase the metastatic potential. 29 Many clinical studies have shown that there is a strong association between L1CAM expression and metastasis in various cancer types. 2-4 The current study shows a comparable association between L1CAM expression and advanced stage, lymph node involvement, and metastasis in both EECs and NEECs. Interestingly, there was no strong association between L1CAM expression and myometrial and cervical invasion, and although it was not scored separately in our study, the pathologists did not notice a clear localization
of L1CAM at the invasive front of the carcinomas. These findings are in accordance with the hypothesis that L1CAM induces EMT-like changes, but only plays a role in metastasis, and not invasion. However, several previous studies analyzing the association between L1CAM expression and clinicopathological variables present conflicting results concerning whether or not there is an association between L1CAM expression and the presence of LVSIs, deep myometrial invasion, and cervical invasion.15–18 Future studies focusing on the function of L1CAM will likely provide more insight in the possible association between L1CAM and these processes of invasion and metastasis.

Future perspectives
Accumulating data associating L1CAM expression in stage I EECs with a poor outcome should have treatment implications. Moreover, this study shows that L1CAM expression in advanced-stage EECs is associated with poor outcome as well, and there was a trend towards more residual disease after treatment, which is in line with L1CAM expression in ovarian carcinoma, which has been associated with restricted tumor resectability.13 Although this would seem to support the use of chemotherapy, L1CAM expression in other cancer types has been shown to be associated with chemotherapy resistance.15, 16, 27 Given the fact that a substantial number of advanced-stage EECs, and the majority of the NEECs are L1CAM positive, and a growing number of these carcinomas are treated with chemotherapy, studying the issue of resistance of L1CAM-positive tumors to chemotherapy has a high priority. In addition, the use of anti-L1CAM treatment might be an interesting future option.36 This study also found a strong association between L1CAM expression and the presence of other markers of poor prognosis, most notably non-endometrioid histology, grade 3 histology, and nodal disease. Once it is established that L1CAM expression in preoperative biopsies is in accordance with final pathology, incorporation into currently used preoperative prediction models might improve the selection of patients requiring a lymphadenectomy, and help pathologists to identify high-risk carcinomas, especially those with non-endometrioid histology.

In light of the recently proposed subdivision of endometrial carcinomas into four subgroups based on the molecular profile, it would be interesting to analyze the L1CAM expression in these four groups.5 The studies of both Dellinger et al. and Van Gool et al. looked at L1CAM expression in the mRNA data from the TGCA database, but unfortunately they did not describe the expression of L1CAM relative to the four proposed subgroups.17, 19 If either L1CAM expression or the proposed genetic subdivision are to be used in the future management of endometrial carcinomas, it is imperative to know how these markers are related to each other.

Strengths and weaknesses
This is the largest study to date including all endometrial carcinoma types. Complete surgical staging was performed in the majority of cases, limiting the risk of under diagnosis, and a minimal follow-up of 36 months was required, limiting the risk of missing disease recurrences and deaths. As this was a retrospective study, there has been no standardized treatment protocol, and there is the risk for selection bias. There was no centralized pathology review in this study, but all slides were from large referral hospitals with dedicated gynecological pathologists. This makes the results of this study applicable to daily practice in such hospitals.

Conclusion
In conclusion, this study shows the prognostic value of L1CAM expression in stage I EECs and advanced-stage EECs, but not in NEECs. L1CAM expression was associated with the presence of nodal disease, non-endometrioid histology, grade 3 histology, LVSIs, and with a high risk of distant disease recurrence. Implementation of L1CAM expression in clinical practice could improve the postoperative selection of high-risk carcinomas. Both the value of L1CAM expression in the preoperative selection of high-risk carcinomas and the consequences of L1CAM expression on the use of and response to chemotherapy should be studied.
CHAPTER 7

References


CHAPTER 8

Combined value of ER, PR, and L1CAM expression in predicting endometrial carcinoma recurrences: an ENITEC collaboration study

Abstract
Background
Endometrial carcinoma mortality is mainly caused by recurrent disease, and the value of various immunohistochemical markers in predicting recurrences has been studied. Loss of the estrogen (ER) and progesterone receptor (PR), and expression of L1CAM are promising markers, but their combined value has not been studied yet.

Materials and Methods
Expression of ER, PR and L1CAM was immunohistochemically determined in 293 endometrial carcinomas from 11 collaborating ENITEC centers. ER, PR, or L1CAM staining was considered positive or negative when expressed by >10% or ≤10% of the tumor cells, respectively. The association between these markers and clinicopathological markers, and their combined value in predicting survival were calculated.

Results
Expression of ER and PR was lost in 19% and 28% of the cases, respectively, and L1CAM was expressed in 18%. All three markers were associated with advanced stage, high grade, non-endometrioid histology, lymphovascular space invasion (LVSI), and reduced disease-free survival. Only advanced stage, loss of PR, and lymphovascular space invasion were independently associated with reduced disease-free survival in multivariate analysis. A prognostic model including these three markers was superior to models including only histological or immunohistochemical markers.

Conclusion
Loss of ER and PR, and expression of L1CAM are associated with high risk characteristics. For predicting recurrences, loss of PR appears to be more important than loss of ER or expression of L1CAM. A prognostic model including stage, PR expression, and LVSI instead of the traditional model including histological markers could improve the identification of high risk carcinomas.

Introduction
Endometrial carcinoma is the most common gynecological malignancy in developed countries, and its incidence is increasing. These carcinomas are traditionally divided into endometrioid, and non-endometrioid endometrial carcinomas, based on their histology. Endometrioid carcinomas are assumed to arise from hyperplastic endometrium under the influence of unopposed estrogen stimulation, and generally have a favorable prognosis. Non-endometrioid carcinomas on the other hand are assumed to arise from atrophic endometrium less dependent of estrogen stimulation, and have a worse outcome.

Primary treatment for endometrial carcinomas consists of a hysterectomy and bilateral salpingo-oophorectomy, and additionally a lymphadenectomy in high grade endometrioid, and non-endometrioid carcinomas. Adjuvant treatment is recommended based on FIGO stage, histological type, tumor grade, myometrial invasion, lymphovascular space invasion (LVSI), and the age of the patient.

Despite these recommendations, 15-20% of the carcinomas which are assumed to be low risk will recur, whereas 50% of the assumed high risk carcinomas will not. As the majority of the disease related mortality is caused by distant recurrences, additional markers are needed to identify cases at risk, and subsequently individualize the treatment. Well studied markers that might be of value in improving the identification of high risk cases are immunohistochemical loss of the estrogen receptor (ER) and progesterone receptor (PR), and expression of the L1 cell adhesion molecule (L1CAM). Expression of ER and PR is common in the estrogen dependent endometrioid carcinomas, whereas loss of ER and PR, and expression of L1CAM are more common in the non-endometrioid carcinomas.

