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MOLECULAR PROFILING OF ENDOMETRIAL CARCINOMA

CLOSING IN ON PERSONALIZED MEDICINE

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ACADEMIC DISSERTATION
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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

I Pasanen A, Tuomi T, Isola J, Staff S, Butzow R, Loukovaara M. L1 Cell Adhesion Molecule as a Predictor of Disease-Specific Survival and Patterns of Relapse in Endometrial Cancer. Int J Gynecol Cancer 2016;26:1465–1471.

II Pasanen A, Loukovaara M, Tuomi T, Butzow R. Preoperative Risk Stratification of Endometrial Carcinoma: L1CAM as a Biomarker. Int J Gynecol Cancer 2017;27:1318–1324.

III Pasanen A, Ahvenainen T, Pellinen T, Vahteristo P, Loukovaara M, Butzow R. PD-L1 Expression in Endometrial Carcinoma Cells and Intratumoral Immune Cells: Differences Across Histologic and TCGA-based Molecular Subgroups. Am J Surg Pathol 2019;44:174–181.

IV Pasanen A, Loukovaara M, Butzow R. Clinicopathological significance of deficient DNA mismatch repair and MLH1 promoter methylation in endometrioid endometrial carcinoma. Mod Pathol 2020; 33:1443–1452.

V Pasanen A, Loukovaara M, Ahvenainen T, Vahteristo P, Butzow R. Differential impact of clinicopathological risk factors within the two largest TCGA-related molecular subgroups of endometrial carcinoma (submitted)

The publications are referred to in the text by their Roman numerals. These articles have been reprinted with the permission of their copyright holders.

ABSTRACT

Cancer treatment is moving towards precision medicine based on therapy that is tailored to patient and tumor characteristics. The current clinicopathological risk assessment methods of endometrial carcinoma (EC) are not optimal and both under- and overtreatment occur. To provide more objective tools for personalized treatment, research efforts focus on the molecular landscape of EC. A landmark study by the Cancer Genome Atlas (TCGA) research group defined a novel histotype-independent classification of EC based completely on molecular features.

This thesis consists of five retrospective studies that were conducted to investigate the relevance of various molecular biomarkers in EC. Emphasis was placed on L1 cell adhesion molecule (L1CAM), a promising and relatively novel prognostic factor of EC. In addition, we examined the clinicopathological significance of *MLH1* methylation status in mismatch repair deficient (MMRd) EC. Further, we profiled the expression of an immunotherapy target molecule, programmed death ligand 1 (PD-L1), within histological and molecular subgroups of EC. Lastly, we investigated the differential impact of individual risk factors across the two largest TCGA-based molecular subgroups of EC. The studies were based on a cohort of 842 patients who were surgically treated for EC at the Department of Obstetrics and Gynecology, Helsinki University Hospital, between January 2007 and December 2012.

In the first study, including 805 patients, we examined the prognostic significance of L1CAM in the postoperative setting of EC. L1CAM positivity determined by immunohistochemistry was associated with several poor clinicopathological prognostic factors. In addition, L1CAM expression was associated with more frequent distant (extra-abdominal) relapses and poor prognosis in the subgroup of endometrioid EC but not in non-endometrioid EC.

The second study investigated the value of L1CAM in preoperative risk stratification. Immunohistochemistry for L1CAM was conducted on endometrial biopsies of 241 EC patients. Preoperative tumoral L1CAM positivity was associated with poor prognostic features including lymph node involvement/advanced stage. However, integrating L1CAM with the conventional risk assessment models did not improve the capability of predicting nodal dissemination or distant metastases.

In the third study, multiplex fluorescent immunohistochemistry-based PD-L1 scorings were performed on 804 EC samples. PD-L1 expression was more frequent in intratumoral immune cells (27.7%) than in carcinoma cells (8.6%). With the combined positive score (CPS) method, 19.4% of the samples were positive for PD-L1. Within the molecular subgroups, *POLE*-mutated and MMRd tumors were more likely to present abundant T-cell infiltrates, CPS

positivity, and PD-L1 expression in immune cells than the other molecular groups (p53 abnormal and no specific molecular profile, NSMP). Non-endometrioid carcinomas and advanced stage tumors were more likely to display PD-L1 positivity than endometrioid carcinomas and early-stage tumors, respectively. Finally, we performed concordance analysis between multiplex and conventional chromogenic immunohistochemistry, where CPS outperformed the scoring systems based on a single cell type (carcinoma cells or immune cells).

In the fourth study, we classified 682 endometrioid ECs on the basis of MMR protein expression and *MLH1* promoter methylation status. MMR immunohistochemistry identified 35.8% of the cases as MMR-deficient and the majority (76%) of these were linked to *MLH1* methylation. MMR deficiency correlated with several negative clinicopathological prognostic factors. Methylated phenotype was associated with older age and larger tumor size. Methylated MMRd phenotype predicted poor disease-specific survival compared with MMR-proficient EC, but the difference with non-methylated MMRd EC was not significant. We found no association between methylation status and quantity of intratumoral T-cells or PD-L1 expression.

In the fifth study, TCGA-based molecular classification was performed for 535 cases of endometrioid EC. The original TCGA survival curves were reproduced with *POLE* mutated EC having an excellent prognosis, followed by NSMP, MMRd, and the more aggressive p53 abnormal EC. In multivariable analysis, survival difference between NSMP and MMRd groups became non-significant after adjusting for principal clinicopathological risk factors, suggesting that the negative prognostic effect of MMRd reflects the more frequent presence of conventional risk factors in this subgroup. Survival analyses were also performed separately for the two largest molecular subgroups, MMRd (n=264) and NSMP (n=206), in order to identify subgroup differences in the prognostic effect of single clinicopathological and molecular risk factors. Interaction analysis confirmed a significantly stronger impact of high grade (G3) and p16 hyperexpression in the NSMP group than in the MMRd group.

This thesis identifies molecular markers that may improve clinical management of EC patients. We confirmed the value of L1CAM as a postoperative prognostic marker in the endometrioid subtype of EC. By contrast, our results do not support integrating L1CAM into current lymphadenectomy stratification algorithms, as this offers no advantages relative to conventional methods. If TCGA-based molecular classifiers were to be introduced in the preoperative treatment algorithms, the role of L1CAM might need to be re-evaluated within this context. We demonstrated the clinicopathological significance of methylation profiling in MMRd EC. Differences in the impact of risk factors and in PD-L1 expression within TCGA subgroups supports the adoption of a molecular subgroup-specific study approach when formulating treatment algorithms for patients with EC.

ABBREVIATIONS

AI	aromatase inhibitor
AUC	area under curve
BMI	body mass index
CI	confidence interval
CPS	combined positive score
EC	endometrial carcinoma
ER	estrogen receptor
FIGO	International Federation of Gynecology and Obstetrics
HR	hazard ratio
IHC	immunohistochemistry
L1CAM	L1 cell adhesion molecule
LVSI	lymphovascular space invasion
MMR	mismatch repair
MMRd	mismatch repair deficient
MRI	magnetic resonance imaging
MSI	microsatellite instability
NSMP	no specific molecular profile
p53abn	p53 abnormal
PD-L1	programmed death ligand 1
POLE	polymerase epsilon
PR	progesterone receptor
TCGA	The Cancer Genome Atlas
TMA	tissue microarray
WHO	World Health Organization
WT	wild type

INTRODUCTION

Endometrial carcinoma (EC) is a pathogenetically and prognostically heterogeneous group of diseases. Early-stage EC generally carries an excellent prognosis and may be cured by surgery alone. Total hysterectomy and bilateral salpingo-oophorectomy form the cornerstone of the treatment. Patients with a significant risk of disseminated disease also undergo pelvic and para-aortic lymphadenectomy and may receive adjuvant chemo- and/or radiotherapy. Recently, immunotherapy has emerged as an ancillary treatment option for cancer patients, but the indications are not fully established for EC.

Preoperative risk assessment stratifies EC patients with regard to lymph node dissection. Whether lymphadenectomy itself offers therapeutic effects is unclear, but foremost it serves staging purposes [1, 2]. Postoperative risk stratification based on clinicopathological parameters including nodal status, guides the selection of adjuvant therapy. Extensive surgery and adjuvant therapies entail complications and not all patients benefit from them [3, 4]. Current risk stratification methods have limitations in identifying patients requiring intensive treatment. Approximately 10% of patients with a preoperatively determined low-risk EC shift to the high-risk category after the final pathological evaluation, indicating a risk of incomplete surgical staging and under-treatment [5]. On the other hand, 80% of patients subjected to lymphadenectomy eventually present with no nodal dissemination [6]. In the postoperative setting, up to 10% of patients with a presumably low or intermediate-risk disease experience recurrence [7].

Molecular classifiers of EC offer more objective tools for risk stratification. In 2013, The Cancer Genome Atlas (TCGA) consortium identified four prognostically and pathogenetically distinct subgroups of EC: *POLE* ultramutated, microsatellite instability (MSI) hypermutated, copy-number low, and copy-number high [8]. How these molecular subclasses and other risk factors should be integrated in the EC risk assessment algorithms and whether the four molecular subgroups should be treated as separate disease entities remain to be established. This thesis explores various prognostic and predictive factors of EC and investigates their relevance within the TCGA-based molecular subgroups.

REVIEW OF THE LITERATURE

EPIDEMIOLOGY

Cancer of the uterine corpus is the fourth most common cancer in females in the Western world [9]. Lifetime risk of EC is approximately 3% and this malignancy accounts for 4% of all cancer deaths in women [9]. The vast majority (95%) of uterine cancers are endometrial carcinomas, i.e. they develop in the epithelial compartment of the uterine mucosa (endometrium). The remaining 5% include sarcomas and other rare malignancies.

The median age at EC diagnosis is 62 years [10]. Approximately 20–25% of endometrial carcinomas occur in premenopausal patients and 5% occur in patients younger than 40 years [11]. Most women are diagnosed at an early stage i.e. before the cancer has spread outside the uterus, when the disease carries a good prognosis [12]. The 5-year relative survival rate of all EC patients is approximately 80% (Finnish Cancer Registry 2017).

RISK FACTORS

Many of the known risk factors of EC are related to unopposed estrogen exposure. The effects of hyperestrogenism and/or progesterone deficiency reflect the proliferative and growth suppressive responses that estrogen and progesterone physiologically exert on the endometrium.

The increased risk of EC associated with reproductive factors, such as nulliparity, early menarche, and late menopause, supports the relationship between cancer risk and greater premenopausal exposure to estrogens [13]. Other hormone-related risk factors are obesity, polycystic ovary syndrome, and diabetes, which also share various metabolic disturbances (hyperinsulinemia, insulin resistance, ovarian hyperandrogenism) [14, 15]. Obesity is associated with higher levels of circulating estrogens in postmenopausal women and with lower progesterone levels in premenopausal women [14]. The hyperestrogenic state related to obesity is mainly produced by increased extraovarian aromatization of androgens to estrogens occurring in the adipose tissue. Chronic anovulatory cycles resulting from any endocrine disorder (including obesity and polycystic ovary syndrome) cause prolonged progesterone deficiency and unopposed estrogen exposure. Rarely, hyperestrogenism may be caused by estrogen-producing tumors (mainly granulosa cell tumor of the ovary).

