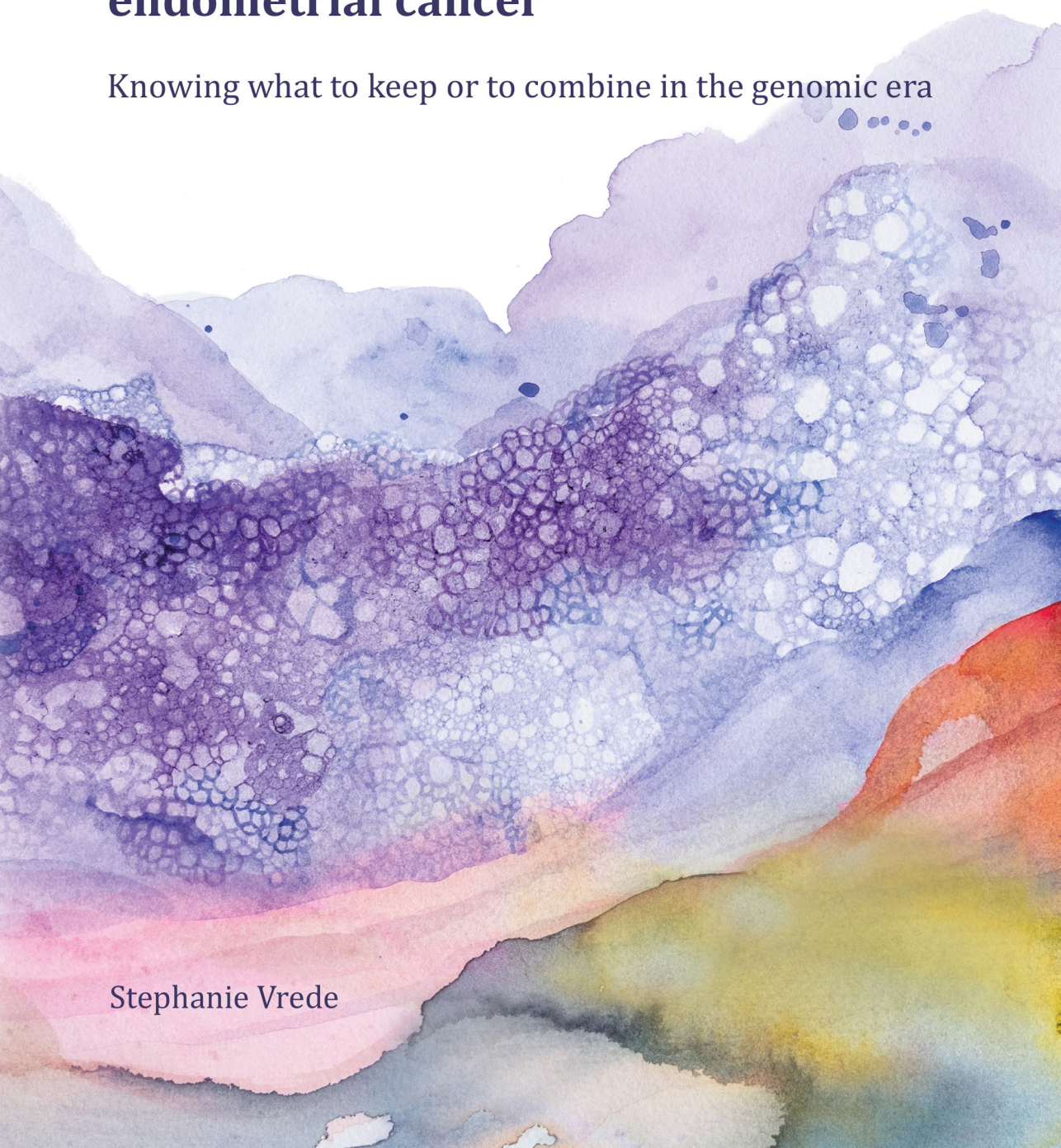
Further refinement in the prognostic evaluation of patients with endometrial cancer

Knowing what to keep or to combine in the genomic era



Stephanie Vrede

Further refinement in the prognostic evaluation of patients with endometrial cancer

Knowing what to keep or to combine in the genomic era

Stephanie Vrede

The research presented in this thesis was carried out at the department of Obstetrics and Gynaecology of the Radboud university medical center, Nijmegen, the Netherlands

For reasons of consistency within the thesis, some terms and abbreviations have been standardized throughout the text and might therefore slightly differ from the original publications.

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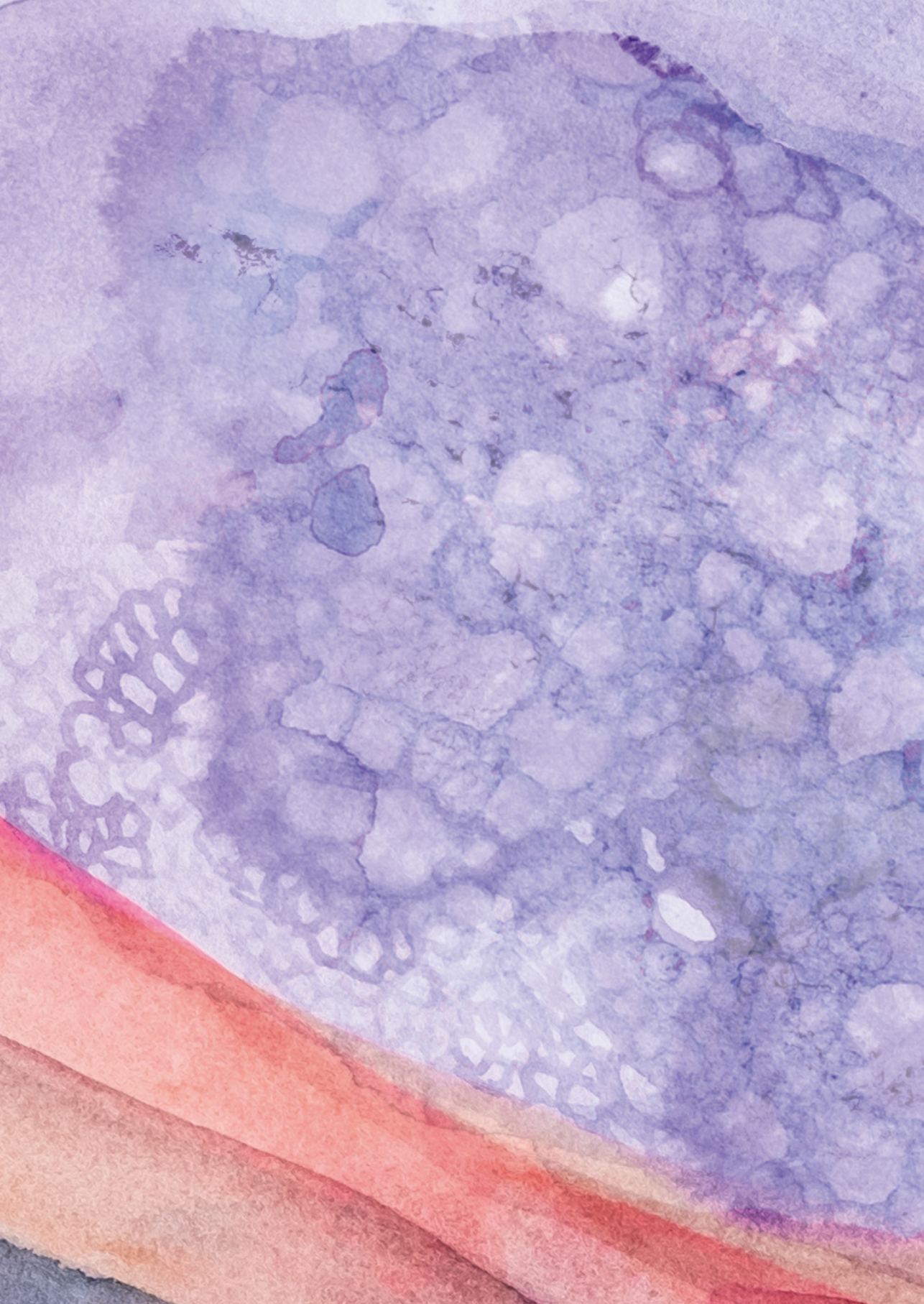


CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE THESIS

INTRODUCTION

Epidemiology

Endometrial cancer (EC) is the most common gynecological malignancy in industrialized countries, like Europe and North America. The incidence is rising due to advanced life expectancy and increasing obesity.[1] In 2020 worldwide, 417,367 women were diagnosed with EC and 97,370 people died from this cancer.[2] Various risk factors are identified for EC and summarized in **Table 1**. Low-income countries have a lower incidence of EC, because most risk factors are less present.

Within the Netherlands, the reported incidence of EC in 2020 was 2069, with a mortality rate of 559 women, both have increased over the past years.[3] The majority of patients diagnosed with EC are between 60-74 years old (50%) and a third of the patients is older than 75 years.[3]

Table 1. Risk factors for endometrial cancer

Increasing factors	
Long-term exposure to unopposed estrogens	Increasing Age
	Obesity
	Nulliparity
	Polycystic ovary syndrome
	Early menarche/late menopause
	Hormone replacement therapy without progestogens
Others	Tamoxifen use for breast cancer
Genetic	First-degree relative with endometrial cancer
	Lynch-syndrome
Decreasing factors	
	Grand multiparity
	Increased physical activity
	Oral conceptive and/or hormone replacement therapy (combination of estrogen & progestogens)
	Smoking

Diagnosis

Among EC patients, most women present with abnormal or postmenopausal bleeding as an early symptom.[4] The diagnostic work-up consists of gynecological examination including cervical cytology and transvaginal ultrasonography (TVU) to measure endometrial thickness. Thickened endometrium, defined as >4.0 mm, or recurrent abnormal postmenopausal bleeding that occurs six weeks after a first episode within a year, requires histological evaluation by either endometrial sampling, hysteroscopic biopsy or dilatation and

curettage (D&C). Performing office-based endometrial sampling as a first-line diagnostic procedure is recommended. When histopathological findings are inconclusive to rule out cancer, hysteroscopic biopsy or D&C is recommended.[5] The obtained tissue is subjected to histopathological evaluation including tumor typing and grading.[6]

Histology

Historically, EC was subdivided into two histopathological subtypes, type 1 and type 2 EC (**Figure 1**). Type 1, comprising grade 1 and 2 endometrioid EC (EEC), is associated with high immunohistochemical (IHC) expression of estrogen receptor (ER) and a favorable prognosis. Type 2, comprising of grade 3 EEC and non-endometrioid EC (NEEC), generally shows low ER expression, mainly *TP53* mutation and an unfavorable prognosis.[7]

Non-endometrioid histology includes most commonly serous and clear cell histology.[8] Serous carcinoma has a poor prognosis with extra-uterine disease in 37% of EC patients.[9] Uterine clear cell carcinoma (CCC) also often presents with extra-uterine disease (40-45%), has a high recurrence rate (50% at 3-years) and a 5-year overall survival of only 63%.[8, 10] Besides pure also mixed EC occurs, being a tumor composed of two or more different histological types of EC, for example components of endometrioid, serous and/or clear cell histology.[11] The mixed form of uterine CCC can display apart from clear cell, endometrioid and/or serous carcinoma histological components.[12] It is questioned whether pure CCC presents with another molecular and IHC background compared to the mixed form of uterine CCC, and affecting clinical outcome.

The most recent ESGO/ESTRO/ESP (European Society of Gynaecological Oncology/ European SocieTy for Radiotherapy and Oncology/European Society of Pathology guideline) and WHO (World Health Organization) Classification of Tumors, recommends a modified binary FIGO (Federation International of Gynecology and Obstetrics) grading, considering both FIGO grade 1 and 2 lumped as low-grade EC and FIGO grade 3 EEC, and NEEC as high-grade EC.[6, 12, 13] Most patients (80%) are diagnosed with low-grade EC and an overall favorable prognosis with a 5-year survival rate of 85%. About 20% of the patients are diagnosed with high-grade EC, associated with increased risk of regional or distant metastases and have a poor prognosis with a 5-year survival rate of 58%.[4]

Numerous studies state that preoperative endometrial sampling is poorly to moderately correlated with final tumor grade and histological subtype.[14-17] Within a meta-analysis, the lowest concordance was found for grade 2 EC (only 61.0%).[17] Since the primary treatment of EC is mainly based on preoperative tumor histology, disagreement in grading between preoperative and final diagnosis may therefore result in either under- or overtreatment and subsequently impact outcome.[18, 19] Currently, sampling errors and interobserver variability

are considered the most important explanations for this disagreement.[17, 20-23] Besides, it remains unclear whether the amount of diagnostic tissue impacts the concordance.

Immunohistochemical biomarkers

In recent years, several IHC biomarkers have been studied in EC to improve diagnosis and prognostication of which ER, progesterone receptor (PR), p53 and L1 cell adhesion molecule (L1CAM) appear the most relevant. Examples of expression patterns of these biomarkers are shown in **Figure 2**.[30-37] Positive ER/PR expression is associated with favorable outcome and low risk of lymph node metastasis (LNM). Negative ER/PR expression is associated with the opposite.[37, 38] Our research group recently demonstrated that a revised three-tiered ER and PR risk classification improves prognostication over the commonly used cutoff value of 10% for ER and/or PR positivity: 0-10% with most unfavorable outcome, 20-80% with intermediate outcome and 90-100% with most favorable outcome.[39] *TP53* is the most frequently mutated gene, causing dysfunction of p53 tumor suppressor protein, playing an important role in cell proliferation, apoptosis, DNA repair and genomic stability. Overexpression of p53 or null-expression is associated with an unfavorable outcome. [40-42] The transmembrane L1CAM is critical for epithelial to mesenchymal transition (EMT) and cancer initiating cell (CIC) formation which may result in chemotherapy resistance.[32, 43] Positive L1CAM tumor expression is associated with a poor outcome in EC.[32, 44-47] Currently, most of these easy accessible IHC biomarkers are not yet used in the risk classifications for primary and secondary treatment.

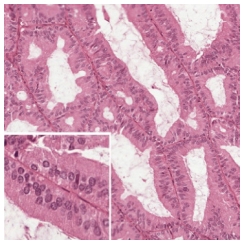
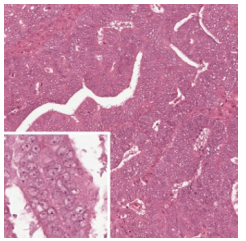
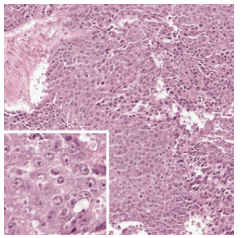
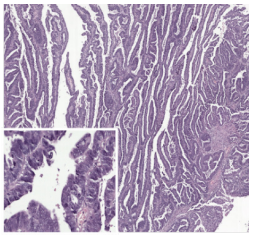
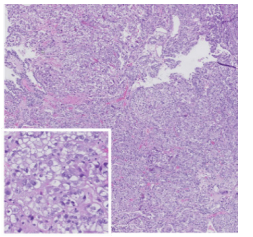
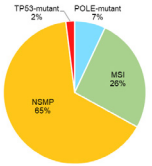
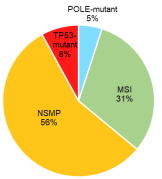
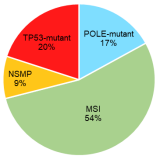
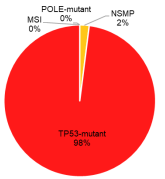
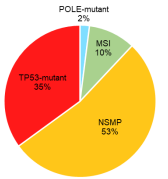
	Endometrioid		Non-endometrioid		
	Type 1		Type 2		
	Grade 1 EC	Grade 2 EC	Grade 3 EEC	Serous	Clear cell
A.					
B.					
C.	Low-grade Incidence 80% Age 40-60 years Obesity High ER/PR expression *PTEN*, *PIK3CA* or *KRAS* mutation (NSMP) Favorable prognosis		High-grade Incidence 20% Age 60-80 years Generally low ER/PR expression Unfavorable prognosis		

Figure 1: *A.* Examples of type 1; grade 1 EC and grade 2 EC and examples of type 2; grade 3 EEC, serous and clear cell EC. B. The pie charts showing the distribution of the molecular subgroups within the different histology's. C. The characteristics of type 1 EC and type 2 EC.

Abbreviations: *POLE*, polymerase epsilon; *TP53*, tumor protein 53; MSI, microsatellite instable; NSMP, no specific molecular profile; ER, estrogen receptor; PR, progesterone receptor; *PTEN*, phosphatase and tensin homolog; *PIK3CA*, phosphatidylinositol 3-kinase; KRAS, kristen ras sarcoma viral oncogene

Immunohistochemical expression

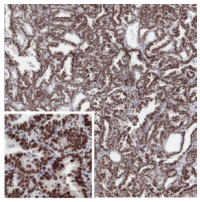
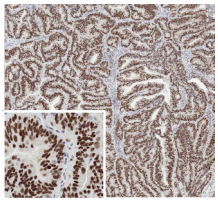
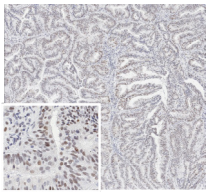
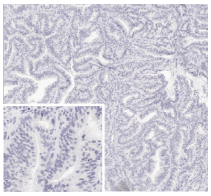
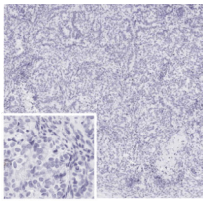
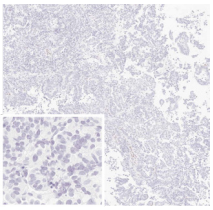
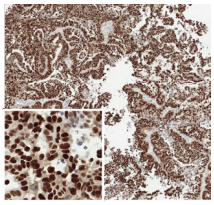
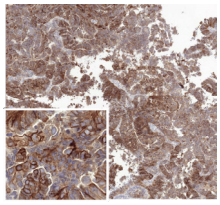
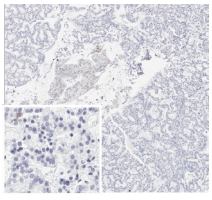
ER expression	*PR expression*	*P53 expression*	*L1CAM expression*
ER positive	**PR positive**	**p53 wildtype**	**L1CAM negative**
A. 	C. 	E. 	H. 
ER negative	**PR negative**	**p53 overexpression**	**L1CAM positive**
B. 	D. 	F. 	I. 
		p53 null-expression	
		G. 	

Figure 2. Immunohistochemical expression of ER, PR, p53 and L1CAM. A/C. Positive ER/PR expression with a cutoff >1 or 10%. B/D. negative ER/PR expression with a cutoff ≤1 or 10%. E. p53-wildtype when there is no *TP53* mutation. F. p53 overexpression when there is nuclear accumulation of p53 protein caused by a missense mutation of *TP53*. G. p53 null-expression with a frameshift or nonsense mutation of *TP53*. H. Negative L1CAM expression with a cutoff <10%. I. Positive L1CAM expression with a cutoff ≥10%.

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; p53, protein 53; L1CAM, L1 cell adhesion molecule

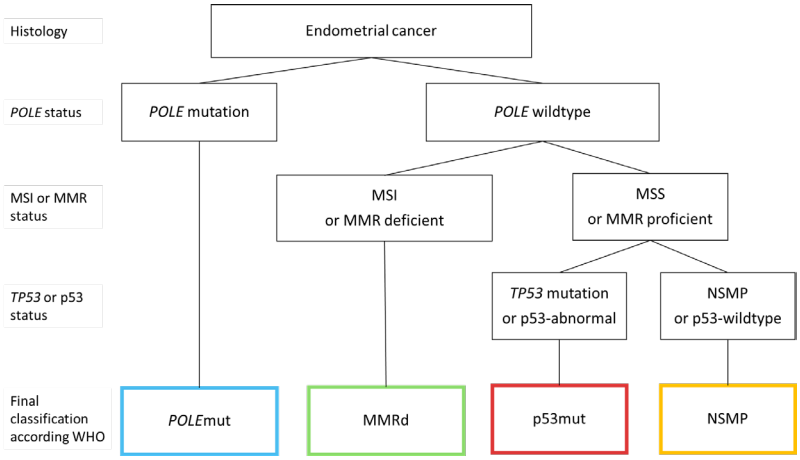
Molecular biomarkers

Recently, The Cancer Genome Atlas (TCGA) defined four important prognostic molecular subgroups in EC based on integrated genomic data: I) ultramutated tumors with polymerase epsilon (*POLE*) mutations, II) hypermutated tumors with microsatellite instability (MSI), III) copy-number-high (CNH) with frequent tumor protein (*TP53*) mutations and, IV) copy-number-low (CNL) (also known as no-specific molecular profile (NSMP)). These four subgroups increase insight in biological tumor behavior based on molecular signature beyond the histological morphological classification of type 1 and 2 EC.[7, 51] Several studies have shown that patients with *POLE* mutation have an excellent outcome in EC. Patients within the MSI or NSMP subgroup are known with intermediate outcome, and patients with *TP53*-mutant tumors have the worst outcome, the latter representing 15% of all EC diagnosis and responsible for 50-70% of all EC-related mortality.[51-54] The diagnostic algorithm and prognostic relevance of these subgroups are shown in **Figure 3A-B**.

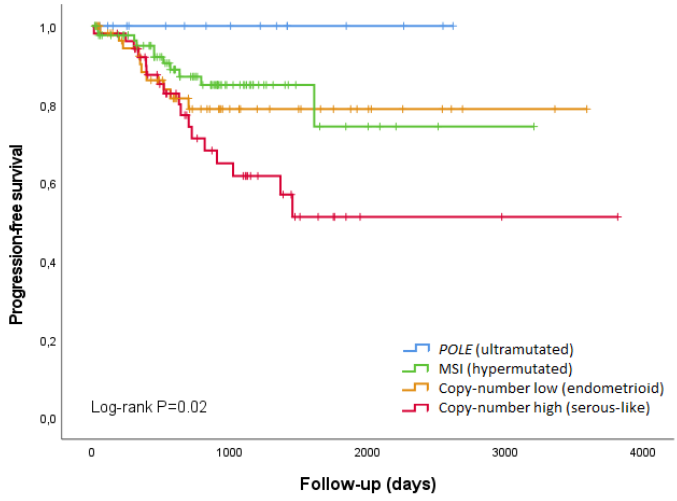
These molecular subgroups have shown to improve prognostication mainly in patients with high-grade EC, probably due to poor interobserver reproducibility of morphological classification and the prognostic and intratumoral heterogeneity of high-grade ECs.[53, 55, 56] Specifically in patients with low-grade EC, the prognostic relevance of molecular classification so far is lacking.

Clinical biomarkers

In addition to the tumor histology and immunohistochemical and/or molecular biomarkers, clinical biomarkers may contribute to an improved risk stratification by reflecting the tumor macro-environment. Endometrial carcinogenesis is characterized by chronic inflammation with elevated pro-inflammatory cytokines and acute phase proteins.[57] Overexpression of inflammatory cytokines could contribute to the development of cancer-related anemia, thrombocytosis and leukocytosis, thus generating a pro-tumorigenic environment.[58-61] Preoperative anemia, thrombocytosis and leukocytosis, as clinical hematological parameters, may contribute to the identification of patients with extended disease and/or aggressive tumor behavior.[46, 62-64] Indeed, they have been associated with advanced-stage (FIGO stage III-IV) and therefore prognostic relevant, however results remain conflicting.[59, 60, 65-70] If these often routinely obtained preoperative hematological parameters may also influence the response to adjuvant therapy still remains to be elucidated.[46, 62, 63]



A.



B.

Figure 3A-B. A. Diagnostic algorithm and final classification according the WHO (World Health Organization) classification of Female Genital tumors. B. Progression-free survival of the four molecular subgroups according to The Cancer Genome Atlas (TCGA).[51]

Abbreviations: *POLE*, polymerase epsilon mutant; MMRd, mismatch repair deficient; MSI, microsatellite instable; MSS, microsatellite stable; *TP53*, tumor protein 53; p53, protein 53; NSMP, No specific molecular profile.

Preoperative risk stratification model guiding primary surgical treatment of endometrial cancer

Primary surgical treatment according the latest ESGO/ESTRO/ESP guideline is based on preoperative tumor grade, histology and, if indicated imaging. Besides hysterectomy and bilateral salpingo-oophorectomy, additional staging including lymph node surgery (i.e. sentinel lymph node (SLN), lymph node dissection (LND)) is recommended in patients at substantial risk of metastases.[13, 71]

Current models for preoperative prediction of LNM and survival in EC are not optimal.[13, 72] Numerous studies proposed preoperative risk stratification models for LNM.[73-77] However, preoperative risk models including IHC and/or molecular markers are only limited.[46, 78, 79] Within our research group we developed a Bayesian network model, ENDORISK, by integrating easy accessible preoperative markers and patient characteristics showing improved preoperative risk classification in EC.[46] ENDORISK includes preoperative markers like; thrombocytosis, Cancer Antigen 125 (CA125), tumor grade, lymphadenopathy on imaging, atypical endometrial cells in cervical cytology, and IHC expression of p53, L1CAM, ER and PR. It was established to predict preoperatively macro-LNM and outcome accurately.[46]

Postoperative risk stratification model guiding adjuvant treatment of endometrial cancer

For postoperative adjuvant treatment different classifications are used in clinical practice: ESGO/ESTRO/ESP, Postoperative Radiation Therapy for Endometrial Carcinoma (PORTEC) and Gynecologic Oncology Group (GOG) criteria.[13, 80, 81] According to the latest ESGO/ESTRO/ESP guideline, adjuvant treatment is based on risk classification groups incorporating FIGO stage, tumor grade and histology, lympho-vascular space invasion (LVSI), with or without molecular markers.[13]

With the integration of the TCGA-based molecular classification a postoperative risk stratification model appears promising for guidance of adjuvant treatment.[82, 83] Adjuvant therapy tailored to the TCGA groups will be studied in the prospective randomized control RAINBO trial.[83, 84]

AIMS AND OUTLINE OF THE THESIS

Aims

With only a moderate concordance between pre- and postoperative diagnosis, the creation of a more objective molecular classification by the TCGA was most welcome. However, routine molecular profiling comes with high costs, especially for low-income countries. With the introduction of these molecular subgroups, the prognostic relevance of tumor grading has gained less attention, as well as the easily accessible clinical and IHC biomarkers. It is questioned if the use of molecular biomarkers can be optimized by combining with IHC and clinical biomarkers.

In this thesis we aim to evaluate the prognostic relevance of the current histomorphology, IHC and clinical biomarkers within the new era of molecular profiling in EC.

Outline

In **chapter 2**, the amount of preoperative endometrial tissue surface is evaluated to the degree of concordance with final low- and high-grade EC. Furthermore, it is determined whether discordance is influenced by sampling method and may impact outcome.

In **chapter 3,** the prognostic relevance of molecular profiling in patients with low-grade EC is assessed.

In **chapter 4,** the molecular and immunohistochemical features within mixed and pure uterine CCC are investigated and whether this affects clinical outcome.

In **chapter 5**, the added prognostic relevance of preoperative IHC biomarkers to the ESMO-ESGO-ESTRO risk classification groups is investigated.

In **chapter 6**, the relevance of using a three-tiered ER/PR risk model is investigated including the possible additional prognostic relevance within the four molecular subgroups.

In **chapter 7**, the prognostic and predictive relevance of preoperative abnormal hematological parameters in patients with EC is evaluated.

In **chapter 8**, a summary of the results of this thesis and future implications for clinical practice are discussed .

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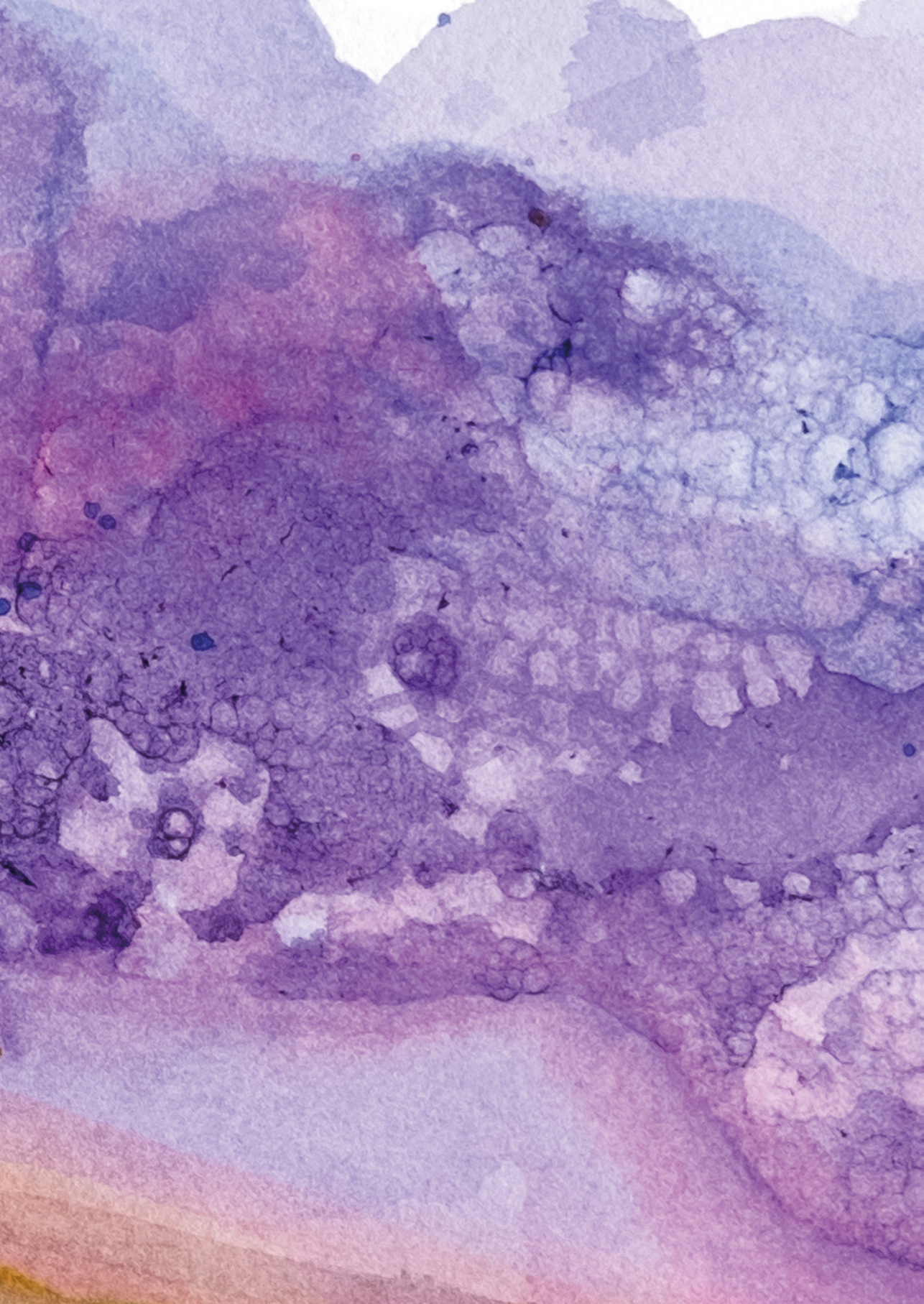

CHAPTER 2

THE AMOUNT OF PREOPERATIVE ENDOMETRIAL TISSUE SURFACE IN RELATION TO FINAL ENDOMETRIAL CANCER CLASSIFICATION

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ABSTRACT

Objective

To evaluate whether the amount of preoperative endometrial tissue surface is related to the degree of concordance with final low- and high-grade endometrial cancer (EC). In addition, to determine whether discordance is influenced by sampling method and impacts outcome.

Methods

A retrospective cohort study within the European Network for Individualized Treatment of Endometrial Cancer (ENITEC). Surface of preoperative endometrial tissue samples was digitally calculated using ImageJ. Tumor samples were classified into low-grade (grade 1-2 endometrioid EC (EEC)) and high-grade (grade 3 EEC + non-endometroid EC).

Results

The study cohort included 573 tumor samples. Overall concordance between pre- and postoperative diagnosis was 60.0%, and 88.8% when classified into low- and high-grade EC. Upgrading (preoperative low-grade, postoperative high-grade EC) was found in 7.8% and downgrading (preoperative high-grade, postoperative low-grade EC) in 26.7%. The median endometrial tissue surface was significantly lower in concordant diagnoses when compared to discordant diagnoses, respectively 18.7 mm^2 and 23.5 mm^2 (P=0.022). Sampling method did not influence the concordance in tumor classification. Patients with preoperative high-grade and postoperative low-grade EC showed significant lower DSS compared to patients with concordant low-grade EC (P=0.039).

Conclusion

The amount of preoperative endometrial tissue surface was inversely related to the degree of concordance with final tumor low- and high-grade. Obtaining higher amount of preoperative endometrial tissue surface does not increase the concordance between pre- and postoperative low- and high-grade diagnosis in EC. Awareness of clinically relevant down- and upgrading is crucial to reduce subsequent over- or undertreatment with impact on outcome.

INTRODUCTION

Endometrial cancer (EC) is the most common gynecological malignancy in industrialized developed countries with an increasing incidence.[1-3] These carcinomas are histopathological classified as either endometrioid endometrial cancer (EEC) or non-endometrioid endometrial cancer (NEEC).[4] Primary surgical treatment for EC consist of hysterectomy and bilateral salpingo-oophorectomy.[5, 6] Additional lymph node surgery, i.e. sentinel lymph node mapping, lymph node dissection or algorithm-based approach for staging, is recommended in patients with increased risk of lymph node metastasis (LNM).[7, 8] The recent ESGO-ESTRO-ESP guideline recommended a modified binary FIGO grading considering both grade 1 and 2 EC together as low-grade EC and grade 3 EC and NEEC as high-grade EC.[9] Most patients are diagnosed with low-grade EC, and generally have a favorable prognosis with a 5-year survival rate of 85.6%.[5] About 20.0% of the patients are diagnosed with high-grade EC, have an overall poor prognosis with a 5-year survival rate of 58.8% and are associated with increased risk of regional or distant metastases.[5, 10]

A meta-analysis has shown only moderate concordance of 67.0% between pre- and postoperative tumor grading.[11] The lowest concordance was found for grade 2 EC (61.0%), and as these are generally classified as low-grade EC, disagreement in grading might impact treatment and outcome since performance of lymph node surgery is generally performed in high-grade EC only.[9, 12, 13] Explanations for discordance on grade include 1) sampling errors leading to missed tumor components, 2) interobserver disagreement due to subjective interpretation of the defined criteria and 3) limited amount of tissue obtained by preoperative endometrial sampling, that might impair assessment of tumor characteristics. In 13-30% of the pipelle endometrial samples, insufficient material requires repeated biopsy for a reliable diagnosis, as in 7.3% of the failed samples women are subsequently diagnosed with EC.[14-17] Interestingly, Visser et al. showed that hysteroscopic biopsies had a higher concordance (89%) compared to samples obtained by dilatation and curettage (D&C) (70%), questioning whether in addition to the amount of tissue, the sampling method may also be relevant.[11]

In a previous study of our research group, we showed that the amount of endometrial tissue surface to classify an endometrial sample as conclusive with high diagnostic accuracy as malignant or non-malignant, was defined by a minimum cut-off level of 35 mm^2.[11, 14] However, this study was not designed to further specify the diagnosis on tumor grade and/or histological subtype. Therefore, in the present study, we aim to evaluate the amount of preoperative endometrial tissue surface in relation to the degree of concordance with final low- and high-grade EC. Furthermore, we investigate whether discordancy in pre- and postoperative grading is influenced by the sampling method and whether discordancy impacts outcome.

METHODS

Patients

The samples of patients were retrospectively collected within the European Network for Individualized Treatment of Endometrial Cancer (ENITEC) from a previous study including 1199 EC patients.[15] Patients were only included when they were diagnosed by an expert gynecological pathologist of the participating hospitals, with complete data on treatment and histopathology. Clinical and pathological data were recorded from the patient files into a database; including patient age, date of diagnosis, preoperative sampling method, surgical treatment, original pre- and postoperative tumor grade and histological subtype, myometrial invasion (MI), cervical invasion (CI), lymphovascular space invasion (LVSI), FIGO (International Federation of Gynecology and Obstetrics) stage, adjuvant treatment, recurrent disease and death.[15] The sole additional inclusion criterion used for this study was the availability of preoperative EC tissue samples, resulting in 598 patients.

Tumor classification

In addition to the FIGO three-tiered tumor grade, EC tissue samples were classified into low- and high-grade EC as recommended by the recent ESGO-ESTRO-ESP guideline and the World health organization (WHO) classification of tumors.[9, 18] Low-grade EC was defined as grade 1 and 2 EEC, and included samples with mucinous histology as well, since prognosis and molecular characterization are similar to low-grade EECs.[15] High-grade EC included grade 3 EEC and NEEC, i.e. serous, clear cell carcinoma, carcinosarcoma and mixed carcinomas.[9, 18] Endometrial tissue samples were defined as upgraded if the preoperative sample was low-grade and postoperative high-grade EC. Downgraded was defined as preoperative high-grade and postoperative low-grade EC. Biopsies initially diagnosed as premalignant, but EC on final hysterectomy specimen were included in this study.

Scoring

All the preoperative endometrial sampling slides were digitalized using Pannoramic Scanner 250 Flash III (3DHISTECH, Budapest, Hungary). As described previously by Reijnen et al., images were saved as a JPEG-compressed file and the area of endometrial tissue was digitally calculated using ImageJ software, selecting only benign, premalignant and malignant endometrial epithelium (*Supplementary Figure S1).*[14] Thresholds 24-bit RGB images based on Hue Saturation and Brightness (HSB) were used to select the endometrial tissue surface, by adjusting the different threshold values to segment the image into the area of interest and the background. The Pannoramic Viewer software was used to examine the original-size digital slide in order to ensure ImageJ correctly selected the proper tissue. Subsequently, analysis was performed on the area selection to count and measure pixels in the threshold images and calculate the total area of endometrial tissue. A set of 50 slides were scored

independently by two investigators (AH, CR) to assess the degree of inter-rater variability and intraclass correlation coefficient (ICC). A set of 90 slides were double-checked by a third investigator (SV) to ensure ImageJ selected the proper tissue.

Statistical analysis

All statistical analyses were performed using IBM Statistical Package for the Social Sciences (SPSS) statistics for Windows, version 25.0 (released 2017, Armonk, NY, United States) and $P<0.05$ was considered statistically significant. For observing within the low- and high-grade classification, the pre- and postoperative tumor diagnosis was specified in individual FIGO tumor grade and histological subtype. These included the original diagnosis (including premalignant tissue); grade 1, grade 2, grade 3 EEC or NEEC. For continuous data that were not normally distributed, the Mann-Whitney U and Kruskal Wallis test were used to compare the differences in median endometrial tissue surface and patient characteristics. Clinicopathological characteristics between dichotomous subgroups were compared using the χ^2 or Fisher's exact test for categorical data. Survival analyses were performed using the Kaplan Meier curves (first 10 years after diagnosis). Disease-specific survival (DSS) was defined as time from date of diagnosis to date of death from EC, all censored by date of last contact.

RESULTS

Patients

From the original cohort of 1199 patients, 644 preoperative biopsies were available, of those 46 patients were excluded because absence of tumor tissue due to insufficient amount of tissue and benign endometrium and 25 because of an unspecified grade on preoperative biopsy, resulting in a total of 573 patients included in this study with a median follow-up of 5.7 years (*Supplementary Figure S2*). Excluded patients did not significantly differ from included patients with respect to tumor histology (*data not shown*). Baseline characteristics for all included patients, classified into postoperative low- and high-grade EC, are summarized in **Table 1**. Among these 573 patients, 462 patients (80.6%) were postoperative low-grade and 111 (19.4%) high-grade EC. The mean age at diagnosis was 64.8 years, most patients were preoperative diagnosed with grade 1 EEC (53.8%) and postoperative FIGO stage I (82.9%). The most used preoperative sampling method was the pipelle (45.2%). Patients diagnosed with postoperative high-grade EC were significantly older, had lower Body Mass Index (BMI), more often LNM, subsequently resulting in more applied adjuvant chemotherapy and chemoradiotherapy compared to patients with low-grade EC.

In *Supplementary Table S1* detailed baseline information about patients diagnosed with postoperative NEEC (n=34) is shown. Most patients with NEEC had serous histology (n=14, 41.2%).

Concordance pre- and postoperative tumor grade and histology

Figure 1 shows the number and percentages of the pre- vs. postoperative individual tumor grade and histological subtype. ***Dark green*** shows the exact concordance between grading and histology, ***light green*** the concordance for the clinically relevant low- and high-grade classification and in ***red*** the clinically relevant discordancy. Overall, of the 573 EC tissue samples, 60.0% (n=345) showed concordant pre- and postoperative tumor grade and histological subtype (***dark green***). The lowest concordance was found for preoperative grade 3 EC (51.4%).

Concordance between pre-and postoperative low- and high-grade EC was found in 88.8% (n=509) patients (***light green*** + ***dark green***). Patients with preoperative low-grade EC showed concordant diagnoses in 92.2% (n=435) and were upgraded to high-grade EC in 7.8% (n=37). Patients with preoperative high-grade EC showed concordant diagnoses in 73.3% (n=74) and were downgraded in 26.7% (n=27).

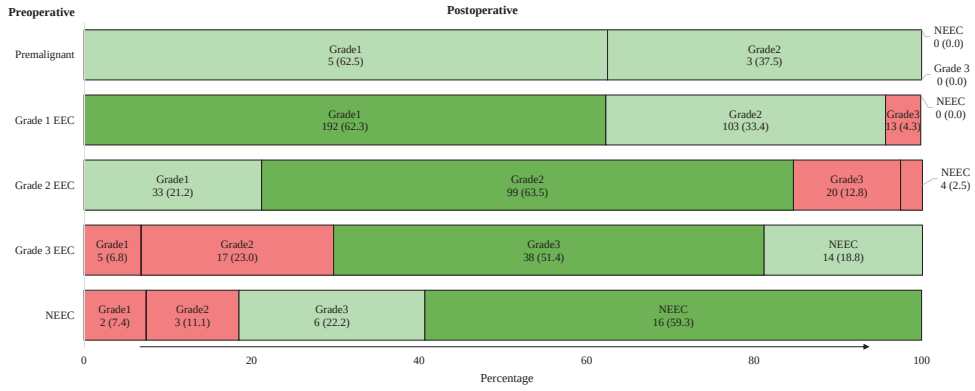

Figure 1. Number and percentages (*n* (%)) of the pre- vs. postoperative individual tumor grade and histological subtype.

Abbreviations: EEC, endometroid endometrial cancer; NEEC, non-endometroid endometrial cancer

Table 1. Baseline characteristics

	Total (***n*=573**)	**Postoperative Low-grade** (***n*=462**)	**Postoperative High-grade** (***n*=111**)	***P***
Age (years)	64.8 ± 9.8	64.1 ± 9.6	66.6 ± 10.0	0.014*
BMI (kg/m²)	30.2 ± 6.7	30.4 ± 6.5	28.7 ± 5.5	0.013*
Preoperative grade				
Premalignant†	8 (1.4)	8 (1.7)	0 (0.0)	<0.001*
1 EEC	308 (53.8)	295 (63.9)	13 (11.7)	
2 EEC	156 (27.2)	132 (28.6)	24 (21.6)	
3 EEC	74 (12.9)	22 (4.8)	52 (46.8)	
NEEC	27 (4.7)	5 (1.1)	22 (19.8)	
Preoperative sampling method				
Pipelle	259 (45.2)	199 (43.1)	60 (54.1)	0.002*
D&C	77 (13.4)	63 (13.6)	14 (12.6)	
Hysteroscopic biopsy	213 (37.2)	189 (40.9)	24 (21.6)	
Not specified	24 (4.2)	11 (2.4)	13 (11.7)	
FIGO stage				
I	475 (82.9)	413 (89.4)	62 (55.9)	<0.001*
II	36 (6.3)	24 (5.2)	12 (10.8)	
III	45 (7.9)	22 (4.8)	23 (20.7)	
IV	17 (2.9)	3 (0.6)	14 (12.6)	
Positive nodes				
No	299 (52.2)	240 (52.0)	59 (53.2)	<0.001*
Pelvic	17 (3.0)	7 (1.5)	10 (9.0)	
Para-aortic	11 (1.9)	2 (0.4)	9 (8.1)	
Both	5 (0.9)	1 (0.2)	4 (3.6)	
Not specified	241 (42.0)	212 (45.9)	29 (26.1)	
Adjuvant treatment				
No	267 (46.7)	238 (51.5)	29 (26.1)	<0.001*
Radiotherapy	263 (46.0)	204 (44.2)	59 (53.2)	
Chemotherapy	17 (3.0)	5 (1.1)	12 (10.8)	
Chemoradiotherapy	25 (4.4)	14 (3.0)	11 (9.9)	
Missing	1 (0.2)	1 (0.2)		

Data is presented in number (%), mean ± standard deviation (SD)

Abbreviations: EEC, endometrioid endometrial cancer; NEEC, non-endometroid endometrial cancer; BMI, Body Mass Index; FIGO, International Federation of Gynecology and Obstetrics

* $P<0.05$

†including simple or complex hyperplasia, with or without atypia.

Table 2. Overview of pre- vs. postoperative tumor grade and histological subtype. Median endometrial tissue surface (mm^2) of endometrial cancer patients are shown. Displayed in dark green are the concordant diagnoses. ***Dark green*** shows the exact concordance between grading and histology, ***light green*** the concordance for the clinically relevant low- and high-grade classification and in ***red*** the clinically relevant discordancy.

		Postoperative				
		Grade 1 EEC	Grade 2 EEC	Grade 3 EEC	NEEC	Total**
Preoperative	Premalignant	7.3 (0.8-8.4)	2.9 (1.0-3.5)	NA	NA	4.4 (0.8-8.4)
	Grade 1 EEC	17.2 (0.2-298.7)	15.6 (0.0-354.0)	16.1 (0.5-145.0)	NA	16.6 (0.0-354.0)
	Grade 2 EEC	35.1 (1.0-251.4)	21.9 (0.6-278.7)	30.0 (1.9-110.9)	18.2 (10.1-30.6)	24.6 (0.6-278.7)
	Grade 3 EEC	42.4 (12.5-94.2)	38.6 (0.4-274.9)	29.7 (0.2-210.2)	16.1 (1.4-81.7)	24.4 (0.2-274.9)
	NEEC	26.7 (9.8-43.6)	16.6 (0.1-44.7)	11.5 (0.9-18.1)	21.1 (0.7-49.4)	14.7 (0.1-49.3)
	Total*	18.7 (0.2-298.7)	19.9 (0.0-354.0)	23.3 (0.2-210.2)	17.6 (0.7-81.7)	

Data is presented in median (range).

Abbreviations: EEC, endometroid endometrial cancer; NEEC, non-endometroid endometrial cancer

* P=0.888 between the total median postoperative endometrial tissue surface

** P=0.063 between the total median preoperative endometrial tissue surface

Median endometrial tissue surface and degree of concordance

An overview of the median endometrial tissue surface related to pre- vs. postoperative tumor grade and histological subtype is shown in **Table 2.** There was no significant difference between the median endometrial tissue surface of the individual tumor grade and histological subtype preoperatively, nor postoperatively, (*P*=0.063 and *P*=0.888, respectively).

The median endometrial tissue surface between concordant (***dark green***) and discordant (***light green*** + ***red***) individual tumor grade and histological subtype showed no significant difference (19.6 mm² vs. 18.6mm², respectively, *P*=0.468). For the clinically relevant low- and high-grade classification, the median endometrial tissue surface for concordant diagnoses (***dark green*** + ***light green***) was significant lower compared to the discordant diagnoses (***red***) (18.7 mm² vs. 23.5 mm², respectively, *P*=0.022) (**Table 2**). In *Supplementary Table S2* the correlation between median endometrial tissue and concordant and discordant diagnoses is shown per included center.

Patients with concordant pre- and postoperative low-grade EC showed lower median endometrial tissue surface compared to preoperative low-grade and postoperative high-grade EC (upgraded), but not significantly (18.4 vs 20.1 mm², *P*=0.335). Patients with concordant pre- and postoperative high-grade EC had significant lower endometrial tissue surface compared to patients with preoperative high-grade and postoperative low-grade EC (downgraded) (20.3 vs 38.6 mm², *P*=0.044) (**Figure 2**).

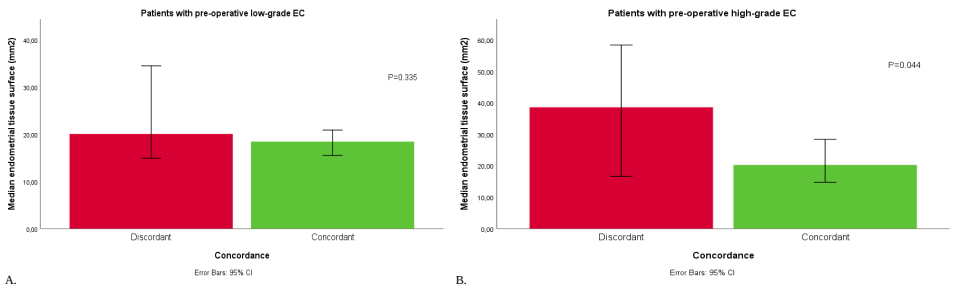

Figure 2 A-B. A. Patients with preoperative low-grade endometrial cancer (EC) and the median endometrial tissue surface for postoperative discordant or concordant diagnoses. B. Patients with preoperative high-grade EC and the median endometrial tissue surface for postoperative discordant or concordant diagnoses.

Sampling method

For 549 (95.8%) patients preoperative sampling method was available. Pipelle endometrial sampling was performed in 47.2%, D&C in 14.0% and hysteroscopic biopsy in 38.8% of the patients with available sampling method (*Supplementary Table S3*). No significant difference was found between the diagnostic sampling methods and the concordance between pre- and postoperative low- and high-grade EC (*P*=0.364), nor for the individual tumor grade and histological subtype (*P*=0.097).

Median endometrial tissue surface for the preoperative sampling method pipelle was 18.6 mm^2, D&C 67.8 mm^2 and hysteroscopic biopsy 15.4 mm^2 (*P*<0.001). All preoperative sampling methods (pipelle, D&C, hysteroscopic biopsy) showed higher median endometrial tissue surface in discordant low-and high-grade diagnoses, compared to concordant low- and high-grade diagnoses. Similar was shown for individual tumor grade and histological subtype diagnoses (*Supplementary Figure S3*).

Concordance, discordance and survival outcome

The DSS of the concordant and discordant diagnoses are shown in **Figure 3A-C. Figure 3A** shows the DSS of the patients with concordant high-grade EC, concordant low-grade EC, and clinically relevant downgraded and upgraded diagnoses (P<0.001). Patients with concordant low-grade EC had a significant superior DSS compared to patients that were downgraded (96.5% and 88.9% respectively, P=0.039) (**Figure 3B**). Patients with concordant high-grade EC had a significant impaired DSS compared to patients that were upgraded (71.4% and 88.6% respectively, P=0.046) (**Figure 3C**).

DISCUSSION

This study assessed whether the amount of preoperative endometrial tissue surface is related to the degree of concordance with final classification of low- and high-grade EC, and whether discordance is influenced by the diagnostic sampling method and impacts outcome. Overall, 60% showed concordant pre- and postoperative tumor grade and histological subtype and there was 88.8% concordance in pre- and postoperative classification into low- and high-grade EC, with 92.2% concordant low-grade, and 73.3% concordant high-grade EC. The median endometrial tissue surface between concordant and discordant individual tumor grade and histological subtype showed no significant difference. Interestingly, concordant diagnoses revealed a significant lower median endometrial tissue surface compared to discordant diagnoses. Furthermore, the sampling method did not influenced the degree of concordance. Finally, patients with preoperative low-grade and postoperative high-grade EC had significant improved DSS compared to patients with concordant high-grade EC.

