Molecular alterations in endometrial cancer: implications for clinical management

Stelloo, E.

Chapter 4

Improved risk assessment by integrating molecular alterations and clinicopathological factors in the PORTEC endometrial cancer trials cohort

Stelloo E, Nout RA, Osse EM, Jürgenliemk-Schulz IJ, Jobsen JJ, Lutgens LC, van der Steen-Banasik EM, Nijman HW, Putter H, Bosse T, Creutzberg CL, Smit VT

Clin Cancer Res. 2016; 22(16): 4215-24

Abstract

Purpose: Recommendations for adjuvant treatment for women with early-stage endometrial carcinoma (EC) are based on clinicopathological features. Comprehensive genomic characterization defined four subgroups: p53-mutant, microsatellite instability (MSI), *POLE*-mutant and no specific molecular profile (NSMP). We aimed to confirm the prognostic capacity of these subgroups in large randomized trial populations, investigate potential other prognostic classifiers, and integrate these into an integrated molecular risk assessment guiding adjuvant therapy.

Experimental design: Analysis of MSI, hotspot mutations in 14 genes including *POLE*, protein expression of p53, ARID1a, β-catenin, L1CAM, PTEN, ER, and PR was undertaken on 947 available early-stage endometrioid ECs from the PORTEC-1 and -2 trials, mostly high-intermediate risk (n=614). Prognostic value was determined using univariable and multivariable Cox proportional hazard models. Areas under the curve of different risk stratification models were compared.

Results: Molecular analyses were feasible in >96% of the patients and confirmed the four molecular subgroups: p53-mutant (9%), MSI (26%), *POLE*-mutant (6%), and NSMP (59%). Integration of prognostic molecular alterations with established clinicopathological factors resulted in a stronger model with improved risk prognostication. Approximately 15% of high-intermediate risk patients had unfavorable features (substantial LVSI, p53-mutant, and/or >10% L1CAM), 50% favorable features (*POLE*-mutant, NSMP being microsatellite stable and *CTNNB1*-wild type), and 35% intermediate features (MSI or *CTNNB1*-mutant).

Conclusions: Integrating clinicopathological and molecular factors improves the risk assessment of patients with early-stage EC. Assessment of this integrated risk profile is feasible in daily practice, and holds promise to reduce both over- and undertreatment.

Introduction

Endometrial cancer (EC) is the most common gynecological cancer in developed countries.[1] Over 50% of women with EC present with early-stage, low-risk disease, and are treated with surgery alone.[2] Adjuvant therapy recommendations are based on the individual patient's risk of disease recurrence using clinicopathological factors such as age, stage, histological subtype, tumor grade, and lymphovascular space invasion (LVSI).[3] EC patients are generally stratified in three risk groups; however, various definitions exist.[4-6] The PORTEC-1 and -2 (PostOperative Radiation Therapy for EC) clinical trials have contributed evidence that adjuvant radiotherapy can be safely omitted in patients with low-intermediate risk features, and that EC patients with high-intermediate risk features can effectively be treated with vaginal brachytherapy.[4,7] Despite this clinicopathological risk stratification considerable over- and undertreatment remains: seven patients with stage I high-intermediate risk EC need to receive vaginal brachytherapy to prevent one recurrence, while 8% of patients develop distant metastases that may have been prevented or delayed with adjuvant chemotherapy. We hypothesized that the clinicopathological risk assessment might be improved by integration of molecular biomarkers predictive of individual tumor behavior.

Many studies addressing the prognostic significance of molecular alterations in EC have focused on one or two biomarkers.[8,9] Integrated genomic characterization by The Cancer Genome Atlas (TCGA) defined four distinct EC subgroups with possible prognostic value.[10] Using methods broadly available in clinical practice these four subgroups can be easily determined by their surrogate markers: p53, microsatellite instability (MSI), and *POLE* resulting in a practically and clinically useful molecular classification tool.[11,12] In relatively small series of unselected ECs, the combination of both the clinicopathological and molecular classification improved the clinicopathological risk assessment.[12] At present it is unclear how other potential molecular prognosticators, such as mutations in *CTNNB1*, *PIK3CA* and L1CAM overexpression should be integrated in the suggested TCGA subgroups.

The aims of this study were to confirm and validate the prognostic significance of the proposed molecular classification tool in early-stage endometrioid ECs (EECs), mainly high-intermediate risk, from two large randomized trials (PORTEC-1 and -2) with mature long-term follow-up data and to investigate whether incorporation of other molecular alterations and established clinicopathological risk factors will result in an improved risk assessment.

