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Immunological, molecular and therapeutic mechanisms in endometrial cancer

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Immunological, molecular and therapeutic mechanisms in endometrial cancer

PhD Thesis

to obtain the degree of PhD at the
 University of Groningen
 on the authority of the
 Rector Magnificus Prof. C. Wijmenga
 and in accordance with
 the decision by the College of Deans.

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 Wednesday 2 October 2019 at 14.30 hours

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CHAPTER 01

INTRODUCTION AND SCOPE OF THIS THESIS

INTRODUCTION AND SCOPE OF THIS THESIS

Our understanding of the molecular mechanisms that drive cancer development, progression and metastasis has vastly increased over the past decades. Besides molecular mechanisms, immunology has been increasingly recognized as an important regulator of cancer development and progression. The research reported in this thesis aims to increase our understanding of the interplay between immunological, molecular and therapeutic mechanisms in endometrial cancer (EC), with the bigger perspective of improving patient outcome.

In **chapter 1**, the current knowledge of immunological and molecular mechanisms of cancer development and progression relevant to this thesis are discussed. Subsequently, we outline the existing knowledge and immunological and molecular mechanisms that drive EC development and progression as well as the current treatment options.

CANCER, A RECENT HISTORY

At the turn of the 21st century, a landmark article by Hanahan et al discussed the contemporary perspective on cancer.¹ Cancer development is a process analogous to Darwinian evolution characterized by six hallmarks, namely: self-sufficiency in growth signals, insensitivity to inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion or metastasis. The relative contribution of each hallmark to cancer development varies between cancer types, and these hallmarks need not occur in a prefixed order. Moreover, the hallmarks of cancer can act in parallel, where a change in one hallmark may facilitate a change in another. The development of each hallmark of cancer is enabled by alterations in key molecular mechanisms, which culminates in the transformation of healthy cells.

A decade later, our view on cancer has expanded from the premise that a tumor is solely a collection of cancerous cells to the current perception that a tumor is composed of both cancerous and stromal cells that form complex interdependent interactions. This mode of organization is comparable to the cellular organization in regular organs.²

In addition, epithelial-to-mesenchymal transition (EMT) has emerged as a mechanism underlying the hallmark of tissue invasion and metastasis in many epithelial cancers. EMT is characterized by a functional change, wherein epithelial tumor cells acquire a mesenchymal phenotype, essential for migration and metastasis. At the cellular level, EMT is characterized by the loss of cell polarity and cell-cell junctions, as well as a reorganization of their cytoskeleton. Besides the important role of EMT in tissue invasion and metastasis, EMT has recently also been associated with the development of resistance to radiotherapy and chemotherapy.³

Furthermore, two enabling characteristics have been recognized: genome instability and tumor-promoting inflammation. Whereas the hallmarks are acquired features, the enabling characteristics are preexisting conditions which enable cancer development. The most prominent characteristic is genetic instability, where defects in the DNA repair mechanisms are associated with an increased occurrence rate of mutations after cell division, culminating in cancer development.

Lastly, two new hallmarks have been introduced: reprogramming of energy metabolism and avoidance of immune destruction. The latter has received much attention in light of the recent development of immunotherapy as a new option for cancer patients. The immune system is able to recognize and destroy tumor cells in a complex process named immune surveillance.⁴ This process consists of seven phases; 1. the release of antigens by the tumor, 2. the presentation of these antigens to the immune system, 3. the priming and activation of immune cells, 4. the trafficking of immune cells to the tumor, 5. the infiltration of the tumor, 6. the recognition of cancer cells, and 7. the destruction of these tumor cells.⁵ Each of these phases offers opportunities for immune escape and for immunomodulation to overcome immune escape. For example, cytotoxic T-cells (CTL) play an important role in the destruction of tumor cells, but some tumors are able to suppress CTL activity by expressing programmed cell death protein ligand 1 (PD-L1) on their cell surface. Therefore, reactivation of CTL using Anti-PD-L1/PD-1 antibodies is a promising new cancer treatment, named checkpoint inhibition. Illustrating the importance of previous phases in the immune response, such as trafficking of immune cells; the effect of anti-PD-L1/PD-1 antibodies, seems limited to cases in which intratumoral CTL are present.⁵ A clinical response is uncommon for cases where the tumor is not infiltrated by CTL. The combined direction of these factors influencing immune response, can be either towards tolerance or immunity.

The use of checkpoint inhibitors is an example of immunotherapy that aims to shift the combined direction towards immunity and the destruction of tumor cells. Roughly, two approaches in immunotherapy can be distinguished.⁶ Firstly, immune enhancement therapy is a general approach aimed at boosting overall immune function. For example, interleukine-2 (IL-2) is a cytokine that stimulates growth of CTL as well as natural killer (NK) cells and is used in the treatment of renal cell cancer and melanoma. Although this approach has proven benefit, response rates are low and immune related adverse events are frequent. The use of checkpoint inhibitors, such as anti-PD-L1/PD-1 antibodies, aims to correct a specific defect in antitumor immunity and is an example of a second approach named immune normalization. Immune normalization implies that particular defects or dysfunctions impeding an immune response are identified and specifically corrected. When compared to immune enhancement, adverse events are less common with this approach. However, it does require extensive understanding of the immunological and molecular aspects of a specific tumor.

The rapid development in our understanding of the immunological and molecular mechanisms in cancer is the result of a continuous drive to improve the clinical outcome

for cancer patients. In line with a shifting focus to patient-centered care, evaluation of disease outcome is increasingly making use of patient-reported indicators, next to indicators such as overall survival and disease free survival. Although there is much discussion on how to implement patient-reported indicators in treatment evaluation, there is no doubt that patient-related indicators are essential to the evaluation of treatment outcome.^{7,8}

ENDOMETRIAL CANCER, A RECENT HISTORY

EC is the most common gynecological cancer in developed countries and the incidence is rising.⁹⁻¹¹ Annually, 2050 new cases are diagnosed in the Netherlands alone. Most patients present at an early stage with postmenopausal vaginal blood loss. A biopsy to confirm the diagnosis is taken depending on findings via ultrasound examination or hysteroscopy. Current treatment options for EC include surgery, radio- and chemotherapy. In cases where curative treatment is deemed appropriate, the primary treatment consists of a hysterectomy with bilateral salphingo-oophorectomy (BSO). Additional lymphadenectomy (LND) infers information on possible lymph node metastasis and is relevant to determine the extent of disease spread. LND for treatment purposes is subject to debate. Surgery extended with LND carries an increased risk of complications and a general survival benefit has not been shown.¹²⁻¹⁴ A large randomized controlled trial (RCT), including 1408 cases with EC, found no benefit of LND in the treatment of EC.¹² In addition, a meta-analysis of available studies on LND in the treatment of EC failed to demonstrate a survival benefit.¹⁴ However, retrospective cohort studies suggest LND could improve survival in specific subgroups with a high risk of recurrence.^{13,15-18} Currently, an RCT evaluating the role of LND in the treatment of EC with a high risk of recurrence is ongoing.¹⁹ At this point, LND is not standard therapy for EC in treatment guidelines.¹⁴ The Dutch guideline recommends to extend surgery with a LND only in cases with a suspicion or high risk of lymph node metastasis.²⁰

After primary treatment, patients are counseled for adjuvant radio- and/or chemotherapy based upon pathological findings and age. Currently, the pathology report includes information on lymphovascular space invasion, disease stage and differentiation grade in cases of endometrioid histology, or histologic subtype in cases of non-endometrioid histology. Lymphovascular space involvement is defined as 'tumor growth into blood vessels or lymphatics'. Disease stage in endometrial cancer is defined by 'local and metastatic spread of the cancer'. In Europe, the International Federation of Gynecology and Obstetrics (FIGO) system is used for staging.²¹ According to the FIGO system, stage I refers to cancer limited to the uterine corpus. The stage increases with increased disease spread and in stage IV disease there is distant metastasis and/or involvement of bladder and/or bowel. The differentiation grade ranges from well differentiated grade 1 to poorly differentiated grade 3 or undifferentiated cancer. The histological subtype can be defined as endometrioid or non-endometrioid endometrial cancer including serous EC, clear cell EC and uterine carcinosarcoma (UCS). UCS, a rare subtype of endometrial cancer, is characterized by an epithelial as well as a mesenchymal histology. Due to this remarkable histology,

this subtype has been suggested as a model for the process of EMT.^{22,23} A higher FIGO stage, non-endometrioid histology and a higher differentiation grade are related to an unfavorable prognosis.

In the counseling process, the risk of recurrence is weighed against the side effect of adjuvant treatment. Patients are stratified into low, intermediate, high-intermediate or high risk of recurrence based on the pathology review.¹⁰ The Dutch guideline separates the intermediate risk group into low- and high- intermediate risk of recurrence. A small majority of cases presents with a low FIGO stage and a low or low-intermediate risk profile in which treatment is limited to surgery. In line with several large RCTs such as the PORTEC 1&2 and ASTEC, adjuvant radiotherapy is recommended in cases with an high-intermediate and high risk profile according to the Dutch guideline.^{10,20,24-26} Adjuvant chemotherapy is considered in advanced disease, non-endometrioid histology or residual tumor.^{27,28}

Although 5-year overall survival of endometrial cancer is between 75-90%, the current therapeutic approach has limitations. Firstly, only a minority of patients receiving adjuvant treatment actually benefit. The current stratification has limited predictive value for recurrence as is illustrated by the PORTEC1 trial where patients with an intermediate risk profile and FIGO stage 1 EC were randomized for radiotherapy or no further treatment.^{24,29,30} Of the patients not receiving further treatment, 14% had recurrent disease compared to 4% of the intervention group. Although this shows a benefit of radiotherapy for this patient group as a whole, improved patient selection could result in less patients receiving radiotherapy with similar or further reduced recurrence rates. Secondly, survival for patients with aggressive histologic subtypes of EC is poor. Despite intensive treatment regimens, 5-year survival for these patients is below 50% and new treatment options are much needed.

Developing insights on the molecular and immunological mechanisms in EC could result in better selection of patients for existing treatment options and the development of new treatment options.^{10,31} For example, studies into the molecular mechanisms underlying EC indicate that in a new classification could improve patient selection for adjuvant treatment.^{23,29,32,33} Traditionally, a distinction is made between type 1 and type 2 EC based on histologic subtype and differentiation grade.³⁴ Type 1 is characterized by an endometrioid histology with grade 1-2 differentiation, whereas Type 2 has either a non-endometrioid histology or a grade 3 differentiated endometrioid histology. Type 2 cancer is more aggressive, and presents with advanced disease and poor prognosis when compared to type 1 EC. To some extent, the classification is reflected by molecular studies that show different expression for various proteins between the two types.^{23,32} However, the molecular characteristics of type 1 versus 2 EC are not mutually exclusive. More recently, a new classification was suggested that includes 4 categories: POLE ultramutated, microsatellite instability (MSI) hypermutated, copy-number low and copy-number high.^{29,33} In each category different molecular mechanisms underlying cancer development are involved and the different categories have a different prognosis.²⁹ For example, POLE ultramutated EC has a favorable prognosis even in cases with poor differentiation that would originally be classified as type 2 cancer.³⁵ The new classification may be more useful to predict

recurrence and currently, a RCT is investigating the value of this classification in selection of patients for adjuvant radiotherapy.³⁶

Molecular studies show EC to be more heterogenous than previously assumed. Similarly, studies into the immunological mechanisms underlying EC show a complex picture. Several immunological variables are related to disease outcome. For example, expression of indoleamine 2,3-dioxygenase (IDO) – an endogenous immunosuppressive enzyme – as well as downregulation of classical human leucocyte antigen 1 (HLA-1) both relate to an unfavorable outcome.³⁷⁻³⁹ Lastly, the presence of intratumoral cytotoxic T-cells has been related to improved survival.^{40,41}

The additional value of immunotherapy in EC has been investigated to a limited extent. POLE ultramutated and MSI hypermutated show increased numbers of TIL but also overexpression of PD1 and PDL-1.⁴² Anti-PD-L1/PD-1 antibodies have shown some promising results in MSI hypermutated EC.^{43,44}

In summary, our understanding of endometrial cancer specifically, has vastly increased over the past decades. Both the molecular and immunological mechanisms underlying cancer development and disease course are increasingly understood, offering novel possibilities for improved selection of patients for existing treatment options. Additionally, there are emerging possibilities for the development of new treatment options. In combination, better stratification and more disease-targeted therapies can contribute to improving the outcome for patients with EC, defined as improved overall survival and/or quality of life. To maintain the progression in EC therapy, we need to keep expanding our knowledge of the immunological and molecular mechanisms that drive EC development and progression, as well as evaluation of existing treatment modalities.

OUTLINE OF THIS THESIS

The studies described in this thesis have been categorized into two parts. **Chapters 2,3, and 4** focus on the immunological and molecular mechanisms of development and progression of EC. **Chapters 5 and 6** focus on the molecular and therapeutic aspects of UCS.

In **chapter 2**, we questioned if selection of patients for adjuvant treatment can be improved using immunological variables and studied the predictive value for recurrence for a multitude of variables. Candidate predictors were selected from a pool of clinicopathological and immunological variables previously shown to relate to prognosis.^{37,41} Next, the predictive value for recurrence was evaluated.

In **chapter 3**, we hypothesized that Human Leukocyte Antigen E (HLA-E) affects survival in EC by interaction with CTL or NK-cells. The expression of HLA-E is related to survival in other solid tumors such as colorectal and breast cancer.⁴⁵⁻⁴⁸ In this study,

expression of HLA-E, presence of NK-cells and CTL, in relation to survival in EC was evaluated.

In **chapter 4**, the relation between molecular and immunological mechanisms in EC is assessed. Frequent mutations such as seen in MSI hypermutated EC might contribute to immune evasion by impairing antigen presentation and T-cell activity.^{4,49} We examined the possibility that immune evasion is facilitated by mutations in Janus Kinase 1 (JAK1), important in cytokine signaling. We studied JAK1 mutation in relation to expression of immune response proteins such as LMP7, TAP1 and Human Leukocyte Antigen class 1 (HLA-1) as well as presence of cytotoxic T-cells.

In **chapter 5**, L1CAM as a possible marker for EMT in UCS is investigated. UCS has been suggested as a model for EMT because of its remarkable histology that includes an epithelial as well as a mesenchymal component. L1CAM is a transmembrane adhesion molecule important for embryonic development and several studies describe a possible role for L1CAM as a marker for an mesenchymal phenotype in EMT.^{22,23,50,51} We therefore questioned if L1CAM can be a marker for the mesenchymal component in UCS and evaluated L1CAM expression by both components.

In **chapter 6**, the extension of surgery for UCS is evaluated in a large retrospective pattern of care study. UCS is an aggressive subtype of EC that more often presents with lymph node metastasis.^{16,17,52} We hypothesized LND offers a survival benefit and evaluated surgery with or without lymphadenectomy, next to adjuvant radio- and chemotherapy in relation to survival in 1140 cases of UCS.

Chapter 7 not only discusses the implications and perspective of each specific research project, but also addresses the broader perspective of future scientific developments.

Chapter 8 provides an English and Dutch summary and is followed by the **appendices**

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CHAPTER

02

PREDICTION MODEL FOR REGIONAL OR DISTANT RECURRENCE IN ENDOMETRIAL CANCER BASED ON CLASSICAL PATHOLOGICAL AND IMMUNOLOGICAL PARAMETERS

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ABSTRACT

BACKGROUND

Adjuvant therapy increases disease free survival in endometrial cancer (EC) but has no impact on overall survival and negatively influences quality of life. We investigated the discriminatory power of classical and immunological predictors of recurrence in a cohort of EC patients and confirmed findings in an independent validation cohort.

METHODS

We reanalysed the data from 355 EC patients and tested our findings in an independent validation cohort of 72 patients with endometrial cancer. Predictors were selected and Harrell's C-index for concordance was used to determine discriminatory power for disease free survival in the total group and stratified for histological subtype.

RESULTS

Predictors for recurrence were FIGO stage, lymphovascular space invasion and numbers of cytotoxic- and memory T-cells. For high risk cancer, FIGO stage and lymphovascular space invasion combined, performed as well as cytotoxic or memory T-cells (C-index 0.70 vs 0.67 and 0.71 respectively). Recurrence was best predicted when FIGO stage, lymphovascular space invasion and numbers of cytotoxic cells were used in combination (C-index 0.82). Findings were confirmed in the validation cohort.

CONCLUSIONS

In high risk endometrial cancer, clinicopathological or immunological variables can predict regional or distant recurrence with equal accuracy, but the use of these variables in combination is more powerful.

INTRODUCTION

Endometrial cancer (EC) is the most common gynaecological cancer in the western world, with yearly over 8,500 new cases in the UK alone.¹ A distinction can be made between low and high risk EC based on histological parameters.²⁻⁴ Low risk EC comprises grade 1-2 endometrioid neoplasms and is most common. High risk EC includes non-endometrioid and high grade endometrioid neoplasms. Whilst high risk EC accounts for just 10% of new cases, it is responsible for more than 40% of deaths from EC.⁴⁻⁶ Molecular studies describe distinct molecular pathways involved in pathogenesis and suggest that low- and high risk EC are manifestations of two different diseases.⁷⁻⁹

There is a clear need to better predict recurrence and disease free survival (DFS) in order to optimise patient-tailored treatment, especially in high risk EC. The mainstay of treatment is hysterectomy and bilateral salpingo-oophorectomy with or without pelvic and para-aortic lymphadenectomy.^{5,10} Adjuvant radiotherapy is advised depending on age, depth of myometrial invasion, tumor grade and the presence of lymphovascular space involvement (LVSI).¹⁰⁻¹⁵ This reduces locoregional recurrence from 15 to 5% but has no impact on overall survival and is associated with considerable toxicity in a substantial proportion of patients. For patients with advanced and/or high grade disease, chemotherapy reduces recurrence outside the pelvis by 5% but has no effect on survival.¹⁶

Many studies show a relationship between tumor infiltrating lymphocytes and cancer behaviour.¹⁷⁻²⁴ In a cohort of 90 EC patients, Kondratiev et al described that low numbers of cytotoxic T cells (CTLs) was related to poor prognosis (HR 2.79). A favourable effect of high numbers of CTLs on progression-free and overall survival in EC patients was confirmed by staining for CD8 positive cells with a hazard ratio (HR) for survival of 0.48 (95% CI 0.26-0.89).²⁰ De Jong et al, also described a strong relation with recurrence for the ratio between CD8 positive cytotoxic T-cells and FoxP3 positive regulatory T-cells with a HR of 0.44 (95% CI 0.23-0.84).²⁰ Downregulated expression of classical Major Histocompatibility Complex (MHC) class I by EC cells also correlates with risk of recurrence.²⁵

In colorectal cancer, an immune score based on tumor infiltrating lymphocytes has been shown to better predict recurrence compared to standard TNM classification.^{19,26-29} The use of tumor infiltrating lymphocytes (TIL) as a predictor on colorectal cancer, together with an already established relation of TIL with disease course has prompted us to investigate these immunological variables and their value in predicting EC recurrence. We examined this possibility by re-analysing existing data and validated our findings in an independent cohort of high risk EC patients.

PATIENTS AND METHODS

PATIENTS

For this study, a new analysis was performed on data pooled from two previous studies.^{20,25} This initial study cohort comprised a consecutive series of 355 EC patients treated at a single institution in the Netherlands between 1984 and 2004. Patients with a previous malignancy or radiotherapy prior to surgery were excluded. Patients received standard care, undergoing hysterectomy followed by adjuvant radiotherapy as stipulated by local and international guidelines.^{11-14,30} Sections were reviewed by an experienced Gynaecologic Pathologist (HH) and classified according to WHO criteria. Low risk EC was defined as grade 1-2 endometrioid cancer. High risk EC was defined as grade 3 or undifferentiated endometrioid cancer or non-endometrioid cancer. Due to the retrospective character of this study, staging according to FIGO 1988 classification was used. Follow up visits were performed for a period of five years, in accordance with local practice. During each follow up visit, clinical history was updated and a physical examination was performed. Data were accrued until September 2011 and entered into a password protected database. Patient identity was protected by study specific patient numbers. According to Dutch legislation no further approval by an Institutional Review Board approval was necessary.

Our validation cohort comprised an international series of 106 high-risk patients selected for grade 3 with deep invasion, advanced stage or serous or clear cell EC. These patients were included from 1985 to 2013 and underwent treatment in Manchester (UK), London (UK), Villejuif (FR), Leiden (NL), Groningen (NL) and were collected as a pilot series in the international TransPORTEC collaboration.³¹ To avoid overlapping cases across the two study cohorts, patients from the Groningen center were excluded, leaving 72 cases for the validation cohort. Clinical follow up was accrued on all patients in the validation cohort until September 2014.

IMMUNOHISTOCHEMISTRY

Details of the staining procedure and the antibodies used in the initial study cohort were described previously.^{20,25} In brief, tissue microarrays (TMAs) were constructed by transferring three core biopsies of 0.6 mm diameter from representative areas of tumor centre to a pre-defined location in a recipient paraffin block. Sections of 4 µm were cut from these blocks and stained using the antibodies summarized in supplementary table 1. CD8 was used as a marker for cytotoxic T-cells, CD45R0 as a marker for memory T-cells and FoxP3 as a marker for regulatory T-cells. MHC class 1 expression was stained using antibodies for a wide range of MHC class 1 heavy chains. Antigen-antibody reactions for FoxP3 was visualized with NovaRED™ (Vector Laboratories, Burlingame) and with 3,3'-diaminobenzidine in all other cases. Tumors were evaluated if more than 20% of at least two cores consisted of tumor material. Slides were scored by two independent observers, blinded for patient characteristics and outcome. Discrepancies were resolved by consensus. Tumor infiltrating cells per core were counted and findings were dichotomized using the median as a cut-off.

We choose to use the median because an optimal threshold is unknown. In studies on prediction of recurrence in colorectal cancer the median was also used as a cut-off.^{19,26-29} As for the ratio cytotoxic/regulatory T-cells, there is also no consensus on an optimal threshold. In a previous study, the median value for the ratio showed a strong relation with survival and we therefore maintained the median as a cut-off.²⁰ In line with previous publications, CD45RO+ cells were classified as either present or not present. For scoring of HLA expression a semiquantitative scale as described in literature was used to categorize expression into normal, partial loss or loss of expression.^{25,32} TMAs have been used in previous histological studies on EC. Fons et al showed a high concordance for protein expression with full slide sections.³³ Concordance for immunological markers was also evaluated for the previous to the study by de Jong et al.²⁰

For the validation cohort, TMAs were constructed in a similar fashion. Sections used for staining CD8 were pre-treated with Ultra CC1 for 52 minutes at 95 °C. Staining for CD45RO and CD8 was performed automatically with Ventana BenchMark® ULTRA IHC/ISH Staining Module according to the manufacturer's instructions. Scoring was again done by two independent observers, blinded for patient characteristics. Discrepancies were resolved by consensus. Examples of staining for CTLs are shown in figure 1.