Loss of ER and PR, and expression of L1CAM have been associated with more aggressive disease, both in endometrioid and non-endometrioid carcinomas. However, although all three markers have been shown to be strong predictors of poor outcome, we only have limited knowledge about their combined value, which makes it difficult to translate these findings into daily clinical practice. The aim of this study therefore is to analyze the relationship between these three markers, and to analyze the combined value of ER, PR and L1CAM in predicting recurrent disease.

Materials and Methods
Patients
Data and tissue were already collected for a study analyzing the value of L1CAM expression in endometrial carcinomas, which included endometrial carcinomas from 11 collaborating European Network for Individualized Treatment of Endometrial Cancer (ENITEC) centers. Cases with any non-endometrioid component were categorized as non-endometrioid. Only cases diagnosed by an expert gynecological pathologist, with complete data on treatment and pathological examination, and at least 36 months of follow-up were included. Clinical and pathological data were recorded from the patient files into a database. From the 1199 cases included in the previous study, 300 were randomly selected using SPSS version 22 (SPSS IBM, New York, NY, U.S.) statistical software.
Table 1. Clinical and pathologic characteristics and disease outcome of all included carcinomas, as well as differences between the ER, PR, and L1CAM subgroups.

<table>
<thead>
<tr>
<th>All patients</th>
<th>ER+</th>
<th>ER-</th>
<th>p</th>
<th>PR+</th>
<th>PR-</th>
<th>p</th>
<th>L1CAM-</th>
<th>L1CAM+</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>293</td>
<td>238 (81%)</td>
<td>55 (19%)</td>
<td>2.21 (72%)</td>
<td>82 (28%)</td>
<td>0.32</td>
<td>240 (82%)</td>
<td>53 (18%)</td>
<td>0.03</td>
</tr>
<tr>
<td>Median age (years)</td>
<td>64 (32-93)</td>
<td>63 (32-93)</td>
<td>66 (37-91)</td>
<td>0.23</td>
<td>63 (32-86)</td>
<td>65 (37-93)</td>
<td>0.32</td>
<td>63 (32-91)</td>
<td>67 (39-93)</td>
</tr>
<tr>
<td>Median follow-up (months)</td>
<td>70 (0-210)</td>
<td>74 (6-210)</td>
<td>56 (3-134)</td>
<td>&lt;0.01</td>
<td>73 (6-210)</td>
<td>56 (3-138)</td>
<td>&lt;0.01</td>
<td>74 (3-210)</td>
<td>53 (5-168)</td>
</tr>
</tbody>
</table>

**Treatment**
- Lymphadenectomy: 170 (58%) vs 138 (58%), p = 1.00
- Positive nodes: 15 (9%) vs 9 (6%), p = 0.09
- Radiotherapy: 155 (53%) vs 124 (52%), p = 0.57
- Chemotherapy: 30 (10%) vs 20 (8%), p = 0.046

**FIGO stage**
- I: 246 (84%) vs 208 (87%), p < 0.01
- II: 16 (5%) vs 12 (5%), p = 0.50
- III: 22 (8%) vs 14 (6%), p = 0.04
- IV: 9 (3%) vs 4 (2%), p = 0.01

**Histology**
- Endometrioid: 270 (92%) vs 227 (95%), p < 0.01
- Non-endometrioid: 23 (8%) vs 11 (5%), p < 0.01

**Myometrial invasion**
- <1/2: 197 (67%) vs 164 (69%), p < 0.01
- ≥1/2: 96 (33%) vs 74 (31%), p < 0.01

**LVSI**
- No: 242 (85%) vs 207 (89%), p < 0.01
- Yes: 42 (15%) vs 26 (11%), p < 0.01

**Markers**
- ER negative: 55 (19%) vs 36 (15%), p < 0.01
- PR negative: 82 (28%) vs 46 (44%), p < 0.01
- L1CAM positive: 53 (18%) vs 31 (13%), p < 0.01

**Outcome**
- Residual disease: 9 (3%) vs 5 (9%), p = 0.01
- Recurrence: 32 (11%) vs 20 (9%), p < 0.01
- Locoregional: 18 (6%) vs 11 (5%), p = 0.02
- Distant: 14 (5%) vs 9 (4%), p = 0.08
- Disease site: 31 (11%) vs 19 (8%), p < 0.01
- Endometrial carcinoma: 20 (7%) vs 11 (5%), p < 0.01

*P-value of the Mann-Whitney U test for continuous, and χ² test for categorical variables, *Median follow-up including deceased patients, *Lymphovascular space invasion, *Including one recurrence with both a locoregional and a distant component
CHAPTER 8

Tissue and staining
Blank 4µm formalin fixed, paraffin embedded sections on Superfrost slides, corresponding to previously stained sections, were immunohistochemically stained for expression of ER and PR. After EDTA antigen retrieval and blocking of endogenous peroxidase with hydrogen peroxide, slides were incubated for one hour with either anti-ER (SP1 RM-9101-S, Thermo Scientific, Waltham, MA, US) or anti-PR (PgR636, Dako, Glostrup, Denmark) diluted 1:80 or 1:500, respectively, in Normal Antibody Diluent (Immunologic, Duiven, the Netherlands). They were subsequently incubated with PowerVision+ Poly-HRP and visualized with PowerVision DAB substrate solution (Leica Biosystems, Buffalo Grove, IL, US). Finally, the slides were counterstained with hematoxylin, dehydrated, and mounted. L1CAM staining was performed for a previous study using a 1:100 diluted L1CAM antibody (purified anti-CD171 (L1) antibody clone 14.10, Biolegend, San Diego, CA, US), and the protocol described above.13

Scoring
L1CAM expression was scored for a previous study. In brief, it was considered to be present when expression was seen in more than 10% of the tumor cells, regardless of histological type and disease stage. All ER and PR slides were scored twice for this study, first by LP and subsequently by either NV or KV. All were blinded for clinical and pathological data and each other’s scores. ER and PR were considered lost when expression was seen in less than 10% of the tumor cells. In case of disagreement, the final score was determined by JB, who was blinded for clinical and pathological data as well as the previous scores.