Methods

Patients and study design

For both PORTEC-1 and -2 trials central pathology review was undertaken, during which formalin-fixed paraffin-embedded (FFPE) tumor material was collected. All tumor samples with confirmed endometrioid histology were included in the current analysis. The design and clinical results of both randomized trials have been published previously.[4,7] In brief, PORTEC-1 (1990-1997) included 714 patients with stage I EC, grade 1 or 2 with deep myometrial invasion, or grade 2 or 3 with superficial invasion. PORTEC-2 (2000-2006) included 427 EC patients with high-intermediate risk features: stage I, age >60 years, grade 1-2 with deep invasion or grade 3 with superficial invasion and stage IIA disease (except grade 3 with deep invasion). The PORTEC study protocols were approved by the Dutch Cancer Society and the medical ethics committees at participating centers. All patients provided informed consent. Data on patient and tumor characteristics, including results of pathology review and outcome, were obtained from the trial databases. The presence of substantial LVSI, diffuse or multifocal LVSI around the tumor, was evaluated and previously reported.[13] The REMARK criteria were followed, wherever possible, throughout this study.[14]

Procedures

For immunohistochemical analyses, all slides were evaluated by two investigators and a gyneco-pathologist, blinded for patient characteristics and outcome. Evaluations were done independently with discrepancies resolved at simultaneous viewing. For DNA analyses, tumor DNA was isolated as previously reported.[11]

p53 expression, MSI, and *POLE* exonuclease domain mutation status were assessed, as described previously, to identify the four molecular EC subgroups.[11] In short, immunohistochemical expression of p53 (clone DO-7, 1:2000; Neomarkers) was scored positive if >50% of the tumor cells showed a strong positive nuclear staining, or when discrete geographical patterns showed >50% tumor cell positivity. Tumors in which no p53 staining of the tumor was observed and cases with only DNA present (n=119) were sequenced for exon 5-8 *TP53* mutations.[15] The MSI status was determined using the Promega MSI analysis system (version 1.2). Tumors with instability in at least two markers were defined as being MSI whereas those showing no instability were classified as being stable (MSS). Tumors in which instability at one repeat was observed or MSI status could not be determined due to poor DNA quality (n=121) were stained manually for the mismatch repair proteins MLH1 (clone ES05, 1:100; DAKO), MSH2 (clone FE11, 1:200, DAKO), MSH6 (clone EPR3945, 1:800, Genetex), and PMS2 (clone EP51, 1:75, DAKO).[11] Both methodologies, MSI assay and mismatch repair protein expression, are highly sensitive methods for the identification of a defective DNA mismatch repair system.[16] Tumors were then considered MSI if tumor cells showed loss of nuclear staining of at least one of the mismatch repair proteins, and MSS if tumor cells showed nuclear positivity for

all mismatch repair proteins. *POLE* exonuclease domain hotspot mutations (named *POLE* mutations throughout this paper) were detected by Sanger sequencing of exon 9 and 13. KASPar competitive allele specific PCR (LGC Genomics) assays were used to screen for *POLE* variants at codons 286, 297 and 411 in tumors with poor DNA quality (n=98, primer sequences are available upon request). Part of these results were previously published.[17]

To assess mutations in other frequently altered genes in EC, we used the Sequenom MassARRAY system and the GynCarta multigene analysis 2.0 (Sequenom) to test for 159 hotspot mutations in *BRAF*, *CDKNA2*, *CTNNB1*, *FBXW7*, *FGFR2*, *FGFR3*, *FOXL2*, *HRAS*, *KRAS*, *NRAS*, *PIK3CA*, *PPP2R1A*, *PTEN* as described previously.[15] Further immunohistochemical analyses were performed for estrogen receptor (ER), progesterone receptor (PR; clone PGR636, 1:200; DAKO), PTEN (clone 6H2.1, 1:200; DAKO), β-catenin (clone 14, 1:1600; BD transduction), and ARID1a (clone PSG-3, 1:800; Santa Cruz) expression. Immunohistochemical procedures were as described previously except for ER expression analysis (clone EP1, DAKO, 1:100, Tris-EDTA pH 9.0, 3,3'-diaminobenzidine+).[11,15] ER and PR were scored positive when at least >10% of tumor cells showed nuclear expression. PTEN, β-catenin, and ARID1a staining were evaluated as described previously.[11,15] In short, PTEN staining was evaluated in three categories as negative, positive and heterogeneous. Activated Wnt-signaling was defined as nuclear staining of ß-catenin. ARID1a was scored as negative, weak positive or strong positive nuclear staining or as 'clonal loss'. Previously published results of immunohistochemical L1CAM expression (clone 14.10, 1:500; Covance Inc.) on the same patients in this study were integrated for analysis.[18] Tumors with >10% positive tumor cells were considered L1CAM positive.