STATISTICAL ANALYSIS

Disease-free survival (DFS) was defined as time until regional or distant recurrence. Local recurrence was not considered an event because radiotherapy reduces local recurrence rate and the indication for radiotherapy is based upon clinicopathological parameters. Disease specific survival (DSS) was defined as time until death of disease. Although DFS was the main objective in this analysis we also performed an analysis on DSS for comparison. Because not all cores were suitable for scoring, missing values for immunological variables varied between 14.4 and 19.2%. Therefore, missing values for all immunological variables were imputed based on correlation structure. Myometrial invasion, FIGO stage, LVSI, nodal status, tumor grade, histological type, age and

Figure 1. examples of IHC for CD8 in high risk EC. (A) tumour with low number of CTL. (B) tumour with high numbers of CTL.

immunological variables were used as predictors (5 imputations). To assess the impact of the imputations on the results, the analysis was also done on the complete cases.

To analyse relations between clinicopathological and immunological variables Chi-square tests or Fishers'exact tests were used. For survival analysis log rank test and Cox regression analysis were performed. To identify predictors for DFS, variables were selected if these had a previously reported relationship with disease course. The following variables were considered: age, FIGO stage, LVSI, myometrial invasion, grade, histological type, HLA-class 1 expression, presence of CD45R0+ cells, high/low numbers of CD8+ cells and high/low ratio CD8+/Foxp3+ cells. Survival analysis was performed and hazard ratios (HR) with a 95% confidence interval were estimated. Predictors for further analysis were selected from the candidate predictors through backward elimination. To this end, a multivariate Cox regression analysis for DFS was performed using all candidate predictors. The least significant variable was left out in the subsequent Cox regression analysis, and the analysis was repeated until only significant variables remained ($P < 0.05$). Backward selection was performed to build a model including only clinicopathological variables, as well as a model including only immunological variables, as well as a model combining both groups of variables. These analyses were performed with SPSS (v20, IBM statistics, Chicago, IL, USA).

To assess the discriminatory power of the three models Harrell's concordance index (C-index) (34) was calculated for each model, using STATA (v 11, Statacorp LP, College Station, TX, USA). This was calculated for the entire cohort and separately for low/high risk EC as well as for endometrioid/non-endometrioid and low(1-2)/high(3) grade subgroups. A C-index close to 0.5 indicates low predictive power, a C-index closer to 1.0 indicates increasing discriminatory power. In the final step the three models were applied to the validation cohort and again the C-index was calculated.

RESULTS

Baseline characteristics, stratified for low/high risk EC, are shown in Table 1. The distribution of variables in the imputed dataset was not different from the original cohort dataset. Relative efficiency of imputation for both cohorts varied between 0.97 and 1.00 indicating that more imputations would not contribute to a more reliable analysis. Median follow up time was 6.0 years for the initial study cohort and 2.2 years for the validation cohort. Median age at diagnosis was 64 and 65 respectively for low/high risk EC and 69 in the validation cohort. Patients from the initial study cohort with high risk EC presented more frequently with unfavourable clinicopathological findings: advanced FIGO stage ($\chi^2=53.0$;df=3;p=0.000), deep myometrial invasion ($\chi^2=22.2$;df=1;p=0.000), LVSI ($\chi^2=42.3$;df=1;p=0.000) and positive lymph nodes ($\chi^2=4.9$;df=1;p=0.027), compared to those with low risk EC. Patients with high risk EC were also more likely to receive adjuvant radiotherapy ($\chi^2=19.6$;df=1;p=0.000). Significantly more patients received adjuvant chemotherapy in the validation cohort compared to high risk patients in the study cohort. Immunological findings in the initial study cohort show that a ratio cytotoxic/regulatory T-cells above the median was seen more often in low risk EC compared to high risk EC (55.8%versus 38.5% respectively ($\chi^2=10.0$;df=1;p=0.002)Memory T-cells were present in 62.3% of low risk and 54.1% of

Table 1 Baseline characteristics of the initial study cohort stratified by low and high risk endometrial cancer (EC), and the high risk validation cohort

	Low risk (n=250)		High risk (n=105)		Difference low and high risk chi ² ; df; p value	Validation cohort (n=72)	
	n	% or IQR	n	% or IQR		n	% or IQR
Median age	64	IQ: 56-73	65	IQ: 56.5-73	0,35; 1; =0,554	69	IQ: 61-76
Treatment							
-Surgery	250	100.0	105	100.0	19,6; 1; <0.001	72	100.0
-Adjuvant radiotherapy	127	50.8	80	76.2	1,0; 1; =0,332	42	56.8
-Chemotherapy	5	2.0	4	3.8	53,0; 3; <0.001	13	17.6
FIGO*							
-Stage 1	164	65.6	32	30.5		35	49.3
-Stage 2	40	16.0	18	17.1		9	12.6
-Stage 3	40	16.0	37	35.2		23	0.32
-Stage 4	6	2.4	18	17.1		4	0.1
Missing						1	
Invasion myometrium*					22.2; 1; <0.001		
<Half	163	65.2	40	38.1		13	19.7
>Half	87	34.8	65	61.9		53	80.3
Missing						6	
Lymph nodes (any)*					4.9; 1; =0,027		
-Negative	67	79.8	46	63.9		**	**
-Positive	17	20.2	26	36.1		9	12.7
Not assessed	166		33				
Lymphovascular invasion*					42.3; 1; <0,001		
-No	194	81,9	48	47,1		23	46.0
-Yes	43	18,1	54	52,9		27	54.0
Missing	13		3			22	
Differentiation grade*					350.2; 3; <0,001		
-Grade 1	159	63.6					
-Grade 2	91	36.4				3	6.7
-Grade 3			1	1.0		5	11.1
-Undifferentiated			98	93.3		37	82.2
Missing			6	5.7		27	
Tumour type					136.4; 3; <0,001		
-Endometrioid	250	100.0	56	53.4		45	68.1
-Serous papillary			18	17.1		9	13.6
-Clear cell			25	23.8		18	27.3
-Undifferentiated			6	5.7		6	
HLA class 1 *					1.6; 1; =0,212		
-Normal expression	110	57.9	44	50.0		-	-
-Loss of expression	85	42.1	44	50.0		-	-
Missing	48		17				
Memory T-cells*†					3.7; 1; =0,056		
-None	69	37,7	41	45,9		38	61,3
-Present	130	62,3	47	54,1		24	38,7
Missing	51		17			12	
Cytotoxic T-cells (CTL)*					0.6; 1; =0,423		
Below median	100	48.0	48	50.7		38	63.3
Above median	112	52.0	44	49.3		22	39.7
Missing	38		13			12	
Ratio Cytotoxic/Regulatory T-cells*†					10,0; 1; =0,002		
<Median	88	44,2	58	61,5		-	-
>Median	113	55,8	33	38,5		-	-
Missing	49		14				

Percentages exclude missing values, FIGO=International Federation of Gynecology and Obstetrics disease classification 1988, n=number, IQR=Interquartile Range, * Significant differences between low and high risk EC in the study cohort, chi2 P<0.005. ** Data on the number of lymph node dissections performed in the validation cohort was unknown. † Values before imputation. Relative efficiency of imputation was between 0.97 and 1.00

high risk EC ($\chi^2=3.7$; $df=1$; $p=0.056$). There was no significant difference in HLA class 1 expression or number of CTLs between low and high risk EC. Immunological findings were similar in the validation cohort. Survival is shown in Table 2. In the initial study cohort, disease recurred in 32% of patients with high risk EC and in 21% of low risk EC ($\chi^2=5.4$; $df=1$; $p=0.020$). The location of recurrence showed a similar pattern in both low and high risk EC. Almost all patients with recurrence of high risk died during follow up. Overall, 44% of patients with high risk EC died of disease during follow up versus 25% of patients with low risk EC ($\chi^2=16.9$; $df=1$; $p=0.000$). For the validation cohort, clinicopathological and immunological findings as well as survival was not significantly different from the high risk subgroup from the initial study cohort.

Backward selection of clinicopathological variables resulted in FIGO stage and LVSI as independent predictors for DFS. In backward selection of immunological variables, only high/low numbers of CTLs remained significant. Analysis of both clinicopathological and immunological variables resulted in presence of memory cells, FIGO stage and LVSI as predictors for DFS. The HR and discriminatory power for DFS of the selected variables for the entire cohort are shown in Table 3. DFS is best predicted by FIGO and LVSI combined (C-index 0.81). Addition of either immunological variable (CTLs or memory cells) to this combination did not improve discriminatory power (C-index 0.83). The discriminatory power of either CTLs or memory T-cells alone as a predictor of recurrence or death was low in the total cohort (C-index of 0.60 and 0.61 respectively).

Table 2. Disease outcome in 355 patients with endometrial cancer (EC) stratified by low and high risk endometrial cancer (EC), and the high risk validation cohort.

	Low risk (n=250)		High risk (n=105)		Difference low and high risk χ^2 ; df ; p value	Validation cohort (n=72)	
	n	%	n	%		n	%
Recurrent disease	52*		34*		5.4; 1; =0.02	33	
-Local	21	40.4	9	26.5			
-Regional	6	11.5	5	14.7		9	24.3
-Distant	25	48.1	20	58.8		24	64.9
Missing						4	
Death	62		46		18.0; 2; <0.001	28	
-Death of disease	29*	46.8	31*	67.4		22	78.9
-Death of other disease	33	53.2	15	32.6		6	21.4
Missing						3	

Percentages exclude missing values, n=number.

Table 3. Hazard ratios (HR) and discriminatory power (C-index) for disease free survival (DFS) in the original cohort (n=355).

	HR (95%CI)	C-index
FIGO, LVSI	4.5(3.3-6.3), 4.4(3.4-5.8)	0.81
Cytotoxic T-cells (CTLs)	0.35(0.27-0.46)	0.60
Memory T-cells	0.44(0.34-0.57)	0.61
FIGO, LVSI, Cytotoxic T-cells (CTLs)	4.1(3.0-5.8), 4.5(3.4-5.9), 0.41(0.32-0.53)	0.83
FIGO, LVSI, Memory T-cells	4.2(3.0-5.8), 4.9(3.7-6.4), 0.39(0.30-0.50)	0.83

FIGO=International Federation of Gynecology and Obstetrics disease classification 1988, LVSI = lymphovascular invasion, HR=Hazard Ratio, CI=Confidence Interval.

Table 4. Hazard ratios (HR) and discriminatory power (C-index) for disease free survival (DFS), stratified by low and high risk EC.

	Low risk (n=250) HR (95% CI)	C-index	High risk (n=105) HR (95% CI)	C-index
FIGO, LVSI	5.1(3.5-7.6), 8.0(5.6-11.3)	0.85	3.8(2.0-7.0), 2.0(1.3-3.0)	0.70
Cytotoxic T-cells (CTLs)	0.61(0.43-0.86)	0.52	0.23(0.15-0.37)	0.67
Memory T-cells	0.90(0.85-0.96)	0.59	0.75(0.66-0.84)	0.71
FIGO, LVSI, Cytotoxic T-cells (CTLs)	5.1(3.4-7.5), 8.1(5.7-11.5), 1.2(0.79-1.7)	0.86	3.2(1.7-5.9), 2.0(1.3-3.1), 0.29(0.19-0.45)	0.79
FIGO, LVSI, Memory T-cells	4.4(2.9-6.7), 7.5(5.1-11.1), 1.2(0.86-1.7)	0.87	1.7(0.9-3.3), 2.9(1.8-4.6), 0.73(0.64-0.83)	0.76

FIGO=International Federation of Gynecology and Obstetrics disease classification 1988, LVSI = lymphovascular invasion, HR=Hazard Ratio, CI=Confidence Interval.

Table 5. Hazard ratios (HR) and predictive value (C-index) for disease free survival (DFS) in the high risk validation cohort (n=72).

	HR (95%CI)	C-index
FIGO, LVSI	3.02(2.23-4.01); 1.00(1.00-1.00)	0.81
Cytotoxic T-cells (CTLs)	0.16(0.11-0.24)	0.60
Memory T-cells	0.42(0.30-0.59)	0.61
FIGO, LVSI, Cytotoxic T-cells (CTLs)	2.51(1.83-3.45); 1.00 (1.00-1.00); 0.17(0.12-0.25)	0.83
FIGO, LVSI, Memory T-cell	2.96(2.17-4.05); 1.00 (1.00-1.00); 0.42(0.29-0.60)	0.83

FIGO=International Federation of Gynecology and Obstetrics disease classification 1988, LVSI = lymphovascular invasion, HR=Hazard Ratio, CI=Confidence Interval.

Results after stratification for low/high risk EC are shown in Table 4. In low risk, FIGO and LVSI were the best predictors for DFS and addition of either CTLs or memory T-cells did not improve discriminatory power. However, in high risk EC, DFS is best predicted by FIGO and LVSI combined with CTLs (Table 4, C-index 0.79). A combination of clinicopathological variables with presence of memory T-cells also performs well (C-index 0.76). Separately, the discriminatory power of either immunological variable was similar to that of FIGO and LVSI combined (C-index of 0.71 versus 0.70).

C-index for the subgroups low versus high grade EC and endometrioid versus nonendometrioid EC was similar to the combination of these subgroups in low versus high risk EC. Both in high grade and in nonendometrioid EC DFS was best predicted by a combination of FIGO and LVSI with CTLs (C-index 0.80 vs 0.85 respectively).

For comparison, DSS was also analysed and showed a similar pattern (data not shown). In high risk EC, clinicopathological variables performed similarly to either CTLs or memory T-cells (C-index 0.63 versus 0.64-0.68). The combination of FIGO, LVSI and CTLs had the highest predictive accuracy for survival (C-index 0.72). Both presence of memory T-cells and low numbers of CTLs, contribute to prediction of DSS (data not shown).

Table 5 shows hazard ratios and the C-index for the validation cohort. The discriminatory power of CTLs below/above the median in the validation cohort equalled that of advanced FIGO stage and LVSI combined (C-index 0.71). The combination increased the discriminatory power (C-index 0.79) confirming the results of the initial study cohort. Furthermore, findings in the subgroup analysis of low versus

high grade EC (C-index 0.84) and endometrioid versus nonendometrioid EC (C-index 0.80) was similar to low versus high risk EC (data not shown).

Figure 2 represents DFS in the validation cohort and confirms the finding in previous studies that a high number of CTLs is related to a favourable disease course. Figure 3 is a boxplot illustrating the distribution of CTLs for patients from the validation cohort, with and without an event in DFS. There was one recurrence out of 19 patients with a CTL count above 50.

Without imputation, and using only cases with all variables complete, the datasets contained 257 and 54 cases for the study and validation cohort respectively. Results were similar to that of the imputed dataset. CTLs performed well as a predictor for regional and distant recurrence in the high risk study cohort and the validation cohort (C-index 0.71 and 0.74 respectively). Combination with FIGO stage and LVSI again increased predictive power with a C-index of 0.80 in the high risk study cohort and 0.83 in the validation cohort.

DISCUSSION

Immunological parameters may be useful to select patients with high risk EC for adjuvant therapy. Low numbers of CTLs is equivalent to FIGO stage and LVSI combined for predicting regional and distant recurrence or death from disease in type 2 EC. A model that combines all three parameters has the highest predictive power in high risk EC.

Currently the selection of patients for adjuvant treatment depends on age, depth of myometrial invasion and histological subtype.¹¹⁻¹⁴ Our data support the role of FIGO stage and LVSI in decision making. Age was not selected in backward selection and we therefore cannot confirm the role of age in decision making. CTLs could contribute to decision making in order to reduce the number of patients receiving

Figure 2. KM-curve showing disease free survival and tumour infiltration by low/high numbers of CTL for 72 patients in the validation cohort.

Figure 3. Number of tumour infiltrating CTL for patients with and without recurrence for 72 patients in the validation cohort.

adjuvant radiotherapy with its associated toxicity, whilst at the same time keeping risk of recurrence as low as possible. Furthermore, a model that accurately predicts distant recurrence could indicate which patient should additionally receive adjuvant chemotherapy, rather than radiotherapy alone.

In theory a high discriminatory power of CTLs and memory T-cells could be explained by the involvement of immune system in development and progression of cancer. The immune system can eliminate tumors by killing of cancer cells.^{23,24} But in doing so a selection of tumor cells will survive by escaping immune surveillance. Presence of memory and especially CTLs may reflect a functional immune response capable of eradication of tumor cells. This immune response consists of a chain of events from antigen presentation and recognition to tumor infiltration and destruction of cancer cells. Tumor infiltration by CTL and memory T-cells indicates that previous steps in the immune response were successful.

Predicting recurrence using immunological variables is a relatively new concept. In colorectal cancer, an immunological prediction model has shown promising results.^{19,26-29} A score derived from number of intratumoral cytotoxic and memory T-cells had a concordance index of 0.65 for DFS and 0.66 for DSS in colorectal cancer, which was higher than that of TNM classification. To our knowledge, we are the first to explore the possibility of an immune prediction score for recurrence in EC. Similarly to what has been shown in colorectal cancer, CTLs and, to a lesser extent, memory T-cells are the best predictors for recurrence in our study. We used the median number of cytotoxic cells per core as the cut-off for distinguishing low and high CTLs density, as was previously described by Galon et al in colorectal cancer.^{19,26-29} However, this cut-off is arbitrary and may not be the optimal threshold for predicting disease recurrence. With a higher cut-off point, discriminatory power for no recurrence will increase. The boxplot (Figure 3) shows that there were only a few recurrences for patients with a high CTLs count. In fact, there was only one recurrence in the 21 cases with a count above 50. Of these 21 patients, 18 had an indication for adjuvant radiotherapy. These data do exemplify that patients with a high number of CTLs may be better off without adjuvant radiotherapy. Future studies are needed to determine the optimal threshold.

We specifically evaluated TIL and HLA-class 1 expression as possible predictors in EC. Other immunological variables such as PD1 were not evaluated but may also contribute in patient selection. We performed immunofluorescent staining for PD1 in sections of endometrial cancer as part of another study but were unable to confirm a relation to survival. Nonetheless, it might be useful to examine a possible contribution of other immunological variables in patient selection.

Radiotherapy reduces local recurrence and is advised for patients with a high risk profile based on clinicopathological variables, and this could be a confounding factor in our study. As patients in our cohorts were treated accordingly, fewer local recurrences were seen, as expected, in those patients who received radiotherapy. We therefore excluded local recurrence when defining disease free survival. We are aware that this may cause a selection bias if local recurrence is related to distant recurrence. Future work should include similar cohorts of patients treated in randomized trials of radiotherapy and/or adjuvant chemotherapy.

Here we describe an innovative statistical approach to establish which immunological predictors can select patients for adjuvant treatment. Multivariate Cox

regression analyses was used to select relevant predictors from a pool of candidate predictors. This analysis also provides relative risk of recurrence in hazard ratios, but gives no information on how well a variables or combination of variables can discriminate between recurrence or no recurrence. Discriminatory power of selected clinicopathological and immunological variables was assessed by calculating a C-index, where a C-index close to 1 is indicative of a strong discrimination between recurrence and no recurrence, and the resultant C-index can also be used to determine the quality of a model.

There are several issues to address in the statistical approach. For example, missing data can be problematic in developing a model and several methods have been described to deal with missing values.³⁵ In this study we choose to impute for missing values because of limitations caused by alternative approaches. The high relative efficiency in our imputation (0.97-1.00) suggests a high concordance between the 5 imputations. Alternatively, missing values could be ignored or deleted. However, this would result in loss of significance and possibly to incorrect estimates of discriminatory power. Also, in our approach, the best predictors were selected by first including all variables and then selecting the best model using backward selection. The advantage of this approach is that the information of the correlation structure between the variables is used in the selection of variables. Another possibility would have been to select the most significant variables from univariate analysis and take these forward into the multivariate analysis. However, selecting variables by significance testing allows for a selection bias and over fitting of a model. Furthermore, including all variables may result in an impractical model.³⁵

We used as our initial study cohort a large, consecutive case series previously described and subsequently updated to include up to 6 years of follow up.^{20,25} Two studies have already described a relationship between immunological variables and disease course. Over fitting may occur when these variables are subsequently tested for prediction of recurrence. Results were therefore tested in an independent, international validation cohort of patients collected in the TransPORTEC collaboration with high-risk EC. Clinicopathological findings in this cohort were similar to the study cohort except for a lower number of patients receiving adjuvant chemotherapy in the latter. Possibly this reflects an international difference in pattern of care between institutions. The validation cohort confirms the findings from our initial study cohort and agrees with previously published studies in EC.^{17,20,22,36} Presence of memory T-cells and high numbers of CTLs in the tumor relates to DFS and DSS. Discriminatory power of high/low numbers of CTLs was similar in both the study and validation cohorts.

In conclusion, here we describe for the first time a prospective statistical approach to select immunological variables as predictors of recurrence in EC. In high-risk EC, recurrence was best predicted by a combination of FIGO stage and LVSI in addition to cytotoxic or memory t-cell numbers. The discriminatory power of both cytotoxic and memory t-cells should be confirmed in a larger cohort, preferably from a randomized controlled trial of high-risk EC.

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CHAPTER 03

THE PROGNOSTIC BENEFIT OF TUMOR INFILTRATING NK CELLS IN ENDOMETRIAL CANCER IS DEPENDENT ON CONCURRENT OVEREXPRESSION OF HLA-E IN THE TUMOR MICROENVIRONMENT

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ABSTRACT

BACKGROUND

Human Leucocyte Antigen- E (HLA-E) has been reported as both a positive and negative prognostic marker in cancer. This apparent discrepancy may be due to opposing actions of HLA-E on tumor-infiltrating immune cells. Therefore, we evaluated HLA-E expression and survival in relation to the presence of intratumoral natural killer cells and cytotoxic T-cells.

METHODS

Tissue micro arrays (TMAs) of endometrial tumors were used for immunohistochemical staining of parameters of interest. The combined impact of clinical, pathological and immune parameters on survival was analysed using log rank testing and Cox regression analyses.

RESULTS

Upregulation of HLA-E was associated with an improved disease free and disease specific survival in univariate analysis (HR 0.58 95%CI 0.37-0.89; HR 0.42 95%CI 0.25-0.73, respectively). In multivariate analysis, the presence of NK-cells predicts survival with a HR 0.28 (95% CI 0.09-0.91) when HLA-E expression is upregulated, but is associated with a worse prognosis when HLA-E expression is normal (HR 13.43, 95% CI 1.70-106.14). By contrast, the prognostic benefit of T cells was not modulated by HLA-E expression.

CONCLUSIONS

Taken together, we demonstrate that the prognostic benefit of NK-cells, but not T-cells, is influenced by HLA-E expression in endometrial cancer and propose a model to explain our observations.