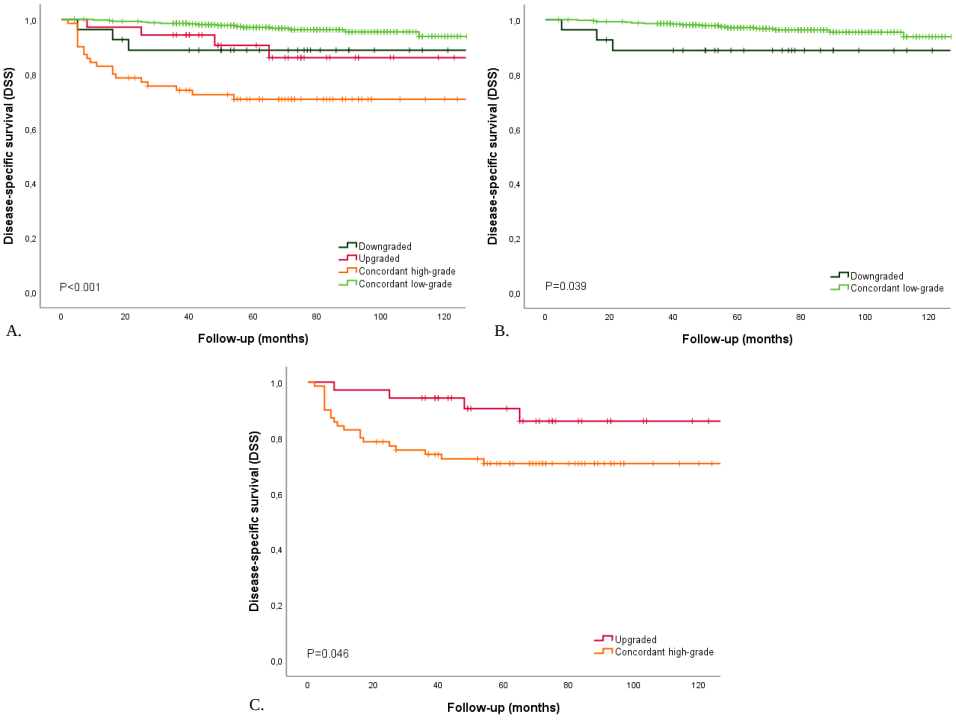

Figure 3 A-C. Kaplan-Meier survival curves of disease-specific survival A. Disease-specific survival of concordant low-grade endometrial cancer (EC), concordant high-grade EC, downgraded and upgraded patients. B. Disease-specific survival of concordant low-grade EC and downgraded patients. C. Disease-specific survival of concordant high-grade EC and upgraded patients.

Numerous studies stated that preoperative endometrial sampling is poorly correlated with final tumor grade and histological subtype.[19-21] On the contrary, Sany et al. mentioned good agreement between preoperative and final pathology with sensitivities of 96.5% for EECs and 86.5% for NEECs.[22] Our study findings are in line with Visser et al. who reported an overall moderate concordance of 67% on tumor grade.[11] Clinically relevant downgrading was reported in 26% of the included patient samples and upgrading in 8%.[11] Our results show similar clinically relevant downgrading of 26.7% and upgrading in 7.8%. Several studies note that the diagnostic consensuses of tumor grade and histological subtype based on morphology alone are overall moderate. Performing immunohistochemical (IHC) markers on preoperative tissue could help to improve the degree of concordance between pre- and postoperative diagnosis, especially for preoperative grade 2 and grade 3 EC with the lowest concordance.[11, 23-26] For preoperative grade 2, a panel of progesterone (PR) and p53 biomarkers has been recommended, and, for grade 3/high-grade EC additional PR, IMP3 and L1CAM.[26] Whether combined pathologic and molecular classification might further improve preoperative classification for high-grade EC needs to be determined.[27]

Our study design is comparable to Reijnen et al. in which the diagnostic accuracy of pipelle endometrial sampling and the amount of endometrial tissue surface for benign, premalignant and malignant tissue was quantified.[14] Reijnen et al. found a positive correlation between the amount of endometrial tissue surface and concordance of diagnosis for premalignant and malignant tissue, furthermore he defined a minimum cut-off of 35 mm^2 to classify an endometrial sample as conclusive. Interestingly, whereas the amount of tissue seems to be important for classifying tissue as premalignant or malignant, in our study, no positive correlation was found when malignant tissue was classified into tumor grade and histology and we did not found a minimum cut-off for concordant grading (*data not shown*). An explanation for this contra-intuitive finding could be interobserver agreement, yet, both studies show a high intraclass correlation coefficient (ICC) (0.98 vs. 0.92 in our study).[14] Another explanation could be sampling bias or a missed tumor component by the pathologist. In our study, three experienced expert gynecological pathologists (JB, HK, KV) performed an explorative analysis in 30 (46.9%) cases with low- vs. high-grade discrepancy. Sampling bias based on heterogeneous and mixed tumors, or only superficial tumor tissue sampling was present in a third of the cases. In two third of the cases the discrepancy was misjudged by the pathologist, by miscalculation of the percentage solid growth or missed tumor component (*data not shown*). So, incorrect classification by the pathologist seems to be present, and will remain in the current diagnostic context. This might partially be resolved by molecular profiling in high-grade EEC as demonstrated by Bosse et al., but will not solve the sampling bias.[28]

The concordance between pre- and postoperative low- and high-grade EC did not significantly differ between the three sampling methods, which is quite comparable to other studies.[11, 29] Illustrating, that more tissue provided with D&C or accurate sampling by hysteroscopic biopsy will not automatically result in more concordant diagnoses.

Accurate preoperative classification of tumor grade and histological subtype is crucial in EC, as this may be directive to the extent of the surgical approach. Consequently, postoperative upgrading will lead to omitted lymph node surgery and/or staging procedure and altered adjuvant therapy, whereas downgrading may result in unnecessarily surgical related complications both impacting clinical outcome.[11] A significant increase of DSS has been found in patients that were postoperatively upgraded, compared to patients with concordant high-grade EC. Furthermore, patients that were downgraded had significant decreased DSS compared to concordant low-grade EC. Both of our findings are in line with Werner et al.[13], and may be explained by the presence of tumor heterogeneity and/or minor mixed morphologic characteristics.[30, 31]

To our knowledge, this is the first study that quantified the amount of endometrial tissue surface by computerized measurement, and related this to the degree of concordance with final tumor grade and histological subtype in EC. The computerized assessment of the endometrial tissue surface was performed in a structured and reproducible fashion with a good interobserver agreement (ICC 0.92, 95% CI 0.80-0.97).

As this was a retrospective study, one limitation could be that there has been no study protocol for the assessment of endometrial tissue. In addition, there might be a selection bias as the original diagnosis and classification of both pre- and postoperative histology was used without centralized pathology review. However, slides were from large referral hospitals and diagnoses were made by expert gynecological-pathologists. The results of this study are therefore applicable to daily practice and, as agreement is in line with previous findings, bias may be therefore considered to be limited. Finally, the small number of patients with serous EC (n=14, 2.4%) could limit the generalizability for this type of EC. Yet, serous carcinoma represents <10% of all ECs[32]. Also, it is known that there is poor interobserver agreement in differentiating serous EC from high-grade EEC based on preoperative histology.[23, 24, 33-36]

Although several studies support the use of a binary grading system (low- vs. high-grade) over the three-tiered FIGO grading system with respect to reproducibility, awareness of clinically relevant down- and upgrading remains crucial.[9, 18, 35, 37, 38] Instead of providing more endometrial tissue, the use of a simple and relatively cheap set of IHC markers, such as p53 (reflecting the most aggressive molecular subgroup of the TCGA), ER/PR and L1CAM, could improve the concordance between pre- and postoperative low- and high-grade EC, and pre- and postoperative individual tumor grade and histological subtype.[26, 39] According to the recent recommendations of the Society of Gynecologic Oncology (SGO), current clinicopathological prognostic parameters (e.g. histology and grade) should guide initial clinical management in EC. Molecular classification, especially *TP53* mutations, may help guide future treatment decisions.[8]

In conclusion, obtaining a higher amount of preoperative endometrial tissue surface does not increase the concordance between pre- and postoperative low- and high-grade classification in EC. Awareness of clinically relevant down- and upgrading is crucial to reduce subsequent over- or undertreatment with impact on outcome.

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SUPPLEMENTARY

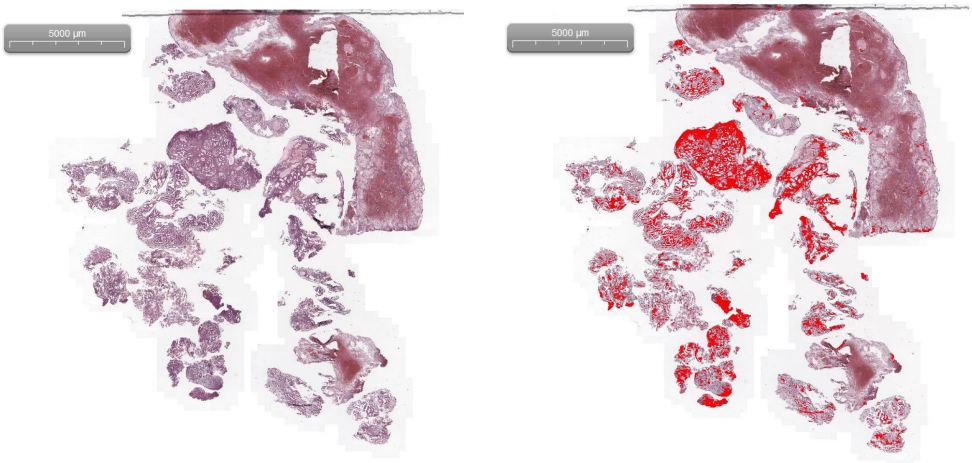


Figure S1. A digitalized 18 mm^2 slide with selection of the endometrial tissue surface

Table S1. Baseline characteristics of patients diagnosed with postoperative NEEC histology and divided by the NEEC subgroups

	NEEC total (*n*=34)	**Serous (n=14)**	**Clear cell (n=8)**	**Carcinosarcoma (n=7)**	**Heterogenous (serous + clear cell) (n=5)**
Age (years)	65.5 (47.0-88.0)	64.0 (51.0-82.0)	65.0 (47.0-81.0)	69.0 (56.0-74.0)	72.0 (61.0-88.0)
BMI ((kg/m^2)	27.0 (18.4-41.5)	27.3 (18.4-37.8)	24.6 (21.2-33.6)	27.0 (22.0-41.1)	29.3 (20.4-41.5)
Preoperative grade					
Premalignant†	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
1	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
2	4 (11.8)	3 (21.4)	0 (0.0)	1 (14.3)	0 (0.0)
3	14 (41.2)	5 (35.7)	3 (37.5)	2 (28.6)	4 (80.0)
NEEC	16 (47.0)	6 (42.9)	5 (62.5)	4 (57.1)	1 (20.0)
FIGO stage surgical					
I	13 (38.2)	6 (42.9)	2 (25.0)	4 (57.1)	1 (20.0)
II	6 (17.6)	0 (0.0)	2 (25.0)	2 (28.6)	2 (40.0)
III	11 (32.4)	6 (42.9)	3 (37.5)	1 (14.3)	1 (20.0)
IV	4 (11.8)	2 (14.2)	1 (12.5)	0 (0.0)	1 (20.0)
Positive nodes					
No	15 (44.1)	6 (42.9)	2 (28.6)	6 (85.7)	1 (50.0)
Pelvic	6 (17.6)	3 (21.4)	2 (28.6)	0 (0.0)	1 (50.0)
Para-aortic	5 (14.7)	3 (21.4)	2 (28.6)	0 (0.0)	0 (0.0)
Both	2 (5.9)	0 (0.0)	1 (14.3)	1 (14.3)	0 (0.0)
Adjuvant treatment					
No	12 (35.3)	3 (21.4)	3 (37.5)	3 (37.5)	3 (60.0)
Radiotherapy	12 (35.3)	6 (42.9)	2 (25.0)	2 (25.0)	1 (20.0)
Chemotherapy	7 (20.6)	4 (28.6)	3 (37.5)	3 (37.5)	0 (0.0)
Chemoradiotherapy	3 (8.8)	1 (7.1)	0 (0.0)	0 (0.0)	1 (20.0)

Data is presented in number (%) and median (range)

Abbreviations: NEEC, non-endometroid endometrial cancer; BMI, Body Mass Index; FIGO, International Federation of Gynecology and Obstetrics

†including simple or complex hyperplasia, with or without atypia.

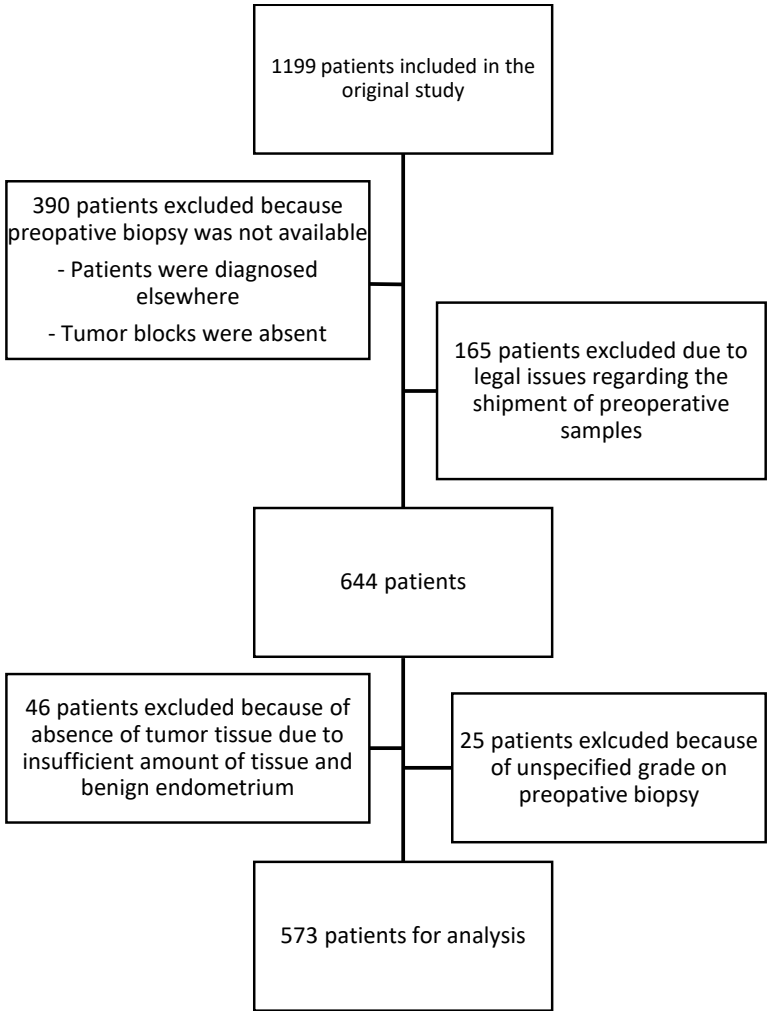


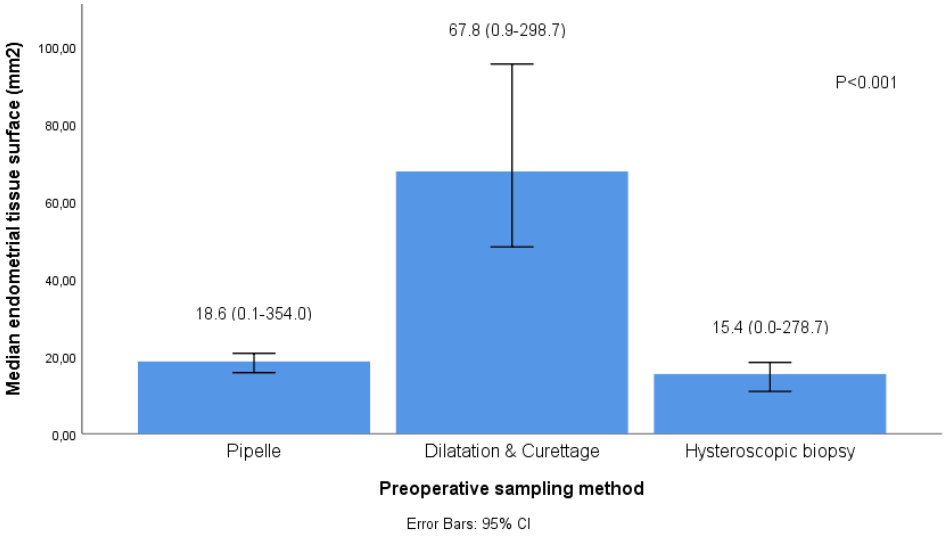
Figure S2. Study flowchart

Table S2. Median endometrial tissue surface (mm^2) for concordant and discordant tumor grade and histological subtype, and low- and high-grade classification per included center.

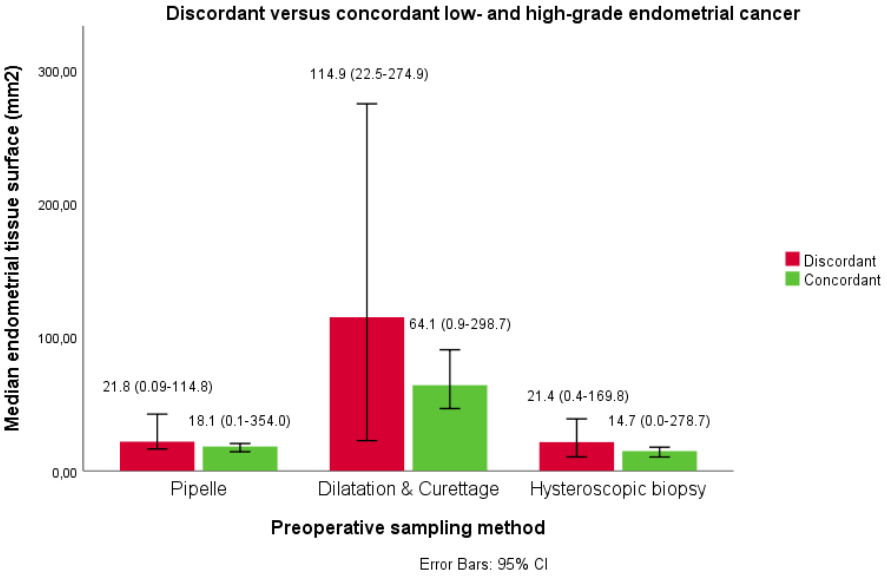
Centers	Concordant tumor grade and histological subtype	Discordant tumor grade and histological subtype	*P*	Concordant low- and high-grade EC	Discordant low- and high-grade EC	*P*
1 (*n*=102)	17.6 (2.4-130.2)	26.0 (0.7-114.8)	0.080	18.0 (0.7-130.2)	38.6 (2.8-114.8)	0.011*
2 (*n*=84)	65.6 (5.2-298.7)	71.8 (9.6-274.9)	0.733	65.5 (5.2-298.7)	94.2 (16.5-274.9)	0.150
3 (*n*=42)	18.4 (1.7-70.9)	22.4 (1.0-78.8)	0.356	20.0 (1.0-78.8)	24.6 (9.7-38.8)	0.664
4 (*n*=34)	32.7 (3.8-210.2)	11.0 (0.0-354.0)	0.071	14.7 (0.9-354.0)	10.1 (0.0-55.9)	0.295
5 (*n*=116)	4.0 (0.1-203.0)	12.5 (0.0-169.8)	0.702	3.3 (0.0-203.0)	31.8 (0.4-169.8)	0.016*
6 (*n*=17)	40.2 (0.7-85.1)	14.9 (5.4-30.9)	0.093	20.6 (0.7-85.1)	15.1 (7.9-22.3)	0.618
7 (*n*=82)	21.9 (0.1-251.5)	9.1 (0.0-98.2)	0.050	11.5 (0.0-251.5)	16.1 (1.5-65.0)	0.527
8 (*n*=63)	17.0 (0.2-278.7)	16.5 (0.1-115.3)	0.884	16.5 (0.0-278.7)	27.7 (2.8-45.7)	0.775
9 (*n*=33)	22.2 (3.7-66.0)	22.6 (0.6-125.2)	1.000	24.5 (0.6-125.2)	16.3 (3.3-38.7)	0.169

Data is presented in median (range)

Abbreviation: EC, endometrial cancer



A.



B.

Figure S3 A-D. A. Median endometrial tissue surface (mm^2) by preoperative sampling method. B. Median endometrial tissue surface (mm^2) for concordant and discordant low- and high-grade diagnosis distributed by sampling method. C. Median endometrial tissue surface (mm^2) for concordant and discordant individual tumor grade and histological subtype distributed by sampling method. D. Median endometrial tissue surface (mm^2) for preoperative individual tumor grade and histological subtype distributed by sampling method.

Table S3. Patient characteristics versus the preoperative sampling method.

	Total (n=549)††	**Pipelle (n=259)**	**D&C (n=77)**	**Hysteroscopic biopsy (n=213)**	***P***
Preoperative					
Premalignant†	8 (1.5)	8 (100.0)	NA	NA	<0.001*
Grade 1 EEC	302 (55.0)	118 (45.6)	42 (54.5)	142 (66.7)	
Grade 2 EEC	150 (27.3)	79 (30.5)	18 (23.4)	53 (24.9)	
Grade 3 EEC	67 (12.2)	41 (15.8)	15 (19.5)	11 (5.2)	
NEEC	22 (4.0)	13 (5.0)	2 (2.6)	7 (3.3)	
Postoperative					
Grade 1 EEC	230 (41.9)	94 (36.3)	40 (51.9)	96 (45.1)	0.002*
Grade 2 EEC	221 (40.3)	105 (40.5)	23 (29.9)	93 (43.7)	
Grade 3 EEC	71 (12.9)	39 (15.1)	11 (14.3)	21 (9.9)	
NEEC	27 (4.9)	21 (8.1)	3 (3.9)	3 (1.4)	
Concordant low- and high-grade EC	488 (88.9)	225 (86.9)	70 (90.9)	20 (90.6)	0.364
Concordant tumor grade and histology subtype	335 (61.0)	168 (64.9)	49 (63.6)	118 (55.4)	0.097
Downgraded (preoperative high- and postoperative low-grade)	26 (4.7)	14 (5.4)	5 (6.5)	7 (3.3)	0.237
Upgraded (preoperative low- and postoperative high-grade)	35 (6.4)	20 (7.7)	2 (2.6)	13 (6.1)	

Data is presented in number (%)

Abbreviations: EC, endometrial cancer; EEC, endometroid endometrial cancer; NEEC, non-endometroid endometrial cancer; D&C, dilatation & curettage.

*p<0.05, †including simple or complex hyperplasia, with or without atypia, †† n=24 missing sampling method

CHAPTER 3

RELEVANCE OF MOLECULAR PROFILING IN PATIENTS WITH LOW-GRADE ENDOMETRIAL CANCER

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ABSTRACT

Importance

Patients with low-grade (grade 1-2) endometrial cancer (EC) are characterized by their favorable prognosis when compared to high-grade (grade 3) EC patients. With the implementation of molecular profiling, the prognostic relevance of tumor grading might lose attention. As most patients present with low-grade EC and have an excellent outcome, it could be questioned whether molecular profiling is valuable in these patients.

Objective

To determine the prognostic relevance of molecular profiling within low-grade EC patients.

Design

In this cohort study, patients were diagnosed with EC between 1994 and 2018, with a median follow-up of 5.9-years. Molecular subgroups were determined by next generation sequencing using single-molecule molecular inversion probes and by immunohistochemistry. Subsequently, cases were classified as: polymerase epsilon (*POLE)*-mutant, microsatellite instable (MSI), tumor protein 53 (*TP53)*-mutant and no-specific molecular profile (NSMP).

Setting

Retrospective multicenter international study.

Participants

Patients diagnosed with all histological subtypes and FIGO (International Federation of Gynecology and Obstetrics) stages of EC. Patients with early-stage EC (FIGO I-II) were only included with known lymph node status.

Exposure

Molecular testing of the four molecular subgroups.

Main outcome and measure

Disease-specific survival (DSS) within the molecular subgroups.

Results

A total of 393 European EC patients were included with a median age of 64.0 (31.0-86.0) years and median BMI 29.1 (18.0-58.3) kg/m^2. Most patients presented with early-stage EC (73.8%), and low-grade EC (53.2%). Of all patients, 8.4% was classified as *POLE*-mutant, 19.8% as MSI, 18.3% as *TP53*-mutant and 53.4% as NSMP. Across all molecular subgroups, patients with low-grade EC had superior 5-year DSS compared to high-grade EC, varying between 90-100% vs. 41-90% (P<.001), respectively. Multivariable analysis in the entire

cohort including age, tumor grade, FIGO stage, lymphovascular space invasion (LVSI), and the molecular subgroups showed that only high-grade EC, *TP53*-mutant, and advanced-stage (FIGO III-IV) were independently associated with reduced DSS (respectively, HR 4.29 (95%-CI 2.15-8.53) *P*<.001, HR 1.76 (1.04-2.95) *P*=.03, HR 4.26 (2.50-7.26) *P*<.001).

Conclusions and relevance

Patients with low-grade EC have an excellent prognosis independent of molecular subgroup. Current data do not support routine molecular profiling in patients with low-grade EC, and demonstrate the importance of primary diagnostic tumor grading and selective profiling in low-grade EC to increase cost-effectiveness.

INTRODUCTION

More than 85% of endometrial cancer (EC) patients present with low-grade histology (grade 1-2) FIGO (International Federation of Gynecology and Obstetrics) early-stage (I-II) endometrioid EC (EEC), and have a favorable prognosis with a 5-year overall survival of 95%.[1, 2] Standard treatment consists of hysterectomy with bilateral salpingo-oophorectomy, including lymph node staging for patients with substantial risk of lymph node metastasis.[2]

The Cancer Genome Atlas (TCGA) defined four important prognostic molecular subgroups in EC based on integrated genomic data: I) ultramutated tumors with polymerase epsilon (*POLE*) mutations, II) microsatellite instability (MSI), III) copy-number-high (CNH) with frequent tumor protein (*TP53*) mutations, IV) copy-number-low (CNL) (also known as no-specific molecular profile (NSMP)). These subgroups increase insight in biological tumor behavior based on molecular signature beyond current morphological classification.[3] Patients with *TP53*-mutant tumors have the worst outcome, representing 15% of all EC diagnosis and responsible for 50-70% of all EC-related mortality.[4, 5]

For decades, tumor grading and FIGO staging have been used to guide primary and adjuvant treatment.[6] Currently, with incorporation of the molecular classification to guide adjuvant treatment, the prognostic relevance of tumor grading has gained less attention.[7] Molecular profiling has shown to improve prognostication mainly in patients with high-grade EC, probably due to poor interobserver reproducibility of morphological classification and the prognostic and intratumoral heterogeneity of high-grade ECs.[5, 8] So far, no data has been reported about the prognostic relevance of molecular profiling specifically in patients with low-grade EC. The aim of this study is to determine the prognostic relevance of molecular profiling within low-grade EC. As most patients present with low-grade EC and have an excellent outcome, we hypothesized that molecular profiling might be less useful in these patients.

MATERIALS AND METHODS

Data source

This retrospective European multicenter study consisted of data out of four previously published studies and one submitted, all published by our research group.[9-13] A baseline overview and flowchart of the included studies is shown in *eTable 1* and *eFigure 1 in the supplement*. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline.

Patients

All patients were surgically treated between 1994 and 2018 (median 2006). Inclusion criteria for this current study were: (I) availability of EC tissue samples (II) patients diagnosed with primary EC with all histological subtypes and FIGO stages, in whom (III) patients were successfully classified according molecular profiling or the Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE)[14] classification. Exclusion criteria were: (I) unknown lymph node (LN) status in FIGO early-stage.

Patients were classified into one of the four molecular subgroups according to the diagnostic algorithm (**Figure 1**); *POLE*-mutant, MSI, *TP53*-mutant and NSMP. Multiple-classifiers were classified as the molecular subgroup with the best prognosis.[15]

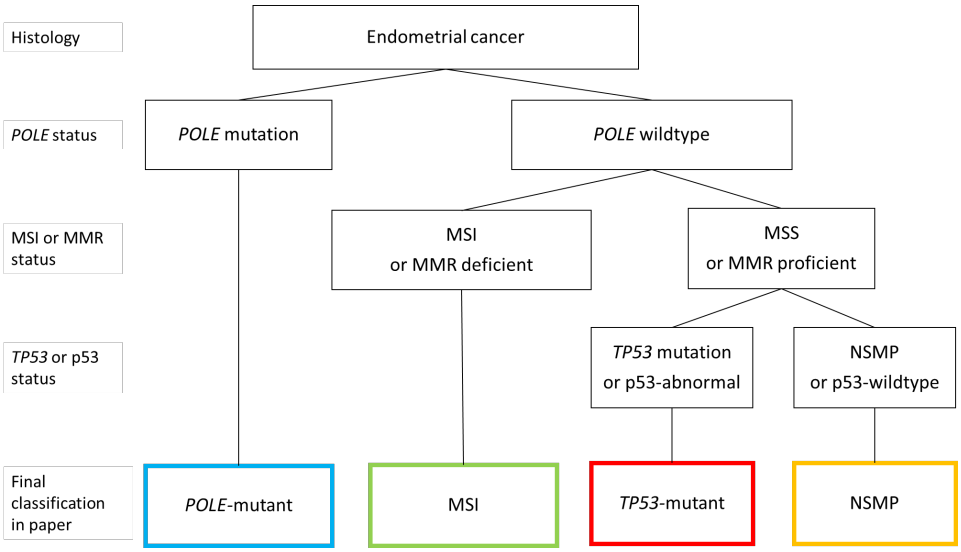

Figure 1. Diagnostic algorithm of patients diagnosed with molecular profiling or with immunohistochemistry.

Abbreviations: *POLE,* Polymerase epsilon; MSI, Microsatellite instability; MMR, Mismatch repair protein, *TP53,* Tumor protein 53; NSMP, No-specific molecular profile

DNA analysis

Representative areas of EC in the surgical specimen were marked and selected for formalin-fixed paraffin-embedded (FFPE) 20 μm thick sections. Slides were cut from these FFPE section and stained with hematoxylin and eosin (H&E). Tumor areas were marked on these slides and the tumor cell percentage was estimated. These specimens were digested overnight at 56°C in TET-lysis buffer (10mmol/L Tris/HCL pH 8.5, 1 mmol/L EDTA pH 8.0, 0.01% Tween-20) with 5% Chelex-100 (Bio-Rad, Hercules, CA) and 0.2% proteinase K, with subsequent inactivation at 95°C for 10 min. After this was centrifugated, the supernatant was transferred into a clean tube. DNA concentration was determined using the Qubit Broad Range Kit (Thermo Fisher Scientific, Waltham, MA).

smMIP design and library preparation

Samples were analyzed with single-molecule Molecular Inversion Probes (smMIPs). The design (Integrated DNA Technologies Leuven, Belgium) as well as the library preparation were previously published.[16] *eMethod in the supplement* shows further detailed information on smMIP design, library preparation and sequencing.

Immunohistochemical staining and scoring

Detailed information about the immunohistochemical staining for p53, PMS2 and MSH6 can be found in the *eMethod in the supplement* and original published studies.[9, 10] In brief, staining for p53 was considered abnormal when more than 80% of tumor cell nuclei showed strong expression (overexpression) or when there was complete absence of nuclear staining (null-expression). Mismatch repair deficiency (MMR-D) was defined as total loss of nuclear staining of PMS2 and/or MSH6, in the presence of a positive internal control.

Statistical analysis

Early-stage was defined as FIGO stage I-II and advanced-stage as FIGO III-IV. Low-grade EC was defined as grade 1 and grade 2 EC and high-grade as grade 3 EEC and non-EEC (NEEC), according to the latest ESGO-ESTRO-ESP and WHO guideline.[2, 17] The included patients in our retrospective cohort received either full lymphadenectomy or no lymphadenectomy, as sentinel lymph node procedure was not routinely incorporated yet.

Statistical analyses were performed on SPSS version 25.0 (released 2017, Armonk, NY, United States) using χ^2, Fisher's exact test, Mann-Whitney U test, Kaplan-Meier survival analysis and univariable and multivariable Cox-regression analysis. SAS version 9.4 was used for survival curves including Hall-Wellner confidence bands. $P<0.05$ was considered statistically significant. The assumption of proportionality for the included variables was tested with log minus log curves and time-dependent covariate (time x covariate). Disease-

specific survival (DSS) was defined as time from date of surgery to date of death from EC, all censored by date of last contact.

We validated our data with the open access database of Kandoth et al. by performing Kaplan-Meier analysis. Method and baseline characteristics can be found in the original article.[3]

RESULTS

Patients

In total, 689 patients were available with successful DNA analysis, of whom 296 (42.9%) were excluded based on unknown LN status in FIGO early-stage (*Efigure 1 in the Supplement)*. Baseline characteristics of the included versus excluded patients are shown in *Etable 2 in the Supplement*. Of the included 393 patients, median age was 64.0 (31.0-86.0) years and median BMI 29.1 (18.0-58.3) kg/m^2 (Table 1). Baseline characteristics of the included patients according to the four molecular subgroups are shown in **Table 1.** Molecular subgroup distribution was as followed: *POLE*-mutant 8.4% (n=33), MSI 19.8% (n=78), *TP53*-mutant 18.3% (n=72) and NSMP 53.4% (n=210). Low- and high-grade EC were equally distributed in patients with *POLE*-mutant and MSI tumors. The majority of patients with *TP53*-mutant tumors were high-grade EC and the majority of NSMP tumors were low-grade EC. The EC-related mortality was highest in the *TP53*-mutant subgroup compared to the other molecular subgroups (45.8% vs. 15.7%, 7.7% and 3.0%).

Table 1. Baseline characteristics of the included study cohort according to the four molecular subgroups

			Total N=393	*POLE*-mutant N=33 (8.4%)	MSI N=78 (19.8%)	*TP53*-mutant N=72 (18.3%)	NSMP N=210 (53.4%)	*P*
Patient characteristic								
Age (years)			64.0 (31.0-86.0)	58.0 (31.0-78.0)	65.0 (43.0-83.0)	64.5 (35.0-82.0)	63.5 (37.0-86.0)	.001
BMI kg/m^2			29.1 (18.0-58.3)	31.3 (18.4-58.3)	29.5 (21.90-46.9)	31.2 (21.2-41.1)	27.0 (18.0-38.9)	.004
Primary treatment								
Lymph node dissection	No		12 (3.1)	0 (0.0)	3 (3.8)	2 (2.8)	7 (3.3)	.26
	Yes		376 (95.7)	33 (100.0)	75 (96.2)	67 (93.1)	201 (95.7)	
		Pelvic	214 (56.9)	21 (63.6)	47 (62.7)	25 (37.3)	121 (60.2)	
		Para-aortic	13 (3.4)	0 (0.0)	1 (1.3)	4 (6.0)	8 (4.0)	
		Pelvic and para-aortic	54 (14.4)	4 (12.1)	8 (10.7)	12 (17.9)	30 (14.9)	
		Unknown which nodes	95 (25.3)	8 (24.2)	19 (25.3)	26 (38.8)	42 (20.9)	
	Unknown		5 (1.3)	0 (0.0)	0 (0.0)	3 (4.2)	2 (1.0)	
Final pathologic characteristics								
Histology	EEC		318 (80.9)	28 (84.8)	69 (88.5)	41 (56.9)	180 (85.7)	<.001
	NEEC		75 (19.1)	5 (15.2)	9 (11.5)	31 (43.1)	30 (14.3)	
Grade	1-2		209 (53.2)	17 (51.5)	41 (52.6)	13 (18.1)	138 (65.7)	<.001
	3		184 (46.8)	16 (48.5)	37 (47.4)	59 (81.9)	72 (34.3)	
MI	<50%		197 (50.1)	13 (39.4)	42 (53.8)	32 (44.4)	110 (52.4)	.14
	>50%		194 (49.4)	19 (57.6)	35 (44.9)	40 (55.6)	100 (47.6)	
	Unknown		2 (0.5)	1 (3.0)	1 (1.3)	0 (0.0)	0 (0.0)	
LVSI	No		304 (77.4)	27 (81.8)	64 (82.1)	41 (56.9)	172 (81.9)	<.001
	Yes		89 (22.6)	6 (18.2)	14 (17.9)	31 (43.1)	38 (18.1)	
Lymph nodes	N0		305 (77.6)	29 (87.9)	68 (87.2)	46 (63.9)	162 (77.1)	.02
	N1		43 (10.9)	1 (3.0)	5 (6.4)	13 (18.1)	24 (11.4)	

Table 1. *Continued*

		Pelvic	18 (41.9)	1 (100.0)	2 (40.0)	6 (46.2)	9 (37.5)	
		Para aortic	7 (16.3)	0 (0.0)	0 (0.0)	4 (30.8)	3 (12.5)	
		Pelvic and para aortic	6 (13.9)	0 (0.0)	2 (40.0)	0 (0.0)	4 (16.7)	
		Unknown which nodes	12 (27.9)	0 (0.0)	1 (20.0)	3 (23.0)	8 (33.3)	
	Nx		40 (10.2)	3 (9.1)	5 (6.4)	13 (18.1)	24 (11.4)	
FIGO stage	Early (I-II)		290 (73.8)	27 (81.8)	68 (87.2)	37 (51.4)	158 (75.2)	<.001
	Advanced (III-IV)		103 (26.2)	6 (18.2)	10 (12.8)	35 (48.6)	52 (24.8)	
Adjuvant treatment								
None			97 (24.7)	6 (18.2)	15 (19.2)	17 (23.6)	59 (28.1)	.02
Radiotherapy			225 (57.3)	20 (60.6)	56 (71.8)	34 (47.2)	115 (54.8)	
	EBRT		67 (29.8)	8 (40.0)	15 (26.8)	16 (47.1)	28 (24.3)	
	VBT		89 (39.6)	6 (30.0)	25 (44.6)	7 (20.6)	7 (20.6)	
	ERBT+VBT		47 (20.9)	5 (25.0)	10 (17.9)	5 (14.7)	5 (14.7)	
	Unknown		22 (9.8)	1 (5.0)	6 (10.7)	6 (17.6)	6 (17.6)	
Chemotherapy			33 (8.4)	2 (6.1)	2 (2.6)	13 (18.1)	16 (7.6)	
Chemoradiation			34 (8.7)	5 (15.2)	4 (5.1)	6 (8.3)	19 (9.0)	
Unknown			4 (1.0)	0 (0.0)	1 (1.3)	2 (2.8)	1 (0.5)	
Mortality								
Recurrence			74 (18.8)	1 (3.1)	12 (15.8)	30 (50.8)	31 (15.4)	<.001
Mortality			90 (22.9)	2 (6.1)	8 (10.3)	38 (52.8)	42 (20.0)	<.001
EC-related mortality			73 (18.6)	1 (3.0)	6 (7.7)	33 (45.8)	33 (15.7)	<.001

Data is presented as No. (%), median (IQR)

Abbreviations: *POLE*, Polymerase epsilon; MSI, Microsatellite instability; *TP53*, Tumor protein 53; NSMP, No-specific molecular profile; EEC, endometrioid endometrial cancer; NEEC, non-endometrioid endometrial cancer; MI, myometrial invasion; LVSI, lymphovascular space invasion; N0, negative lymph nodes, N1, positive lymph nodes; Nx, no information about the lymph nodes; FIGO, Federation International of Gynecology and Obstetrics; EBRT, external beam radition therapy; VBT, vaginal brachytherapy; EC, endometrial cancer.

Outcome

For the independent variables in cox-regression models the proportional hazard assumption was checked. Results of testing the proportional hazard assumption shows that all the variables were satisfied.

The 5-years DSS of the included study cohort showed worst prognosis for *TP53*-mutant and best for *POLE*-mutant tumors (**Figure 2A**). Across all molecular subgroups, patients with low-grade EC had an outstanding 5-year DSS when compared to high-grade EC, varying between 90-100% vs. 41-90% (*P*<.001), respectively (**Figure 2B**). For all the molecular subgroups and patients with grade 1 EC, an excellent 5-year DSS is shown (**Figure 2C**). Patients with grade 2 EC and *TP53*-mutant or NSMP shows a 5-year DSS of 85-95%, respectively (**Figure 2D**). Within the external validation cohort (n=373), survival outcomes were similarly distributed across all the molecular subgroups, with 5-year DSS varying between 98-100% in low-grade EC and 62-100% in high-grade EC (*P*=0.017) (*Efigure 2 in the Supplement)*.

In multivariable analysis of the entire cohort, high-grade EC, *TP53*-mutant and FIGO advanced-stage were independently associated with reduced DSS. Among patients with low-grade EC, FIGO advanced stage was independently associated with a reduced DSS, but none of the molecular subgroups. However, the number of events was low and the estimated HR's were of similar magnitude as in the entire cohort (**Table 2**). Within patients with high-grade EC, only FIGO advanced-stage remained associated as independent prognostic factor for a reduced DSS (*Etable 3 in the Supplement)*.

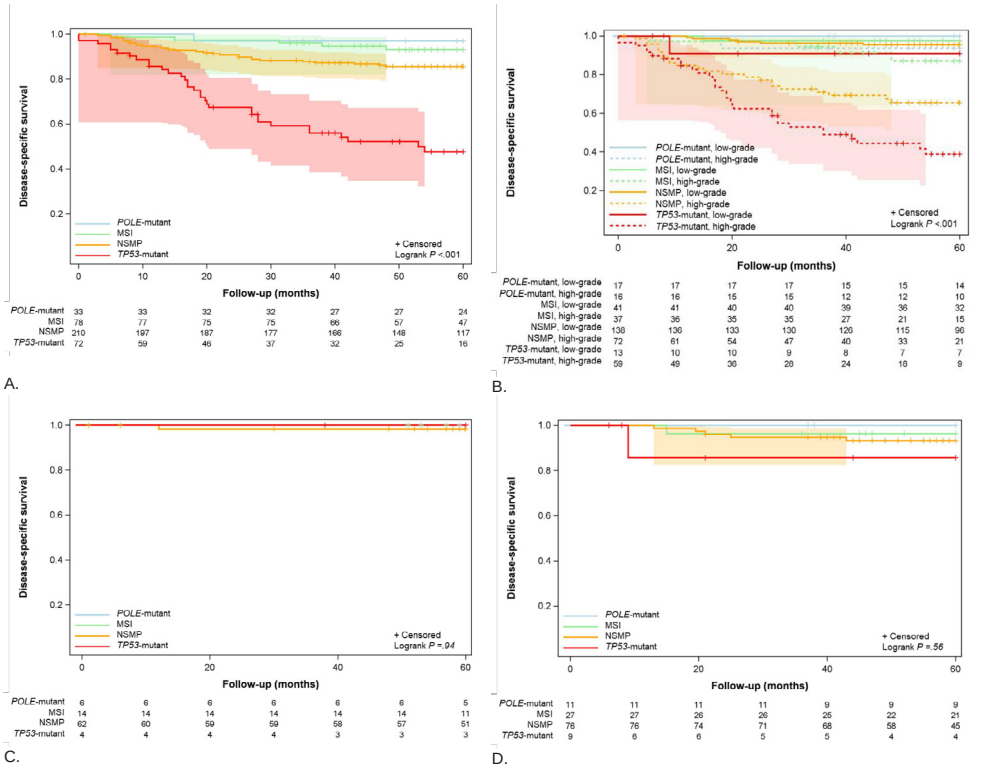

Figure 2A-D. A. 5-year disease-specific survival (DSS) of the molecular subgroups in the entire included cohort. B. 5-year DSS of the molecular subgroups and low- versus high-grade endometrial cancer (EC). C. 5-year DSS of the molecular subgroups within grade 1 EC patients. D. 5-year DSS of the molecular subgroups within grade 2 EC patients.

Abbreviations: *POLE*, Polymerase epsilon; MSI, Microsatellite instability; *TP53*, Tumor protein 53; NSMP, No-specific molecular profile

Table 2. Cox regression univariable and multivariable analysis disease-specific survival (DSS) of the entire cohort and within low-grade EC

Variable	Entire cohort Univariable DSS		Entire cohort Multivariable DSS 73 events		Low-grade EC Univariable DSS		Low-grade EC Multivariable DSS 12 events	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Patient characteristics								
Age (continuous)	1.04 (1.02-1.07)	.001	1.02 (0.99-1.05)	.08	[a]		[a]	
Grade								
1-2	1	<.001	1	<.001	[a]		[a]	
3	7.70 (4.13-14.35)		4.29 (2.15-8.53)					
Molecular subgroup								
POLE-mutant	0.17 (0.02-1.27)	.09	0.16 (0.02-1.16)	.07	0.00 (0.00-0.00)	.99	0.00 (0.00-0.00)	.98
MSI	0.45 (0.19-1.11)	.08	0.51 (0.21-1.22)	.13	0.73 (0.15-3.40)	.69	0.65 (0.13-3.02)	.58
TP53-mutant	4.14 (2.53-6.75)	<.001	1.76 (1.04-2.95)	.03	1.58 (0.19-12.63)	.66	2.94 (0.33-25.83)	.63
NSMP	1		1		1		1	
LVSI								
No	1	<.001	1	.64	1	.30	1	.78
Yes	3.78 (2.37-6.00)		1.13 (0.67-1.88)		2.27 (0.48-10.57)		1.28 (0.24-6.88)	
FIGO								
Stage I-II	1	<.001	1	<.001	1	.01	1	.008
Stage III-IV	7.02 (4.35-11.33)		4.26 (2.50-7.26)		4.57 (1.43-14.56)		5.38 (1.55-18.62)	

Abbreviations: DSS, disease-specific survival; EC, endometrial cancer; HR, hazard ratio; CI, confidence interval; *POLE*, Polymerase epsilon; MSI, Microsatellite instability; *TP53,* Tumor protein; NSMP, No-specific molecular profile, LVSI, lymphovascular space invasion; FIGO, Federation International of Gynecology and Obstetrics.

[a]Cox-regression analysis within low-grade EC patients did not included age and grade as variables.

DISCUSSION

A new era of incorporating molecular profiling in EC to guide adjuvant treatment has started. This study assessed whether molecular profiling is prognostically relevant specifically in patients with low-grade EC. Interestingly, low-grade EC patients had a very favorable 5-years DSS independent of the molecular subgroups, when compared to high-grade EC. Furthermore, high-grade EC as well as *TP53*-mutant and FIGO advanced-stage, were independently associated with a decreased DSS. Within patients with low-grade EC, none of the molecular subgroups seemed independently associated with reduced DSS.

Our study confirmed the excellent prognosis for *POLE*-mutant, good/intermediate for MSI and NSMP, and poor for *TP53*-mutant when analyzing all histological subtypes.[3, 14] Moreover, the present study illustrated that the molecular subgroups were mainly discriminative amongst high-grade EC.[3, 8] So far, no previous studies have evaluated the outcome for the molecular subgroups within patients with low-grade EC. We analyzed the open access data of Kandoth et al.[3] to validate our results.

Molecular profiling has been proposed to perform routinely in all EC patients.[2, 18] However, as the majority of EC patients are diagnosed with low-grade EC, it is questioned whether this strategy is beneficial and cost-effective. Our data on low-grade EC demonstrate that full molecular profiling seems not necessary (except for screening for Lynch syndrome).[19] Multivariate analyses did not show any statistically significant effect of the molecular subgroups within patients with low-grade EC. However, the number of events was low in this subgroup analysis. Analyzing the HR's, the high HR of *TP53*-mutant could possibly still be associated with a reduced DSS in low-grade EC patients. We question whether this is mainly attributed to grade 2 EC as shown in the DSS curve of *TP53*-mutant within grade 2 EC. Poor interobserver reproducibility is mainly observed within grade 2 and 3 EC, in these patients the use of immunohistochemical (IHC) or molecular markers could be recommended, e.g. *TP53* mutation analyses in patients with doubtful low-grade (grade 2) EC.[4, 8, 20, 21] In this way binary grading (low- vs. high-grade) including molecular profiling or immunohistochemistry could be optimized with respect to reproducibility.[2]

Molecular profiling is demanding for health care facilities and comes with high costs, especially challenging in low income countries. The primary clinical management of EC should therefore be guided based on morphological tumor characteristics, consideration of immunohistochemistry in doubtful cases, and selective molecular profiling in high-grade and/or advanced stage patients for guiding adjuvant treatment decisions.[22]

This is the first study to address the prognostic relevance of molecular profiling in low-grade EC. Our study consisted of a large study population, with known LN status in FIGO early-stage to prevent bias by undiagnosed stage III. Furthermore, our results are comparable with the data of the TCGA research network.[3]

A few limitations must be reported due to the retrospective character of the study. First, differences in the methodology between the included cohorts exists. More than 80% was performed with complete molecular profiling and less than 20% with the immunohistochemistry surrogates of molecular profiling according to the ProMisE criteria. Though, immunohistochemistry surrogate analysis has been established as a reliable alternative for molecular profiling.[14] Second, the original diagnosis was used without centralized pathology review, however slides were from large referral hospitals and diagnoses were made by expert gynecological-pathologist. This makes our study applicable to daily practice. Third, race or ethnicity has not been reported in our study. Although we fully agree that these patients' information might be impact outcome in several diseases, within Europe it is not routinely documented in patient files.[23] In order to evaluate whether race and ethnicity might have impacted our results, we performed additional analyses within the Kandoth open access database. Race was not statistically different between low- and high EC patients and between EC-related mortality (data not shown). However, in patients with Black or African American race, *TP53*-mutant appeared to be more frequently present supporting previous findings of Lu et al. that these women more often were diagnosed with non-endometrioid EC.[24] It seems that molecular subgroups overrates the prognostic relevance of race. Fourth, patients were diagnosed between 1994 and 2018, a time spanning over 24 years (median 2006), this could have biased the survival because of different treatment strategies over the time. Including the diagnostic year in the multivariable cox-regression analyses did not change the results of the cox-regression analyses as presented in the result section (data not shown). Finally, although there were significantly more low-grade EC patients in the excluded cases, the DSS for excluded cases showed similar favorable outcome for all molecular subgroups within low-grade EC (*data not shown*).

CONCLUSIONS

Routine molecular profiling is not beneficial in low-grade EC patients due to their excellent prognosis independent of molecular subgroup. Our data demonstrate the importance of primary diagnostic tumor grading and do not support routine molecular profiling in low-grade EC as cost-effective approach.