Statistical analysis

Associations between clinicopathological features and molecular alterations were tested using Chi-square statistics or Fishers exact test in case of categorical and t test or analysis of variance (ANOVA) for continuous variables. Time-to-event analyses were calculated from the date of randomization to date of recurrence (vaginal and/or pelvic for locoregional recurrence, and distant metastases for distant recurrence) or to date of endometrial cancer death (disease specific survival) or to date of death (overall survival) or to date of any recurrence or death (recurrence-free survival); patients who were alive and without recurrence were censored at the date of last follow-up. Survival curves were calculated using the Kaplan–Meier method with log-rank test. Cox proportional hazards models were used to evaluate the prognostic value of each factor. Factors with *P*-values <0.10 were included in a multivariable Cox model with a stepwise method to include in the final model. In the last step, significant factors from the forward selection model (*P*-values <0.05) were included in a final Cox model together with established clinicopathological prognostic factors: age as continuous variable, grade (1-2 vs. 3), LVSI (substantial vs. none or mild), and adjuvant treatment (vaginal brachytherapy, external beam radiotherapy or no additional therapy). Discrimination between the risk

stratification models was quantified using the area under the receiver operating curve with 95% confidence intervals (CIs). All reported p-values were based on two–sided tests with *P*-values <0.05 considered statistically significant (IBM SPSS 20.0).

Results

In total, 947 (83% of randomized patients) EECs from PORTEC-1 and -2 were available (Figure 1). Analysis of classifying alterations (p53, MSI, *POLE*) was successful in 809/836 (97%) cases for which sufficient material was available. For 111 PORTEC-cases only FFPE slides were available for DNA isolation, which provided 52 (47%) additional successfully analyzed cases. Patient, tumor and treatment characteristics did not differ between included, excluded and failed cases (Supplementary Table S1). The median follow-up was 131 months (range 0.2-219.2 months).

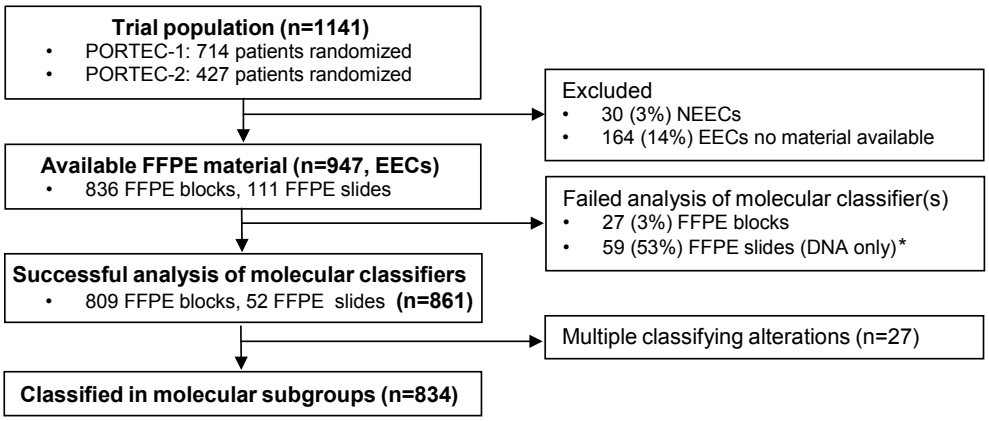

Figure 1. Flow chart of sample analyses. *The majority of cases with incomplete analysis were PORTEC-1 cases from which only FFPE tumor slides were available EEC= endometrioid endometrial cancer; NEEC=non-endometrioid endometrial cancer.

The four molecular subgroups displayed marked differences in clinicopathological characteristics, alterations in potential other classifiers, and clinical outcome (Table 1, Figure 2, Supplementary Table S2). In total, 834 EECs could be classified in one of the four subgroups: 74 (9%) p53-mutant, 219 (26%) MSI, 49 (6%) *POLE*-mutant, and 492 (59%) NSMP. Twenty-seven (3%) tumors were found to have more than one classifying alteration (p53, MSI or *POLE*). p53-mutant tumors were significantly associated with grade 3, loss of hormone receptors, >10% L1CAM expression, *PPP2R1a*, and *FBXW7* mutations. MSI tumors presented more frequently with substantial LVSI, and abnormal ARID1a expression. *POLE* mutations occurred more frequently in younger women, grade 3, and often co-occurred with *PTEN* mutations. In contrast, the NSMP tumors were more frequently grade 1, and *CTNNB1* mutant. The prognosis was unfavorable in the p53-mutant group, intermediate in the MSI and NSMP group, and the *POLE*-mutant group had a favorable prognosis with no local and only

two distant recurrences (Figure 2). In addition, women with a *POLE*- and p53-mutant tumor developed no recurrences (0/7), whereas some of the women with MSI tumors with *POLE* (2/6) or p53 mutation (2/13), or both (1/1) developed recurrences (Supplementary Table S3). Within the four subgroups, distant recurrence and endometrial cancer-related death rates were similar.

Table 1. Clinicopathological characteristics and alterations in potential other molecular classifiers according to the four molecular subgroups in early-stage endometrial cancer (n=834).