As for exogenous steroidal hormones, large population-based studies and meta-analyses provide evidence that oral contraceptive therapy containing estrogen and progestin reduces the risk of EC [16–18]. The contraceptive use of a hormone-releasing intrauterine device also appears to be associated with a decreased risk of EC [19, 20]. Menopausal hormone replacement therapy based on unopposed estrogen is associated with an increased incidence of EC, but the effect of combined hormone replacement therapy is less clear [21]. Selective estrogen receptor modulators (e.g. tamoxifen) used to treat breast cancer patients, increase the risk of uterine pathology (polyps, hyperplasia, carcinoma) [22]. EC risk of breast cancer patients is estimated to increase by 2- to 7-fold in tamoxifen users as compared to non-users [22]. As a result of the differential recruitment of co-regulators, tamoxifen exerts estrogen receptor (ER) antagonistic effects in breast cancer tissue and partial ER agonist effects in the endometrium [23]. In addition, several potential ER-independent carcinogenetic mechanisms of tamoxifen have been described [23]. Aromatase inhibitor (AI) -based endocrine treatment of breast cancer appears to be associated with a significantly lower risk of EC than tamoxifen treatment. AIs may even reduce the risk of EC compared with patients not receiving endocrine treatment [24]. At a hypothetical level, a protective effect of AIs can be expected, given their inhibitory action on estrogen production. As ovarian production of estrogen is not effectively inhibited by AIs, their use is recommended mainly for postmenopausal patients [25].

As regards non-steroidal hormones, hyperinsulinemia is the best-known risk factor for EC. A meta-analysis including 16 studies reported an approximately twofold risk of EC for diabetic compared with non-diabetic patients [15]. Studies included in the meta-analysis were mainly conducted on patients with type II diabetes, but similar results were found in studies restricted to type I diabetes [15]. The association remained significant after controlling for body mass index suggesting a body weight-independent effect. Insulin is a known growth factor, that among many target organs, promotes proliferation in endometrial cells. The effect is both direct via insulin signaling and indirect via regulation of the synthesis of various other proteins. For instance, insulin inhibits the synthesis of insulin-like growth factor (IGF) binding proteins, thereby increasing the level of free circulating IGF-1, a molecule which also has a proliferative effect on endometrial cells. In addition, insulin influences the levels of sex hormones by stimulating androgen synthesis in the ovary and adrenal gland, by enhancing aromatase production in endometrial stroma and by inhibiting the synthesis of circulating sex hormone binding globulin (SHBG) [26, 27]. Androgens are an estrogen reservoir, and therefore, an EC risk factor, but whether they have a direct cancerogenic effect is less clear [26, 28].

Approximately 3–5% of cases of uterine cancer are attributable to a hereditary cause [29–31]. The most common cause of familial EC is Lynch syndrome (formerly hereditary nonpolyposis colorectal cancer). Lynch syndrome is an autosomal dominant cancer predisposition syndrome, caused by deleterious germline mutation of a DNA mismatch

repair (MMR) gene (*MLH1, PMS2, MSH2, MSH6)* or germline deletion of the adjacent epithelial cell adhesion molecule gene (*EPCAM*), which leads to constitutive inactivation by methylation of *MSH2*. In addition, rare cases of hereditary (constitutive) hypermethylation of *MLH1* have been reported [32]. EC is the most common extracolonic manifestation of the syndrome with a lifetime risk of 40–60% in female Lynch syndrome patients [33]. Cowden syndrome is a rare autosomal dominant disorder caused by mutations in the phosphatase and tensin (*PTEN*) tumor suppressor gene. Lifetime risk of EC in female patients with Cowden syndrome is 5–10% [34].

PRECURSOR LESIONS OF ENDOMETRIAL CARCINOMA

Carcinogenesis is a multistep process in which cells accumulate genetic and epigenetic alterations inducing uncontrolled cell proliferation and impaired DNA repair functions, leading to further accumulation of genetic changes. The 2020 World Health Organization (WHO) classification categorizes two histological precursor lesions for EC: endometrial hyperplasia without atypia and atypical hyperplasia/endometrioid intraepithelial neoplasia [35]. Histologically, hyperplastic endometrium is characterized by exaggerated growth of irregular glands with an increase in the gland-to-stroma ratio compared with normal proliferative endometrium.

Many of the genetic alterations described in carcinoma have been reported in co-existing endometrial hyperplasia. Generally, the number of coexisting alterations increases from hyperplastic lesions to carcinoma supporting the theory of multistep tumorigenesis progressing through precursor lesions [36–40]. Rate of progression to carcinoma is 1–3% for non-atypical hyperplasia and 25–33% for atypical hyperplasia. Hyperplasia frequently develops in the setting of long-lasting unopposed estrogen exposure and is considered a risk factor for Bokhman's type I EC (see Histological classification and molecular background below). On the contrary, serous endometrial intraepithelial carcinoma, given its metastatic potential, is not considered a precursor lesion by the WHO 2020 classification, but rather a non-invasive form of carcinoma [35].

HISTOLOGICAL CLASSIFICATION AND MOLECULAR BACKGROUND

In 1983, Bokhman introduced a dualistic model of pathogenetic types of EC based on clinical observations and histopathological characteristics, but not molecular features [41]. "Type I" cancers (65%) were mostly represented by low-grade (G1-2) endometrioid tumors arising in

pre- or perimenopausal women, who often present hyperestrogenism, obesity, and other signs of metabolic syndrome. Typically, these carcinomas follow a favorable course (85.6% 5-year survival rate). "Type II" cancers (35%) follow an estrogen-unrelated pathway and generally develop from atrophic endometrium in postmenopausal women in the absence of signs of endocrine and metabolic disturbances stated above. Type II cancers are typically high-grade carcinomas (G3 endometrioid or non-endometrioid EC). These tumors follow an aggressive clinical course (58.8% 5-year survival rate) [41].

Type I and type II ECs present divergent molecular features. However, tumors with overlapping features exist, and the dualistic classification of Bokhman has been challenged. The most common genetic alteration in type I EC (40–60%) is inactivation of *PTEN* [37, 42, 43]. PTEN acts as a major antagonist of the PI3K–AKT pathway that controls cell proliferation and cell survival. Activating mutations of *PIK3CA*, which is part of the same pathway, are also common (20–40%) [39, 44]. Loss of ARID1A (AT-rich interacting domain-containing protein 1A), a member of a chromatin remodeling complex that regulates transcription, occurs in approximately 30% of endometrioid ECs [45, 46]. Approximately one-third (25–40%) of endometrioid carcinomas present an impaired DNA MMR system caused by a loss of function of one of the MMR genes (*MLH1, PMS2, MSH2, MSH6*). MSI represents phenotypic evidence of an impaired MMR system. In the majority of cases, MMR gene silencing is a sporadic event due to somatic *MLH1* promoter hypermethylation [47, 48]. In approximately half of the remaining cases, i.e. Lynch syndrome suspected cases, a germline mutation can be confirmed [49]. Some of the methylation-negative and germ line-negative (Lynch-like) cases have been shown to harbor biallelic somatic mutations [50–52]. Mutation-induced constitutive *KRAS* activity leading to uncontrolled cell proliferation is found in 18–28 % of endometrioid EC. Alterations in *PTEN*, *PIK3CA, KRAS*, MMR genes and *ARID1a* frequently co-exist, whereas an equally frequent (20–40%), but apparently independent, mutational process involves beta-catenin gene (*CTNNB1*) [8, 44, 53–55]. Beta-catenin is a component of the E-cadherin–catenin unit, and mutations of *CTNNB1* result in aberrant nuclear accumulation of the protein leading to transcriptional activation.

The above molecular aberrations are rare in type II EC, where the most common genetic alteration involves *TP53* gene (90 % of serous carcinomas). P53 is a tumor suppressor protein that blocks cell proliferation and promotes apoptosis when DNA damage occurs in the cell. Cells with inactivated *TP53* suffer from genetic instability at the chromosome level resulting in aneuploidy and numerous copy number changes. Other molecular changes often present in serous carcinomas are altered expression of the cell cycle regulatory gene *p16*, amplification of *Her2/neu* oncogene (40–80%, member of the human epidermal growth factor receptor family) and mutations of *E-cadherin* leading to loss of the homonymous structural protein (60–90%) [56]. High-grade (G3) endometrioid carcinomas often share molecular features with serous carcinomas. Most importantly, they present *TP53* mutations and p16 overexpression relatively frequently (37.5–61.0% and 11.0–30.4%, respectively)

[57–59]. In contrast to serous ECs, they rarely show *Her-2* amplification [57]. Endometrial clear cell carcinoma, which is considered an uncommon type II carcinoma, shares common molecular changes with endometrioid carcinomas, including loss of PTEN, MMR proteins, and ARID1A, but also exhibits alterations in the expression of *TP53* and *p16* [60].

The 2020 WHO classification of tumors of the uterine corpus defines several distinct histological subtypes of EC: endometrioid (≈70%), serous (10%), clear cell (<10%), mixed cell adenocarcinoma (10%, diagnosed and graded as high-grade carcinoma irrespective of the relative proportion of serous or clear cell carcinoma present), carcinosarcoma (5%), and undifferentiated and dedifferentiated carcinoma (2%). Very rare histotypes include squamous cell carcinoma, high-grade neuroendocrine carcinoma, low grade neuroendocrine tumor, mucinous carcinoma (intestinal type), and mesonephric and mesonephric-like adenocarcinoma [35]. Histotypes are distinguished based on tumor architecture, cell morphology and nuclear features. Endometrioid carcinoma presents histological features most closely resembling normal proliferative endometrium and frequent squamous differentiation. Various histological patterns (e.g. secretory, microglandular, mucinous, and spindle cell patterns) are recognized in the classification, but they do not carry prognostic information. Endometrioid ECs are graded according to a grading system developed by the International Federation of Gynecology and Obstetrics (FIGO), which is based on the proportion of non-squamous/non-morular solid tumor (≤5%, 6–50%, >50%, respectively, for G1, G2, G3). The architectural grade is upgraded by one if there is severe nuclear atypia, which should not be associated with features of serous carcinoma (marked nuclear pleomorphism, prominent nucleoli, high nuclear-to-cytoplasm ratio, frayed luminal border, and frequent mitotic figures that are often abnormal). Binary grading classifying G1–2 as low grade and G3 as high grade is recommended [35]. Histology-based classification of EC suffers from poor reproducibility, especially in the diagnosis of high-grade (G3 endometrioid vs. non-endometrioid) carcinomas [59, 61].