INTRODUCTION

Endometrial cancer (EC) is the most common gynaecological cancer in the western world and EC incidence has increased by more than 40% in the last two decades.^{1,2} There is a need for novel treatment options as prognosis has not improved over the years. Such innovative treatment options may come in the form of immunotherapy, exemplified by recent reports on the efficacy of checkpoint inhibitors in EC patients.³ Besides treatment options, cancer immunology could contribute to improved selection of patients at risk of recurrence that would benefit from adjuvant treatment. CD8 positive, cytotoxic T-cells (CTLs) are a strong prognostic biomarker for survival in EC.⁴⁻⁶ In addition, other immune cell populations such as innate Natural Killer (NK)-cells may also be of importance.⁷⁻¹⁰

Recognition of tumor cells by CTLs and NK-cells occurs through binding to Human Leucocyte Antigen (HLA) molecules on the cell surface.^{11,12} For CTLs, specificity is determined via antigen derived peptides presented in the context of classical HLA-Ia molecules. For NK-cells, recognition occurs through interaction of Killer Cell Ig-like receptors with classical HLA-I molecules. In addition, NK-cell activity is modulated by an interaction between HLA-E and NKG2x/CD94 receptors such as NKG2A and NKG2C. HLA-E is expressed in most tissues and inhibits NK-cell activity by binding to the NKG2A/CD94 receptor complex.^{8,13-17} Alternatively, HLA-E activates NK cells by binding to the NKG2C/CD94 receptor complex.^{15,18} In addition, upregulated HLA-E expression in ovarian cancer neutralized the positive relation between CTL and survival.⁴ Similarly, CTL only had a positive effect on survival in HLA-E negative tumors in non-small cell lung carcinoma.¹⁶

Previously, our group reported on the association between downregulated expression of HLA-Ia, unfavorable clinicopathological variables and worse disease specific survival in EC.^{19,20} It is unclear whether HLA-E expression on tumor cells is of prognostic importance in EC. A relation between HLA-E expression and survival has been shown in other cancer types such as colorectal and breast cancer. Both loss of expression as well as upregulated expression of HLA-E turned out to be related to improved survival.^{4,16,21-28} Zeestraten et al linked loss of expression for HLA-E to improved prognosis in colorectal cancer.²⁷ On the other hand, Benevolo et al found upregulated expression of HLA-E to be related to improved survival.²²

We hypothesized that HLA-E expression affects survival in EC, by interacting with NK-cells and/or CTL. We therefore analysed the expression of HLA-E in a well described cohort of 355 EC patients and evaluated the interaction of HLA-E with NK-cells and CTL on disease free and overall survival in EC.

MATERIALS AND METHODS

PATIENTS

In total 355 consecutive patients treated for endometrial cancer at the University Medical Center Groningen between 1984 and 2004 were enrolled as described previously.^{5,20} Patients that had received radiotherapy prior to surgery or treatment for another neoplasm were excluded. Treatment and adjuvant treatment was in conformance with national and international guidelines.^{2,29} Tissue was classified according to FIGO criteria by an experienced gynecologic pathologist and reclassified conform the FIGO 2009 classification where necessary (HH). Follow-up visits included history taking and a physical examination and were performed for a period of 5 years. Follow-up was completed until December 2014 and entered into a password-protected database. Study specific patient numbers were used, untraceable to patient identities. According to Dutch legislation, no approval by an Institutional Review Board is necessary. This cohort is also described in previous investigations on cancer immunology in EC including the role of CTL and this data was also used for this study.^{5,6}

IMMUNOHISTOCHEMISTRY (IHC)

TMA's were constructed as described previously.^{5,20} Three 0.6 mm core biopsies were taken from representative areas of tumor centre and transferred to a blank paraffin block. Recipient blocks were placed in an incubator at 37° Celsius for 15 minutes to facilitate adhesion. For staining, sections of 4 µm were cut and applied to APES-coated slides.

For this study, sections were stained for HLA-E and NK cells. To this end, sections were treated with xylene and rehydrated through graded concentrations of ethanol. Antigens were retrieved by heating slides for 15 minutes in citrate buffer (pH 6.0) in a microwave oven followed by blocking of endogenous peroxidase activity by immersion in a 0.3% H₂O₂ solution for 30 minutes. Sections were stained for HLA-E using the monoclonal mouse anti-human HLA-E Clone MEM-E/02 diluted 1:400 (Abcam, Cambridge, UK) as a primary antibody. For staining of NK cells, the NKp46 receptor was chosen as a marker because of its high specificity and because of its expression on both activated and non-activated NK cell.^{30,31} The monoclonal mouse anti-human NKp46 clone 195314 diluted 1:25 (R&D Systems, Abingdon, UK) was used. Staining for HLA-E was performed at room temperature for 60 min, while staining for NKp46 was performed overnight at 4°C. Sections were subsequently incubated with secondary antibody RAMpo and tertiary antibody GARpo diluted 1:100 for 30 minutes each. Antigen-antibody complex was visualized using 3,3'-diaminobenzidine, and slides were counterstained with hematoxylin. Sections were dehydrated through graded concentrations of ethanol and coverslipped. As a reference, HLA-E expression was also stained in normal endometrium. Full slide, 4 µm sections of tissue from 7 postmenopausal women after hysterectomy for pelvic organ prolapse were stained for HLA-E using the same procedure as described above.

EVALUATION OF HISTOLOGY.

Slides were independently scored by two observers, blinded to clinicopathological findings and disease course. TMA cores were only analysed when two or more cores contained more than 20% tumor tissue. Presence of NK cells was evaluated by manually counting the total number NKp46 positive cells in the tumor stroma. Numbers were recalculated into average number of cells per core of 100% tumor. As there was a clear binary distribution for NK-cell infiltration, this variable was categorized as absent or present.

HLA-E expression in tumor cores was assessed using a semiquantitative scale using the percentage of positive cells and the intensity of staining to evaluate expression as described elsewhere.³² The percentage of cells was scored as 0 for 0%, 1 for 1-5%, 2 for

Figure 1. Examples of immunohistochemical staining for HLA-E and NKp46. (A) HLA-E expression in normal endometrium. (B) Weak positive staining for HLA-E. (C) Strong positive staining for HLA-E. (D) Presence of NK cells.

5-25%, 3 for 25-50%, 4 for 50-75% and 5 for >75%. The intensity of staining was scored in four categories with 0 indicating absence of staining and 3 indicating strong staining. The average sum of these scores per core was calculated. There is limited available data on HLA-E expression in normal endometrium. The Human Protein Atlas describes mostly weak expression of HLA-E limited to glandular cells.³³ In order to determine whether tumor expression of HLA-E was normal, upregulated or lost, we used full slide sections of 7 postmenopausal women as a reference. In these slides, HLA-E expression scored weak positive in all cases and expression was limited to glandular cells. For this study, HLA-E expression was therefore dichotomized into normal and upregulated expression using the lowest quartile (2.5) as a cut off.

STATISTICAL ANALYSIS

HLA-E expression (normal versus upregulated) and the presence or absence of NK cells was compared to clinicopathological variables as well as immunological variables reported previously by our group.^{5,6,20} Immunological variables were categorized conform previous publications. The number of CTL was dichotomized into below and above the median of 15 cells per core. HLA-G expression was dichotomized into normal and loss of expression. HLA-1 expression was a composite of HLA-A/B/C and B2M expression and divided into normal, partial loss and total loss of expression. Disease free survival (DFS) was defined as time until first recurrence. Disease specific survival (DSS) was defined as time until death of disease.

Survival analysis was performed using Log Rank testing and Cox regression analysis. Variables for multivariate Cox regression analysis were selected by forward selection from univariate analysis, i.e. variables that were significant in a model including that variable were entered into the multivariate analysis. To test a possible interaction of HLA-E expression with either NK-cells or CTL, a multivariate survival analysis was performed including an interaction term.³⁴ Addition of an interaction term reveals modification of a predictor by another variable. Clinicopathological variables other than FIGO stage were not related to survival in this multivariate analysis. Therefore, only FIGO stage was included in the final analysis. To investigate a similar interaction for HLA-E and CTL we performed a multivariate analysis including an interaction term for CTL and HLA-E expression. SPSS software, version 22.0 was used (SPSS Inc., Chicago, IL, USA).

RESULTS

HLA-E expression on cancer cells and presence of intratumoral NK-Cells

Figure 1 shows examples of histological staining for HLA-E expression and NK-cells on Tissue Micro Arrays (TMAs). After scoring for HLA-E expression on tumor material, images were compared to the human protein atlas and full section staining of HLA-E expression in 7 postmenopausal woman that served as a control. Conform previous descriptions, HLA-E expression was limited to the endometrium and absent in stromal tissue in EC as well as in the seven controls. HLA-E Expression in EC was

Table 1. Relationship between clinicopathological and immunological variables

	HLA-E expression (n=313)		chi ² p value	Presence of NK cells (n=303)		chi ² p value
	Normal n=65(%)	Upregulated n=248(%)		Absent n=179 (%)	Present n=124 (%)	
Age			1.14			2.13
≤ 60	28 (43.1)	89 (35.9)	0.286	74 (41.3)	41 (33.1)	0.144
> 60	37 (56.9)	159 (64.1)		105 (58.7)	83 (66.9)	
FIGO 2009			27.66			1.50
-Stage 1	17 (26.2)	152 (61.3)	<0.001	89 (49.7)	70 (56.5)	0.683
-Stage 2	19 (29.2)	35 (14.1)		34 (19.0)	19 (15.3)	
-Stage 3	20 (30.8)	49 (19.8)		43 (24.0)	26 (21.0)	
-Stage 4	9 (13.8)	12 (4.8)		13 (7.3)	9 (7.3)	
Invasion myometrium			10.52			0.24
<Half	25 (38.5)	151 (60.9)	0.001	98(54.7)	69 (55.6)	0.877
>Half	40 (61.5)	97 (39.1)		81 (45.3)	55 (44.4)	
Lymph node status*			2.15			0.02
-Negative	21 (61.8)	80 (74.8)	0.143	57 (69.5)	36 (70.6)	0.895
-Positive	13 (38.2)	27 (25.2)		25 (30.5)	15 (29.4)	
Lymphovascular invasion			21.06			0.85
-No	27 (45.8)	183 (76.3)	< 0.001	115 (68.0)	87 (73.1)	0.355
-Yes	32 (54.2)	57 (23.8)		54 (32.0)	32 (26.9)	
Differentiation grade			9.61;			0.045
-Grade 1 & 2	36 (55.4)	186 (75.0)	0.003	125 (69.8)	88 (71.0)	0.832
-Grade 3 & Undifferentiated	29 (44.6)	62 (25.0)		54 (30.2)	36 (29.0)	
Tumour type			4.28			0.47
-Endometrioid	52 (80.0)	222 (89.5)	0.039	154 (86.0)	110 (88.7)	0.494
-Non endometrioid	13 (20.0)	26 (10.5)		25 (14.0)	14 (11.3)	
HLA class 1			35.56			0.20
-Normal expression	43 (79.6)	76 (34.7)	< 0.001	71 (45.2)	45 (42.5)	0.657
-Loss of expression	11 (20.4)	143 (65.3)		86 (54.8)	61 (57.5)	
HLA-G expression			9.53			0.69
- Loss	48 (77.4)	133 (55.9)	0.002	111 (63.8)	69 (59.0)	0.407
- Normal	14 (22.6)	105 (44.1)		63 (36.2)	48 (41.0)	
Cytotoxic T-cells			13.88			0.32
< Median (<15)	42 (71.2)	97 (43.9)	< 0.001	85 (52.1)	53 (48.6)	0.569
> Median (≥15)	17 (28.8)	124 (56.1)		78 (47.9)	56 (51.4)	
Natural Killer Cells			0.01			
- Absent	38 (59.4)	136 (58.6)	0.914	-	-	
- Present	26 (40.6)	96 (41.4)				

* Lymphnode dissection or sampling was performed in 156 cases. FIGO=International Federation of Gynecology and Obstetrics, n=number.

either similar or increased compared to normal endometrium, and expression was therefore dichotomized into normal- and upregulated expression. NK-cell infiltration was predominantly intratumoral with a clear binary distribution and was categorized as either present or absent. Staining for HLA-E expression could be evaluated in 313 cases, NK cells in 303 and CTL in 304 cases.

Table 1 shows clinicopathological characteristics in relation to HLA-E expression and NK-cells. Normal HLA-E expression was observed in 65 cases of EC (21%) and upregulation in 248 cases (79%) of EC. Upregulated HLA-E expression was related to favourable clinicopathology variables such as lower FIGO staging ($P<0.001$), low grade differentiation ($P=0.003$), absence of lymphovascular space invasion (LVSI, $P<0.001$) and endometrioid histology ($p=0.039$). Concerning immunological variables, upregulated HLA-E expression related to CTL above the median. Upregulated HLA-E expression was also associated with normal expression of classical HLA-class I ($P<0.001$) and HLA-G ($P<0.002$). Supplementary table 1 shows the relation between

HLA-E expression and the HLA-1 subunits: HLA-A, HLA-B/C and B2M. Normal HLA-E was associated with decreased expression of HLA-A and HLA-B/C but not B2M. NK cells were present in 41% of cases (N=124). There was no relationship between the presence of NK cells and clinicopathological or immunological variables.

SURVIVAL ANALYSIS

Median follow-up time was 6.2 years (Inter Quartile Range: 2.4-10.5). Figure 2 A and B illustrate a favourable relation of upregulated HLA-E expression with DFS and DSS respectively. Table 2 shows the univariate Cox regression analysis of DFS and DSS. HLA-E upregulation was a prognostic biomarker for DFS as well as DSS (hazard ratio (HR) 0.58; 95% confidence interval (CI) 0.37-0.89; $P=0.013$ and HR 0.42; 95%CI 0.25-0.73; $P=0.002$, respectively). For DFS, FIGO stage, myometrial invasion, lymph node status, differentiation grade, LVSI and histological type (endometrioid; nonendometrioid) were also significant predictors ($P<0.05$). In line with previous publications, CTL above the median was related to DFS a HR of 0.42 (95% CI 0.27-0.63, $P<0.001$). Presence of NK cells did not relate to survival in univariate analysis. As for DSS, FIGO stage, myometrial invasion, lymph node status, LVSI, differentiation grade, histological type and CTL again related to survival ($P<0.05$, table 2).

STRATIFIED SURVIVAL ANALYSIS

To investigate a possible interaction of HLA-E expression with NK-cells or CTL, survival analysis for NK-cells and CTL was stratified for HLA-E expression. Alternatively, survival analysis for HLA-E expression was stratified for presence/absence of NK cells or CTL below/above the median. Figure 2 shows the KM curves for the complete cohort and relevant subgroups. There was no significant relation between HLA-E expression and DFS when NK cells were absent (fig. 2C). On the other hand, figure 2D shows a more distinct relation of HLA-E expression and survival when NK cells were present (HR 0.30, 95%CI 0.16-0.59).

Conversely, in the subgroups of normal and upregulated HLA-E expression, NK cells predict DFS although in opposite direction. Fig. 2E and 2F illustrate a trend for presence of NK cells in relation to survival in cases with upregulated versus normal HLA-E expression. In short, with upregulated HLA-E expression, survival is better for patients with NK cells; in case of normal HLA-E expression, survival is worse when NK cells are present. Findings for DSS were similar, where upregulated HLA-E expression related to survival only in presence of NK-cells (Log Rank, $P<0.001$, data not shown).

In survival analysis stratified for CTL below/above the median, HLA-E expression was not related to DFS (supplementary fig. 1 A and B). CTL below/above the median on the other hand, was related to survival despite the level of HLA-E expression (supplementary fig 1 C and D).

Figure 2. Kaplan Meier curves for survival. (A) HLA-E expression and DFS. (B) HLA-E expression and DSS. (C) HLA-E expression and DFS, NK cells absent. (D) HLA-E expression and DFS, NK cells present. (E) Presence of NK cells and DFS, normal HLA-E expression. (F) Presence of NK cells and DFS, upregulated HLA-E expression. DFS=Disease Free Survival, DSS=Disease Specific Survival

MULTIVARIATE SURVIVAL ANALYSIS

Following the stratified analysis, the interaction of HLA-E with NK-cells was evaluated in a multivariate Cox regression analysis including all significant clinicopathological variables from the univariate analysis. Additionally, in order to investigate a possible interaction between HLA-E and NK-cells, presence of NK-cells was included in the analysis with and without an interaction term.³⁴ Addition of an interaction term reveals modulation of an effect of one variable by another. In multivariate analysis without an interaction term, only FIGO stage related to DFS (Table 3). However, including an interaction term for HLA-E expression and presence of NK-cells reveals a relation between presence of NK cells and DFS that is modulated by HLA-E expression. Representing an increased risk of recurrence with presence of NK-cells and normal HLA-E expression, presence of NK-cells is a significant predictor for recurrence in multivariate analysis (HR 13.43 95%CI 1.70-106.14; P=0.014). Representing a reduced risk of recurrence with presence of NK-cells and upregulated HLA-E expression, the interaction term is a significant predictor for recurrence (HR 0.28; 95%CI 0.09-0.91; P=0.035). Likelihood ratio testing comparing models with and without an interaction

Table 2. Predictors of disease free (DFS) and disease specific (DSS) survival in EC in univariate analysis (n=355).

	Univariate Analysis for DFS			Univariate Analysis for DSS		
	HR	(95% CI)	p value	HR	(95% CI)	p value
Age	1.01	(1.00-1.03)		1.02	(1.00-1.04)	0.074
FIGO 2009			<0.001			<0.001
-Stage 1		Reference			Reference	
-Stage 2	2.22	(1.22-4.04)	0.009	3.11	(1.29-7.52)	0.012
-Stage 3	4.40	(2.72-7.10)	<0.001	7.75	(3.84-15.64)	<0.001
-Stage 4	8.72	(4.90-15.64)	<0.001	20.20	(9.44-43.21)	<0.001
Invasion myometrium						
<half		Reference			Reference	
>half	2.66	(1.79-3.95)	<0.001	3.63	(2.14-6.17)	<0.001
Lymph node status*						
-Negative		Reference			Reference	
-Positive	3.05	(1.83-5.09)	<0.001	3.47	(1.83-6.56)	<0.001
Lymphovascular invasion						
-No		Reference			Reference	
-Yes	4.18	(2.82-6.20)	<0.001	4.87	(2.92-8.13)	<0.001
Differentiation grade			<0.001			<0.001
-Grade 1		Reference			Reference	
-Grade 2	1.59	(0.95-2.65)	0.076	2.34	(1.18-4.64)	0.015
-Grade 3	2.68	(1.69-4.25)	<0.001	3.77	(2.01-7.10)	<0.001
-Undifferentiated	6.36	(2.24-18.02)	0.001	13.54	(4.48-40.95)	<0.001
Tumour type						
-Endometrioid		Reference			Reference	
-Non endometrioid	2.40	(1.52-3.79)	<0.001	3.70	(2.18-6.28)	<0.001
HLA-E expression						
-Loss		Reference			Reference	
-Normal	0.58	(0.37-0.89)	0.013	0.42	(0.25-0.73)	0.002
Cytotoxic T-cells						
< median (<15)		Reference			Reference	
> median (≥15)	0.42	(0.27-0.63)	<0.001	0.32	(0.18-0.57)	<0.001
Natural Killer Cells						
- Absent		Reference			Reference	
- Present	0.85	(0.56-1.29)	0.445	0.97	(0.57-1.65)	0.914

* Lymphnode dissection or sampling was performed in 156 cases. FIGO=International Federation of Gynecology and Obstetrics, HR=Hazard Ratio, CI=Confidence Interval.

Table 3. Multivariate analysis for disease free survival. Analysis including presence of NK-cells with and without effect modulation by HLA-E expression (n=355).

	HR	(95% CI)	p value
Without an interaction term:			
-Presence of NK-cells	1.56	(0.90-2.72)	0.115
-HLA-E expression	1.00	(0.55-1.82)	0.997
-FIGO stage 2009	2.07	(1.47-2.92)	<0.001
-Myometrial invasion	1.13	(0.57-2.25)	0.721
-Lymphnode status	0.85	(0.46-1.58)	0.608
-Lymphovascular space invasion	1.74	(0.87-3.51)	0.119
-Differentiation grade	1.08	(0.75-1.56)	0.664
-Histologic type	1.48	(0.71-3.09)	0.292
With an interaction term (HLA-E and NKcells):			
-Presence of NK-cells	13.43	(1.70-106.14)	0.014
-HLA-E expression	1.76	(0.76-4.07)	0.185
-FIGO stage 2009	2.07	(1.46-2.92)	<0.001
-Myometrial invasion	1.19	(0.60-2.38)	0.619
-Lymphnode status	0.98	(0.52-1.84)	0.943
-Lymphovascular space invasion	1.63	(0.81-3.28)	0.173
-Differentiation grade	1.08	(0.75-1.56)	0.668
-Histologic type	1.43	(0.68-2.99)	0.341
-NK cells present * HLA-E expression	0.28	(0.09-0.91)	0.035

FIGO=International Federation of Gynecology and Obstetrics, HR=Hazard Ratio, CI=Confidence Interval.

Table 4. Multivariate analysis for disease free survival. Analysis including cytotoxic T-cells below and above the median, with and without effect modulation by HLA-E expression (n=355).

	HR	(95% CI)	p value
Without an interaction term:			
-Cytotoxic T-cells below/above median	0.62	(0.34-1.17)	0.142
-HLA-E expression	1.14	(0.62-2.12)	0.673
-FIGO stage 2009	1.95	(1.35-2.81)	<0.001
-Myometrial invasion	1.07	(0.53-2.16)	0.845
-Lymphnode status	1.01	(0.53-1.94)	0.970
-Lymphovascular space invasion	1.49	(0.73-3.01)	0.273
-Differentiation grade	1.20	(0.82-1.75)	0.353
-Histologic type	1.43	(0.69-2.96)	0.336
With an interaction term (HLA-E and NKcells):			
-Cytotoxic T-cells below/above median	0.20	(0.01-4.25)	0.309
-HLA-E expression	1.00	(0.50-2.02)	0.995
-FIGO stage-2009	1.98	(1.37-2.86)	<0.001
-Myometrial invasion	1.09	(0.55-2.19)	0.802
-Lymphnode status	1.02	(0.54-1.95)	0.943
-Lymphovascular space invasion	1.51	(0.75-3.05)	0.252
-Differentiation grade	1.20	(0.82-1.76)	0.337
-Histologic type	1.37	(0.66-2.92)	0.402
-Cytotoxic T-cells below/above median * HLA-E expression	1.89	(0.36-9.92)	0.451

FIGO=International Federation of Gynecology and Obstetrics, HR=Hazard Ratio, CI=Confidence Interval

term confirmed the interaction of HLA-E expression and the number of NK-cells ($P < 0.001$, data not shown). Besides FIGO stage, all other variables did not relate to DFS in this analysis.

Table 4 shows a similar multivariate analysis including CTL below/above the median with and without an interaction term. CTL did not relate to DFS in this analysis. In multivariate analysis for DSS, with and without interaction terms, FIGO stage was the only significant predictor (data not shown).

DISCUSSION

The presence of NK cells relates to survival, either favourable or unfavourable, depending on HLA-E expression. HLA-E expression relates to survival but only when NK cells are present. It is therefore likely that this relation is mediated through an interaction with NK-cells. We found no evidence of an interaction of HLA-E expression with CTL.