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SUPPLEMENTARY

eTable 1. Baseline characteristics of the four previous published studies and one submitted

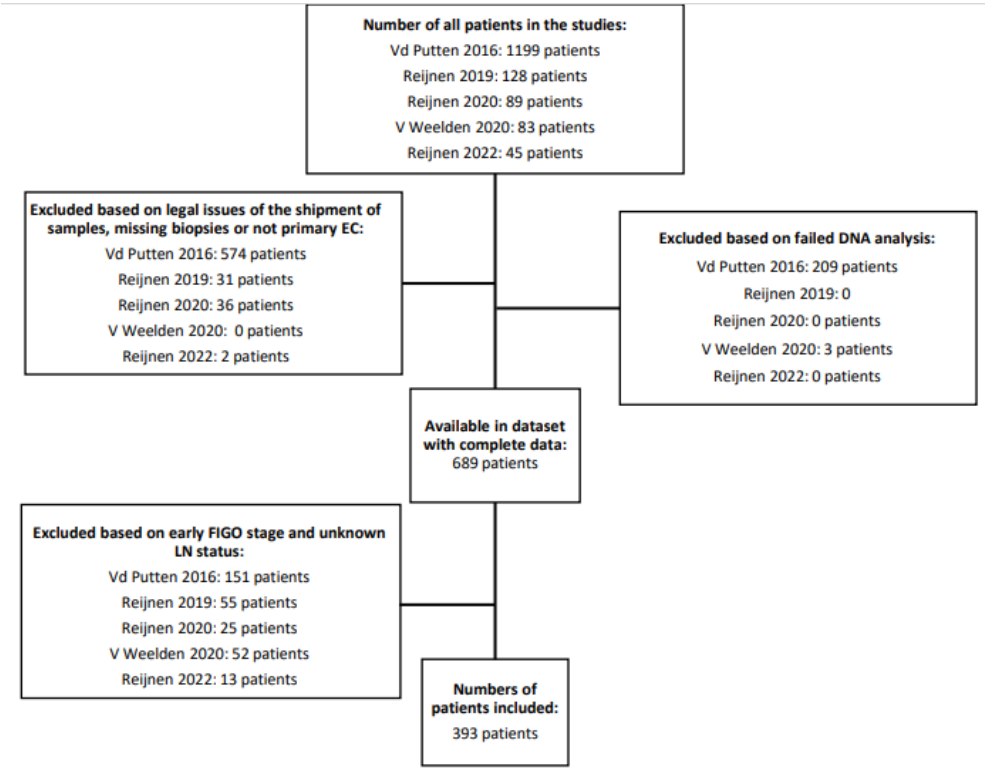
		Van der Putten et al. 2016[1]	Reijnen et al. 2019[2]	Reijnen et al. 2020[3]	Van Weelden et al. 2020[4]	Reijnen et al. 2022[5]
Study characteristics						
N included		265	42	28	28	30
Diagnostic classification based on		Molecular profiling	ProMisE	ProMisE	Molecular profiling	Molecular profiling
Median follow-up (months)		76.0 (0.0-197.0)	41.0 (14.0-87.0)	35.5 (0.0-197.0)	37.5 (3.0-21.0)	34.5 (3.0-168.0)
Demographics		European	European	Dutch	European	Dutch
Patient characteristic						
Age (years)		63.0 (34.0-86.0)	66.0 (50.0-82.0)	57.0 (31.0-81.0)	66.0 (45.0-82.0)	67.0 (49.0-77.0)
Primary treatment						
Lymph node dissection	No	11 (4.2)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.3)
	Yes	254 (95.8)	42 (100.0)	28 (100.0)	24 (85.7)	28 (93.3)
	Pelvic	190 (74.8)	0 (0.0)	0 (0.0)	6 (25.0)	18 (64.3)
	Para-aortic	4 (1.6)	0 (0.0)	0 (0.0)	2 (8.3)	7 (25.0)
	Pelvic and para-aortic	45 (17.7)	0 (0.0)	0 (0.0)	9 (37.5)	3 (10.7)
	Unknown which nodes	15 (5.9)	42 (100.0)	28 (100.0)	7 (29.2)	0 (0.0)
	Unknown	0 (0.0)	0 (0.0)	0 (0.0)	4 (14.3)	1 (3.3)
Final pathologic characteristics						
Histology	EEC	26 (9.8)	42 (100.0)	20 (71.4)	17 (60.7)	0 (0.0)
	NEEC	239 (90.2)	0 (0.0)	8 (28.6)	11 (39.3)	30 (100.0)
Grade	1-2	183 (69.1)	0 (0.0)	18 (64.3)	8 (28.6)	0 (0.0)
	3	82 (30.9)	42.0 (100.0)	10 (35.7)	20 (71.4)	30 (100.0)

Molecular	POLE-mut	26 (9.8)	3 (7.1)	3 (10.7)	0	1 (3.3)
	MSI	51 (19.2)	15 (35.7)	2 (7.1)	4 (14.3)	6 (20.0)
	TP53-mut	31 (11.7)	9 (21.4)	8 (28.6)	16 (57.1)	8 (26.7)
	NSMP	157 (59.2)	15 (35.7)	15 (53.6)	8 (28.6)	15 (50.0)
MI	<50%	154 (58.1)	4 (9.5)	16 (57.1)	11 (39.3)	12 (40.0)
	>50%	111 (41.9)	36 (85.7)	12 (42.9)	17 (60.7)	18 (60.0)
	Missing	0 (0.0)	2 (4.8)	0 (0.0)	0 (0.0)	0 (0.0)
LVSI	No	219 (82.6)	27 (64.3)	26 (92.9)	14 (50.0)	18 (60.0)
	Yes	46 (17.4)	15 (35.7)	2 (7.1)	14 (50.0)	12 (40.0)
Lymph nodes	N0	231 (87.2)	42 (100.0)	0 (0.0)	16 (57.1)	16 (53.3)
	N1	23 (8.7)	0 (0.0)	0 (0.0)	8 (28.6)	12 (40.0)
	Pelvic	14 (60.9)	0 (0.0)	0 (0.0)	4 (50.0)	0 (0.0)
	Para-aortic	4 (17.4)			3 (37.5)	0 (0.0)
	Pelvic and para-aortic	5 (21.7)	0 (0.0)	0 (0.0)	1 (12.5)	0 (0.0)
	Unknown which nodes	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	12 (100.0)
	Nx	6 (2.3)	0 (0.0)	28 (100.0)	4 (14.3)	2 (6.7)
FIGO stage	Early (I-II)	223 (84.2)	42 (100.0)		13 (46.4)	12 (40.0)
	Advanced (III-IV)	42 (15.8)	0 (0.0)	28 (100.0)	15 (53.6)	18 (60.0)
Adjuvant treatment						
None		66 (24.9)	12 (28.6)	4 (14.3)	6 (21.4)	9 (30.0)
Radiotherapy		163 (61.5)	25 (59.5)	7 (25.0)	15 (53.6)	15 (50.0)
	EBRT	53 (32.5)	8 (32.0)	0 (0.0)	6 (40.0)	0 (0.0)
	VBT	72 (44.2)	12 (48.0)	0 (0.0)	5 (33.3)	0 (0.0)
	ERBT+VBT	38 (23.3)	5 (20.0)	0 (0.0)	4 (26.7)	0 (0.0)
	Unknown	0 (0.0)	0 (0.0)	7 (100.0)	0 (0.0)	15 (100.0)

Chemotherapy	9 (3.4)	1 (2.4)	14 (50.0)	5 (17.9)	4 (13.3)
Chemoradiation	27 (10.2)	1 (2.4)	2 (7.1)	2 (7.1)	2 (6.7)
Unknown	0 (0.0)	3 (7.1)	1 (3.6)	0 (0.0)	0 (0.0)
Mortality					
Recurrence	34 (12.8)	13 (31.0)	9 (32.1)	12 (42.9)	6 (20.0)
Mortality	40 (15.1)	11 (26.2)	12 (42.9)	14 (50.0)	13 (43.3)
EC-related mortality	29 (10.9)	9 (21.4)	9 (32.1)	13 (46.4)	13 (43.3)

Data is presented as No. (%), median (IQR)

Abbreviations: *POLE,* Polymerase epsilon; MSI, Microsatellite instability; *TP53,* Tumor protein 53; NSMP, No-specific molecular profile; EEC, endometrioid endometrial cancer; NEEC, non-endometrioid endometrial cancer; MI, myometrial invasion; LVSI, lymphovascular space invasion; N0, negative lymph nodes, N1, positive lymph nodes; Nx, no information about the lymph nodes; FIGO, Federation International of Gynecology and Obstetrics; EBRT, external beam radition therapy; VBT, vaginal brachytherapy; EC, endometrial cancer.



eFigure 1. Study flowchart

Abbreviations: EC, Endometrial Cancer; LN, Lymph node

eMethod. Detailed information on DNA analysis, smMIP design and library preparation, sequencing and immunochemistry analysis

DNA analysis

Representative areas of EC in the surgical specimen were marked and selected for formalin-fixed paraffin-embedded (FFPE) 20 μm thick sections. Slides were cut from these FFPE section and stained with hematoxylin and eosin (H&E). Tumor areas were marked on these slides and the tumor cell percentage was estimated. These specimens were digested overnight at 56°C in TET-lysis buffer (10mmol/L Tris/HCL pH 8.5, 1 mmol/L EDTA pH 8.0, 0.01% Tween-20) with 5% Chelex-100 (Bio-Rad, Hercules, CA) and 0.2% proteinase K, with subsequent inactivation at 95°C for 10 min. After this was centrifugated, the supernatant was transferred into a clean tube. DNA concentration was determined using the Qubit Broad Range Kit (Thermo Fisher Scientific, Waltham, MA).

smMIP design and library preparation

The panel consisted of 10 genes important for EC oncogenesis (ARID1A, CTNNB1, ERBB2, KRAS, MTOR, NRAS, PIK3CA, PTEN, POLE, TP53). The smMIPs were designed in a tilling manner for hotspots in oncogenes and all coding as well as splice site consensus sequences of tumor suppressor genes (TSGs), with preferential targeting of both strand by two independent smMIPs. All the smMIP probes are constructed by an extension and ligation probe arm (40 bp long) with a 112 bp gap and a common backbone sequence for PCR-based library amplification. The backbone and ligation probe arm are connected by means of an 8 bp degenerate sequence (8xN) serving as a Unique Molecular Identifier (UMI, "single-molecule tag"). Following, the smMIP probes were mixed and phosphorylated with 1 μl of T4 polynucleaotide kinase (M0201; New England Biolabs). The molecular ratio between gDNA and smMIPs was set at 1:3,200 for each individual smMIP and the standard genomic DNA input was set at 100ng. A capture mix was made (volume 25 μl) with the phosphorylated smMIP pool, 1 unit of Ampligase DNA ligase (A0110K; EpiBio, Madison, WI) and Ampligase Buffer (A1905B, DNA ligase buffer), 3.2 units of Hemo Klentaq (M0332; New England Biolabs), 8 mmol of dNTPs (28-4065-20/-12/-22/-32; GE Healthcare, Little Chalfont, UK) and 100 ng of genomic DNA in a 20 μl volume. This capture mix was denatured at 95°C for 10 min and subsequently incubated for probe hybridization, extension and ligation for 18hr at 60°C. To perform the exonuclease treatment, Exonuclease 1 (10 units; M0293; New England Biolabs) and III (50 units; M0206; New England Biolabs) and Ampligase Buffer was added to the capture mix after cooling (total of 27 μl). This mix was incubated at 37°C for 45 min, with subsequent inactivation at 95°C for 2 min. From the 27 μl, 20 μl was used for PCR in at total volume of 50 μl including a common forward primer, bar-coded reverse primers, and iProof high fidelity master mix (1725310, Bio-Rad, Veenendaal, the Netherlands). The resulting PCR products were then pooled and purified with 0.8x volume of Agencourt Ampure XP Beads (a63881, Beckman Coulter, Woerden, the Netherlands).

Sequencing

The purified libraries were denatured and diluted to 1.2pmol/l, and then sequenced on a NexSeq500 device (Illumina, San Diego, CA) using the manufacturer's instructions (300 cycles High Output sequencing kit, v2), resulting in 2x150bp paired-end reads. All Bcl files were converted to fastq files and bar-coded reads were then demultiplexed. Single-molecule-directed assembly of the duplicate reads was conducted generating consensus ('unique') reads with the software Sequence Pilot (version 4.4.0; JSI medical system, Ettenheim, Germany).

Variants were annotated as 'malignant', 'likely malignant', 'unknown significance', 'likely benign' and 'benign' using amongst others publicly available databases such as ClinVar (https://www.ncbi.nlm.

nih.gov/clinvar/), The Clinical Knowledgebase (CKB, https://ckb.jax.org/), Cancer Genome Interpreter (CGI, https://www.cancergenomeinterpreter.org/home), the Catalog of Somatic Mutations in Cancer (COSMIC, https://cancer.sanger.ac.uk/cosmic), OncoKB (https://www.oncokb.org/), Varsome (https://varsome.com/). The three first categories were taken into consideration and included known activating hotspot mutations for the oncogenes, and missense, nonsense, frameshift and splice site mutations for the included TSGs. Intronic mutations were excluded with exception of splice site sequences. To determine whether sufficient DNA molecules were sequenced to reliably exclude mutation, a cumulative binomial distribution was used for calculating the required unique read depths, above a certain mutant allele frequency with a certainty of >95%.[6] These required read depts were assessed in the context of estimated tumor percentage cells by microscopy.

Immunohistochemical staining

For p53 staining, antigen retrieval (30 minutes, pH 6·7) and blocking of endogenous peroxidase with hydrogen peroxide was performed. Subsequently, slides were incubated with p53 antibody (clone DO-7 + BP53-12, dilution 1:600). Powervision+ Poly-HRP was used and visualization was accomplished by using PowerVision DAB substrate solution (Leica Biosystems, Buffalo Grove, IL, US). Counterstaining was performed with hematoxylin, slides were dehydrated and mounted.

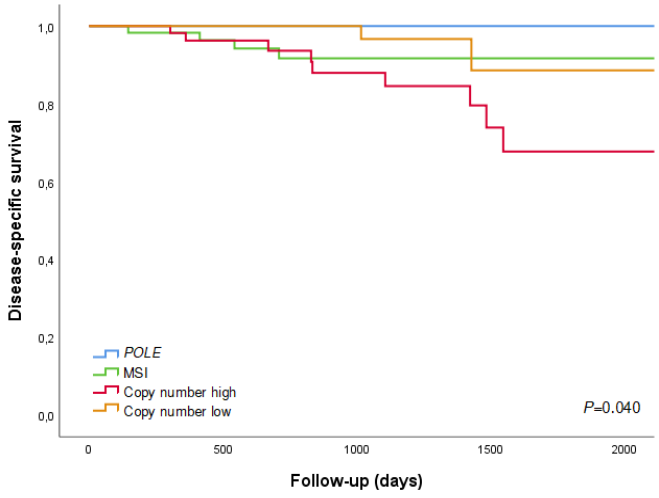
For PMS2 and MSH6 staining, antigen retrieval with EnVision FLEX High pH Target Retrieval Solution, and blocking of endogenous peroxidase with hydrogen peroxide was performed. After, slides were incubated with anti-MSH6 (clone EPR3945 1:400, Abcam, Cambridge, UK) or anti-PMS2 (clone A16-4 dilution 1:20, BD Biosciences , San Jose, CA). Incubation was performed with EnVision FLEX and visualized with High pH visualization system. Counterstaining was performed with hematoxylin, slides were dehydrated and mounted.

eTable 2. Baseline characteristics of the included versus excluded patients

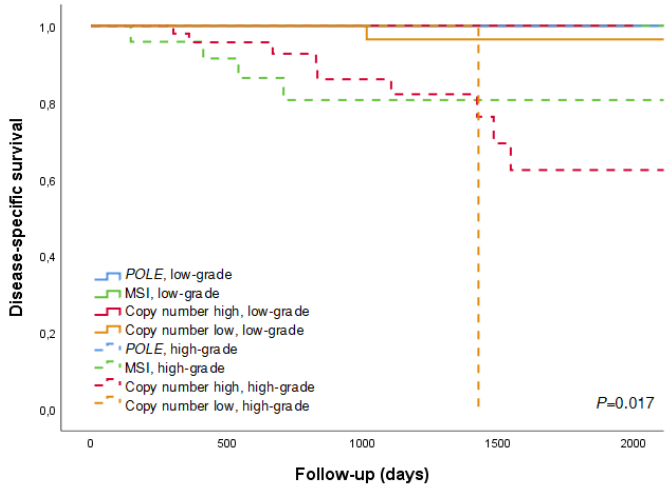
		Included N=393	Excluded N=296	*P*
Patient characteristic				
Age (years)		63.0 (31.0-82.0)	64.5 (35.0-93.0)	.09
Pathologic characteristics				
POLE-mutant		33 (8.4)	14 (4.7)	.001
MSI		78 (19.8)	79 (26.7)	
TP53-mutant		72 (18.3)	29 (9.8)	
NSMP		210 (53.4)	174 (58.8)	
Histology	EEC	318 (80.9)	275 (92.9)	<.001
	NEEC	75 (19.1)	21 (7.1)	
Grade	1-2	209 (53.2)	217 (73.3)	<.001
	3	184 (46.8)	79 (26.7)	
MI	<50%	197 (50.1)	178 (61.0)	.006
	>50%	194 (49.4)	114 (39.0)	
	Unknown	2 (0.5)		
LVSI	No	304 (77.4)	238 (80.4)	.33
	Yes	89 (22.6)	58 (19.6)	
Adjuvant treatment				
None		97 (24.7)	148 (50.3)	<.001
Radiotherapy		225 (57.3)	124 (42.2)	
Chemotherapy		33 (8.4)	17 (5.8)	
Chemoradiation		34 (8.7)	5 (1.7)	
Unknown		4 (1.0)		
Mortality				
Recurrence		74 (18.8)	38 (12.8)	.013
Mortality		90 (22.9)	55 (18.6)	.17
EC-related mortality		73 (18.6)	26 (8.8)	<.001

Data is presented as No. (%), median (IQR)

Abbreviations: *POLE,* Polymerase epsilon; MSI, Microsatellite instability; *TP53,* Tumor protein 53; NSMP, No-specific molecular profile; EEC, endometrioid endometrial cancer; NEEC, non-endometrioid endometrial cancer; MI, myometrial invasion; LVSI, lymphovascular space invasion; EC, endometrial cancer.



Numbers at risk					
POLE	17	12	9	4	3
MSI	65	46	23	16	11
Copy number high	60	43	28	13	5
Copy number low	90	68	30	10	7



Numbers at risk					
Low-grade					
POLE	9	7	5	1	1
MSI	40	27	14	10	7
Copy number high	7	6	4	3	1
Copy number low	84	64	28	10	7
High-grade					
POLE	8	5	4	3	2
MSI	25	19	9	6	4
Copy number high	53	37	24	10	4
Copy number low	6	4	2	0	0

eFigure 2A-B. Disease-specific survival curves of the validation cohort A. The 5-years disease-specific survival (DSS) of the molecular subgroups in the entire cohort. B. 5-years DSS of the molecular subgroups and low- versus high-grade endometrial cancer (EC).

Abbreviations: *POLE*, Polymerase epsilon; MSI, Microsatellite instability; *TP53*, Tumor protein 53; NSMP, No-specific molecular profile

eTable 3. Cox regression univariable and multivariable analysis of disease-specific survival (DSS) in high-grade EC patients

Variable	**Univariable DSS**		**Multivariable DSS** 61 events	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Molecular subgroup				
POLE-mutant	0.15 (0.02-1.09)	.06	0.19 (0.02-1.46)	.12
MSI	0.27 (0.09-0.77)	.02	0.45 (0.16-1.24)	.12
TP53-mutant	1.93 (1.13-3.28)	.02	1.70 (0.99-2.91)	.05
NSMP	1		1	
LVSI				
No	1	.002	1	.67
Yes	2.23 (1.34-3.68)		1.12 (0.65-1.92)	
FIGO				
Stage I-II	1	<.001	1	<.001
Stage III-IV	5.67 (3.30-9.73)		4.05 (2.24-7.29)	

Abbreviations: DSS, disease-specific survival; HR, hazard ratio; CI, confidence interval; *POLE*, Polymerase epsilon; MSI, Microsatellite instability; *TP53*, Tumor protein; NSMP, No-specific molecular profile, LVSI, lymphovascular space invasion; FIGO, Federation International of Gynecology and Obstetrics.

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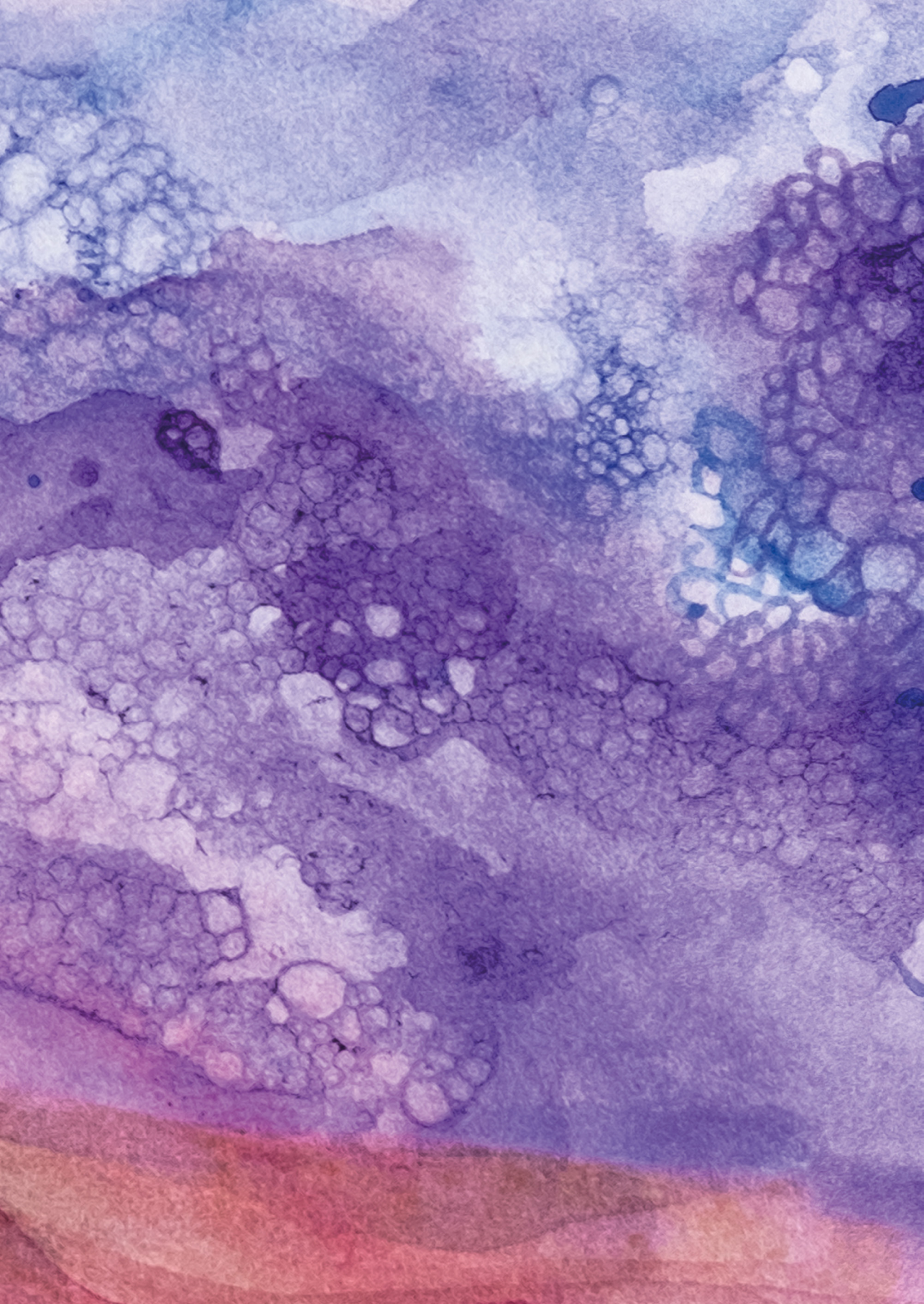

CHAPTER 4

PURE AND MIXED CLEAR CELL CARCINOMA OF THE ENDOMETRIUM: A MOLECULAR AND IMMUNOHISTOCHEMICAL ANALYSIS STUDY

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ABSTRACT

Background

Uterine clear cell carcinoma (CCC) consists of either pure clear cell histology but can also display other histological components (mixed uterine CCCs). In this study, the molecular and immunohistochemical background of pure and mixed uterine CCC was compared. Secondly, it was evaluated whether histological classification and molecular background affected clinical outcome.

Methods

A retrospective multicenter study was performed comparing pure uterine CCCs (n=22) and mixed uterine CCCs (n=21). Targeted next-generation sequencing using a twelve-gene targeted panel classified cases as polymerase-ε(*POLE*) mutated, microsatellite instable (MSI), *TP53* wildtype or *TP53* mutated. Immunohistochemistry was performed for estrogen receptor, progesterone receptor, L1CAM, MSH6 and PMS2.

Results

The following molecular subgroups were identified for pure and mixed uterine CCCs respectively: *POLE* mutated 0% (0/18) and 6% (1/18); MSI in 6% (1/18) and 50% (9/18); *TP53* wildtype in 56% (10/18) and 22% (4/18); *TP53* mutated in 39% (7/18) and 22% (4/18) (p=0.013). Patients with mixed CCCs had improved outcome compared to patients with pure CCCs. Frequent *TP53* mutations were found in pure CCCs and frequent MSI in mixed CCCs, associated with clinical outcome.

Conclusion

Pure and mixed uterine CCCs are two entities with different clinical outcomes, which could be explained by different molecular backgrounds. These results underline the relevance of both morphological and molecular evaluation, and may assist in tailoring treatment.

INTRODUCTION

Endometrial carcinoma (EC) is the fourth leading cancer in female patients in Europe, with 121 600 new cases and 29 600 deaths in 2018 [1]. The most common histological type of EC is endometrioid endometrial carcinoma (EEC), which accounts for 80% of all cases and generally shows a favorable prognosis [2]. Less than 5% of EC consists of uterine clear cell carcinoma (CCC), an aggressive subtype of non-endometrioid endometrial carcinoma (NEEC) [3-5]. Uterine CCC is frequently diagnosed in older, postmenopausal women, with 40-45% presenting with extra-uterine disease [6, 7]. The 5-year overall survival is 63%, compared to 83% in the EEC population [8]. The Society of Gynecologic Oncology recommends comprehensive surgical staging and the use of adjuvant radiotherapy and/or chemotherapy for patients with uterine CCC, given the high incidence of recurrence [9, 10].

In addition to pure clear cell histology, it is not uncommon for uterine CCC to display other histological components. These so-called mixed uterine CCCs usually show an additional component of endometrioid or serous carcinoma [11]. In serous EC, it is known that carcinomas with mixed histology have a significantly better prognosis than patients with serous histology only [12]. Whether mixed uterine CCCs display a better clinical outcome than pure uterine CCCs remains unclear.

Major advances in characterization of the molecular background of ECs have been made in recent years [13]. The Cancer Genome Atlas (TCGA) has defined four distinct molecular subtypes each with prognostic relevance. These molecular subtypes have been modified for clinical use by, amongst others, the Proactive Molecular Risk classifier for Endometrial Cancer (ProMisE) classification [14, 15]. Molecular profiling has demonstrated to be supportive in high grade EC, yet for CCC, data are limited. Molecular subtypes in EC include an "ultramutated" subgroup with mutations in the exonuclease domain of *polymerase-ε* (*POLE*) and an excellent prognosis; a "hypermutated" subgroup with microsatellite instability (MSI); a "copy-number high" subgroup characterized by *TP53* mutations and generally unfavorable outcome; and the copy number-low subgroup [13]. Recent studies have shown that pure uterine CCC is a molecularly heterogeneous disease which encompasses different molecular subtypes [16-20]. Due to this heterogeneous molecular background, clinical behavior and prognosis of uterine CCC may be more varied than generally thought, which could have consequences for the extent of (adjuvant) therapy.

The primary aim of this study was to identify and compare the molecular and immunohistochemical (IHC) background of pure and mixed uterine CCC. The secondary aim was to evaluate whether histological classification, molecular and IHC features affect clinical outcome.

MATERIAL AND METHODS

Patient cohort

The nationwide Dutch database of histopathology and cytopathology (PALGA) was used to search for all patients diagnosed with uterine CCC between January 1990 and December 2020 at the Radboud university medical center and the Canisius Wilhelmina Hospital Nijmegen, The Netherlands [21]. Patients were excluded when having less than 10% clear cell component, when not receiving a surgical treatment and when no histological tumor tissue could be retrieved for IHC/molecular analysis.

Data collection

Clinicopathological data was collected regarding age at diagnosis, body mass index (BMI), cancer antigen 125 (CA-125), cervical cytology, preoperative endometrial sampling, extend of primary surgical approach, stage, adjuvant treatment, and follow-up data. Stage of disease was based on the 2009 International Federation of Gynecology and Obstetrics (FIGO) endometrial cancer criteria [22].

Histopathological review

Hematoxylin & eosin (H&E) slides of the hysterectomy specimens were systematically reviewed by two pathologists with special interest in gynecology (J.B., H.K.), being blinded to any clinical or histological data. Histological review included classification of tumor histology, an estimation of percentages of the different components if present, depth of myometrial invasion (MI) and the presence of cervical stromal invasion (CI). Slides were screened for the presence of lymphovascular space invasion (LVSI). Diagnosis was made according to the 2020 World Health Organization (WHO) guidelines and a tumor was classified as mixed when it contained at least 2 different histological components, regardless of component percentage [11]. To support the diagnosis mixed uterine CCC, IHC stains were used in doubtful cases according to the 2020 WHO guidelines. For each case, an H&E slide with representative tumor tissue was selected and marked off for the purpose of DNA extraction in parallel unstained slides. In case of mixed uterine CCC, the different components were marked off separately, if possible.

Immunohistochemical staining

Immunohistochemical staining was performed for estrogen receptor (ER), progesterone receptor (PR), L1 Cell Adhesion Molecule (L1CAM), PMS2 and MSH6 (*Supplementary A*). For ER and PR, the number of stained tumor nuclei was scored. Cases were dichotomized, using 10% as a cut-off value. For L1CAM, the number of tumor cells showing membranous expression was scored and dichotomized, using 10% as a cut-off value. Mismatch repair

deficiency (MMRd) was defined as total loss of nuclear staining of either PMS2 or MSH6, in the presence of a positive internal control.

DNA extraction and library preparation

Representative tumor tissue was selected by means of microdissection from 8 x 10 µm thick formalin-fixed, paraffin-embedded (FFPE) sections. In case of mixed histology, both components were microdissected separately. Next, tissue was digested at 56°C for at least 16 hours in the presence of TET-lysis buffer (1M Tris/HCL pH 8.5, 0.5M EDTA pH 8.0, 20% Tween-20) with 5% Chelex- 100 (Bio-Rad, Hercules, CA) and 10% Proteinase K (Qiagen, Valencia, CA), followed by inactivation at 95°C for 10 minutes. Twice, the supernatant was transferred to a clean tube after centrifugation at 14 000 xg for 10 minutes. DNA concentration was determined using the Qubit 1x dsDNA High Sensitivity assay kit (Thermo Fisher Scientific, Waltham, MA, US). The isolated DNA was stored at -20°C. The samples were analyzed with single-molecule Molecular Inversion Probes (smMIPs, Integrated DNA Technologies, Leuven, Belgium) [23]. The panel consisted of twelve relevant genes involved in EC oncogenesis as well as a number of genes informative for ProMisE classification *(AKT1, ARID1A, CTNNB1, ERBB2, FGFR2, KRAS, MTOR, NRAS, PIK3CA, PTEN, POLE, TP53, Supplementary B*), in addition to markers for microsatellite instability (MSI). Targeted sequencing with smMIPs was performed as previously described [23]. All smMIPs were designed in a tiling manner for hotspots in oncogenes and all coding as well as splice site consensus sequences of tumor suppressor genes (TSGs), with preferential targeting of both strands by two smMIPs. The smMIP probes were constructed by an extension and ligation probe arm (40bp long) with a 112bp gap and a common backbone sequence for PCR-based library amplification. The ligation probe arm and backbone were connected by means of an 8bp degenerate sequence (8xN) serving as a Unique Molecular Identifier (UMI, 'single molecule tag'). The smMIP probes were mixed and phosporylated with 1µL of T4 polynucleotide kinase (M0201; New England Biolabs, Ipswich, MA, US) per 25µL of 100 µmol/L smMIPs and ATP-containing G4 DNA ligase buffer (B0202, New England Biolabs). The molecular ratio between gDNA and smMIPs was set at 1:3200 for each individual smMIP and the standard genomic DNA input was 100ng. Next, a capture mix was made (volume: 25µL) with the phosporylated smMIP pool, 1 unit of Ampligase DNA ligase (A0110K; EpiBio, Madison, WI) and Ampligase Buffer (A1905B, DNA ligase buffer), 3.2 units of Hemo Klentaq (M0332; New England Biolabs), 8mmol of dNTPs (28-4065-20/-12/-22/-32; GE Healthcare, Little Chalfont, UK) and 100ng of genomic DNA in a 20µL volume. This capture mix was denatured at 95ºC for 10 minutes and subsequently incubated for probe hybridization, extension and ligation at 60ºC for 18 hours. After cooling, to perform exonuclease treatment, Exonuclease I (10 units; M0293; New England Biolabs) and III (50 units; M0206; New England Biolabs) and Ampligase Buffer was added to the capture mix (total of 27µL). The mix was incubated at 37ºC for 45 minutes, with subsequent inactivation at

95ºC for two minutes. Twenty µL was then used for PCR in a total volume of 50µL including a common forward primer, bar-coded reverse primers, and iProof high fidelity master mix (1725310, Bio-Rad, Veenendaal, The Netherlands). The resulting PCR products were pooled and purified with 0.8x volume of Agencourt Ampure XP Beads (A63881, Beckman Coulter, Woerden, The Netherlands).

Sequencing and analysis

The purified libraries were sequenced on a NexSeq500 instrument (Illumina, San Diego, CA). The Sequence Pilot software (version 4.4.0; JSI Medical Systems) was used to demultiplex the bar-coded reads and create consensus ('unique') reads to minimize sequencing errors. Variant calling was performed and variants were annotated as benign, likely benign, unknown, likely pathogenic or pathogenic using publicly available databases such as The Clinical Knowledgebase (CKB, https://www.jax.org/clinical-genomics/ckb), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), Cancer Genome Interpreter (CGI, https://www.cancergenomeinterpreter.org/home) and the Catalog of Somatic Mutations in Cancer (COSMIC, cancer.sanger.ac.uk/cosmic) [24]. The last three categories were considered relevant and consisted of known activating hotspot mutations for the oncogenes[25], and frameshift, nonsense, missense, and splice-site mutations for the tumor suppressor genes. The following molecular subgroups were identified based on these sequencing results: *POLE* mutated, MSI, *TP53* wildtype and *TP53* mutated. In case of double classifiers (for example, *POLE* and *TP53* mutation), the tumor was classified as previously described. It was previously shown that there is an excellent correlation between *TP53* mutational status and p53 IHC, and we therefore decided not to perform additional IHC in this study [26].

Statistical analysis

For statistical analyses, Statistical Package for the Social Sciences (SPSS), version 25.0 (IBM, New York, NY, USA) was used. Clinicopathological differences between subgroups were compared using the Fisher's exact test and χ^2 for discrete variables and the Mann-Whitney U test for continuous variables. Survival analyses were performed using the Kaplan Meier (KM) curves and univariable and multivariable Cox-regression analysis. A recurrence was defined as first sign of relapse after a 6-month disease-free interval after initial surgery. Disease-free survival (DFS) was calculated from the date of initial surgery until the date of recurrence, whereas overall survival (OS) was calculated from the date of initial surgery until the date of death or, for surviving patients, to the date of last follow-up. Disease-specific survival (DSS) was calculated from the date of primary treatment to the date of death caused by the disease or, for surviving patients, to the date of the last follow-up.

RESULTS

Patients

A total of 72 patients were identified of which 29 were excluded (n=8 after pathology review, n=13 due to insufficient tissue, n=2 because of missing follow-up and n=6 due to palliative treatment). A total of 43 patients were included in the analysis, of which 22 (51%) were pure uterine CCC and 21 (49%) mixed uterine CCC. Median age was 70 years (range 48 – 88) and did not differ between patients with pure or mixed uterine CCC (**Table 1**). The second histological component in mixed uterine CCC consisted of endometrioid histology in 16 patients (76%), serous histology in 3 patients (14%) and endometrioid + serous histology in 2 patients (10%). In patients with pure uterine CCC, 12 patients (55%) presented with FIGO stage III-IV disease, compared to 6 patients (29%, p = 0.084) with mixed uterine CCC. As shown in **Table 2**, ER an PR IHC stains were reflective of the mixed components as the clear cell component was mostly negative for ER and PR and the other histological components were mostly positive. The use of adjuvant therapy did not differ significantly between groups. In patients with pure uterine CCC, 11 patients (50%) died in the follow-up period, compared to 4 patients (19%, p = 0.033) with mixed uterine CCC.

Table 1. Baseline characteristics

	All (n=43)	Pure (n=22)	Mixed (n=21)	*P*
Age (years)	70 (49-88)	71 (49-84)	68 (51-88)	0.780
BMI (kg/m^2)	27 (19-50)	26 (19-40)	28 (20-50)	0.621
CA-125 at diagnosis (IU/mL)	19 (2-508)	22 (9-508)	16 (2-175)	0.259
Follow-up (months)	34 (3-194)	22 (3-168)	41 (3-194)	0.174
Second histological component				
Endometrioid			16 (76)	
Serous			3 (14)	
Endometrioid + serous			2 (10)	
Myometrial invasion				0.393
<50%	18 (42)	8 (36)	10 (48)	
>50%	24 (56)	14 (64)	10 (48)	
Unknown	1 (2)	0	1 (5)	
Cervical stroma invasion				0.162
Present	18 (42)	12 (55)	6 (29)	
Not present	24 (56)	10 (46)	14 (67)	
Unknown	1 (2)	0	1 (5)	
Lymph nodes				0.435
Negative	17 (40)	8 (36)	9 (43)	
Positive pelvic nodes	4 (9)	3 (14)	1 (5)	
Positive para-aortal nodes	9 (21)	6 (27)	3 (14)	
Unknown	13 (30)	5 (23)	8 (38)	
FIGO stage				
Stage I-II	25 (58)	10 (46)	15 (71)	0.084
Stage III-IV	18 (42)	12 (55)	6 (29)	
Adjuvant therapy				
Radiotherapy	21 (51)	10 (46)	11 (52)	0.932
Chemotherapy	5 (12)	3 (14)	2 (10)	
Chemoradiotherapy	2 (5)	1 (5)	1 (5)	
None	14 (33)	8 (36)	6 (29)	
Unknown	1 (2)		1 (5)	
Residual disease				
Yes	9 (21)	7 (32)	2 (10)	0.072
No	34 (79)	15 (68)	19 (91)	
Recurrence*				
Yes	6 (18)	4 (27)	2 (11)	0.095
No	28 (82)	11 (73)	17 (90)	

Table 1. *Continued*

Deceased				
Yes	15 (35)	11 (50)	4 (19)	0.033
No	28 (65)	11 (50)	17 (81)	
Deceased EC-related				0.065
Yes	14 (33)	10 (46)	4 (19)	
No	29 (67)	12 (55)	17 (81)	

P-values were obtained using the Mann-Whitney U test, Fisher's exact test and χ^2. Values are presented as median (range) or number (%). BMI, body mass index; CA-125, cancer antigen 125; EC, endometrial cancer. * excluding patients with residual disease

Table 2. Immunohistochemical staining

	Pure	**Mixed**	
		Clear cell component	**Other component**
Estrogen receptor			
Positive	5 (24)	11 (52)	21 (100)
Negative	16 (76)	10 (48)	0
Progesterone receptor			
Positive	1 (5)	6 (29)	16 (84)
Negative	21 (96)	15 (71)	3 (16)*
L1CAM			
Negative	5 (23)	6 (29)	16 (76)
Positive	17 (77)	15 (71)	5 (24)
PMS2			
Positive	22 (100)	20 (95)	20 (95)
Negative	0	1 (5)	1 (5)
MSH6			
Positive	21 (95)	14 (67)	14 (67)
Negative	1 (5)	7 (33)	7 (33)

* Not assessable in 2 cases. L1CAM, L1 cell adhesion molecule.

Molecular patterns

Of 43 patients, 8 were excluded for analyses due to either poor quality DNA and/or failed sequencing, leaving 35 patients for analysis. In one patient with mixed uterine CCC/serous carcinoma, a *POLE* hotspot mutation (c.857C>G) was found (both components could not be separately extracted, Table 3). Within the pure uterine CCCs, one patient had a tumor with MSI (5%), while 9 patients (43%) with mixed uterine CCC (p=0.004) showed MSI. In 7 patients (41%) with pure uterine CCC, a *TP53* mutation was found, which was also the case for the mixed uterine CCCs (39%). Within the *TP53* mutated cases, MSI was present in

two tumors (both mixed uterine CCCs). The single *POLE* mutated (mixed) uterine CCC in addition harbored a *TP53* mutation. Other mutation frequencies did not differ significantly between pure and mixed CCCs (Table 3).

Within the whole study group, the following molecular subgroups were identified: *POLE* mutated in 1 patient (3%); MSI in 10 patients (28%); *TP53* wildtype in 14 patients (39%) and *TP53* mutated in 11 patients (31%). Analyzing pure and mixed uterine CCCs separately, the *POLE* mutated subgroup was found in respectively 0% and 6% of patients; the MSI subgroup in 6% and 50%; the *TP53* wildtype subgroup in 56% and 22%; and the *TP53* mutated subgroup in 39% and 22%, respectively (p = 0.013, **Table 3**).

In ten patients with mixed uterine CCC, both components could be sequenced separately (**Figure 1**). In eight patients, at least one shared mutation was found (*Supplementary C*). A total of 13 mutations were found in both components (three *ARID1A*; one *ERRB2*; three *PIK3CA*; three *PTEN*; three *TP53*). A total of 26 mutations were only found in one of the components and can be seen as unique variants (one *AKT1*; twelve *ARID1A*; one *MTOR*; one *NRAS*; five *PIK3CA*; four *PTEN*; two *TP53*). Excluding patients with *POLE* mutated and MSI tumors, six mutations were found in both components, and only four mutations were only found in one of the components.

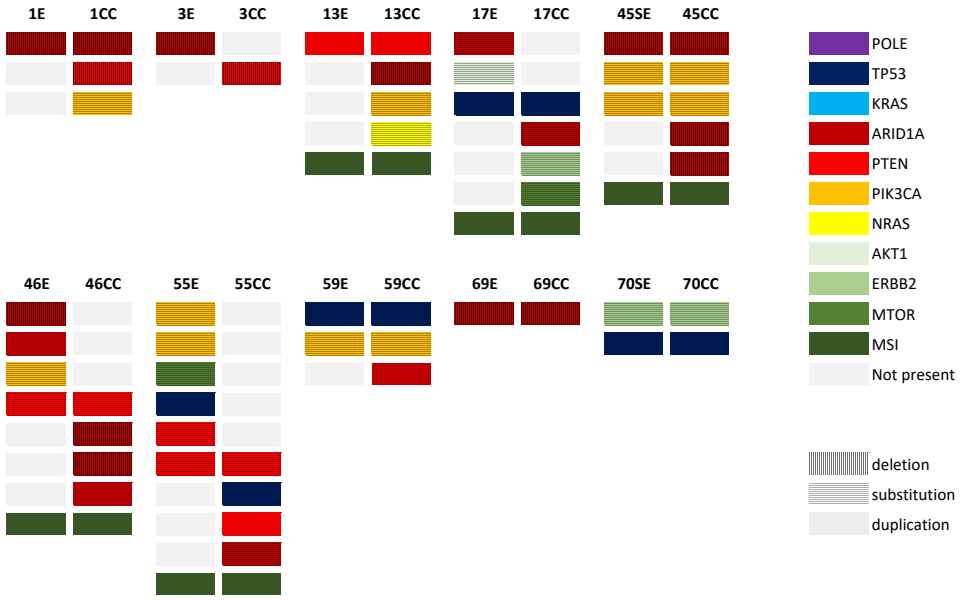


Figure 1. Display of all next-generation sequencing derived mutated genes in ten cases with mixed uterine clear cell carcinomas (CCCs) in which both component were sequenced separately. The colors indicate specific genes (see legend).

Abbreviations: CC, clear cell; E, endometrioid; SE, serous; MSI, microsatellite instable.

Table 3. Molecular patterns of pure and mixed clear cell carcinomas

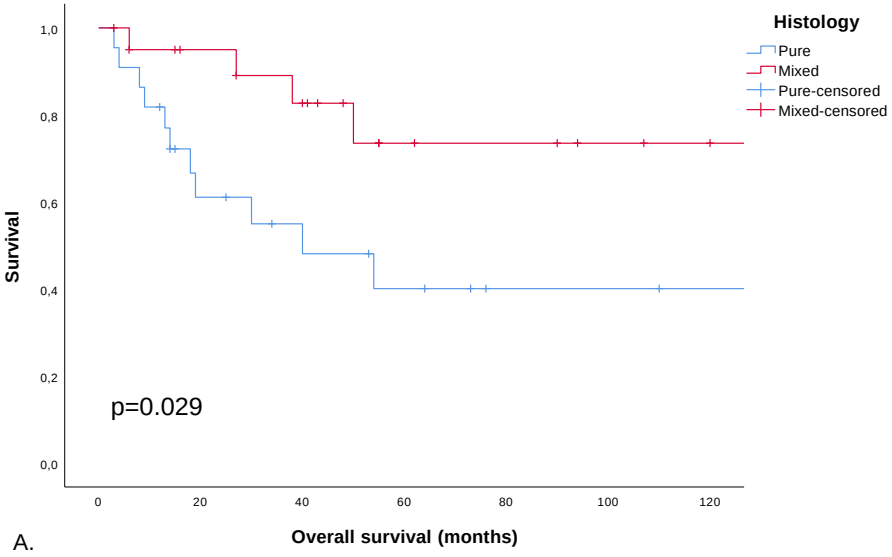
	Pure CC	Mixed	P
AKT			1.000
Wildtype	16 (94)	17 (94)	
Mutated	1 (6)	1 (6)	
ARID1A			0.318
Wildtype	10 (59)	7 (39)	
Mutated	7 (41)	11 (61)	
CTNNB1			1.000
Wildtype	16 (94)	17 (94)	
Mutated	1 (6)	1 (6)	
ERBB2			1.000
Wildtype	15 (88)	16 (89)	
Mutated	2 (12)	2 (11)	
KRAS			
Wildtype	17 (100)	16 (89)	0.486
Mutated	0	2 (11)	
MTOR			0.229
Wildtype	17 (100)	15 (83)	
Mutated	0	3 (17)	
NRAS			1.000
Wildtype	16 (94)	17 (94)	
Mutated	1 (6)	1 (6)	
PIK3CA			0.289
Wildtype	13 (77)	10 (56)	
Mutated	4 (24)	8 (44)	
POLE			1.000
Wildtype	17 (100)	17 (94)	
Mutated	0	1 (6)	
PTEN			
Wildtype	14 (82)	9 (50)	0.075
Mutated	3 (18)	9 (50)	
TP53			1.000
Wildtype	10 (59)	11 (61)	
Mutated	7 (41)	7 (39)	
MSI			**0.004**
No	21 (96)	12 (57)	
Yes	1 (5)	9 (43)	
Molecular subgroup			**0.013**
POLE mutated	0	1 (6)	
MSI	1 (6)	9 (50)	
TP53 wildtype	10 (56)	4 (22)	
TP53 mutated	7 (39)	4 (22)	

Immunohistochemical staining patterns

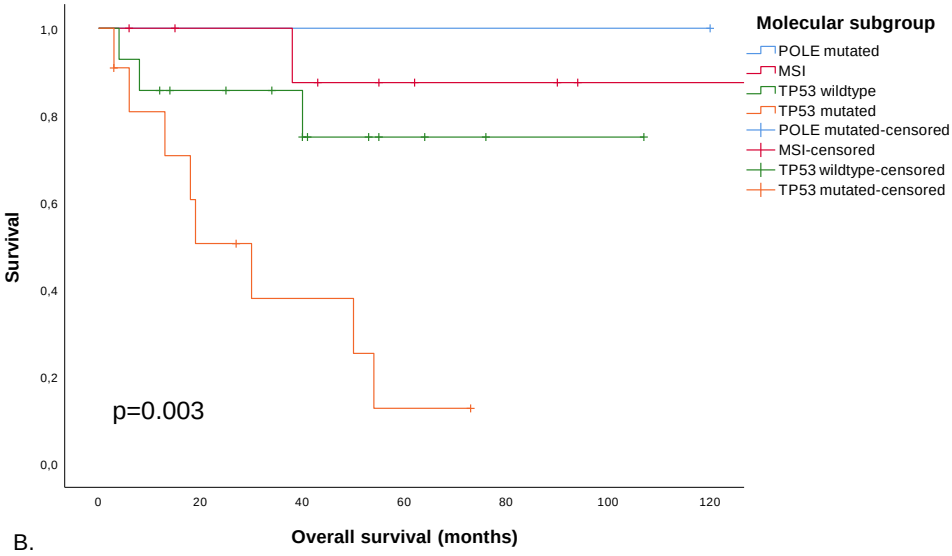
In **Table 2** immunohistochemical staining patterns are shown. The clear cell component in mixed uterine CCC was ER positive in 52% of patients, compared to 24% in pure uterine CCCs (p = 0.111). PR was positive in 29% of mixed uterine CCCs, compared to 5% in pure uterine CCCs (p = 0.046); while L1CAM was positive in 71% compared to 77% in mixed versus pure uterine CCCs respectively (p = 0.736). Within the endometrioid/serous component, loss of hormone receptors and L1CAM positivity was seen less frequently: ER positivity in 100%; PR positivity in 84%; L1CAM positivity in 24%. PMS2 staining was deficient in one case. Loss of MSH6 was present in 29% of mixed CCC, compared to 5% in pure uterine CCCs (p = 0.046). In case of mixed uterine CCCs, MSH6 and PMS2 expression was concordant in both components. MMR immunohistochemical and MSI results were concordant in all cases, except for one patient with a mixed uterine CCC showing MSI but intact immunohistochemical expression of both MSH6 and PMS2.

Outcome

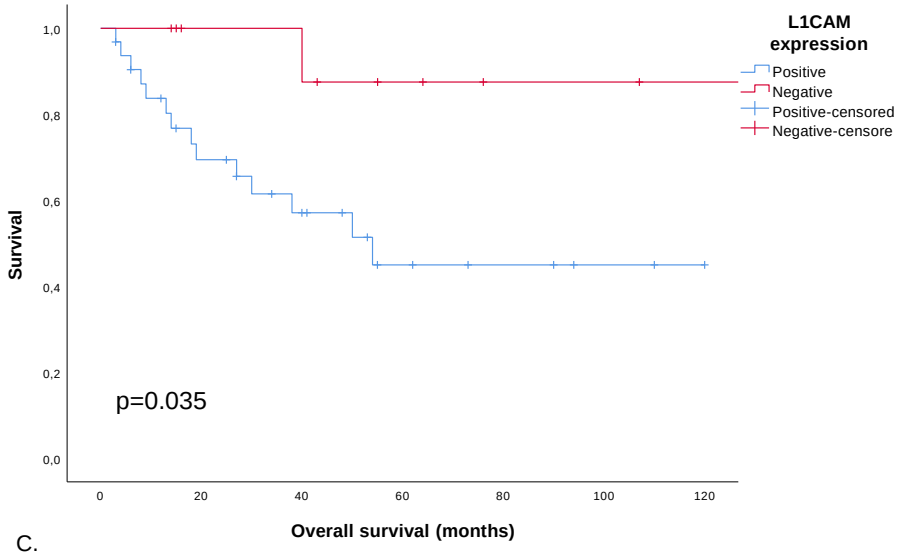
Figure 2A shows that patients with mixed uterine CCC had a superior OS compared to patients with pure uterine CCC (log-rank test: p = 0.029), which is also the case for DSS and PFS (*Supplementary D*, p = 0.045 and = 0.034, respectively). As can be appreciated from **Figure 2B**, OS was inferior in the *TP53* mutated subgroup (log-rank test: p = 0.003) whereas patients with *POLE* mutations and MSI showed very favorable outcome. DSS and PFS were inferior in the *TP53* mutated subgroup as well (*Supplementary D*, p = 0.001 and p = 0.022). Remarkably, patients with negative L1CAM had a superior OS (p = 0.035, **Figure 2C**), as well as a superior DSS and PFS (*Supplementary D*, e molecular subgroups were correlated with OS, DSS and PFS (**Table 4A-C**). In multivariable Cox regression analysis however, histology was not correlated with outcome, whereas molecular subgroups were correlated with OS and DSS.


Histology
Pure
Mixed
Pure-censored
Mixed-censored
Survival
1,0
0,8
0,6
0,4
0,2
0,0
p=0.029
0
20
40
60
80
100
120
Overall survival (months)

A.


Molecular subgroup
POLE mutated
MSI
TP53 wildtype
TP53 mutated
POLE mutated-censored
MSI-censored
TP53 wildtype-censored
TP53 mutated-censored
Survival
1,0
0,8
0,6
0,4
0,2
0,0
p=0.003
0
20
40
60
80
100
120
Overall survival (months)

B.



C.

Figure 2. Kaplan Meier curves displaying overall survival according to histology (A), molecular subgroup (B), and L1CAM-expression (C)

Table 4A. Overall survival (OS) by histology in univariable and multivariable Cox regression analysis.

	Univariable		Multivariable	
	HR 95% CI	*P*	HR 95% CI	*P*
Histology				
Pure				
Mixed	0.30 (0.10 – 0.94)	0.04	0.69 (0.15 – 3.18)	0.64
Age	1.02 (0.96 – 1.08)	0.60	-	
FIGO				
I/II				
III/IV	5.3 (1.66 – 16.8)	0.01	2.39 (0.62 – 9.19)	0.21
TCGA				
TP53 wildtype				
POLE mutated	No events	-	No events	-
MSI	0.37 (0.04 – 3.55)	0.39	0.61 (0.05 – 6.79)	0.69
TP53 mutated	4.69 (1.24 – 17.8)	0.02	3.94 (1.00 – 15.6)	0.05

Abbreviations: DSS, disease-specific survival; EC, endometrial cancer; HR, hazard ratio; CI, confidence interval; *POLE*, Polymerase epsilon; MSI, Microsatellite instability; *TP53*, Tumor protein; NSMP, No-specific molecular profile, LVSI, lymphovascular space invasion; FIGO, Federation International of Gynecology and Obstetrics.

Table 4B. Disease-specific survival (DSS) by histology in univariable and multivariable Cox regression analysis.

	Univariable			Multivariable		
	HR	95% CI	*P*	HR	95% CI	*P*
Histology						
Pure						
Mixed	0.32	(0.10 – 1.04)	0.06	1.05	(0.20 – 5.60)	0.95
Age	1.00	(0.94 – 1.07)	0.91	-		
FIGO						
I/II						
III/IV	7.23	(1.99 – 26.3)	0.01	3.84	(0.83 – 17.8)	0.09
TCGA						
TP53 wildtype						
POLE mutated	No events		-	No events		
MSI	0.54	(0.05 –6.00)	0.62	0.93	(0.07 – 11.8)	0.95
TP53 mutated	7.15	(1.51 – 33.9)	0.01	6.50	(1.24 – 34.2)	0.03

Abbreviations: DSS, disease-specific survival; EC, endometrial cancer; HR, hazard ratio; CI, confidence interval; *POLE*, Polymerase epsilon; MSI, Microsatellite instability; *TP53,* Tumor protein; NSMP, No-specific molecular profile, LVSI, lymphovascular space invasion; FIGO, Federation International of Gynecology and Obstetrics.