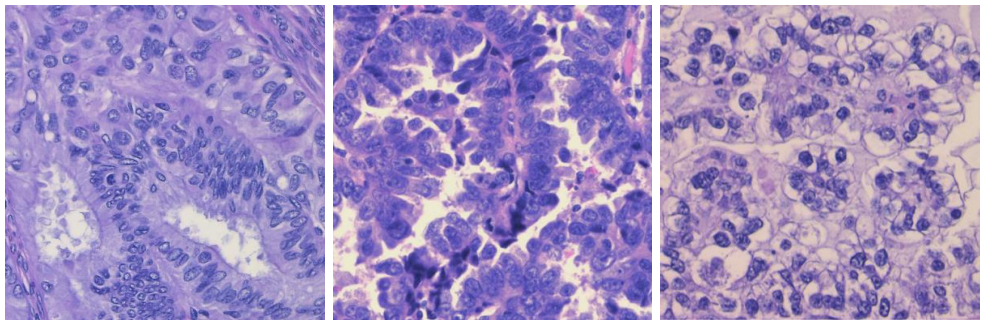

Figure 1. Representative images of endometrial carcinoma histotypes: a) G1 endometrioid carcinoma, b) serous carcinoma, c) clear cell carcinoma.

TCGA MOLECULAR CLASSIFICATION OF ENDOMETRIAL CARCINOMA

TCGA consortium performed genomic, transcriptomic, and proteomic characterization of 373 ECs and identified four prognostically distinct molecular subgroups: *POLE* ultramutated (7% of cases), MSI hypermutated (28%), copy-number low (39%), and copy-number high (26%) tumors (Figure 2) [8].

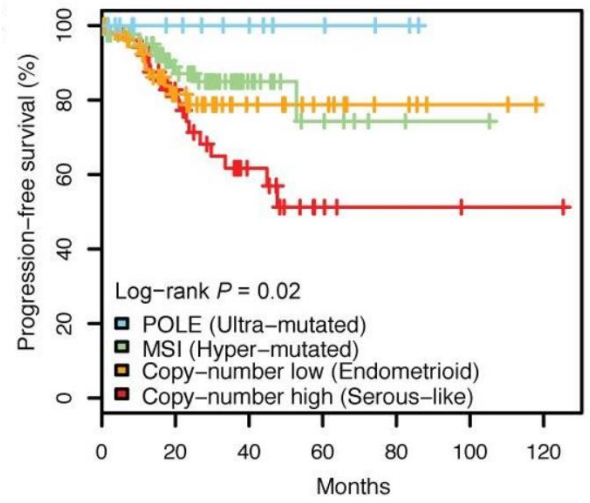

Figure 2. TCGA survival curves for EC (Cancer Genome Atlas, 2013) With permission from Nature.

The analyses of tumor mutational burden and somatic copy number alterations require methodologies that are laborious, expensive, and unsuitable for clinical application. Subsequently, two research groups replicated the TCGA survival curves with classifiers based on more pragmatic surrogate markers, i.e. the "Leiden classification" by the PORTEC group and the "Proactive Molecular Risk Classifier for Endometrial Cancer" (ProMisE) by the Vancouver group. These molecular classifiers are based on targeted *POLE* exonuclease domain mutational analysis as a surrogate for ultramutated EC, MMR markers for the hypermutated subgroup and p53 aberration for the copy-number high subgroup. In the Leiden model, MMR status is assessed by MSI analysis validated by MMR immunohistochemistry (IHC). P53 status is defined by IHC, followed by *TP53* mutational testing in indeterminate cases [62]. The ProMisE model is based on MMR protein and p53 IHC [63]. Tumors not presenting any of the above alterations are classified as no specific molecular profile/p53 wild type (NSMP/p53wt), a surrogate for the copy-number low group. In the original TCGA model and in ProMisE, tumors are classified in a stepwise fashion. The major difference between these proposed decision trees lies in the order in which tumors are subcategorized. In the original TCGA algorithm, the first step of the decision tree separates the *POLE* ultramutated subgroup. *POLE* wild type tumors are further categorized

according to the MSI status and microsatellite stable tumors according to copy number alterations. In the ProMisE model, the first subgroup assignment is based on the MMR status (Figure 3). Tumors with intact MMR proteins undergo *POLE* mutational analysis, and *POLE*wt tumors are classified as p53 abnormal (p53abn) or p53wt. In the Leiden algorithm, all molecular markers are determined for each sample, and cases with multiple molecular alterations are discarded from the classification. By contrast, in the original TCGA classification and in the ProMisE classification, tumors may present multiple molecular alterations, although this occurs rarely.

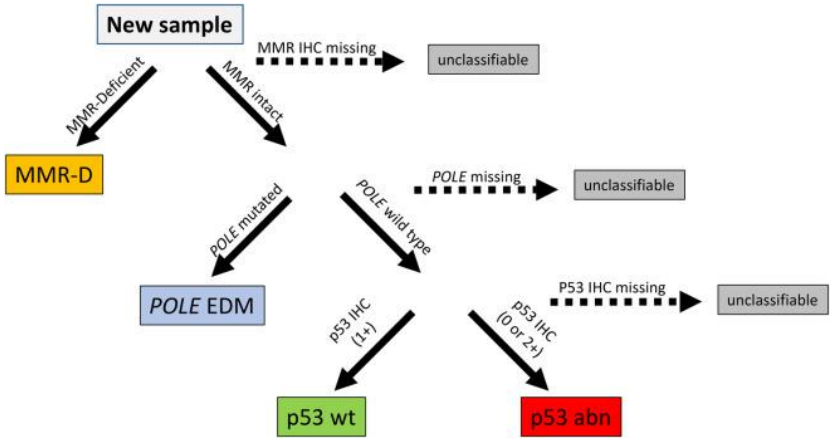

Figure 3. ProMisE molecular classification of endometrial carcinoma. Talhouk et al. (2016). With permission from Gynecol Oncol Res Pract.

POLE ultramutated/*POLE* mutant/*POLE* exonuclease domain mutated endometrial carcinoma

DNA polymerase ε exonuclease domain recognizes and removes mispaired nucleotides during DNA replication and deleterious mutation of *POLE* leads to ultrahigh mutational rates (232×10^{-6} mutations/Mb) [8, 63]. *POLE* mutated (*POLE*mut) ECs relatively frequently (40%) have high-grade (G3) endometrioid morphology [64]. Regardless of the high mutational frequency and the high grade of differentiation, *POLE*mut EC appears to have an excellent prognosis [8, 62, 63]. It is not known whether the good prognosis reflects an intrinsic quality of the disease or an increased sensitivity to adjuvant treatment [65, 66].

MSI hypermutated/MSI/MMR-deficient endometrial carcinoma

An impaired mismatch repair system leads to genomic instability and high mutational rates (18×10^{-6} mutations/Mb). Microsatellite unstable ECs generally exhibit favorable endometrioid histotype but are also associated with negative prognostic factors, including advanced stage, high grade of differentiation, lymphovascular space invasion (LVSI), and myometrial invasion [67–70].

Studies on colorectal cancer have provided evidence that MSI phenotype predicts resistance to 5-fluorouracil chemotherapy [71, 72]. By contrast, MSI/MMRd phenotype is associated with good response to immune check-point inhibitor treatment in many cancer types including EC [73–76]. In EC, MMR status may also correlate with response to adjuvant radiotherapy [77, 78].

Along with prognostic and predictive implications, MSI/MMR analysis is a useful screening test for Lynch syndrome. PCR-based MSI analysis and MMR IHC can be used to determine MMR deficiency. When loss of MLH1 is observed by IHC, methylation analysis may be conducted to exclude patients with a methylation-linked (presumably sporadic) disease from genetic counseling and germline mutation analysis. Limited data have been published regarding clinicopathologic differences between sporadic and hereditary or methylated and non-methylated MMRd ECs.

Copy-number low/No specific molecular profile, NSMP/p53wt endometrial carcinoma

Copy-number low tumors are mainly low-grade endometrioid carcinomas with a relatively low mutational frequency (2.9×10^{-6} mutations/Mb). Characteristically, they present frequent (52%) mutations of the β-catenin encoding gene*, CTNNB1*. Other mutations with relatively high frequency are *PTEN*, *PIK3CA*, *ARID1A,* and *KRAS* [8]. NSMP tumors have an intermediate prognosis, similar or somewhat better than that of MSI tumors [8, 63, 79].

Copy-number high/p53 mutant/p53 abnormal endometrial carcinoma

Most serous carcinomas and approximately one fourth of high-grade endometrioid ECs cluster into this tumor group that is characterized by extensive somatic copy number alterations, frequent *TP53* mutations and a relatively low overall mutational rate ($2.3\text{x}10^{-6}$ mutations/Mb) [8]. *P53*-mutated phenotype independently predicts poor survival and is

associated with the frequent presence of other poor prognostic factors, which further aggravate the prognosis [80, 81].

CURRENT METHODS OF RISK STRATIFICATION AND TREATMENT PLANNING FOR ENDOMETRIAL CARCINOMA

Preoperative risk stratification and surgical treatment of endometrial carcinoma

The standard primary treatment of EC consists of total hysterectomy and salpingo-oophorectomy complemented with pelvic and para-aortic lymphadenectomy in selected cases. Individual treatment strategies are tailored according to the estimated risk of extrauterine dissemination and the patient's comorbidities. Preoperative risk stratification of EC is based on tumor histology, local extent of the tumor (depth of myometrial invasion and cervical stromal invasion) and distant spread, including lymph nodal metastases. Vaginal ultrasonography and pelvic magnetic resonance imaging (MRI) are used to estimate the depth of myometrial invasion and the presence of cervical stromal invasion; MRI can also be used for assessment of regional lymph nodes. Whole-body computed tomography scan is a standard method for the detection of distant metastases at many institutions, although its cost-effectiveness as a universal preoperative imaging technique remains questionable [3]. Preoperative biopsy is taken to determine the histotype (endometrioid vs. non-endometrioid carcinoma) and grade of differentiation in the case of endometrioid carcinoma. Lymphadenectomy may be omitted in patients with a low risk of lymphatic dissemination, i.e. patients with G1-2 endometrioid carcinoma with invasion of less than one-half of the myometrial thickness [82]. In fact, two randomized trials suggest that patients with early-stage EC do not benefit from routine lymphadenectomy, although these studies have been challenged [1, 2, 83]. As lymph node status indisputably affects the prognosis of EC, lymphadenectomy completes surgical staging. Consequently, a failure to recognize a high-risk EC preoperatively leads to omission of lymphadenectomy, incomplete surgical staging, and possibly omission of necessary adjuvant treatments.