Statistical analysis
Cohen’s kappa value was calculated to assess the interobserver variability of scoring ER and PR expression. Clinical and pathological differences between cases with and without ER, PR, and L1CAM expression were calculated using the χ2 and Fisher’s exact tests for categorical, and the Mann-Whitney U test continuous variables. The association between ER, PR, and L1CAM expression and other known risk factors was calculated using univariate logistic regression analysis, expressed as odds ratios (OR) and 95% confidence intervals (95%CI). Correlation between ER, PR, and L1CAM expression was calculated as well.

Using Cox regression analysis, the association between several risk factors, including ER, PR and L1CAM expression, and reduced 10-year disease-free survival was calculated, expressed as Hazard Ratios (HR) and 95% CI. A multivariate analysis was performed including covariates related mortality, were more common in the cases with loss of ER and PR, and those with expression of L1CAM. Only locoregional recurrences were more common in cases with ER loss, whereas distant recurrences were more common in cases with L1CAM expression. Both locoregional and distant recurrences were more common in cases with loss of PR. Regression analysis (Table 2) shows statistically significant associations between these three immunohistochemical markers and clinicopathological markers of poor prognosis. There was a particularly strong association between L1CAM expression and non-endometrioid histology (OR 24.2, 95%CI 5.1-47.6) and LVSI. Cases with loss of PR and cases with L1CAM expression also had deep myometrial invasion more often. Additionally, residual disease after surgery, recurrent disease, overall mortality, and disease related mortality, were more common in the cases with loss of ER and PR, and those with expression of L1CAM. Only locoregional recurrences were more common in cases with ER loss, whereas distant recurrences were more common in cases with L1CAM expression.

Statistical differences were considered significant at a two-sided p-value ≤0.05. SPSS version 22 (SPSS IBM, New York, NY, U.S.) statistical software was used to perform the statistical analyses.

Results
After immunohistochemically staining for ER and PR, 7 of the 300 cases were excluded because those slides did not contain endometrial carcinoma tissue. The kappa values of ER and PR scoring were 0.74 and 0.80, respectively, and revision was necessary in 26 (9%) and 24 (8%) cases, respectively.

Clinical and pathological details of the remaining 293 cases, including 236 stage I endometrioid, 34 advanced stage endometrioid, and 23 non-endometrioid carcinomas, are shown in Table 1. This table also shows the characteristics of the subgroups with ER or PR loss, and L1CAM expression. Loss of ER and PR, and expression of L1CAM were found in 55 (19%), 82 (28%), and 53 (18%), respectively. As shown in Table 1, cases with loss of ER or PR, and cases with L1CAM expression significantly more often had advanced stage disease, high grade endometrioid disease, non-endometrioid histology, and LVSI. Cases with loss of PR and cases with L1CAM expression also had deep myometrial invasion more often. Additionally, residual disease after surgery, recurrent disease, overall mortality, and disease related mortality, were more common in the cases with loss of ER and PR, and those with expression of L1CAM. Only locoregional recurrences were more common in cases with ER loss, whereas distant recurrences were more common in cases with L1CAM expression. Both locoregional and distant recurrences were more common in cases with loss of PR.

Regression analysis (Table 2) shows statistically significant associations between these three immunohistochemical markers and clinicopathological markers of poor prognosis. There was a particularly strong association between L1CAM expression and non-endometrioid histology (OR 24.2, 95%CI 5.1-47.6) and LVSI. Statistically significant associations between expression of the markers were also found, with correlation coefficients for ER/PR, ER/L1CAM, and PR/L1CAM of 0.60, -0.27, and -0.46, respectively.

Table 2. Associations between loss of ER, loss of PR, and L1CAM expression with several clinical and pathological characteristics, expressed as Odds Ratios and 95% confidence intervals. Significant associations are bold.

<table>
<thead>
<tr>
<th>FIGO stage 3 or 4</th>
<th>ER-</th>
<th>PR-</th>
<th>L1CAM+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-endometrioid</td>
<td>3.8 (1.7-8.3)</td>
<td>3.2 (1.5-6.8)</td>
<td>3.4 (1.5-7.5)</td>
</tr>
<tr>
<td>High grade†</td>
<td>6.2 (3.3-11.8)</td>
<td>8.2 (4.4-15.2)</td>
<td>10.7 (5.5-20.9)</td>
</tr>
<tr>
<td>Lymphovascular</td>
<td>3.6 (1.8-7.5)</td>
<td>3.7 (1.9-7.2)</td>
<td>4.9 (2.4-10.1)</td>
</tr>
<tr>
<td>Myometrial invasion</td>
<td>1.4 (0.8-2.7)</td>
<td>2.0 (1.2-3.3)</td>
<td>2.6 (1.4-4.7)</td>
</tr>
</tbody>
</table>

†Both high grade endometrioid and non-endometrioid carcinomas
Results from the Cox regression analysis are shown in Figure 1. All included markers, except age > 60 years, were associated with reduced 10-year disease-free survival in the univariate analysis. In the subsequent multivariate analysis, only advanced stage disease, loss of PR, and the presence of LVSI, were significantly associated with reduced 10-year disease-free survival.

Figure 1. Cox regression analysis, which shows the association between several risk factors and reduced disease-free survival. The Hazard Ratio (HR) and corresponding 95% Confidence Interval (95%CI) of the univariate analysis are depicted by the dotted lines and the normal text. All risk factors significantly associated with reduced disease-free survival in univariate analysis were included in a multivariate Cox regression analysis, depicted by the uninterrupted lines and the bold text.

Additionally, we explored the presence of the histological and immunohistochemical markers (ranked based on the HRs in Figure 1) in the individual recurrences in this study, including 18 locoregional, and 14 distant recurrences. As shown, recurrent case number 1-5, including two distant recurrences, were not identified by any of the markers. The value of various combinations of histological and/or immunohistochemical markers in predicting recurrences was also explored, as depicted in Figure 2 and Table 3. The traditional histological model was able to identify 14 recurrences, including 11 with a distant component, but also considered 38 cases that did not develop a recurrence to be at risk. When only the three immunohistochemical markers were used, 16 recurrences were identified, including 8 distant recurrences, but 38 cases that did not develop a recurrence were considered to be at risk. When histological and immunological markers were combined, 22 recurrences, including 12 distant recurrences, were identified, but 73 cases which did not develop a recurrence were also considered to be at risk. Finally, when using the three markers identified by Cox regression analysis, 14 recurrences were identified, including 11 distant recurrences, and 15 cases which did not develop a recurrence were considered to be at risk.

Table 3. Characteristics of various possible prognostic models.