Table 4C. Progression-free survival (RFS) by histology in univariable and multivariable Cox regression analysis.

	Univariable			Multivariable		
	HR	95% CI	*P*	HR	95% CI	*P*
Histology						
Pure						
Mixed	0.31	(0.10 – 0.99)	0.05	0.59	(0.14 – 2.56)	0.49
Age	0.96	(0.91 – 1.02)	0.22	-		
FIGO						
I/II						
III/IV	8.58	(2.39 – 30.8)	0.01	4.92	(1.06 – 22.9)	0.04
TCGA						
TP53 wildtype						
POLE mutated	No events		-	No events		-
MSI	0.32	(0.04 – 2.89)	0.31	0.98	(0.08 – 11.9)	0.99
TP53 mutated	3.29	(0.95 – 11.5)	0.06	2.93	(0.80 – 10.7)	0.10

Abbreviations: DSS, disease-specific survival; EC, endometrial cancer; HR, hazard ratio; CI, confidence interval; *POLE*, Polymerase epsilon; MSI, Microsatellite instability; *TP53,* Tumor protein; NSMP, No-specific molecular profile, LVSI, lymphovascular space invasion; FIGO, Federation International of Gynecology and Obstetrics.

DISCUSSION

The primary aim of this study was to identify and compare the molecular and IHC background of pure and mixed uterine CCC in association with clinical outcome. Interestingly, all TCGA subgroups were observed within this cohort, in line with previous findings showing that uterine CCCs are molecularly heterogeneous [19]. Only one mixed uterine CCC with a *POLE* mutation was found with an excellent outcome. In pure uterine CCCs, no *POLE* mutations were found, in line with a previous study by Hoang et al [19]. DeLair et al, however, comprehensively sequenced a cohort of 32 pure CCCs and did find two patients with pathogenic *POLE* mutations [18]. Interestingly, in our study, mixed uterine CCCs were found to be MSI frequently, whereas pure uterine CCCs were mainly microsatellite stable. DeLair et al found MMRd in 19%, whereas Hoang et al found MSI in none of the evaluated tumors.

The secondary aim was to evaluate whether histological classification, molecular and IHC features affect clinical outcome. In Kaplan-Meier analysis, outcome was correlated to histology, molecular subgroups and L1CAM status. In multivariable Cox regression analysis, molecular status was correlated to OS and DSS, whereas histology was not. These results may suggest that differences in outcome between pure and mixed uterine CCCs may rather be explained by distinct molecular background. *TP53* mutations were found more often in pure uterine CCC, which could be contributive to their dismal prognosis [19, 27]. *POLE* mutations and MSI, associated with improved outcome, were on the other hand observed in mixed uterine CCCs more frequently.

Previously, it was shown by Köbel et al. that mixed uterine CCCs harbor a superior prognosis compared to pure uterine CCCs [20]. Also in serous ECs it is known that mixed serous ECs harbor a superior prognosis compared to pure serous ECs [12]. In this previous study however it was not investigated whether differences in prognosis could be explained by molecular signatures.

The oncogenesis of mixed tumors has not been fully elucidated. In ten patients with mixed uterine CCCs, we sequenced both histological components separately and found that eight tumors harbored at least one shared mutation in both components. Also, in nine tumors 'non-shared' mutations were found, most frequently *ARID1A* mutations. Most 'non-shared' mutations were found in patients with *POLE* mutated or MSI tumors. A previous study has shown that both components in mixed CCCs harbored shared mutations, but also showed significant molecular heterogeneity and non-shared mutations. [28] These data are indicative that both components may evolve from a single clone but diverge along the way by obtaining new and unique mutations. *POLE* mutated and MSI tumors are considered as ultra/hypermutated tumors due to the acquirement of an extremely high burden of secondary mutations due to

deficient DNA repair mechanisms [13]. Even though the number of patients was limited, the observation that *POLE* mutations and MSI were seen almost exclusively in mixed tumors could indicate that these tumors actually have a high burden of secondary acquired (shared and non-shared) mutations that lead to morphological divergence, dedifferentiation and the presence of different histological components.

A recent meta-analysis included 136 uterine CCCs (114 pure and 22 mixed) and found similar rates of molecular subgroups: 4% *POLE* mutated, 11% MSI, 50% *TP53* wildtype and 35% *TP53* mutated in pure CCCs [29]. In mixed CCCs, no *POLE* mutations were found, whereas 59% was MSI, and only 18% was *TP53* mutated. In our study, similar rates of *TP53* mutated tumors were found (39% and 22%, respectively), as well as frequent MSI in mixed uterine CCCs (50%). This meta-analysis also showed a favorable outcome in *POLE* mutated and MSI tumors, which supports recent recommendations by the European Society of Gynaecological Oncology (ESGO), European Society for Radiotherapy and Oncology (ESTRO) and the European Society of Pathology (ESP), encouraging molecular classification in all endometrial carcinomas, especially high-grade tumors [10]. In the present study, we have analyzed immunohistochemical patterns within both components. In case of loss of one of the MMR proteins, absence of the protein was always seen in both components. In contrast, loss of ER and/or PR, and L1CAM positivity was discrepant in most cases, and was seen more often in the CCC component. These findings suggest that loss of hormone receptors, as well as L1CAM expression is obtained in a more advanced stage within tumor progression. Compared to literature, showing ER expression in 0 – 16% of uterine CCC cases, we found a somewhat higher prevalence (24%) in pure uterine CCCs, even though ER was only focally positive in 4/5 cases [17, 30, 31]. PR expression was found in only 1 patient (5%), which is in line with literature. In mixed uterine CCCs, frequency of ER and PR expression was surprisingly high in the clear cell components of mixed uterine CCCs (52% and 29%). Previous papers have shown that mixed CCCs can display unexpected IHC staining patterns, including (patchy) ER/PR expression, which may be attributed to the fact that these tumors arise from a single clone and subsequently diverge [28, 32].

As a potential target for HER2 directed antibody therapy, *ERBB2* mutations could be of interested for uterine CCCs. A pathogenic *ERBB2* mutation was found in four cases (12%), which is in line with literature showing *ERBB2* mutations in 11% of patients [18].

In our study, L1CAM expression was frequent and associated with impaired survival. Two previous studies did not find a correlation between L1CAM expression and impaired survival, possibly due to a limited sample size [16, 33]. L1CAM is a transmembrane protein that is involved in increasing invasiveness, motility and metastatic potential, and has been found to be a poor prognostic factor in several cancers [34, 35]. More recently, L1CAM positivity was found to be

associated with resistance to platinum-based chemotherapy in high-risk EC [36]. The number of CCCs in that study was limited underlining the need for studies investigating the association between L1CAM expression and therapy responsiveness within this particular subgroup.

We have performed a comprehensive molecular, immunohistochemical and clinical analyses in a series of both pure and mixed uterine CCCs. However, there are some limitations. Due to the rare nature of these tumors, the number of patients within this series was limited. Also, because of the retrospective nature of the study and the use of FFPE tumor tissue, quality of the extracted DNA was variable, and DNA sequencing was unsuccessful in some cases. Of the 21 mixed uterine CCCs, it was possible to extract the DNA of both histological components separately in 10 cases. In the other cases, both histological components merged into one another and could not be isolated separately.

Concluding, we observed different molecular background between pure and mixed uterine CCCs. *TP53* mutations were found more frequently in patients with pure CCCs, and MSI was found more frequently in mixed uterine CCCs. An improved clinical outcome was found in patients with mixed uterine CCCs, compared to patients with pure uterine CCCs. Inferior outcome in pure CCCs may be explained by frequent *TP53* mutations, whereas superior outcome in mixed CCCs may be explained by frequent occurrence of MSI. These results underline the relevance of both morphological and molecular evaluation, and may assist in tailoring treatment.

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SUPPLEMENTARY

Supplementary A. Detailed information on immunohistochemical staining

For ER and PR, antigen retrieval (97 ºC for 30 minutes in Tris/EDTA buffer pH 9 [Envision FLEX Target Retrieval Solution High pH, DAKO, Agilent Technologies, Santa Clara, CA, United States]) and blocking of endogenous peroxidase with hydrogen peroxide were performed. Subsequently, slides were incubated with: ER antibody (clone EP1 GA084, DAKO, Agilent Technologies, Santa Clara, CA, United States) and PR antibody (clone, Pgr 1294 GA090, DAKO, Agilent Technologies, Santa Clara, CA, United States). Envision FLEX/HRP (DAKO, Agilent Technologies, Santa Clara, CA, United States) was used and visualization was performed using Envision FLEX DAB+ Chromogen (DAKO, Agilent Technologies, Santa Clara, CA, United States).

For L1CAM, EDTA (95 ºC for 10 minutes in Tris-EDTA buffer pH 9) and blocking of endogenous peroxidase with hydrogen peroxide were performed. Subsequently, slides were incubated with: L1CAM antibody (purified anti-CD171, clone 14.10, Biolegend, San Diego, CA, US, dilution 1:100). Powervision+ Poly-HRP was used and visualization was performed using PowerVision DAB substrate solution (Leica Biosystems, Buffalo Grove, IL, US).

Immunohistochemical analysis of the mismatch repair (MMR) proteins PMS2 and MSH6 was performed. In short, blank 4µm formalin-fixed, paraffin-embedded (FFPE) sections were cut on Superfrost+ glass slides. After antigen retrieval with EnVision FLEX High pH Target Retrieval Solution, and blocking of endogenous peroxidase with hydrogen peroxide, all slides were incubated with anti-MSH6 (clone EPR3945 1:400, Abcam, Cambridge, UK) or anti- PMS2 (clone A16-4 dilution 1:20, BD Biosciences, San Jose, CA). Subsequently, they were incubated with EnVision FLEX and visualized with High pH visualization system according to the manufacturer's instructions for use. Counterstaining was performed with hematoxylin, and the slides were dehydrated and mounted.

Supplementary B. All gene regions targeted by the smMIP panel

Gene	Exon	Targeted codons	Positions	RefSeq ID	Ensembl ID
AKT1	3	E17	c.47-5 to c.86	NM_005163	ENST00000555528
ARID1A	1 to 20	M1-Stop2286	c.1 to c.6858	NM_006015.5	ENST00000324856
CTNNB1	3	D32-S45	c.53 to c.146	NM_001904.3	ENST00000349496
ERBB2	20	Y772-Y781	c.2308-1 to c.2357	NM_004448	ENST00000269571
FGFR2	6 to 9	I217-V392	c.649 to c.1174	NM_000141.5	ENST00000358487
	11 to 14	K485-L627	c.1453 to c.1879		
KRAS	2	G12-G13	c.9 to c.71	NM_004985.4	ENST00000311936
	3	A59-Q61	c.122 to c.215		
	4	K117, A146	c.291-5 to c.357		
			c.402 to c.450+5		
MTOR	30	D1458-E1489	c.4371 to c.4469+5	NM_004958.3	ENST00000361445
	39	A1789-A1820	c.5365-5 to c.5460		
	43	A1971-L1995	c.5911-5 to c.5985		
	47	Q2194-L2220	c.6580 to c.6662+5		
	53	M2404-D2433	c.7210 to c.7300+5		
	56	G2484-T2509	c.7448-5 to c.7527		
NRAS	2	G12, G13 A59, Q61	c.-17-5 to c.64	NM_002524	ENST00000369535
	3	K117, A146	c.161 to c.245		
	4		c.312 to c.450+5		
PIK3CA	2	S66 – I117 Y317 –	c.195 to c.352 c.947 to	NM_006218.3	ENST00000263967
	5	K353 E418 – M441	c.1059 c.1252 to c.1323		
	8	D520 – H554	c.1558 to c.1664		
	10				
	21	S1015 – N1068	c.3058 to c.3207		
POLE	9 to 14	D268-E491	c.802-5 to c.1473+5	NM_006231.3	ENST00000320574
PTEN	1 to 9	M1-Stop404	c.1 to c.1210+5	NM_000314.6	ENST00000371953
TP53	2 to 11	>95% of all coding and splice sequences (-5/+5)	c.1 to c.1180+5	NM_000565.5	ENST00000269305

Supplementary C. Sequencing results

Study number	Variant	Variant allele frequency	*N* of mutant reads	Variant class
1EEC	ARID1A:c.2178-2187del p.(Arg727fs)	70	468	4
1CC	ARID1A:c.2178-2187del p.(Arg727fs)	52	64	4
1CC	PIK3CA:c.3140A>G p.(His1047Arg)	52	182	5
1CC	PTEN:c.113del p.(Pro38fs)	33	68	4
1	Microsatellite stable			
2	TP53:c.396G>C p.(Lys132Asn)	49	46	4
2	Microsatellite stable			
3EEC	ARID1A:c.1181del p.(Pro394fs)	47	14	4
3CC	PTEN:c.428del p.(Gly143fs)	62	26	4
3	Microsatellite stable			
4	No (potentially) pathogenic variants			
	Microsatellite stable			
5	No (potentially) pathogenic variants			
	Microsatellite stable			
6	AKT1:c.49G>A p.(Glu17Lys)	73	462	5
6	ARID1A:c.6301_6302dup p.(Asp210fs)	28	38	4
6	CTNNB1:c.94G>T p.(Asp32Tyr)	21	108	5
6	TP53:c.658T>G p.(Tyr220Asp)	70	142	4
6	Microsatellite stable			
8	PTEN:c.697C>T p.(Arg233*)	58	1096	4
8	Microsatellite stable			
10	ARIDA1:c.1353_1354del p.(Pro452fs)	34	104	4
10	Microsatellite stable			
11	No (potentially) pathogenic variants			
	Microsatellite stable			
13EEC	PTEN:c.723dup p.(Glu242*)	30	92	4
13CC	PTEN:c.723dup p.(Glu242*)	30	92	4
13CC	ARID1A:c.5548del p.(Asp1850fs)	29	28	4
13CC	NRAS:c.3G>A p.(Thr2_Met67del)	31	214	5
13CC	PIK3CA:c.3140A>G p.(His1047Arg)	30	182	5
13	Microsatellite instable			
15	PTEN:c.405dup p.(Cys136fs)	50	134	4

Supplementary C. Continued

15	PTEN:c.635-16_636del p.(?)	55	88	4
15	TP53:c.537T>G p.(His179Gln)	80	86	4
15	Microsatellite stable			
17EEC	ARID1A:c.3219G>A p.(Try1073*)	17	24	4
17EEC	AKT1:c.49G>A p.(Glu17Lys)	22	50	5
17EEC	TP53:c.817C>T p.(Arg273Cys)	61	289	4
17CC	TP53:c.817C>T p.(Arg273Cys)	61	289	4
17CC	ARIDA1:c.3826C>T p.(Arg1276*)	22	14	4
17CC	ERBB2:c.2524G>A p.(Val842Ile)	39	142	5
17CC	MTOR:c.5395G>A p.(Glu1799Lys)	41	966	5
17	Microsatellite instable			
18	No (potentially) pathogenic variants			
	Microsatellite stable			
19	PIK3CA:c.1636C>A p.(Gln546Lys)	19	669	5
19	PIK3CA:c.1035T>A p.(Asn345Lys)	52	48	5
19	POLE:c.857C>G p.(Pro286Arg)	36	566	5
19	PTEN:c.1021T>G p.(Phe341Val)	63	376	5
19	MTOR:c.6644C>A p.(Ser2215Tyr)	42	196	5
19	MTOR:c.7513C>T p.(Arg2505*)	28	248	5
19	TP53:c.339C>A p.(Phe113Leu)	16	381	5
19	Microsatellite stable			
20	ARID1A:c.2808del p.(Ser936fs)	39	56	4
20	PIK3CA:c.3140A>G p.(His1047Arg)	21	22	5
20	TP53:c.338T>G p.(Phe113Cys)	16	43	5
20	Microsatellite stable			
21	ARID1A:c.6420del p.(Phe2141fs)	30	128	4
21	KRAS:c.34G>T p.(Gly12Cys)	18	96	5
21	PTEN:c.437dup p.(Leu146fs)	32	426	4
21	PIK3CA:c.278G>T p.(Arg93Leu)	26	42	4
21	Microsatellite instable			
27	KRAS:c.35G>A p.(Gly12Asp)	47	30	5
27	PTEN:c.800del p.(Lys267fs)	35	22	4
27	Microsatellite instable			
29	TP53:c.406C>T p.(Gln136*)	5	10	4
29	ARIDA1:c.3826C>T p.(Arg1276*)	6,8	10	4
29	Microsatellite stable			
31	No (potentially) pathogenic variants			
	Microsatellite stable			

Supplementary C. Continued

33	ERBB2:c.2524G>A p.(Val842Ile)	29	142	5
33	ERBB2:c.2047C>T p.(Arg683Trp)	5	10	5
33	TP53:c.414del p.(Lys139fs)	56	194	4
33	TP53:c.1024c>T p.(Arg342*)	22	202	4
33	Microsatellite stable			
41	ERBB2:c.2493+1G>A p.(?)	5,1	10	4
41	TP53:c.801del p.(Asn268fs)	35	68	4
41	TP53:c.342_344delins p.(Leu114fs)	34	37	4
41	Microsatellite stable			
43	TP53:c.488A>G p.(Tyr163Cys)	63	460	4
43	Microsatellite stable			
44	ARID1A:c.598C>T p.(Gln200*)	36	24	4
44	ARID1A:c.6092dupA p.(Tyr2031*)	26	190	4
44	NRAS:c.182A>G p.(Gln61Arg)	26	110	5
44	PIK3CA:c.1633G>A p.(Glu545Lys)	26	598	5
44	Microsatellite stable			
45SER	ARID1A:c.3977del p.(Pro1326fs)	32	56	4
45SER	PIK3CA:c.1636C>G p.(Gln546Glu)	16	492	5
45SER	PIK3CA:c.1633G>A p.(Glu545Lys)	12	102	5
45CC	ARID1A:c.6420del p.(Phe2141fs)	18	128	4
45CC	ARID1A:c.3977del p.(Pro1326fs)	32	32	4
45CC	PIK3CA:c.1636C>G p.(Gln546Glu)	14	492	5
45CC	PIK3CA:c.1633G>A p.(Glu545Lys)	17	586	5
45CC	ARID1A:c.183del p.(Ala62fs)	25	38	4
45	Microsatellite instable			
46EEC	ARID1A:c.5548del p.(Asp1850fs)	50	222	4
46EEC	ARID1A:c.3524dup p.(Leu1176fs)	35	36	4
46EEC	PIK3CA:c.1031T>C p.(Val344Ala)	22	32	5
46EEC	PTEN:c.71A>G p.(Asp24Gly)	47	166	4
46CC	ARID1A:c.3216del p.(Lys1072fs)	6,1	14	4
46CC	ARID1A:c.437del p.(Pro146fs)	41	32	4
46CC	ARID1A:c.5548dup p.(Asp1850fs)	33	12	4
46CC	PTEN:c.71A>G p.(Asp24Gly)	63	316	4
46	Microsatellite instable			
47	TP53:c.405C>T p.(Cys135*)	53	36	4
47	Microsatellite stable			
55EEC	PIK3CA:c.1030G>A p.(Val344Met)	55	1880	5
55EEC	PIK3CA:c.3139C>T p.(His1047Tyr)	57	1472	5

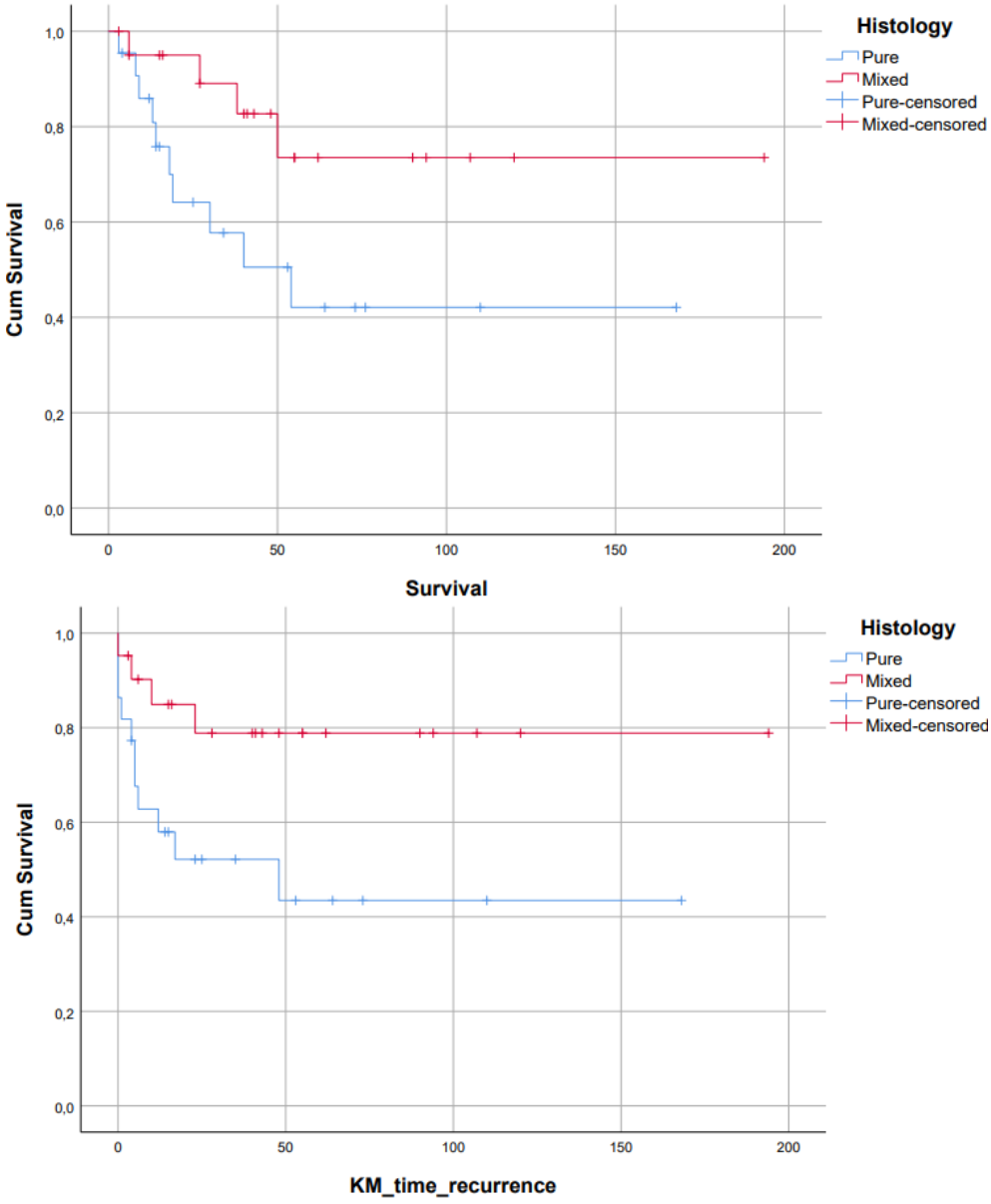
Supplementary C. Continued

55EEC	MTOR:c.5395G>A p.(Glu1799Lys)	40	966	5
55EEC	PTEN:c.697C>T p.(Arg233*)	58	1096	4
55EEC	PTEN:c.517C>T p.(Arg173Cys)	48	420	4
55EEC	TP53:c.1009C>T p.(Arg337Cys)	44	200	4
55CC	TP53:c.916C>T p.(Arg306*)	41	1628	4
55CC	PTEN:c.697C>T p.(Arg233*)	58	930	4
55CC	PTEN:c.800dup p.(Asp268fs)	37	532	4
55CC	ARID1A:c.3424C>T (c.Gln1142*)	42	754	4
55	Microsatellite instable			
58	ARID1A:c.5693del p.(Pro1898fs)	7,1	54	4
58	ARID1A:c.5548del p.(Asp1850fs)	50	222	4
58	CTNNB1:c.121A>G p.(Thr41Ala)	18	510	5
58	PTEN:c.956_959del p.(Thr319fs)	32	334	4
58	PTEN:c.209+4_209+7del p.(?)	29	634	5
58	Microsatellite instable			
59EEC	PIK3CA:c.1633G>A p.(Glu545Lys)	26	598	5
59EEC	TP53:c.743G>A p.(Arg248Gln)	47	373	4
59CC	PIK3CA:c.1633G>A p.(Glu545Lys)	18	192	5
59CC	TP53:c.743G>A p.(Arg248Gln)	19	146	4
59CC	ARID1A:c.1501C.T p.(Gln501*)	21	118	4
59	Microsatellite stable			
66	PIK3CA:c.1258T>C (c.Cys420Arg)	78	603	5
66	Microsatellite stable			
68CC	PTEN:c.1003C>T p.(Arg335*)	51	108	5
68CC	ARID1A:c.2896G>T p.(Glu966*)	36	98	4
68	Microsatellite stable			
69EEC	ARID1A:c.2951del p.(Lys984fs)	30	122	4
69CC	ARID1A:c.2951del p.(Lys984fs)	30	120	4
69	Microsatellite stable			
70SER	ERBB2:c.929C>T p.(Ser310Phe)	23	859	5
70SER	TP53:c.859G>T p.(Glu287*)	38	930	4
70CC	ERBB2:c.929C>T p.(Ser310Phe)	13	254	5
70CC	TP53:c.859G>T p.(Glu287*)	20	214	4
70	Microsatellite stable			
71	PIK3CA:c.1637A>G p.(Gln546Arg)	21	1732	5
71	Microsatellite stable			
72	ARID1A:c.6420del p.(Phe2141fs)	30	90	4
72	ARID1A:c.5548del p.(Asp1850fs)	36	28	4

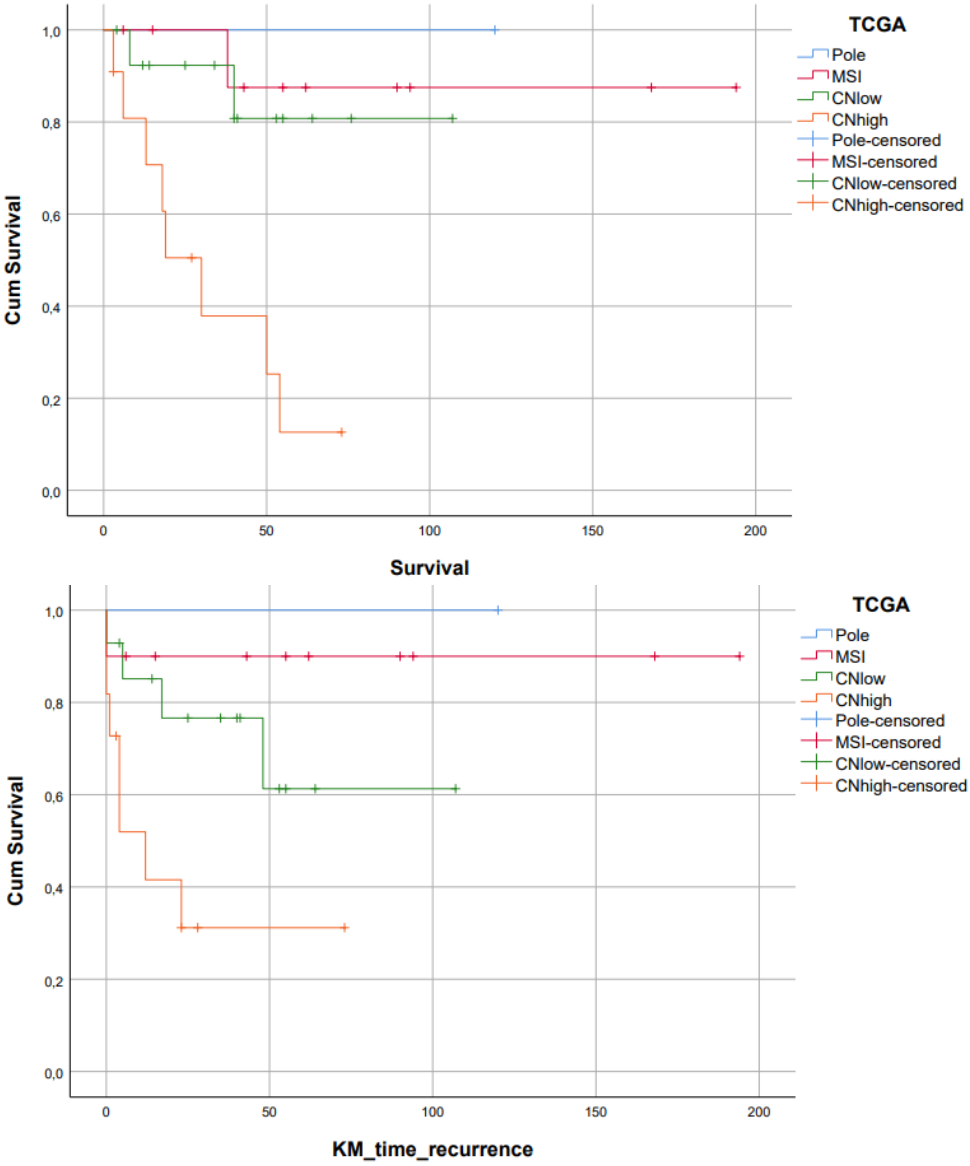
Supplementary C. Continued

72	PIK3CA:c.1624G>A p.(Glu542Lys)	21	298	5
72	Microsatellite instable			

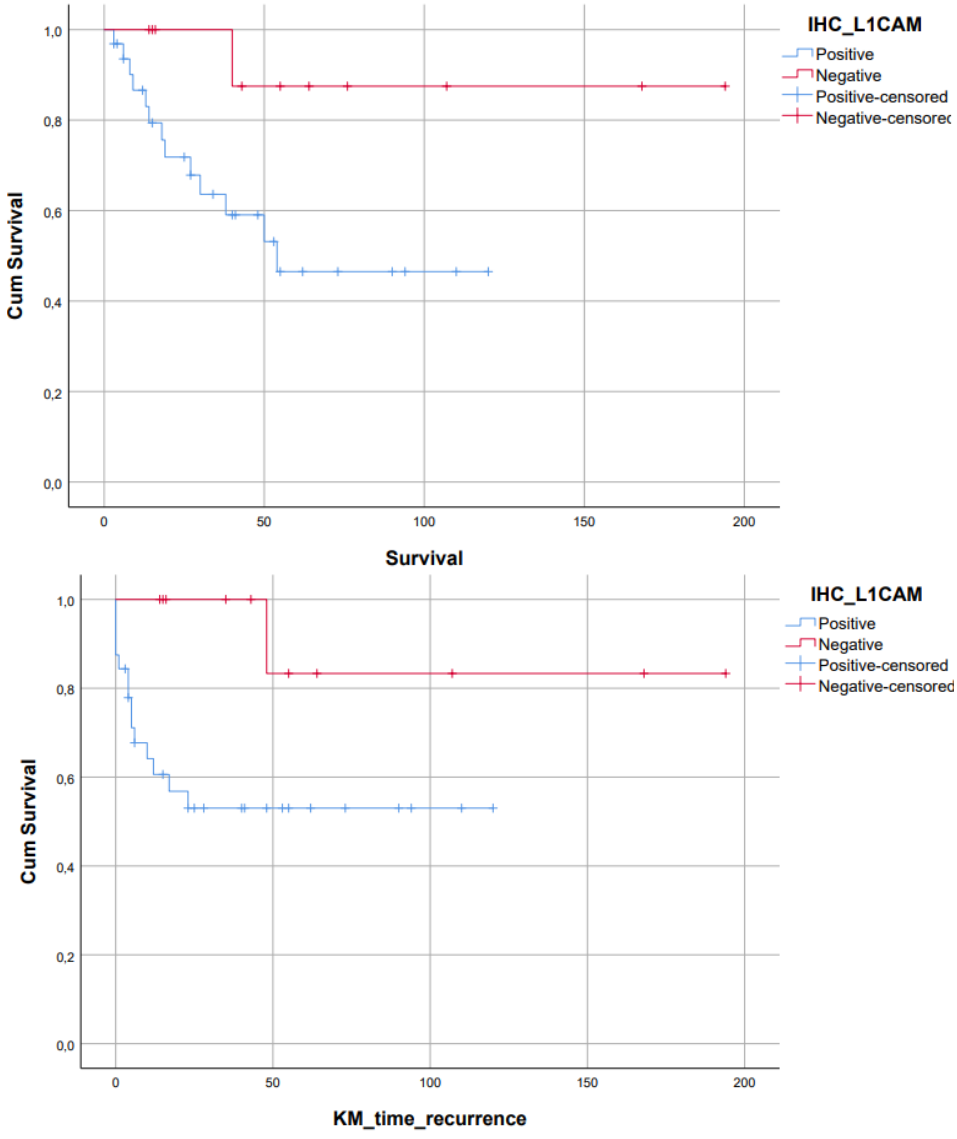
Supplementary D.

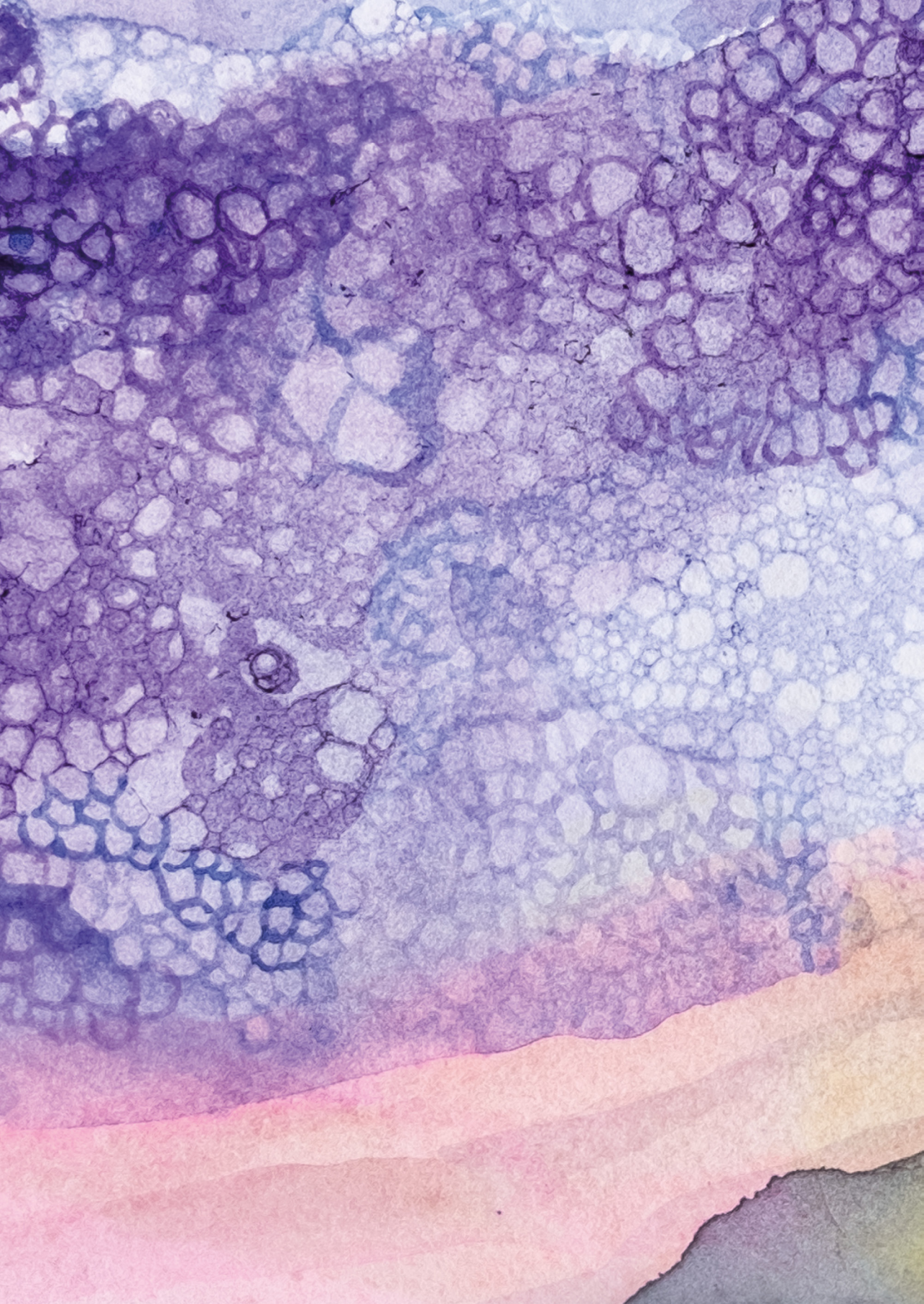


Supplementary D. Continued



Supplementary D. Continued





CHAPTER 5

IMMUNOHISTOCHEMICAL BIOMARKERS ARE PROGNOSTIC RELEVANT IN ADDITION TO THE ESMO-ESGO-ESTRO RISK CLASSIFICATION IN ENDOMETRIAL CANCER

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ABSTRACT

Objective

Preoperative immunohistochemical (IHC) biomarkers are not incorporated in endometrial cancer (EC) risk classification. We aim to investigate the added prognostic relevance of IHC biomarkers to the ESMO-ESGO-ESTRO risk classification and lymph node (LN) status in EC.

Methods

Retrospective multicenter study within the European Network for Individualized Treatment of Endometrial Cancer (ENITEC), analyzing preoperative IHC expression of p53, L1 cell-adhesion molecule (L1CAM), estrogen receptor (ER) and progesterone receptor (PR), and relate to ESMO-ESGO-ESTRO risk groups, LN status and outcome.

Results

A total of 763 EC patients were included with a median follow-up of 5.5-years. Abnormal IHC expression was present for p53 in 112 (14.7%), L1CAM in 79 (10.4%), ER- in 76 (10.0%), and PR- in 138 (18.1%) patients. Abnormal expression of p53/L1CAM/ER/PR was significantly related with higher risk classification groups, and combined associated with the worst outcome within the 'high and advanced/metastatic' risk group. In multivariate analysis p53-abn, ER/PR- and ESMO-ESGO-ESTRO 'high and advanced/metastatic' were independently associated with reduced disease-specific survival (DSS). Patients with abnormal IHC expression and lymph node metastasis (LNM) had the worst outcome. Patients with LNM and normal IHC expression had comparable outcome with patients without LNM and abnormal IHC expression.

Conclusion

The use of preoperative IHC biomarkers has important prognostic relevance in addition to the ESMO-ESGO-ESTRO risk classification and in addition to LN status. For daily clinical practice, p53/L1CAM/ER/PR expression could serve as indicator for surgical staging and refine selective adjuvant treatment by incorporation into the ESMO-ESGO-ESTRO risk classification.

INTRODUCTION

Endometrial cancer (EC) is the most common gynecologic malignancy in industrialized countries and the incidence is rising due to advanced life expectancy and obesity[1]. In general, patients diagnosed at an early stage have a favorable prognosis. Yet, about 20% of patients with clinical early stage disease have a poor outcome[2, 3]. ECs are histologically classified into type 1, comprising endometrioid EC (EEC) with a favorable prognosis, and type 2, comprising of non-endometrioid EC (NEEC) most commonly with serous-, carcinosarcoma- or clear cell histology and unfavorable prognosis[4].

Currently used risk classifications systems are based on clinicopathological risk factors, and guide primary- and/or adjuvant treatment. Different EC risk classifications are used in clinical practice: the European Society for Medical Oncology - European Society of Gynaecological Oncology - European SocieTy for Radiotherapy & Oncology (ESMO-ESGO-ESTRO), Post-operative Radiation Therapy for Endometrial Carcinoma (PORTEC) and Gynecologic Oncology Group (GOG) criteria[1, 5-7]. All these risk classifications stratify into 'low, low-intermediate, intermediate, high-intermediate, high or advanced/metastatic' based on tumor grade, stage, histology, and age (GOG and PORTEC)[5-8]. The ESMO-ESGO-ESTRO risk classification can be used preoperatively to guide the need for lymph node (LN) directed surgery, and postoperatively to define adjuvant treatment. Recently, we published the ENDORISK model showing improved preoperative risk classification in EC with easy accessible biomarkers integrated in a Bayesian network[9]. This personalized network included immunohistochemical (IHC) expression of p53, L1 cell-adhesion molecule (L1CAM), estrogen receptor (ER), progesterone receptor (PR), and clinical preoperative biomarkers and was established to predict lymph node metastasis (LNM) and outcome preoperatively.

The Cancer Genome Atlas (TCGA) identified four important prognostic molecular subgroups based on integrated genomic data[10], in which patients with p53-abn had the poorest outcome[11-13]. Integration of molecular profiling according to the TCGA in the ESMO risk classification was evaluated by Talhouk et al. and showed high prevalence of p53-abn in the ESMO 'high' risk group. However, for the other ESMO risk groups molecular profiling was not discriminative[11].

The integration of molecular profiling appears promising in guiding adjuvant treatment[14]. However, routine molecular profiling in each patient is expensive, and as most patients have a good outcome with hysterectomy and bilateral salpingo-oophorectomy only, a cost-effective stepwise approach might be a suitable alternative. It is hypothesized that the use of preoperative IHC biomarkers such as p53, L1CAM and ER/PR, is not only valuable in guiding primary surgical approach (e.g. ENDORISK), yet also adjuvant treatment in daily clinical

practice. Despite their prognostic relevance for LNM and survival in EC, none of these were studied in relation to the postoperative ESMO-ESGO-ESTRO risk classification groups or to LN status[13, 15-19]. Therefore, our primary aim was to investigate the added prognostic relevance of preoperative IHC biomarkers, p53/L1CAM/ER/PR, to the postoperative ESMO-ESGO-ESTRO risk classification groups in EC. Secondary, the added prognostic relevance of these IHC biomarkers to LN status in EC.

MATERIALS AND METHODS

Study cohort

Within the European Network for Individualized Treatment of Endometrial Cancer (ENITEC), a retrospective multicenter cohort study was performed. The patients were surgically treated between February 1995 and August 2013 at one of the 10 participating ENITEC centers and were identified from a previously published cohort[9, 20]. Only patients diagnosed by an expert gynecological pathologist with complete clinical and pathological data and follow-up of at least 36 months were included, yielding 1199 patients out of ten European hospitals.

Pathological characteristics

Preoperative tumor grade and histology were used for analysis, combined with IHC staining of p53, L1CAM, ER and PR according to ENDORISK[9]. Detailed information on tissue processing and IHC analysis is shown in *Supplementary S1 method*.

Scoring of the IHC was performed twice by assessors blinded to pathological and clinical characteristics (N.V., H.K., J.B., K.v.d.V., C.R.). Disagreements in scoring were solved in a consensus meeting with all assessors. For p53, staining was considered abnormal/aberrant (p53-abn) when more than 80% of tumor cell nuclei showed strong expression (over-expression) or when there was complete absence of nuclear staining (null-expression). For L1CAM, the number of tumor cells showing membranous expression was scored and dichotomized, using 10% as a cut-off value. For ER and PR, the number of stained tumor nuclei was scored. Cases were also dichotomized, using 10% as a cut-off value. L1CAM expression was considered abnormal when >10% of tumor cells were positive (L1CAM+), ER and/or PR expression was considered abnormal when <10% nuclear staining was present (ER/PR-).

Postoperative ESMO-ESGO-ESTRO risk classification

Five subgroups were identified based on postoperative tumor stage, tumor histology, grade, myometrial invasion (MI) and presence of lymphovascular space invasion (LVSI): low, intermediate, high-intermediate, high and advanced/metastatic risk group[5].

Lymph node status

For LN status three subgroups were defined: histologically confirmed LNM (N1), LN sampled by lymphadenectomy and histologically negative (N0), and LN status unknown (Nx) if no lymphadenectomy was performed. Sentinel lymph node (SLN) procedure was allowed but not performed in this study cohort. For the relation of IHC biomarkers and LN status, including the survival analysis, patients with LN status unknown (Nx) were excluded for analysis.

Outcome measurements

Our primary aim was to define the added prognostic relevance of preoperative IHC biomarkers, p53/L1CAM/ER/PR, to the ESMO-ESGO-ESTRO risk classification groups in EC. Secondary, the added prognostic relevance of these IHC biomarkers to LN status in EC.

Statistical analysis

For statistical analyses, Statistical Package for the Social Sciences (SPSS), version 25.0 (IBM, New York, NY, USA) was applied. The results were considered significant if *P*-value was less than 0.05 ($P<0.05$). For the association of IHC expression with the ESMO-ESGO-ESTRO risk classification groups, the Mantel-Haenszel chi^2 test for trend was used.

Survival analyses were performed using the Kaplan Meier curves (first 10 years after diagnosis) and univariate and multivariate Cox-regression. Recurrence-free survival (RFS) was defined as time from surgery to time of recurrence from EC disease, and disease-specific survival (DSS) was defined as time from date of surgery to date of death from EC, all censored by date of last contact. The definition of ER/PR was defined as either ER or PR negative and/or positive. The ESMO-ESGO-ESTRO risk classification in the survival analysis was dichotomized: 'low, intermediate and high-intermediate' and 'high and advanced/metastatic'. This dichotomy was used, as the 'high and advanced/metastatic' ESMO-ESGO-ESTRO risk classification groups included all cases with LNM. Associations were calculated as hazard ratio (HR) with corresponding 95% confidence interval (CI) and *P*-value.

RESULTS

Study cohort

A total of 1199 patients were included from ten European hospitals. For the current study only patients with available preoperative endometrial biopsies were included. Samples with insufficient tumor tissue were excluded, resulting in 763 patients with a median follow-up of 5.5 years[9]. Baseline patient- and tumor characteristics of included patients were not significantly different when compared with excluded patients (*data not shown*).

Clinicopathological characteristics of the study cohort are shown in **Table 1**. Mean patient age in the study population was 65 years. Most patients presented preoperatively with lowgrade (1-2) EC and endometrioid histology, 71.4% and 89.4% respectively. Pre-operative IHC expression of p53-abn was present in 112 (14.7%), L1CAM+ in 79 (10.4%) and ER/PR- in 151 (19.8%) patients. IHC staining was unsuccessful in *N*=67 cases for p53, *N*=19 for L1CAM, *N*=1 for ER and *N*=6 for PR. Lymphadenectomy was performed in 493 (64.6%) patients of whom 53 (10.7%) patients had LNM (N1). Adjuvant treatment was administered in 347 (45.6%) patients, of which 81.6% received radiotherapy (RT). A total of 105 (13.8%) patients developed recurrent EC disease and 102 (13.4%) patients died of whom 61 (59.8%) due to EC.

Stratification of the study cohort according to the ESMO-ESGO-ESTRO risk classification is shown in **Table 1**. A total of 169 (22.1%) EC patients were classified as 'high' risk.

Immunohistochemical expression in relation to the ESMO-ESGO-ESTRO risk classification

In **Figure 1** abnormal IHC expression of p53, L1CAM, ER and PR is shown in relation to the ESMO-ESGO-ESTRO risk classification groups. Increased abnormal IHC expression was related to higher risk classification groups ($P<0.001$), with the highest frequency of p53-abn, L1CAM+, ER- or PR- in the ESMO-ESGO-ESTRO 'high and advanced/metastatic' subgroups.

Table 1. Baseline clinicopathological characteristics of study cohort

		Total ***N*=763**
Patient characteristics		
Age (years)		65.2 ± 10.2
BMI (kg/m^2)		29.9 ± 6.7
Preoperative histology		
Tumor grade	1-2	545 (71.4)
	3	109 (14.4)
	Not classified	108 (14.2)
Histology	Endometrioid	682 (89.4)
	Non-endometrioid	39 (5.1)
	Not specified	42 (5.5)
Biomarker expression	p53-abnormal	112 (14.7)
	L1CAM positive	79 (10.4)
	ER/PR negative	151 (19.8)

Table 1. *Continued*

Postoperative histology		
Tumor grade	1-2	607 (79.6)
	3	156 (20.4)
Histology	Endometrioid	714 (93.6)
	Non-endometrioid	49 (6.4)
FIGO stage	I-II	675 (88.5)
	IA	428 (56.1)
	IB	196 (25.7)
	II	51 (6.7)
	III - IVB	88 (11.5)
	IIIA	20 (2.6)
	IIIB	4 (0.5)
	IIIC	43 (5.6)
	IVA	2 (0.3)
	IVB	19 (2.5)
Lymph node status	Positive (N1)	53 (6.9)
	Negative (N0)	440 (57.7)
	Unknown† (Nx)	270 (35.4)
ESMO-ESGO-ESTRO risk classification	Low	366 (48.0)
	Intermediate	140 (18.3)
	High-Intermediate	68 (8.9)
	High	169 (22.1)
	Advanced/Metastatic	20 (2.6)
Adjuvant treatment		
None		415 (54.4)
Radiotherapy		283 (37.1)
	VBT	112 (39.6)
	EBRT	104 (36.7)
	EBRT+VBT	93 (32.9)
Chemotherapy		38 (5.0)
Chemoradiation		26 (3.4)
Not specified		1 (0.1)
Outcome		
Recurrence		105 (13.8)
	Local	25 (23.8)
	Regional	9 (8.6)
	Distant	69 (65.7)
	Not classified	2 (1.9)
Mortality	Overall	102 (13.4)
	EC-related	61 (8.0)

Data is presented in number (%), mean ± standard deviation (SD).

EC, endometrial cancer; ER/PR, estrogen receptor/progesterone receptor; ESMO-ESGO-ESTRO, European Society for Medical Oncology - European Society of Gynaecological Oncology - European SocieTy for Radiotherapy & Oncology; EBRT, external beam radiotherapy; FIGO, Federation International Gynaecology Obstetric; L1CAM, L1 cell-adhesion molecule; *N*, number; VBT, vaginal beam therapy

†no lymphadenectomy performed

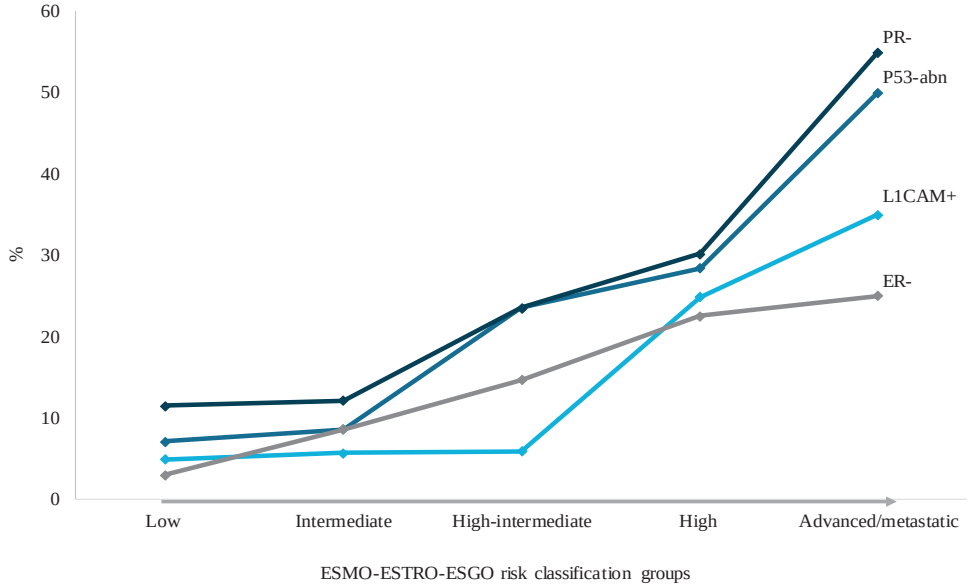

Figure 1. Immunohistochemical expression of p53, L1CAM and ER/PR in relation to the ESMO-ESGO-ESTRO risk classification.

Abbreviations: L1CAM, L1 cell-adhesion molecule; ER/PR, estrogen receptor/progesterone receptor; ESMO-ESGO-ESTRO, European Society for Medical Oncology - European Society of Gynaecological Oncology - European SocieTy for Radiotherapy & Oncology.

Immunohistochemical expression in addition to ESMO-ESGO-ESTRO risk classification

The RFS according to the ESMO-ESGO-ESTRO risk groups and IHC expression of p53, L1CAM and ER/PR in the ESMO 'high and advanced/metastatic' risk group are shown in **Figure 2**. The ESMO-ESGO-ESTRO risk classification group 'high and advanced/metastatic' are significantly associated with poor RFS (p<0.001) (**Figure 2A**). Within the 'high and advanced/metastatic' risk group, patients with abnormal IHC expression of; p53, L1CAM and ER/PR, p53 and L1CAM, L1CAM and ER/PR, and only ER/PR have the lowest RFS, compare to patients with abnormal expression of; p53 and ER/PR, only p53 and only L1CAM (**Figure 2B**). Detailed survival curves of the ESMO-ESGO-ESTRO risk groups in relation to IHC expression are demonstrated in **Figure 3A-C**. Patients with abnormal IHC expression (p53-abn, L1CAM+ or ER/PR-) and ESMO-ESGO-ESTRO risk group 'high and advanced/metastatic' show the lowest RFS compared with the other subgroups.