To avoid complications of standard pelvic lymphadenectomy (e.g. lymphoedema, lymphoceles), sentinel lymph node mapping has been introduced in EC treatment. In a large meta-analysis, sentinel node mapping and conventional lymphadenectomy presented comparable rates of detection of metastatic para-aortic nodes and rates of nodal or overall recurrence [84]. For the more common pelvic nodal involvement, sentinel lymph node assessment may even provide superior detection rates to full lymphadenectomy [84]. This may be due to pathological ultrastaging methods applied in sentinel protocols.

Postoperative risk stratification and adjuvant therapy of endometrial carcinoma

The FIGO staging system for EC changed from clinical to surgical in 1988 and the surgical staging system was revised in 2009 (Table 1) [85, 86].

Table 1. FIGO 2009 staging system for endometrial carcinoma

Stage	**Description**
Stage I ▪ IA ▪ IB	Tumor confined to the corpus uteri No or less than half myometrial invasion Invasion equal to or more than half of the myometrium
Stage II	Tumor invades cervical stroma, but does not extend beyond the uterus
Stage III ▪ IIIA ▪ IIIB ▪ IIIC IIIC1 IIIC2	Local and/or regional spread of the tumor Tumor invades the serosa of the corpus uteri and/or adnexae Vaginal and/or parametrial involvement Metastases to pelvic and/or para-aortic lymph nodes Positive pelvic nodes Positive para-aortic lymph nodes with or without positive pelvic lymph nodes
Stage IV ▪ IVA ▪ IVB	Tumor invades bladder and/or bowel mucosa, and/or distant metastases Tumor invasion of bladder and/or bowel mucosa Distant metastases, including intra-abdominal metastases and/or inguinal lymph nodes

Positive cytology is reported separately without changing the stage. Adapted from Pecorelli et al. (2009).

The selection of adjuvant therapy is based on postoperative risk assessment that, in addition to FIGO stage, takes into account tumor histology (low-grade endometrioid, high-grade endometrioid, non-endometrioid), LVSI, and patient's age. Risk groups according to the consensus conference of the European Society for Medical Oncology (ESMO), European Society for Radiotherapy and Oncology (ESTRO), and European Society for Gynecological Oncology (ESGO) are depicted in Table 2 [82]. Patients with low (or intermediate) -risk disease may forgo adjuvant therapies. Patients with early-stage endometrioid carcinoma with uterine risk features (G3, ≥50% myometrial invasion, LVSI) generally receive either vaginal brachytherapy or external pelvic radiotherapy. The latter is often preferred when lymphadenectomy has not been performed. Patients with a non-endometrioid EC or

advanced endometrioid cancer often undergo multimodality treatment with radiation and chemotherapy.

Table 2. Risk groups to guide use of adjuvant therapy for EC

Risk group	Description
Low	Stage I endometrioid, grade 1-2, <50% myometrial invasion, LVSI negative
Intermediate	Stage I endometrioid, grade 1-2, ≥50% myometrial invasion, LVSI negative
High-intermediate	Stage I endometrioid, grade 3, <50% myometrial invasion, regardless of LVSI status
	Stage I endometrioid, grade 1-2, LVSI unequivocally positive, regardless of depth of invasion
High	Stage I endometrioid, grade 3, ≥50% myometrial invasion, regardless of LVSI status
	Stage II
	Stage III endometrioid, no residual disease
	Non-endometrioid (serous or clear-cell or undifferentiated carcinoma or carcinosarcoma)
Advanced	Stage III residual disease and stage IVA
Metastatic	Stage IVB

Adapted from ESMO-ESGO-ESTRO guidelines (Colombo et al., 2016).

Immunotherapy in endometrial carcinoma

Immunotherapy stimulates the body's own immune system to eliminate tumor cells. Currently approved forms of immunotherapy mainly involve immune checkpoints, i.e. cellular pathways that regulate immunological responses. As an example of physiological immune check-point function, cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) maintains self-tolerance by blocking potentially autoreactive T-cells from being activated [87]. The programmed death 1 (PD-1) pathway is active in early stages of T-cell maturation in the thymus and later peripherally, where it attenuates the function of previously activated T-cells [87]. In addition to these crucial regulatory molecules, many other pathways modulate the amplitude and duration of an immune response. In cancer immunotherapy,

reactivation of silent antitumoral T-cells can be obtained by removing inhibitory signaling with immune check-point inhibitors.

Various phase II studies have reported promising results in EC patients treated with PD-1/PD-L1 inhibitors [73–76]. A trial including many cancer types (only 1 EC) provided evidence that tumors expressing PD-L1 protein more likely benefit from anti-PD1/PD-L1 treatment [88]. Various scoring systems exist, and they evaluate PD-L1 expression in carcinoma cells and immune cells, separately or in combination [89–91]. Cell type-specific scoring methods may suffer from limited reproducibility, as discerning PD-L1-positive carcinoma cells and intratumoral immune cells may be difficult [92]. The optimal cut-off for PD-L1 positivity needs to be determined specifically for each tumor type. Reported PD-L1 positivity rates in EC show wide variation in different studies (0.9-44.3%), presumably due to different antibodies used to detect PD-L1 expression and different scoring methods [93-105].

Tumors with a heavy mutational burden, i.e. *POLE*mut and MSI ECs, display the highest numbers of activated cytotoxic tumor infiltrating lymphocytes, often expressing PD-1 and PD-L1 [94, 98, 105, 106]. This “T-cell inflamed PD-L1-positive” phenotype possibly predicts favorable response to immunotherapy [107-109]. At present, the Food and Drug Administration has approved MMR deficiency and MSI as predictive biomarkers for anti-PD-1/PD-L1 immune check-point therapy [110].

INTEGRATING MOLECULAR AND CLINICOPATHOLOGICAL FACTORS IN RISK ASSESSMENT MODELS

There is evidence that integrated risk models including both molecular and conventional risk factors, outperform the current clinicopathological approach [79, 111]. An ongoing randomized trial (PORTEC-4a, NCT03469674) is recruiting patients with stage I-II EC and compares adjuvant treatment assignments based on standard clinicopathological risk factors alone or in combination with molecular risk factors (TCGA markers and L1 cell adhesion molecule (L1CAM)).

L1CAM promotes neuronal migration and differentiation during the development of the nervous system. In the cancer microenvironment, it facilitates tumor cell motility, invasion, and metastasis [112]. L1CAM has been shown to predict poor prognosis in many cancer types [113]. In a large study conducted on stage I endometrioid EC, L1CAM expression ≥10% independently predicted poor disease-free and overall survival with impressive hazard ratios (HRs, HR=16.33 for recurrence, HR=15.01 for death) even after adjustment for age, grade of differentiation, disease stage, and conventional risk group [114]. Further studies corroborated the prognostic value of this molecule [115, 116]. Studies including ECs of all

stages provide evidence of a correlation between L1CAM expression and advanced stage including nodal dissemination of the disease, but the value of L1CAM in preoperative stratification to lymphadenectomy is unknown [116, 117].

In the pre-TCGA era, several other biomarkers, such as ER, PR, ki-67, stathmin, and ASRGL1, have been shown to have prognostic value in EC, but large prospective studies demonstrating their prognostic utility have not been conducted, and they are currently not in clinical use [118-124].

AIMS OF THE STUDY

This study was designed to explore the prognostic value of molecular biomarkers and to provide data for future EC risk stratification efforts integrating TCGA-based molecular subclasses, conventional clinicopathological risk factors and ancillary molecular markers.

Specific objectives were as follows:

I To investigate the prognostic value of L1CAM expression in an unselected EC cohort. Specifically, to evaluate the relationship between L1CAM expression, relapse patterns, and disease-specific survival separately for endometrioid and non-endometrioid EC.

II To determine whether preoperative analysis of L1CAM can improve the stratification of EC patients to lymphadenectomy. Further, to address the correlation between tumoral L1CAM expression and soluble L1 detected in serum.

III To explore the prevalence of PD-L1 positivity in endometrial carcinoma cells and intratumoral immune cells. Further, to demonstrate eventual differences in PD-L1 expression profiles across histological and TCGA-based molecular subgroups of EC.

IV To examine prognostic and clinicopathological differences between methylation-linked and non-methylated MMRd ECs.

V To evaluate eventual differences in the prognostic impact of known clinicopathological risk factors and molecular biomarkers within the two largest TCGA-based molecular subgroups (NSMP and MMRd) of EC.

MATERIALS AND METHODS

PATIENTS AND TUMOR SAMPLES

We used a single-center database to identify all patients who received primary surgical treatment for EC at the Department of Obstetrics and Gynecology, Helsinki University Hospital between January 2007 and December 2012 (n=965). Formalin-fixed paraffin-embedded (FFPE) hysterectomy samples of 842 (87.3%) patients were available and suitable for the construction of a tumor tissue microarray (TMA). All high-grade endometrioid and non-endometrioid cases were reviewed by an experienced gynecopathologist. LVSI was defined as the unequivocal presence of tumor cells within an endothelial-lined space outside the invasive border. Starting in November 2014, we collected preoperative blood samples from voluntary EC patients. The final number of patients, sample types, and methodologies of each study are shown in Table 3. The study was approved by the Institutional Review Board and National Supervisory Authority for Welfare and Health (Valvira).

Table 3. Summary of materials and methods in Studies I-V

	Cohort size	**Sample type**	**Histotype**	**Methodology**
Study I	805	TMA	All	IHC
Study II	241 40	Aspiration biopsy/curettage Serum	All	IHC ELISA
Study III	804	TMA	All	IHC Direct sequencing
Study IV	682	TMA	Endometrioid	IHC Direct sequencing MS-MLPA
Study V	470	TMA	Endometrioid	IHC Direct sequencing

IHC=immunohistochemistry, ELISA=enzyme-linked immunosorbent assay, MS-MLPA= methylation-specific multiplex ligation-dependent probe amplification

TREATMENT PROTOCOLS

In 2007-2011, routine pelvic lymphadenectomy was recommended for all patients. Selective para-aortic lymphadenectomy was performed in surgically eligible patients considered to be

at elevated risk for lymphatic dissemination. Risk stratification was based on preoperative endometrial histology and on gross visual inspection of myometrial invasion during operation. From January 2012 onward, routine pelvic lymphadenectomy was abandoned and in patients with low-risk EC, i.e. G1-2 endometrioid carcinoma with <50% myometrial invasion assessed by magnetic resonance imaging, lymphadenectomy was omitted. Lymphadenectomy rate was 70.7% over the whole study population. Triage to adjuvant therapy was based on histology, stage, and type of surgery (execution or omission of lymphadenectomy). In patients with stage I-II endometrioid carcinoma, vaginal brachytherapy or whole pelvic radiotherapy was administered. Patients with stage III-IV endometrioid carcinoma or non-endometrioid EC of any stage received chemotherapy or a combination of chemotherapy and radiotherapy.