<table>
<thead>
<tr>
<th>Model</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV*</th>
<th>NPV*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Histological</td>
<td>44%</td>
<td>85%</td>
<td>27%</td>
<td>92%</td>
</tr>
<tr>
<td>2. IHC</td>
<td>50%</td>
<td>85%</td>
<td>30%</td>
<td>93%</td>
</tr>
<tr>
<td>3. Combined</td>
<td>69%</td>
<td>71%</td>
<td>15%</td>
<td>95%</td>
</tr>
<tr>
<td>4. Cox regression</td>
<td>44%</td>
<td>94%</td>
<td>48%</td>
<td>93%</td>
</tr>
</tbody>
</table>

*Positive predictive value, **Negative predictive value

Table 3 shows the test characteristics (sensitivity, specificity, positive predictive value, and negative predictive value) which were calculated based on the numbers above. Compared to the histological markers, the immunohistochemical markers had an equal specificity, and a superior sensitivity and positive predictive value. When all histological and immunohistochemical markers were combined, the sensitivity increased, at the cost of a decreased specificity and positive predictive value. Finally, the model using the three
markers which were significantly associated with reduced disease-free survival in the Cox regression analysis had a sensitivity equal to that of the traditional histological markers, and a much higher specificity and positive predictive value than any of the other combinations. The negative predictive value of all four combinations was almost the same.

Discussion

This study of a clinically representative cohort of 293 endometrial carcinomas shows that loss of ER, loss of PR, and expression of L1CAM are associated with clinicopathological markers of poor prognosis. Advanced FIGO stage, loss of PR, and LVSI were associated with recurrent disease in multivariate Cox regression analysis. When used to predict recurrences, the three significant markers from the Cox regression analysis were superior to using only histological or immunohistochemical markers.

Expression of ER, PR, and L1CAM separately

ER and PR play an important role in the development of endometrial carcinomas, and loss of these markers is a characteristic of tumor progression.1, 4, 10 Consequently, many studies have shown that loss of these receptors is associated with a reduced disease-free and overall survival, as summarized in a recent meta-analysis.12 In our study, loss of ER and PR were associated with advanced stage disease, non-endometrioid histology, high tumor grade, and LVSI. Even though there are slight variations in the associations and the strength of the associations found by previous studies, they are generally in line with our findings, indicating that loss of these markers is associated with markers of poor prognosis like non-endometrioid or high grade histology, advanced stage, lymph node metastasis, and LVSI.13-22 As expected, L1CAM expression was associated with advanced stage, non-endometrioid histology, high grade endometrioid histology, and LVSI.12, 13, 15, 17 However, in contrast to previous studies, we did not find an independent association between L1CAM expression and reduced disease-free survival. The percentage of recurrences, especially those with a distant component, was lower in the randomly selected cohort compared to the original one.13 Although this might be an explanation for the fact that L1CAM, which is assumed to be a strong marker of distant spread, was not associated with recurrent disease, the percentage of (distant) recurrences is comparable to that of two other studies which did find L1CAM a strong marker of distant spread, was not associated with recurrent disease, the percentage and overall survival, as summarized in a recent meta-analysis. In our study, loss of ER and PR play an important role in the development of endometrial carcinomas, and loss of these markers is a characteristic of tumor progression. Consequently, many studies have shown that loss of these receptors is associated with a reduced disease-free and overall survival, as summarized in a recent meta-analysis.12 In our study, loss of ER and PR were associated with advanced stage disease, non-endometrioid histology, high tumor grade, and LVSI. Even though there are slight variations in the associations and the strength of the associations found by previous studies, they are generally in line with our findings, indicating that loss of these markers is associated with markers of poor prognosis like non-endometrioid or high grade histology, advanced stage, lymph node metastasis, and LVSI. However, in contrast to previous studies, we did not find an independent association between L1CAM expression and reduced disease-free survival. The percentage of recurrences, especially those with a distant component, was lower in the randomly selected cohort compared to the original one.13 Although this might be an explanation for the fact that L1CAM, which is assumed to be a strong marker of distant spread, was not associated with recurrent disease, the percentage of (distant) recurrences is comparable to that of two other studies which did find L1CAM expression to be of prognostic value.6, 11, 12, 21 Moreover, we found an association between L1CAM expression and reduced disease-free survival in univariate analysis, indicating that the other markers included in the multivariate regression are superior.

Combined value of ER, PR, and L1CAM expression

Our study is one of the very few which have studied the combined value of these three markers. We found that there is a strong association between expression of ER and PR, but the associations between expression of ER and PR and L1CAM were only moderate and weak, respectively. A previous study analyzed the correlation between expression of these three markers in endometrial carcinomas, but not their combined prognostic value.12 Contrary to our findings, loss of ER and PR was observed in all L1CAM positive cases included in that study. This might be explained by the fact that this study did not use a cut-off value for L1CAM expression, but also included tumor foci with loss or ER and PR, and L1CAM expression. For our study, we did not perform such an in-depth analysis, but rather used the clinically relevant cut-off of 10%.

Interestingly, although all three markers were associated with reduced disease-free survival in univariate Cox regression analysis in our study, only loss of PR was an independent predictor of reduced disease-free survival. This is in line with multiple previous studies which have analyzed the additional value of ER and PR immunohistochemistry, and concluded that loss of PR was the most important sign of tumor progression.14-18 The strong prognostic value of loss of PR might be explained by the fact that progesterone is a strong tumor suppressor, and by the fact that loss of PR has been shown to play a role in epithelial to mesenchymal transition (EMT), the process which is assumed to increase invasive and migratory capacities of cancer cells, and therefore assumed to be a driver of cancer progression.6, 15, 25, 26 Progesterone lowers the levels of multiple molecules involved in EMT, as well as the activity of the Wnt/β-catenin pathway, which plays an important role in EMT.19 Interestingly, the expression of PR was shown to be lower in metastatic endometrial carcinoma lesions compared to the corresponding primary tumor, which suggests that loss of PR plays a role in EMT.19, 21 Expression of L1CAM has also been related to both distant spread and EMT.12, 19, 21 However, these studies show that expression of L1CAM is dependent on TGFβ signaling, and Wnt/β-catenin activity, both of which are inhibited by progesterone.6, 15, 25, 26 This suggests that loss of PR precedes L1CAM expression, and might be a possible explanation for the fact that L1CAM expression is not an independent predictor in our study. To our knowledge, only one other prognostic study included all three markers.14 That study analyzed the expression of these (and other) markers in relation to molecularly defined endometrial carcinoma subgroups, as proposed by The Cancer Genome Atlas research network.14, 23 These four distinct subgroups are the ultramutated and copy-number low groups, containing mainly endometrioid carcinomas with an intermediate prognosis, the copy-number high group, containing non-endometrioid and high grade endometrioid carcinomas with an unfavorable prognosis, and the hypermutated group, containing high grade endometrioid carcinomas with, unexpectedly, an excellent prognosis.23 Loss of ER and PR, and expression of L1CAM were mainly found in the copy-number high and hypermutated groups, and were associated with high grade disease, deep myometrial invasion, and LVSI.14 All three markers were shown to be associated with reduced survival in univariate regression analyses. Unfortunately, it was not further analyzed whether these markers were independent prognostic markers.