The DSS according to the ESMO-ESGO-ESTRO risk classification groups, and detailed survival curves of the ESMO-ESGO-ESTRO risk group in relation to the IHC expression were comparable to the RFS (*Supplementary Figure S1 A and Figure S2 A-C).* Within the ESMO 'high and advanced/metastatic' risk group, patients with abnormal IHC expression of; p53, L1CAM and ER/PR, p53 and L1CAM, p53 and ER/PR and only ER/PR have the lowest DSS compare to patients with abnormal expression of; L1CAM and ER/PR, only p53 and only L1CAM (*Supplementary Figure S1 B).*

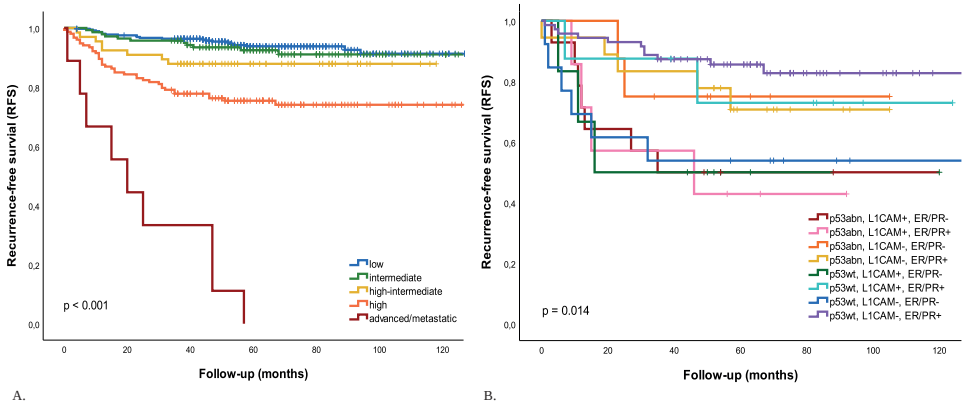

Figure 2 A-B. A. RFS for the ESMO-ESGO-ESTRO risk groups. B. RFS for the ESMO-ESGO-ESTRO 'high and advanced/metastatic' risk group in relation to p53, L1CAM and ER/PR expression.

Abbreviations: ESMO-ESGO-ESTRO, European Society for Medical Oncology - European Society of Gynaecological Oncology - European SocieTy for Radiotherapy & Oncology; p53abn, p53-abnormal; p53wt, p53-wildtype; L1CAM, L1 cell-adhesion molecule; ER/PR, estrogen receptor/progesterone receptor; RFS, recurrence-free survival.

Prognostic relevance of immunohistochemical expression in relation to the ESMO-ESGO-ESTRO risk classification

Multivariate analysis was performed for the prognostic relevance of IHC expression in relation to the ESMO-ESGO-ESTRO risk classification groups. The ESMO-ESGO-ESTRO classification 'high and advanced/metastatic' risk was independently associated with reduced RFS (HR 3.11 [CI 1.93-5.02] *P*<0.001)(**Table 2**). P53-abn, ER/PR- and ESMO-ESGO-ESTRO classification 'high and advanced/metastatic' risk were independently associated with reduced DSS (HR 1.88 [CI 1.00-3.51] *P*=0.048, HR 2.74 [CI 1.48-5.07] *P*=0.001 and HR 5.69 [CI 3.03-10.67] *P*<0.001, respectively) (**Table 3**).

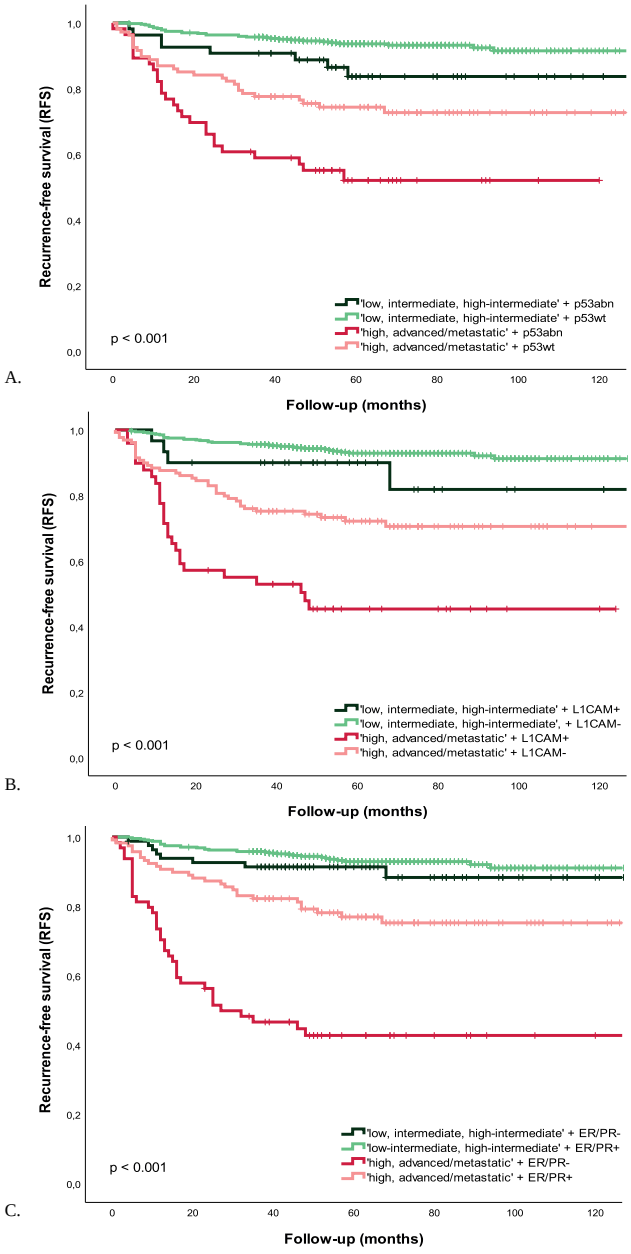

Figure 3 A-C. A. RFS for ESMO-ESGO-ESTRO risk groups and p53-expression. B. RFS for ESMO-ESGO-ESTRO risk groups and L1CAM-expression C. RFS for ESMO-ESGO-ESTRO risk groups and ER/PR-expression.

Abbreviations: ESMO-ESGO-ESTRO, European Society for Medical Oncology - European Society of Gynaecological Oncology - European SocieTy for Radiotherapy & Oncology; p53abn, p53-abnormal; p53wt, p53-wildtype; L1CAM, L1 cell-adhesion molecule; ER/PR, estrogen receptor/progesterone receptor; RFS, recurrence-free survival.

Table 2. Univariate and multivariate Cox regression analysis of RFS

Variable	Univariate RFS			Multivariate RFS		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
ESMO-ESGO-ESTRO risk classification						
'Low - Intermediate - High-intermediate' vs 'High – Advanced/metastatic'	3.92	2.52-6.08	<0.001*	3.11	1.93-5.02	<0.001*
Immunohistochemical markers						
p53-abnormal	2.94	1.83-4.72	<0.001*	1.58	0.92-2.71	0.097
L1CAM+	4.27	2.63-6.92	<0.001*	1.78	0.98-3.21	0.058
ER/PR-	3.16	2.03-4.91	<0.001*	1.54	0.90-2.63	0.115

Abbreviations: CI, confidence interval; ER/PR, estrogen receptor/progesterone receptor; ESMO-ESGO-ESTRO, European Society for Medical Oncology-European SocieTy for Radiotherapy & Oncology-European Society of Gynaecological Oncology ; HR, Hazard ratio; L1CAM, L1 cell-adhesion molecule; RFS, Recurrence-free survival.

* $p<0.05$

Table 3. Univariate and multivariate Cox regression analysis of DSS

Variable	Univariate DSS			Multivariate DSS		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value
ESMO-ESGO-ESTRO risk classification						
Low - Intermediate - High-intermediate' vs 'High – Advanced/metastatic'	6.93	3.82-12.57	<0.001*	5.69	3.03-10.67	<0.001*
Immunohistochemical markers						
p53-abnormal	4.44	2.56-7.69	<0.001*	1.88	1.00-3.51	0.048*
L1CAM +	5.43	3.19-9.26	<0.001*	1.17	0.59-2.31	0.656
ER/PR-	5.49	3.30-9.12	<0.001*	2.74	1.48-5.07	0.001*

Abbreviations: CI, confidence interval; DSS, disease-specific survival; ER/PR, estrogen receptor/progesterone receptor; ESMO-ESGO-ESTRO, European Society for Medical Oncology-European SocieTy for Radiotherapy & Oncology-European Society of Gynaecological Oncology ; HR, Hazard ratio; L1CAM, L1 cell-adhesion molecule.

* $p<0.05$

Immunohistochemical expression in relation to lymph node status

The LN status in relation to abnormal IHC expression (p53-abn, L1CAM+ or ER/PR-) is shown in *Supplementary Figure S3*. LNM was observed in 21.4% of p53-abn, 31.3% of L1CAM+, and 20% of ER/PR- cases. Survival outcome curves (RFS and DSS) of abnormal and normal IHC expression in relation to LN status (N1/N0) is shown in *Supplementary Figure S4 A-C and S5 A-C.* Patients with LNM (N1) and p53-abn or L1CAM+ had significantly decreased RFS/DSS compared to patients having LNM (N1) and normal IHC expression (p53-wt or L1CAM-), or patients without LNM (N0) and normal/abnormal IHC expression ($p<0.001$). No significant reduction in RFS was seen in patients with LNM (N1) and ER/PR- compared with patients having LNM (N1) and ER/PR+ (*Supplementary Figure*

S4 C). Patients with LNM (N1) and ER/PR- had significantly reduced DSS compared with patients having LNM (N1) and ER/PR+ (*Supplementary Figure S5 C).* Patients without LNM (N0) and abnormal IHC expression (p53-abn, L1CAM+ or ER/PR-) had similar RFS/DSS compared with patients with LNM (N1) and normal IHC expression (p53-wt, L1CAM- or ER/PR+) (*Supplementary Figure S4 A-C and S5 A-C).*

DISCUSSION

In this study the added prognostic relevance of the pre-operative IHC expression of p53, L1CAM and ER/PR, to the ESMO-ESGO-ESTRO risk classification groups is demonstrated. Significantly increased abnormal IHC expression is observed in higher risk classification groups. Within the 'high and advanced/metastatic' risk group, patients with a combination of abnormal IHC expression had the poorest outcome (RFS and DSS). ER/PR-, p53-abn, and ESMO-ESGO-ESTRO 'high and advanced/metastatic' risk group were independently associated with decreased DSS. Furthermore, abnormal IHC expression had added prognostic relevance to LN status. Patients with abnormal IHC expression and LNM had most dismal outcome. Interestingly, patients without LNM and abnormal IHC expression showed comparable RFS/DSS as, patients with LNM and normal biomarkers. This indicated that the IHC biomarkers p53, L1CAM and ER/PR have prognostic relevance to the ESMO-ESGO-ESTRO risk classification groups and to patients with and without LNM.

Our findings of p53-abn as important prognosticator is in line with the TCGA data that have been validated by multiple other research groups[10, 21]. The percentage of p53-abn in our study cohort (12.0%) in patients with endometroid histology was comparable to the original TCGA paper (11.4%)[10]. Instead of using *p53* sequencing, we used easy accessible p53 IHC staining comparable to the ProMisE classification system, which was shown to be a good surrogate biomarker for *p53* mutations[11, 13, 22]. Talhouk et al. studied the prevalence and prognostic relevance of p53-abn in the ESMO risk classification, and observed a high prevalence of p53-abn in the ESMO 'high' risk group in line with our findings[13]. The fact that integration of ProMisE/TCGA in the ESMO risk classification did not show significant difference in outcome, is contrary to our results in which p53-abn had added prognostic value in the 'high and advanced/metastatic' risk group. This could be explained by the use of the ESMO 2013 guideline in the study of Talhouk et al. compared with the ESMO-ESGO-ESTRO 2016 guideline used in our study[13].

In addition to p53-abn, L1CAM+ is an established prognosticator in EC as observed in our study[16, 20, 23]. The percentage of L1CAM+ cases was slightly lower in our study compared with other studies[16, 19, 20, 23, 24]. This might be related to the fact that preoperative analysis was

used instead of final tumor sections in which L1CAM can be expressed focally and/or at the invasive front predominantly[25]. Our results are in line with a study reporting that patients with L1CAM+ had significantly reduced survival also in the ESMO-ESGO-ESTRO 'high and advanced/metastatic' risk group compared with normal L1CAM expression[24]. A more recent study reported reduced overall survival (OS) and progression-free survival (PFS) in patients with FIGO stage III and L1CAM+ when compared with L1CAM- patients[26]. This is in line with our results since FIGO stage III is included in the 'high' risk group.

Multiple studies have investigated ER/PR expression in relation to outcome in EC reporting conflicting results[18, 27, 28]. In our study, ER/PR- was not significantly related to RFS in the multivariate analysis, contrary to previous studies[18, 27, 28]. In line with the study by Trovik et al. ER/PR- was related to DSS and LNM[17]. In a previous ENITEC study of our study group, mainly loss of PR predicted disease recurrence[18]. Biologically, loss of ER is preceded by loss of PR and therefore PR might be the most relevant to outcome. In the current study, we did not analyze ER/PR separately. Interestingly, loss of PR was mainly present in the 'advanced/metastatic' risk group, underlining the possible relevance for distant spread. The expression of ER/PR was studied in relation to the different TCGA groups and although ER and PR biomarkers were both predictive for outcome in the univariate analysis, only the ProMisE subtypes maintained significant associated with outcome in the multivariate analysis. It was suggested that the prognostic significance of single biomarkers could be explained by being a covariable with the ProMisE molecular subtype[16]. Similar was shown in the study of Stelloo et al[29]. Due to the used cut-offs for ER/PR of 5% and 1% respectively in one study, the prognostic value might have been underestimated when compared with the 10% cut-off that was used in our study and by Trovik et al[16, 17]. Stelloo et al. did used the 10% cut-off, however they only included early stage EEC patients hampering comparison to our study[29].

There is an ongoing debate about routine surgical staging with LN dissection or sampling in EC, especially after the introduction of molecular profiling. LN status as determined by either lymphadenectomy or SLN remains an important prognosticator for survival and guiding adjuvant treatment in the current ESMO-ESGO-ESTRO risk classification[5, 30-33]. The study of Ouldamer et al. concluded, that even patients within the 'high-intermediate' risk group should receive systematic nodal staging for a significant better survival[34]. This is in line with recent paper of Weelden et al. that demonstrated that patients with FIGO IIIA-B had significant improved outcome if LN were sampled and negative[35]. Our results show the prognostic relevance of IHC expression in addition to LN status, similar to other studies[17, 19]. The importance of both IHC biomarkers and LN status is shown since patients with LNM and normal IHC expression had comparable RFS/DSS with patients without LNM and abnormal IHC expression.

To our knowledge the prognostic relevance of integrating IHC biomarkers in the ESMO-ESGO-ESTRO risk classification groups has not been studied so far. Yet, there are some limitations that need to be addressed. First, in addition to the IHC biomarkers, we did not include the well-established final histopathological markers related to the prognosis. However, as expected the preoperative abnormal biomarker expression of p53, L1CAM and ER/PR are significant associated with grade 3, NEEC, LVSI, MI and cervical stromal invasion (CSI) *(data not shown)*. Abnormal preoperative biomarkers could therefore serve as surrogate biomarkers for these final histopathological risk factors. Second, as we used p53 IHC expression as indicator for *p53*-abn without information on *POLE* or mismatch repair deficient (*MMR-D*) status, we might have slightly overestimated the number of patients with p53-abn. However, as multiple classifiers are only present in 3% of the cases, it is unlikely that this has influenced our findings[36]. Finally, inherent to the retrospective character of the study, differences in outcome might be explained by the fact that adjuvant treatment was not uniformly applied. The majority (80-100%) of the patients with LNM and abnormal IHC expression received chemotherapy (CT) or chemo- and radiotherapy (CTRT) as adjuvant treatment, compared with 55% for patients with LNM and normal IHC expression (*data not shown*). This difference in percentage could be explained by patients being treated according final tumor stage and histology in different ENITEC centers in Europe, i.e. patients that received more often radiotherapy mainly had endometrioid histology, whereas those with adjuvant chemotherapy more often non-endometrioid histology. Thus, patients having worst outcome most frequently received CT or CTRT instead of RT alone, and this does therefore not explain the specifically worse outcome in these patients.

The strength of this multicenter study is the large patient cohort, and well-documented and long-term follow-up. Although primary and adjuvant treatment was not uniformly applied, the current study reflects actual clinical practice facilitating implementation.

For this study we focused on the IHC expression in the preoperative setting, as the risk of extended disease and LNM appears mainly associated with p53-abn and significantly less with the other TCGA groups[12]. We expect that molecular profiling will be incorporated in future treatment planning[37-40]. The study of Leslie et al. revealed that patients with *TP53* mutation had significant better PFS with adjuvant chemotherapy + bevacizumab when compared to chemotherapy + temsirolimus, this significant difference was not shown in patients with *TP53* wildtype[41]. This illustrated the relevance of TCGA with respect to the adjuvant treatment. However, routine molecular analysis is expensive and requires fully equipped laboratory, therefore a step-wise approach could bridge this gap, and contribute to selective molecular profiling in 'high' risk EC patients[13]. Cosgrove et al. showed that even selective molecular profiling in patients with only EEC histology could provide additional prognostic information. This step-wise approach could be used combined with the Lynch

syndrome screening panel and so refining the choice of adjuvant treatment[42]. The recently published ENDORISK model demonstrates that preoperative identification for patients at risk for LNM can be significantly improved by incorporating clinical and IHC biomarkers into a Bayesian network[9]. Although we fully endorse the integration of both clinical and IHC biomarkers, in clinical practice we often have to deal with incomplete data. This current study showed that the IHC biomarkers could serve as indicator for LN directed surgery and either IHC biomarkers or molecular profiling or a combination, could be used as refinement for selective adjuvant treatment by being incorporated in the ESMO-ESGO-ESTRO risk classification and being used next to LN status. These results should be further validated in an prospective study with an independent cohort.

CONCLUSION

Concluding, preoperative IHC biomarkers are important prognostic markers within the ESMO-ESGO-ESTRO risk classification groups and in addition to LN status. For daily clinical practice, integrating IHC expression of p53/L1CAM/ER/PR into the ESMO-ESGO-ESTRO risk classification groups may be valuable in guiding surgical staging, and identifying patients who would benefit from specific adjuvant treatment.

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SUPPLEMENTARY

Supplementary S1 Method
Detailed information of immunohistochemical analysis

Immunohistochemical staining
For PR and ER, antigen retrieval (97 ºC for 30 minutes in Tris/EDTA buffer pH 9 [Envision FLEX Target Retrieval Solution High pH, DAKO, Agilent Technologies, Santa Clara, CA, United States]) and blocking of endogenous peroxidase with hydrogen peroxide were performed. Subsequently, slides were incubated with: PR antibody (clone, Pgr 1294 GA090, DAKO, Agilent Technologies, Santa Clara, CA, United States) an ER antibody (clone EP1 GA084, DAKO, Agilent Technologies, Santa Clara, CA, United States). Envision FLEX/HRP (DAKO, Agilent Technologies, Santa Clara, CA, United States) was used and visualization was performed using Envision FLEX DAB+ Chromogen (DAKO, Agilent Technologies, Santa Clara, CA, United States).

For L1CAM, EDTA (95 ºC for 10 minutes in Tris-EDTA buffer pH 9) and blocking of endogenous peroxidase with hydrogen peroxide was performed. Thereafter, slides were incubated with: L1CAM antibody (purified anti-CD171, clone 14.10, Biolegend, San Diego, CA, US, dilution 1:100). Powervision+ Poly-HRP was used and visualization was accomplished by using PowerVision DAB substrate solution (Leica Biosystems, Buffalo Grove, IL, US).

For p53 staining, antigen retrieval (30 minutes, pH 6·7) and blocking of endogenous peroxidase with hydrogen peroxide was performed. Subsequently, slides were incubated with p53 antibody (clone DO-7 + BP53-12, dilution 1:600). Powervision+ Poly-HRP was used and visualization was accomplished by using PowerVision DAB substrate solution (Leica Biosystems, Buffalo Grove, IL, US). Counterstaining was performed with hematoxylin, slides were dehydrated and mounted.

For the subgroup of patients from the Haukeland university hospital, Bergen, Norway (166 patients), staining protocol differed slightly from the above mentioned staining protocols. Tissue microarrays (TMAs) were constructed for all endometrial biopsies with three tissue cylinders from each case. Microwave antigen retrieval (750 W for 10 and 350 W for 15 min) in Tris–EDTA buffer pH 9 was performed before peroxidase blocking (Dako S-2032) for 5 minutes and incubation with: Oestrogen Receptor α (ER) (Dako M7047) diluted 1:50, Progesterone Receptor (PR) (Dako M3569) diluted 1:150 both for 30 min, purified anti-CD171 antibody clone 14.10 (Biolegend, San Diego, CA, US) diluted 1:100; and tumor protein 53 (p53) (Dako M7001) diluted 1:1000 for 60 min. Subsequently, the

EnVision+Mouse HRP labelled polymer secondary antibody with DAB+ (K4006) was used. Slides were counterstained with Dako Automation Haematoxylin.

Scoring of immunohistochemistry

For ER and PR, the number of stained tumor nuclei regardless of staining intensity was scored, and subsequently cases were dichotomized, using 10% as a cut-off value. For L1CAM, the number of tumor cells showing membranous expression was scored and cases were dichotomized, using 10% as a cut-off value. For p53, staining was considered abnormal when there was complete absence of nuclear staining (null-expression) or when more than 80% of tumor cell nuclei showed strong expression (overexpression).

For the Bergen subgroup, scoring was performed at the Haukeland university hospital and differed slightly from the Radboud staining protocols. In short, both intensity and area of positive tumour cells were scored. The intensity was scored from 0 (no staining) to 3 (strong), and the area as 0, 1 (<10%), 2 (10–50%) and 3 (51–100%). From this, a staining index (0–9) was calculated as the product of intensity and area. If heterogeneity was seen for the three tissue cylinders of each case, the three cylinders were given one overall averaged score. For L1CAM staining, index was dichotomized using ≥ 4 as a cut-off for positive expression. For ER staining index $\leqslant 3$ and PR staining index 0 was defined as negative expression. Pathologic expression of p53 (high) was defined as staining index $\geqslant 4$.

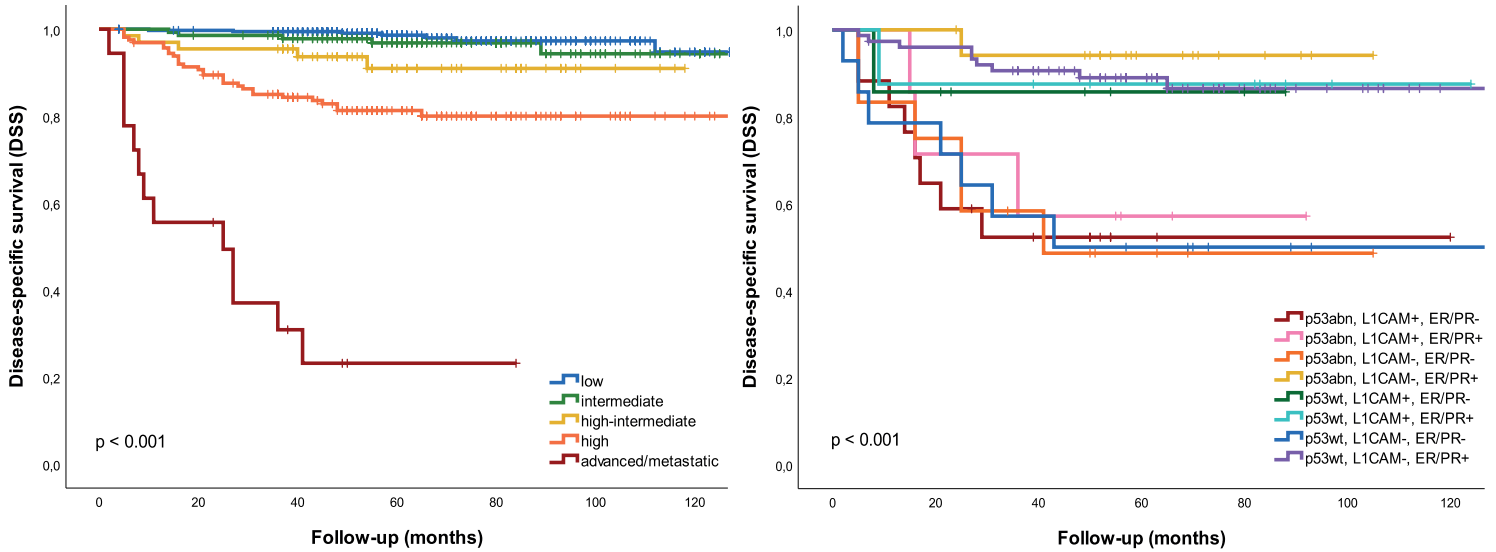


Figure S1 A-B. A. DSS for the ESMO-ESGO-ESTRO risk groups. B. DSS for the ESMO-ESGO-ESTRO 'high and advanced/metastatic' risk group in relation to p53, L1CAM and ER/PR expression.

Abbreviations: ESMO-ESGO-ESTRO, European Society for Medical Oncology - European Society of Gynaecological Oncology - European SocieTy for Radiotherapy & Oncology; ER/PR, estrogen receptor/progesterone receptor; L1CAM, L1 cell-adhesion molecule; p53abn, p53-abnormal; p53wt, p53-wildtype; DSS, disease-specific survival.

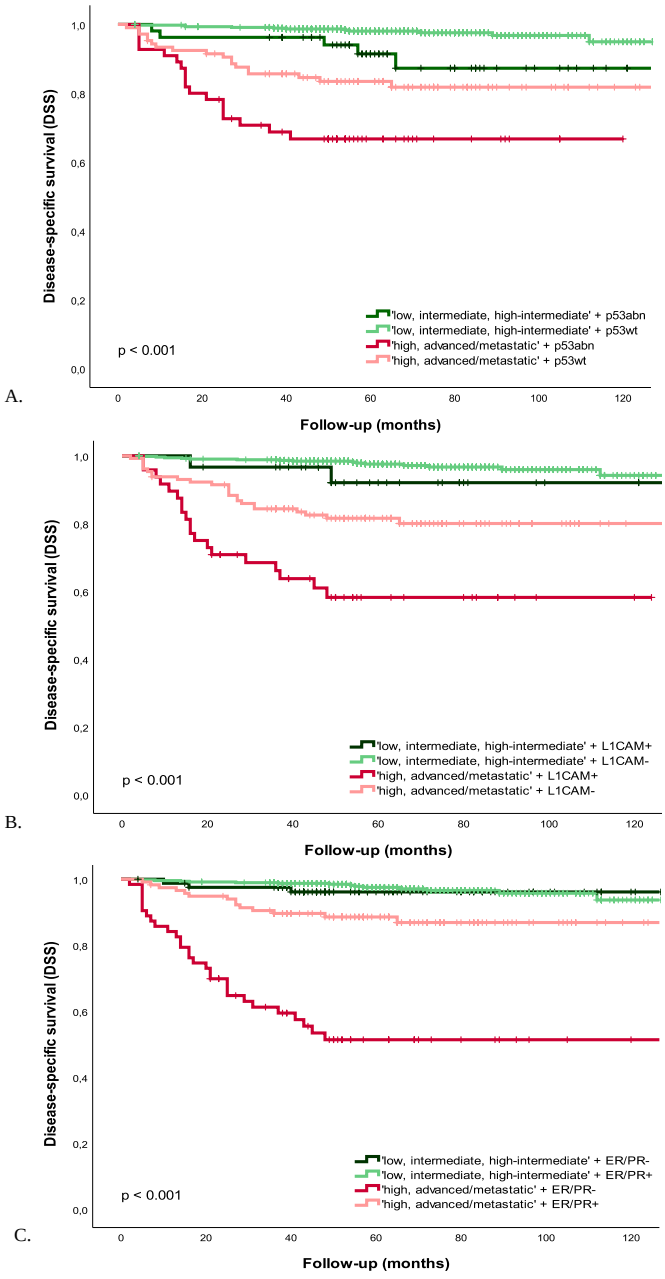


Figure S2 A-C. A. DSS for ESMO-ESGO-ESTRO risk groups and p53-expression. B. DSS for ESMO-ESGO-ESTRO risk groups and L1CAM-expression C. DSS for ESMO-ESGO-ESTRO risk groups and ER/PR-expression.

Abbreviations: ESMO-ESGO-ESTRO, European Society for Medical Oncology - European Society of Gynaecological Oncology - European SocieTy for Radiotherapy & Oncology; ER/PR, estrogen receptor/progesterone receptor; L1CAM, L1 cell-adhesion molecule; p53abn, p53-abnormal; p53wt, p53-wildtype; DSS, disease-specific survival.

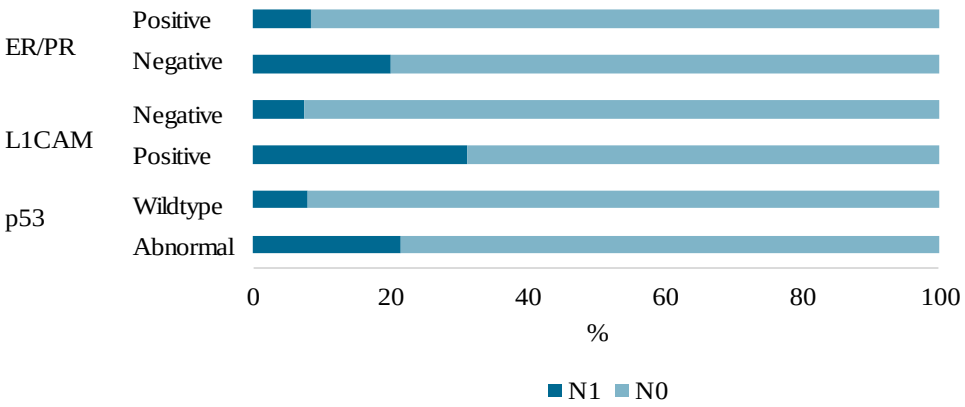


Figure S3. Positive and negative lymph node status in relation to abnormal and normal expression of p53, L1CAM and ER/PR.

Abbreviations: ER/PR, estrogen receptor/progesterone receptor; L1CAM, L1 cell-adhesion molecule; N1, lymph node metastasis; N0, no lymph node metastasis.

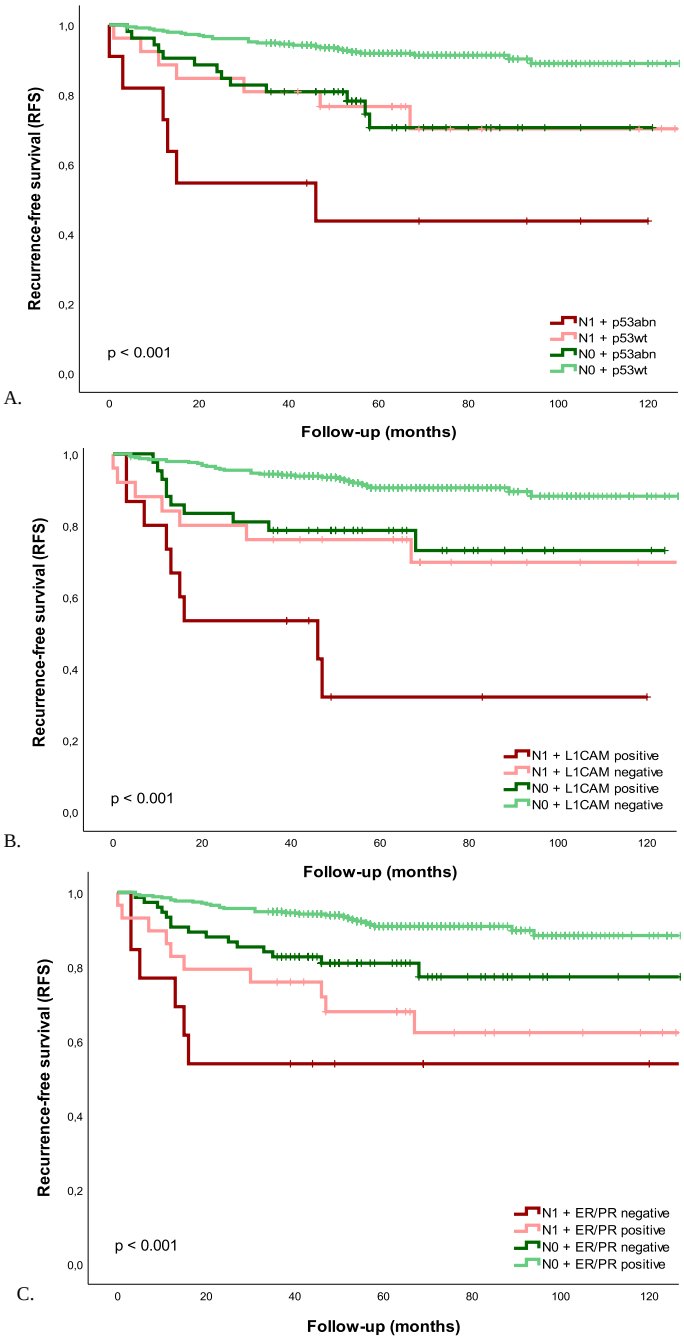


Figure S4 A-C. A. RFS for p53-abn and p53-wt with N1 and N0. B. RFS for LICAM+ and L1CAM- with N1 and N0. C. RFS for ER/PR+ and ER/PR- with N1 and N0.

Abbreviations: ER/PR, estrogen receptor/progesterone receptor; L1CAM, L1 cell-adhesion molecule; N1, lymph node metastasis ; N0, no lymph node metastasis; p53abn, p53-abnormal; p53wt, p53-wildtype; RFS, recurrence-free survival.

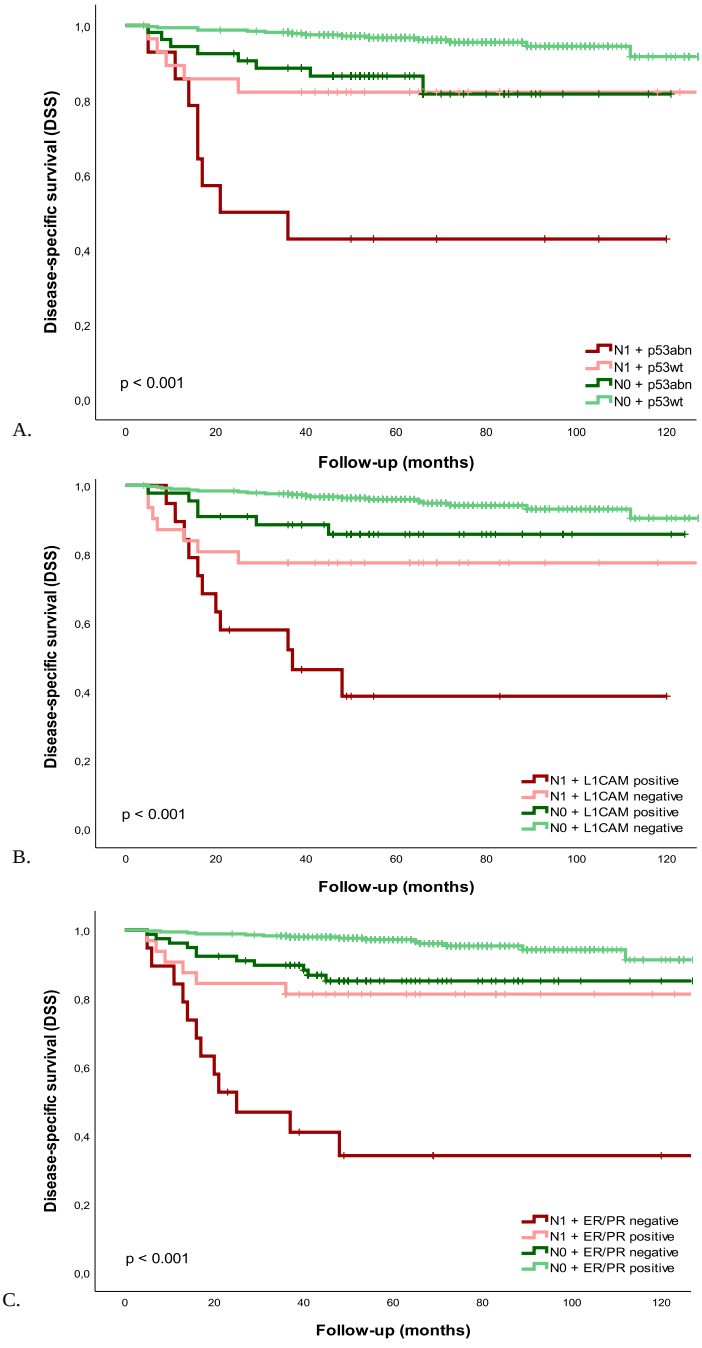
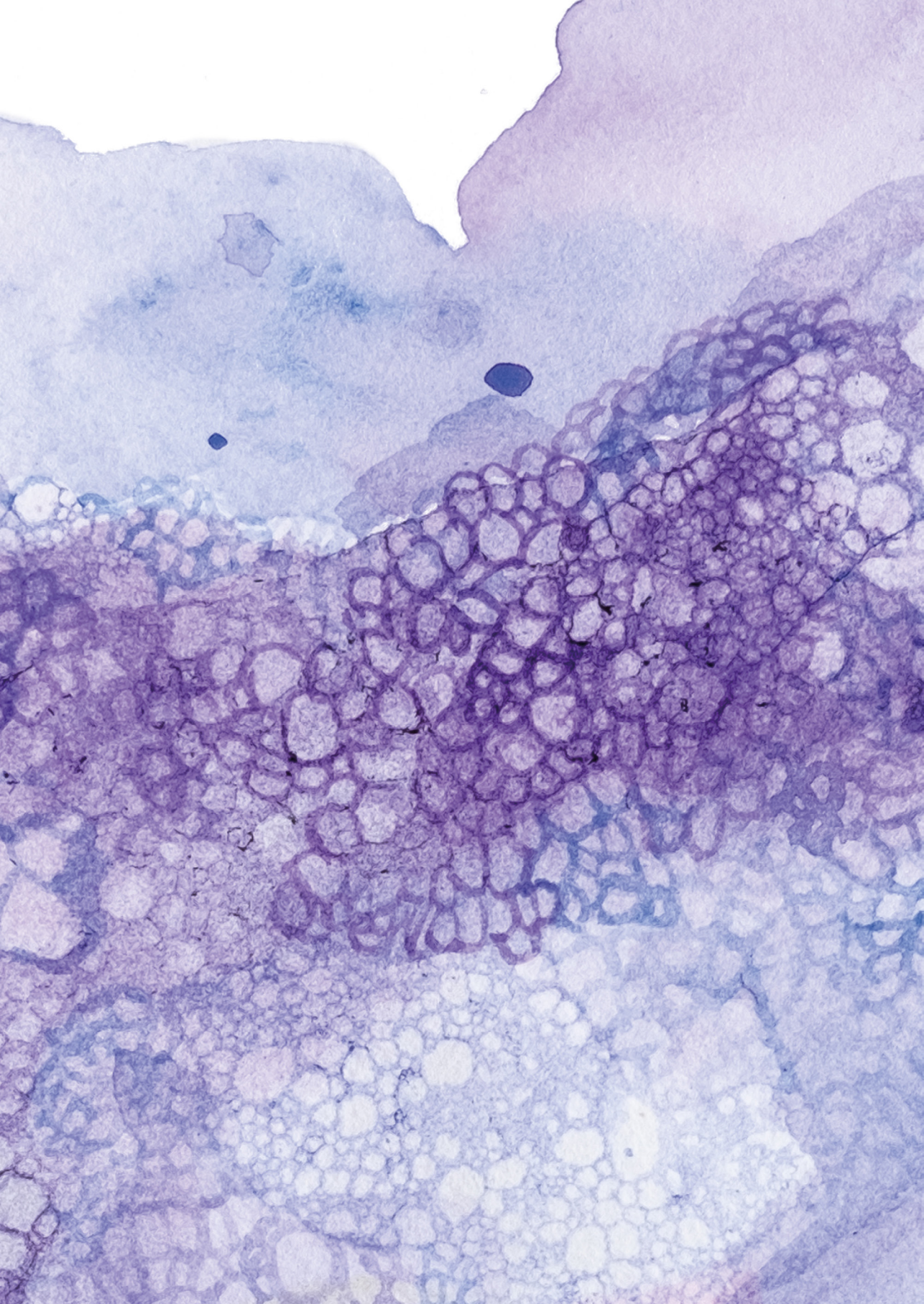


Figure S5 A-C. A. DSS for p53-abn and p53-wt with N1 and N0. B. DSS for LICAM+ and L1CAM- with N1 and N0. C. DSS for ER/PR+ and ER/PR- with N1 and N0

Abbreviations: ER/PR, estrogen receptor/progesterone receptor; L1CAM, L1 cell-adhesion molecule; N1, lymph node metastasis ; N0, no lymph node metastasis; p53abn, p53-abnormal; p53wt, p53-wildtype; DSS, disease-specific survival.



CHAPTER 6

HORMONAL BIOMARKERS REMAIN PROGNOSTICALLY RELEVANT WITHIN THE MOLECULAR SUBGROUPS IN ENDOMETRIAL CANCER

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Submitted

ABSTRACT

Objective

The prognostic relevance of hormonal biomarkers in endometrial cancer (EC) has been well-established. A refined three-tiered risk model for estrogen receptor (ER)/progesterone receptor (PR) expression was shown to improve prognostication. This has not been evaluated in relation to the molecular subgroups. This study aimed to evaluate the ER/PR expression within the molecular subgroups in EC.

Methods

A retrospective multicenter cohort study was performed and data from the European Network for Individualized Treatment centers and Vancouver, Canada were used. ER/PR immunohistochemical expression was grouped as: ER/PR 0-10%, 20-80% or 90-100%. Molecular subgroups were determined with full next-generation sequencing or combined with immunohistochemistry: *POLE*mut, mismatch repair deficient (MMRd), p53mut and no-specific molecular profile (NSMP).

Results

A total of 739 patients were included (median follow-up 5.0 years). Tumors were classified as *POLE*mut in 9.1% (*N*=67), MMRd in 27.6% (*N*=204), p53mut in 20.8% (*N*=154) and NSMP in 42.5% (*N*=314). Among all molecular subgroups, patients with ER/PR 90-100% expression revealed the best disease-specific survival (DSS). Within p53mut, PR 90-100% expression showed a 5-year DSS of 100.0%. ER expression is prognostic more relevant in MMRd and NSMP tumors while PR expression in p53mut and NSMP tumors. Across all molecular subgroups, PR 0-10%, p53mut, lympho-vascular space invasion and FIGO stage III-IV remained independent prognostic for reduced DSS Whereas PR 90-100% and *POLE*mut remained independent prognostic for improved DSS.

Conclusion

We demonstrated that ER/PR expression remain prognostically relevant within the molecular subgroups, and that a three-tiered cutoff refines prognostication. These data support incorporating routine evaluation of ER/PR expression in clinical practice.

INTRODUCTION

Historically, endometrial cancer (EC) was divided into two histopathological subtypes.[1] Type 1 EC includes low-grade (grade 1 and 2) endometrioid EC (EEC), represents the majority (80%) of patients, and is associated with obesity and good prognosis. Type 1 EC is considered to be hormone driven with high expression of estrogen (ER) and progesterone receptors (PR).[2] Type 2 EC represents high-grade tumors (grade 3 EEC and non-endometrioid EC (NEEC)), generally have low ER expression and an unfavorable prognosis.[1, 3] Despite the overall good prognosis of type 1 EC, mortality in absolute numbers is higher in type 1 compared to type 2 EC.[4]

Hormone receptor expression (ER/PR) are prognostic biomarkers that predict lymph node metastasis (LNM) and outcome.[5-7] The current used cutoff for ER/PR expression within EC is adopted from breast cancer studies, and most frequently considered positive if >1% or >10% expression.[8, 9] In an earlier study we evaluated different cutoff values for ER/PR expression using the subgroups 0-10% with unfavorable outcome, 20-80% with intermediate outcome and 90-100% with favorable outcome. This revised three-tiered risk classification model was shown to improve prognostication over the mostly used cutoff of 10%.[10]

The Cancer Genome Atlas (TCGA) classified patients with EC into four important prognostic subgroups based on their genomic molecular signature: I) ultramutated tumors with polymerase epsilon (*POLE*) mutations, II) hypermutated tumors with microsatellite instability (MSI) , III) copy-number-high (CNH) tumors with frequent tumor protein (*TP53*) mutations, IV) copy-number-low (CNL) tumors (also known as no-specific molecular profile (NSMP)).[11] The Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) is a surrogate diagnostic algorithm using low cost clinically applicable immunohistochemistry (IHC); mismatch repair deficient (MMRd) instead of MSI and p53 instead of *TP53*.[12, 13]

The histopathological subtypes (type 1 and 2) are present within all molecular subgroups. Type 1 (EEC histology) is mainly represented by the *POLE*mut, MMRd and NSMP subgroup, with positive ER/PR expression. Type 2 (NEEC histology) is mainly represented by the p53mut subgroup, with generally negative ER/PR expression.[11]

In this era of molecular profiling, the relevance of hormonal biomarkers needs to be redefined. Earlier study demonstrated that ER status was still important for the outcome of EC patients regardless of risk class and p53 or MMR status.[14] Within the NSMP subgroup loss of ER and/or PR expression (<1% and <10%) was shown to be an important prognosticators for EC, but this was not found in the other molecular subgroups.[15-17] So far, it has not been investigated whether the previously mentioned three-tiered ER/PR risk model[10], has prognostic impact in the different molecular subgroups. Therefore, we studied the prognostic relevance of the

three-tiered ER/PR classification within the molecular subgroups in EC. It is hypothesized that this three-tiered model refines prognostication within all molecular subgroups.

MATERIALS AND METHODS

A retrospective multicenter cohort study has been performed. Data was used from the European Network for Individualized Treatment (ENITEC) centers and Vancouver, Canada. Data from four previously published and one unpublished cohort were collected, resulting in 978 patients (flowchart *Supplementary Figure S1*).[10, 12, 13, 18, 19] Patients were treated between 1994-2019 (median 2007) and data on clinicopathological characteristics and outcome were collected.

Inclusion criteria were: (I) availability of ER/PR immunohistochemistry, (II) patients successfully classified with either full next-generation sequencing (NGS) or NGS combined with IHC according to ProMisE[12]. An exclusion criteria was: missing follow-up. Patients were aligned according to the diagnostic algorithm in **Figure 1** and final classified according to the World Health organization (WHO) classification of Female Genital tumors[20]; *POLE*mut, MMRd, p53mut and NSMP.

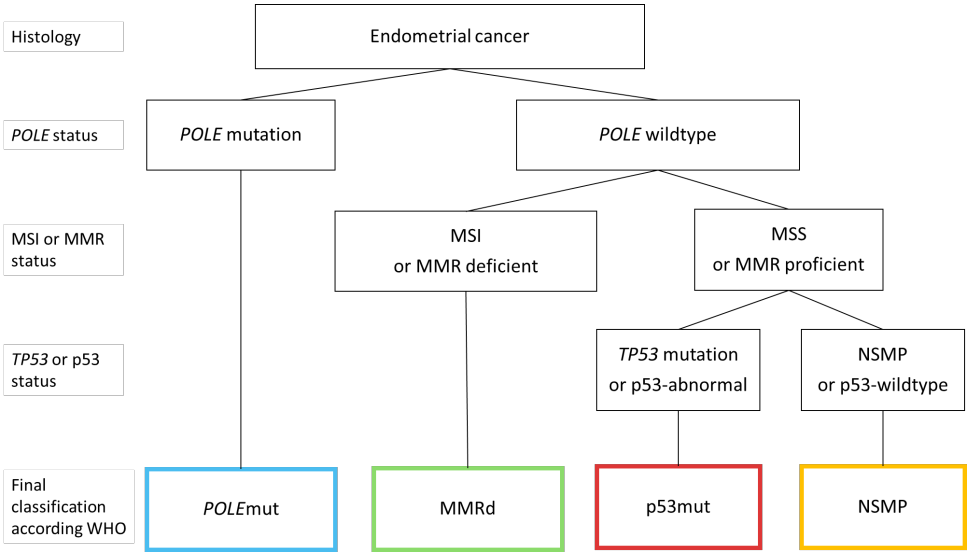

Figure 1. Diagnostic algorithm of patients diagnosed with full next-generation sequencing or combined with immunohistochemistry, and the final classification according to the World Health organization (WHO) classification of female genital tumors.

Abbreviations: *POLE,* Polymerase epsilon; MSI, Microsatellite instability; MMRd, Mismatch repair deficient; *TP53,* Tumor protein 53; p53mut, p53-mutant; NSMP, No-specific molecular profile

DNA analysis

The molecular subgroups included in this study were determined by either full NGS or according to ProMisE. Both methods have been described previously[13, 21] and details are provided for the different cohorts in the *Supplementary Method S1*. Briefly, for molecular profiling by full NGS, DNA was isolated from formalin-fixed paraffin-embedded (FFPE) tumor blocks. Next, DNA was sequenced by NGS with single-molecule Molecular Inversion Probes (smMIPs).[22] For the detection of MSI, 55 MSI markers were tested according to the previously published design.[23] Multiple-classifiers were classified as the molecular subgroup with the best prognosis.[24] For the molecular subgroups determined according ProMisE criteria, *POLE*mut analysis was performed by MiSeq, Sanger or NGS.[13, 19]

Immunohistochemical analysis

IHC was performed on 4 um FFPE tumor sections for the ENITEC centers and tissue microarrays (TMA) for Vancouver cohort, as described previously and detailed in the *Supplementary method S1*.[10, 13, 17, 19] In brief, antibodies specific to MSH6, PMS2, p53, ER and PR were used. Staining for p53 was considered abnormal when more than 80% of tumor cell nuclei showed strong expression (*overexpression*) or when there was complete loss of nuclear staining (*null-expression*) with a positive internal control. Mismatch repair deficiency (MMRd) was defined as complete absence of nuclear staining of PMS2 and/or MSH6, in the presence of a positive internal control. For the TMAs, staining for individual MMR proteins and ER/PR was repeated on whole sections whenever there was equivocal, uninterpretable, or aberrant staining. ER and PR expression was determined by estimating the percentage of positive nuclei in the whole invasive tumor area by 'eyeballing'. Scoring for ER and PR expression within the included cohorts was performed by two assessors (pathologists and researchers, who were trained by an expert gynecologic-pathologist), reviewing discrepancies in a consensus meeting.[10, 19] The ER/PR risk groups were defined as: ER/PR 0-10% as high-risk, ERPR 20-80% as intermediate risk and 90-100% as low risk.[10] Percentages were scored by the pathologist as 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. Some percentages were scored in between, e.g. 85%, these were rounded off into the nearest category (so 85% was categorized as 90%). When ER and PR were taken together, the subgroups were defined as ER+PR 0-10%, ER+PR 20-80%, ER+PR 90-100% and discordant. Patients were grouped 'discordant' if the ER and PR percentages were not aligned in the same risk group (e.g. ER 10% and PR 90%).

Primary objective

To study the prognostic relevance of the three-tiered ER/PR risk classification within the molecular subgroups in EC.

Statistical analysis

The molecular subgroups were compared with the dichotomous clinicopathological characteristics using the χ^2 or Fisher's exact test for categorical data, and the non-parametric Mann-Whitney U-test for continuous variables. Survival analyses were performed using Kaplan-Meier curves and univariable and multivariable Cox-regression analysis. Associations are shown as hazard ratio (HR), 95% CI and *P*-value. The including covariates in multivariable analysis are the main known prognostic biomarkers in EC. Myometrial invasion (MI) was excluded because this is already included in FIGO stage and grade was excluded because this is represented by ER/PR expression. Disease-specific survival (DSS) was defined as time from date of diagnosis to date of death by EC all censored by date of last contact. The results were considered significant with *P*-value less than 0.05. Statistical Package for the Social Sciences, version 27.0 (IBM, New York, NY, USA) was used for statistical analyses.