	Total n=834 (100%)	p53-mutant n=74 (8.9%)	MSI n=219 (26.3%)	*POLE*-mutant n=49 (5.9%)	NSMP n=492 (59.0%)	*P*-value
Age, years						
Mean (range)	68 (41-90)	69 (51-86)	69 (43-89)	62 (46-81)	68 (41-90)	0.000
< 60	138 (16.5)	7 (9.5)	35 (16.0)	19 (38.8)	77 (15.7)	
60-70	360 (43.2)	32 (43.2)	89 (40.6)	18 (36.7)	221 (44.9)	0.001
> 70	336 (40.3)	35 (47.3)	95 (42.4)	12 (24.5)	194 (39.4)	
Grade						
1-2	724 (86.8)	48 (64.9)	135 (83.6)	33 (73.4)	457 (92.9)	0.000
3	110 (13.2)	26 (35.1)	36 (16.4)	13 (26.6)	35 (7.1)	
Myometrial invasion						
<50%	251 (30.1)	35 (47.3)	71 (32.4)	25 (51.0)	120 (24.4)	0.000
>50%	583 (69.9)	39 (52.7)	148 (67.6)	24 (49.0)	372 (75.6)	
LVSI*						
Absent/Focal	784 (95.5)	70 (94.6)	194 (91.1)	47 (100)	473 (97.1)	0.002
Substantial	37 (4.5)	4 (5.4)	19 (8.9)	0	14 (2.9)	
Risk group						
Low	242 (29.0)	22 (29.7)	62 (28.3)	24 (49.0)	134 (27.2)	
High-intermediate	546 (65.5)	44 (59.5)	143 (65.3)	23 (46.9)	336 (68.3)	0.013
High	46 (5.5)	8 (10.8)	14 (6.4)	2 (4.1)	22 (4.5)	
Treatment						
NAT	241 (28.9)	17 (23.0)	63 (28.8)	16 (32.7)	145 (29.5)	
EBRT	409 (49.0)	38 (51.3)	113 (51.6)	25 (51.0)	233 (47.4)	0.688
VBT	184 (22.1)	19 (25.7)	43 (19.6)	8 (16.3)	114 (23.1)	
Mutations**						
CDKN2A	2 (0.2)	0	0	0	2 (0.4)	0.707
CTNNB1	157 (19.5)	5 (7.0)	19 (9.0)	8 (17.0)	125 (26.3)	0.000
FBXW7	40 (5.0)	8 (11.3)	13 (6.1)	1 (2.1)	18 (3.8)	0.032
FGFR2	80 (9.9)	2 (2.8)	20 (9.4)	0	58 (12.2)	0.007
KRAS	139 (17.3)	7 (9.9)	43 (20.3)	3 (6.4)	86 (18.1)	0.042
NRAS	25 (3.1)	1 (1.4)	8 (3.8)	0	16 (3.4)	0.456
PIK3CA	261 (32.4)	17 (23.9)	70 (33.0)	24 (51.1)	150 (31.6)	0.019
PPP2R1a	39 (4.8)	12 (16.9)	6 (2.8)	1 (2.1)	20 (4.2)	0.000
PTEN	349 (43.4)	15 (21.1)	106 (50.0)	34 (72.3)	194 (40.8)	0.000
Altered protein expression***						
>10% L1CAM	44 (5.6)	27 (39.7)	5 (2.4)	1 (2.6)	16 (3.4)	0.000
<10% ER	38 (5.0)	16 (24.2)	5 (2.5)	4 (10.5)	13 (2.8)	0.000
<10% PR	81 (10.6)	25 (39.1)	19 (9.4)	9 (23.7)	28 (6.1)	0.000
loss/clonal ARID1a	329 (45.4)	17 (27)	123 (63.7)	13 (35.1)	176 (40.8)	0.000
loss/hetero. PTEN	395 (51.5)	28 (43.1)	130 (64.4)	19 (48.7)	218 (47.3)	0.000
nuclear β-catenin	184 (23.6)	7 (10.6)	34 (16.3)	3 (7.7)	140 (30.1)	0.000

* Degree of LVSI unknown for 13 (1.6%) cases. ** Mutation analysis failed for 29 (3.5%) cases. ***Immunohistochemical analysis failed, or no available FFPE slides for 111 (13%) ARID1a, 56 (6.7%) β-catenin, 73 (9%) ER, 68 (8%) PTEN, 73 (9%) PR. LVSI=lymphovascular space invasion, NAT=no additional treatment, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy, hetero.= heterogeneous.