FOLLOW-UP PROTOCOL AND ENDPOINTS

The standard follow-up protocol consisted of medical check-ups scheduled every 3-4 months for the first year and thereafter every 6-12 months for a minimum of 3 years. The physical examination was complemented with imaging studies when alarming symptoms/findings emerged. A biopsy confirmed the endometrial origin of relapse in patients with additional non-uterine primary tumors. Follow-up data were collected from institutional medical records or from primary physicians at the referring institutions. Missing data were collected from death certificates obtained from Statistics Finland.

The main outcome of interest was disease-specific survival, defined as the time from surgery to death from EC. In Study I, cancer relapses were classified as follows: isolated vaginal relapse, retroperitoneal relapse (i.e. metastasis in pelvic and/or para-aortic lymph nodes, including those with a concomitant vaginal relapse or a lymph node metastasis extending to the groin), intraperitoneal relapse (including those with a metastasis in the vagina or in regional lymph nodes), and extra-abdominal relapses (metastases in the lung, liver, skin, brain etc., including those with concomitant metastasis in any other site).

TMA AND IMMUNOHISTOCHEMISTRY

Histological slides were reviewed by a pathologist, and representative areas of vital carcinoma tissue were marked on the slides. Four 0.8-mm cores were drawn from the corresponding area of the paraffin blocks and were inserted in the recipient TMA block with a manual tissue microarrayer (Beecher MTA-1, Beecher Instruments Inc., Sun Prairie, WI, USA). Details on the antibodies, dilutions and scoring are shown in Table 4.

Table 4. Details of the immunohistochemistry and scoring

	Clone/Cat.no.	**Dilution**	**Source**	**Cut-off (localization)**
L1CAM	14.10	1:40	Covance	≥ 10% (membranous)
MLH1	ES05	1:50	Dako	Complete or clonal loss (nuclear)
PMS2	EPR3947	1:25	Epitomics	Complete or clonal loss (nuclear)
MSH2	G219-1129	1:400	BD Biosciences	Complete or clonal loss (nuclear)
MSH6	EPR3945	1:200	Abcam	Complete or clonal loss (nuclear)
p53	DO-7	1:500	Dako	Null or strong and diffuse (nuclear)
ARID1a	HPA005456	1:200	Sigma-Aldrich	Complete or clonal loss (nuclear)
ERa	SP1	R-T-U	Roche/Ventana	< 10% (nuclear)
PR	16	1:50	Novocastra	< 10% (nuclear)
PD-L1 (chromogenic)	SP263	R-T-U	Ventana	≥ 1% (membranous)
PD-L1 (multiplex)	E1L3N	1:200	CST	≥ 1% (membranous)
CD3	MA1-82041	1:750	ThermoFisher	membranous
CD163	ab188571	1:200	Abcam	membranous
PanEpi: panCK panCK E-cadherin	 AE1/AE3 C-11 36	 1:100 1:150 1:200	 InVitrogen Abcam BD Biosciences	 cytoplasmic cytoplasmic membranous
Beta-catenin	CAT-5H10	1:400	Zymed	Any nuclear staining
E-cadherin	HECD-1	1:200	Invitrogen	Complete or clonal loss (membranous)
p-16	E6H4	R-T-U	CINtec Histology	Block type strong and diffuse hyperexpression (nuclear and cytoplasmic)
c-erbB2	4B5	R-T-U	Roche/Ventana	2+/3+ hyperexpression (membranous)

Chromogenic immunohistochemical stainings were performed at the Huslab Pathology Laboratory, Helsinki, Finland, with a few exceptions. L1CAM (preoperative samples) and ARID1a were stained at the Research Laboratories of the Departments of Obstetrics and Gynecology and Medical Genetics, Biomedicum, Helsinki, and L1CAM (TMA slides) at the Institute of Biomedical Technology, University of Tampere, Finland. Chromogenic PD-L1 stainings were performed at Fimlab Laboratoriot Oy, Tampere, Finland. Immunohistochemcal scoring was carried out either by traditional microscopy or digitalized microscopy using WebMicroscope Software (Fimmic Oy).

Multiplex fluorescent immunohistochemistry was performed at the Institute for Molecular Medicine Finland (FIMM), Helsinki. Five-channel fluorescent images were acquired using Metafer 5 scanning and imaging platform (MetaSystems, Alltlussheim, Germany). Image analysis was carried out by a pathologist. PD-L1 expression was scored separately for carcinoma cells, intratumoral immune cells (macrophages and T-lymphocytes), and combined positive scoring (CPS, i.e. the percentage of all PD-L1-positive cells relative to carcinoma cells).

For stainings where an internal control was applicable (MMR protein, p53, ARID1a, ER and PR), samples with a completely negative internal control were discarded. All slides were scored blinded to the clinical data. The concordance between the staining results on TMA and whole sections was tested with L1CAM, which is known to present a heterogeneous staining pattern. Representative microphotographs of the principal immunostainings are depicted in the original publications I, III and IV.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

We performed ELISA to measure the level of soluble L1 in serum samples from 17 patients with an immunohistochemically verified L1CAM-positive and 23 patients with an L1CAM-negative EC. Blood fractionation was carried out by centrifugation for 10 minutes at 2000g. We used a commercial ELISA kit (LS-F24209; LifeSpan Biosciences Inc., Seattle, WA, USA). Sandwich ELISA was performed on standards, controls, and samples according to the manufacturer's instructions. We ran test reactions on serial dilutions and the optimal serum dilution was 1:2000. Duplicate wells were run for each sample. The absorbance at 450 nm was measured by an automatic ELISA reader (Multiskan EX; Thermo Fisher Scientific). Results were expressed in nanogram per milliliter according to the established standard curve. The limit of detection was 93.75 to 6000 pg/mL.

POLE AND *KRAS* MUTATIONAL ANALYSIS BY DIRECT SEQUENCING

For DNA extraction, a pathologist identified representative areas of formalin-fixed paraffin-embedded tumor tissue, which were then macrodissected. DNA was extracted by the proteinase K/phenol-chloroform method. Direct sequencing was performed to identify *POLE* exonuclease domain hot spot mutations in exon 9 (c.857C>G, p.P286R; c.890C>T, p.S297F), exon 13 (c.1231G>C, p.V411L) and exon 14 (c.1366G>C, p.A456P) and *KRAS* hot spot mutations in exon 2 (c.34G>T, p.G12C; c35G>A, p.G12D; c.37G>T, p.G13T; c.38G>A, p.G13D). PCR products were sequenced on an ABI3730xl Automatic DNA Sequencer at the Institute for Molecular Medicine Finland (FIMM). Sequence graphs were analyzed both manually and with Mutation Surveyor (Softgenetics, State College, PA, USA).

METHYLATION-SPECIFIC MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION (MS-MLPA)

MLH1 promoter methylation status in Deng promoter regions C and D was determined by MS-MLPA using the SALSA MMR MS-MLPA Kit ME011 (MRC-Holland) on 250ng of DNA from each sample. All MS-MLPA reactions, analyses, and calculations of methylation dosage ratios were done according to the manufacturer's instructions. Reaction products were separated by capillary electrophoresis on an ABI 3730 Automatic DNA sequencer (Applied Biosystems) and analyzed using GeneMapper 5.0 genotyping software (Applied Biosystems). We considered a promoter to show methylation if the methylation dosage ratio was >0.15, corresponding to 15% of methylated DNA, in either region C or region D or both.

STATISTICAL ANALYSIS

Data were analyzed using SPSS version 25 software (IBM Corp., Armonk, NY, USA). Statistical significance was set at $P < 0.05$ in all studies.

In Study I, logistic regression analysis was performed to compute odds ratios along with 95% confidence intervals (CIs) for the associations between L1CAM expression and various clinicopathological risk factors and relapse patterns. We applied the Kaplan-Meier method and log rank test to compare differences in disease-specific survival between patients with L1CAM-positive and -negative ECs. The association of L1CAM expression and disease-specific survival within ESMO-ESGO-ESTRO risk groups was assessed by univariate Cox regression analysis. Multivariable Cox proportional hazard model was applied to estimate the effects

of various risk factors on disease-specific survival. Multiple imputation was used to account for missing data.

In Study II, Cohen κ statistic was used to measure the concordance between L1CAM stainings in preoperative and hysterectomy samples of individual patients. Logistic regression was used to test for associations between L1CAM expression in preoperative samples and various categorical risk parameters. Differences regarding serum levels of sL1 (continuous variable) in patients with L1CAM-positive and negative tumors were analyzed using the Mann-Whitney U test. Logistic regression analysis estimated the effect (odds ratios) of selected parameters (L1CAM expression, tumor dimension, preoperative histotype, and myometrial invasion) on the risk of having advanced (stage IIIc/IV) disease. These odds ratios were rounded and summed to form risk scores according to alternative risk models. The discriminating abilities of the models were evaluated in two-tailed receiver operating characteristic curve analyses.

In Study III, Pearson χ^2 test and Fisher exact test (two-sided) were used for comparisons of PD-L1 expression and various categorical variables (e.g. histological and molecular subgroups, disease stage). Kaplan-Meier method and log rank test were applied to compare differences in disease-specific survival according to histological subtype, molecular subgroups, quantity of intratumoral T-cell infiltrates, and PD-L1 positivity. Cohen κ statistic was used to measure the concordance rate between PD-L1 scorings obtained by conventional chromogenic immunohistochemistry and multiplex fluorescent immunohistochemistry.

In Study IV, Pearson χ^2 test and Fisher exact test (two-sided) were used to investigate associations between MMR phenotype and various categorical variables (e.g. clinicopathological risk factors, immunological and various other biomarkers). The contribution of each risk factor to disease-specific survival was determined by simple and multivariable analyses by the Cox proportional hazard model. Kaplan-Meier method and log rank test were applied to compare differences in DSS according to MMR phenotype.

In Study V, Pearson χ^2 test and Fisher exact test (two-sided) were used to investigate associations between molecular subgroups (NSMP, MMRd) and categorical variables (e.g. clinicopathological risk factors and various biomarkers). Kaplan-Meier method and log rank test were applied to compare differences in disease-specific survival according to molecular group and single risk factors. Multivariable analyses by the Cox proportional hazard model were used to investigate the independent effect of individual factors within each molecular group. In order to test statistical significance of differential impacts across the molecular groups, interaction terms including molecular group and individual risk factors were introduced in a multivariable model adjusting for pertinent clinicopathological factors and the main effects of the interaction term.

RESULTS

Pertinent clinicopathological characteristics of the study cohort are shown in Table 5.

Table 5. Clinicopathologic data (n = 842).