RESULTS

In total, 978 patients with known and classifiable ER/PR IHC status were available for molecular analysis. Only patients with a successful molecular analysis were included, resulting in 747 EC patients. In which 8 patients were excluded due to missing follow-up, leading to a total of 739 patients included in this study (Flowchart *Supplementary Figure S1*). A baseline overview of each included cohort is shown in *Supplementary Table 1*. The baseline characteristics of the entire cohort are shown in **Table 1**. Median age was 65.0 (31.0-93.0) years, median BMI 29.0 (15.8-66.2) kg/m^2 and median follow-up 60.0 (1.0-283.0) months. The majority of the patients revealed EEC histology 80.4% (*N*=594), grade 1-2 EEC 53.5% (*N*=394) and FIGO stage I-II 75.5% (*N*=558). A minority of patients was diagnosed with ER+PR expression 0-10% or 90-100% (respectively, 16.8% (*N*=124) and 17.1% (*N*=126)). A total of 251 patients (34.0%) was not aligned to one of the three risk groups and classified as 'discordant'. Most discordant cases are located in patients with ER 20-80% + PR 0-10% expression (13.4%), and PR 20-80% + ER 90-100% expression (12.3%) (*data not shown*).

Tumors were classified as *POLE*mut in 9.1% (*N* =67), MMRd in 27.6% (*N* =204), p53mut in 20.8% (*N* =154), and NSMP in 42.5% (*N* =314), in line with the original TCGA paper[11]. The majority of patients within the *POLE*mut, MMRd and NSMP subgroups had EEC histology (respectively, 88.1%, 91.7%, 92.7%), whereas the majority of patients within the p53mut subgroup had NEEC histology (63.0%).

Table 1. Baseline

		Total *N* = 739	*POLE*mut *N* = 67 (9.1)	MMRd *N* = 204 (27.6)	p53mut *N* = 154 (20.8)	NSMP *N* = 314 (42.5)	*P*
Patient characteristics							
Age (years)		65.0 (31.0-93.0)	57.3 (34.0-93.3)	65.1 (42.0-87.0)	72.6 (35.0-93.0)	62.9 (35.0-88.0)	<0.001*
BMI (kg/m^2)		29.0 (15.8-66.2)	27.6 (18.4-58.3)	29.1 (15.8-62.0)	28.4 (17.5-46.2)	29.6 (15.8-66.2)	0.142
Postoperative histology							
Histology	EEC	594 (80.4)	59 (88.1)	187 (91.7)	57 (37.0)	291 (92.7)	<0.001*
	NEEC	145 (19.6)	8 (11.9)	17 (8.3)	97 (63.0)	23 (7.3)	
Grade	1-2	394 (53.3)	30 (44.8)	102 (50.0)	22 (14.3)	240 (76.4)	<0.001*
	3	345 (46.7)	37 (55.2)	102 (50.0)	132 (85.7)	74 (23.6)	
MI[a]	<50%	400 (54.1)	33 (50.0)	107 (52.5)	75 (50.0)	185 (59.3)	0.172
	>50%	332 (44.9)	33 (50.0)	97 (47.5)	75 (50.0)	127 (40.7)	
LVSI	No	474 (64.1)	37 (55.2)	115 (56.4)	78 (50.6)	244 (77.7)	<0.001*
	Yes	265 (35.9)	30 (44.8)	89 (43.6)	76 (49.4)	70 (22.3)	
Lymph nodes	N0	231 (31.3)	31 (46.3)	61 (29.9)	38 (24.7)	101 (32.2)	0.170
	N1	54 (7.3)	3 (3.0)	13 (6.4)	15 (9.7)	20 (6.3)	
	Nx	454 (61.4)	34 (50.7)	129 (63.2)	101 (65.6)	190 (39.5)	
FIGO stage[a]	Early	558 (75.5)	60 (90.9)	154 (75.9)	87 (56.5)	257 (82.6)	<0.001*
	Advanced	176 (23.8)	6 (9.1)	49 (24.1)	67 (43.5)	54 (17.4)	
Hormonal receptor expression							
ER	0-10	142 (19.2)	16 (23.9)	39 (19.5)	55 (36.2)	32 (10.2)	<0.001*
	20-80	354 (47.9)	36 (53.7)	98 (49.0)	74 (48.7)	146 (46.6)	
	90-100	236 (31.9)	15 (22.4)	63 (31.5)	23 (15.1)	135 (43.1)	
PR	0-10	241 (32.6)	21 (31.3)	60 (29.7)	101 (66.9)	59 (18.8)	<0.001*
	20-80	344 (46.5)	39 (58.2)	108 (53.5)	38 (25.2)	159 (50.8)	
	90-100	148 (20.0)	7 (10.4)	34 (16.8)	12 (7.9)	95 (30.4)	

Table 1. *Continued*

ER+PR	0-10	124 (16.8)	13 (19.4)	30 (14.7)	54 (35.1)	27 (8.6)	<0.001*
	20-80	238 (32.2)	29 (43.3)	69 (33.8)	29 (18.8)	111 (35.4)	
	90-100	126 (17.1)	6 (9.0)	29 (14.2)	10 (6.5)	81 (25.8)	
	Discordant	251 (34.0)	19 (28.4)	76 (37.3)	61 (39.6)	95 (30.3)	
Adjuvant treatment[a]							
None		287 (38.8)	24 (36.4)	75 (36.9)	46 (30.1)	142 (45.7)	<0.001*
Radiotherapy		247 (33.4)	25 (37.9)	73 (36.0)	35 (22.9)	114 (36.7)	
Chemotherapy		77 (10.4)	8 (12.1)	19 (9.4)	34 (22.2)	16 (5.1)	
Chemoradiation		122 (16.5)	9 (13.6)	36 (17.7)	38 (24.8)	39 (12.5)	
Outcome							
Recurrence		198 (26.8)	4 (6.1)	58 (29.1)	72 (51.4)	64 (20.5)	<0.001*
Mortality		216 (29.2)	8 (11.9)	57 (27.9)	83 (53.9)	68 (21.7)	<0.001*
EC-related mortality		152 (20.6)	1 (1.5)	38 (19.4)	67 (45.3)	46 (14.9)	<0.001*

Data is presented in number (%), median (IQR).

Abbreviations: *N*, number; *POLE*mut, polymerase epsilon mutant; MMRd, mismatch repair deficient; p53, protein 53; NSMP, No specific molecular profile; EEC, endometrioid endometrial cancer; NEEC, non-endometrioid endometrial cancer; LVSI, lympho-vascular space invasion; N0, negative lymph nodes, N1, positive lymph nodes; Nx, no information about the lymph nodes; FIGO, International Federation of Gynecology and Obstetrics, ER, estrogen receptor; PR, progesterone receptor; EC, endometrial cancer

[a] Missing data for 7 cases MI, 5 cases for FIGO stage, 6 for adjuvant treatment

*$P<0.05$

Outcome ER or PR expression

Figure 2A-E shows the 5-year DSS curve of the three-tiered ER risk classification within the entire cohort and the molecular subgroups. In the entire cohort, patients with ER 90-100% expression showed a significantly better DSS when compared to ER 20-80% (P<0.001) and ER 0-10% (P<0.001). Patients with ER 20-80% had a significant higher 5-year DSS compared to ER 0-10% (P<0.001) (**Figure 2A**). Across all molecular subgroups, patients with ER 90-100% expression showed the most favorable 5-year DSS (**Figure 2B-E**). Within *POLE*mut EC, patients with ER 90-100%, 20-80% and 0-10% revealed no significantly different 5-year DSS (respectively, 100.0%, 100.0% and 92.0%) For MMRd tumors, patients with ER 90-100% and 20-80% or 0-10% revealed significantly different 5-year DSS (respectively, 96.0% vs 80.0% P=0.017 and 96.0% vs 71.0% P=0.002) (**Figure 2C**). Within patients with p53mut no significant differences in 5-year DSS were found between the three ER subgroups (**Figure 2D**). Within NSMP tumors, patients with ER 0-10% had a significant worst 5-year DSS of 48.0% compared to ER 90-100% (96.0%, P<0.001) and compared to ER 20-80% (88.0%, P<0.001)). (**Figure 2E**).

Figure 3A-E shows the 5-year DSS curve of the three-tiered PR risk classification within the entire cohort and the molecular subgroups. In the entire cohort, patients with PR 90-100% expression showed a significantly better DSS when compared to PR 20-80% (P=0.003) and PR 0-10% (P<0.001). Patients with ER 20-80% had a significant higher 5-year DSS compared to ER 0-10% (P<0.001) (**Figure 3A**). Across all molecular subgroups, patients with PR 90-100% expression showed the most favorable 5-year DSS and PR 0-10% the worst (**Figure 3B-E**). Within *POLE*mut and MMRd tumors, no significant different 5-year DSS was revealed within the three subgroups of PR expression (**Figure 3B-C**). Patients with p53mut EC and PR 90-100% had a 5-year DSS of 100%, this was significantly different compared to PR 20-80% (62.0%, P=0.032) and PR 0-10% (48.0% P=0.006) (**Figure 3D**). Within NSMP tumors, patients with PR 90-100% had an excellent 5-year DSS of 98.0%, for PR 20-80% the 5-year DSS was 88.0% and PR 0-10% showed the worst 5-year DSS of 56.0%. All were significantly different from each other (**Figure 3E**).

Across all molecular subgroups, PR 0-10%, p53mut, lympho-vascular space invasion (LVSI) and FIGO stage III-IV remained independent prognostic for reduced DSS. Whereas PR 90-100% and *POLE*mut remained independent prognostic for improved DSS (**Table 2**).

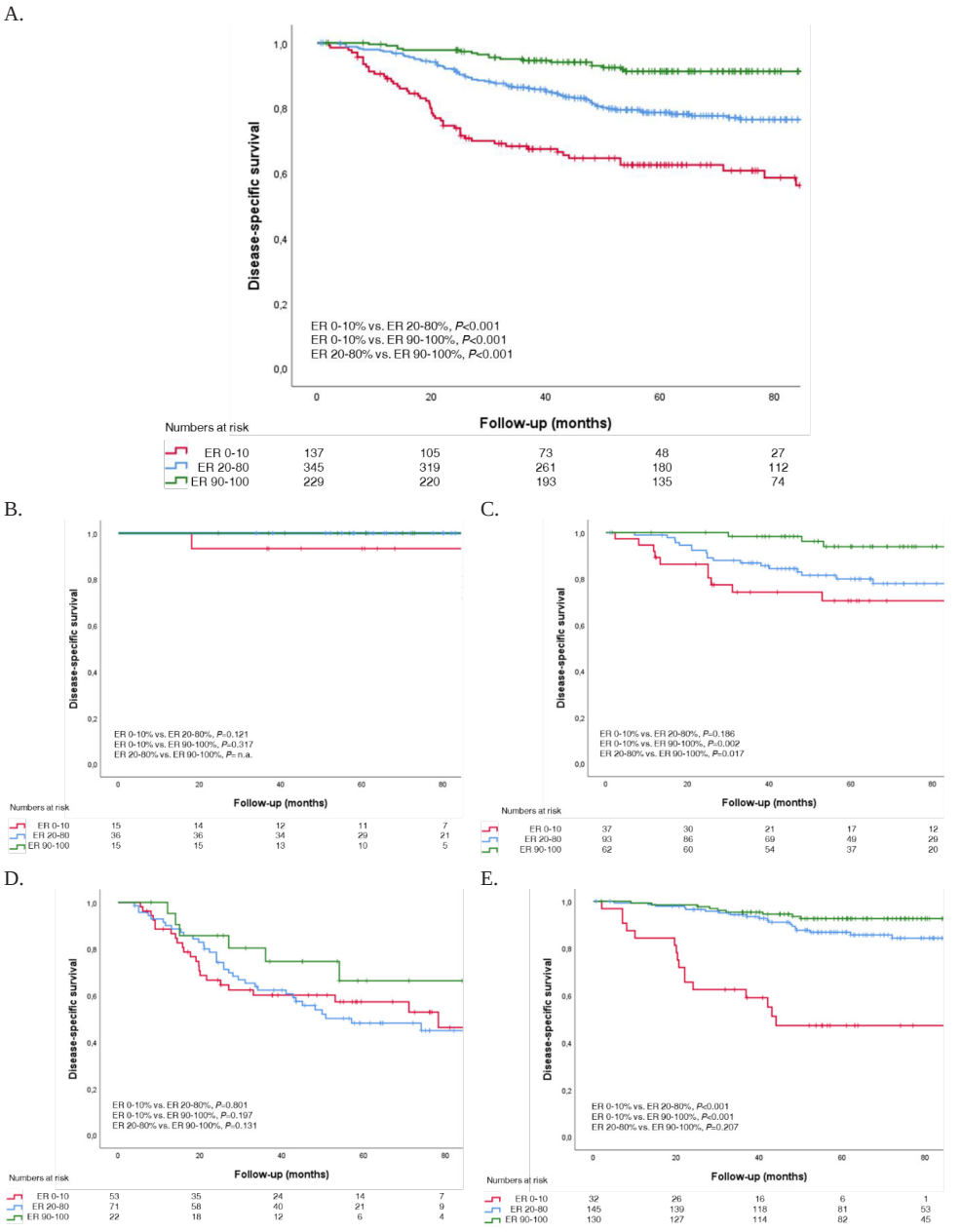

Figure 2 A-E. A. 5-year disease-specific survival (DSS) of the ER three-tiered risk model within the entire cohort. B. 5-year DSS of the ER three-tiered risk model within *POLE*mut patients. C. 5-year DSS of the ER three-tiered risk model within MMRd patients. D. 5-year DSS of the ER three-tiered risk model within p53mut patients. E. 5-year DSS of the ER three-tiered risk model within NSMP patients.

Abbreviations: ER, estrogen receptor; *POLE*, Polymerase epsilon; MMRd, Mismatch repair deficient; p53mut, p53-mutant; NSMP, No-specific molecular profile

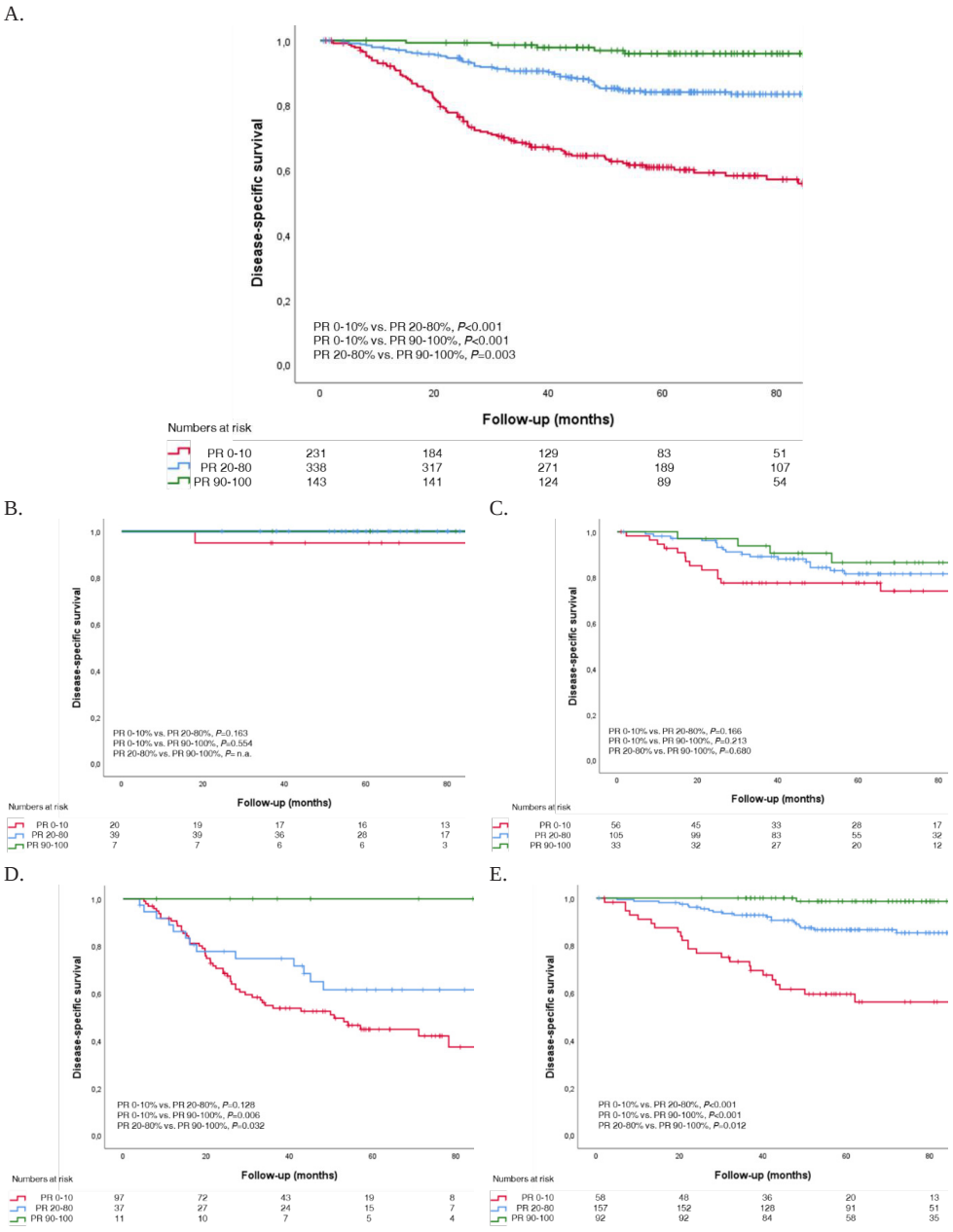

Figure 3 A-E. A. 5-year disease-specific survival (DSS) of the PR three-tiered risk model within the entire cohort. B. 5-year DSS of the PR three-tiered risk model within *POLE*mut patients. C. 5-year DSS of the PR three-tiered risk model within MMRd patients. D. 5-year DSS of the PR three-tiered risk model within p53mut patients. E. 5-year DSS of the PR three-tiered risk model within NSMP patients.

Abbreviations: PR, progesterone receptor; *POLE*, Polymerase epsilon; MMRd, Mismatch repair deficient; p53mut, p53-mutant; NSMP, No-specific molecular profile

Table 2. Cox regression univariable and multivariable analysis disease-specific survival (DSS)

Variable	Univariable		Multivariable	
			141 events	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
ER cutoff				
ER 0-10%	2.16 (1.51-3.08)	<0.001	1.20 (0.80-1.79)	0.371
ER 20-80%	1		1	
ER 90-100%	0.41 (0.24-0.69)	<0.001	0.82 (0.48-1.41)	0.487
PR cutoff				
PR 0-10%	3.09 (2.19-4.35)	<0.001	1.71 (1.11-2.62)	0.014*
PR 20-80%	1		1	
PR 90-100%	0.34 (0.16-0.71)	0.005	0.41 (0.17-0.95)	0.039*
Molecular subgroup				
*POLE*mut	0.09 (0.01-0.65)	0.017*	0.06 (0.00-0.50)	0.007*
MMRd	1.37 (0.89-2.10)	0.154	0.79 (0.49-1.26)	0.321
p53mut	4.09 (2.81-5.97)	<0.001*	1.65 (1.07-2.53)	0.022*
NSMP	1		1	
LVSI				
No	1	<0.001*	1	0.003*
Yes	4.14 (2.96-5.78)		1.86 (1.23-2.84)	
FIGO				
Stage I-II	1		1	<0.001*
Stage III-IV	6.17 (4.45-8.55)	<0.001*	2.83 (1.89-4.20)	

Abbreviations: DSS, disease-specific survival; EC, endometrial cancer; HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PR., progesterone receptor; *POLE*mut, polymerase epsilon mutant; MMRd, mismatch repair deficient; p53, protein 53; NSMP, No specific molecular profile; LVSI, lympho-vascular space invasion; FIGO, Federation International of Gynecology and Obstetrics.

** P<0.05*

Outcome ER+PR expression combined

Supplementary Figure S2A-E shows the 5-year DSS curve of the three-tiered ER+PR combined risk classification within the entire cohort and the molecular subgroups. The 5-year DSS was significantly different between the three ER+PR risk classification groups (*Supplementary Figure S2A*). Patients with p53mut EC and ER+PR 90-100% had a 5-year DSS of 100%, and patients with ER+PR 20-80% and 0-10% had comparable outcome as ER+PR 0-10% (respectively, 55.0% and 44.0%). The 5-year DSS between ER+PR 0-10% and 90-100% was significantly different (*Supplementary Figure S2D).* Within NSMP tumors, patients with ER+PR 90-100% had an excellent 5-year DSS of 98.0%, for ER+PR 20-80% the 5-year DSS was 84.0% and ER+PR 0-10% showed the worst 5-year DSS of 43.0%. All were significantly different from each other (*Supplementary Figure S2E*). In the entire cohort

and within *POLE*mut, MMRd and NSMP subgroup, the patients grouped as 'discordant', showed comparable outcomes as patients with ER+PR 20-80% expression, Within p53mut EC the outcome was in line with the outcome of ER+PR 0-10% expression. (*Supplementary Figure S2A-E).*

Across all molecular subgroups and ER+PR risk groups, ER+PR 0-10%, p53mut, lympho-vascular space invasion (LVSI) and FIGO stage III/IV remained independent prognostic factors for reduced DSS. ER+PR 90-100% and *POLE*mut were independent prognostic factors for improved DSS (*Supplementary Table S2).*

DISCUSSION

In this large retrospective multicenter cohort study we confirmed the relevance of using a three-tiered ER/PR risk classification that refined the prognostic relevance across the molecular subgroups. Among all molecular subgroups, patients with ER/PR 90-100% expression revealed the best 5-year DSS. Interestingly, patients with PR 90-100% and with p53mut EC revealed an excellent 5-year DSS. In multivariable analyses, PR 0-10% was an independent prognostic factor for reduced DSS and PR 90-100% an independent prognostic factor for improved DSS. Combining ER+PR, 0-10% ER+PR expression was an independent prognostic factor for reduced DSS, while ER+PR 90-100% for improved DSS.

In EC, numerous studies have already shown the importance of ER and PR expression in relation to predicting LNM and outcome, regardless of risk class.[5-7, 14, 25] However, no uniform cutoff is applied within EC. In an earlier study, we defined a three-tiered risk classification for ER/PR expression to improve prognostication specifically in patients with EC.[10] The current study confirmed the additional value of using this three-tiered risk classification when compared to the commonly used cutoff of 1% or 10%.

The relevance of ER/PR expression within all molecular subgroups was not fully elucidated until this study. Comparable to our data, early studies observed higher PR expression within the NSMP subgroup and low PR expression in p53mut tumors.[11, 17] In addition, our study shows the relevance of hormonal biomarkers within the MMRd, p53mut and NSMP subgroups. Vermij et al. confirmed the significance of ER status within the NSMP high-risk EC. Comparable to our study, patients with ER expression <10% showed the worst outcome compared to ER >10%. Contrary to our findings, they found no prognostic impact of ER in the other molecular subgroups (especially MMRd) which might be explained by their cut-off of 1-10%.[15] Jamieson et al. used ER and tumor grade to subclassify the NSMP subgroup. Low-risk NSMP was identified as low-grade EC and ER >1% with favorable outcome, and

high-risk NSMP as high-grade EC and ER <1% expression with unfavorable outcome.[16] Which confirms the relevance of ER within the NSMP subgroup. Our study revealed also the additional relevance of PR expression within the NSMP and p53mut subgroup, contrary to the other studies which might again by explained by the use of a three-tiered risk classification.[15] Interestingly, patients with p53mut EC and PR 90-100% expression showed an excellent 5-year DSS of 100%, since all these patients had EEC histology, the importance of both morphology and IHC in addition to molecular subgroups within EC is illustrated. Patients within p53mut or NSMP EC and PR 0-10% show the worst outcome. Early studies indicated that PR <10% expression was predominantly present in the 'advanced/metastatic' ESGO risk group and predicting disease recurrence in patients and increased risk of death. This is in line with our findings in multivariable regression analysis, were PR expression 0-10% is more correlated with decreased DSS compared to ER expression 0-10%. Due to the used cutoffs for ER and PR of 1% or 10% the prognostic relevance within the molecular subgroups might have been underestimated when compared with the three-tiered ER/PR risk classification in our study.[15, 17]

In clinical practice generally both ER and PR IHC expression are determined, therefore, understanding the prognostic relevance of both ER/PR expression within the molecular subgroups is interesting. Early studies indicated that both ER/PR provide additional prognostic information, comparable with our study.[5, 7, 10, 17] Combining ER+PR shows ER+PR 0-10% as an independent prognostic marker for reduced DSS and ER+PR 90-100% as an independent prognostic marker for improved DSS. Combining ER+PR expression within the three-tiered risk classification will create a remaining subgroup, in this paper classified as discordant. For clinical practice, when the ER+PR subgroup is discordant in patients with *POLE*mut, MMRd or NSMP EC, the prognosis is in line with an intermediate prognosis. Within p53mut, the prognosis is in line with decreased prognosis (comparable to high risk 0-10% expression).

The strengths of this retrospective study are the large number of included cases from multiple centers, including ER and PR immunohistochemistry and representing all tumor grades and FIGO stages. Second, by including ER/PR expression both and combined these results are highly relevant for clinical practice Furthermore, this is the first study to analyze a three-tried ER/PR risk classification within all molecular subgroups.

Some limitations need to be addressed. First, the mortality rate of *POLE*mut patients is low, possibly hampering interpretation on the impact of ER/PR expression within this specific subgroup. Second, technical allocation of the molecular subgroups differed slightly. However, either full NGS or use of ProMiSe criteria (combination of NGS and IHC) are repeatedly validated as comparable techniques and representative for the daily practice in Europe and Canada.[26, 27] Third, a relative amount of patients were excluded due to unsuccessful molecular

profiling, perhaps as an result of using older archival tumor samples for DNA testing. Fourth, race or ethnicity has not been reported in our study. Although we fully agree that these patients' information might be impact outcome in several diseases[28], within Europe it is not routinely documented in patient files.[29] Fifth, using patients between 1994-2019 could have biased the survival because of different treatment strategies over the time. However, the death caused by EC has not been reduced or increased over the 25 years in our study cohort (*data not shown*), therefore we believed this has not biased our results. Finally, according to the ProMiSe criteria the order of molecular subgroup allocation within the Vancouver cohort is different compare to the original TCGA cohort, in which MMRd testing is followed by *POLE* testing.[11, 13] The distribution of MMRd that also include *POLE*mut varies, patients with *POLE*mut and MMRd have comparable prognosis to *POLE*mut.[30] Therefore a different allocating order could bias the outcome. However, in the original ProMiSe cohort, no MMRd patients are present with also *POLE*mut. Within the cohorts from the ENITEC centers the order of molecular testing was in line with the original TCGA cohort.[10, 11, 18, 19]

This study demonstrates the prognostic importance of ER and PR biomarkers within the era of molecular profiling. Future prospective studies need to focus on response to hormonal treatment within the molecular subgroups. Currently, an international randomized control trial has been started to refine the adjuvant treatment in endometrial cancer based on molecular features (RAINBO trial), in which one arm includes patients with NSMP EC (ClinicalTrials.gov Identifier: NCT05255653). Patients with ER positive expression will receive RT and hormonal treatment. However, only the presence of ER expression is part of the inclusion criteria, and the cutoff for positivity is not specified. Furthermore, in order to increase response to hormonal treatment, a different cutoff for ER and PR might be indicated as suggested by a recent paper in which a cutoff of 50% was suggested.[31]

CONCLUSION

Our study demonstrated the prognostic relevance of ER and PR expression within the molecular subgroups of patients with EC and that the use of a three-tiered risk classification refines prognostication. These data support incorporating routine evaluation of ER/PR expression in clinical practice.

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SUPPLEMENTARY

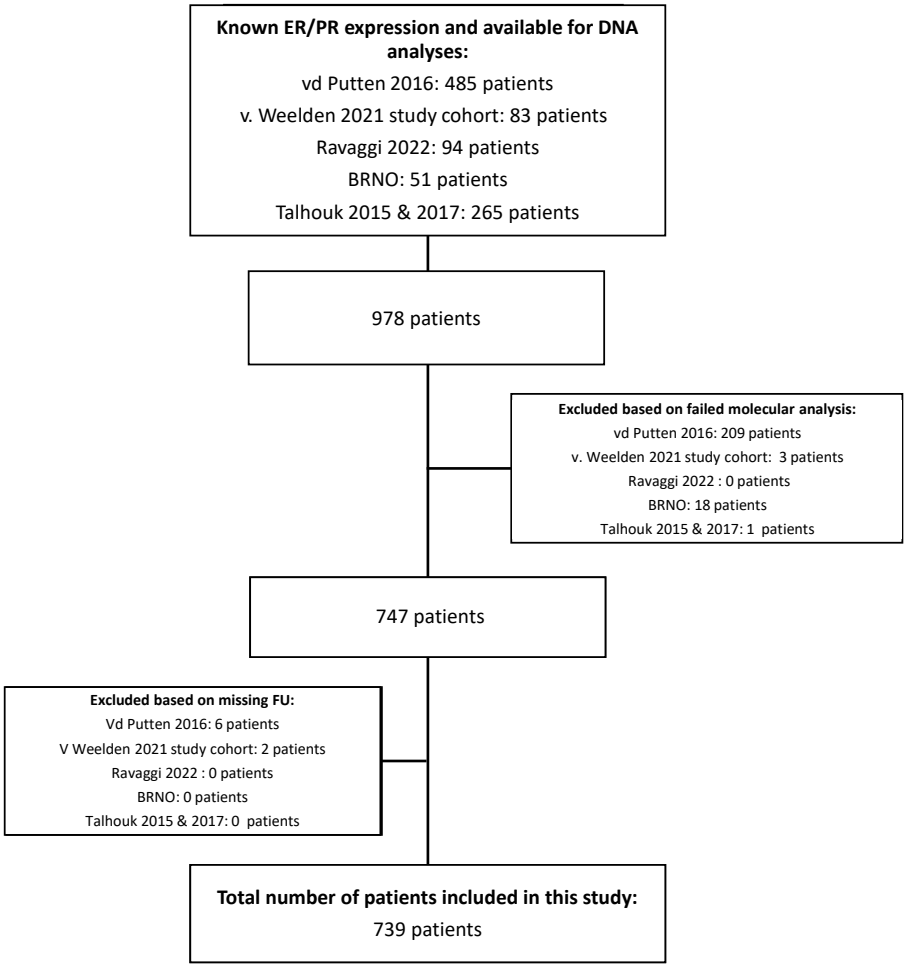


Figure S1. Flowchart of the included patients from the five cohorts.

Table S1. Baseline characteristics of the four previous published cohorts and one unpublished cohort

		ENITEC centers				Vancouver cohort
		Van der Putten et al. 2016	**Van Weelden et al. 2020**	**Ravaggi et al. 2022**	**BRNO**	**Talhouk et al. 2015 and 2017**
Study characteristics						
N included		305	40	94	37	263
Molecular subgroups determined by		Full NGS	Full NGS	ProMisE	ProMisE	ProMisE
DNA sequencing by		smMIP by NGS	smMIP by NGS	NGS	Sanger	MiSeq and Sanger
Order of molecular subgroup allocation according to		TCGA	TCGA	TCGA	TCGA	ProMisE
Median follow-up (months)		71.0 (4.0-198.0)	52.5 (2-123)	53.5 (9.0-217.0)	53.0 (6.0-129.0)	55.2 (1.0-283.0)
Demographics		European	European	European	European	Canadian
Patient characteristic						
Age (years)		63.0 (34.0-93.0)	64.0 (49.0-82.0)	65.0 (31.0-90.0)	71.0 (47.0-85.0)	65.5 (35.0-93.0)
Final pathologic characteristics						
Histology	EEC	282 (92.5)	33 (82.5)	69 (73.4)	29 (78.4)	181 (68.8)
	NEEC	23 (7.5)	7 (17.5)	25 (26.6)	8 (21.6)	82 (31.2)
Grade	1-2	234 (76.7)	27 (67.5)	17 (18.1)	21 (56.8)	95 (36.1)
	3	71 (23.3)	13 (32.5)	77 (81.9)	16 (43.2)	168 (63.9)
ER	0-10	27 (8.9)	7 (17.5)	34 (36.2)	6 (16.2)	68 (25.9)
	20-80	151 (49.5)	23 (57.5)	37 (39.4)	13 (35.1)	130 (49.4)
	90-100	127 (41.6)	10 (25.0)	16 (17.0)	18 (48.6)	65 (24.7)
	Missing	0 (0.0)	0 (0.0)	7 (7.4)	0 (0.0)	0 (0.0)
PR	0-10	48 (15.7)	12 (30.0)	47 (50.0)	9 (24.3)	125 (47.5)
	20-80	164 (53.8)	22 (55.0)	35 (37.2)	12 (32.4)	111 (42.2)
	90-100	91 (29.8)	6 (15.0)	8 (8.5)	16 (43.2)	27 (10.3)
	Missing	2 (0.7)	0 (0.0)	4 (4.3)	0 (0.0)	0 (0.0)

Table S1. Continued

Molecular	*POLE*mut	27 (8.9)	3 (7.5)	15 (16.0)	0 (0.0)	22 (8.4)
	MMRd	65 (21.3)	14 (35.0)	34 (35.2)	14 (37.8)	77 (29.3)
	P53mut	32 (10.5)	11 (27.5)	25 (26.6)	8 (21.6)	78 (29.7)
	NSMP	181 (59.3)	12 (30.0)	20 (21.3)	15 (40.5)	86 (32.7)
MI	<50%	191 (62.6)	26 (65.0)	19 (20.2)	19 (51.4)	145 (55.1)
	>50%	114 (37.4)	14 (35.0)	75 (79.8)	18 (48.6)	111 (42.2)
	Missing	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (2.7)
LVSI	No	52 (17.0)	11 (27.5)	9 (9.6)	3 (8.1)	149 (56.7)
	Yes	253 (83.0)	29 (72.5)	85 (90.4)	34 (91.9)	114 (43.3)
Lymph nodes	N0	164 (53.8)	9 (22.5)	47 (50.0)	11 (29.7)	0 (0.0)
	N1	19 (6.2)	4 (10.0)	31 (33.0)	0 (0.0)	0 (0.0)
	Nx	122 (40.0)	27 (67.5)	16 (17.0)	26 (70.3)	263 (100.0)
FIGO stage	Early (I-II)	270 (88.5)	31 (77.5)	40 (42.6)	37 (100.0)	180 (68.4)
	Advanced (III-IV)	35 (11.5)	9 (22.5)	54 (57.4)	0 (0.0)	78 (29.7)
	Missing	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (1.9)
Adjuvant treatment						
None		122 (40.0)	24 (60.0)	1 (1.1)	18 (48.6)	122 (46.4)
Radiotherapy		153 (50.2)	11 (27.5)	30 (31.9)	19 (51.4)	34 (12.9)
Chemotherapy		10 (3.3)	3 (7.5)	29 (30.9)	0 (0.0)	35 (13.3)
Chemoradiation		20 (6.6)	2 (5.0)	34 (36.2)	0 (0.0)	66 (25.1)
Unknown		0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	6 (2.3)

Table S1. Continued

Mortality					
Recurrence	44 (14.4)	9 (22.5)	51 (54.3)	16 (43.2)	73 (27.8)
Mortality	50 (16.4)	9 (22.5)	48 (51.1)	15 (40.5)	94 (35.7)
EC-related mortality	30 (9.8)	6 (15.0)	42 (44.7)	13 (35.1)	61 (23.2)

Data is presented as No. (%), median (IQR)

Abbreviations: NGS, Next-Generation Sequencing; EEC, endometrioid endometrial cancer; NEEC, non-endometrioid endometrial cancer; ER, estrogen receptor; PR, progesterone receptor; *POLE*mut, polymerase epsilon mutant; MMRd, mismatch repair deficient; p53, protein 53; NSMP, No specific molecular profile; MI, myometrial invasion; LVSI, lymphovascular space invasion; N0, negative lymph nodes, N1, positive lymph nodes; Nx, no information about the lymph nodes; FIGO, Federation International of Gynecology and Obstetrics; EBRT, external beam radition therapy; VBT, vaginal brachytherapy; EC, endometrial cancer; smMIP, single-molecule Molecular Inversion Probes; NGS, next-generation sequenting.

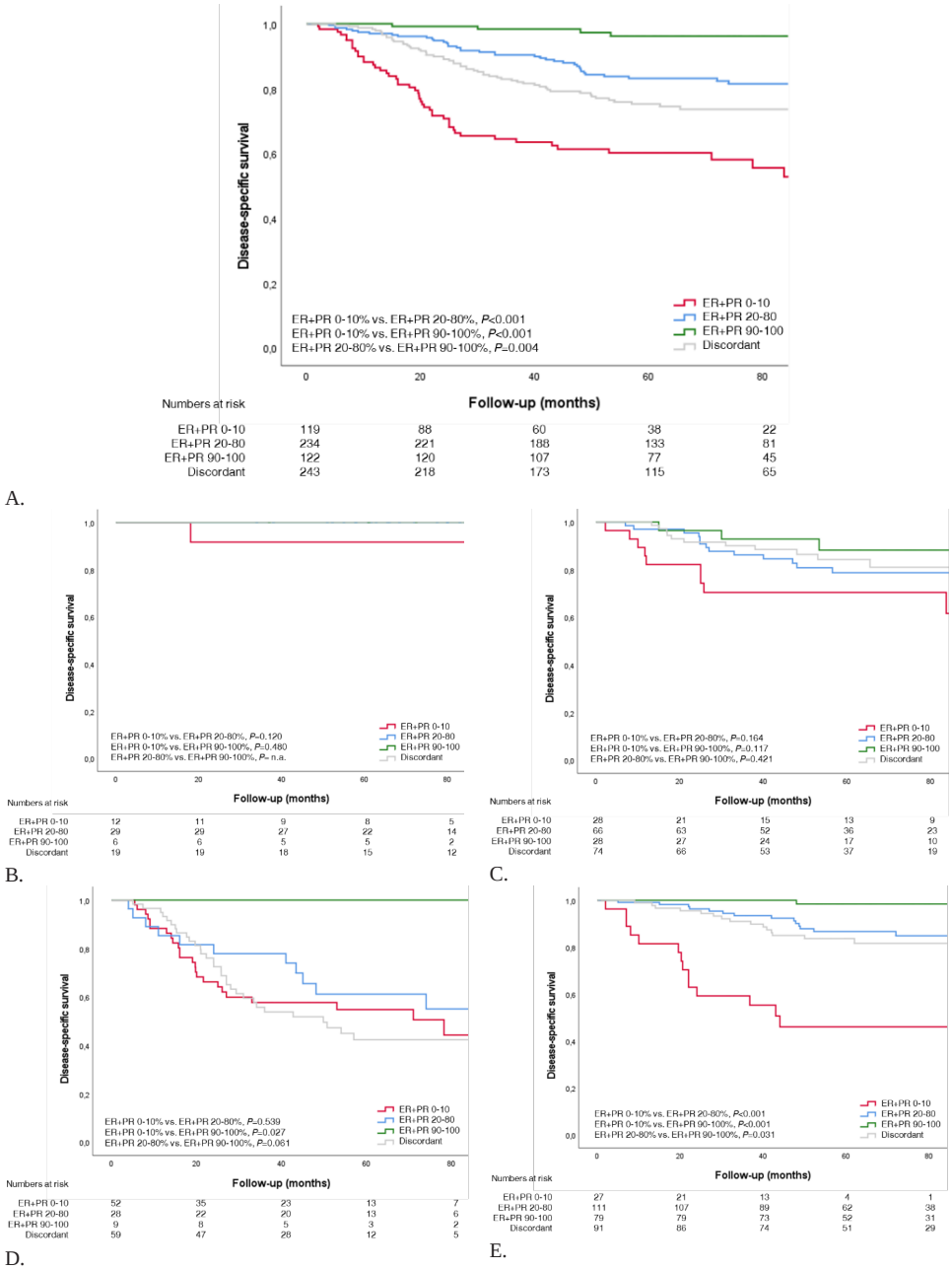


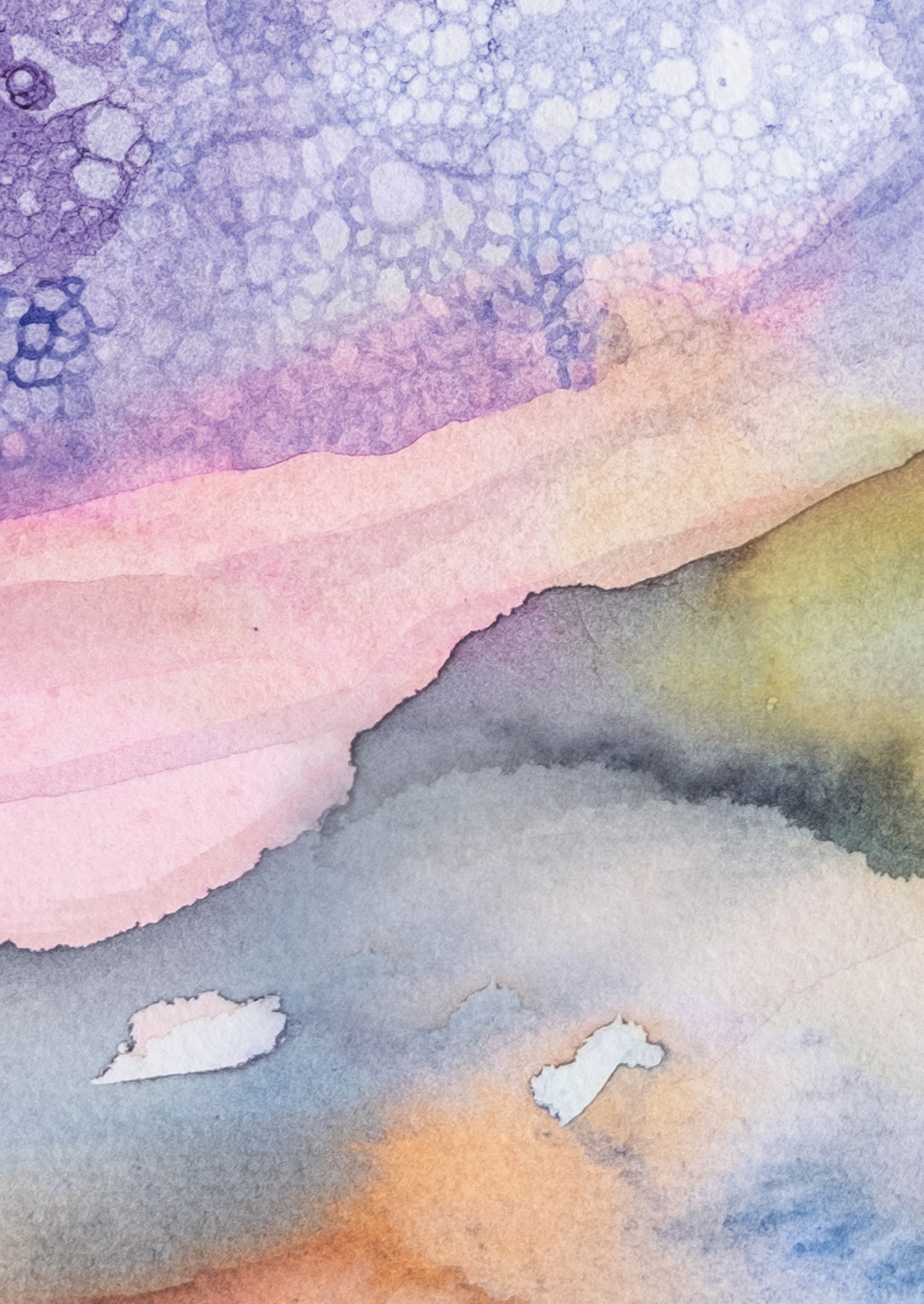
Figure S2 A-E. A. 5-year disease-specific survival (DSS) of the ER+PR three-tiered risk model within the entire cohort. B. 5-year DSS of the ER+PR three-tiered risk model within *POLE*mut patients. C. 5-year DSS of the ER+PR three-tiered risk model within MMRd patients. D. 5-year DSS of the ER three-tiered risk model within p53mut patients. E. 5-year DSS of the ER+PR three-tiered risk model within NSMP patients.

Abbreviations: ER, estrogen receptor; PR, progesterone receptor; *POLE,* Polymerase epsilon; MMRd, Mismatch repair deficient; *TP53,* p53mut, p53-mutant; NSMP, No-specific molecular profile

Table S2. Cox regression univariable and multivariable analysis disease-specific survival (DSS)

Variable	Univariable DSS		Multivariable DSS 150 events	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value
ER+PR cutoff				
ER+PR 0-10%	2.11 (1.43-3.09)	<0.001*	1.54 (1.03-2.28)	0.032*
ER+PR 20-80%	0.67 (0.44-1.00)	0.052	0.91 (0.59-1.38)	0.656
ER+PR 90-100%	0.21 (0.09-0.47)	<0.001*	0.38 (0.17-0.86)	0.020*
Discordant	1		1	
Molecular subgroup				
*POLE*mut	0.09 (0.01-0.65)	0.017*	0.07 (0.00-0.50)	0.008*
MMRd	1.37 (0.89-2.10)	0.154	0.94 (0.60-1.46)	0.780
p53mut	4.09 (2.81-5.97)	<0.001*	2.02 (1.34-3.06)	<0.001*
NSMP	1		1	
LVSI				
No	1	<0.001*	1	<0.001*
Yes	4.14 (2.96-5.78)		2.11 (1.41-3.15)	
FIGO				
Stage I-II	1		1	<0.001*
Stage III-IV	6.17 (4.45-8.55)	<0.001*	2.95 (2.00-4.33)	

Abbreviations: DSS, disease-specific survival; EC, endometrial cancer; HR, hazard ratio; CI, confidence interval; *POLE*mut, polymerase epsilon mutant; MMRd, mismatch repair deficient; p53, protein 53; NSMP, No specific molecular profile; LVSI, lympho-vascular space invasion; FIGO, Federation International of Gynecology and Obstetrics.



CHAPTER 7

ABNORMAL PREOPERATIVE HEAMATOLOGICAL PARAMETERS IN ENDOMETRIAL CANCER; REFLECTING TUMOUR AGGRESSIVENESS OR REDUCED RESPONSE TO RADIOTHERAPY?

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ABSTRACT

Background

In endometrial cancer (EC), preoperative anaemia, thrombocytosis and leucocytosis appear to be associated with worse prognosis. It remains unclear whether these parameters solely reflect tumour aggressiveness, or also impact response to adjuvant treatment. Therefore, our primary aim is to evaluate the prognostic relevance of anaemia, thrombocytosis and leucocytosis on survival in EC. Secondary, to explore their predictive relevance in response to radiotherapy in EC.

Methods

A retrospective multicentre cohort study was performed within 10 hospitals. Preoperative haematological parameters were defined as: Anaemia – haemoglobin <7.45mmol/L(<12 g/Dl), thrombocytosis – platelets >400x10^9 platelets/L, leucocytosis – leukocytes >10x10^9/L. The relationship of haematological parameters with clinicopathological characteristics, ESGO/ESTRO/ESP risk groups and survival were evaluated. Furthermore, the predictive value of abnormal haematological parameters was determined on response to adjuvant radiotherapy and specifically for the ESGO/ESTRO/ESP intermediate-risk group solely receiving radiotherapy.

Results

A total of 894 patients were included with a median follow-up of 4.5 years. Anaemia was present in 103 (11.5%), thrombocytosis in 79 (8.8%) and leucocytosis in 114 (12.7%) patients. The presence of anaemia or thrombocytosis was significantly associated with ESGO/ESTRO/ESP high-risk (respectively, *P*=0.002 and *P*=0.041). In the entire cohort, anaemia remained independently associated with decreased disease-specific survival (HR 2.31, 95% CI (1.19-4.50), *P*=0.013) after adjusting for age, the abnormal haematological parameters and ESGO/ESTRO/ESP risk groups. In patients that were treated with adjuvant radiotherapy (n=239), anaemia was associated with significant reduced 5-year disease-specific and recurrence-free survival (*P*=0.005 and *P*=0.025, respectively). In ESGO/ESTRO/ESP intermediate risk patients that received solely vaginal brachytherapy (n=74), anaemia was associated with reduced disease-specific survival (*P*=0.041).

Conclusion

Current data demonstrate the importance of preoperative anaemia as independent prognostic factor in patients with EC. Moreover, anaemia seems to be associated with reduced response to radiotherapy. Prospective validation in a larger study cohort is needed to verify anaemia as predictive biomarker for radiotherapy.

INTRODUCTION

Endometrial cancer (EC) is the most common gynaecologic malignancy in industrialized countries with incidence rates rising due to aging and obesity. Most patients are diagnosed with low-grade EC (grade 1-2 endometrioid EC), and generally have a favourable prognosis.[1] Around 20% of patients are diagnosed with high-grade EC (grade 3 endometrioid EC and non-endometrioid EC), have an overall poor prognosis and are associated with an increased risk of regional or distant metastases.[1] Currently, primary surgical treatment is based on preoperative tumour grade and histology. Yet, in some patients fertility preservation could be considered based on: (I) grade 1 tumour histology, (II) tumour restricted to the endometrium by imaging, (III) no contra-indications of hormonal treatment, (IV) counselling about not the standard care of EC, including the risks. Patients with high-grade EC or with deep myometrial invasion are not recommended for fertility preservation treatment due to high risk of nodal metastasis.[2, 3] Immunohistochemical or molecular markers could be additional helpful facilitate decision making for fertility-sparing treatment.[2-6]

According to the recent ESGO/ESTRO/ESP (European Society of Gynaecological Oncology – European SocieTy for Radiotherapy and Oncology – European Society of Pathology) guideline, adjuvant treatment is based on risk classification groups incorporating FIGO (Federation International of Gynecology and Obstetrics) stage, tumour grade and histology, lymphovascular space invasion (LVSI) and with or without molecular markers.[7] Often routinely obtained preoperative clinical biomarkers, such as haematological parameters, may contribute to identification of patients with extended disease and/or aggressive tumour behaviour that might respond differently to adjuvant therapy.[8-10]

Endometrial carcinogenesis is characterized by chronic inflammation with elevated pro-inflammatory cytokines and acute phase proteins.[11] Overexpression of inflammatory cytokines could contribute to the development of cancer-related anaemia, thrombocytosis and leucocytosis, and could generate a pro-tumorigenic environment.[12-15] Preoperative abnormal haematological parameters like anaemia, thrombocytosis and/or leucocytosis, have been shown to be associated with FIGO advanced-stage and unfavourable outcome, however results remain conflicting.[13, 14, 16-21]

Several studies showed an adverse impact of anaemia to radiotherapy (RT) response in solid tumours, explained by the fact that anaemia is proposed to be a surrogate maker for tumour hypoxia.[9, 22] Hypoxia is very common in solid tumours and leads to cellular stress response, which allows tumour cells to survive. In addition, these hypoxic conditions may also protect tumour cells from downstream DNA breaks and lethality induced by radiotherapy.[23, 24] Within gynaecological tumours, leucocytosis was also observed to have an adverse predictive impact

on RT response.[10] So far, no studies reported the impact of thrombocytosis on RT in solid tumours.

Based on conflicting results in outcome of abnormal preoperative haematological parameters in EC, we aim to evaluate the prognostic relevance of anaemia, thrombocytosis and leucocytosis on survival. Second, we aim to explore the predictive relevance of these abnormal haematological parameters on response to adjuvant RT. We hypothesize that patients with anaemia, thrombocytosis and/or leucocytosis have reduced survival due to advanced-stage EC, and anaemia might have negative impact on response to adjuvant RT.

MATERIAL AND METHODS

Study cohort

A multicentre cohort study was performed with a combination of prospective and retrospectively collected data in patients diagnosed with EC. This study is a collaboration between the Netherlands and the United Kingdom (UK) by which data of nine hospitals in the Netherlands (PIpelle Prospective ENDOmetrial carcinoma (PIPENDO) cohort[25]) and one in the UK[26] were merged. The design and patient cohort of both cohorts, including 946 patients in total (PIPENDO and UK), have been published previously.[25, 26] A study flowchart is shown in the **Figure 1**. This study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline.

Data collection

All patients were surgically treated between 2006-2015. For the Dutch participating hospitals patient characteristics, postoperative tumour histology, grade and FIGO staging were collected prospectively.[25] Preoperative haemoglobin level, platelet- and leukocyte counts were collected retrospectively from hospital records. For the UK centre, all clinicopathological characteristics and preoperative haematological parameters were collected retrospectively.[26] Regarding to the data collection of nodal status, in the Netherlands and UK surgical staging is selectively performed in patients with preoperative high-grade histology (grade 3 endometrioid EC and non-endometrioid EC) and in case of clinical suspicion of extended disease, according to the Dutch and British EC guideline.[27, 28]

The sole additional inclusion criteria used for this study was that patients were only included if at least one of the three preoperative haematological parameters was conducted ≤ 6 weeks prior to surgery, resulting in 896 patients.