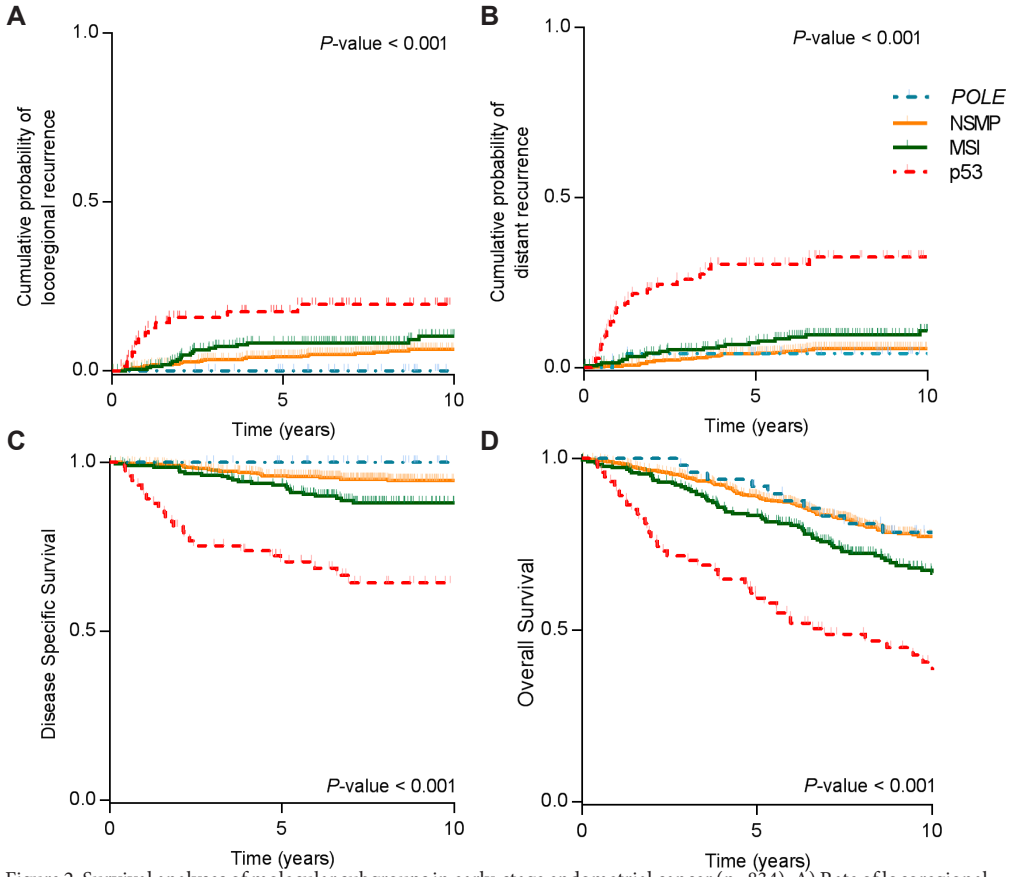

Figure 2. Survival analyses of molecular subgroups in early-stage endometrial cancer (n=834). A) Rate of locoregional recurrences, B) rate of distant recurrences, C) disease specific survival, and D) overall survival.

The prognostic value of the molecular subgroups and additional molecular alterations was evaluated in univariable analysis and multivariable analysis with the clinicopathological factors (age, grade, depth of myometrial invasion, LVSI) and treatment, both in the whole population (Supplementary Table S4) and in an analysis restricted to cases with high-intermediate risk features (Table 2-univariable analysis, Table 3-multivariable analysis). In both analyses, p53-mutant and substantial LVSI were the strongest prognostic factors for locoregional-, distant recurrence, and overall survival, while >10% L1CAM expression was prognostic for distant recurrence and overall survival. After excluding cases with favorable (*POLE*-mutant) and unfavorable factors (substantial LVSI, p53-mutant and >10% L1CAM), a final analysis found MSI prognostic for distant recurrence and overall survival, and *CTNNB1* exon 3 mutation status prognostic for distant recurrence (Table 3, Supplementary Table S4). Univariable prognostic factors, *FGFR2* mutation and loss of hormone receptor expression, lost its significance in multivariable analysis in the presence of other (un)favorable prognostic factors. Univariable analysis in 242 ECs with low-risk features showed a higher rate of locoregional and distant recurrences and lower overall survival in the eight patients with >10% L1CAM, and a trend for p53-mutant patients (Supplementary Table S5).

Table 2. Univariable analysis of clinicopathological characteristics, molecular subgroups, and potential other molecular classifiers in high-intermediate risk early-stage endometrial cancer (n=546).