The unfavourable effect of normal HLA-E expression on prognosis is in concordance with studies in other cancers.^{16,22,23,26,27} It corresponds to the commonly acknowledged theory that decreased or increased expression of HLA-E provokes an immune response.^{11,15,18} Upregulated HLA-E expression in response to viral infection or cancer development, may facilitate an adequate immune response whereas loss of expression contributes to cell lysis by NK cells. There are also studies that show a worse prognosis for tumors with upregulated HLA-E expression.^{4,16} The effect of HLA-E expression on survival in these studies could be mediated by CTL. Upregulated HLA-E expression neutralizes the favourable effect of CTL. As CTL are also an important prognostic biomarker in EC we investigated this possibility, but were unable to demonstrate an interaction between HLA-E and CTL.

Our findings suggest an interaction between HLA-E expression and NK-cells. A relation between HLA-E expression and survival was limited to cases with NK cells present. Further support comes from the multivariate regression analysis. By adding an interaction term, the analysis reveals a modulating effect of HLA-E expression on NK-Cells. When HLA-E expression is upregulated, NK-cells have a favourable effect on survival. Presence of NK-cells has an unfavourable effect when HLA-E expression is normal.

There are several possible explanations for our findings. First, different subsets of NK cells may be involved. For example, presence of different subsets have been noticed in response to cytomegalovirus infection where NKG2C positive cells show enhanced IFN- γ production and reduced NKG2A expression.^{35,36} Secondly, these subsets may be shaped the local environment of MHC molecules and cytokines, including HLA-E expression.³⁶ Finally, NK-cell activity may be influenced by a difference in affinity of NKG2x receptors for HLA-E. Affinity of HLA-E for the NKG2A receptor is higher than for the NKG2C receptor.^{14,18,37} Figure 3 illustrates this possible mechanism. In case of normal expression, HLA-E binds mainly to NKG2A and inhibits NK-cell activity. In case of increased expression, NKG2A receptors would become saturated and the binding distribution would shift towards binding to the activating NKG2C receptor. This activation may in turn lead to enhanced IFN- γ production and decreased NKG2A

Figure 3. Illustration of a possible mechanism for an inhibitory changing to an activating effect on NK-cell activity with upregulation of HLA-E expression.

expression. This mechanism would also explain the favourable effect on survival for decreased HLA-E expression in cancer where absence of HLA-E expression would lead to NK-cell activation.

An alternative explanation for our findings could be a difference in peptide sequence presented by HLA-E with upregulated expression.^{18,38-40} With normal expression, HLA-E mostly presents so called leader protein sequences from the classical HLA-Ia molecules. Upregulated HLA-E, induced by stress, also binds other protein sequences possibly causing changes in the affinity for or interaction with NKG2a/c receptors.

A third explanation could be that upregulated HLA-E expression is merely a characteristic of an immunogenic tumor. HLA-E upregulation is related to CTL above the median in this cohort as well as a low FIGO stage. However, this seems unlikely considering the results of the multivariate analysis including FIGO stage.

Considering the effect of intratumoral NK-cells on survival, most studies describe low numbers of NK-cells and no relation to survival.⁸⁻¹⁰ For EC, previous reports also describe low number of NK-cells and do not address survival. We suggest that NK-cells are relevant to disease course depending on HLA-E expression. The strong effect of NK-cells on survival by presence of just a few cells is a remarkable finding that can be explained by the effective killing strategy of NK-cells where one cell is able to destroy many target cell, e.g. tumor cells.

Remarkably, the presence of NK cells negatively affects DFS with normal HLA-E expression. Apparently, the combination of normal expression and presence of NK-cells represents an unfavourable immune response in tumors that have developed extensive immune escape mechanisms. For example, normal HLA-E expression relates to less tumor infiltrating lymphocytes and loss of expression for HLA1a, both markers for a suboptimal immune response. Furthermore, NK cells are known to influence an immune response both positively and negatively through release of cytokines such as IFN- γ .⁹ With normal HLA-E expression this activating effect may be lost. The combination of normal HLA-E expression in presence of NK-cells could amplify a hindered immune response.

Although this study supports an important role of HLA-E expression in disease course through an interaction with NK cells, there are limitations to consider. First, there was a large confidence interval in the multivariate analysis including with interaction term. This is likely due to missing values for both HLA-E expression and NK cells. Secondly, samples of tumor tissue are used for TMA construction and heterogeneity

within the tumor cannot be excluded. Finally, our findings could be further supported by simultaneously staining for HLA-E, NK-Cells and NKg2a/c receptors. We have attempted this but were unable to provide histological proof.

A strength of this study is the use of a large, well described, consecutive case cohort. This cohort has been used for several previous studies. Besides clinicopathological data, the dataset also contains information on immunological variables. Furthermore, long follow up data were available with a median follow up of 6.2 years.

In conclusion, we describe a relation between presence of NK-cells on survival that is influenced by HLA-E expression. Conversely, we find a favourable effect of upregulated HLA-E expression survival when NK cells are present. We propose a mechanism explaining our findings that is in agreement with common understanding of immunology but has not been described in tumor immunology. Preferably, future studies should not only confirm our findings but should also focus on the mechanism involved. If confirmed, this relation offers possibilities for prediction of recurrence and selection of patients for adjuvant treatment. Further knowledge of the intratumoral NK-cell population and interaction with HLA-E also offers possibilities for novel treatment options.

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SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. Relationship between HLA-E expression and expression of HLA-1 subunits: HLA-A, HLA-BC and B2M.*

	Normal n=65(%)	HLA-E expression (n=313) Upregulated n=248(%)	chi ² p value
HLA-A expression (n=293)			
-Total Loss	42 (70,0)	60 (25,8)	41,442
-Partial Loss	15 (25,0)	131 (56,2)	<0,001
-Normal	3 (5,0)	42 (18,0)	
HLA-BC expression (n=293)			
-Absent	39 (63,9)	56 (24,1)	40,31
-Weak	10 (16,4)	32 (13,8)	<0,001
-Moderate	11 (18,0)	122 (52,6)	
-Strong	1 (1,6)	22 (9,5)	
B2M expression (n=289)			
-Total Loss	2 (3,4)	3 (1,3)	4,819
-Partial Loss	14 (24,1)	33 (14,3)	0,090
-Normal	42 (72,4)	195 (84,4)	

* Expression of HLA-A, HLA-BC and B2M in this cohort was reported previously (Bijen et al, 2010). n=number.

Supplementary figure 1. Kaplan Meier curves for DFS. (A) HLA-E expression and DFS, cytotoxic T-cells below the median. (B) HLA-E expression and DFS, cytotoxic T-cells above the median. (C) cytotoxic T-cells below/above the median and DFS, normal HLA-E expression. (D) cytotoxic T-cells below/above the median and DFS, upregulated HLA-E expression.

CHAPTER

04

MICROSATELLITE INSTABILITY DERIVED JAK1 FRAMESHIFT MUTATIONS ARE ASSOCIATED WITH TUMOR IMMUNE EVASION IN ENDOMETRIOID ENDOMETRIAL CANCER

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ABSTRACT

JAK1 frameshift mutations may promote cancer cell immune evasion by impeding upregulation of the antigen presentation pathway in microsatellite unstable endometrial cancers (ECs). This study investigated the JAK1 mutation frequency, its functional implication in immune evasion and its prognostic significance in microsatellite unstable EC. Microsatellite instability and three microsatellite repeats within JAK1 were analysed in 181 ECs. Sixty-two (34%) ECs showed microsatellite instability, of which 22 (35%) had a JAK1 mutation. LMP7, TAP1 and HLA class I protein expression and the presence of CD8-positive T-cells were analyzed in the microsatellite unstable ECs. JAK1 mutant microsatellite unstable ECs showed impaired upregulation of LMP7 ($P=0.074$) and HLA class I ($P<0.001$), validated using RNAseq data of the TCGA. TAP1 expression and presence of CD8-positive T-cells were not related to JAK1 mutations. In 198 additional microsatellite unstable ECs, the JAK1 mutation frequency was confirmed but no prognostic significance was found. For, JAK1 wildtype ($n=135$, 72%) and mutant ($n=52$, 28%) ECs, 10-year recurrence free rates were 84% and 77% ($P=0.301$). These observations show that JAK1 mutations are highly frequent in microsatellite unstable EC, not associated with survival, but are associated with impaired upregulation of LMP7 and HLA class I and may therefore facilitate immune escape.

INTRODUCTION

About 30% of endometrial cancers, predominantly of endometrioid histology, can be molecularly characterized by microsatellite instability (MSI). MSI is a hypermutable phenotype caused by the loss of DNA mismatch repair (MMR) activity mostly due to sporadic MLH1 promoter hypermethylation.¹ Tumors that exhibit this phenotype have numerous insertions and deletions also in coding microsatellites causing frameshift mutations and loss of protein function. The coding microsatellite-containing genes frequently affected by MSI are believed to be involved in progression of MSI tumors.² Some target genes, such as BAX, are altered in diverse MSI tumor types (e.g. colorectal- and ovarian cancer), whereas others, such as JAK1, have a very restricted occurrence in MSI endometrial cancers.³⁻⁶ MSI endometrial cancers show a remarkably high number of JAK1 frameshift mutations that may have clinical implications.^{4,5}

JAK1 plays a role in the JAK/STAT pathway, which is activated by cytokines such as IFN γ , that influence several cellular processes such as cell growth and immune response.⁷⁻⁹ Ren et al. have shown that JAK1 mutant gynecological cancer cell lines were defective in interferon gamma (IFN γ) induced STAT1 tyrosine phosphorylation and thereby impede upregulation of antigen processing machinery components such as LMP2 and TAP1.¹⁰ Impaired antigen processing and presentation due to hindered expression of LMP and/or TAP proteins are associated with lack of HLA class I upregulation and resistance to cytotoxic T-cell mediated lysis.^{11,12} HLA class I expression has been reported as a prognostic marker in endometrial cancer patients.¹³⁻¹⁵ and upregulation of HLA class I was frequently impaired in MSI endometrial cancers.¹⁴⁻¹⁶ The high rate of JAK1 mutations in MSI endometrial cancer is suggestive of an adaptation favoring tumor survival by blocking the JAK/STAT pathway activity, and impeding an adequate immune response.

MSI tumors exhibit a high number of somatic mutations that could facilitate an immune response by presentation of neo-antigen-epitopes in the context of HLA class I molecules. Programmed death 1 expressed on cytotoxic T-cells is a checkpoint involved in immune suppression. Checkpoint inhibitors, as potential mechanism for T-cell activation, recently showed promising results in treatment of mismatch repair deficient tumors independent of tumor origin.¹⁷ However, JAK1 mutations and other mechanisms involved in impeding antigen presentation and expression of antigen processing machinery components in MSI endometrial cancers may interfere with new treatment regimens for MSI tumors such as the programmed death 1 inhibitor pembrolizumab.^{17,18}

In this study, MSI and JAK1 mutation status were analyzed in a study cohort of 181 tissue samples of endometrial cancer patients with the aim to evaluate that the JAK1 locus is frequently affected by MSI, and to determine its functional implication in immune evasion by analyzing expression of antigen presenting machinery components and the presence of cytotoxic T-cells specifically in MSI endometrial cancers. Finally, the effect of JAK1 mutation status on survival was evaluated in a large independent cohort of 198 MSI endometrial cancer patients with mature long-term follow-up from the PORTEC-1 and -2 clinical trials.^{19,20}

METHODS

PATIENTS

DNA analysis and immunohistochemical staining was performed on a study cohort of 181 endometrial cancers with endometrioid histology, treated at the University Medical Center Groningen between 1985-2004 or at the University Medical Center Leiden between 2000-2013. Classification and grading was done according to the World Health Organization criteria and staging was according to FIGO guidelines (2009). No follow-up data was available for this study cohort.

To validate our findings regarding the JAK1 mutation frequency in MSI endometrial cancer, an independent cohort of 198 MSI early-stage endometrial cancers derived from the randomized PORTEC-1 and -2 clinical trials was used.^{19,20} To estimate the impact of JAK1 mutation on survival the same cohort of 198 MSI endometrial cancers was used.²⁶

MSI AND JAK1 MUTATION STATUS

DNA was isolated as previously described.²⁷ The MSI status of each tumor was determined using the Promega MSI analysis system (version 1.2, Promega). Tumors with instability in at least two markers were defined as being high-frequency MSI whereas those showing no instability or instability in one marker were classified as being stable (MSS). JAK1 frameshift mutations (k142fs, p430fs, k860fs) were detected by Sanger sequencing. The following primers were used: exon 5-F: 5'-GTCACATCTGGGTCCCCTTTGCCAC-3', exon 5-R: 5'-CACAACTCCAGTTCTCTCTGGGC-3', exon 9-F: 5'-GTCGAGGAGGCCTGTCCTTTGTGC-3', exon 9-R: 5'-ACACGGGCTCTCTGCACACC-3', exon 19-F: 5'-GTATCGACTGCCTTTCACTCTG-3', exon 19-R: 5'-CTTACCTCTCCCAAGTCACGG-3'.

IMMUNOHISTOCHEMISTRY

Formalin-fixed paraffin embedded 4-µm tissue sections of MSI endometrial cancers (n=58) with sufficient tumor tissue were immunohistochemically stained for expression of LMP7, TAP1, HLA class I (HCA2 and HC10) and CD8 (marker of cytotoxic T-cells). Sections were deparaffinized in xylene, rehydrated in graded concentration of ethanol and microwave antigen retrieval was performed in 10 mM citrate pH 6.0 (LMP7, TAP1), 10 mM Tris/1 mM EDTA pH 9.0 (LMP7, TAP1, HCA2, HC10 and CD8) before staining. Endogenous peroxidase was blocked by incubation in a 0.3% hydrogen peroxide solution. LMP7 and TAP1 were stained using anti-LMP7 mouse monoclonal 1B3 (Novus Biologicals) and anti-TAP1 rabbit polyclonal H300 (Santa Cruz) as primary antibodies by incubation overnight at 4°C (dilution 1:100 and 1:50 respectively). HLA class I was stained using HCA2 and HC10 as previously described.^[16] Antigen-antibody reactions were visualized using 3,3'-diaminobenzidine (DAB) and slides were counterstained with hematoxylin.

EVALUATION OF IMMUNOHISTOCHEMISTRY

Two observers blinded to clinicopathological features, MSI and JAK1 mutation status independently evaluated the stained slides. Expression of LMP7 and TAP1 was scored using a semiquantitative scale as described previously.^{13,28} This score is based on the percentage of cells stained and the intensity of staining. The percentage of cells was scored on a 6 point scale with 0 for 0%, 1 for 1-5%, 2 for 5-25%, 3 for 25-50%, 4 for 50-75% and 5 for 75-100%. The intensity was scored on a 4 point scale with 0 indicating absence of staining and 3 indicating strong staining. The expression of LMP7, and TAP1 was categorized in impaired (score 0-2), normal (score 3-6) and upregulated (score 7-8) expression. For analysis of HLA class I expression, the percentage of tumor cells with membranous HCA2 and HC10 staining was quantified as previously described.¹⁶ The expression of HLA class I was defined as follows: impaired HLA class I expression: less than 5% of tumor cells expressing both HCA2 and HC10, normal HLA class I expression: less than 5% of tumor cells expressing either of the markers, and upregulated HLA class I expression: 5% or more expressing both markers.

The number of CD8-positive T-cells was calculated using the average number of stained cells in 8 fields at 40x magnification. The average was calculated for four locations: intraepithelial at the tumor center, intraepithelial at the invasive margin, intrastromal in the tumor center and intrastromal at the invasive margin. For statistical analysis values for CD8 were dichotomized using the median as a cut off.

THE CANCER GENOME ATLAS (TCGA) RNASEQ DATA

Details of the TCGA RNAseq analysis have been previously reported.¹ Level 3 RSEM normalized RNA data profiled using the Illumina HiSeq RNAseq v2 were retrieved at the TCGA data portal. MSI events, differences in length of microsatellites, in 30 MSI EC patients were reported by Kim et al.⁴ In total, 25 MSI endometrial cancers with both RSEM normalized and DNA slippage event data were informative for analysis.

STATISTICAL ANALYSIS

JAK1 mutation status was compared between cases with and without microsatellite instability using Chi-square tests. Similarly, Chi-square tests were used to detect differences in expression of LMP7, TAP1, HLA class I and number of CD8-positive T-cells below or above the median for cases with and without JAK1 mutation. The non-parametric Mann-Whitney test was used for all comparisons of continuous data and Spearman's rho to analyze correlation between variables. RNAseq data was visualized by unsupervised clustering using RStudio.

To evaluate the impact of JAK1 mutation status on survival in MSI endometrial cancer patients that participated in the randomized PORTEC-1 and -2 clinical trials, time-to-event analyses were calculated from the date of randomization to date of recurrence (vaginal, pelvic and/or distant recurrence) or to date of death (overall survival); patients who were alive and without recurrence were censored at the date of

last follow-up. Survival curves were calculated using the Kaplan–Meier method with log-rank test.

Analyses were performed using SPSS (v20, IBM statistics, Chicago, IL, USA).

RESULTS

Of the 181 endometrial cancers from the study cohort, MSI was detected in 62 (34%) cases, in nine cases MSI status remained unclear due to technical failure. Twenty-two (35%) MSI endometrial cancers had a JAK1 frameshift mutation, mainly at position K860, whereas only 3 of 110 (3%) MSS endometrial cancers had a JAK1 mutation ($P<0.001$, Supplementary Table 1). Two of these three JAK1 mutant MSS cases showed focal loss of MLH1 protein expression in part of the tumor as a result of MLH1 promoter hypermethylation. There were no significant differences in age, FIGO stage, differentiation grade or tumor type between JAK1 wildtype and mutant MSI endometrial cancers (Table 1). However, JAK1 mutations were associated with deeper myometrial invasion ($P=0.030$; odds ratio 3.500, 95% confidence interval 1.102–11.116).

The functional implication of JAK1 in tumor immune evasion was analyzed by expression analysis of TAP1, LMP7, HLA class I and presence of CD8-positive T-cells in the MSI endometrial cancers (Table 2). Distribution of TAP1 expression was similar for both JAK1 wildtype and mutant ($P=0.151$). Upregulation of LMP7 was impaired in JAK1 mutant tumors, although not statistically significant ($P=0.074$). Upregulation of HLA class I was significantly impaired in JAK1 mutant tumors ($P<0.001$). The expression of

Table 1. Clinicopathological characteristics of 58 MSI endometrial cancers of the study cohort and 187 MSI endometrial cancers of the PORTEC cohort according to JAK1 mutation status.

	Study cohort		<i>p</i> value	PORTEC cohort		<i>p</i> value
	JAK1wt n=36 (62%)	JAK1mut n=22 (38%)		JAK1wt n=135(72%)	JAK1mut n=52(28%)	
Age			1.000			0.914
≤ 60 years	18 (50.0)	11 (50.0)		19 (14.1)	7 (13.5)	
>60 years	18 (50.0)	11 (50.0)		116 (85.9)	45 (86.5)	
Tumor type			0.430			0.534
Endometrioid	35 (97.2)	22 (100)		134 (99.3)	52 (100)	
Serous	1 (2.8)	0 (0.0)		1 (0.7)	0	
FIGO (2009)*			0.650			0.270
-Stage 1	22 (61.1)	11 (52.4)		135 (100)	52 (100)	
-Stage 2	3 (8.3)	4 (19.0)		0	0	
-Stage 3	10 (27.8)	5 (23.8)		0	0	
-Stage 4	1 (2.8)	1 (4.8)		0	0	
Differentiation grade*			0.885			0.725
-Grade 1	13 (36.1)	7 (31.8)		77 (57.0)	33 (63.5)	
-Grade 2	14 (38.9)	10 (45.5)		31 (23.0)	10 (19.2)	
-Grade 3	9 (25.0)	5 (22.7)		27 (20.0)	9 (17.3)	
Invasion myometrium			0.030			0.172
<Half	21 (55.3)	6 (28.6)		45 (33.3)	12 (23.1)	
>Half	15 (41.7)	15 (71.4)		90 (66.7)	40 (76.9)	

* one missing value in this cohort. FIGO=International Federation of Gynecology and Obstetrics, JAKwt=JAK wildtype, JAKmut=JAK mutation, n=number.

Table 2. Expression of antigen processing machinery components TAP1, LMP7 and HLA class I in JAK1 wildtype and mutant MSI endometrial cancers.

	JAK1wt N=36 (62%)	JAK1mut N=22 (38%)	<i>p</i> value
TAP1*			0.151
-Impaired/Normal	24 (68.6)	18 (85.7)	
-Upregulated	11 (31.4)	3 (14.3)	
LMP7*			0.074
-Impaired/Normal	8 (22.9)	10 (45.5)	
-Upregulated	27 (77.1)	12 (54.5)	
HLA class I			<0.001
-Impaired	7 (19.4)	12 (54.5)	
-Normal	3 (8.3)	6 (27.3)	
-Upregulated	26 (72.2)	4 (18.2)	

* 1 missing value. JAKwt=JAK wildtype, JAKmut=JAK mutation, n=number.

HLA class I was related to LMP7 expression in contrast to TAP1 ($P=0.001$ vs. $P=0.381$). Presence of CD8-positive T-cells was not related to JAK1 mutation (Figure 1).

In order to validate these findings in an independent cohort, RNAseq data was used from 25 TCGA MSI endometrial cancers with analyzed DNA slippage events. The 13 JAK1 mutant endometrial cancers showed significantly lower expression of TAP1 (2.1-fold, $P<0.001$), LMP7 (3.0-fold, $P<0.001$), and HLA class I (2.5-fold, $P<0.001$) in comparison to JAK1 wildtype endometrial cancers (Figure 2). Consistent with the results in our study cohort, JAK1 mutation status did not correlate with CD8 expression ($P=0.112$).

Two patient cohorts derived from the PORTEC-1 and -2 randomized trials with MSI early-stage endometrial cancers ($n=198$) with long-term mature follow-up data were used to investigate a possible prognostic effect of JAK1 frameshift mutations. In this independent cohort, fifty-two (28%) of MSI endometrial cancers had a JAK1 mutation (Supplementary table 1). No significant differences were found between JAK1 mutation status and clinicopathological characteristics, and the association of JAK1 mutation and deep myometrial invasion could not be confirmed (Table 1). This discrepancy could be explained by the fact that this cohort consisted of significant more tumors with deep myometrial invasion compared to the study cohort (Supplementary Table 2). For JAK1 wildtype and mutant endometrial cancers, 10-year recurrence free rates were 84% versus 77%, respectively ($P=0.301$) and 10-year overall survival was 64.4% and 63.5% ($P=0.716$) (Figure 3). Neither did subanalysis (e.g. in grade 3 cancers, or when analyzing only pelvic and distant recurrences) show a significant difference in outcome between JAK1 wildtype and mutant endometrial cancer patients. Survival analysis within the TCGA microsatellite unstable endometrial cancers with known JAK1 mutation status ($n=30$) showed neither a survival benefit for JAK1 wildtype tumors (data not shown).