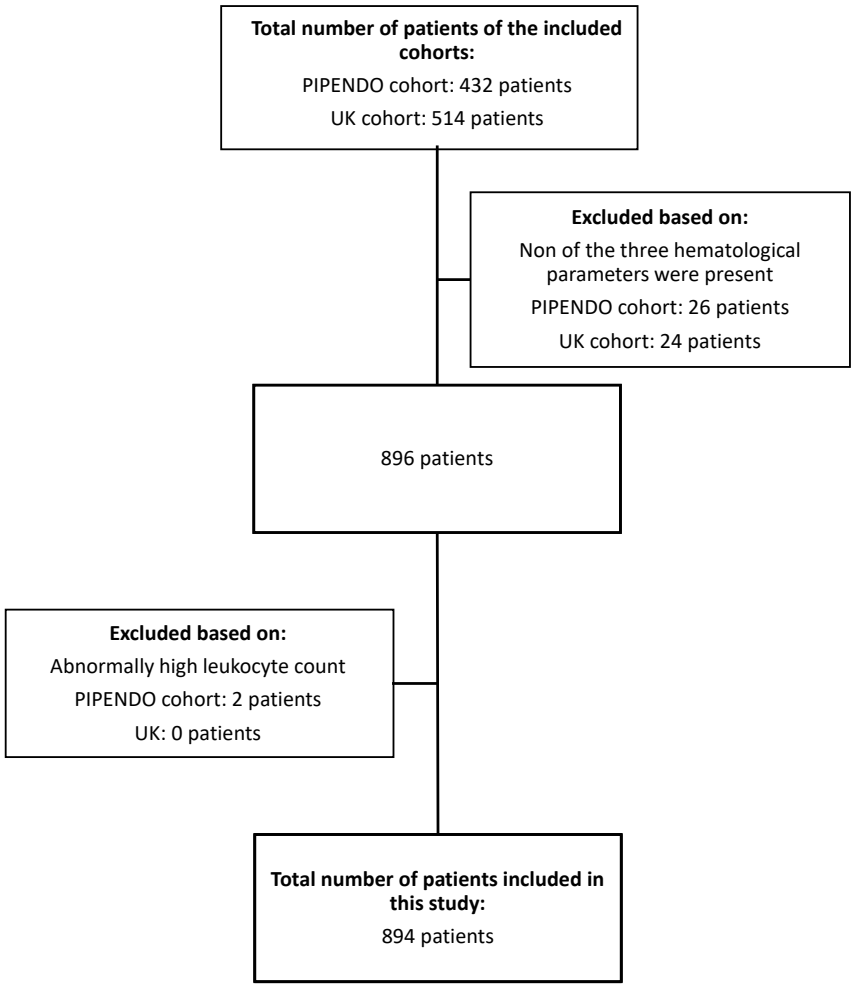


Figure 1. Study flowchart

Statistical analysis

The haematological parameters were analysed as a dichotomous value, with defined cut-offs. Anaemia was defined according to the World Health Organization as haemoglobin level <7.45mmol/L (<12g/Dl)[29], thrombocytosis as platelet counts >400x10^9 /L according multiple studies involving gynaecologic malignancies[13], and leucocytosis as leukocyte counts >10x10^9/L.[15]

The risk classification groups were classified according to the ESGO/ESTRO/ESP guideline; low, intermediate, high-intermediate, high and advanced/metastatic risk group.[7] To explore the response on RT, all patients who received solely adjuvant RT were included for the

second analysis. To further refine response of RT and in order to prevent treatment bias by including patients who were not treated according to the recent guideline, patients classified as ESGO/ESTRO/ESP intermediate risk were only included (flowchart secondary analysis *Figure S1*). According to the guideline, these patients are recommended to receive adjuvant vaginal brachytherapy (VBT)[7], whereas other risk classification groups include observation or combined chemoradiotherapy.

For statistical analyses, Statistical Package for the Social Sciences, version 25.0 (IBM, New York, NY, USA) was applied. The results were considered significant with *P*-value less than 0.05 ($P<0.05$). Clinicopathological characteristics between dichotomous haematological subgroups were compared using the χ^2 or Fisher's exact test for categorical data, and the non-parametric Mann-Whitney U-test for continuous variables. Association between exposure and outcome are shown as odds ratio (OR), 95% Confidence Interval (CI) and *P*-value. Survival analyses were performed using Kaplan-Meier curves and univariable and multivariable Cox-regression. Associations are shown as hazard ratio (HR), 95% CI and *P*-value. Disease-specific survival (DSS) was defined as time from date of diagnosis to date of death by EC and recurrence-free survival (RFS) was defined as time from surgery to time of recurrence from EC disease, all censored by date of last contact. Within the survival analysis, patients with abnormal haematological parameters and an increased or reduced/decreased DSS or RFS were compared to the reference group, patients with normal haematological parameters.

RESULTS

Patients

A total of 896 EC patients were included with a least one haematological parameter. Two patients had abnormally high leukocyte count (>50x10^9/L) due to chronic lymphatic leukaemia and unknown cause, these patients were excluded, resulting in 894 EC patients (54.8% British and 45.2% Dutch) included in this study with a median follow-up of 4.5 years (range 0-10 years) (**Figure 1**). Clinicopathological characteristics of the study cohort are shown in **Table 1**. Median age was 65.9 (27.2-93.8) years and median body mass index 29.7 (16.4-60.9) kg/m^2. Of 653 (73.0%) EC patients all three haematological parameters were available. Median preoperative haemoglobin level was 8.4 mmol/L, median platelet count 298.3x10^9 platelets/L and median leukocyte count 8.1x10^9/L. Anaemia was present in 103 (11.3%), thrombocytosis in 79 (8.6%) and leucocytosis in 114 patients (12.5%). Most patients were diagnosed with low-grade (grade 1-2), FIGO stage I-II and endometrioid EC (respectively, 69.4%, 90.2% and 82.2%). Lymphadenectomy was performed in 205 patients (22.9%) of whom 34 (16.5%) had lymph node metastasis. Adjuvant treatment was administered in 344 patients (38.5%). A total of 239 patients (69.5%) received RT of which 132 patients (55.2%) VBT and 107

patients (44.8%) external beam radiation therapy with or without VBT. Hundred and twenty-four patients (13.9%) developed recurrent EC, and 160 patients (17.9%) were deceased of which 99 (61.8%) deaths were directly related to EC.

Preoperative haemoglobin-, platelet- and leukocyte level in relation to clinicopathological characteristics are shown in **Table 2**. Haemoglobin level was measured in 894 (100.0%), platelet count in 721 (80.6%) and leukocyte count in 667 patients (74.6%). Patients with anaemia were significantly associated with grade 3 EC (OR 1.81, 95% CI 1.18-2.79), LVSI (OR 1.61, 95% CI 1.00-2.57), and ESGO/ESTRO/ESP high risk (OR 2.11, 95% CI 1.30-3.42). The presence of thrombocytosis was significantly associated with LVSI (OR 1.77, 95% CI 1.04-2.99), and ESGO/ESTRO/ESP high risk (OR 1.78, 95% CI 1.02-3.11). Leucocytosis was significantly associated with ESGO/ESTRO/ESP advanced/metastatic risk (OR 2.72, 95% CI 1.06-6.97).

Table 1. Baseline clinicopathological characteristics

Patient characteristics		**Total (n=894)**
Age (years)		65.9 (27.2-93.8)
BMI (kg/m^2)		29.7 (16.4-60.9)
Serum values		
Haemoglobin mmol/L		8.4 (3.9-10.6)
Haemoglobin <7.45mmol/L		103 (11.3)
Platelets x10^9 /L		298.3 (13.9-781.0)
Platelets >400x10^9		79 (8.6)
Leukocytes x10^9/L		8.1 (2.2-33.5)
Leukocytes >10x109/L		114 (12.5)
Final tumour histology		
Tumour Grade	1-2	620 (69.4)
	3	274 (30.6)
Histology	Endometrioid	735 (82.2)
	Non-endometrioid	159 (17.8)
LVSI	Yes	177 (19.8)
	No	717 (80.2)
FIGO stage	Early (I-II)	806 (90.2)
	Advanced (III-IV)	88 (9.8)
Lymph node status	Positive (N1)	34 (3.8)
	Negative (N0)	171 (19.1)
	Unknown† (Nx)	689 (77.1)
ESGO/ESTRO/ESP risk groups	Low	409 (45.7)
	Intermediate	159 (17.8)
	High-intermediate	162 (18.1)
	High	140 (15.7)
	Advanced/metastatic	24 (2.7)
Adjuvant treatment		
None		550 (61.5)
RT	VBT	132 (14.8)
	EBRT (+/- VBT)	107 (11.9)
CT+CRT		100 (11.2)
Other		5 (0.6)
Outcome		
Recurrence	Yes	124 (13.9)
	No	770 (86.1)
Mortality	Overall	160 (17.9)
	EC-related	99 (11.1)

Data is presented in numbers (%) or median (IQR).

Abbreviations: Number (n), Federation International Gynecology Obstetric (FIGO), European Society of Gynaecological Oncology (ESGO), European SocieTy for Radiotherapy and Oncology (ESTRO), European Society of Pathology (ESP), Radiotherapy (RT), Vaginal brachytherapy (VBT), External beam radiation therapy (EBRT), Chemotherapy (CT), Chemoradiation (CRT), Endometrial cancer (EC)

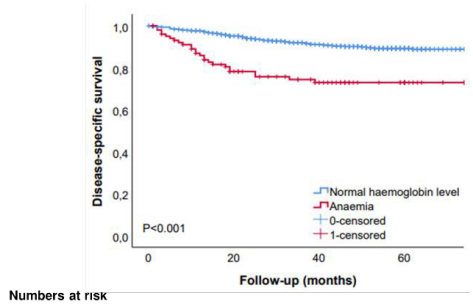
†no lymphadenectomy performed

Table 2. Clinicopathological characteristics in relation to haemoglobin-, leukocytes- and thrombocytosis-level

		Normal haemoglobin (n=791)	Anaemia (n=103)	*P*	Normal platelets (n=642)	Thrombocytosis (n=79)	*P*	Normal leukocytes (n=553)	Leucocytosis (n=114)	*P*
Patient characteristics										
Age		65.7 (27.2-91.0)	68.2 (33.8-93.8)	0.246	66.0 (31.2-93.8)	64.0 (27.2-90.7)	**0.017***	66.0 (31.2-93.8)	65.0 (27.2-86.0)	0.386
Final tumour histology										
Tumour grade	1-2	560 (70.8)	60 (58.3)	**0.009***	448 (69.8)	52 (65.8)	0.471	383 (69.3)	81 (71.1)	0.705
	3	231 (29.2)	43 (41.7)		194 (30.2)	27 (34.2)		170 (30.7)	33 (28.9)	
Endometrioid		656 (82.9)	79 (76.7)	0.120	531 (82.7)	62 (78.5)	0.353	455 (81.7)	94 (82.5)	0.856
Non-endometrioid		135 (17.1)	24 (23.3)		111 (17.3)	17 (21.5)		101 (18.3)	20 (17.5)	
LVSI	Yes	149 (18.8)	28 (27.2)	**0.046***	121 (18.8)	23 (29.1)	**0.031***	109 (19.7)	26 (22.8)	0.454
	No	642 (81.2)	75 (72.8)		521 (81.2)	56 (70.9)		444 (80.3)	88 (77.2)	
ESGO/ESTRO/ESP risk groups										
Low risk		372 (47.0)	37 (35.9)	**0.033***	309 (48.1)	31 (39.2)	0.135	270 (48.8)	53 (46.5)	0.650
Intermediate risk		146 (18.5)	12 (11.7)	0.088	105 (16.4)	7 (8.9)	0.083	77 (13.9)	17 (14.9)	0.782
High-intermediate risk		140 (17.7)	22 (21.4)	0.364	114 (17.8)	17 (21.5)	0.413	101 (18.3)	17 (14.9)	0.393
High risk		114 (14.4)	27 (26.2)	**0.002***	97 (15.1)	19 (24.1)	**0.041***	92 (16.6)	20 (17.5)	0.813
Advanced/metastatic		19 (2.4)	5 (4.9)	0.148	17 (2.6)	5 (6.3)	0.073	13 (2.4)	7 (6.2)	**0.031***
Adjuvant treatment										
None		499 (63.1)	51 (49.5)	**0.009***	412 (64.2)	42 (53.2)	0.066	361 (65.2)	64 (56.1)	0.069
RT	VBT	122 (15.4)	10 (9.7)	0.124	87 (13.6)	3 (3.8)	**0.013***	58 (10.5)	15 (13.2)	0.406
	EBRT +/- VBT	87 (11.0)	20 (19.4)	**0.012***	75 (11.6)	14 (17.7)	0.124	69 (12.5)	17 (14.9)	0.480
CT+CRT		79 (10.0)	21 (20.4)	**0.002***	65 (10.1)	19 (24.0)	**<0.001***	62 (11.3)	17 (15.0)	0.260
Other		4 (0.5)	1 (1.0)	0.459	3 (0.5)	1 (1.3)	0.372	3 (0.5)	1 (0.8)	0.528

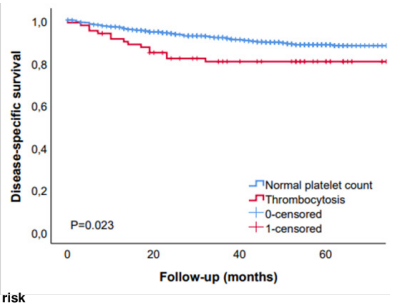
Data is presented in numbers (%), median (range), * $P<0.05$

Abbreviations: number (n), Lymphovascular space invasion (LVSI), European Society of Gynaecological Oncology (ESGO), European SocieTy for Radiotherapy and Oncology (ESTRO), European Society of Pathology (ESP), Radiotherapy (RT), Vaginal brachytherapy (VBT), External beam radiation therapy (EBRT), Chemotherapy (CT), Chemoradiation (CRT)



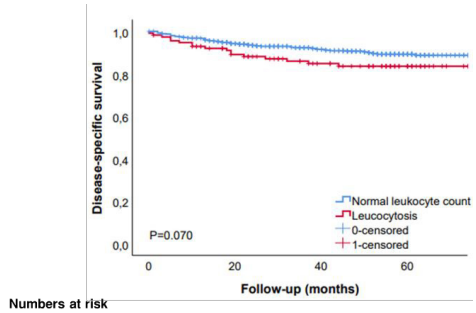
Numbers at risk				
Normal haemoglobin level	791	683	517	277
Anaemia	103	67	48	31

A.



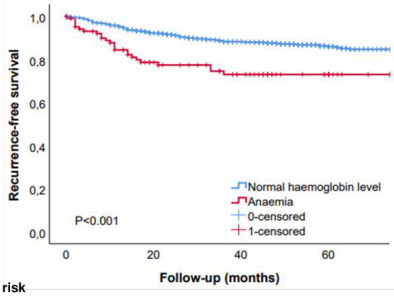
Numbers at risk				
Normal platelet count	642	542	397	224
Thrombocytosis	79	64	51	38

B.



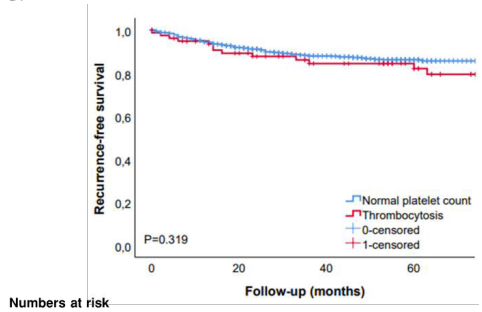
Numbers at risk				
Normal leukocyte count	553	468	341	207
Leucocytosis	114	91	71	45

C.



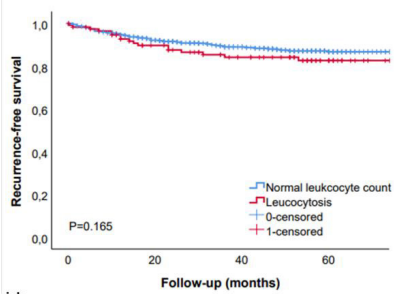
Numbers at risk				
Normal haemoglobin level	791	654	495	269
Anaemia	103	63	45	29

D.



Numbers at risk				
Normal platelet count	642	518	380	217
Thrombocytosis	79	62	48	36

E.



Numbers at risk				
Normal leukocyte count	553	451	327	200
Leucocytosis	114	86	67	42

F.

Figure 2 A-F. 5-year disease-specific survival (DSS) and recurrence-free survival (RFS) of patients with normal and abnormal haematological parameters. A. 5-year DSS of patients with and without anaemia. B. 5-year DSS of patients with and without thrombocytosis. C. 5-year DSS of patients with and without leucocytosis. D. 5-year RFS of patients with and without anaemia. E. 5-year RFS of patients with and without thrombocytosis. F. 5-year RFS of patients with and without leucocytosis.

Prognostic outcome

The 5-year DSS and RFS of preoperative anaemia, thrombocytosis and leucocytosis are shown in **Figure 2A-F**. Patients with anaemia had a significant reduced 5-year DSS and RFS compared to patients with normal haemoglobin level (respectively, $P<0.001$ and $P<0.001$) (**Figure 2A, 2D**). Patients with thrombocytosis showed significant reduced 5-year DSS compared to normal platelet count ($P=0.023$), no difference was found for RFS (**Figure 2B, 2E**). For patients with leucocytosis compared with normal leukocyte count, no significant difference in DSS and RFS was found (**Figure 2C, 2F**).

In multivariable analysis after adjusting for age, the three abnormal haematological parameters and the ESGO/ESTRO/ESP risk groups, only anaemia, age and ESGO/ESTRO/ESP high- and advanced/metastatic risk groups remained independently associated with a reduced DSS. None of the haematological parameters were independently associated with a decreased RFS (**Table 3**).

Predictive outcome

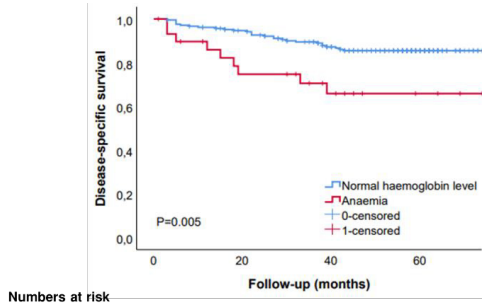
The 5-year DSS and RFS of the preoperative haematological parameters in all patients who received solely adjuvant RT are shown in **Figure 3A-F**. Anaemia was associated with a significant decreased DSS and RFS compared to normal haemoglobin level (respectively, $P=0.005$ and $P=0.025$) (**Figure 3A, 3D**). Thrombocytosis and leucocytosis did not significantly impact the response to RT (**Figure 3B, 3C, 3E, 3F**). The 5-year DSS and RFS of the haematological parameters within patients classified as ESGO/ESTRO/ESP intermediate risk who received solely VBT are shown in *Figure S2A-E***.** Patients with anaemia had a significant decreased DSS compared to normal haemoglobin level ($P=0.041$), this was not significant for the RFS ($P=0.214$). No significant difference in DSS and RFS were found for patients with thrombocytosis or leucocytosis, however numbers were low.

Table 3. Cox regression univariable and multivariable analysis of disease-specific survival (DSS) and recurrence-free survival (RFS)

Variable	Univariable DSS		Multivariable DSS Event 66		Univariable RFS		Multivariable RFS Event 78	
	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Patient characteristics								
Age (continuous)	1.04 (1.02-1.06)	<0.001*	1.03 (1.00-1.06)	0.009*	1.04 (1.01-1.05)	<0.001*	1.03 (1.00-1.05)	0.022*
Haematological parameters								
Anaemia	3.19 (2.02-5.02)	<0.001*	2.31 (1.19-4.50)	0.013*	2.32 (1.49-3.60)	<0.001*	1.71 (0.91-3.19)	0.091
Thrombocytosis	1.90 (1.08-3.34)	0.025*	1.06 (0.49-2.30)	0.872	1.31 (0.73-2.36)	0.363	0.70 (0.32-1.55)	0.382
Leucocytosis	1.65 (0.95-2.86)	0.074	1.37 (0.74-2.55)	0.312	1.45 (0.85-2.44)	0.168	1.53 (0.84-2.74)	0.159
ESGO/ESTRO/ESP risk groups								
Low	1		1		1		1	
Intermediate	7.59 (3.01-19.12)	<0.001*	3.06 (0.98-9.53)	0.053	6.20 (3.06-12.55)	<0.001*	2.38 (0.88-6.39)	0.087
High-intermediate	3.90 (1.38-10.96)	0.010*	1.59 (0.44-5.65)	0.472	5.23 (2.56-10.89)	<0.001*	3.43 (1.44-8.16)	0.005*
High	32.66 (13.99-76.23)	<0.001*	18.3 (7.64-43.94)	<0.001*	22.21 (11.64-42.37)	<0.001*	16.78 (8.05-34.83)	<0.001*
Advanced/metastatic	101.97 (40.33-257.79)	<0.001*	72.1 (27.36-189.97)	<0.001*	36.59 (15.79-84.74)	<0.001*	33.68 (13.15-86.29)	<0.001*

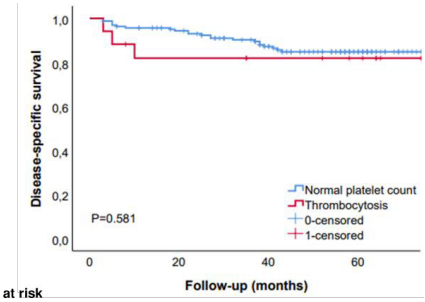
Abbreviations: Disease-specific survival (DSS), Recurrence-free survival (RFS), Hazard ratio (HR), Confidence interval (CI), European Society of Gynaecological Oncology (ESGO), European SocieTy for Radiotherapy and Oncology (ESTRO), European Society of Pathology (ESP),

* $P<0.05$



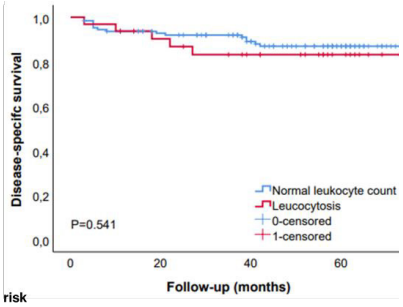
Numbers at risk				
Normal haemoglobin level	209	186	151	65
Anaemia	30	20	14	9

A.



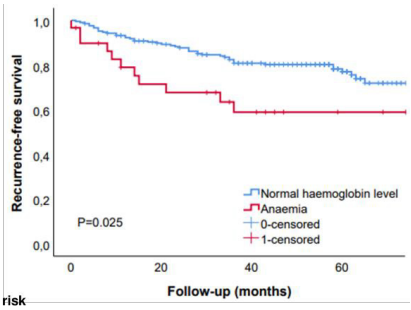
Numbers at risk				
Normal platelet count	162	142	112	55
Thrombocytosis	17	13	11	9

B.



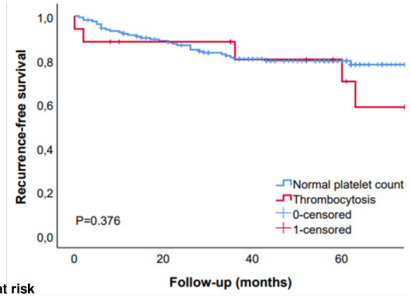
Numbers at risk				
Normal leukocyte count	127	110	89	50
Leucocytosis	32	26	20	11

C.



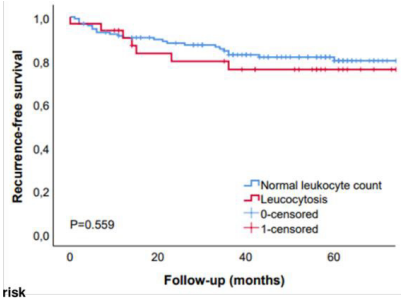
Numbers at risk				
Normal haemoglobin level	209	175	141	63
Anaemia	30	19	13	8

D.



Numbers at risk				
Normal platelet count	162	133	105	54
Thrombocytosis	17	13	10	8

E.



Numbers at risk				
Normal leukocyte count	127	106	84	48
Leucocytosis	32	23	19	10

F.

Figure 3 A-F. 5-year disease-specific survival (DSS) and recurrence-free survival (RFS) of patients with normal and abnormal haematological parameters within patients with solely adjuvant radiotherapy (RT). A. 5-year DSS of patients with and without anaemia in patient with adjuvant RT. B. 5-year DSS of patients with and without thrombocytosis in patients with adjuvant RT. C. 5-year DSS of patients with and without leucocytosis in patients with adjuvant RT. D. 5-year RFS of patients with and without anaemia with adjuvant RT. E. 5-year RFS of patients with and without thrombocytosis with adjuvant RT. F. 5-year RFS of patients with and without leucocytosis with adjuvant RT.

DISCUSSION

In this study, the prognostic and predictive relevance of preoperative abnormal haematological parameters in patients with EC was evaluated. Anaemia was identified as an independent prognostic factor for DSS, along with age and ESGO/ESTRO/ESP 'high- and advanced/metastatic' risk. Furthermore, anaemia seemed an overall predictive factor for response to adjuvant RT, and specifically for patients with ESGO/ESTRO/ESP intermediate risk who received solely VBT.

Although most patients with EC present with postmenopausal bleeding as an early symptom, this rarely causes anaemia at diagnosis. Hence, the development of cancer-related anaemia in EC is more likely caused by inflammatory cytokines which results in a shortened survival of red blood cells, suppression of erythroid progenitor cells, impaired iron utilization, and inadequate erythropoietin (EPO) production.[12, 30] Anaemia in patients with an absolute or relative EPO deficiency seems to be more aggressive in solid tumours.[31] Therefore, it is suggested that preoperative anaemia in EC could be a biomarker of tumour burden and/or aggressive tumour behaviour.[30, 31] In our study cohort we observed that patients with anaemia were significantly more often allocated to ESGO/ESTRO/ESP high risk group, grade 3 EC, and the presence of LVSI. In both univariable and multivariable DSS analysis, we found anaemia as independent prognostic factor. To our knowledge, the presence of anaemia has so far not been related to the ESGO/ESTRO/ESP risk groups. Previous studies did show a significantly higher prevalence of anaemia in patients classified into the ESGO/ESTRO/ESP high risk group; FIGO advanced-stage, grade 3 EC and LVSI.[21] The 5-year RFS was significantly reduced in patients with anaemia compared to those without anaemia. However, anaemia was not an independent prognostic factor for the RFS, comparable to the findings of Wilairat et al. (2012)[32]

Cancer-related anaemia may also cause tumour hypoxia, which may lead to a reduced response to RT.[9, 22-24] Normally, hypoxia will lead to an EPO increase, however due to the cancer-associated inflammation the EPO production is insufficient and the iron metabolism is impaired. VBT is given for local control of the tumour and EBRT could be applied to control locoregional recurrence.[24] In patients within our study who received RT and even with solely VBT within the ESGO/ESTRO/ESP intermediate risk group, anaemia was correlated with a significantly reduced DSS. However, numbers were low and therefore multivariable analysis was not achievable. So far, no other studies including EC patients have been performed to compare our findings.

Three recent meta-analyses published the clinicopathological and/or prognostic significance of preoperative thrombocytosis in EC.[13, 14, 18] In line with our findings, a significant association of thrombocytosis with FIGO advanced-stage, LVSI and grade 2-3 EC was found.[13, 18] The

prognostic relevance, however, still remains conflicting in EC studies, probably due to different used cut-off values for thrombocytosis.[13, 14, 18] Comparable to our study, Njolstad et al. (2013) found a significant reduced DSS of patients with thrombocytosis.[16] However, thrombocytosis as dichotomous value instead of continuous platelet count was not found as independent factor for DSS and RFS.[13] The pathophysiological mechanism between tumour behaviour and preoperative thrombocytosis is not fully elucidated.[18] The overexpression of inflammatory cytokines results in an increase of megakaryocyte maturation which causes increased platelet production.[33] Some hypothesize that platelets infiltrate tumour tissue and contribute to tumour growth by secreting pro-angiogenic factors and pro-tumorigenic factors, while others suggest a platelet-cancer interaction facilitating cancer cell migration, which contributes cancer metastasis.[34]

The impact of leucocytosis on tumour behaviour may also be explained by upregulation of inflammatory cytokines and hematopoietic growth factor through tumour cells, thus promoting enhanced inflammation, leucocytosis, angiogenesis and tumour cell proliferation.[11, 35] We observed a significant association between leucocytosis and the ESGO/ESTRO/ESP advanced/metastatic risk group in our study cohort, however leucocytosis was not significant in univariable and multivariable analysis. A recent meta-analysis found a correlation between leucocytosis and FIGO advanced-stage[20], of whom only one study performed a multivariable analysis for RFS with comparable results as our study.[19]

Due to the pro-angiogenic factors induced with elevated platelet and leukocyte count, its suspected that angiogenesis will lead to a better drug or oxygen access to tumour cells, however there is a lack of homogeneity of vasculature density in different parts of the same tumour which could affect outcome and response to adjuvant treatment.[9] Although we did not observe impact of thrombocytosis and/or leucocytosis on response to RT, included numbers were low. In patients with cervical cancer leucocytosis was related to poor response to RT, but due to differences in carcinogenesis it may be difficult to compare those results with EC.[10]

As shown in this study, some patients are diagnosed with EC during their reproductive years. So far, haematological parameters in solely young women with EC has not been studied. It might be relevant in future studies to evaluate whether these haematological biomarkers could additionally assist in fertility-sparing strategies (including hormonal, surgical and assisted reproductive technologies) in young women with EC.

For hormonal treatment, progestin is recommended as first-line therapy based on the antiproliferative effect of the endometrium. Using hormones in stage IA EEC until completion of childbearing has not been associated with decreased oncologic outcomes compare to women who underwent hysterectomy (97.5% 5-year overall survival).[2, 4]

Molecular or immunohistochemical markers could help predicting response to hormonal treatment.[4, 5] Conservative surgical treatment consists of hysteroscopic resection followed by oral or intra-uterine devise of progestin. Those women achieved the highest complete remission rate compared with other fertility-sparing strategies.[2] Ovarian preservation needs to be considered in the fertility preserving approach, including other assisted reproductive technologies i.e. oocyte/embryo cryopreservation or ovarian tissue cryopreservation. In which closed vitrification systems are, based on the current available evidence, the safest option for cryopreserved cells.[2, 36]

There are some limitations inherent to the retrospective design. First, adjuvant treatment was not uniformly applied which could lead to differences in outcome. Second, due to the fact that most of our labs do not run routine complete blood count, platelet- and leukocyte count were not available for all included patients. Finally, complete molecular data according The Cancer Genome Atlas is not available for the patients in this cohort. However, within a subset of the PIPENDO cohort, we do have immunohistochemistry of p53 and mismatch repair proteins. Within patients with p53-abnormal, anaemia was associated with significant reduced DSS and RFS compared to patients with normal haemoglobin (*data not shown*).

To our knowledge, this is the first study that addressed the relationship of all three, often routinely obtained, preoperative abnormal haematological parameters with clinicopathological characteristics and univariable and multivariable outcome in EC. Other strengths of this study includes its multicentre design resulting in the largest patient cohort to date, and a well-documented and long follow-up period.

Future studies in a prospective study design, may determine the prognostic and/or predictive value of preoperative abnormal haematological markers (more specific anaemia) in addition to the molecular markers in EC. When confirmed, studies should explore in more detail the cause between for example anaemia and impaired prognosis. Furthermore, the value of haematological parameters in young women who are eligible for fertility-sparing strategies needs to be further elucidated.

CONCLUSION

Our data demonstrated the independent prognostic impact of preoperative anaemia in patients with EC. In addition, anaemia seems to be associated as predictive biomarker for response to radiotherapy. It remains unclear whether preoperative anaemia reflects tumour aggressiveness or reduced response to radiotherapy. So, prospective validation in a larger study cohort is needed to verify anaemia as predictive biomarker for radiotherapy.

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SUPPLEMENTARY

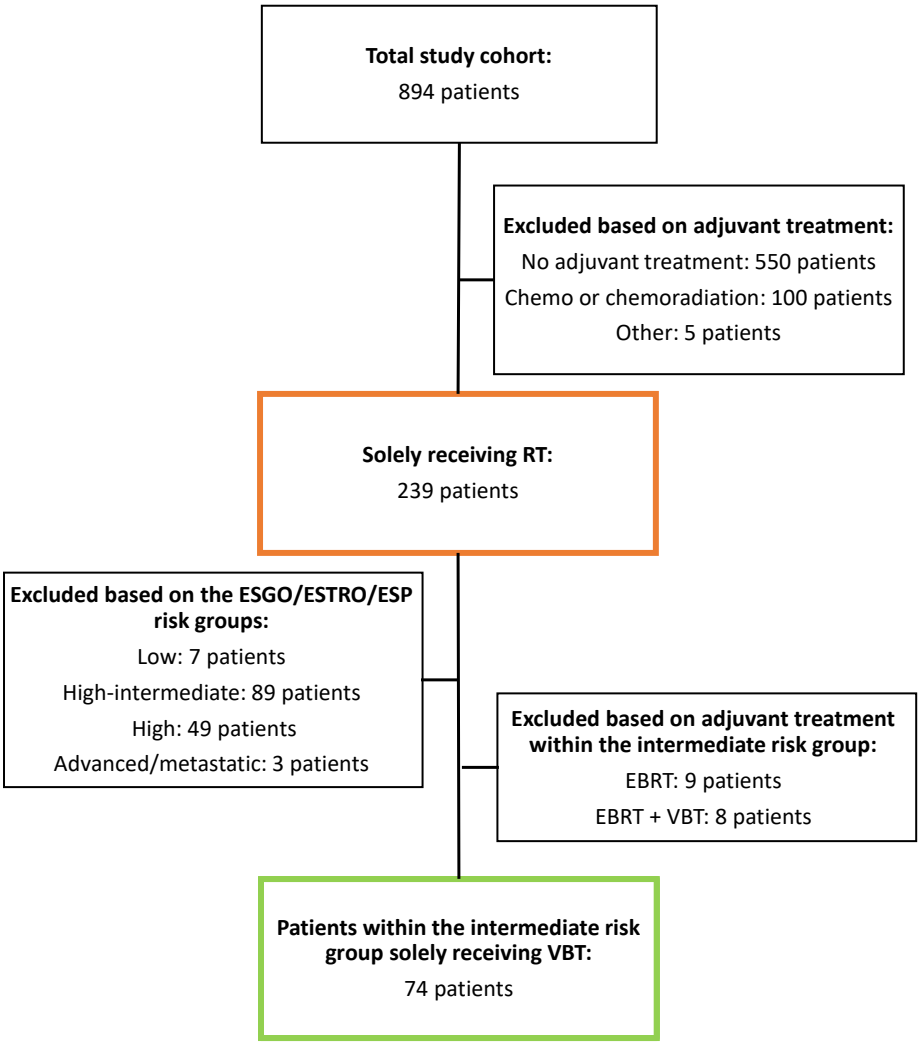


Figure S1. Flowchart secondary analysis

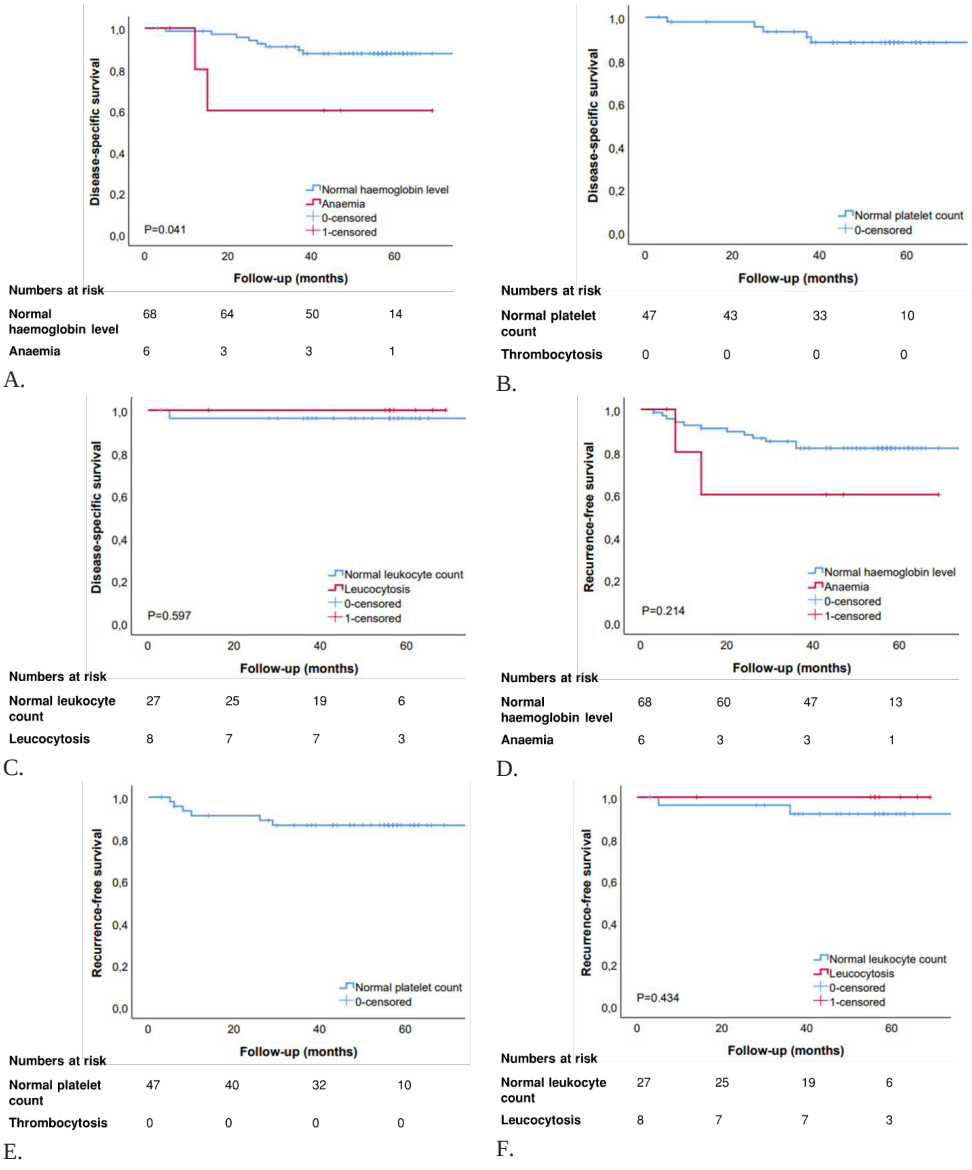
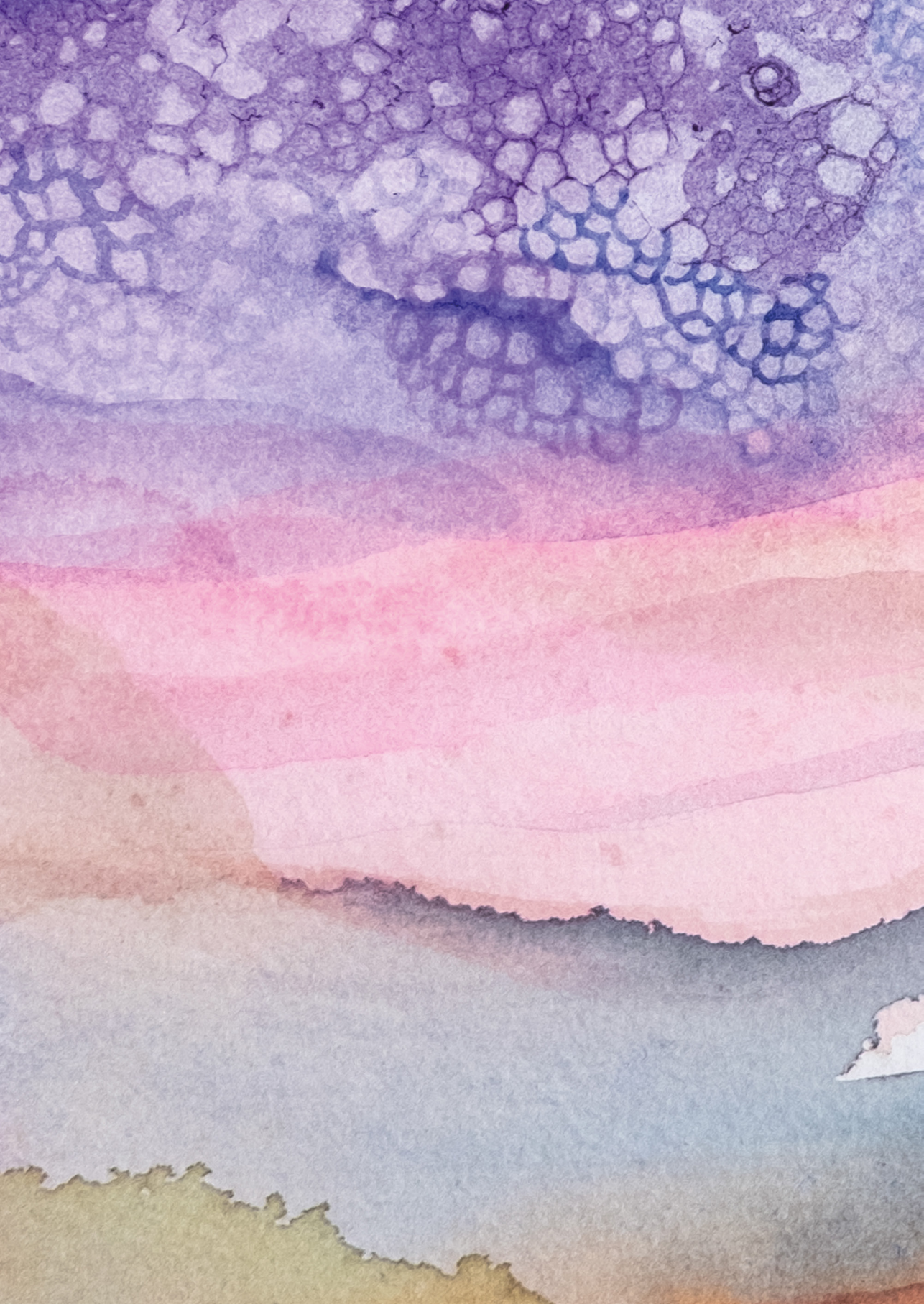


Figure S2 A-F. 5-year disease-specific survival (DSS) and recurrence-free survival (RFS) of patients with normal and abnormal haematological parameters within patients classified as intermediate risk who received VBT. A. 5-year DSS of patients with intermediate risk and VBT, and with and without anaemia. B. 5-year DSS of patients patients with intermediate risk and VBT, and with and without thrombocytosis. C. 5-year DSS of patients with intermediate risk and VBT, and with and without leucocytosis. D. 5-year RFS of patients with intermediate risk and VBT, and with and without anaemia. E. 5-year RFS of patients with intermediate risk and VBT, and with and without thrombocytosis. F. 5-year RFS of patients with intermediate risk and VBT, and with and without leucocytosis.



CHAPTER 8

GENERAL DISCUSSION

DISCUSSION

In this thesis, the aim was to evaluate the prognostic relevance of histomorphology, immunohistochemical (IHC) and clinical biomarkers within the new era of molecular profiling in endometrial cancer (EC). In this general discussion the main findings and clinical relevance of the different studies are presented. Furthermore, a clinical decision tool for primary treatment is proposed optimizing the use of biomarkers beholding current histomorphology, decreasing diagnostic costs and further improving prognostication of EC patients.

Diagnosis of endometrial cancer

EC is commonly diagnosed by endometrial sampling (pipelle biopsy, hysteroscopic biopsy or dilatation and curettage (D&C). Previous studies indicated that preoperative diagnoses based on endometrial sampling is only moderately (≤67%) correlated with final tumor grade and histological subtype.[1-4] The lowest concordance was found for grade 2 EC (61.0%).[4] Explanations for discordance on histological diagnosis include 1) the limited amount of tissue obtained by preoperative endometrial sampling 2) sampling errors leading to missed tumor components, 3) interobserver disagreement due to subjective interpretation of defined criteria. In **chapter 2** it is shown, that the amount of preoperative endometrial tissue surface is not related to the degree of concordance with final classification in low- and high-grade EC, based on retrospective analysis of a large study cohort. Interestingly, concordant diagnoses revealed a significant lower median endometrial tissue surface compared to discordant diagnoses. Even the sampling method (pipelle biopsy, hysteroscopic biopsy or D&C) did not influence the degree of concordance between pre- and postoperative diagnoses. Therefore, sampling errors that occur due to heterogeneous and/or mixed tumors or sampling of only superficial tumor tissue, will remain and cannot be resolved by the use of a different sampling method. Based on the study of Mota et al. genetic analysis out of pipelle biopsy material (uterine aspirates) might be a solution, even in samples that are not histologically classifiable. With this approach tumor heterogeneity might be captured as well.[5]

In some cases discordance of grading can be caused by a misjudgment of the percentage solid growth or a missed tumor component. Unintentional misjudgment by the pathologist, including interobserver disagreement, may be reduced by the binary grading classification and/or the additional use of immunohistochemical (IHC) or molecular biomarkers. The use of a binary grading system (low- vs. high-grade EC) is preferred by the most recent ESGO/ESTRO/ESP (European Society of Gynaecological Oncology / European SocieTy for Radiotherapy and Oncology / European Society of Pathology) guideline and World Health organization (WHO) classification of Female Genital tumors over the FIGO (International Federation of Gynecology and Obstetrics) three-tiered grading system with respect to reproducibility.[4, 6-8]

This new grading system considers both grade 1 and 2 EC lumped together as low-grade EC and grade 3 endometrioid EC (EEC) and non-endometrioid EC (NEEC) as high-grade EC.[9, 10] We confirmed that, by using this binary classification, the concordance between pre- and postoperative diagnosis resulted in an improved percentage of 88.8%. However, this binary classification inherently covers the poor interobserver reproducibility specifically for grade 2 EC. This may still impact treatment decisions when the correct diagnosis is either low- or high-grade EC. The standard use of a simple and relatively cheap set of IHC markers is therefore recommendable.[4, 11-15] This set includes the most studied IHC markers over the years: estrogen receptor (ER), progesterone receptor (PR), L1 cell adhesion molecule (L1CAM) and p53. ER/PR are well-known prognostic hormonal biomarkers that predict LNM and outcome in EC.[16, 17] Additionally, positive L1CAM expression is also an established prognosticator for LNM and outcome in EC.[18-23] Finally, p53 is one of the most well-known IHC markers for several tumors; abnormal expression of p53 (overexpression or null-expression) is associated with an unfavorable outcome, representing 15% of all EC diagnosis and responsible for 50-70% of all EC-related mortality.[24-30] Furthermore, it is associated with NEEC histology and LNM.[20-22, 31]

In our clinical oncology network, routine evaluation of PR and p53 is now recommended in preoperative grade 2 EC. Preliminary results of ongoing research shows, that patients with preoperative grade 2 EC, p53 abnormal and/or PR negative expression, have a worse prognosis comparable to high-grade EC. Underlining the fact that patients with doubtful low-grade EC, such as grade 2 EC might benefit from routine IHC to improve binary grading into low and high, and is expected to improve reproducibility.[9, 30, 32, 33]

Preoperative risk stratification

Primary treatment of EC consists of hysterectomy with bilateral salpingo-oophorectomy with or without lymph node assessment. Approximately 10% of all patients have lymph node metastases (LNM), which can be predicted and/or diagnosed by an algorithm directing approach for staging, integrated risk classification, routinely sentinel lymph node (SLN) mapping or full lymph node dissection (LND).[9, 34, 35] According to the most recent ESGO/ESTRO/ESP guideline, surgical treatment is based on determining preoperative tumor grade and histology by endometrial biopsy. In addition to grade, deep myometrial invasion (MI) is an important pathological finding that is associated with increased risk of LNM.[34] Preoperatively, MI may be detected with TVU or MRI.[9, 36] Reported sensitivity and specificity of TVE for deep MI are 71-85% and 72-90%, respectively. For contrast enhanced MRI, the sensitivity and specificity for deep MI are 33-100% and 44-100%, respectively. Depending on clinical and pathological risk factors, imaging for detection of metastatic disease is considered.[9] LN surgery is particularly recommended in high-grade EC, and may be considered in low-grade EC. Routine lymphadenectomy in low-grade EC has so far not shown to improve overall and

recurrence-free survival.[37] Low-grade EC has generally a favorable prognosis with a 5-year survival rate of 85-95%.[9, 38, 39] Due to the overall favorable outcome of patients with low-grade EC, standard histomorphology analysis remains the cornerstone in the preoperative risk stratification of EC. In **chapter 3** the relevance of molecular profiling within patients with low-grade EC has been analyzed. It was shown that patients with low-grade EC have an excellent prognosis independent of their molecular subgroup. This was confirmed by external validation within the study cohort of Kandoth et al. In multivariable analyses high-grade EC was independently associated with decreased DSS along with *TP53*mut and FIGO stage III-IV, again supporting the relevance of tumor grading in EC. Patients with preoperative grade 2 EC and *TP53*mut did show a decreased disease-specific survival compared to grade 1 EC and *TP53*mut, confirming our previous hypothesis that so called doubtful preoperative grade 2 EC should be considered as high-grade EC.

Approximately 20.0% of the patients are diagnosed with high-grade EC (grade 3 EEC and NEEC), with an overall poor prognosis (5-year survival rate of 58.8%), which is mainly attributed to the presence of regional and/or distant metastases and have a high risk of recurrence even after adjuvant treatment.[38, 40] Discordance for high-grade EC is likely supported by poor interobserver reproducibility of diverse morphological classification, different NEEC subtypes and the intratumoral heterogeneity of NEEC subtypes (mixed tumors). Molecular classification has shown to improve prognostication in high-grade tumors and an IHC panel for high-grade EC with PR and IMP3 has shown to improve concordance.[14, 32, 41] The improved prognostication by molecular classification in high-grade EC was confirmed in **chapter 3** and **chapter 4.** Furthermore the prognostic and intratumoral heterogeneity of one of the NEEC subtypes (clear cell carcinoma (CCC)) was shown in **chapter 4**. We demonstrated that pure and mixed CCC are two entities with different molecular background and clinical outcome. Frequent *TP53* mutations were found in pure uterine CCCs whereas microsatellite instability (MSI)/mismatch repair deficient (MMRd) was more frequently present in mixed uterine CCCs. This may explain the different outcome in pure vs. mixed uterine CCC. Results were comparable to Köbel et al., in which patients with mixed uterine CCC had improved outcome, compared to patients with pure uterine CCC.[42] Similar findings were reported in serous EC in which mixed serous EC had a superior prognosis compared to pure serous EC.[43] Expected that the etiology and pathogenesis of mixed serous EC differs from the pure serous EC. These studies support that histomorphology remains the cornerstone and that molecular classification or IHC is of additional value in high-grade EC patients to refine prognostication. In **Table 1** the association of morphological and clinical characteristics with immuno- and molecular markers is shown.

Table 1. Known association of immune- and molecular markers with pathological and clinical characteristics in endometrial cancer; high, medium, low and unknown association.

	ER+	ER-	PR+	PR-	L1CAM+	P53/*TP53*mut	*POLE*mut	MSI/MMRd	NSMP
Grade 1 EEC	High	Low	High	Low	Low	Low	Medium	Medium	High
Grade 2 EEC	High	Low	High	Low	Low	Medium	Medium	Medium	High
Grade 3 EEC	Low	Medium	Low	Medium	High	High	High	Medium	Low
NEEC	Low	High	Low	High	High	High	Low	Low	Low
LVSI	Low	High	Low	High	High	High	Unknown	Unknown	Unknown
MI >50%	Low	Medium	Low	High	High	High	Unknown	Unknown	Unknown
CA 125 >35 kU/l	Unknown	Unknown	Unknown	Unknown	High	High	Unknown	Unknown	Unknown
LNM	Low	High	Low	High	High	High	Unknown	Unknown	Unknown

Abbreviations: EEC, endometrioid endometrial cancer; NEEC, non-endometrioid endometrial cancer; LVSI, lympho-vascular space invasion; MI, myometrial invasion; CA 125, cancer antigen 125; LNM, lymph node metastasis; ER, estrogen receptor; PR, progesterone receptor; L1CAM, L1 cell adhesion molecule, *TP53*mut, tumor protein 53 mutant, *POLE*mut, polymerase epsilon mutant; MSI, microsatellite instable; MMRd, mismatch repair deficient; NSMP, no specific molecular profile.