		Locoregional Recurrence 42 events			Distant Recurrence 50 events			Overall Survival 182 events		
	Total n	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (cont.)	546	1.035	0.988-1.085	0.145	1.015	0.972-1.060	0.508	1.085	1.062-1.110	0.000
Grade										
1-2	492	1			1			1		
3	54	1.784	0.751-4.239	0.190	3.038	1.552-5.945	0.001	1.741	1.149-2.639	0.009
Myometrial invasion										
<50	62	1			1			1		
>50	484	0.539	0.239-1.216	0.137	0.304	0.161-0.574	0.000	0.586	0.392-0.877	0.009
LVSI										
Absent/mild	507	1			1			1		
Substantial	28	3.733	1.567-8.891	0.003	4.895	2.368-10.121	0.000	2.791	1.668-4.432	0.000
Given treatment										
NAT	113	1			1			1		
EBRT	276	0.311	0.150-0.642	0.002	0.873	0.425-1.796	0.713	0.833	0.595-1.167	0.289
VBT	157	0.546	0.257-1.157	0.114	1.108	0.511-2.401	0.796	0.745	0.480-1.157	0.190
Molecular subgroup										
NSMP	336	1			1			1		
p53	44	6.787	3.069-15.012	0.000	11.083	5.629-21.821	0.000	4.861	3.098-7.073	0.000
MSI	143	2.476	1.182-4.776	0.015	2.220	1.180-4.447	0.025	1.853	1.329-2.584	0.000
POLE	23	-	-	0.970	0.869	0.116-6.532	0.891	0.907	0.367-2.237	0.832
CTNNB1										
No mutation	433	1			1			1		
Mutation	101	0.575	0.225-1.467	0.247	0.934	0.453-1.929	0.854	0.669	0.438-1.023	0.063
FBXW7										
No mutation	512	1			1			1		
Mutation	22	0.666	0.091-4.848	0.688	0.530	0.073-3.847	0.531	1.569	0.827-2.977	0.168
FGFR2										
No mutation	468	1			1			1		
Mutation	66	0.746	0.256-2.095	0.578	0.296	0.072-1.219	0.092	0.556	0.316-0.979	0.042
KRAS										
No mutation	453	1			1			1		
Mutation	81	0.998	0.419-2.379	0.997	1.322	0.639-2.734	0.452	1.035	0.686-1.561	0.871
NRAS										
No mutation	519	1			1			1		
Mutation	15	-	-	0.430	-	-	0.398	0.635	0.231-1.690	0.354
PIK3CA										
No mutation	358	1			1			1		
Mutation	176	0.572	0.272-1.201	0.140	0.814	0.436-1.516	0.516	0.921	0.668-1.271	0.618
PPP2R1A										
No mutation	504	1			1			1		
Mutation	30	1.599	0.492-5.203	0.435	2.128	0.842-5.375	0.110	1.640	0.932-2.888	0.086
PTEN										
No mutation	305	1			1			1		
Mutation	229	0.908	0.484-1.702	0.763	0.517	0.277-0.965	0.038	0.797	0.588-1.080	0.144
L1CAM										
<10%	496	1			1			1		
>10%	30	3.283	1.283-8.404	0.013	7.718	3.993-14.917	0.000	3.763	2.379-5.953	0.000
ER										
>10%	499	1			1			1		
<10%	21	3.547	1.259-9.993	0.017	6.194	2.882-13.310	0.000	2.139	1.183-3.865	0.012
PR										
>10%	465	1			1			1		
<10%	51	2.828	1.297-6.165	0.009	5.684	3.042-10.622	0.000	2.096	1.379-3.188	0.001
ARID1a										
Positive	249	1			1			1		
Loss/clonal	228	0.792	0.423-1.483	0.467	0.827	0.455-1.503	0.533	0.878	0.643-1.200	0.415
PTEN										
Positive	232	1			1			1		
Loss/hetero.	283	0.979	0.529-1.812	0.946	0.988	0.553-1.765	0.967	1.043	0.769-1.414	0.788
β-catenin										
Membrane	399	1			1			1		
Nuclear	126	0.758	0.350-1.643	0.483	0.846	0.420-1.704	0.640	0.704	0.471-1.051	0.086

Cont.=continuous, hetero.=heterogeneous.

Table 3. Multivariable analysis on the prognostic role of the clinicopathological characteristics, molecular subgroups, and potential other molecular classifiers in high- intermediate risk (HIR) endometrial cancers (EC) (n=546) and in a subset of HIR EC without substantial LVSI, >10% L1CAM, p53 and *POLE* mutation (n=443).

All cases of HIR EC (n=546)

	Locoregional Recurrence 41 events			Distant Recurrence 46 events			Overall Survival 170 events		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (Cont.)	1.032	0.984-1.081	0.197	1.016	0.972-1.062	0.469	1.076	1.051-1.101	0.000
Grade									
1-2	1			1			1		
3	0.203	0.021-1.946	0.167	0.162	0.029-0.904	0.038	0.262	0.057-1.204	0.085
Myometrial invasion									
<50%	1			1			1		
>50%	0.201	0.024-1.678	0.138	0.126	0.026-0.624	0.011	0.254	0.058-1.101	0.067
LVSI									
Absent/mild	1			1			1		
Substantial	3.190	1.301-7.821	0.011	4.303	1.833-10.09	0.001	2.637	1.542-4.509	0.000
Treatment									
NAT	1			1			1		
EBRT	0.277	0.133-0.574	0.001	1.154	0.498-2.677	0.738	0.897	0.623-1.292	0.559
VBT	0.466	0.212-1.027	0.058	1.134	0.465-2.769	0.782	0.707	0.445-1.123	0.142
Molecular subgroup									
NSMP	1			1			1		
p53	7.340	3.168-17.00	0.000	5.766	2.400-13.85	0.000	3.777	2.364-6.037	0.000
MSI	2.319	1.105-4.866	0.026	2.154	1.022-4.540	0.044	1.879	1.307-2.700	0.001
POLE	-	-	0.973	0.883	0.113-6.890	0.906	1.105	0.394-3.101	0.850
L1CAM									
<10%				1			1		
>10%				4.303	1.833-10.09	0.001	2.462	1.453-4.170	0.001