Future perspectives

Recurrences are an important cause of endometrial carcinoma related mortality, but so far we are not able to reliably predict disease recurrences based on the currently used histological prognostic model.3, 11 Many immunohistochemical and genetic additions to the currently used predictive model have been proposed, but none of these have been translated to daily clinical practice.3, 6-10 The current study is the first to study the combined value of immunohistochemical expression of ER, PR and L1CAM, three very promising prognostic markers. Interestingly, only loss of PR was shown to be an independent predictor of reduced disease-free survival, along with advanced FIGO stage and LVSI. When combined into a prognostic model, these three markers were superior to the traditional histological model. The value of this new prognostic model should be validated. However, this study also highlights the fact that, even when adding ER, PR, and L1CAM expression, we are still unable...
to predict a substantial number of recurrences, while we consider a substantial number of cases which will not develop a recurrence to be at risk. Future studies are warranted to clarify whether these recurrences can be predicted with other markers. At the moment, the extent of surgery is based on the histological classification of small amounts of preoperatively obtained tumor tissue. Histological markers like myometrial invasion and LVSI, which play an important role in the postoperative prognostic model, cannot be reliably determined in endometrial biopsies. However, we found a strong association between loss of ER and PR, and expression of L1CAM, and the presence of histological markers of poor prognosis. Moreover, a prognostic model including only these three easily reproducible, immunohistochemical markers was shown to be superior to the traditional model (Table 3). The combined value of these markers in preoperative biopsies might therefore be valuable in the early identification of high risk carcinomas, and should be studied. Furthermore, all three markers have previously been implied to play a role in EMT, which might explain their prognostic value. More studies are needed to understand this process and the markers involved, because this will help us understand the mechanism between expression or loss of these markers, and poor prognosis. Finally, treatment aimed specifically at these markers might be of value in personalizing the management of endometrial carcinomas.

Strengths and weaknesses
This is the first study analyzing the combined value of histological markers, and combined immunohistochemical expression of ER, PR, and L1CAM in a clinically representative, well described cohort of endometrial carcinomas. Complete surgical staging was performed in the majority of cases, reducing the risk of incorrectly diagnosed early stage carcinomas, and a minimal follow-up of 36 months was required, reducing the risk of missing disease recurrences. Given the low event rate, the results of the (Cox) regression analyses should be interpreted with caution. However, because this is the first study to include all of these markers, and most previous studies including only ER and PR were smaller, these findings are still a strong indicator which can be used to design future (prospective) studies. As this was a retrospective study, there has been no standardized treatment protocol, and there is the risk for selection bias. There was no centralized pathology review in this study, but all slides were from large referral hospitals with dedicated gynecological pathologists.

Conclusion
Immunohistochemical loss of ER and PR, and expression of L1CAM were shown to be easily reproducible markers, which were associated with other markers of poor outcome. Out of these three markers, only loss of PR was an independent predictor of reduced disease-free survival. A prognostic model including the FIGO stage, PR expression, and LVSI, was shown to be superior to the currently used model including only histological markers, and this model should be validated. However, additional markers are needed to selectively predict all recurrences. Moreover, the value of these markers in the preoperative diagnosis of endometrial carcinomas, as well as the treatment consequences of expression of these markers, should be further studied.

References


"When people thought the earth was flat, they were wrong. When people thought the earth was spherical, they were wrong. But if you think that thinking the earth is spherical is just as wrong as thinking the earth is flat, then your view is worser than both of them put together.”

Categorizing endometrial carcinomas

Following surgical treatment of endometrial carcinomas, guidelines for the management of endometrial carcinomas recommend adjuvant radiotherapy based on tumor histology and differentiation grade, depth of myometrial invasion, and presence of lymphovascular space invasion. However, despite management based on the presence of these markers, 15–20% of the carcinomas which are considered to be low risk, and 50% of the carcinomas which are considered to be high risk will still recur. Moreover, radiotherapy is aimed at preventing locoregional recurrences, and does not reduce disease related mortality, which is caused by distant spread. It is therefore important to improve the identification of carcinomas at risk of distant recurrent disease. In this thesis we explored the predictive value of molecular markers, as well as possible improvements to the currently used histological markers. Previously, low grade endometrioid carcinomas adjacent to atrophic endometrium were shown to have a worse prognosis than those adjacent to hyperplastic endometrium. In Chapter 2 and 3 we show that the endometrioid carcinomas adjacent to atrophic endometrium are characterized by the absence of KRAS mutations, and immunohistochemical loss of E-cadherin. Loss of the estrogen receptor (ER) and progesterone receptor (PR) were associated with distant disease spread in Chapter 4. Furthermore, we have shown that expression of the L1 cell adhesion molecule (L1CAM), and loss of ER and PR are strong and independent prognostic markers in clinically representative cohorts of endometrial carcinomas (Chapter 7 and 8). In Chapter 5 we show that the recurrence rate of stage I endometrioid carcinomas is very high if lymphovascular space invasion is present. Most of these recurrences had a distant component, which consequently resulted in disease related mortality in the majority of those cases. In order to prevent distant recurrences, it should be studied whether systemic therapy is indicated in the presence of lymphovascular space invasion. Despite the fact that many other publications have also shown that lymphovascular space invasion, L1CAM expression, and loss of ER and PR are mainly associated with distant recurrences, lymphovascular space invasion is only used as a marker in recommended adjuvant radiotherapy, and immunohistochemical markers are only used to support the histological findings. This thesis illustrates that translating new findings to daily clinical practice is challenging. Based on the findings in Chapter 7, and several other studies which have analyzed the additional value of L1CAM expression alone, L1CAM immunohistochemistry should be incorporated in daily clinical practice, since it was shown to be a stronger predictor of poor outcome than several currently used prognostic markers. However, by adding just two biomarkers, as demonstrated in Chapter 8, it already becomes more complex, and loss of PR appears to be an more important than L1CAM expression. However, this study included only three new markers, whereas many more biomarkers which could be of value in daily clinical practice have been described. Implementing biomarkers in daily clinical practice is a challenge in all fields of medicine: although around 150.000 biomarkers been described in literature, less than 100 are currently used. The question, then, is how these large numbers of promising biomarkers can be incorporated in a prognostic model, and translated to daily clinical practice. Although there have been some studies relating multiple (bio)markers to each other, these included a limited number of (bio)markers, and were performed in single institutions. In order to reliably validate large numbers of biomarkers a very large number of samples and patient data, collected and handled in a standardized fashion, is required. As illustrated by the findings
in Chapter 6, there are at the moment major differences in the collection and handling of tissue. The centralized validation of markers in a large cohort of data and tissue collected in a standardized way by all centers involved in biomarker research might therefore be of great value in translational research. Subsequently, these centers could also work together in designing prospective studies which test new markers or different management strategies of endometrial carcinomas. Currently, several studies with significant overlap have been initiated, complicating not only the inclusion, but the applicability of their findings to daily clinical practice as well.