Surgical approach

In patients with increased risk for LNM, additional LN surgery is recommended to guide tailored adjuvant therapy. Full LND is associated with substantial surgical morbidity. SLN mapping has emerged as a feasible, safe and accurate alternative to full LND in EC.[44, 45] The introduction of SLN has many advantages over full LND, however there remain few challenges. A recent review shows a bilateral detection rate of only 60%, when using a cervical injection with indocyanine green.[44] Patients with failure of bilateral SLN mapping, still require side specific LND.[46, 47] Proper preoperative non-invasive risk stratification of truly low-risk patients for LNM reduces unnecessary referrals to oncology centers, operating time and ultra-staging, hence decreasing health care costs and surgical related morbidity.

Postoperative risk stratification

For decades, tumor grading, histological subtype and surgical FIGO staging have been used to guide adjuvant treatment choices.[48] In the presence of LNM, adjuvant therapy results in a 5-year survival rate of 65% compared to 5-10% if LNM remain undetected and untreated.[49-54]

In 2014, the first joint European Society for Medical Oncology (ESMO), European SocieTy for Radiotherapy & Oncology (ESTRO) and European Society of Gynaecological Oncology (ESGO) consensus conference was held, resulting in the ESMO-ESGO-ESTRO 2016 risk classification groups (low / intermediate/ high-intermediate / high / advanced / metastatic). Hormonal biomarkers, p53, and L1CAM, being reported as having prognostic value in observational studies, were not incorporated in the 2016 ESMO-ESGO-ESTRO risk classification groups. However, in this thesis (**chapter 5)** it is demonstrated that the IHC biomarkers p53, L1CAM and ER/PR are prognostically relevant in the ESMO-ESGO-ESTRO 2016 risk classification groups and in addition to LN status. This is in accordance with multiple other studies which have investigated IHC markers in relation to LNM, outcome and the latest ESGO/ESTRO/ESP 2020 classification in EC.[15-17, 55, 56]

In the latest ESGO/ESTRO/ESP 2020 guideline the molecular subgroups are incorporated into risk classification groups for guidance of adjuvant treatment. However, some critical notes remain, the predictive relevance of molecular subgroups is mainly extracted from retrospective studies, hampering translation to the current clinical context and its cost-benefit. Furthermore, the guideline did not include hormonal biomarkers or L1CAM expression, which are shown prognostic highly relevant in the NSMP subgroup.[18, 56-59] Within the molecular subgroups the historical histopathological subtypes according to Bokhman (type 1 and type 2) are present.[38, 60] Type 1, EEC histology with mostly positive ER/PR expression, is mainly represented by the *POLE*mut, MSI and NSMP subgroup. Type 2, NEEC histology with generally negative ER/PR expression, is mainly represented by the *TP53*mut subgroup.[60] In **Chapter 6** it is illustrated that hormonal biomarkers remain prognostically

relevant within the molecular subgroups, particularly in p53mut and NSMP subgroup. In this study, a predefined cutoff for ER/PR expression, with improved prognostication, was used. Cutoff 0-10% with unfavorable outcome, 20-80% with intermediate outcome and 90-100% with favorable outcome.[55] Among all molecular subgroups, patients with ER+PR 0-10% expression showed the worst DSS and ER+PR 90-100% expression an excellent 5-year DSS, interestingly even within p53mut tumors. In multivariable analyses, ER+PR 0-10% was in addition to p53mut, lympho-vascular space invasion (LVSI) and FIGO stage an independent prognostic factor for reduced DSS. ER+PR 90-100 was in addition to *POLE*mut an independent prognostic factor for improved DSS. These data confirms our hypothesis that ER/PR expression would be preferably divided in three subgroups instead of the mostly used ≤1% and >1% or ≤10% and >10%, and underline the retained relevance in addition to the molecular subgroups, potentially guiding adjuvant treatment such as hormonal therapy as proposed in the RAINBO trial.[61]

Cancer-related anemia may cause tumor hypoxia, which could lead to a reduced response to radiotherapy (RT).[62-65] Hypoxia is very common in solid tumors and leads to cellular stress response, which allows tumor cells to survive. In addition, these hypoxic conditions may also protect tumor cells from downstream DNA breaks and lethality induced by radiotherapy.[64, 65] Therefore, it is hypothesized that preoperative anemia in EC could be a biomarker of tumor burden and/or aggressive tumor behavior.[66, 67] Within gynecological tumors, leukocytosis was also observed to have an adverse predictive impact on RT response.[68] In **chapter 7** the predictive relevance of hematological parameters within EC was analyzed. Patients with anemia and adjuvant RT had a significantly reduced DSS compared to patients with a normal hemoglobin. However, numbers were low and multivariable analysis could therefore not be performed. These data suggest that more research is required in EC patients assessing the effect of anemia on adjuvant RT treatment and incorporation of anemia in the risk stratification models for adjuvant treatment.

FUTURE PERSPECTIVES

A PERSONALIZED APPROACH

Clinical biomarkers

In addition to tumor histomorphology, IHC and molecular biomarkers, patient characteristics may be important within EC patients regarding to outcome. Therefore, it will be clinically relevant to incorporate them within risk classification groups aiming a refined personalized approach. EC has the strongest association with obesity, and about 50% of EC diagnosis can be attributed to obesity with an enormously increased incidence with a body mass index (BMI) between 30-35 kg/m^3.[69, 70] Risk of LNM may differ within the different BMI subgroups.[71] BMI also differs in the molecular subgroups, showing *POLE*mut having the lowest BMI and NSMP the highest, which at least also links tumor biology to patient environment.[72] Leukocytosis is frequently observed in obese patients, and could be a surrogate biomarker of obesity. Adipose tissue establishes a pro-inflammatory environment, stimulating carcinogenic cellular proliferation pathways.[25,73] Overexpression of pro-inflammatory cytokines could also contribute to the development of cancer-related anemia and thrombocytosis, and may therefore be related with tumor progression and LNM.[74-76] However, a clear answer about the relation between those patient characteristics (macro-environment) and tumor progression (micro-environment) still remains unclear and needs to be elucidated. In **chapter 7** we evaluated in addition to the predictive relevance, the prognostic relevance of preoperative abnormal hematological parameters i.e. anemia, thrombocytosis and leukocytosis, in EC. Previously, those abnormal hematological parameters have been associated with FIGO stage III-IV and unfavorable outcome.[76-83] In this study, anemia was mainly the most important prognosticator and was identified as an independent prognostic factor for DSS. Anemia could therefore be a clinical biomarker for aggressive tumor behavior. It would be interesting to evaluate the relevance of this easy clinical biomarker in addition to the molecular subgroups.

In addition to obesity, and anemia, race may also be considered for the risk classification subgroups. A recent study shows that the mortality of EC is increasing more in black women compared to white women, possibly because these women have higher incidence rates of NEEC versus EEC, the cause of which is still unclear and needs to be elucidated.[69] Hence future studies should evaluate whether racial disparities impact molecular subgroups in EC. Early studies in breast cancer revealed racial differences in outcome as result of an interplay between intrinsic and extrinsic factors. Intrinsic factors with germline genetics (including different molecular subgroups) and extrinsic factors includes environmental/lifestyle factors, both affecting the tumor biology.[84, 85]

Combining morphology and biomarkers

Within our research group we developed a Bayesian network (ENDORISK) that can be used for preoperative risk stratification. This model includes preoperative variables; tumor grade, IHC biomarkers (ER, PR, L1CAM, p53), clinical biomarker (thrombocytosis), suspected lymph nodes on imaging, atypical endometrial cells in cervical cytology and cancer antigen (CA)125 level. Furthermore, the model includes postoperative variables; MI, LVSI, postoperative tumor grade and adjuvant treatment.[21] The current ENDORISK model has been validated and demonstrated to properly identify patients with risk of LNM (area under the curve (AUC) 0.81), with a false-negative rate of 1.6% in those with very low risk of LNM (<5%).[21] Validation in two independent cohorts already resulted in the similar AUC and false-negative rate.[86, 87] Involving patients with SLN biopsy, did not affect the accuracy of ENDORISK.[87]

An advantage of Bayesian networks such as the ENDORISK-model is, it is a dynamic machine learning based computational model. Including the molecular subgroups is currently ongoing research, p53 mutant and wildtype were already included, so only *POLE* and MSI/MMR will be added (**Figure 1**). It will be interesting to evaluate the impact of multiple classifiers in patients with high-grade EC (for example p53mut and *POLE*) with respect to their risk of LNM and outcome.[88]

In addition to the molecular subgroups and in line with the latest ESGO/ESTRO/ESP 2020 guideline and study of Creasman et al., preoperative MI will be included for the prediction of LNM.[9, 34] **Figure 1** shows a proposal for a revised ENDORISK model as a diagnostic algorithm for optimalisation of personalized primary treatment and it shows the doctors' user interface.

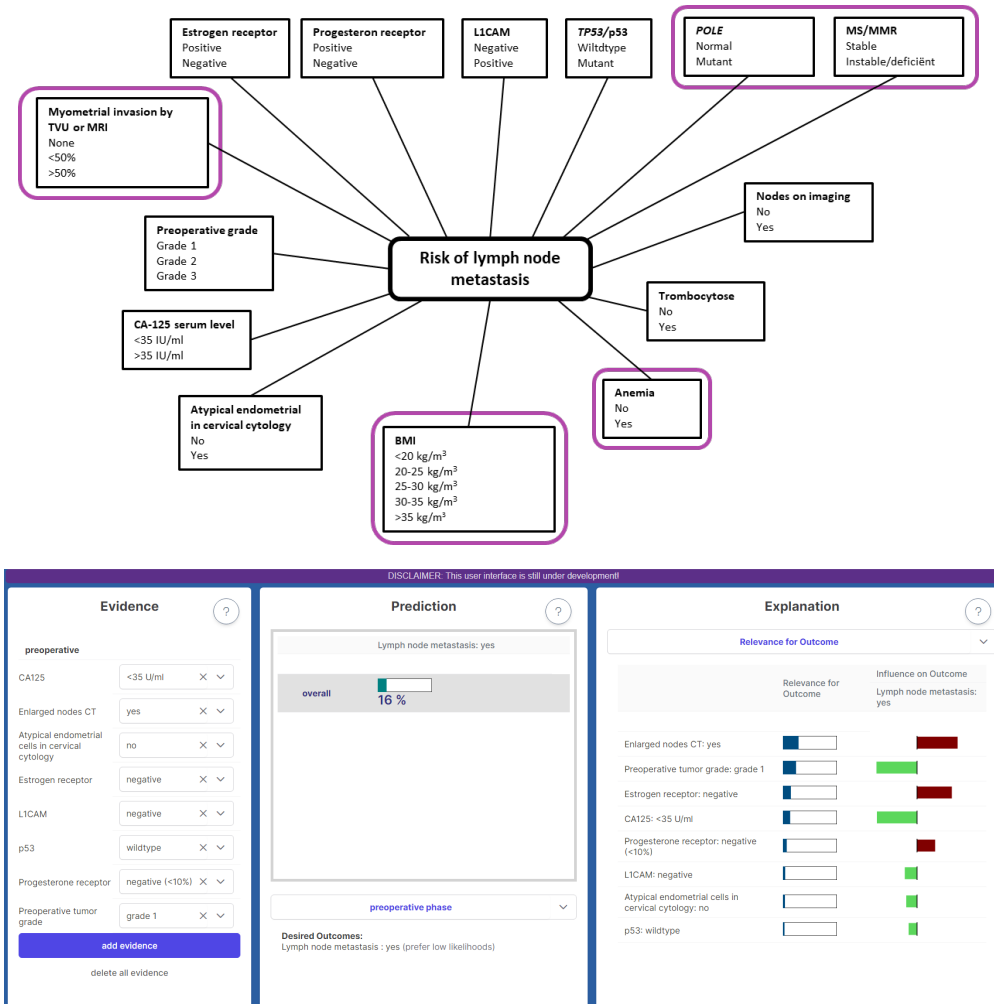


Figure 1. A. Revised ENDORISK-model for optimalisation of primary treatment. Circled in purple, the variables that will be added to the current ENDORISK-model. B. Current ENDORISK-model user interface for doctors, available at www.endorisk.eu

Abbreviations: L1CAM, L1 cell adhesion molecule*; TP53*, tumor protein 53; POLE, polymerase epsilon; MS, microsatellite; MMR, mismatch repair; TVU, transvaginal ultrasound; MRI, magnetic resonance imaging; LNM, lymph node metastasis; CA125, cancer antigen; BMI, Body mass index

Proposed clinical decision tool for primary treatment of endometrial cancer

A clinical decision tool including a diagnostic algorithm for EC is proposed, retaining histomorphology and optimizing the use of clinical, IHC and/or molecular markers, to improve prognostication thereby limiting health-care costs. **Figure 2** shows a proposed clinical decision tool for the primary treatment of EC. Grade 1 EC has an overall good prognosis, so extra diagnostics in these patients are not necessary.

Incorrect classification by the pathologist, especially for grade 2 and high-grade EC, including interobserver disagreement, could be resolved by standard using IHC or molecular biomarkers, as explained earlier. However, in the future most likely grading issues may be resolved by using artificial intelligence (AI) as assisted tool, which appears already very successful for grading prostate cancer in biopsies.[89-92]

Primary treatment for patients with grade 2 or high-grade EC could be tailored with the revised ENDORISK model. This will determine the risk of LNM and could be used as a clinical decision tool for patients and doctors, to choose for primary treatment: hysterectomy and bilateral salpingectomy with or without additional SLN or full LND. **Figure 2** shows a proposed primary treatment model of the different risk subgroups of LNM, percentages based on Creasman et al.[34] In a shared decision concept patients and doctors may of course individualize this proposition, for instance based on comorbidity factors.

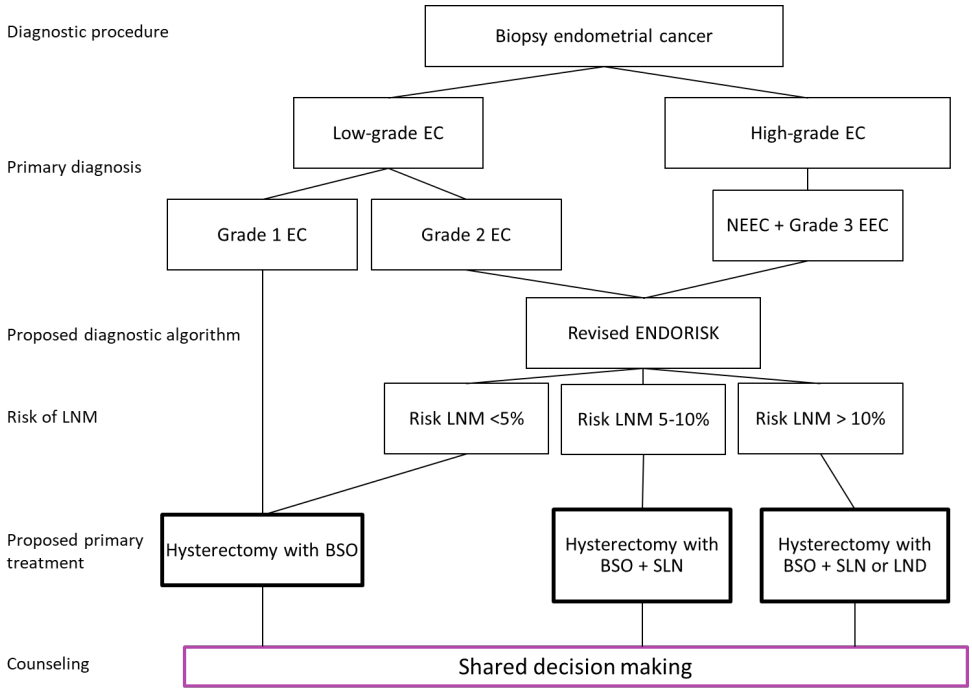


Figure 2. Clinical decision tool for the primary treatment of endometrial cancer

Abbreviations: EC, endometrial cancer; NEEC, non-endometrioid endometrial cancer; EEC, endometrioid endometrial cancer; LNM, lymph node metastasis; BSO, bilateral salpingectomy; SLN, sentinel lymph node; LND, lymph node dissection

Proposition diagnostic algorithm adjuvant treatment of endometrial cancer

For the diagnostic algorithm of adjuvant treatment, molecular subgroup analysis is needed. The large prospective RAINBO trial has recently started to evaluated different treatment strategies for the different molecular subgroups.[61, 93] Unfortunately, patients characteristics, tumor grade, L1CAM expression and both hormonal biomarkers are lacking in the four trails defining treatment strategies. In my opinion, first determination of FIGO stage, grade and histology should be performed and molecular analysis can be used to further refine adjuvant treatment in high-risk EC. In low-grade early stage EC the benefit of molecular classification as well as treatment strategy needs to be elucidated. In addition, it needs to be clarified what the effect is of different adjuvant treatment strategies in the disparities of patient characteristics e.g. performing a study taking into consideration anemia and/or race, in relation to the molecular subgroups to define adjuvant treatment strategies. Future development must also include shared decision making with the patient since most patients with EC are vulnerable and unable to tolerate each type of adjuvant treatment. The revised ENDORISK-model could provide additional information, by including the adjuvant treatment to analyze the 5-year recurrence-free survival risk (**Figure 3**).

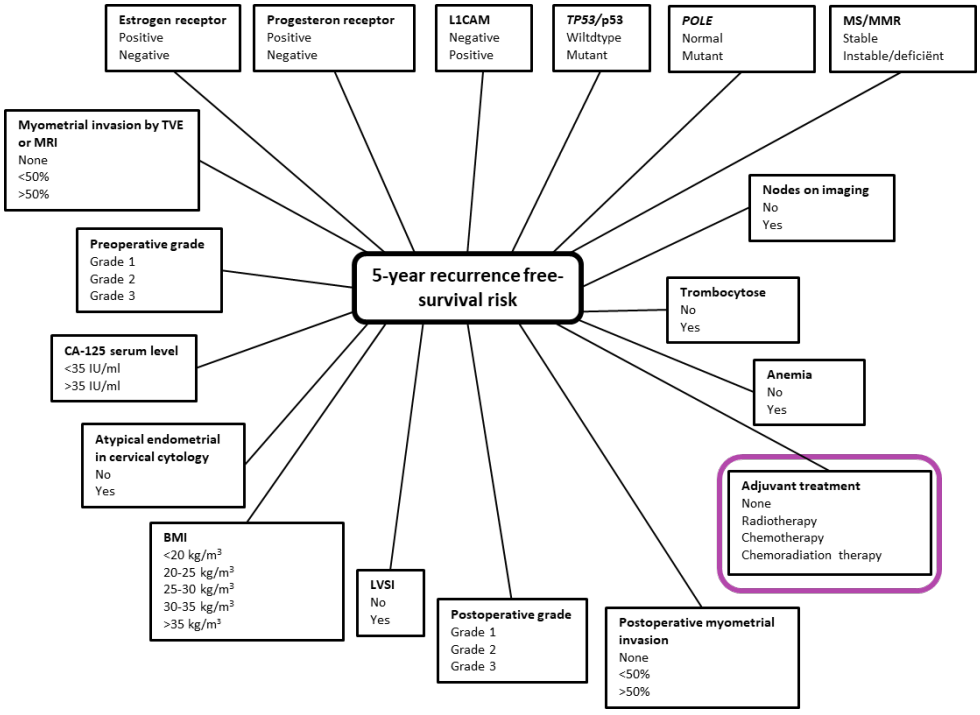


Figure 3. Revised ENDORISK-model including adjuvant treatment (circled in purple) to analyze the 5-year recurrence free survival risk.

Abbreviations: L1CAM, L1 cell adhesion molecule; TP53, tumor protein 53; POLE, polymerase epsilon; MS, microsatellite; MMR, mismatch repair; TVU, transvaginal ultrasound; MRI, magnetic resonance imaging; LNM, lymph node metastasis; CA125, cancer antigen; BMI, Body mass index; LVSI, lympho-vascular space invasion

CONCLUSION

A personalized risk stratification model including a combination of classic histomorphology, IHC markers, clinical markers and recently proposed molecular markers (**Figure 4**) appears to be the best approach in the diagnostic work-up of EC patients thereby reducing health costs. Thereafter, with the presented clinical decision tool an evidence based approach specific for primary and adjuvant treatment is proposed which may be tailored to the individual patient, giving her an optimal choice.

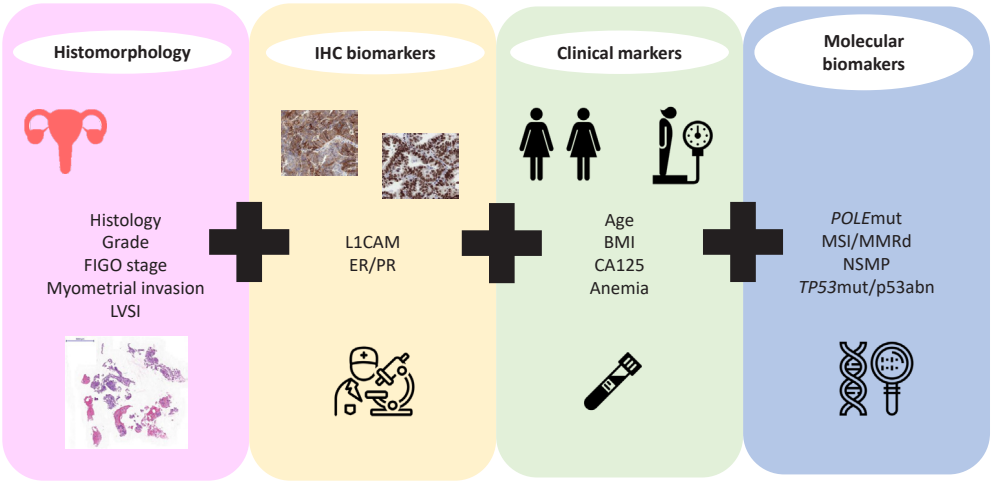


Figure 4. Combining histomorphology, IHC biomarkers, clinical markers and molecular biomarkers should be the best approach in the diagnostic work-up for EC patients.

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics; LVSI, lympho-vascular space invasion; IHC, immunohistochemical; L1CAM, L1 cell adhesion molecule; ER, Estrogen receptor; PR, progesterone receptor; BMI, Body mass index; CA125, cancer antigen; *POLE*, polymerase epsilon; MSI, microsatellite instable; MMRd, mismatch repair deficient; *TP53*, tumor protein 53

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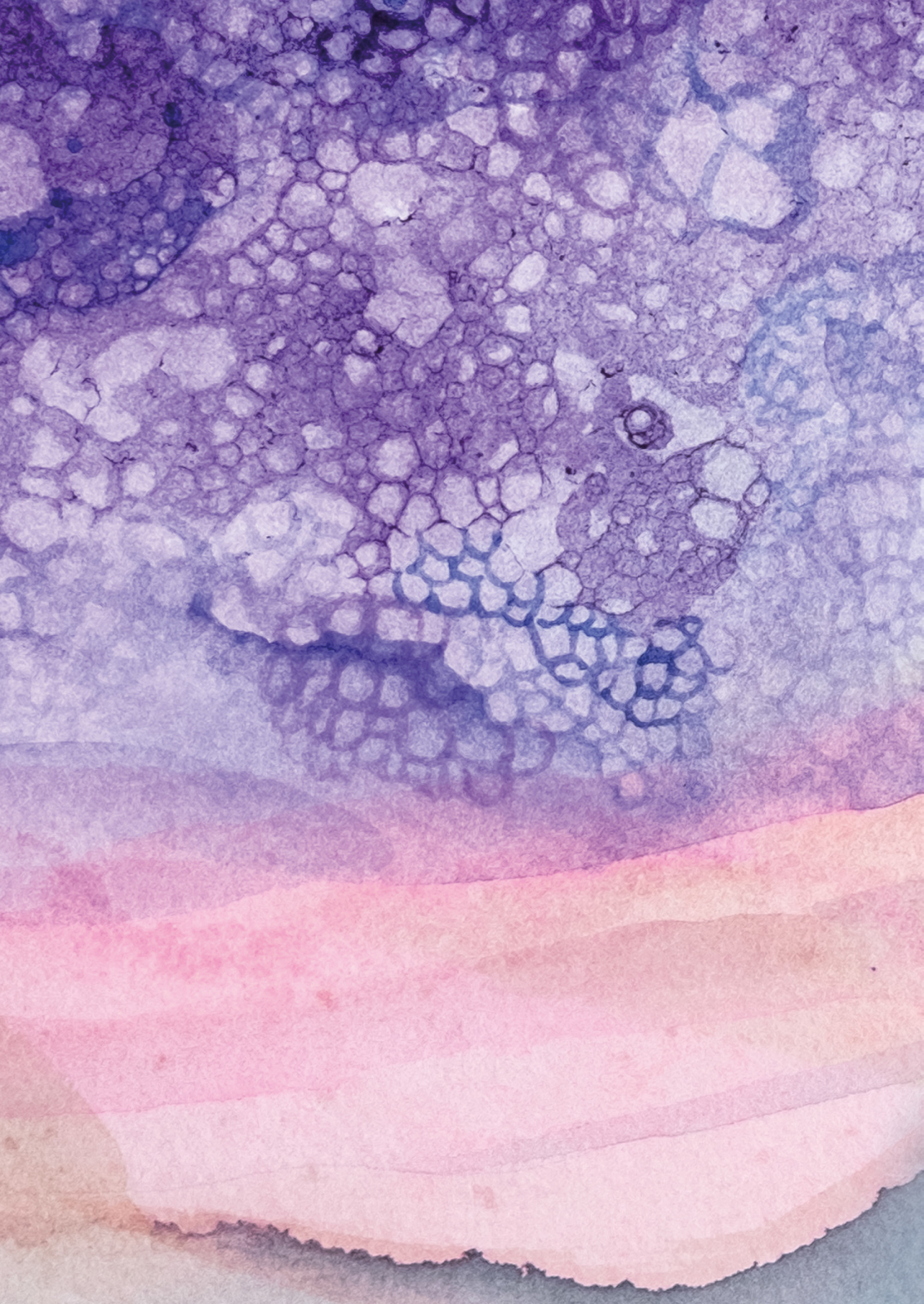

CHAPTER 9

SUMMARY / SAMENVATTING

SUMMARY

In this thesis, we investigated the role of classical tumor morphology and existing biomarkers in the context of the new era of molecular classification. This to optimize the use of all available biomarkers and apply the best care for patients with endometrial cancer (EC).

There is only moderate concordance between pre- and postoperative histological diagnosis in EC (67%) which may lead to under- and overtreatment. In **Chapter 2,** we investigated whether the amount of preoperative tissue and the sampling method (pipelle biopsy, dilation & curettage or hysteroscopy biopsy) affects the concordance. For this purpose, within the ENITEC (European Network of Individual Treatment in Endometrial Cancer) network, 573 tumor samples were collected in which we measured the area of preoperative endometrial tissue by digital imaging of the sections. In 60.0% of the samples, there was agreement between pre- and postoperative tumor grade and histology. When using binary grading, i.e. low- (grade 1 and 2 EC) and high-grade EC (grade 3 and non-endometrioid EC) the agreement was 88.8%. The amount of preoperative endometrial tissue surface was unrelated to the concordance of pre- and postoperative diagnosis. In contrast, the amount of endometrial tissue surface was significantly lower when pre- and postoperative diagnoses were concordant (18.7 mm^2 vs. 23.5 mm^2 , respectively $P=0.022$). The sampling method was also unrelated to the degree of concordance between pre- and postoperative diagnosis.

Immunohistochemical (IHC) or molecular markers can be used to improve the agreement between pre- and postoperative diagnosis. In **Chapter 3**, we investigated the added value of molecular classification within the group of patients with low-grade EC in an ENITEC retrospective cohort. The four molecular subgroups were determined by next-generation sequencing (NGS) or a combination of NGS and IHC: (1) polymerase epsilon (*POLE)* mutation, (2) microsatellite instable (MSI), (3) *TP53* mutation and (4) no specific molecular profile (NSMP). A total of 393 patients were included, *POLE* mutation was present in 8.4%, MSI in 19.8%, *TP53* mutation in 18.3% and NSMP in 53.4%. In all molecular subgroups, patients with low-grade EC had an excellent 5-year disease-specific survival of 85-100%, and this was confirmed in the external validation cohort. Patients with high-grade EC had a significantly worse 5-year disease-specific survival within *TP53* mutation and NSMP subgroup (40% and 68%, respectively). In the multivariable analysis, high-grade EC, *TP53* mutation and FIGO stage III-IV were independently associated with reduced disease-specific survival.

The relevance of molecular classification in high-grade tumors was demonstrated in **Chapter 4**. We investigated whether pure clear cell carcinoma (CCC) differs from mixed type CCC in terms of molecular, IHC markers and prognosis. In a retrospective cohort study of 43 patients

(n=22 pure CCC and n=21 mixed type CCC), molecular classification was determined by NGS. In addition, IHC staining for the hormone receptors, L1 cell adhesion molecule (L1CAM) and the mismatch repair deficiency (MMRd) proteins (MSH6 and PMS2) were performed. Patients with pure CCC had significantly more frequent *TP53* mutations whereas patients with mixed type had more frequent MSI/MMRd and high progesterone receptor expression (>10%). In addition, patients with pure CCC had a significantly worse prognosis than the mixed CCC. In multivariable analysis *TP53* mutation was a significant predictor for reduced disease-specific survival, whereas histology (pure and mixed CCC) was not significant.

The ESMO-ESGO-ESTRO 2016 (European Society for Medical Oncology - European Society of Gynecological Oncology - European SocieTy for Radiotherapy & Oncology) risk classification groups aim to identify patients for tailoring adjuvant therapy. IHC biomarkers with proven prognostic relevance within EC were not included in these risk groups. In **Chapter 5**, we investigated whether preoperative IHC biomarkers were prognostically relevant in addition to lymph node status and within the ESMO-ESGO-ESTRO 2016 risk groups in a retrospective ENITEC cohort study (n=763). Patients with positive lymph node status and abnormal expression of IHC showed significantly reduced recurrence-free survival (p53-abnormal, L1CAM+) and significantly reduced disease-specific survival (p53-abnormal, L1CAM+, estrogen and progesterone receptor -). In the 'high and advanced/metastatic' risk group, patients with abnormal IHC expression had a significantly lower recurrence-free survival compared with normal IHC expression. Within the multivariate analyses, abnormal expression of p53, <10% expression of the hormonal biomarkers, and the ESMO-ESGO-ESTRO risk group 'high and advanced/metastatic' were independent prognostic factors for the disease-specific survival. Thus, in addition to lymph node status and within the ESMO-ESGO-ESTRO 2016 risk groups, the biomarkers remained prognostically relevant.

In **Chapter 6,** the role of hormonal biomarkers within molecular classification was investigated in a retrospective study within the ENITEC cohort and a study cohort of Vancouver (Canada). Molecular subgroups were determined using NGS or a combination of NGS and IHC. The expression of hormonal biomarkers was divided into three risk groups: estrogen + progesterone receptor (ER+PR) expression 0-10%, 20-80% and 90-100%, and 'discordant' when ER/PR expression were not in the same risk group. A total of 739 patients were included, *POLE* mutation present in 9.1%, MMRd in 27.6%, p53 mutation in 20.8% and NSMP in 42.5%. In the complete cohort, patients with hormonal expression of 0-10% had significantly the worst 5-year disease-specific survival, 20-80% an intermediate and 90-100% the best. Within the p53 mutation, MSI and NSMP subgroup, the 5-year disease-specific survival of the different hormonal risk groups was significantly different. Within the multivariate analysis, hormone receptor expression 0-10%, p53 mutation, lymph-vascular

space invasion and FIGO staging were independently associated for reduced disease-specific survival. Hormone expression of 90-100% and *POLE* mutation were independently associated with improved disease-specific survival.

In **Chapter 7,** the prognostic and predictive value of preoperative abnormal hematologic parameters (anemia, thrombocytosis and leukocytosis) was determined among 894 patients with EC. Patients with anemia had significantly reduced 5-year disease-specific and recurrence-free survival and patients with thrombocytosis a reduced 5-year disease-specific survival. In multivariate analysis, anemia, age and the ESGO/ESTRO/ESP 'high and advanced/metastatic' risk group were found to be independently associated with reduced disease-specific survival. Within patients who received adjuvant radiotherapy (n=239) preoperative anemia was found to be significantly associated with decreased disease-specific and recurrence-free survival. Patients with preoperative anemia within the ESGO/ESTRO/ESP 'intermediate' risk group who had received vaginal brachytherapy, showed significantly reduced disease-specific survival. It was concluded that preoperative anemia can possibly be considered as a prognostic marker and probably also as a predictive marker in response to radiotherapy.

In **Chapter 8,** the studies in this thesis are discussed and put into perspective with the most recent findings in literature. Finally, results are translated into clinical recommendations and a diagnostic decision tree to optimize individualized care of patients with endometrial carcinoma.

SAMENVATTING

In dit proefschrift hebben we de rol van klassieke morfologische tumor kenmerken en bestaande biomarkers onderzocht in de context van het nieuwe tijdperk van moleculaire classificatie. Dit met als doel om optimaal gebruik te maken van alle beschikbare markers en deze in te zetten voor de beste zorg van patiënten met endometriumcarcinoom (EC).

Er is een matige overeenkomst tussen pre- en postoperatieve histologische diagnose bij het EC (67%) hetgeen kan leiden tot onder- en overbehandeling. In **hoofdstuk 2** hebben we onderzocht of de hoeveelheid preoperatief weefsel en de methode van biopsie (pipelle biopsie, dilatatie & curettage of hysteroscopie biopsie) van invloed is op de concordantie. Hiervoor zijn binnen het ENITEC ('European Network of Individual Treatment in Endometrial Cancer') netwerk 573 tumor samples verzameld waarbij we middels digitale coupe beeldvorming de oppervlakte van preoperatief endometriumweefsel hebben gemeten. In 60.0% van de samples was er een overeenkomst tussen pre- en postoperatieve tumorgraad en histologie. Overeenkomst tussen pre- en postoperatieve laag- (graad 1 en 2 EC) en hooggradig EC (graad 3 en non-endometrioïd EC) was bij 88.8% van de patiënten het geval. Er werd geen relatie gevonden tussen de hoeveelheid preoperatief endometrium oppervlakteweefsel en de correcte diagnose. In tegendeel, de hoeveelheid oppervlakte endometriumweefsel was significant lager wanneer pre- en postoperatieve diagnoses wel overeenkwamen (18.7 mm^2 vs. 23.5 mm^2, respectievelijk P=0.022). Ook de methode van biopsie was niet gerelateerd aan de mate van overeenkomst tussen pre- en postoperatieve diagnose.

Voor het verbeteren van de overeenkomst tussen pre- en postoperatieve diagnoses kan gebruik worden gemaakt van immunohistochemische (IHC) of moleculaire markers. In **hoofdstuk 3** hebben we in een retrospectieve studie binnen ENITEC onderzocht wat de toegevoegde waarde is van de moleculaire classificatie binnen de groep patiënten met laaggradig EC. De vier moleculaire subgroepen werden bepaald middels 'next-generation sequencing (NGS)' (ook wel mutatie-analyse genoemd) of een combinatie van NGS en IHC; (1) polymerase epsilon (*POLE)* mutatie, (2) microsatelliet instabiel (MSI), (3) *TP53* mutatie en (4) 'no specific moleculair profile' (NSMP). In totaal werden 393 patiënten geïncludeerd, *POLE* mutatie was aanwezig in 8.4%, MSI in 19.8%, *TP53* mutatie in 18.3% en NSMP in 53.4%. Binnen alle moleculaire subgroepen hadden patiënten met een laaggradig EC een excellente 5-jaars ziekte-specifieke overleving van 85-100%. Dit werd bevestigd in het externe validatie cohort. Patiënten met een hooggradig EC hadden een beduidend slechtere 5-jaars ziekte-specifieke overleving binnen *TP53* mutatie en NSMP subgroep (respectievelijk 40% en 68%). In de multivariate analyse waren hooggradig EC, *TP53* mutatie en FIGO stadium III-IV onafhankelijke voorspellers voor een lagere ziekte-specifieke overleving.

Het belang van moleculaire classificatie in hooggradige tumoren is bevestigd in **hoofdstuk 4**. Hier hebben we onderzocht of *pure* clear cell carcinoom (CCC) verschilt van de *mixed* type CCC op gebied van moleculaire, IHC markers en prognose. In een retrospectieve cohortstudie van in totaal 43 patiënten (n=22 pure CCC en n=21 gemixte CCC) werd de moleculaire classificatie bepaald middels NGS. Daarnaast werden IHC kleuringen voor de hormoon receptoren, L1 cel adhesie molecule (L1CAM) en de 'mismatch repair deficiency'(MMRd) eiwitten (MSH6 en PMS2) verricht. Patiënten met een *pure* CCC hadden significant vaker *TP53* mutaties en patiënten met een *mixed* type vaker MSI/MMRd en een verhoogde progesteron expressie >10%, daarnaast hadden patiënten met *pure* CCC een significant slechtere prognose dan de *mixed* CCC. De multivariate analyse liet zien dat met name *TP53* mutatie een belangrijke significante variabele is voor een verminderde ziekte-specifieke overleving, histologie (*pure* en *mixed* CCC) was hierbij niet significant.

De ESMO-ESGO-ESTRO 2016 (European Society for Medical Oncology - European Society of Gynaecological Oncology - European SocieTy for Radiotherapy & Oncology) risicoclassificatie groepen hebben als doel patiënten te identificeren voor afstemmen van adjuvante therapie. IHC biomarkers met een bewezen prognostische relevantie binnen EC zijn hierbij niet meegenomen. In **hoofdstuk 5** hebben we in een retrospectieve studie binnen ENITEC onderzocht of preoperatieve IHC biomarkers prognostisch relevant zijn naast de klierstatus en binnen de ESMO-ESGO-ESTRO 2016 risicogroepen. In totaal werden 763 patiënten geïncludeerd voor analyse. Patiënten met een positieve klierstatus en een abnormale expressie van IHC lieten een significant verminderde recidiefvrije overleving zien (p53-abnormal, L1CAM+) en een significant verminderde ziekte-specifieke overleving (p53-abnormal, L1CAM+, oestrogeen en progesteron receptor -). Patiënten met een abnormale IHC expressie en de 'high' en 'advanced/metastatic' risicogroep lieten een significant lagere recidiefvrije overleving zien in vergelijking met normale IHC expressie in die risicogroep. Binnen de multivariate analyses van de ziektespecifieke overleving waren abnormale expressie van p53 en de hormonale biomarkers naast de 'high' en 'advanced/metastatic' onafhankelijke prognostische factoren. De onderzochte biomarkers zowel als de lymfklierstatus zijn dus prognostisch relevant binnen de ESMO-ESGO-ESTRO 2016 risicogroepen.

In **hoofdstuk 6** hebben we in een retrospectieve studie binnen het ENITEC cohort en een cohort uit Vancouver (Canada), onderzocht wat de rol van hormonale biomarkers is binnen de moleculaire classificatie. Moleculaire subgroepen werden bepaald middels NGS of een combinatie van NGS en IHC. De expressie van de hormonale biomarkers werd onderverdeeld in drie risicogroepen: oestrogeen + progesteron receptor (ER+PR) expressie 0-10%, 20-80% en 90-100%. Wanneer de oestrogeen en progesteron receptor expressie niet in dezelfde risicogroep vielen, werden ze 'discordant' genoemd. In totaal werden 739 patiënten geïncludeerd, *POLE* mutatie aanwezig in 9.1%, MMRd in 27.6%, p53 mutatie

in 20.8% en NSMP in 42.5%. In het complete cohort hadden patiënten met een hormonale expressie van 0-10% significant de slechtste 5-jaars ziektespecifieke overleving, 20-80% een gemiddeld en 90-100% de beste. Binnen de p53 mutatie, MSI en NSMP subgroep was de 5-jaars ziektespecifieke overleving van de drie hormonale risicogroepen significant verschillend van elkaar. Binnen de multivariate analyse was de hormonale receptor expressie 0-10% naast p53 mutatie, lymfevatinvasie en FIGO-stadering een onafhankelijke factor voor een verminderde ziektespecifieke overleving. Hormoon expressie van 90-100% en *POLE* mutatie waren onafhankelijke factoren voor een verbeterde ziektespecifieke overleving.

In **hoofdstuk 7** is de prognostische en predicatieve waarde van preoperatieve abnormale hematologische parameters (anemie, trombocytose en leukocytose) binnen EC bepaald middels een retrospectieve multicenter studie. In totaal zijn voor de prognostische analyses 894 patiënten geïncludeerd. Patiënten met preoperatieve anemie hadden een significant verminderde 5-jaars ziektespecifieke en recidiefvrije overleving en patiënten met trombocytose een significant verminderde 5-jaars ziektespecifieke overleving. In een multivariate analyse bleek anemie, leeftijd en de ESGO/ESTRO/ESP 'high' en 'advanced/ metastatic' risicogroep onafhankelijke factoren voor een verminderde ziektespecifieke overleving. Patiënten die adjuvante radiotherapie kregen (n=239) en preoperatief een anemie hadden, bleken significant een kortere ziektespecifieke en recidiefvrije overleving te hebben. Patiënten met een preoperatieve anemie binnen de ESGO/ESTRO/ESP 'intermediate' risicogroep die vaginale brachytherapie hadden gekregen, toonden een significant verminderde ziektespecifieke overleving. Preoperatieve anemie kan daarmee als een prognostische marker worden gezien en mogelijk ook als predicatieve marker in respons op radiotherapie.

In **hoofdstuk 8** worden de studies van dit proefschrift bediscussieerd en gerelateerd aan de meest recente bevindingen in de literatuur. Tot slot worden de resultaten vertaald naar klinische aanbevelingen en een diagnostische beslisboom om de zorg voor de individuele patiënt met een endometriumcarcinoom te optimaliseren.

APPENDIX

RESEARCH DATA MANAGEMENT

PHD PORTFOLIO

CURRICULUM VITAE

LIST OF PUBLICATIONS

DANKWOORD

RESEARCH DATA MANAGMENT

Ethics and privacy

This thesis is based on the results of human studies (or existing data from published papers), which were conducted in accordance with the principles of the Declaration of Helsinki. All collected patient material is coded and pseudonymized with an individual study number, according to the protocol 'Code for proper use of human tissue'.

The medical ethical committee Radboudumc CMO, Nijmegen, the Netherlands has given approval to conduct the studies in chapter 2 through 7 and concluded that these studies fall not under the Medical Research Involving Human Subjects Act (WMO). Chapter 2 through 7 were also approved by the Institutional Review Board of all participating centers.

Data collection and storage

Some of the data for chapter 7 was collected through electronic Case Report Forms (eCRF) using CASTOR EDC. From Castor EDC data were exported to SPSS version 25.0 (IBM, New York, NY, USA). Data for the other chapters was added to existing datasets in SPSS 25.0 (IBM, New York, NY, USA) of previously published studies.

The data of the PhD project is stored according the FAIR principles on the Radboudumc department server: H:\Onderzoek\ONCO-Endorisk\Stephanie and under H:\Onderzoek\ONCO-Endorisk\Biomarkers endometrial cancer. There will be no patient information on my private computer. Study material (sections, blocks and DNA) of patients are anonymized stored at the pathology department.

Availability of data

The studies of chapter 2 through 5 and 7 are published open access, chapter 6 is submitted. The data will be archived for 15 years after termination of the study. Reusing the data for future research is only possible after a renewed permission of the medical ethical review board and participants. The anonymous datasets that were used for analysis are available from the corresponding author upon reasonable request.

PHD PORTFOLIO

Name: Stephanie Vrede
Department: Obstetrics and Gynaecology
PhD period: 01-10-2019 – 01-10-2022
PhD Supervisor(s): Prof. dr. R.F.P.M Kruitwagen, dr. J.M.A Pijnenborg
PhD Co-supervisor(s): dr. M.P.L.M. Snijders, dr. J. Bulten

Training activities	Hours
Courses	
- Introduction day Radboudumc (2020)	6
- RIHS PhD introduction course for PhD candidates (2020)	15
- Radboudumc - eBROK course (2020)	42
- Training kwalitatief onderzoek (2020) IQ Healthcare	16
- Projectmanagement for PhD candidates (2020)	56
- Statistics for PhD candidates using SPSS (2021)	56
- Radboudumc scientific integrity (2021)	20
- RU - Design and illustration (2021)	26
Seminars	
- PhD retreat (2019)	16
- Themamiddag Endometrium tumorwerkgroep gynaecologie regio Nijmegen (2020)	3
- Organiseren webinar voor Gynaecologie-Oncologie Nederland (2022)	24
Symposia and Conferences	
- European Society of Gynaecological Oncology (ESGO) conference, Athens (2019)^	40
- CCBIO mini symposium, Endometrial Cancer (2020)	2
- European network of individual treatment in endometrial cancer (ENITEC) Virtual meeting (2020)^^	24 36
- International Gynecologic Cancer Society (IGCS) Virtual conference (2020)^	12
- European network of individual treatment in endometrial cancer (ENITEC) Virtual meeting (2021)^^	24
- European Society of Gynaecological Oncology (ESGO) conference, Prague (2021)	18
- European network of individual treatment in endometrial cancer (ENITEC) meeting, Porto (2022)^^	22
- European Society Medical Oncology (ESMO) conference, Valencia (2022)^^	40
- International Gynecologic Cancer Society (IGCS) conference, New York (2022)^^	12
- Nederlandse Vereniging voor Obstetrie en Gynaecologie (NVOG) Gynaecongres, Arnhem (2022)^^	
Teaching activities	
Lecturing	
- Monthly education of interns at the gynaecology department (2020-2023)	36
- Lecture on recent developments in endometrial cancer for residents (2020)	6
Supervision of internships / other	
- Supervision of master student Medical Biology (2020)	20
- Supervision of HBO bachelor life sciences student (2021)	15
- Supervision of master student Medical Biology (2022)	30
Total	**617**

^ indicates poster presentation

^^ indicates oral presentation

CURRICULUM VITAE

Stephanie Willemina Vrede werd geboren op 22 februari 1991 te Utrecht. Zij groeide op samen met haar ouders Richard en Sonja, en broertje Jeremy. Zij behaalde haar VWO diploma op het Cals College te Nieuwegein. Na haar middelbare school heeft Stephanie eerst 2 jaar de studie Biomedische Wetenschappen te Utrecht gevolgd. Daarna werd zij ingeloot voor de studie Geneeskunde te Utrecht. Tijdens haar bachelor Geneeskunde, heeft zij ook haar bachelor Biomedische Wetenschappen afgerond. Zij sloot haar master Geneeskunde af met een keuzecoschap gynaeco-pathologie in het UMC Utrecht en een oudste-coschap Gynaecologie in het Diakonessenhuis te Utrecht. Na het behalen van haar artsendiploma ging ze aan de slag als arts-assistent niet in opleiding (ANIOS) gynaecologie in het Elisabeth-Tweesteden ziekenhuis te Tilburg. Via deze plek kwam ze uiteindelijk in contact met dr. Hanny Pijnenborg waarna haar promotieonderzoek aan de afdeling Gynaecologische-Oncologie van het Radboudumc begon. Dit onder leiding van prof. dr. Kruitwagen, dr. Hanny Pijnenborg, dr. Marc Snijders en dr. Hans Bulten.

In februari 2023 heeft Stephanie haar klinische werkzaamheden hervat en is zij weer begonnen als ANIOS gynaecologie in het Diakonessenhuis. Momenteel woont zij in Utrecht samen met haar vriend Jaap.

LIST OF PUBLICATIONS

Vrede SW, van Weelden WJ, Visser NCM, Bulten J, van der Putten LJM, van de Vijver K, Santacana M, Colas E, Gil-Moreno A, Moiola CP, Mancebo G, Krakstad C, Trovik J, Haldorsen IS, Huvila J, Koskas M, Weinberger V, Bednarikova M, Hausnerova J, van der Wurff AA, Matias-Guiu X, Amant F, Snijders MPLM, Küsters-Vandevelde HVN, Reijnen C, Pijnenborg JMA. Immunohistochemical biomarkers are prognostic relevant in addition to the ESMO-ESGO-ESTRO risk classification in endometrial cancer. *Gynecol Oncol.* 2021 Jun;161(3):787-794. doi: 10.1016/j.ygyno.2021.03.031. Epub 2021 Apr 12. PMID: 33858677.

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Vinklerová P, Ovesná P, Hausnerová J, Pijnenborg JMA, Lucas PJF, Reijnen C, **Vrede SW,** Weinberger V. External validation study of endometrial cancer preoperative risk stratification model (ENDORISK). *Front Oncol.* 2022 Aug 3;12:939226. doi: 10.3389/fonc.2022.939226. PMID: 35992828; PMCID: PMC9381832.

Vrede SW, Donkers H, Reijnen C, Smits A, Visser NCM, Geomini PM, MD, Huy Ngo H, van Hamont D, Pijlman BM, Vos MC, Snijders MPLM, Kruitwagen R, Bekkers RLM, Galaal K, Pijnenborg JMA. Abnormal preoperative hematological parameters in Endometrial Cancer; reflecting tumor aggressiveness or reduced response to radiotherapy? *Journal of Obstetrics and Gynaecology*. In press; doi: 10.1080/01443615.2023.2294332

SUBMITTED

Vrede SW, van Weelden WJ, Bulten J, Gilks B.C, Teerenstra S, Huvila J, Matias-Guiu X, Gil-Moreno A, Asberger J, Sweegers S, van der Putten LJM, Küsters-Vandevelde HVN, Reijnen C, Colas E, Hausnerová J, Weinberger V, Snijders MPLM, Vinklerova P, Ravaggi A, Odicino F, Bignotti E, McAlpine JN, Kruitwagen R, Pijnenborg JMA. Hormonal biomarkers remain prognostically relevant within the molecular subgroups in endometrial cancer

DANKWOORD

Ik ben enorm trots op het resultaat van de afgelopen jaren wat uiteindelijk heeft geleid tot dit proefschrift. Dit was niet gelukt zonder de steun en toeverlaat van velen. Hierin wil ik iedereen enorm bedanken en een aantal mensen in het bijzonder.

Geachte dr. Pijnenborg, Beste Hanny, zonder jou was dit absoluut niet mogelijk geweest. De groei die ik heb meegemaakt in mijn promotietraject is mede te danken aan jou. Jij hebt mij dieper over het onderwerp laten nadenken en mijn academische vaardigheden doen vergroten. Ik waardeer je enorme passie en enthousiasme voor het onderzoek en de snelheid waarop je op mails en papers reageert. Heel erg bedankt voor je kritische noot tijdens de onderzoekmomenten, maar ook de gezelligheid daarbuiten!

Geachte prof. dr. Kruitwagen, Beste Roy, je bent pas later betrokken geraakt bij mijn onderzoek, maar ik was erg blij dat je instapte. Hierdoor werd de juiste focus gelegd voor de afronding van mijn proefschrift. Bedankt voor je input op de laatste artikelen en het manuscript!

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Geachte dr. Snijders, Beste Marc, we hebben elkaar niet veel in real life gezien, maar je was goed bereikbaar via de mail en leverde snel feedback op mijn papers. Waarbij in het begin de feedback werd geleverd in caps lock met soms een ander kleurtje, was je aan het eind gelukkig vertrouwd geraakt met de functie ‘wijzigingen bijhouden’. Bedankt voor de belangrijke klinische relevante blik op mijn papers!

Geachte leden van de manuscriptcommissie, prof dr. Ligtenberg, prof. dr. Verheij en prof. dr. Zweemer, bedankt voor het plaatsnemen in de commissie en het beoordelen van mijn manuscript.

Geachte dr. van der Drift, Beste Miep, bedankt dat jij mijn mentor was tijdens dit promotieonderzoek! Ik heb erg veel gehad aan onze meetings en vond het fijn om te weten dat ik altijd bij je kon aankloppen.

Geachte dr. Küsters-Vandevelde en dr. Visser, Beste Heidi en Nicole, ik heb jullie vaak lastig gevallen als pathologen van andere centra, maar altijd kreeg ik een snelle enthousiaste reactie

en waren jullie zeer bereidwillig om te helpen. Heel erg bedankt daarvoor! Sommige papers en figuren waren zonder jullie hulp een lastige opgave geworden.

Lieve Sanne, bedankt voor al jouw werk bij het verwerken van de samples in het lab. Helaas ben je nu niet meer werkzaam in ons research team, maar gelukkig altijd nog laagdrempelig te bereiken. Bedankt voor alles!

Dear ENITEC members and other foreign co-authors, thank you for the great collaboration and your contribution to the scientific papers.

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