HIR EC without substantial LVSI, >10% L1CAM, p53 and *POLE* mutation (n=443)

	Locoregional Recurrence 27 events			Distant Recurrence 23 events			Overall Survival 127 events		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (Cont.)	1.063	1.000-1.130	0.052	1.007	0.944-1.074	0.837	1.102	1.070-1.134	0.000
Grade									
1-2	1			1			1		
3	0.060	0.004-0.842	0.037	0.350	0.033-3.765	0.387	0.409	0.050-3.381	0.407
Myometrial invasion									
<50%	1			1			1		
>50%	0.076	0.009-0.668	0.020	0.099	0.011-0.859	0.036	0.277	0.037-2.070	0.211
Treatment									
NAT	1			1			1		
EBRT	0.249	0.106-0.585	0.001	0.862	0.329-2.262	0.764	0.806	0.541-1.201	0.289
VBT	0.181	0.054-0.605	0.005	0.511	0.139-1.877	0.312	0.559	0.312-1.003	0.051
Molecular subgroup									
NSMP	1			1			1		
MSI	1.816	0.815-4.048	0.145	2.520	1.049-6.051	0.039	1.672	1.146-2.438	0.008
CTNNB1									
No mutation				1					
Mutation				2.959	1.234-7.098	0.015			

Cont.=continuous.

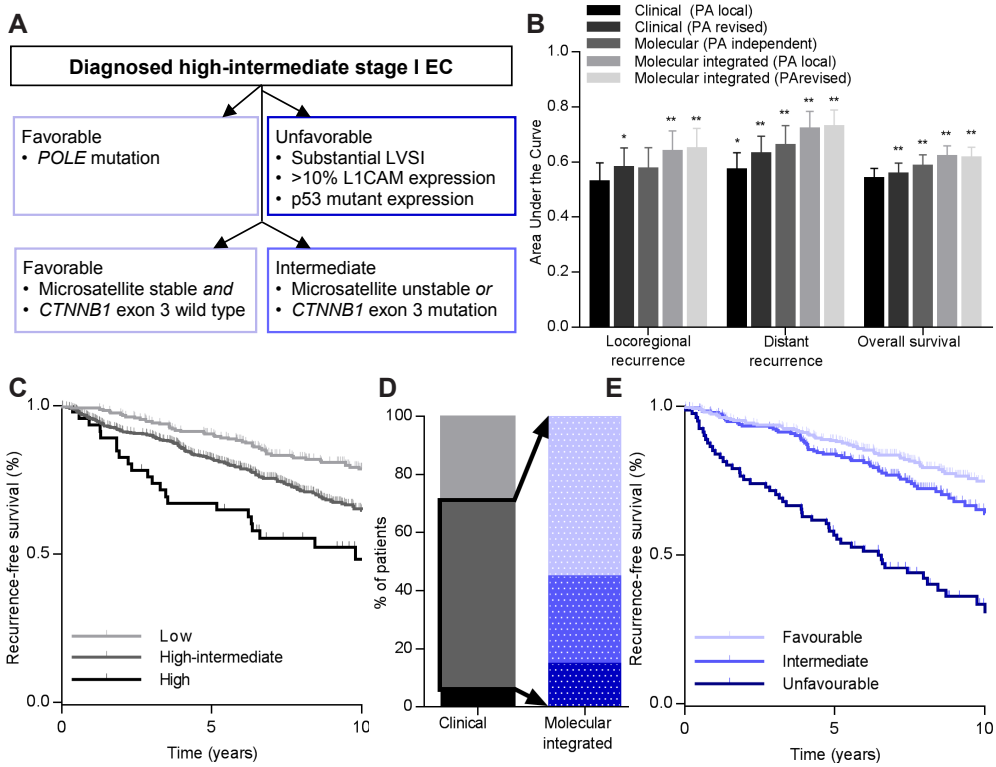

Figure 3. Molecular integrated risk assessment. A) Flow chart of the molecular integrated risk model. B) Area under the curve for the clinical- and molecular-, and molecular integrated risk assessment, with and without central pathology review (*P-value<0.05, **P-value<0.01). C) Recurrence-free survival of clinical risk assessment in early-stage endometrial cancer (n=834, P-value<0.001). D) Bar chart of the proportion of clinically low-, high-intermediate-, and high-risk patients based on central pathology review (left) and the proportion of clinically high-intermediate risk patients reclassified into favorable, intermediate and unfavorable molecular integrated risk groups. E) Recurrence-free survival of molecular integrated risk assessment in early-stage high-intermediate risk endometrial cancer (n=546, P-value<0.001).