Figure 1. Quantification of CD8-positive T-cells in intraepithelial and intrastromal components in the center of the tumor (CT) and invasive margin (IM) in JAK1 wildtype (WT) and mutant (MUT) MSI endometrial cancers.

Figure 2. Heatmap of RNA expression of genes encoding for antigen machinery components. Case numbers 1-12 represent JAK1 wildtype and 13-25 JAK1 mutant MSI endometrial cancers. A broader analysis independent of MSI status was previously shown by Kim et al.

DISCUSSION

This research shows that frameshift mutations in JAK1 frequently and almost exclusively occur in MSI endometrial cancers. JAK1 mutations were associated with impaired upregulation of antigen presenting machinery proteins LMP7 and HLA class I. The association of JAK1 mutations with limited expression of the antigen presentation pathway was validated using RNAseq data of the TCGA MSI endometrial cancers. Impaired upregulation of HLA class I expression interferes with tumor lysis by cytotoxic T-cells, and therefore JAK1 mutations may facilitate an immune escape. However, no effect was observed of JAK1 mutation status on recurrence rate and overall survival in a large independent cohort of 198 MSI endometrial cancers. These findings suggest a functional role for JAK1, although with no prognostic value which suggests that JAK1 mutations are pivotal to cancer initiation and/or maintenance, in an intriguing tissue-specific manner.

The overall JAK1 mutation frequency of 28% in our large series of MSI endometrial cancers is in line with findings from two recent smaller studies.^{4,5} Both studies also showed that JAK1 frameshift mutations in MSI tumors are tissue specific and significantly less important in colorectal cancers as compared with endometrial cancers. Similarly, Ren et al. identified JAK1 frameshift mutations mainly in gynecological cancers, primarily in endometrial and cervical cancer, when sequencing more than 3,000 tumors from various human tissues.¹⁰ Altogether, the occurrence of JAK1 mutation specifically in endometrial cancer is suggestive of a positive selection for this mutation in endometrial cancer.

To date, there is very little evidence for the functionality of JAK1 frameshift mutations in endometrial cancer. An in vitro study with one endometrial cancer and

Figure 3. Clinical outcome of 198 MSI endometrial cancer stratified by JAK1 mutations status. A) Overall recurrence rate and B) Overall survival. WT= wildtype; MUT=mutant.

two ovarian cancer cell lines harboring JAK1 frameshift mutations demonstrated that JAK1 mutations impede STAT1 phosphorylation and upregulation of antigen presenting machinery components LMP2 and TAP1.¹⁰ Kim et al. have shown that JAK1 mutations were associated with hampered JAK/STAT signaling and lymphocyte activation.⁴ These findings suggest that JAK1 mutations have a negative effect on tumor immune surveillance. Our study now confirmed impaired upregulation of LMP7 and HLA class I with no effect on TAP1 expression and the number of CD8-positive T-cells in JAK1 mutant endometrial cancer tissue samples. The findings on protein expression were validated using RNAseq data of TCGA MSI endometrial cancers, except for TAP1. TAP1 gene expression may not be equal to its protein expression because of the small number of cases with RNAseq data (n=25) or due to the (post-)translational process into proteins. The lack of correlation between JAK1 mutations and CD8-positive T-cells might be explained by the fact that recruitment and migration of T-cells do not rely on recognition of peptides presented by HLA class I molecules. In addition, previous studies have also shown a high correlation between LMP7 expression and HLA class I expression, but not with LMP2 or TAP1/2.^{21,22} This further strengthens the argument that JAK1 mutations favor immune escape via the JAK/STAT signaling, although, this study did not evaluate the activation status of the JAK/STAT signaling by phospho-STAT1 expression.

The association of JAK1 mutations and lack of HLA class I upregulation in MSI endometrial cancers with no effect on clinical course was an unanticipated finding of our study. Bijen et al. and Yakabe et al. reported impaired upregulation of HLA class I as a prognostic marker for survival in endometrial cancer patients.^{13,15} Of note, a large proportion of HLA class I negative endometrial cancers are MSI.¹⁴ Therefore, separate analysis of MSI and MSS tumors would be of interest to determine the prognostic impact of HLA class I. This study indirectly showed no effect of HLA class I expression on survival via the JAK1 mutation status in a large cohort of MSI, early-stage endometrial cancers. HLA class I expression was not analyzed on the precious PORTEC tissue samples as this will only validate previous findings of Ren et al., the study and TCGA cohort and the fact that JAK1 mutations have no effect on survival will remain. However, the relatively good prognosis of this cohort of early-stage cancers needs to be taken into account. The previous studies were performed in heterogeneous groups of endometrial cancers consisting of different histologic subtypes and different FIGO stages.¹³⁻¹⁵ Therefore, the prognostic role remains to be determined in higher risk endometrial cancers. These results, in contrast to the finding that JAK1 mutations favor immune evasion, may suggest a lack of negative selection of JAK1 mutations specifically in endometrial cancer.

Considering the process of immune surveillance in cancer, it is likely that JAK1 mutant tumors are still recognizable to cells of the innate immune system (natural killer cells) or that JAK1 wildtype tumors have encountered other mechanisms to evade immune-mediated killing.^{11,12} However, low numbers of natural killer cells that lack an association with HLA class I expression were observed in endometrial cancer (unpublished data by Versluis et al.). On the contrary, IFN γ production might be responsible for a CD4-positive T-cell-mediated antitumor immunity.²⁴ Furthermore, JAK1 wildtype tumors may not demonstrate a survival benefit because of other strategies to dampen immune response such as upregulation of anti-apoptotic molecules, expression of immune-

inhibitory ligands or secretion of immunosuppressive cytokines. Nevertheless, our findings imply that JAK1 mutations may exert in part its oncogenic effects by immune escape, but we cannot exclude other contributions of JAK1 in the JAK/STAT signaling.⁷

Better understanding of the antigen-specific immune responses and tumor microenvironment may guide immunotherapy. Recently, immune checkpoint blockade were reported as promising therapies for tumors with a high mutational load, including mismatch repair deficient endometrial cancers, as a result of an increased neo-antigen specific T-cell response.¹⁷ JAK1 mutations in MSI endometrial cancers may interfere with the T-cell response due to impaired HLA class I or PD-L1 expression.^{17,18} In melanoma, one patient without a clinical response to PD-1 blockade and increased T-cell response showed a loss of function JAK1 mutation that unables PD-L1 upregulation. All other fifteen melanoma patients did not show genetic alterations in the interferon receptor signaling pathway.¹⁸ These limited data suggest that JAK1 mutations may be used as negative selective predictive biomarker for immune blockade therapy. However, additional studies are required on the immune microenvironment of JAK1 mutant endometrial cancers (e.g. PD-L1 expression).

We have identified a high frequency of JAK1 mutations in MSI endometrial cancers in two relatively large series of MSI endometrial cancers. In addition, JAK1 mutations may have a negative effect on tumor immune surveillance due to lack of HLA class I upregulation on the cell surface. It must be noted that it remains unclear why JAK1 mutations are limited to gynecological cancers and mainly to MSI endometrial cancer. However, B2M frameshift mutations, also leading to immune escape via loss of HLA class I expression, frequently occur in MSI colorectal cancer and are a rare phenomenon in MSI endometrial cancer.^{14,24} In addition, Xiong et al. showed that JAK1 inhibition is associated with cell cycle arrest and apoptosis in colorectal cancer.²⁵ No effect on cell viability upon IFN γ was found in JAK1 mutant gynecological cell lines.¹⁰ In conclusion, we confirmed the remarkably high frequency of JAK1 mutations and associations with impaired upregulation of antigen presenting machinery components in MSI endometrial cancers, which suggest a functional role for JAK1 in an intriguing tissue-specific manner.

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SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. JAK1 mutation frequency in 181* endometrial cancers and 198 MSI endometrial cancers.**

	MSI n=62 (36.0%)	MSS n=110 (64.0%)	p value
JAK1 mutation status			<0.001
-Wildtype	40 (64.5)	107 (97.3)	
-K142fs	1 (1.6)	1 (0.9)	
-P430fs	8 (12.9)	0	
-K860fs	11 (17.8)	2 (1.8)	
-P430fs & k860fs	2 (3.2)	0	
	MSI N=187 (%)		
JAK1 mutation status			
-Wildtype	135 (72.2)		
-K142fs	1 (0.5)		
-P430fs	7 (3.7)		
-K860fs	40 (21.4)		
-K142fs & K860fs	1 (0.5)		
-P430fs & k860fs	3 (1.6)		

* Failed analysis for 9 cases. ** Failed analysis for 11 cases. n=number.

Supplementary Table 2. Comparison of the clinicopathological characteristics of the MSI endometrial cancers included in the study cohort and PORTEC cohort.

	Study cohort N=62 (100%)	PORTEC cohort N=198 (100%)	p value
Age			<0.001
≤ 60	30 (48.4)	27 (13.6)	
> 60	32 (51.6)	171 (86.4)	
FIGO 2009			<0.001
-Stage 1	33 (57.9)	198 (100)	
-Stage 2	7 (12.3)	0	
-Stage 3	15 (26.3)	0	
-Stage 4	2 (3.5)	0	
Invasion myometrium			0.025
<half	29 (47.5)	63 (31.8)	
>half	32 (52.5)	135 (68.2)	
Differentiation grade			0.560
-Grade 1	21 (33.9)	116 (58.6)	
-Grade 2	27 (43.5)	44 (22.2)	
-Grade 3	14 (22.6)	38 (19.2)	
Tumour type			0.384
-Endometrioid	61 (98.4)	197 (99.5)	
-Serous papillary	1 (1.6)	1 (0.5)	

FIGO=International Federation of Gynecology and Obstetrics, n=number.

CHAPTER 05

L1CAM EXPRESSION IN UTERINE CARCINOSARCOMA IS LIMITED TO THE EPITHELIAL COMPONENT AND MAY BE INVOLVED IN EPITHELIAL-MESENCHYMAL TRANSITION

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ABSTRACT

Uterine carcinosarcoma (UCS) has been proposed as a model for epithelial-mesenchymal transition (EMT), a process characterized by a functional change facilitating migration and metastasis in many types of cancer. L1CAM is an adhesion molecule that has been involved in EMT as a marker for mesenchymal phenotype. We examined expression of L1CAM in UCS in a cohort of 90 cases from four different centers. Slides were immunohistochemically stained for L1CAM and scored in 4 categories (0%, <10%, 10-50% and >50%). A score of more than 10% was considered positive for L1CAM. The median age at presentation was 68.6 years and half of the patients (53.3%) presented with FIGO stage 1 disease. Membranous L1CAM expression was positive in the epithelial component in 65.4% of cases. Remarkably, expression was negative in the mesenchymal component. In cases where both components were intermingled expression limited to the epithelial component was confirmed by a double stain for L1CAM and keratin. Expression of L1CAM did not relate to overall or disease free survival. Our findings suggest L1CAM is either not a marker for the mesenchymal phenotype in EMT, or UCS is not a good model for EMT.

INTRODUCTION

Uterine carcinosarcomas (UCS) is a rare subtype of endometrial cancer (EC) with a poor prognosis. Approximately 60% of cases present with advanced disease and recurrence occurs in approximately 50% of patients.¹⁻⁴ Accordingly, 5-year survival ranges from 33-39%. Histology is peculiar because it contains both an epithelial as well as a mesenchymal component.¹⁻⁵ Various models have been proposed to explain this feature ranging from collision of two separate and original components to conversion of a common monoclonal neoplasm of epithelial (carcinomatous) origin.⁵⁻⁸ Currently, most studies support the theory of conversion of an endometrial carcinoma into a cancer consisting of an epithelial and a mesenchymal component.

Because UCS contains both an epithelial and mesenchymal component, UCS has been suggested as a model for epithelial-mesenchymal transition (EMT).^{5,9-14} EMT is a process that is characterized by a functional change in tumor cells that facilitates migration and metastasis.^{11,15} By loss of cell polarity and cell-cell junctions, and by reorganization of their cytoskeleton, tumor cells acquire the ability to migrate. This transformation is thought to be essential to metastasis in gynecologic cancers as well as other tumors.^{13,16} In a study on 76 cases of UCS, Castilla et al found a different expression of epithelial and mesenchymal markers between the two components. Expression of E-cadherin for example was limited to the epithelial component.

A possible marker for a mesenchymal phenotype is L1 cell adhesion molecule (L1CAM), a transmembrane adhesion molecule important for embryonic development.¹⁷ The extracellular domain interacts with other binding molecules and integrins expressed on either the same or other cells. The cytoplasmic tail mainly interacts with cytoskeletal proteins. Several studies support involvement of L1CAM in EMT.¹⁸⁻²¹ Exposure of both endometrial cancer (EC) and breast cancer cell lines to transforming growth factor B1 (TGF-B1), a known inducer of EMT, results in increased expression of L1CAM.^{19, 21} Huszar et al. found that increased expression of L1CAM relates to reduced membranous expression of E-cadherin. L1CAM could be a marker for a mesenchymal phenotype of EMT in UCS.

Expression of L1CAM previously described as immunohistochemical staining of more than 10%, is absent in normal endometrium.²² In addition, expression of L1CAM is related to metastasis and poor prognosis in many cancers, including EC.^{17,22-28} In early stage EC with endometrioid histology, expression of L1CAM is related to unfavorable pathological findings, distant recurrence and poor survival.^{22,23} Correspondingly, in high grade EC and non-endometrioid EC (NEEC), L1CAM expression is related to unfavorable pathological findings such as advanced stage and lymphovascular space invasion (LVSI) as well as distant recurrence but not survival.²⁶⁻²⁸

We hypothesize that L1CAM could be involved in EMT in UCS as a marker for the mesenchymal phenotype and may relate to metastasis. Therefore, we aimed to evaluate L1CAM expression in a large cohort of 90 UCS patients assembled in 4 oncologic centers participating in the European Network for Individualized Treatment of Endometrial Cancer (ENITEC) collaboration. Expression was evaluated separately in the epithelial and the mesenchymal component and related to Patient and tumor characteristics and survival.

METHODS

STUDY POPULATION

4µm formalin fixed slides were assembled from 4 separate centers (Haukeland University hospital Bergen, Norway; University hospital Lleida, Spain; Leiden University Medical Center, The Netherlands; and University Medical Center Groningen, the Netherlands). Sufficient material from 88 primary tumors and 10 metastases was available. With an overlap of 8 cases, this added up to a total of 90 cases with UCS diagnosed between 1980 and 2012 to be included in this study. Data concerning age at diagnosis, International Federation of Gynecology and Obstetrics (FIGO) stage as well as Patient and tumor characteristics concerning age at diagnosis, histology of both components (homologue or heterologue for the epithelial component), differentiation grade, LVSI, were delivered by each participating center. All material was revised at the University Medical Center Groningen by HH and MV blinded from findings at the primary center. For some cases clinicopathological information was missing and could not be retrieved from the slides. For example when information on lymphovascular space involvement was missing. Follow up data was provided by each center in an anonymized dataset and completed until October 2014. Datasets were combined into a final password-protected database. Patient identity was protected by study specific patient numbers. Informed consent was obtained according to local protocol in each participating center.

HISTOLOGY

Slides, 4µm formalin fixed paraffin embedded sections were immunohistochemically stained as described by Bosse et al 2014.²³ "Briefly, formalin fixed paraffin-embedded (FFPE) tissue blocks were cut into 4 µm slides and mounted on Starfrost slides. Antigen retrieval was achieved by microwave oven procedure in 10 mmol/L citrate buffer, pH 6. Sections were incubated overnight with primary monoclonal antibodies against L1CAM (CD171, clone 14.10, SIG-3911, Convince Inc., lot number D13KF03087, Biolegend, San Diego, USA) in a 1:500 dilution. Sections were incubated and stained for 30 min using a secondary antibody (Poly-HRP-GAM/R/R; DPV0110HRP; Immunological Immunological, Duiven, The Netherlands). The slides were counterstained with Mayer's Haematoxylin, dehydrated and mounted. Omission of the primary antibody was used as a negative control and a highly L1CAM-expressing serous ovarian cancer as an external positive control."

In 8 cases it was difficult to differentiate between L1CAM expression by the epithelial or mesenchymal components as both components were intermingled. Therefore, these selected cases were double stained for both L1CAM and cytokeratin-8 as expression of cytokeratin is limited to the epithelial component. Tissue sections were dewaxed in xylene and hydrated in a graded series of alcohol to tap water. Antigen retrieval was performed in 10 mM EDTA pH 8.0 in a microwave at 400 Watt for 15 min. Endogenous peroxidase was blocked by incubating sections for 30 minutes

in 1% H₂O₂. Primary antibody against L1CAM (anti-CD171, Biolegend, Sig-3911-1000, San Diego, USA) was applied in a 1:500 dilution and left to incubate overnight at 4°C. Subsequently, tissue sections were incubated with secondary antibody (RAM-HRP; Dako, Glostrup, Denmark) and tertiary antibody (GAR-HRP; Dako, Glostrup, Denmark), both diluted at 1:100, for 30 minutes each at room temperature. L1CAM staining was visualized with 3,3'-diaminobenzidine. After washing the sections in demineralized water, they were incubated with the antibody against cytokeratin-8 (CAM 5.2, BD Biosciences, #345779) diluted 1:5 for 1 hour at room temperature. Sections were then incubated with secondary antibody (RAMbio; Dako) and tertiary antibody (streptavidin-AP; Dako), both diluted 1:300, for 30 minutes each at room temperature. Cytokeratin staining was visualized using Fast Blue BB/Naphthol-AS-MX-phosphate (Sigma, F3378) for 30 minutes at room temperature. Sections were rinsed with demineralized water and mounted with Kaiser's glycerol gelatine (109242; Merck). Omission of the primary antibody was used as a negative control and tonsil tissue as a positive control.

EVALUATION OF IMMUNOHISTOCHEMISTRY

Evaluation of immunohistochemistry was performed by two independent investigators (MV and AP), blinded for clinical outcome data. Expression of L1CAM in either component was scored as the percentage of positive membranous staining and categorized into 4 groups: 0%; 0-10%; 10-50% or >50% as described by Meier et al.²⁹ Conform previous publications, tumors with more than 10% expression of L1CAM were considered positive.^{23,29} There was no disagreement in evaluation of the mesenchymal component. There was disagreement in evaluation of 21 cases of the carcinomatous component mainly on a score of either 3 or 4. Disagreement was resolved by consensus. Location of L1CAM staining was scored as expression at the tumor center, tumor margin or diffusely over the tumor.

STATISTICAL ANALYSIS

All statistical analyses were done by using SPSS (version 23, IBM statistics, Chicago USA). For analysis, age was dichotomized into 65 and below versus above 65 years as this is an important cut-off in treatment of endometrial cancer. Recurrence was classified as local, pelvic or distant. Disease free survival (DFS) was defined as time until any recurrence or death of disease, disease specific survival (DSS) as time until death of disease. Associations between patient and tumor characteristics were compared using logistic regression analyses. For survival analysis, a relation between L1CAM expression and DFS, DSS or distant recurrence was examined using Cox regression analyses. Patients without evidence of disease were censored at last date of follow up.

RESULTS

POPULATION

Tissue from the primary tumor was available in 88 cases. In 10 cases, metastatic tissue was available. With an overlap of 8 cases (primary as well as metastatic tissue), this added up to a total of 90 cases to be evaluated. Not all tissue contained both components. The epithelial component was available in 81 cases, the mesenchymal component in 83 cases. Patient- and tumor characteristics are shown in table 1. The median age at diagnosis was 68.6 years (IQR 62.0-75.5). A small majority of cases presented with FIGO stage 1 disease (53.3%), most other cases presented with stage 3a or 3c disease (14.4 vs 12.2 %). LVSI was observed in 53.8%, and non-endometrioid histology of the epithelial component was 56.5%.

IMMUNOHISTOCHEMISTRY

Figure 1 shows examples of L1CAM expression in both components. Expression was not limited to either the tumor center or margin.

Table 2 shows the results of L1CAM staining. The epithelial component was L1CAM positive in a majority of cases (65.4%). Remarkably, the mesenchymal component

Table 1. Patient and tumor characteristics in 90 cases of uterine carcinosarcoma (UCS).

	n	% or IQR
Median age (years)	68.6	62.0-75.5
FIGO stage (2009)		
-I & II	50	55.4
-III & IV	40	44.4
Myometrial invasion		
<half	43	53.1
>half	38	46.9
-Unknown		9
Lymph node status		
-Negative	36	78.3
-Positive	10	21.7
-Unknown		44
Lymphovascular space invasion (LVSI)		
-No	24	36.4
-Yes	42	63.6
-Missing		24
Differentiation grade carcinoma component		
-Grade 2	6	8.3
-Grade 3	63	87.5
-Undifferentiated	3	4.2
-Unknown		18
Histologic type carcinoma component		
-Endometrioid	27	39.1
-Non-endometrioid	42	60.9
-Unknown		21
Histologic type sarcoma component		
-Homologue	39	53.4
-Heterologue	34	46.6
-Unknown		17

n=number, *IQR*=Interquartile Range.

Figure 1. L1CAM staining in carsinosarcoma. (A) L1CAM expression scored as 0%, (B) L1CAM expression scored as less than 10%, (C) L1CAM expression scored as 10-50%, (D) L1CAM expression scored as more than 50%. All figures are presented at x10 magnification.

Table 2. Expression of L1CAM in both components in 88 primary UCS tumors and 7 metastatic tumors

	Epithelial component, primary tumor n(%)	Mesenchymal component, primary tumor n(%)	Epithelial component, metastasis n(%)	Mesenchymal component, metastasis n(%)
0% expression	16 (20.2)	72 (86.7)	1 (12.5)	4 (80)
<10 % expression	11 (15.2)	11 (13.3)	3 (37.5)	1 (20)
10-50% expression	22 (27.8)		4 (50.0)	
>50% expression	30 (38.0)			
Component not available	9	5	2	5

n=number

Figure 2. Examples of double stain for L1CAM (brown) and keratin (blue). (A) HE stain of case 8. This case was originally scored as L1CAM expression >50% in the epithelial component and 0% in the mesenchymal component. (B) double stain for L1CAM and keratin in case 8. (C) HE stain for case 9. This case was originally scored as L1CAM expression >50% in the epithelial component and 0% in the mesenchymal component. (D) double stain for L1CAM and keratin in case 9. (E) HE stain for case 14. This case was originally scored as L1CAM expression >50% in the epithelial component and <10% in the mesenchymal component. (F) double stain for L1CAM and keratin in case 14. All figures are presented at x10 magnification.

was determined as negative (<10%) for L1cam in all cases. In 86.7% no expression was found. In 13.3 %, L1CAM expression was scored less than 10 percent. In some of these cases, both components were intermingled and therefore difficult to distinguish. To differentiate between L1CAM expression by either component, a double stain for L1CAM and keratin was performed in 8 cases. In all 8 cases, L1CAM expression was limited to cells expressing keratin. Figure 2 shows examples of double staining for L1CAM.