The second question is whether we need a completely new prognostic model, like for example the completely genomic categorization of endometrial carcinomas as proposed by The Cancer Genome Atlas research network.33 However, this focus disregards the fact that the markers which are currently used in the guidelines are not wrong, but rather incomplete. Both this thesis and many of the previous biomarker studies show a strong association between these new markers and the currently used prognostic markers, indicating that both are expressions of the same underlying processes. Instead of trying to develop new models, we should keep improving the model with new findings, as demonstrated or proposed by several studies.19, 38, 39, 41

**Profiling endometrial carcinomas**

Even after improvement, prognostic models will be limited to categorizing endometrial carcinomas. The question is whether these categories are a good reflection of the intricate processes that cause endometrial carcinomas to progress, which is assumed to be driven by endothelial-mesenchymal transition (EMT).42-44

As shown in Figure 1, this complex process which results in an invasive and metastatic phenotype is driven by multiple pathways.45, 46 Interestingly, many findings presented in the current thesis can be explained by our knowledge of EMT. In Chapter 2 and 3 we show that endometrioid carcinomas adjacent to atrophic endometrium, which have a worse prognosis than those adjacent to hyperplastic endometrium, are characterized by loss of E-cadherin and mutations in the PI3K/Akt and Wnt pathways. These pathways both play an important role in EMT, and in the subsequent loss of E-cadherin, one of the most important characteristics of EMT, which causes endometrial carcinoma cells to become more invasive.47 Loss of E-cadherin has been previously associated with lymph node metastasis, deep myometrial invasion, and advanced disease stage.48, 49 We also observed (Chapter 4 and 8) that loss of ER and PR is associated with lymphogenous and hematogenous spread, and subsequently with worse disease outcome. Loss of ER and PR have also been shown to be a characteristics of EMT.45 L1CAM expression, which was associated with advanced stage, lymph node involvement, and metastasis (Chapter 7 and 8), has also been associated with EMT.50, 51 Finally, lymphovascular space invasion, which was shown to be a strong prognostic marker in Chapter 5, 7, and 8, is likely caused by EMT.45

Our current knowledge of EMT might also give explanations between some of the associations found in this thesis. We found, for example, a strong associations between L1CAM expression and loss of ER and PR, which were previously shown to be characteristics of EMT, and the presence of lymphovascular space invasion, which was previously shown to be a result of EMT.41, 44 It has been previously shown that progesterone, which is a strong tumor suppressor, lowers the activity of the Wnt/β-catenin pathway, and inhibits transforming growth factor beta (TGF-β) signaling.51, 52 Other studies have shown that Wnt/β-catenin activity and TGF-β signaling play an important role in L1CAM expression.45-53 However, this process is not yet completely understood. For example, L1CAM expression only appears to play a role in increasing the metastatic properties of endometrial carcinoma cells, whereas EMT also plays a role in increasing the invasive properties.54 Moreover, in Chapter 4 we show that increased expression of PTEN and p16 is associated with intra-abdominal spread of endometrial carcinomas. These markers do not play a significant role in EMT, and EMT therefore does not appear to be responsible for intra-abdominal metastasis.45

Understanding the role of EMT in individual carcinomas is complicated by the question whether the process depicted in Figure 9.1 applies to all endometrial carcinomas, as it has previously been hypothesized that in every individual carcinoma there is only one active tumor-driving pathway.55 Moreover, endometrial carcinomas can have mixed histology and mixed molecular profiles, and small, more aggressive parts of the carcinoma might in fact be completely responsible for progression and metastasis.56, 57

The influence of the micro- and macroenvironment on tumor progression is also not very well understood.52 It was, for example, shown that PIK3CA mutations were more common in endometrioid carcinomas if the patient had a low body mass index.52 Interestingly, in Chapter 2 and 3 we show that these mutations are more common in endometrioid carcinomas with adjacent atrophic endometrium. Patients with these carcinomas had a lower body mass index, and a worse prognosis than patients with an endometrioid carcinoma adjacent hyperplastic endometrium.53 Endometrial hyperplasia is assumed to be caused by high estrogen levels, which are often related to a high body mass index, illustrating the possibility that differences between patients could have molecular effects in their carcinomas.54
Identification of endometrial carcinoma progression, which causes the majority of the disease mortality, will require a thorough understanding of EMT.1,3,10-20,64 Complete profiling of all macroscopical, microscopical, and molecular characteristics of individual cases, but especially understanding the combined role of these markers in disease progression and recurrence, might make it possible to reliably predict the prognosis of individual carcinomas. Moreover, this approach could also lead to truly personalized management of endometrial carcinomas, as these mechanisms might be targeted using anti-L1CAM, progesterone, or even treatment specifically aimed at metastases.1,4,6-8

Conclusion

Scientific theories, including prognostic models for endometrial carcinomas, should not be considered to be wrong, but rather incomplete.1 Endometrial carcinoma research has been a quest for finding molecular markers which are superior to the currently used histological ones. However, these histological characteristics are often closely linked to the molecular characteristics, and have proven to be very useful in the management of endometrial carcinomas. The real challenge therefore is not the discovery of superior models, but rather the continuous improvement of the currently used model. Ultimately, the prognostic models should become profiles which include macroscopic, microscopic, and molecular aspects of individual cases. These profiles will no longer be restricted to categorizing cases into prognostic groups, but will be a complete reflection of the mechanisms that are at play within the carcinoma. Based on understanding these mechanisms, it will be possible to accurately predict the behavior of individual tumors. Especially predicting and preventing (possibly by targeted therapy of these mechanisms) distant spread should be studied, as this is the most important cause of endometrial carcinoma mortality.