Age (years, mean ± SD)	67.5 ± 10.6
Pelvic lymphadenectomy (number of cases, %)	469 (55.7)
Pelvic-aortic lymphadenectomy (number of cases, %)	125 (14.8)
Histology (number of cases, proportion)	
Endometrioid carcinoma	745 (88.5)
Clear cell carcinoma	35 (4.2)
Serous carcinoma	29 (3.4)
Undifferentiated carcinoma	15 (1.8)
Carcinosarcoma	17 (2.0)
Neuroendocrine carcinoma	1 (0.1)
Grade (number of cases, percent), endometrioid only	
Grade 1	425 (57.0)
Grade 2	209 (28.1)
Grade 3	111 (14.9)
Molecular classification [a] (number of cases, proportion)	
*POLE*mut	37 (7.2)
MMRd	191 (37.1)
NSMP	218 (42.3)
p53ab	69 (13.4)
FIGO 2009 stage (number of cases, proportion)	
IA	458 (54.4)
IB	177 (21.0)
II	57 (6.8)
IIIA	39 (4.6)
IIIB	7 (0.8)
IIIC1	48 (5.7)
IIIC2	26 (3.1)
IVA	0 (0)
IVB	30 (3.6)
Adjuvant therapy (number of cases, proportion)	
No adjuvant therapy	122 (14.5)
Vaginal brachytherapy	408 (48.5)
Whole pelvic radiotherapy	121 (14.4)
Chemotherapy	84 (9.9)
Chemotherapy and whole pelvic radiotherapy	107 (12.7)
Cancer recurrence	
No	652 (77.4)
Yes	190 (22.6)

[a] only cases with all molecular data available (n=515)

STUDY I: L1 CELL ADHESION MOLECULE AS A PREDICTOR OF DISEASE-SPECIFIC SURVIVAL AND PATTERNS OF RELAPSE IN ENDOMETRIAL CANCER

In the whole cohort, 15% (121/805) of the tumors were L1CAM-positive (defined as ≥10% of the carcinoma cells). We found a statistically significant association between L1CAM expression and the presence of several poor prognostic variables (advanced stage, nodal dissemination, high-grade endometrioid/non-endometrioid histology, LVSI, cervical stromal invasion, positive peritoneal cytology, and age >65 years). L1CAM expression predicted poor disease-specific survival in endometrioid EC but not in non-endometrioid EC (Figure 4). Multivariable survival analysis confirmed the role of L1CAM as an independent poor prognostic factor after controlling for all principal clinicopathologic risk factors. During the median follow-up time of 51 (range 1-98) months, a relapse was diagnosed in 11.2% (68/606) of the patients with stage I cancer. Distant (extra-abdominal) metastases were more frequent in L1CAM-positive than L1CAM-negative stage I endometrioid carcinomas (P<0.0001). The frequency of other types of metastasis (vaginal, retroperitoneal and intraperitoneal) did not significantly differ between L1CAM-positive and -negative cases.

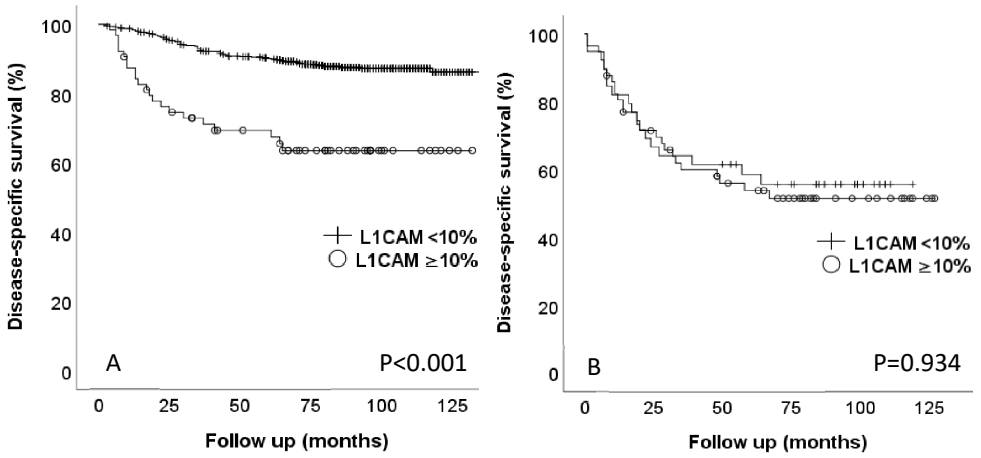

Figure 4. Kaplan-Meier curves for disease-specific survival according to L1CAM expression in A) endometrioid EC (n=735) and B) non-endometrioid EC (n=70).

STUDY II: PREOPERATIVE RISK STRATIFICATION OF ENDOMETRIAL CARCINOMA: L1CAM AS A BIOMARKER

Of the 241 preoperative EC samples, 64 (26.6%) were L1CAM-positive. L1CAM positivity was more frequent in G3 endometrioid (27.6%) and non-endometrioid (68.4%) carcinomas than in G1-2 endometrioid carcinoma (22.3%, P < 0.0001). L1CAM expression in paired pre- and postoperative carcinoma samples showed moderate concordance (as defined by Landis and Koch, kappa=0.586, P < 0.0001) [125].

Preoperative L1CAM positivity correlated with the presence of negative prognostic factors (non-endometrioid histology, lymph node involvement, older age, advanced stage and positive peritoneal cytology). Integrating L1CAM into risk assessment models based on histotype, myometrial invasion and/or tumor diameter did not significantly improve the ability of the models to predict advanced stage (Table 6). Tumoral expression of L1CAM and serum levels of L1 did not show significant correlation (P = 0.786).

Risk assessment model	AUC (95% CI)	P (two-tailed)
1. HRH-TD5cm-MI33%-L1CAM	0.879 (0.828-0.930)	
2. HRH-TD5cm-MI33%	0.870 (0.813-0.928)	0.882 vs. model 1
3. HRH-TD5cm-L1CAM	0.852 (0.778-0.925)	
4. HRH-TD5cm	0.818 (0.730-0.906)	0.613 vs. model 3
5. HRH-TD2cm-MI50%-L1CAM	0.841 (0.777-0.905)	
6. HRH-TD2cm-MI50%	0.805 (0.717-0.894)	0.602 vs. model 5
7. HRH-MI50%-L1CAM	0.833 (0.770-0.896)	
8. HRH-MI50%	0.759 (0.659-0.859)	0.289 vs. model 7

Table 6. Areas under curve (AUC) for risk models predicting stage IIIC–IV endometrial carcinoma. HRH=high risk histology (endometrioid G3/non-endometrioid), MI=myometrial invasion, L1CAM=L1 cell adhesion molecule, TD=tumor diameter.

STUDY III: PD-L1 EXPRESSION IN ENDOMETRIAL CARCINOMA CELLS AND INTRATUMORAL IMMUNE CELLS: DIFFERENCES ACROSS HISTOLOGIC AND TCGA-BASED MOLECULAR SUBGROUPS

In our unselected cohort of ECs, PD-L1 positivity (≥1% of the cells) was more frequent in immune cells (27.7% of the cases) than in carcinoma cells (8.6%). CPS ≥ 1% was detected in 19.4% of the samples.

Molecular, histological, and disease stage-based subgroups of EC showed significant differences in PD-L1 expression profiles. Among the TCGA subgroups, *POLE*mut and MMRd tumors exhibited higher rates of PD-L1 expression determined by immune cell score and CPS (Figure 5) and were more likely to display the PD-L1 inflamed phenotype (PD-L1 expression associated with dense T-cell infiltrates, P<0.001). No differences were found between the subgroups regarding PD-L1 expression in carcinoma cells. Within histological subgroups, non-endometrioid carcinomas presented more frequent PD-L1 positivity in immune cells and CPS. Relative to early-stage disease, advanced carcinomas were more likely to display PD-L1 positivity in carcinoma cells and CPS.

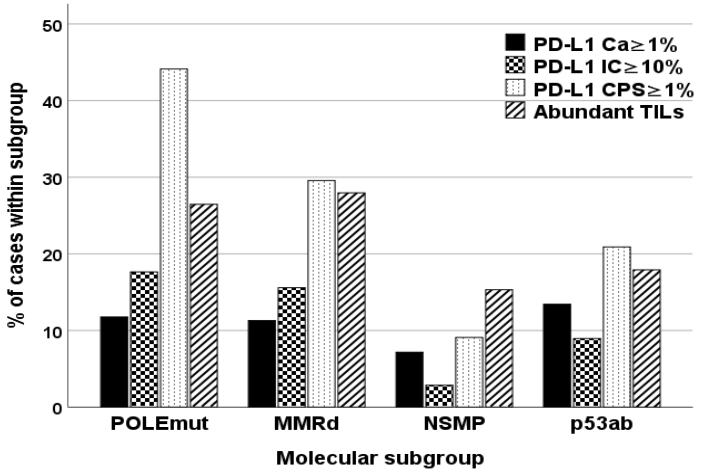

Figure 5. Frequency of PD-L1 positivity in carcinoma cells (p=0.366), ICs (p<0.001), CPS (p<0.001) and presence of heavy T cell infiltrates (p=0.014) according to molecular subgroups. POLEmut = mutated *POLE*, MMRd= MMM deficient, NSMP = no specific molecular type, p53ab = p53 aberrant. Ca=carcinoma cells, ICs=immune cells, CPS=combined positive score, TILs=tumor infiltrating lymphocytes. With permission from Am J Surg Pathol.

PD-L1 expression did not correlate with outcome. In the concordance analysis between multiplex IHC and conventional chromogenic IHC, CPS outperformed the scoring system based on carcinoma cell positivity, which requires discerning positive carcinoma cells and immune cells (κ=0.540 for CPS and κ=0.279 for carcinoma cell scoring). This result indicates that when using conventional chromogenic IHC for PD-L1 scorings, CPS provides more accurate scorings.

STUDY IV: CLINICOPATHOLOGICAL SIGNIFICANCE OF DEFICIENT DNA MISMATCH REPAIR AND *MLH1* PROMOTER METHYLATION IN ENDOMETRIOID ENDOMETRIAL CARCINOMA

In our cohort of 682 unselected endometrioid ECs, 244 (35.8%) of the cases presented loss of MMR protein expression determined by IHC. The frequencies of specific patterns of protein loss were as follows: MLH1+PMS2 in 29.8%, PMS2 in 0.9%, MSH2+MSH6 in 1.3%, MSH6 in 2.8%, and multiple abnormalities in 0.9% of cases. Of the 244 MMRd ECs, approximately 75% were associated with *MLH1* promoter methylation.

We found an association between MMR deficiency and older age, high grade of differentiation (G3), advanced stage (II-IV), larger tumor size, abundant tumor infiltrating lymphocytes, PD-L1 positivity in immune cell score and CPS, p53wt, negative L1CAM, and ARID1a loss. MMRd-Met phenotype correlated with older age and larger tumor size and predicted poor disease-specific survival in the whole cohort (Figure 6). In the MMRd subgroup, significant survival differences were not found between patients with methylated tumors and those with non-methylated tumors. Univariate analysis restricted to MMRd cases showed significant correlations between poor disease-specific survival and disease stage II-IV, high grade (G3), deep myometrial invasion, LVSI, tumor size ≥ 2cm, positive peritoneal cytology, ER negativity, and L1CAM positivity.