PATIENT AND TUMOR CHARACTERISTICS IN RELATION TO L1CAM EXPRESSION

Table 3 shows patient and tumor characteristics stratified for L1CAM expression. Logistic regression showed no relation between patient- or tumor characteristics and L1CAM expression. L1CAM expression Survival in the overall cohort was poor with a median DFS of 2.83 years (95%CI 0.80-4.87) and DSS of 3.30 (1.10-5.50). Known prognostic variables related to DFS in univariate cox regression analysis: FIGO stage (HR 1.67 95%CI 1.30-2.14), myometrial invasion (HR 1.80 95%CI 0.96-3.34) and differentiation grade (HR 3.81 95%CI 1.13-12.80). LVSI was not a significant predictor for DFS. Findings were similar for DSS. Expression of L1CAM did not relate to either DFS (HR 1.48 95%CI 0.74-3.00) or DSS (HR 1.48 95%CI 0.74-3.00). There was also no relation between L1CAM expression and distant recurrence (HR1.02 95%CI 0.45-2.30).

Table 3. Patient and tumor characteristics and L1CAM expression. Data exclude missing values.

	L1CAM negative n(%)	L1CAM positive n(%)	Odds ratio (95% CI)
Age			
≤ 65	12 (44.4)	19 (37.3)	Reference
>65	15 (55.6)	32 (62.7)	0.74 (0.29-1.19)
FIGO stage (2009)			
-I & II	18 (66.7)	28 (53.8)	Reference
-III & IV	9 (33.3)	24 (46.2)	0.58 (0.22-1.54)
Myometrial invasion			
<half	14 (53.8)	26 (54.2)	Reference
>half	12 (46.2)	22 (45.8)	1.01 (0.39-2.64)
Lymph node status			
-Negative	14 (87.5)	21 (72.4)	Reference
-Positive	2 (12.5)	8 (27.6)	0.38 (0.07-2.03)
Lymphovascular space invasion (LVSI)			
-No	13 (58.1)	29 (67.4)	Reference
-Yes	9 (40.9)	14 (32.6)	1.43 (0.50-4.15)
Differentiation grade carcinoma component			
-Grade 2	3 (13.0)	3 (7.0)	Reference
-Grade 3	18 (18.3)	39 (90.7)	2.17 (0.40-11.80)
-Undifferentiated	2 (8.7)	1 (2.3)	0.50 (0.03-8.92)
Histologic type carcinoma component			
-Endometrioid	11 (52.4)	15 (35.7)	Reference
-Non-endometrioid	10 (47.6)	27 (64.3)	0.5 (0.17-1.46)
Histologic type sarcoma component			
-Homologue	12 (50.0)	24 (57.1)	Reference
-Heterologue	12 (50.0)	18 (42.9)	1.33 (0.49-3.65)

n=number

DISCUSSION

L1CAM is frequently expressed in UCS, but expression in UCS is limited to the epithelial component. Expression does not relate to patient characteristics, recurrence or survival.

Expression of L1CAM in UCS is in accordance with other studies on L1CAM expression in aggressive histologic subtypes of EC such as non-endometrioid EC. These studies describe an L1CAM expression of more than 55% and no relation with survival.^{22,23,27} We also find a high percentage of L1Cam positive tumors and no relation between L1CAM expression and survival. A strong relation between expression and survival is therefore unlikely. An explanation for the absence of a relation could be the already poor survival in cohorts of NEEC and the and the high expression of L1CAM in NEEC as compared to EEC as suggested by van der Putten et al.²⁷ Remarkably, L1CAM expression is not expressed in the mesenchymal component. Our working hypothesis was that L1CAM expression could act as a marker for a mesenchymal component in UCS. Several other studies describe increased expression of L1CAM after induction of EMT. In endometrial cancer cell lines, TGF- β 1 induces expression of L1CAM and EMT.^{19,21} Exposure to TGF- β 1 results in increased expression of L1CAM through upregulation of the EMT transcription factor Slug. In turn, expression of L1Cam relates to cell migration and invasion.²¹ TGF- β 1 induced L1CAM expression is related to decreased expression of E-Cadherin and E-cadherin expression is limited to the epithelial component in UCS.^{10,19} Considering our findings, L1CAM is either not a marker for the mesenchymal phenotype in EMT, or UCS is not a good model for EMT.

However, this does not exclude a relevant contribution of L1CAM expression in an early stage of EMT where cancer cells gain motility and invasive properties. This could be by direct function as an adhesion molecule or indirect by acting as a signaling molecule.^{17,20} At a later stage, motility and invasive properties may become less relevant when tumor cells form a secondary tumor.

A strength of this study is a large cohort of 90 UCS cases made possible through an international collaboration. The resulting population covers well the diversity of this type of cancer as can be seen in table 1. Another strength is the stepwise approach with a double stain for cases where histology proved difficult. A limitation is the retrospective nature of the study.

In conclusion, we describe a large cohort of 90 cases with UCS where the majority of tumors, stained positive for L1CAM but where expression is limited to the epithelial component. L1CAM could be involved in development of UCS and EMT at an early stage, but is not a marker for the mesenchymal phenotype. Our study provides further insight into the possible mechanism of EMT and metastasis. Expression of L1CAM did not relate to unfavorable patient and tumor characteristics, recurrence or survival

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COMPLIANCE WITH ETHICAL STANDARDS

The experiments reported here were carried out in agreements with the Declaration of Helsinki principles and in agreement with local legislation at each participating center.

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CHAPTER 06

LYMPHADENECTOMY AND ADJUVANT THERAPY IMPROVE SURVIVAL IN UTERINE CARCINOSARCOMA, A LARGE RETROSPECTIVE COHORT STUDY

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ABSTRACT

OBJECTIVE

Uterine carcinosarcoma is a rare, aggressive subtype of endometrial cancer. Treatment consists of hysterectomy, bilateral salpingo-oophorectomy and lymphadenectomy. The survival benefit of lymphadenectomy in relation to adjuvant radio- and/or chemotherapy, is unclear. We evaluated the impact of lymphadenectomy on survival in relation to adjuvant therapy in uterine carcinosarcoma.

METHODS

Retrospective data of 1140 cases was combined from the Netherlands Cancer Registry and the Dutch nationwide pathology network (PALGA). LND was defined as removal of any nodes. Additionally, cases where ten nodes or less ($LND \leq 10$) or more than 10 nodes ($LND > 10$) were removed, were analyzed separately. Adjuvant therapy was evaluated as radiotherapy, chemotherapy or radiochemotherapy. Associations were analyzed by chi-square, log rank testing and cox regression analysis.

RESULTS

Overall survival (OS) was improved for TAH-BSO with $LND > 10$ (HR 0.62 95%CI 0.47-0.83). Adjuvant therapy related to OS with an HR 0.64 (95%CI 0.54-0.75) for radiotherapy, an HR 0.65 (95%CI 0.48-0.88) for chemotherapy and an HR 0.25 (95%CI 0.13-0.46) for radiochemotherapy. Additionally, adjuvant treatment related to OS when lymph nodes are positive (HR 0.22 95%CI 0.11-0.42), but not when nodes are negative.

CONCLUSION

Lymphadenectomy relates to improved survival when more than 10 nodes are removed. Adjuvant therapy improves survival when LND is omitted, or when nodes are positive.

INTRODUCTION

Uterine carcinosarcoma (UCS) is a rare and aggressive histological subtype of endometrial cancer (EC). Incidence is approximately 5 cases per 1.000.000 person years, and 5-year survival is between 32-39%.¹⁻⁴ Primary treatment consists of a total abdominal hysterectomy with bilateral salpingo-oophorectomy (TAH-BSO) and lymphadenectomy (LND).

LND as a treatment tool is still under debate.⁴⁻⁶ Some relatively small studies describe no benefit of LND.^{7,8} Several larger retrospective cohort studies describe an improved survival for patients in whom LND was performed.^{2,9,10} This is in accordance with studies on other aggressive subtypes of EC like high grade endometrial and serous endometrial cancer. Two of these studies evaluated the number of nodes that were removed in relation to survival as it seems plausible that there may not be an optimal treatment effect when only a few nodes are removed. Nemani et al found no significant difference between removal of more or less than 12 nodes.² Conversely, Temkin et al found survival to be improved when more than 11 nodes are removed.⁹

A limitation of the studies mentioned above is that findings were not corrected for adjuvant radio- and/or chemotherapy.^{2,9,10} This may influence findings as adjuvant radiotherapy (RT), chemotherapy (CT) and combined radiochemotherapy (RCT) may improve survival in UCS patients.¹¹⁻¹⁴ It is therefore unknown how LND, radiotherapy and chemotherapy together relate to survival in UCS.

The aim of this study is to evaluate the role of LND with or without radio- and/or chemotherapy in a large retrospective cohort of 1140 patients diagnosed with UCS undergoing primary surgery with a curative intent.

METHODS AND MATERIALS

DATA COLLECTION AND STUDY POPULATION

Retrospective data were obtained from the Netherlands Cancer Registry (NCR) and the nationwide network and registry of histo- and cytopathology in the Netherlands (PALGA).¹⁵ The NCR contains data on all newly diagnosed cancers, including patient, tumor and treatment characteristics. Data of the NCR were combined with data from PALGA by a coding system connecting the two databases. Data were delivered in password protected sets, not traceable to the individual person. According to Dutch law, no further ethical approval is required. Patients with UCS were included between January 1, 1993 and December 31, 2012. Follow-up was completed until December 31, 2013. Inclusion criteria were patients diagnosed with uterine carcinoma as identified with corresponding ICD-O-3 codes (C54; C55 combined with morphological codes 8950, 8951 or 8980). In total, 1310 patients were identified to be diagnosed with UCS according to NCR database. However, 170 patients did not undergo primary surgery defined as TAH-BSO with or without LND, leaving 1140 patients available for further analysis.

DATA PROCESSING

Both sources, NCR and PALGA, were combined into one database. Inconsistencies between PALGA and NCR were resolved using PALGA as reference as this dataset reflects most accurately the pathology rapport. An exception was made for disease stage as the pathology review lacks information on lymph node status when LND was omitted. Therefore, disease stage was classified according to the FIGO 2009 classification and based on NCR. Distant metastasis was defined as distant metastasis including intra-abdominal metastasis, to be described at the time of UCS-related surgery. Recurrence of disease after surgery needed to be confirmed by histology. Lymphadenectomy was defined as removal of any number of nodes (LND). To further evaluate the relevance of the number of nodes removed, a distinction was made between removal of ten lymph nodes or less ($LND \leq 10$) and removal of 11 nodes or more ($LND > 10$). Adjuvant treatment was documented as radiotherapy, chemotherapy or radiochemotherapy. There was no information as to the timing of adjuvant treatment with respect to other treatment. Age at diagnosis was divided into 70 and below or 71 and above in line with previous publications.¹⁻⁴

STATISTICAL ANALYSIS

For descriptive statistics the median and interquartile range (IQR) where appropriate are given. Overall survival (OS) was defined as time until death with a maximum of five years. Disease free survival (DFS) was defined as time until recurrence or death with a maximum of five years. The chronological order of events for patients starts with surgery, followed by pathology review and possibly adjuvant treatment. Therefore we first analyzed the value of LND followed by analysis of all three types of adjuvant treatment (RT, CT and RCT). Finally we evaluated the value of adjuvant treatment in relation to lymph node status for cases where LND was performed. FIGO stage, presence of metastasis, myometrial invasion and age were included into multivariable analysis. To minimize the chance of bias, clinicopathological variables with more than 10% missing values such as lymphovascular space invasion were excluded from the survival analysis. Statistical analysis was performed with SPSS 22 (IBM, USA, Chicago). Relations between variables were tested by Chi-square testing. Log rank and Cox regression analysis were used for survival analysis and calculation of hazard ratios (HR). P values of 0.05 or less were considered significant.

RESULTS

PATIENT CHARACTERISTICS

Median age at diagnosis was 70 years (IQR 62-77). The clinicopathological characteristics are shown in table 1. The majority (64%) of patients was diagnosed with early stage disease (FIGO 1-2). In 247 (21.7%) patients, lymph nodes were removed in addition to a TAH-BSO. The median number of nodes removed was 12 (IQR 3-18, data

Table 1. Clinicopathological characteristics for 1140 patients with uterine carcinosarcoma stratified by type of surgery

	TAH-BSO n=893 (%)	TAH-BSO and LND n=247 (%)	p value
Age			<0.001
≤ 70	418 (46.8)	148 (59.9)	
> 70	475 (53.2)	99 (40.1)	
FIGO 2009			<0.001
-Stage 1	539 (63.5)	131 (54.1)	
-Stage 2	47 (5.5)	7 (2.9)	
-Stage 3	138 (16.3)	82 (33.9)	
-Stage 4	125 (14.7)	22 (9.1)	
Unknown	44	5	
Invasion myometrium			0.355
<Half	417 (53.1)	111 (49.6)	
>Half	369 (46.9)	113 (50.4)	
Unknown	107	23	
Lymph node status*			
-Negative	-	172 (69.2)	
-Positive	-	75 (30.4)	
Distant metastasis			0.070
-No	748 (88.1)	225 (92.2)	
-Yes	101 (11.9)	19 (7.8)	
Unknown	44	3	
Lymphovascular invasion			0.064
-No	92 (26.4)	50 (34.7)	
-Yes	257 (73.6)	94 (65.3)	
Unknown	544	57	
Adjuvant Therapy			<0.001
-None	443 (49.6)	75 (30.4)	
-Radiotherapy	358 (40.1)	126 (51.0)	
-Chemotherapy	73 (8.2)	31 (12.6)	
-Chemoradiation	19 (2.1)	15 (6.1)	

TAH-BSO = hysterectomy and bilateral salpingo-oophorectomy; LND = lymphadenectomy with removal of any number of nodes; n=number. Percentages were calculated without missing values.

not shown). LND related to higher FIGO stage and age below 70 ($P<0.001$). In 75 out of 247 cases nodes were positive. When comparing $LND\leq 10$ with $LND>10$, the percentage of positive nodes was not significantly higher when less nodes were removed (35% versus 25%, $P=0.106$). Patients from whom lymph nodes were removed more often received adjuvant treatment ($P<0.001$).

Table 2 shows clinicopathological characteristics stratified for adjuvant treatment. Of 622 patients that received adjuvant treatment, 77.8% received radiotherapy (RT), 16.7% received chemotherapy (CT) and 5.5% received combined radiochemotherapy (RCT). RT consisted of external beam RT (EBRT) in 90.1% of cases. Adjuvant treatment related to age above 70, increased FIGO stage, myometrial invasion, lymph node status, metastasis as well as lymphovascular space involvement (LVSI).

SURVIVAL ANALYSIS

In the complete cohort of 1140 cases, median OS was 2.03 years (95% CI 1.76-2.30) and median DFS was 1.53 years (95% CI 1.32-1.76). Histologically proven recurrence was present in 302 (26.5%) cases. Distant recurrence was more common than pelvic or

Table 2. Clinicopathological characteristics for 1140 patients with uterine carcinosarcoma stratified for adjuvant treatment

	No adjuvant treatment n=518 (%)	Adjuvant RT n=484 (%)	Adjuvant CT n=104 (%)	Adjuvant RCT n=34 (%)	p value
Age					<0.001
≤ 70	205 (39.6)	291 (60.1)	74 (71.2)	28 (82.4)	
> 70	313 (60.4)	193 (35.9)	30 (28.8)	6 (17.6)	
FIGO 2009					<0.001
-Stage 1	317 (64.7)	334 (71.5)	8 (8.0)	11 (33.3)	
-Stage 2	20 (4.1)	31 (6.6)	2 (2.0)	1 (3.0)	
-Stage 3	81 (16.7)	84 (18.0)	37 (37.0)	17 (51.5)	
-Stage 4	72 (14.7)	18 (3.9)	53 (53.0)	4 (12.1)	
Unknown	28	17	4	1	
Invasion myometrium					0.034
<Half	256 (56.8)	223 (50.2)	36 (42.9)	13 (41.9)	
>Half	195 (43.2)	221 (49.8)	48 (57.1)	18 (58.1)	
Unknown	67	40	20	3	
Lymph node status*					<0.001
-Negative	53 (70.7)	103 (81.7)	9 (29.0)	7 (46.7)	
-Positive	22 (29.3)	23 (18.3)	22 (71.0)	8 (53.3)	
Not Sampled	443	358	73	19	
Distant metastasis					<0.001
-No	421 (81.3)	424 (87.6)	26 (25.0)	22 (64.7)	
-Yes	97 (18.3)	60 (12.4)	78 (75.0)	12 (35.3)	
Unknown	0	0	0	0	
Lymphovascular invasion					<0.001
-No	70 (35.9)	62 (27.3)	5 (9.4)	5 (27.8)	
-Yes	125 (64.1)	165 (72.7)	48 (90.6)	13 (72.2)	
Unknown	323	257	51	16	

RT = radiotherapy; CT = chemotherapy; RCT = radiochemotherapy; n = number. Percentages were calculated without missing values

Figure 1. Median Overall Survival (OS) (95%CI) in years for 1140 patients with uterine carcinosarcoma (UCS) according to treatment. TAH-BSO = hysterectomy and bilateral salpingo-oophorectomy; LND = lymphadenectomy with removal of any number of nodes; n=number.

local recurrence (55.6% versus 10.9% and 32.1% respectively $P<0.01$, data not shown). Frequency and location of recurrence were not related to FIGO stage or age ($P>0.05$).

Figure 1 provides a general overview of the distribution of various treatment modalities next to the median OS per subgroup. As expected, median OS was better for cases where lymph nodes were removed and turned out negative. In this group there was no difference in median OS for cases that did or did not receive adjuvant treatment (Median OS: 4.15 95%CI 2.83-6.20 and 5.00 95%CI 4.49-5.51 respectively). When nodes were positive, median OS was much shorter with 0.60 years (95%CI 0.09-1.10) for cases that did not receive adjuvant treatment and 2.37 years (95%CI 1.52-3.12) for cases that did receive adjuvant treatment. Median OS for patients with surgery limited to TAH-BSO was also shorter with 1.36 (95%CI 1.04-1.68) years for patients without adjuvant treatment and 2.01 years (95%CI 1.74-2.46) for patients with adjuvant treatment. Supplementary figure 1 shows similar results for median DFS with a significant effect of adjuvant treatment for cases where no nodes were removed or nodes turned out to be positive.

UNIVARIATE SURVIVAL ANALYSIS

Figure 2 shows Kaplan Meier curves for OS according to extent of surgery and adjuvant treatment. LND related to improved OS (logrank $P<0.001$, figure 2A). Figure 2B shows survival according to the extent of surgery with a distinction between $LND\leq 10$ and $LND>10$. Survival for TAH-BSO and $LND\leq 10$ was similar to survival for TAH-BSO without LND. $LND>10$ related to improved survival. As a cut off of 10 nodes is arbitrary we also analyzed OS for different cut off values (removal of 8 or 12 nodes) and found similar results (data not shown).

Figure 2C zooms in on 893 cases where surgery was limited to TAH-BSO and illustrates an advantage of adjuvant treatment in this subgroup ($P<0.001$). In univariate Cox regression analysis, RT and RCT but not CT related to improved OS (HR 0.64 95%CI 0.55-0.75; HR 0.32 95%CI 0.18-0.57 and HR 1.20 95%CI 0.95-1.52 respectively, $P<0.001$). On the other hand, when LND was performed and nodes were positive, adjuvant treatment also related to improved survival (logrank $P<0.001$, fig 2D). For cases where nodes were negative, there was no relation between adjuvant treatment and survival.

Of the clinicopathological variables with less than 10% missing values, lower FIGO stage (HR1.47 95%CI 1.39-1.57), lesser myometrial invasion (HR1.86 95%CI 1.69-2.16), no distant metastasis (HR2.69 95%CI 2.19-3.31), and age below 70 (HR 2.16 95%CI 1.59-2.11) related to improved OS.

MULTIVARIABLE ANALYSIS

Table 3 shows the results for multivariable analysis for OS in 1140 cases that received TAH-BSO with or without LND. Corrected for adjuvant therapy, FIGO stage, age below/above 70, myometrial invasion, distant metastasis, $LND>10$ was an independent predictor for OS (HR 0.65 95%CI 0.48-0.87). $LND\leq 10$ did not relate to OS (HR 0.83 95%CI 0.65-1.05). Adjuvant therapy also related to improved OS. Radiotherapy and

Figure 2. Kaplan Meier for Overall Survival (OS) according to treatment. A) OS and extent of surgery TAH-BSO or TAH-BSO with lymph node dissection (LND) (n=1140). B) OS and extent of surgery TAH-BSO, TAH-BSO and removal of 10 nodes or less ($LND \leq 10$) or removal of more than 10 nodes ($LND > 10$) (n=1140). C) OS and adjuvant treatment for surgery limited to TAH-BSO (n=893). D) OS and adjuvant therapy for cases with lymph node dissection, nodes positive (n=75). E) OS and adjuvant treatment for cases with lymph node dissection, nodes negative (n=172)

Table 3. Multivariable Cox regression analysis for overall survival extend of surgery, adjuvant therapy and clinicopathological variables n=1140

	HR (95%CI)	p value
Extend of surgery		
-TAH-BSO	Reference	
-TAH-BSO and LND≤10	0.83 (0.65-1.05)	0.124
-TAH-BSO and LND>10	0.67 (0.50-0.89)	0.006
Adjuvant Therapy		
-None	Reference	
-Radiotherapy	0.65 (0.55-0.77)	<0.001
-Chemotherapy	0.66 (0.49-0.89)	0.006
-Chemoradiation	0.25 (0.14-0.47)	<0.001
Age		
≤ 70	Reference	
> 70	1.58 (1.35-1.84)	<0.001
FIGO 2009		
-Stage 1	Reference	
-Stage 2	1.60 (1.13-2.29)	0.009
-Stage 3	2.17 (1.76-2.68)	<0.001
-Stage 4	2.48 (1.69-3.65)	<0.001
Invasion myometrium		
<Half	Reference	
>Half	1.61 (1.36-1.90)	<0.001
Distant metastasis		
-No	Reference	
-Yes	1.47 (0.99-2.18)	0.054

TAH-BSO = hysterectomy and bilateral salpingo-oophorectomy; LND≤10= lymph node dissection with 10 nodes or less; LND>10= lymph node dissection with more than 10 nodes; n=number, HR = Hazard Ratio.

chemotherapy had a similar HR of 0.64 (95%CI 0.54-0.75) and 0.65 (95%CI 0.48-0.88) respectively. Radiochemotherapy had a HR of 0.25 (95%CI 0.13-0.46). Results were similar for DFS with LND>10 and adjuvant treatment relating to improved DFS (data not shown)

Table 4 shows the subgroup analyses stratified for lymph node status. In accordance with findings in univariate analysis, adjuvant treatment did not relate to OS when nodes were negative. However, when nodes were positive, adjuvant treatment related to improved OS with HR 0.17 (95%CI 0.07-0.39) for RT; HR 0.40 (95%CI 0.19-0.84) for CT and HR 0.04 (0.03-0.18) for RCT.