References


Summary

Endometrial carcinoma is the most common gynecological malignancy in the developed world, and its incidence is increasing. As described in Chapter 1, these carcinomas are traditionally classified as either endometrioid or non-endometrioid, based on the tumor histology. Endometrioid carcinomas are assumed to arise from hyperplastic endometrium under the influence of unopposed estrogen stimulation, and generally have a favorable prognosis. Non-endometrioid carcinomas on the other hand are assumed to arise from atrophic endometrium, and have a worse outcome. However, this dualistic model appears to be a simplification of the molecular mechanisms which drive the carcinogenesis and progression of endometrial carcinomas. In addition, prognostic models used in the management of endometrial carcinomas are mainly based on histological criteria, and their predictive value could be improved by reevaluating the currently used histological markers, and by adding new (bio)markers.

Although endometrioid carcinomas are assumed to arise from hyperplastic endometrium, in 20% only atrophic endometrium is found adjacent to these carcinomas, which is associated with a worse prognosis. Because this does not fit within the dualistic histological model, we explored possible molecular differences between these carcinomas, and non-endometrioid carcinomas in Chapter 2. As expected, endometrioid carcinomas were shown to be characterized by expression of the estrogen receptor (ER) and progesterone receptor (PR), and in some cases loss of MLH1 and PMS2, whereas non-endometrioid carcinomas are characterized by expression of L1CAM and p53, and loss of E-cadherin. However, compared to the endometrioid carcinomas with adjacent hyperplastic endometrium, those with adjacent atrophic endometrium had reduced E-cadherin expression, and less \textit{KRAS} mutations. To further explore these differences, the genetic profiles of benign and hyperplastic endometrium, endometrial carcinomas, and endometrium adjacent to carcinomas were studied in Chapter 3. This chapter confirms that \textit{KRAS} mutations are prevalent in endometrioid carcinomas with adjacent hyperplastic endometrium, whereas those adjacent to atrophic endometrium were characterized by \textit{PIK3CA} and \textit{CTNNB1} mutations. Although both simple and complex hyperplasia with nuclear atypia are considered to be premalignancies of endometrioid carcinomas, we found most mutations in complex hyperplasia. The clinical application of molecular markers in identifying endometrial carcinogenesis has yet to be studied.

We also studied the molecular background of endometrial carcinoma progression. In Chapter 4, we explored the immunohistochemical profiles of primary endometrial carcinomas without metastases, with either intra-abdominal, lymphogenous, or hematogenous metastases, and of the corresponding metastases. Loss of ER and PR were shown to be associated with lymphogenous and hematogenous spread of endometrial carcinomas. In contrast, increased expression of PTEN and p16 was found in intra-abdominal metastases. Trends we observed were loss of E-cadherin, and increased expression of L1CAM in the metastases. This not only supports the role of these markers in endometrial carcinoma metastasis, but also the hypothesis that different markers play a role in different types of spread. These markers might be of value for the identification of endometrial carcinomas at risk of metastasizing.

In Chapter 5 and 6 we further explored the role of the traditional histological markers in predicting the prognosis. The recurrence rate of early stage endometrioid carcinomas was shown to be high if lymphovascular space invasion was present (Chapter 5). Most of these
might in time be able to truly predict the prognosis of individual carcinomas, based on their knowledge about EMT with a growing database of centrally validated (bio)markers, we associated with endothelial-mesenchymal transition (EMT), which appears to play a crucial role in various models. Improving the model might be facilitated by validating all new markers in a representative cohort of endometrial carcinomas, which has been centrally collected by all centers involved in this research. Moreover, many of the markers discussed in this thesis have previously been studied in the context of endometrial carcinoma, and their use could be facilitated by implementing these markers into the currently used histological model.

In Chapter 7 of this thesis, the value of immunohistochemical L1CAM expression in a clinically representative cohort of endometrial carcinomas is studied. We found a strong association between L1CAM expression and advanced stage, nodal disease, and non-endometrioid histology. Specifically, the association between L1CAM expression and non-endometrioid histology was very strong. We showed that expression of L1CAM was a very strong predictor of poor outcome in both early and advanced stage endometrioid carcinomas, but not in the non-endometrioid carcinomas. Subsequently, the combined value of expression of L1CAM, and loss of ER and PR was studied in Chapter 8. The value of various combinations of histological and/or immunohistochemical markers in predicting recurrent disease was explored. The three easily reproducible immunohistochemical markers were shown to have a slightly higher sensitivity than the currently used histological model, and a similar specificity. However, a combination of advanced stage, LVSI, and loss of PR had the same sensitivity as the currently used histological model, and a much higher specificity. Future studies should further validate which is the best combination for daily clinical practice, but adding one or more of these easily reproducible immunohistochemical markers to the currently used histological model appears to be valuable.

Chapter 9 discusses these findings in relation to previous publications, as well as the future direction of endometrial carcinoma research. At the moment, none of the new markers discussed in this thesis, as well as in other publications, have been incorporated in diagnostic models used in daily clinical practice. Implementing these markers might be facilitated by aiming to improve the currently used model instead of only trying to discover new markers of models. Improving the model might be facilitated by validating all new markers in a cohort which has been centrally collected by all centers involved in endometrial carcinoma research. Moreover, many of the markers discussed in this thesis have previously been associated with endothelial-mesenchymal transition (EMT), which appears to play a crucial role in endometrial carcinoma progression. Future research should further explore the mechanisms which cause progression, and the markers involved. If we can combine the knowledge about EMT with a growing database of centrally validated (bio)markers, we might in time be able to truly predict the prognosis of individual carcinomas, based on their unique marker profiles.

CHAPTER 10

Summary / Samenvatting

Het endometriocarcinoom is de meest voorkomende gynaecologische maligniteit in onderveste landen, en de incidentie neemt toe. In Hoofdstuk 1 wordt besproken dat deze carcinomen onderverdeeld worden in de endometriocarciomen en de non-endometriocarcinomen, afhankelijk van de histologie. Het wordt aangenomen dat endometriocarcinomen, gestimuleerd door oostrogenen, hun oorsprong vinden in hyperplastisch endometrium, en over het algemeen een goede prognose hebben. Non-endometriocarcinomen, daarentegen, vinden hun oorsprong in atrofisch endometrium, en hebben een slechtere prognose. Deze onderverdeling lijkt echter een versimpeling te zijn van de moleculaire mechanismen die verantwoordelijk zijn voor het ontstaan en de progressie van endometriocarcinomen. Ook de prognostische modellen die gebruikt worden in de behandeling van endometriocarcinomen maken voornamelijk gebruik van een beperkte set historische criteria. De waarde van deze modellen zou verbeterd kunnen worden door de histologische markers die nu gebruikt worden te verbeteren, en er nieuwe (bio)markers aan toe te voegen.