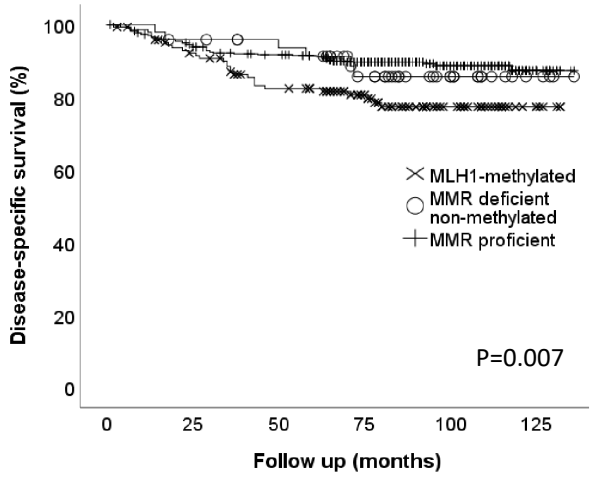

Figure 6. Kaplan-Meier curves for disease-specific survival according to MMR phenotype.

MMR=mismatch repair

STUDY V: DIFFERENTIAL IMPACT OF CLINICOPATHOLOGICAL RISK FACTORS WITHIN THE TWO LARGEST TCGA-RELATED MOLECULAR SUBGROUPS OF ENDOMETRIAL CARCINOMA

Patients with MMRd EC were more likely to be >65 years of age and their tumors were more frequently high grade, PR-negative and E-cadherin-negative than patients with NSMP EC. NSMP cases more frequently showed nuclear beta-catenin positivity.

In Kaplan-Meier analysis, disease-specific survival curves according to TCGA-based molecular subgroups segregated expectedly; *POLE*mut cases had an excellent outcome, followed by NSMP and MMRd, with p53abn presenting the worst prognosis (P=0.001). In multivariable analysis adjusted for stage, grade, myometrial invasion, LVSI, and adjuvant treatment, the effect of the molecular class (NSMP vs MMRd) became non-significant (P=0.101).

In subgroup-specific Cox regression analysis adjusted for confounders, high grade (G3) and p16 hyperexpression remained significant predictors of poor disease-specific survival in NSMP. In the MMRd group, advanced disease stage, deep myometrial invasion, LVSI, and loss of E-cadherin were independent predictors. In the interaction analysis, the effect of G3 and p16 was significantly modified by the molecular subgroup with a stronger effect in the NSMP group (P=0.016 and P=0.033 for the interaction term, respectively) than in the MMRd group. Univariate disease-free survival curves according to molecular subgroup, grade of differentiation and p16 expression are depicted in Figure 7.

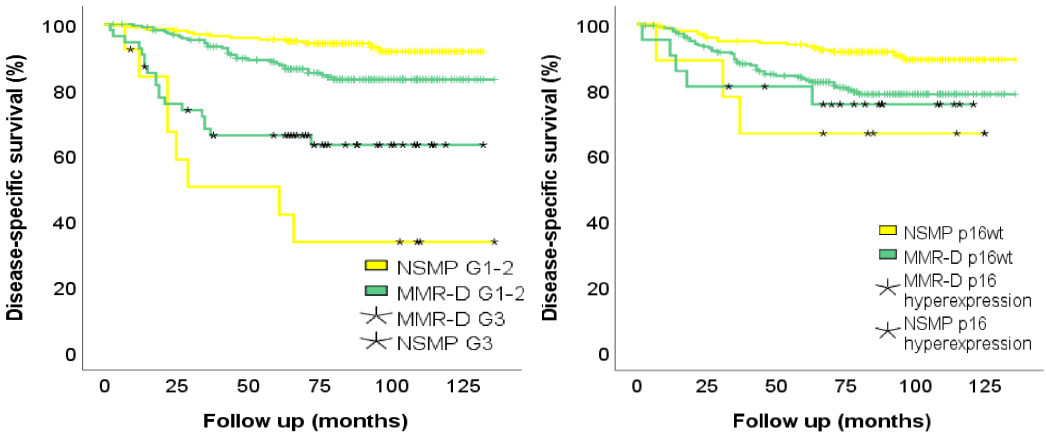

Figure 7. Kaplan-Meier curves for disease-specific survival according to TCGA-based molecular subgroups, grade of differentiation (left), and p16 expression (right). Yellow=NSMP; green=MMRd, wt=wild type.

DISCUSSION

The goal of personalized medicine is to offer optimal treatment to each patient. Targeted management of EC requires accurate risk stratification methods, which will reduce under and overtreatment in both operative and postoperative settings. Knowledge of the molecular events potentially involved in the pathogenesis of individual carcinomas may also facilitate the selection of a specific treatment modality. This study explores the prognostic value of L1CAM as both a pre- and postoperative marker. Further, we investigated the prognostic impact of single risk factors across the two largest TCGA-based subgroups (NSMP and MMRd) and the significance of *MLH1* methylation status in MMRd EC. Lastly, we profiled the expression of PD-L1, an immunotherapy target molecule, across histological and TCGA subclasses.

L1CAM IN PREOPERATIVE RISK ASSESSMENT OF ENDOMETRIAL CARCINOMA

As patients with clinically early-stage EC appear not to benefit from routine lymphadenectomy, biomarkers discerning patients with a high risk of nodal metastasis could help avoid unnecessary radical surgery with a significant complication risk [1, 2]. Currently the decision on lymphadenectomy is mainly based on the estimated depth of myometrial invasion, presence of cervical stromal invasion, and tumor histotype determined by an endometrial biopsy or curettage specimen.

With regard to histotype, the concordance between preoperative samples and final hysterectomy samples is only moderate [5]. In our whole study cohort, 12.7% of the preoperative low-risk (G1-2 endometrioid carcinoma) cases were upgraded to high-risk histology in postsurgical assessment (unpublished data). Similarly, 19.0% of the preoperative high-risk histotypes were downgraded in hysterectomy samples (unpublished data). In the population of Study II, the concordance between preoperative and postoperative assessment of L1CAM positivity was slightly superior to that of high-risk histotype (κ =0.586 and κ =0.551, respectively). By comparison, an earlier study found excellent agreement (κ=0.86) between pre- and postoperative samples categorized by ProMisE molecular classifier [126]. We observed a higher frequency of L1CAM positivity in preoperative samples (26.6%) than in hysterectomy samples (15% in the TMA). Our TMA-based result is comparable to the prevalence of L1CAM positive tumors (17%) observed in a large non-TMA study including ECs of all stages and histotypes [117]. This finding suggests that biopsy-related sampling error may have caused overestimation of L1CAM positive cases.

Finally, we investigated L1CAM positivity in preoperative EC samples regarding its capability to predict the need for lymphadenectomy. We found an association between preoperative tumoral L1CAM positivity and nodal metastases, which has been confirmed in a later study [127]. However, incorporating L1CAM into conventional preoperative stratification models did not improve the capacity of the model to predict advanced disease. Further studies are needed to explore the value of L1CAM within molecular-based stratification methods should these prove to be superior to conventional preoperative algorithms.

L1CAM IN POSTOPERATIVE RISK ASSESSMENT OF ENDOMETRIAL CARCINOMA

In the postoperative setting, further stratification of patients is needed to select appropriate adjuvant treatment. At present, risk assessment is based on disease stage and histotype, grade, and presence or absence of LVSI in early-stage disease. As histotype, grading and LVSI suffer from limited reproducibility, molecular markers may offer more objective tools for this stratification [59, 61, 128].

Our results corroborated the findings of previous studies where L1CAM appeared to be a strong prognostic factor in EC [114-116]. L1CAM positivity was more frequent in non-endometrioid (53.5%) than endometrioid (10.4%) subtype of EC. Nonetheless, our subgroup analysis and the study by Van der Putten et al. [117] demonstrated a prognostic effect only in endometrioid EC, not in non-endometrioid cases. Given the compelling data on L1CAM, it has been incorporated into the molecular treatment algorithm of the ongoing PORTEC-4 trial, which also includes TCGA-based molecular classes [129]. In the trial algorithm applied on early-stage ECs, L1CAM is considered a high-risk feature along with LVSI and p53abn.

L1CAM is a membrane glycoprotein that belongs to the immunoglobulin superfamily and its membranous form functions as a cell surface adhesion molecule [130]. In addition, cleavage enzymes and exosomes can release the ectodomain of L1CAM from cell surface into the surrounding matrix, where it appears to promote cell migration [130] Soluble L1 has been detected in serum samples from cancer patients, but not in samples from healthy subjects, suggesting a potential role of L1 as a diagnostic or follow-up marker [131]. In our cohort, the concentrations of soluble L1 in the serum samples did not correlate with tumoral L1CAM status.

DIFFERENTIAL IMPACT OF RISK FACTORS WITHIN TCGA-BASED MOLECULAR SUBCLASSES OF ENDOMETRIAL CARCINOMA

In 2013, TCGA established four new pathogenetically and prognostically distinct molecular subclasses of EC. Later studies have demonstrated significant differences between the groups regarding response to adjuvant radiotherapy, chemotherapy, expression of immunotherapy target molecules, and homologous recombination deficiency [77, 78, 94, 95, 98, 105, 132, 133]. These findings suggest that molecular subgroups should be taken into account when selecting adjuvant treatment.

The present study corroborated the findings of the original TCGA study and later related studies, where the prognosis was excellent in the *POLE*mut group, intermediate in the NSMP and MMRd groups, and poor in the aggressive p53abn subgroup of EC [8, 79, 80, 134]. In a previous meta-analysis including six TCGA classification-based studies, the relationship between molecular group and survival appeared to be independent of clinicopathological risk factors in the POLEmut group [81]. By contrast, the negative effect of MMRd (compared with NSMP) was related to clinicopathological risk factors, which are more frequently present in this subgroup [80, 81]. P53abn remained an independent predictor of survival after adjustment for other factors, but the effect was aggravated by the frequent presence of other negative prognostic factors. In this cohort, a similar result was seen, as the impact of the molecular group (MMRd vs NSMP) became insignificant after adjustment for the main clinicopathological risk factors.