DISCUSSION

In this large cohort study, LND relates to improved survival specifically in those cases where more than 10 nodes are removed. Adjuvant therapy improves survival when LND is omitted with a similar effect for either RT or CT. Possibly, the combination of RCT has a cumulative effect. When LND is performed, adjuvant treatment relates to improved survival when nodes are positive but not when nodes are negative.

The finding that removal of lymph nodes improves survival is in accordance with previous publications.^{2,5,6,10} In the largest study, Nemani et al, evaluated the role of lymph node dissection in 1855 patients with stage I-III UCS using data from the Surveillance, Epidemiology, and End Results (SEER) program in the United States. For

the 57% of cases where lymph nodes were removed, overall survival was improved. However, there was no relation between the number of nodes removed and survival when a cut off of 12 nodes was used. This is different from our findings where we describe a different outcome between $LND \leq 10$ and $LND > 10$. This may be due to case selection as lymph nodes were removed in less cases (22%) in our cohort. In our cohort nodes were positive in 30% of cases where this was 14% in the SEER cohort. Temkin et al describe 47 cases of UCS where at least 1 node was removed and also found improved survival for cases where more than 11 nodes were removed (9). Improved survival for the subgroup $LND > 10$ could be explained by a higher probability of removal of all metastatic nodes when more than 10 nodes are removed. Another explanation could be that these patients are cared for by more specialized surgeons or cancer centers.

When LND is performed, adjuvant treatment may improve survival when nodes are positive, but not when nodes are negative. Apparently cases with positive nodes are at increased risk of recurrence despite LND. This finding corresponds to the recent findings of the PORTEC-3 trial that investigated the benefit of adjuvant treatment in other histologic types of high risk endometrial cancer.¹⁶ A clinical consequence could be to omit adjuvant treatment when nodes are negative. Preferably, these findings are confirmed in a randomized trial on adjuvant treatment in UCS. Nonetheless, it is an argument in favor of LND for staging purposes at this point. As to the type of adjuvant treatment, Wright et al evaluated adjuvant RT in 1819 patients with early stage UCS also from the SEER database.¹² In accordance with our findings, adjuvant RT improved overall survival, but only in cases where a LND had been omitted. In another study including 2461 cases from the SEER database adjuvant treatment related to improved survival for cases with advanced disease.¹¹ This may be because of lymph node metastasis in advanced disease as we found an improved survival for patients with positive nodes. The relation remained significant in a multivariate analysis including FIGO stage.

A few small studies describe a possible survival advantage for adjuvant CT, similar to RT. A small retrospective study on 111 patients with early stage UCS, CT was related to improved survival.¹³ Survival was similar for CT and RT and further improved for RCT. In an even smaller sample of 49 cases, RCT had a positive effect on survival, similar to that of RT alone.¹⁷ In our large retrospective cohort we also found a survival advantage for CT when LND is omitted or nodes are positive. The hazard ratio's for both CT and RT are in similar range, in accordance with a randomized study comparing adjuvant radiotherapy with chemotherapy.¹⁸ This study randomized 232 patients with stage I-IV UCS and found no significant difference in survival.

As with other retrospective studies, our study has limitations related to study design. For example patients who received lymphadenectomy had FIGO IIIc in 25.1% of cases versus 1.1% of patients without LND ($P < 0.001$). Likely, this is because of upstaging for cases that received LND and this complicates comparison of these two groups. Additionally, the relationship between adjuvant treatment and survival in this cohort is similar for both RT and CT. However, the patient characteristics for these two groups are different. For example, the type of adjuvant therapy related to FIGO stage (table 2). Cases presenting with early stage disease more often received RT whereas CT was more common in cases presenting with advanced stage disease. Another issue is the cut off value of 10 nodes to differentiate between lymph node sampling and

debulking. Assuming that LND improves outcome, it is likely that this effect is related to the number of nodes removed. We set the cut off at ten nodes but we are aware that this is arbitrary. As mentioned above, we found a similar relation with survival when using a cut off of 8 or 12 nodes.

A strength for this study is the use of a large cohort of patients including information on both radio- and chemotherapy. In addition, the use of two national registries as a source for our data improves reliability of this data.

In conclusion, we describe a survival advantage for LND in patients with UCS. In our cohort, the survival benefit is limited to cases where more than 10 nodes were removed (LND>10). Adjuvant therapy improves survival when surgery is limited to hysterectomy with bilateral salpingo-oophorectomy. When LND is performed, the effect of adjuvant treatment is limited although adjuvant treatment does relate to improved survival when nodes are positive. Considering the type of adjuvant therapy, survival for RT and CT is similar whereas RCT may further improve survival. Our findings can be used in counseling of patients with newly diagnosed UCS where LND can improve survival especially where more than 10 nodes are removed. It remains unclear whether adjuvant treatment improves survival when lymph nodes are negative. When nodes are positive adjuvant treatment is likely to further improve survival.

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SUPPLEMENTARY TABLES AND FIGURES

Supplementary figure 1. Median disease free survival (DFS; 95%CI) according to treatment in 1140 patients with uterine carcinosarcoma (UCS). TAH-BSO = hysterectomy and bilateral salpingo-oophorectomy; LND = lymphadenectomy with removal of any number of nodes; n=number.

CHAPTER 07

IMPLICATIONS AND PERSPECTIVES

IMPLICATIONS AND PERSPECTIVES

This thesis describes the complex interplay between immunological, molecular and therapeutic mechanisms in EC. The current chapter discusses the implications and perspectives of the research in this thesis for future research and care for patients with EC. The first part of this chapter discusses the immunological and molecular mechanisms of EC relating to **chapters 2, 3 and 4**. In **chapter 2**, we found that CTL is a valuable marker to improve selection of patients for adjuvant treatment. In **chapter 3**, we described a new immunological mechanism in EC and illustrated a relation between HLA-E expression and survival depending on presence of NK-cells. In **chapter 4**, we demonstrated a relation between JAK1 mutations and impaired upregulation of antigen-presenting machinery in microsatellite unstable EC. The second part of this chapter discusses molecular and therapeutic aspects of uterine carcinosarcoma (UCS) relating to **chapters 5 and 6**. In chapter 5, we found expression of L1CAM to be limited to the epithelial component in UCS, an unexpected finding. In Chapter 6, we found further evidence supporting LND in the surgical treatment of UCS. The third part of this chapter discusses general implications of recent scientific developments concerning care for patients with EC as well as for cancer research. The chapter ends with some final remarks and a conclusion.

MOLECULAR AND IMMUNOLOGICAL ASPECTS OF ENDOMETRIAL CANCER

As mentioned in the introduction, a majority of patients with EC receiving adjuvant treatment do not benefit and there is a need to improve patient selection. In **chapter 2**, we investigated if the selection of patients for adjuvant therapy can be improved by the use of immunological markers. We assessed predictive value of intratumoral cytotoxic, regulatory and helper T-cells as well as HLA-1 expression for recurrence of disease compared to and in combination with classical predictors such as FIGO stage and lymphovascular space involvement (LVSI). The results show that the presence of tumor-infiltrating T-cells (CTL) is an important determinant to be included in prediction models for the time-to-recurrence. Of all the clinicopathological variables included in the analysis, a combination of FIGO stage, LVSI and tumor-infiltrating CTL best predicted recurrence. A higher FIGO stage, presence of LVSI and low numbers of tumor-infiltrating CTL related to a higher risk of regional and distant recurrence. The possibility of an immune model to predict recurrence has also been examined in colorectal cancer among other tumors.¹ In a follow up of their findings, this research group recently constructed an immunoscore assay predicting recurrence in colorectal cancer.² The immunoscore assay is based on the presence of cytotoxic T-cells in the tumor and in the invasive margin. In this well designed study, 1336 cases were randomly assigned to a training set or internal validation. Next, the findings were tested in an external validation set of 978 cases. Several models based on clinicopathological findings with or without an immunoscore were tested. Models with an immunoscore performed better in predicting disease free survival with a C-index of 0.66 (95% CI 0.61-0.71) and 0.67 (0.62-0.72) as compared to a C-index of 0.58 (95% CI 0.54-0.63) for a model without an immunoscore. The authors demonstrate that the immunoscore assay is a

reliable predictor of recurrence in colorectal cancer and suggest its implementation in the classification of colorectal cancer. Implementation would result in the use of this model in future RCTs to evaluate new treatment regimens for colorectal cancer followed by inclusion of an immunoscore in treatment guidelines and clinical practice.

Whether a predictive model in EC should include immunological markers, molecular markers or both remains a topic for further investigation. There is evidence suggesting that a molecular profile better relates to survival than an immunological profile in EC.³ However, immunological variables in this study were either added together in a cluster or used as a numerical variable. In addition, prediction of recurrence was not the aim of this study. Based on our research, the next step would be to confirm our findings in a retrospective cohort of EC patients taken from a RCT in EC as suggested in **chapter 2**. In addition to classical predictive variables, such as LVSI or FIGO stage, the molecular profile should be included in the analysis. Subsequently, a score can then be constructed based on the relevant variables and tested in a separate study. The study method would be comparable to the study performed in colorectal cancer. As for the immunological markers, the immunoscore in colorectal cancer uses both CTL in the tumor as well as in the tumor margin.² Although we did not analyze the predictive value of CTL in the invasive margin in **chapter 2**, intratumoral CTL alone may be a better candidate for a prognostic model. As mentioned in the introduction, immune surveillance depends on a successful chain of events and immune escape can occur at any one or more weak links. Intratumoral CTL alone may better represent CTL that contribute to tumor destruction and may better differentiate between cancer tolerance and immunity. Therefore, intratumoral CTL may be a better candidate for an immune model as these cells. In addition to CD8 as a marker for CTL, staining for CD103 can facilitate identification of intratumoral CTL. In a recent study by our group, CD8+CD103+ cells were preferentially located in the tumor.⁴ Staining for CD8 and CD103 would facilitate the scoring process and make implementation of a model more feasible. Another consideration is possible staining for activated or suppressed CTL. In theory, activated CTL would represent a favorable immune response and better predict cases that will not have a recurrence and could omit adjuvant therapy. Recent studies indicate CD39 and CD137 may be useful markers for activated CTL.^{5,6} Additionally, patients with suppressed CTL may benefit more from immunotherapy with an anti-PD-L1/PD-1 antibody instead of radiotherapy. It can even be considered to investigate immunotherapy administered before surgery as neoadjuvant treatment. A recent study shows that tumor response to checkpoint inhibitors is more favorable when immunotherapy is administered before surgery in stage III melanoma.⁷

Besides the use of immunological markers in patient selection for adjuvant treatment, understanding of immunological mechanisms in EC can provide options for new treatment. In **chapter 3**, we hypothesized that HLA-E affects survival in EC by interaction with CTL or NK-cells and evaluated expression of HLA-E, presence of NK-cells and CTL in relation to survival. We describe a relation between intratumoral NK-cells and survival, depending on HLA-E expression. In patients with upregulated HLA-E expression, presence of NK cells is related to improved survival in multivariate analysis. Conversely, in patients with normal HLA-E expression presence of NK cells related to poor survival. This interaction was not observed between HLA-E expression

and CTL. This illustrates the complexity of immune function and opens a new window for further research, for example into NK-cell immunotherapy.

Currently, immunotherapy is mainly focused on modulating activity of CTL and NK-cell immunotherapy is still in its infancy.^{8,9} Enhancement of a general NK-cell response using IL-2 has low response rates and frequent immune-related adverse effects. Similarly, although transfusion of NK-cells from a donor is safe, the response rate is limited by the poor capacity of NK-cells to persist in the recipient. Our findings underline the importance of a setting facilitating activated, intratumoral NK-cells to destroy tumor cells. Presence of intratumoral NK-cells alone may not be sufficient to destroy tumor cells in cases where NK-cell activity is suppressed by normal expression of HLA-E. Here, the possible use of anti-NKG2a antibodies as a new checkpoint inhibitor is of particular interest. Blocking of the NKG2a receptor could shift the balance and restore NK-cell activity in EC with normal HLA-E expression. In a recent study, anti-NKG2a antibodies promoted anti-tumor activity of both NK-cells and CTL in mouse tumor models.¹⁰ Currently, a phase II dose range study is investigating the use of an antibody for gynecologic malignancies including EC.¹¹ If this antibody proves beneficial for EC patients, it would be interesting to investigate the effect size in relation to HLA-E expression. Also, a possible effect of anti-NKG2a antibodies on CTL activity should be investigated. Even though there was no relation between HLA-E activity, presence of CTL and survival in our study, recent studies do suggest that anti-NKG2a antibodies can restore CTL activity independent of HLA-E expression.^{10,12}

Investigation of immunological mechanisms also requires research into the interaction between immunological and molecular mechanisms. Frequent mutations such as seen in MSI hypermutated EC might contribute to immune evasion by impairing antigen presentation and T-cell activity. **In chapter 4**, we examined the possibility that immune evasion is facilitated by mutations in JAK1 by the molecular analysis of JAK1 mutations and immunohistochemical staining of antigen processing machinery components such as LMP7, TAP1 and HLA-1 as well as presence of CTL. JAK1 mutations were frequent in MSI hypermutated EC and mutations related to impaired expression of HLA-1. Interestingly, JAK1 mutations occur predominantly in MSI hypermutated EC. This is suggestive of a clinical relevance of JAK1 mutations in disease course for this type of EC. MSI hypermutated EC are more immunogenic due to increased numbers of neoantigen production.¹³⁻¹⁵ This is supported by the observation of increased immune cell infiltration in MSI hypermutated EC compared to microsatellite stable EC.¹⁶ This increases a selective advantage for tumor cells that can avoid immune destruction, for example by JAK1 mutation. Although JAK1 mutations were not related to survival, mutations did relate to impaired expression of antigen processing machinery especially HLA-1. In turn, impaired HLA-1 expression is related to poor outcome as was previously reported.¹⁷ It could therefore be that our sample size was too small to show an effect of JAK1 mutation on survival. A future study investigating this theory in a larger sample is recommended. Conversely, it could be that JAK1 mutations are just one of several ways for tumors to avoid immune destruction. Immune escape mechanisms in MSI hypermutated EC other than JAK1 mutations would also explain the absence of a relation between JAK1 mutation and survival in our cohort. Future

studies should investigate the complex interplay between immunological and molecular mechanisms in larger samples, investigating more variables.

Such a study could investigate an alternative mechanism involving JAK1: a possible relation between JAK1 mutation and HLA-E expression or NK-cell activity as discussed in **chapter 3**. Activation of the JAK1/STAT1 pathway by interferon- γ (IFN- γ) not only increases expression of LMP, Tap and HLA-1 but also of HLA-E (18). This was not investigated in **chapter 4**. Therefore, we analyzed 40 overlapping cases of MSI hypermutated EC from the datasets in **chapter 3** and **4**. HLA-E expression was normal in 11 percent of JAK1 wildtype EC versus 31 percent in JAK1 mutated EC. The difference in this small sample was not significant. Although we only investigated HLA-E in a limited patient cohort to data, the vast increase in HLA-E expression in JAK1 mutated EC suggest disease relevance and warrants future investigation in a larger sample sizes and including other immunological variables.

MOLECULAR AND THERAPEUTIC ASPECTS OF UTERINE CARCINOSARCOMA (UCS)

UCS is a rare and aggressive subtype of EC with poor survival. Despite an urgent need to better understand molecular and therapeutic aspects, investigation has been limited due to the low incidence. UCS has a remarkable histology with both an epithelial and mesenchymal component. Also, it has a high rate of metastasis in which EMT could play an important role. With its remarkable histology, UCS could provide a model for EMT and this was studied in **chapter 5** where L1CAM expression as a possible marker for the mesenchymal component was investigated. L1CAM is frequently expressed in UCS but expression is limited to the epithelial component in primary tumors as well as in metastasis, an unexpected finding. L1CAM is not a marker for the mesenchymal phenotype in UCS and whether UCS is a suitable model for EMT remains to be seen.

Further investigating EMT is important as it relates to both metastasis and response to therapy and could provide points of engagement for development of new treatments. Understanding EMT in the context of UCS can still contribute to this and future research should investigate expression of markers for both phenotypes as well as transcription factors involved in EMT induction such as SNAIL 1 and 2, ZEB 1 and 2 and TWIST. There are several issues relevant to these new studies. Firstly, EMT may not be a one-way transformation, but a transitional process where cells move through a spectrum of intermediary phases.¹⁹ Recent evidence suggests that cells oscillate between an epithelial and mesenchymal phenotype directed by epigenetic, transcriptional and post transcriptional mechanisms. Intermediate phenotypes have also been demonstrated. For example, reversible shifts between both phenotypes were observed in circulating tumor cells of breast cancer patients.²⁰ The bimodal histology of UCS with 2 distinctive components suggests that there may not be a clear EMT spectrum observable in UCS. Secondly, EMT is increasingly perceived as a focal, rather than global, event.¹⁹ Therefore, essential observations could be missed when investigating single tumor sections. To increase understanding of UCS within the context of EMT, further investigations could evaluate the process of EMT as well as the relation to treatment response. An alternative area of interest is a relation between

EMT and immunology. There is recent evidence suggesting involvement of EMT in immune escape.²¹ For example, in non-small cell lung cancer (NSCLC) loss of antigen presentation is related to a mesenchymal phenotype.²² In addition, a mesenchymal phenotype is related to decreased lysis by NK-cells.²³

In order to better understand the therapeutic aspects in UCS, we investigated the survival benefit of LND in UCS, a treatment that is still under debate. In **chapter 6**, treatment outcome after surgery with or without lymphadenectomy in relation to adjuvant radio- and chemotherapy was evaluated. LND improves survival when more than 10 nodes are removed. Adjuvant treatment was related to improved survival when lymph nodes were positive or when LND was omitted. Other retrospective studies that evaluate the role of LND for this specific patient population have similar findings.²⁴⁻²⁷ It could be argued that the level of evidence of these retrospective cohort studies is higher than that of RCTs, despite possible selection bias. The available RCTs do not specifically investigate a survival benefit of LND in UCS.^{29,29} In UCS, it seems advisable to extend surgery with LND for staging as well as survival purposes until better evidence to the contrary is presented. This could come from the STATEC trial that is ongoing.³⁰ This RCT randomizes patients with a high risk of recurrence (grade 3 endometrioid EC, serous EC, clear cell EC, undifferentiated EC and UCS) who will undergo surgery with or without LND. Depending on the lymph node status of trial participants, adjuvant treatment is determined. Even though the findings of this study may be extrapolated to UCS, the study is not designed to specifically address this subgroup. This is with good reason, as the low incidence of UCS would be a challenge to such an RCT. This challenge can be addressed using information technology and collaboration between institutions as is discussed in the next part of this chapter.

IMPLICATIONS FOR CARE AND RESEARCH

Contemporary care for patients with EC was described in the introduction and is summarized in figure 1a. In short, three phases can be distinguished: diagnosis, surgical treatment and adjuvant treatment. In the diagnostic phase, a biopsy provides information on histology and differentiation grade. Primary treatment consists of hysterectomy and BSO with or without LND and provides additional information on FIGO stage and LVSI. Adjuvant treatment can consist of radio- and/or chemotherapy depending on patient selection, based on pathologic findings.

Based on current knowledge and the research in this thesis, future care for patients with EC can become much more differentiated. Selection of patients for treatment can be improved by taking into account molecular and immunological markers. New treatment options such as immunotherapy will likely become standard care. Checkpoint inhibitors like anti-PD-L1/PD-1 antibodies may just be the start of many new treatment options aimed to correct a specific defect in immune surveillance. New treatments can be developed to correct other defects and a combination of these therapies can be used to correct several defects. As our understanding of the immunological and molecular aspects develops, options to influence these mechanisms will multiply. Just like the amount of pixels on our television screens have increased over the years, providing us with ever more detailed images and increased

Figure 1A. Summarized overview of contemporary care for patients with endometrial cancer. TAH-BSO = hysterectomy and bilateral salpingo-oophorectomy.

understanding of immunological and molecular aspects of EC will allow us to zoom in on the specific needs of the individual patient.

This is summarized in figure 1b. In comparison to the current situation in figure 1a, the possibilities at diagnosis, surgical treatment and adjuvant treatment are likely to increase. Additionally, neoadjuvant treatment, treatment after diagnosis and before surgery, may be implemented.

An example of further improving patient selection for surgical treatment is already provided by the ongoing PORTEC4-a trial.³¹ This trial investigates a molecular classification of EC into 4 categories to select patients for surgery with or without lymphadenectomy based on information obtained from endometrial biopsy. In theory, a new classification could be used in combination with an immunoscore. However, it is important to realize that reliability of an immunological assessment of a biopsy may be limited by a sampling error. Currently, pathologic assessment of endometrial biopsies is concordant with post-operative findings in 90%.³² Distribution of immunological predictors, such as CTL in a tumor, is heterogenous, further increasing the risk of sampling error.^{33,34} Besides information from endometrial biopsy, patient characteristics could contribute to patient selection and counseling. The transition from paper to electronic medical files allows for easy integration of data. This could include complex data such as an immune and/or molecular profile besides relatively simple variables such as age and body mass index.

Similarly, counseling for adjuvant treatment could be improved by including an immunological as well as a molecular profile in addition to classical clinicopathological

Figure 1B. Summarized overview of possible future care for patients with endometrial cancer. TAH-BSO = hysterectomy and bilateral salpingo-oophorectomy; FIGO=International Federation of Gynecology and Obstetrics; LVSI=lymphovascular invasion.

variables that are currently used. Finally, immunotherapy is added in figure 1b, as a possibility for both neoadjuvant and adjuvant treatment for EC. Patients with a specific molecular profile, such as MSI-hypermutated EC, or a specific immunological profile with intratumoral, suppressed CTL, could benefit from immunotherapy.³⁵⁻³⁷ There is limited evidence suggesting that immunotherapy is more effective when administered before surgical treatment.⁷

The improvement of patient stratification based on clinical findings, immunological and molecular biomarkers and the resulting choice in treatment options is a continuous development, greatly facilitated by current developments in information technology (IT). IT has shown rapid development in the past decades offering the possibility to process large quantities of cases and variables. Although a clear definition is lacking, this is often referred to as “big data”, and the number of studies analyzing datasets with large numbers of cases and variables is rising steeply.³⁸ Generalizing the use of electronic medical files offers the possibility of data-reuse, reanalysis and the generation of clinical studies with unprecedented sample sizes. Big data can be used to complement RCTs to investigate rare cancers, such as UCS. In addition, technologic development offers the possibility for molecular and immunological studies to investigate large numbers of variables in large sample sizes, enabling focus on complex interactions.

FINAL REMARKS

Besides an unprecedented impact on society, the technological developments are also likely to profoundly affect the position and role of researchers and physicians, and this warrants some final remarks. In his popular scientific books, Yuval Noah Harari speculates about the effects of technological development.³⁹ As technology allows us to become smarter and stronger, and live longer and healthier lives, several challenges arise. These challenges include how we will deal with the limited resources of our planet and who will have access to this technology and are important subjects for public debate. The scientific community should actively participate in this debate.