Hoewel aangenomen wordt dat endometriocarcinomen voortkomen uit hyperplastisch endometrium, wordt er in ongeveer 20% van de gevallen alleen atrofisch endometrium gevonden naast endometriocarcinen, die dan een slechtere prognose blijken te hebben. Omdat dit niet past binnen het dualistische model hebben we in Hoofdstuk 2 de moleculaire kenmerken van endometriocarcinen naast hyperplastisch endometrium, endometriocarcinen naast atrofisch endometrium, en non-endometriocarcinen onderzocht. Zoals verwacht vonden we in de endometriocarcinen expressie van ER en PR, en in sommige casus verlies van MLH1 en PMS2, en in de non-endometriocarcinen expressie van L1CAM en p53, en verlies van E-cadherine. Daarnaast vonden we dat de endometriocarcinen naast atrofisch endometrium, vergeleken met die naast hyperplastisch endometrium, een slechtere prognose hebben. Hoewel we in Hoofdstuk 3 de genetische profielen van benignie en hyperplastisch endometrium, endometriocarcinen, en non-endometriocarcinen onderzocht. De bevindingen in dit hoofdstuk bevestigen dat KIRS mutaties veel voorkomen in endometriocarcinen naast hyperplastisch endometrium, terwijl PIK3CA en CTNNB1 mutaties juist vaker voorkomen in endometriocarcinen naast atrofisch endometrium. Hoewel aangenomen wordt dat zowel simpele als complexe hyperplasie met nucleaire atypie voorstadia zijn van endometriocarcinen, vonden we de meeste mutaties in complexe hyperplasie. Het moet nog onderzocht worden hoe moleculaire markers toegespitst kunnen worden in het identificeren van het ontstaan van endometriocarcinen.

Daarnaast hebben we gekeken naar de moleculaire achtergrond van progressie van endometriocarcinen. In Hoofdstuk 4 onderzochten we de immunohistochemische profielen van primaire endometriocarcinen zonder metastasen, met intra-abdominale metastasen, met lymphogene of hematogene metastasen, en van de corresponderende metastasen. Verlies van ER en PR waren geassocieerd met lymfogene en hematogene metastasering, terwijl verlies van PTEN en p16 vooral gevonden werd in intra-abdominale metastasen. Daarnaast vonden we verlies van E-cadherine en expressie van L1CAM in de metastasen, maar dat was niet significant. Deze bevindingen laten niet alleen zien dat deze
markers een rol spelen in het metastaseren van endometriumcarcinomen, maar ook dat verschillende markers een rol spelen in verschillende soorten metastasering.

In Hoofdstuk 5 en 6 hebben we de rol van enkele traditionele histologische markers in het voorspellen van de prognose verder onderzocht. De recidiefkans van vroeg stadium endometrioïde carcinomen was hoog als er invasie van de lymfe- en/of bloedvaten gevonden werd (Hoofdstuk 5). De meeste van deze recidieven bevonden zich op afstand, en deze veroorzaakten een aanzienlijk deel van de ziekte gerelateerde sterfte. De recidiefkans van vroeg stadium endometrioïde carcinomen was hoog als er invasie van de lymfe- en/of bloedvaten gevonden werd (Hoofdstuk 5).

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In Hoofdstuk 7 van dit proefschrift onderzochten we de waarde van immunohistochemische expressie van L1CAM in een klinisch representatief cohort endometriumcarcinomen. Er bleken sterke associaties te zijn tussen L1CAM expressie en gevorderd stadium, lymfekliermetastasen, non-endometrioïde histologie, hoge tumorgraad, invasie van de lymfe- en/of bloedvaten, en recidieven op afstand. De associatie tussen L1CAM expressie en non-endometrioïde histologie in het bijzonder was zeer sterk. L1CAM expressie voorspelde een slechte prognose in zowel de vroege en gevorderde endometrioiden, maar niet in de non-endometrioïde carcinomen. In Hoofdstuk 8 hebben we vervolgens onderzocht wat de gecombineerde toegevoegde waarde was van L1CAM expressie, en verlies van ER en PR. De waarde van verschillende combinaties van histologische en/of immunohistochemische markers werd verkend. De drie immunohistochemische markers hadden een hogere sensitiviteit het voorspellen van recidieven dan het traditionele histologische model, en een gelijkwaardigere specificiteit. Een combinatie van een gevorderd stadium, invasie van de lymfe- of bloedvaten, en verlies van PR, daarentegen, had een veel hogere specificiteit en een gelijkwaardige sensitiviteit. Het moet onderzocht worden welke combinatie het beste toegepast kan worden in de dagelijkse klinische praktijk, maar het lijkt erop dat het waardevol is om een of meerdere van deze gemakkelijk te bepalen immunohistochemische markers aan het model toe te voegen.

De bevindingen in dit proefschrift worden in Hoofdstuk 9 gerelateerd aan de literatuur, en de toekomst van het onderzoek naar endometriumcarcinomen wordt besproken. Op dit moment worden de markers die in dit proefschrift besproken worden, noch de vele markers die in de literatuur beschreven worden gebruikt in de dagelijkse klinische praktijk. Het vertalen van deze bevindingen zou verbeterd kunnen worden als het onderzoek meer gericht is op het verbeteren van het bestaande model, in plaats van op het ontdekken van nieuwe markers en modellen. Het verbeteren van het bestaande model kan vergemakkelijkt worden door de toegevoegde waarde van nieuwe markers te onderzoeken in een centraal cohort, verzameld door alle centra die betrokken zijn in onderzoek naar het endometriumcarcinoom. Daarnaast blijken zeer veel van de markers die in dit proefschrift beschreven zijn een rol te spelen in endotheliale-mesenchymale transitie (EMT), het proces dat verantwoordelijk is voor de progressie van endometriumcarcinomen. Toekomstige studies zouden dit proces, en de markers die daarin een rol spelen verder moeten ophelderen. Door kennis over EMT te combineren met een steeds completerere database van centraal gevalideerde (bio)markers kunnen we wellicht in de toekomst de prognose van individuele carcinomen zeer gericht voorspellen op basis van het unieke profiel van dat carcinoom.
Bibliography


*Both authors contributed equally

Curriculum Vitae

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