Optimal treatment algorithms will presumably integrate molecular classifiers with selected clinicopathological factors. The value of single risk factors, including ancillary molecular markers, will need to be re-examined within the context of molecular subgroups. Along with TCGA subclasses and L1CAM, the ongoing PORTEC-4 trial has incorporated *CTNNB1* (β-catenin) in the treatment algorithm [129]. Our results suggest that also p16 expression may have prognostic value in NSMP EC. In a previous study on ovarian carcinomas, strong and diffuse (block type) p16 hyperexpression appeared to predict poor survival in endometrioid and clear cell carcinoma and both hyperexpression and complete absence of p16 expression (as opposed to heterogeneous staining) were associated with poor survival in low-grade serous carcinoma [135]. Prognostic significance of these various staining patterns is unknown in EC. As p16 negativity was extremely rare (1.7%) in our samples and it did not improve the ability to discern outcomes, we only considered strong and diffuse p16 hyperexpression as an abnormal staining result.

Given the heterogeneity of the molecular EC subclasses, ideal risk assessment algorithms may differ between the molecular subgroups. Therefore, molecular group-specific research efforts are needed to clarify the relevance of individual risk factors within each subgroup. To provide proof-of-concept data for future studies, we examined the impact of established risk

factors and ancillary biomarkers within NSMP and MMRd subclasses of EC. The observed interaction effect between molecular class and single risk factors demonstrates the importance of treating molecular subclasses of EC as separate disease entities, within which the relative weights of various risk factors need to be determined. Multi-cohort studies with higher statistical power may help reveal ulterior interaction effects between molecular groups and other factors.

SIGNIFICANCE OF *MLH1* METHYLATION STATUS IN MMRD ENDOMETRIAL CARCINOMA

MMRd carcinomas may arise from two distinct pathogenetic pathways, i.e. methylation or mutation of MMR genes. Lynch syndrome-associated EC is typically driven by mutation. The majority of sporadic cases are associated with *MLH1* promoter methylation, although biallelic somatic mutations occur. Future studies will establish whether the *MLH1* methylated phenotype of EC is a manifestation of a general hypermethylation tendency (CpG island methylator phenotype), as described for colorectal carcinoma [136]. In MMRd colorectal carcinoma, differences between Lynch syndrome and sporadic cases have been reported as regards histology, molecular features, and (possibly age-dependent) prognostic effect [137-140]. Little is known about the clinicopathological significance of the pathogenetic processes underlying the MMRd phenotype in EC.

In this study cohort, methylated MMRd phenotype correlated with older age and larger tumor size. Methylation-linked MMRd independently predicted poor disease-specific survival compared with MMR intact EC, but we did not find significant survival differences between methylated and non-methylated MMRd EC. In previous studies, *MLH1* methylated EC appeared to present weaker tumoral T-cell inflammation than mutation-linked (hereditary) MMRd EC [108, 141]. In our study, a higher quantity of tumor infiltrating T-lymphocytes and PD-L1 expression correlated with MMR deficiency in general, but not with *MLH1* methylation status. Further studies are needed to explore eventual correlations between MMR and *MLH1* methylation status and sensitivity to adjuvant therapies.

IMMUNOTHERAPY IN ENDOMETRIAL CARCINOMA: PD-L1

Promising results have been obtained from phase I-II clinical trials investigating the use of anti PD-1/PD-L1 agents in advanced EC. Overall response rates of 13-57% have been reported with mild adverse effects (e.g. fatigue, pruritus, pyrexia, and anorexia) [75, 142, 143]. Several ongoing phase I-II trials for advanced or recurrent EC investigate different anti

PD-1 agents in monotherapy (NCT02628067, NCT02899793, NCT02728830, NCT03241745, NCT03474640, NCT02715284). Future studies will also clarify whether the therapeutic effect can be enhanced by combining various modalities of immunotherapy, chemotherapy and radiotherapy. In fact, an ongoing trial is investigating the combination of anti PD-1 with traditional chemotherapy (NCT02549209) and anti PD-L1 agent with an anti CTLA-4 antibody (NCT03015129). Two recruiting phase III trials are exploring the combination of anti-PD-L1 therapy with the conventional chemotherapy treatment (NCT03914612, NCT03981796).

Cancer trials have demonstrated a correlation between PD-L1 expression levels and the efficacy of anti-PD-1/PD-L1 immunotherapy in various cancer types [88, 144, 145]. In trials conducted mainly on non-endometrial cancer types, response rates to pembrolizumab (anti-PD1 agent) appear to correlate with PD-L1 expression levels in carcinoma cells, whereas the response to atezolizumab (anti-PD-L1 agent) appears to correlate with PD-L1 positivity on immune cells rather than carcinoma cells [88, 145]. The reported prevalence of PD-L1 positivity in EC varies considerably between studies (0.9% to 48% regarding expression in carcinoma cells). Along with different cut-offs for IHC, different antibody clones explain some of the variation since considerable interassay variation has been reported [146, 147]. Further, conventional chromogenic IHC for PD-L1 suffers from limited reproducibility especially with cut-offs for positivity as low as 1% [92]. This problem is related to the difficulty of discerning PD-L1 positive carcinoma cells from PD-L1-positive immune cells (mainly macrophages). To overcome this limitation, we adopted multiplex IHC, which provides cell type-specific staining of PD-L1, leading to more accurate scores. As observed in our study and others [95, 96, 99], intratumoral immune cells display more frequent (27.7%) PD-L1 positivity than carcinoma cells (8.6%). In particular, strong positivity in carcinoma cells was rare (0.5%). Further, we performed concordance analysis between conventional and multiplex IHC using various scoring methods, i.e. separate scoring of carcinoma cell and immune cell positivity and combined positivity. Combined scoring showed better concordance, suggesting it may improve reproducibility of conventional IHC scorings, as multiplex IHC is often not available in routine diagnostics. As optimal scoring methods and cut-offs may be tumor type-specific, future studies should consider various scoring methods when estimating the response rates of EC patients receiving immunotherapy.

Clinical trials have reported particularly promising (>50%) overall response rates to immune check-point inhibition in patients with MMRd tumors, including MMRd EC [74–76, 142]. MMRd tumors are considered highly immunogenic due to their elevated mutational frequency and the abundance of predicted neoantigens and T-cell infiltration. An analogous immunological microenvironment has been observed in *POLE*mut EC [94]. Our study corroborated these findings, as we observed a T-cell-inflamed PD-L1-positive phenotype more frequently in the above molecular subgroups. As *MLH1* methylation status of MMRd tumors did not correlate with immunological features, methylation analysis may not provide further indications for immunotherapy. Due to their favorable prognosis, patients with

*POLE*mut EC may not require adjuvant therapy. The need for alternative treatment options is more obvious in the aggressive non-endometrioid ECs, where PD-L1 positivity was more frequent (in immune cells and CPS) than in endometrioid EC.

STUDY STRENGTHS AND LIMITATIONS

Our large and unselected study cohort includes patients with ECs of all histotypes and disease stages. Histological diagnosis was reviewed by an experienced gynecopathologist. Patients were treated in a single center with well-defined diagnostic and operative standards. The relatively high lymphadenectomy rate (70.7%) reduced the risk of understaging due to occult nodal disease. Follow-up data were adequately updated and follow-up times were long. As in any retrospective study, selection bias may have been produced by data collection from a single tertiary referral center. However, the principal clinicopathological characteristics of the cohort were comparable to other large unselected cohorts, excluding substantial sampling bias [148, 149]. Comprehensive clinicopathological data enabled multivariable regression models to control for confounding at the analysis stage. Exhaustively documented causes of death allowed us to measure disease-specific survival instead of the less specific overall survival. In an EC study this is particularly important since the prognosis of the disease is relatively good, but the patients are often elderly and have critical comorbidities. In fact, in EC studies investigating overall survival rather than disease-specific survival, approximately half of the deaths are attributed to causes other than EC [150].

TMA-based methodology allowed us to perform numerous immunohistochemical stainings on a large number of samples. Our TMA included tumor samples from 87.3% of the patients forming the unselected study cohort. Remaining cases were mainly discarded due to tumor size too small to biopsy. Thus, the proportion of early stage (stage Ia) disease resulted slightly underestimated. The challenge with the TMA method lies in the difficulty of assessing heterogeneous stainings. However, it has been shown that TMAs based on three core biopsies provide representative results in over 95% of the cases even for heterogeneous stainings [151-153]. To increase sensitivity, specificity, and reproducibility, we included four separate cores from each tumor in our TMA. κ statistics confirmed a high concordance between our TMA and corresponding whole-section staining results with regard to a notoriously heterogeneous antigen, L1CAM.

FUTURE PROSPECTS

Given the limitations of current risk stratification methods and the compelling results reported by various research groups for TCGA classification-based molecular methods, molecular biomarkers will presumably be incorporated into the risk stratification algorithms of EC. The combination of conventional risk factors and ancillary molecular markers forming the optimal treatment algorithm may differ between TCGA molecular classes. Thus, future studies will need to consider potential modifying effects that molecular subgroups exert on the impact of single risk factors. In order to achieve high statistical power, molecular subgroup-specific analyses necessitate large sample sizes, which could be provided by multi-cohort studies.

Whether performing molecular classification preoperatively offers advantages over postoperative testing, remains to be established. Further, the relationship between TCGA molecular classes and sensitivity to chemotherapy, radiotherapy and immunotherapy needs to be assessed in prospective trials. Similarly, further studies investigating the role of methylation status in predicting treatment responses in MMRd EC are warranted. Whether the excellent prognosis of *POLE*mut EC is attributed to the indolent nature of the disease or to higher sensitivity to adjuvant treatment must be clarified in order to omit potentially unnecessary therapies. Without a doubt, molecular profiling of EC has introduced a new era of precision medicine.

CONCLUSIONS

This study explored molecular biomarkers that could be used for prognostication and personalized treatment planning of EC. The results can be summarized as follows:

I Tumoral L1CAM expression is associated with the presence of aggressive features and occurrence of distant relapses in EC. L1CAM expression independently predicts poor survival in endometrioid EC, but not in non-endometrioid EC.

II L1CAM positivity detected in a preoperative EC sample is associated with the presence of negative prognostic factors including lymph node metastases. However, integrating L1CAM into conventional risk models does not improve the ability to stratify patients to lymphadenectomy.

III *POLE*mut and MMRd present more frequent PD-L1 positivity and abundant T-cell infiltrates compared with other TCGA subgroups of EC. Differential PD-L1 expression in molecular subclasses of EC suggests that in future studies the response to immunotherapy should be examined in a subclass-specific manner. A combined positive scoring method of chromogenic PD-L1 stainings may be more accurate than cell type-specific scoring methods.

IV In endometrioid EC, the MMRd phenotype correlates with the presence of conventional risk factors (older age, high grade, advanced stage, larger tumor diameter). *MLH1* methylation predicts poor disease-specific survival compared with MMR intact EC; however, within MMRd ECs, methylation status does not have a significant effect on disease-specific survival. Methylation status does not correlate with immunological features.

V Molecular subgroups modify the prognostic effect of single risk factors. Subgroup-specific studies are needed to determine the relative impact of each factor in order to formulate an optimal treatment algorithm for each molecular class of EC.

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