In regards to the position and role of health care workers, it is suggested that technology will increasingly outperform humans in the heuristics of medicine. In his latest book, Harari argues that computers will soon be able to decipher algorithms better than doctors.⁴⁰ Be it in 10 or 100 years, IT algorithms are likely to surpass humans in diagnosing, and possibly even in treating, patients. In addition, computers never tire and are not biased by emotion and, through the internet, computers are easily updated whereas humans are not. Although the effects of these developments are uncertain, it is clear that change will come and this will require physicians, scientists and humans alike to do what we are good at: adapt and collaborate. “The single greatest constant of history is that everything changes”, and we are masters of change.³⁹ In medicine, adaptation dictates that even more attention is paid to communication, leadership, collaboration, health advocacy et cetera, in addition to careful diagnosis and treatment. As technology develops, patients will look for guidance and for help, to make sense of all the available information, and choose the treatment that suits them specifically. Doctors already have an important role in this and this will become increasingly more important.

Adaptation and collaboration also implies that we change our research strategies. With the possibility of analyzing large data sets, comes the need to collaborate in data gathering and analysis. Even now, institutions that finance research increasingly require researchers to make datasets available to other researchers. Similarly, some scientific journals no longer require a subscription, but are digitally available as an open access journal. A next step would be to create open access datasets and biobanks to collaboratively make optimal use of available data and material.

A challenge for both medicine and research is to direct technological development in such a way that it does indeed live up to its promise and contributes to improved health care. For example, not all electronic medical file systems are similar and not all systems record the same information in a similar fashion. Furthermore, new reliable statistical and computational methods are needed that are suitable for analysis of big data. At the same time, patient confidentiality needs to be protected. If we want to benefit from the possibilities of technological development, these issues will need to be resolved.

CONCLUSION

In this thesis, we have shown that immunological markers can improve patient selection for adjuvant treatment in EC. We have deepened our understanding of immunological and molecular mechanisms in EC and UCS, as well as evaluated the value of LND in UCS. These investigations are a small part of a larger scientific progress that is already resulting in improved selection of patients for adjuvant treatment, and new, immunological, treatment options. As this progress occurs, the clinical research landscape is changing with technological developments, such as big data and global clinical collaborations. The future challenge, therefore, is not only to further increase our knowledge of immunological, molecular and therapeutic mechanisms in EC, but also to deal with the challenges that technological developments entail.

The way we will face these developments, will determine the future for patients with EC.

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CHAPTER 08

RESEARCH SUMMARY & NEDERLANDSE SAMENVATTING

RESEARCH SUMMARY

Improving outcome for patients with endometrial cancer (EC) has fueled research into immunological and molecular mechanisms that drive EC development, as well as into the treatment of EC. This has resulted in some exciting developments. Increased understanding of molecular mechanisms have resulted in a new classification of EC to better select and treat patients. Immunology is increasingly recognized as an important regulator of cancer development, and immunotherapy is becoming a standard treatment modality in a number of cancer types. The research reported in this thesis aimed to increase our understanding of the interplay between immunological, molecular and therapeutic mechanisms in endometrial cancer in order to further improve outcome in patients with endometrial cancer.

Endometrial cancer (EC) is the most common gynecological cancer in developed countries and 2050 new cases are diagnosed in the Netherlands each year. Currently, primary treatment consists of surgery including hysterectomy and bilateral salpingo-oophorectomy (BSO). Additional lymphadenectomy (LND) to determine spread of disease is important but also relates to complications. A therapeutic role of LND is subject to debate. After surgery, a selection of patients will be counseled for adjuvant treatment consisting of either radio- and/or chemotherapy. In order to optimize use of adjuvant treatment, it is important to select patients with a high risk of recurrent disease. Currently, a risk stratification is based on pathological findings and age. Pathological findings include differentiation grade, histological subtype and disease stage. Although the risk stratification is helpful in counseling of patients, many patients still receive unnecessary adjuvant treatment. Improved selection of patients for existing treatment options as well as development of new treatment options are pivotal to improve outcome. This has directed research to investigate molecular and immunological mechanisms and to evaluate existing treatment options. Traditionally, a distinction is made between type 1 and type 2 endometrial cancer based on histologic subtype and differentiation grade. However, molecular studies suggest that EC is more heterogenous and suggest classification into four categories: POLE ultramutated, microsatellite instability (MSI) hypermutated, copy-number low and copy-number high. Molecular classifications may be more useful in predicting recurrence and response to treatment. Immunological studies revealed several mechanisms relating to survival. A well-known example is the relation between intratumoral cytotoxic T-cells and improved survival. Conversely, tumors that are able to avoid immune destruction have a poor prognosis. Restoration of immune function by so-called checkpoint inhibitors is an exciting development. This immunotherapy is a new treatment option that has shown promising results for several cancers including EC. Improving treatment outcome requires further research into immunological, molecular and therapeutic aspects of EC, in order to improve selection of patients for treatment, and to develop new treatment options. The studies described in this thesis aim to contribute to this research.

In **chapter 2**, the selection of patient for adjuvant treatment, using immunological variables, was studied. Candidate predictors were selected from a pool of clinicopathological and immunological variables previously shown to relate to disease

recurrence. Predictive value for recurrence was evaluated in several statistical models. Of all the clinicopathological variables included in the analysis, a combination of disease stage, lymphovascular space involvement (LVSI) and tumor infiltrating CTL best predicted recurrence. This demonstrates that presence of tumor infiltrating T-cells (CTL) is an important candidate to be included in a prediction model to predict recurrence. Further research could aim to construct and implement an immunoscore to facilitate selection of patients for treatment.

In **chapter 3**, we investigated immunological variables relevant to disease course. Expression of Human Leukocyte Antigen E (HLA-E) is related to survival in solid tumors, such as colorectal and breast cancer. This relation could be mediated by CTL or NK-cells and could offer points of engagement for new treatment options. In this study, we evaluated expression of HLA-E, presence of NK-cells and CTL, in relation to survival in EC. In patients with upregulated HLA-E expression, presence of NK cells was related to improved survival. Conversely, in patients with normal HLA-E expression, presence of NK cells related to poor survival. This remarkable finding illustrates the complexity of immune function and offers opportunities for development of new immunotherapeutic modalities.

Chapter 4 studies the relation between molecular and immunological mechanisms in EC. Frequent mutations such as seen in MSI hypermutated EC could contribute to immune evasion by impairing antigen presentation and T-cell activity. We examined the frequency of mutation in the cytokine receptor Janus Kinase 1 (JAK1) in relation to expression of immune response proteins such as LMP7, TAP1 and Human Leukocyte Antigen class 1 (HLA-1) as well as presence of cytotoxic T-cells. JAK1 mutations were frequent in MSI hypermutated EC and mutations related to impaired expression of HLA-1. Therefore, JAK-1 mutations could be an important mechanism to avoid immune destruction in MSI-hypermutated EC. Future research should further determine the relevance of mutations of JAK-1 for immune escape.

In **Chapter 5**, we investigated molecular mechanisms in UCS. UCS has been suggested as a model for EMT and L1 cell adhesion molecule (L1CAM) could serve as a marker for the mesenchymal phenotype. Expression of L1CAM by both components was evaluated in a large international cohort of UCS cases. Expression was limited to the epithelial component in primary tumors as well as in metastasis. Therefore, L1CAM is either not a marker for the mesenchymal phenotype or UCS is not a suitable model for EMT. New studies can further investigate UCS as a model for EMT.

In **Chapter 6**, the extension of surgery for UCS is evaluated in a large retrospective pattern of care study. UCS is an aggressive subtype of EC that more often presents with lymph node metastasis and lymphadenectomy could offer a survival benefit. We evaluated surgery with or without lymphadenectomy, next to adjuvant radio- and chemotherapy in relation to survival in 1140 cases of UCS. LND related to improved survival when more than 10 nodes were removed. Adjuvant treatment was related to improved survival when lymph nodes were positive or when LND was omitted. It

seems advisable to extend surgery with LND for staging as well as survival purposes until better evidence to the contrary is presented.

The research in this thesis contributes to increased understanding of the immunological, molecular and therapeutic aspects of endometrial cancer. This field of research is rapidly evolving. In part, this is further facilitated by technological development and initiatives to share information. As a result, there is an opportunity to improve outcome for patients with endometrial cancer

NEDERLANDSE SAMENVATTING

Het verbeteren van de ziekte uitkomst voor patiënten met kanker is een belangrijke drijfveer voor kanker onderzoek. Dit boeiende onderzoeksveld heeft veel vooruitgang geboekt in de laatste decennia en ons begrip van de moleculaire mechanismen die een rol spelen bij kanker is enorm toegenomen. Daarbij wordt steeds duidelijker dat het immuunsysteem een belangrijke rol speelt bij het ontstaan en het beloop van kanker. Immunotherapie is een nieuwe vorm van therapie die steeds vaker wordt toegepast naast de reeds bestaande mogelijkheden van chirurgie en radio- en/of chemotherapie. Het onderzoek in dit proefschrift heeft tot doel de kennis van immunologische, moleculaire en therapeutische aspecten van baarmoederslijmvlieskanker te vergroten met het achterliggende streven de ziekte uitkomst te verbeteren.

Baarmoederslijmvlieskanker, of endometrium carcinoom (EC) is de meest voorkomende gynaecologische kanker in hoge inkomenslanden. Per jaar worden ongeveer 2050 vrouwen in Nederland gediagnosticeerd met de aandoening. De eerste behandeling bestaat op dit moment uit een operatie waarbij de baarmoeder, eileiders en eierstokken worden verwijderd. Het verwijderen van lymfklieren, ook wel lymfadenectomie genoemd, geeft belangrijke informatie over de mate waarin de ziekte is verspreid. Anderzijds, verhoogd een lymfadenectomie de kans op complicaties. Of een lymfadenectomie ook de overleving van patiënten verbetert staat ter discussie. Na een operatie kan aanvullende behandeling worden gestart in de vorm van radio- en/of chemotherapie om een recidief van de kwaadaardigheid te voorkomen. Beide vormen gaan gepaard met bijwerkingen en risico's. Het is daarom belangrijk om een inschatting te maken van de kans op een recidief. Binnen de huidige richtlijnen worden patiënten ingedeeld in een risicocategorie aan de hand van bevindingen bij pathologisch onderzoek en leeftijd. Het pathologisch onderzoek geeft informatie over differentiatie graad, histologisch subtype en uitbreiding van de ziekte (FIGO stadium). Een hoge differentiatiegraad, een niet-endometrioid subtype evenals een gevorderd ziekte stadium zijn gerelateerd aan een hogere kans op een recidief. Omgekeerd zijn een lage differentiatiegraad, een endometrioid subtype en een beperkt ziekte stadium gerelateerd aan een lage kans op een recidief en is aanvullende behandeling soms niet nodig. Hoewel deze risico categorieën van toegevoegde waarde zijn bij het advies omtrent aanvullende behandeling kan de selectie van patiënten voor aanvullende therapie nog worden verbeterd. Van de patiënten die volgens de huidige richtlijn aanvullende therapie wordt geadviseerd zou een groot deel ook zonder de aanvullende therapie geen recidief krijgen. Deze patiënten worden dus onnodig behandeld. Anderzijds, zijn er ook patiënten die ondanks de aanvullende therapie toch een recidief krijgen. Betere selectie van patiënten voor aanvullende therapie is evenals het ontwikkelen van nieuwe therapie belangrijk om de ziekte uitkomst te verbeteren. Vanuit dit oogpunt is veel onderzoek gedaan naar de immunologische en moleculaire mechanismen bij kanker en naar evaluatie van bestaande behandelingen.

Traditioneel wordt onderscheid gemaakt tussen type 1 en type 2 EC op basis van de differentiatie graad en het histologisch subtype. Type 1 wordt gedefinieerd als kanker van een endometrioid subtype met graad 1-2 differentiatie en heeft een relatief gunstige prognose. Type 2 wordt gedefinieerd als kanker van een niet-endometrioid subtype en/of kanker waarbij de differentiatiegraad wordt omschreven

als slecht of niet gedifferentieerd. Type 2 EC heeft een relatief slechte prognose. Onderzoek schetst echter een meer complex en heterogeen beeld van EC recent werd een nieuwe classificatie voorgesteld dat is gebaseerd op moleculaire kenmerken: POLE ultramutated, microsatellite instability (MSI) hypermutated, copy-number low and copy-number high. Deze moleculaire classificatie is mogelijk beter geschikt om patiënten te selecteren voor aanvullende behandeling. Naast moleculaire mechanismen bij EC blijkt ook het immuunsysteem een belangrijke rol te spelen. Het immuunsysteem kan kankercellen opsporen en vernietigen. Zo is bijvoorbeeld de aanwezigheid van witte bloedcellen in kanker gerelateerd aan een betere prognose. Omgekeerd kan kanker eigenschappen ontwikkelen om aan het immuunsysteem te ontsnappen. Een voorbeeld hiervan is onderdrukking van witte bloedcellen door tumorcellen. Het wegnemen van die eigenschap kan de immuunreactie herstellen en de prognose verbeteren. Een veelbelovend voorbeeld zijn de zogeheten checkpoint inhibitors. Deze medicatie kan onderdrukte witte bloedcellen reactiveren waardoor de immuunreactie kan worden hersteld. Deze immuuntherapie is een nieuwe behandelmogelijkheid die snel terrein wint. Het wordt bij verschillende vormen van kanker al in praktijk gebruikt en ook de resultaten van onderzoek bij EC zijn veelbelovend.

Om de ziekte uitkomst voor patiënten met EC te verbeteren is meer kennis nodig van de immunologische en moleculaire mechanismen bij EC en is het belangrijk om het gebruik van bestaande therapieën te verbeteren. De studies in dit proefschrift dragen daar aan bij.

In **hoofdstuk 2** wordt onderzocht of selectie van patiënten voor aanvullende behandeling kan worden verbeterd met behulp van immunologische variabelen. Klassieke variabelen die zijn gerelateerd aan de kans om recidief van de ziekte zoals differentiatiegraad, FIGO stadium of lymfovasculaire invasie (LVSI) evenals immunologische variabelen zoals tumor infiltrerende lymfocyten werden meegenomen in de analyse. De voorspellende waarde voor een recidief was het hoogst voor een combinatie van FIGO stadium, LVSI en T-lymfocyten in de tumor. Deze studie laat daarmee zien dat tumor infiltrerende T-lymfocyten een belangrijke voorspeller is voor een recidief en bij kan dragen aan een verbeterde selectie van patiënten voor aanvullende behandeling. Verder onderzoek kan worden gericht op het ontwikkelen en implementeren van een immuunscore om patiënten te selecteren voor een behandeling.

In **hoofdstuk 3** zijn we op zoek gegaan naar nieuwe immunologische mechanismen die relevant zijn voor overleving. Human Leukocyte Antigen E (HLA-E) is een eiwit dat op de celwand van normale cellen wordt gepresenteerd. Cellen met verlaagde of verhoogde expressie worden door het immuunsysteem aangevallen. Dit gebeurt door twee soorten witte bloedcellen: natural killer cellen (NK-cellen) en cytotoxische T-cellen (CTL). In andere vormen van kanker relateert expressie van HLA-E aan overleving. In deze studie werd expressie van HLA-E onderzocht evenals tumor infiltratie met NK-cellen en CTL en werd dit gerelateerd aan overleving. In patiënte met een verhoogde expressie van HLA-E was tumor infiltratie door NK-cellen gerelateerd aan betere overleving. Omgekeerd was in patiënten met normale HLA-E expressie de

aanwezigheid van NK-cellen gerelateerd aan een slechtere overleving. Dit illustreert de complexiteit van het immuunsysteem en biedt mogelijkheden voor onderzoek naar immunotherapie die gebruik maakt van NK-cellen.

Hoofdstuk 4 beschrijft ons onderzoek naar de relatie tussen immunologische en moleculaire mechanismen bij EC. Een manier om aan het immuunsysteem te ontsnappen is verminderde antigeen presentatie door de tumorcellen. Dit zou tot stand kunnen komen door een mutatie in de cytokine receptor Janus Kinase 1 (JAK1). Deze mutatie komt vaker voor in EC tumoren die volgens de nieuwe classificatie wordt geclassificeerd als MSI-hypermuted. Deze groep wordt gekenmerkt door veel mutaties in het DNA en verminderde antigeen presentatie. In dit onderzoek werd de frequentie van JAK1 mutaties onderzocht in relatie tot expressie van eiwitten betrokken bij antigeen presentatie; LMP7, TAP1 en Human Leukocyte Antigen klasse 1 (HLA-1). JAK1 mutaties kwamen relatief vaak voor in MSI-hypermuted EC en was gerelateerd aan verminderde expressie van HLA-1. Er was geen relatie tussen JAK1 mutaties en overleving. Mutaties in JAK1 kan desondanks een bijdrage leveren aan immuun ontsnapping.

In **hoofdstuk 5** wordt een bijzondere, zeldzame vorm van EC onderzocht. Een carcinosaroom van de uterus (UCS) is een histologisch subtype van EC met een slechte prognose. De histologie is eveneens bijzonder omdat deze vorm van kanker zowel een epitheliale als een mesenchymale component bevat. Het zou daarmee een goed model kunnen zijn voor epitheliale mesenchymale transitie (EMT). EMT is een proces waarbij een cel epitheliale kenmerken inruilt voor mesenchymale kenmerken waardoor de cel kan migreren en de kanker kan uitzaaien. L1 cell adhesion molecule (L1CAM) is een mogelijke marker voor een mesenchymal fenotype. In dit onderzoek werd expressie van L1CAM onderzocht in beide componenten van UCS. Verrassend genoeg, was expressie van L1CAM beperkt tot de epitheliale component. L1CAM is daarmee geen goede marker voor de mesenchymale component of UCS is geen goed model voor EMT. Nieuwe studies kunnen de mogelijk van UCS als model voor EMT verder onderzoeken.

UCS presenteert zich relatief vaak met lymfklier metastasen en mogelijk is er een overlevingsvoordeel van een lymfadenectomie (LND) bij de chirurgische behandeling. Het onderzoek in **hoofdstuk 6** is een grote retrospectieve cohort studie waarin de relatie tussen LND, aanvullende radio- en/of chemotherapie en overleving. LND is gerelateerd aan een betere overleving wanneer meer dan 10 lymfklieren werden verwijderd. Aanvullende therapie was gerelateerd aan betere overleving bij patiënten waar geen LND werd verricht, of wanneer de ziekte was uitgezaaid naar de lymfklieren. Het lijkt raadzaam om een LND te verrichten bij chirurgische behandeling van UCS tot studies met een hogere bewijskracht het tegendeel bewijzen.

De onderzoeken in dit proefschrift dragen bij aan het vergroten van de kennis van immunologische, moleculaire en therapeutische aspecten van baarmoederslijmvlieskanker. Het is een onderzoeksveld dat snel in ontwikkeling is. Dit wordt deels gefaciliteerd door technologische ontwikkelingen en initiatieven

om informatie te delen. Hiermee is het mogelijk de uitkomst van patiënten met baarmoederslijmvlieskanker te verbeteren.

APPENDICES

CURRICULUM VITAE, LIST OF PUBLICATIONS, DANKWOORD

CURRICULUM VITAE

Marco Versluis werd op 12 november 1977 geboren in Putten als derde zoon van Evert Versluis en Ina Versluis-Bijl. Samen met zijn twee broers groeide Marco op in Nijkerk. In 1996 behaalde hij zijn VWO examen in Harderwijk. Datzelfde jaar begon hij aan de studie biologie aan de Vrije Universiteit in Amsterdam. Onderdeel van deze opleiding was een tweetal wetenschappelijke stages. Tijdens de eerste stage bestudeerde Marco het zanggedrag van zebravinken en tijdens de tweede het circadiane ritme bij ratten. Na het behalen van zijn master biologie begon Marco in 2001 de opleiding geneeskunde aan de Vrije Universiteit in Amsterdam. Hij rondde, met een oudste en keuze coschap in Maun General Hospital in Botswana, zijn opleiding af. In 2006 nam hij zijn artsenbul in ontvangst.

Gezondheidszorg in de tropen had, na zijn werk in Botswana, zijn aandacht. Na het artsexamen begon hij daarom aan de opleiding tot tropenarts in het Deventer Ziekenhuis op de afdeling gynaecologie (opleider : Dr. P.J.Q. van der Linden). Het vakgebied sprak zo aan dat in 2008 succesvol werd gesolliciteerd voor de opleiding tot gynaecoloog in de onderwijs- en opleidingsregio Groningen. De opleiding werd doorlopen in respectievelijk Zwolle (opleider: Dr. H.H. de Haan), Groningen (opleider: Prof. Dr. M.J.E. Mourits) en Leeuwarden (opleider: Dr. T. Spinder). Marco wilde zich tijdens zijn opleiding ook verder verdiepen en ontwikkelen in wetenschappelijk onderzoek. Zo werd er in 2012 al een begin gemaakt met het onderzoek in dit proefschrift.

Sinds 2014 is Marco werkzaam als gynaecoloog, met aandachtsgebied algemene gynaecologie, binnen het Universitair Medisch Centrum Groningen. Naast onderzoek en patiëntenzorg in de academische kliniek, zet hij zich actief in voor de opleiding geneeskunde, voor de artsen van de toekomst. Hij is niet alleen een gepassioneerd docent (Docent van het Jaar 2018), maar is ook nauw betrokken bij de organisatie en (door)ontwikkeling van de opleiding. Naast course director is Marco staflid van de learning community Global Health. Geneeskunde over de grens heeft nog steeds zijn interesse. Wanneer de omstandigheden het toelaten zet hij zijn onderwijs- en klinische expertise in om de moeder- en kindzorg in Masanga Hospital in Sierra Leone (gebied met zorgschaarste) te verbeteren.

Marco woont met zijn vrouw Ruth Admiraal en hun twee zonen, Bram (2008) en Gijs (2009) in Groningen.

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DANKWOORD

Wat begon als een project voor persoonlijke ontwikkeling, is uitgelopen op een promotie met dit proefschrift. Het is een mooie reis geweest. Zoals elke reis, met hoogte en dieptepunten. Ik heb daarbij veel steun ontvangen en wil iedereen die daar aan heeft bijgedragen bedanken.

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Beste Hans, toen ik tijdens mijn opleiding tot gynaecoloog op zoek ging naar persoonlijke ontwikkeling en wetenschappelijke verdieping, ben ik bij veel collega's langs gelopen. Bij jou vond ik wat ik zocht; een klein project zonder verplichtingen. Ik vond het super om op deze manier meer wetenschappelijke ervaring en kennis op te doen. Bij gesprekken over de wetenschappelijke dilemma's van het onderzoek, was er ook altijd ruimte voor andere onderwerpen. We bespraken literatuur, politiek, leiderschap en gaandeweg is de gewoonte ontstaan om elkaar af en toe zonder reden een boek te geven. Ik waardeer onze gesprekken en hoop de bijzondere gewoonte ook na dit proefschrift voort te zetten. Heel veel dank.

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