Based on the outcomes of multivariable analysis a molecular integrated risk assessment was defined that combines clinicopathological and molecular risk factors (Figure 3A). Substantial LVSI, p53-mutant and >10% L1CAM tumors were designated unfavorable, while in the remaining cases both MSI and *CTNNB1* mutant were distinguished from the favorable group of *POLE*-mutant tumors and NSMP tumors being MSS and *CTNNB1* wild type (Figure 3A). Since PORTEC-1 included patients that are currently considered low risk, and central pathology review in both trials identified additional low- and high-risk cases, the area under the curve (AUC) was estimated for the molecular integrated risk assessment taking these different starting points into account (Figure 3B-C). Compared to the original pathology reports, central pathology review or molecular classification improved the AUC. However, AUCs of the integrated molecular risk assessment showed a substantial improvement, without additional improvement when using findings of central pathology review. Approximately 15% of the high-intermediate risk patients had unfavorable features, and 50% had favorable

features leaving 35% intermediate (Figure 3D-E). In tumors with unfavorable features, targetable alterations were found: 65% *PI3K/AKT* alterations, 9% *FBXW7* mutations, 7% *FGFR2* mutations, 28% L1CAM positivity 78% ER positivity, and 61% PR positivity.

Discussion

In 834 early-stage EECs from two randomized trials (PORTEC-1 and -2) with mature long-term follow-up, the prognostic impact of the four molecular subgroups, originally proposed by the TCGA, was confirmed.[10] Clinically applicable molecular analysis methods for surrogate markers were used and proved feasible in >96% of EEC patients. Integration of prognostic molecular alterations with established clinicopathological factors results in a stronger risk assessment. As a consequence, within the high- intermediate risk population, who are currently thought to be relatively homogenous with regard to clinical outcomes, approximately 15% patients with a marked unfavorable and 50% with a favorable prognosis could be identified.

L1CAM, p53, and LVSI were consistent independent prognostic factors for distant recurrence, overall and disease specific survival. p53-mutant tumors exhibit a high degree of genomic instability linked to tumor progression, and invasion by upregulation of p53-mutant target genes, and *TP53* mutation is well known for its prognostic impact in EC.[8,10] LVSI, especially when quantified as substantial, and L1CAM have similar strong negative prognostic value. LVSI strongly increases the risk of tumor spread via lymphatics and capillaries. L1CAM is known to enhance motility and migration of tumor cells. Both were recently published in this same population as single risk factors[13,18] and by Zeimet *et al.*[19], but were now confirmed to be independent prognostic factors in an integrated analysis. In contrast, patients with *POLE*-mutant or MSS and *CTNNB1* wild type tumors displayed a more favorable prognosis. The favorable outcome of *POLE*-mutant ECs with their striking mutation burden may be explained by an increased immunogenicity, and became evident in EC recently.[20,21] *CTNNB1* mutations result in activation of Wnt signalling contributing to tumor progression, abnormal expression of cell proliferation, and progression genes. Similarly to our results, a previous report showed that ECs carrying a *CTNNB1* mutation characterize a more aggressive subset within low-grade early-stage EEC.[10,22] The prognostic importance of MSI has been controversial, although the strongest association with poor clinical outcome has been observed in early-stage EC similar to our observation.[23] This report integrates a large number of single prognostic factors in the context of clinical trial material resulting in a comprehensive overview.

In this large cohort, only few (3%) tumors had multiple classifying alterations (e.g. *POLE* and MSI). Classification of this small subset would require further analyses, such as mutational load and copy-number status. Supek *et al.* reported that colorectal and stomach tumors

with both MSI and *POLE* mutation had an overall mutational load similar to MSI tumors, whereas two out of three MSI/*POLE* endometrial tumors had a much higher mutational load and different mutational signature.[24] Furthermore, Shinbrot *et al.* showed that the *TP53* gene is frequently affected by *POLE* mutation induced strand-specific mutations.[25] These data support that mutational load, mutation signature, and pattern may be useful for molecular classification of rare tumors that present with combinations of MSI, *POLE*, or *TP53* mutations. With the advent of next generation sequencing technologies, these can be easily analyzed.

Several molecular alterations, such as hormone receptor expression, *CTNNB1* and *FGFR2* mutations, have been previously reported as having prognostic potential in single biomarker studies.[8,9,13,19,22,26,27] Some univariable factors, *FGFR2* mutation and hormone receptor status, lost significance in multivariable analysis. This may be due to the fact that *FGFR2* mutations were equally frequent in MSI and NSMP ECs, and that hormone receptor loss was mainly found in p53-mutant and L1CAM positive ECs but was also frequently observed in *POLE*-mutant ECs.[10,26-28] MSI, p53 and L1CAM proved stronger independent prognosticators in this analysis. *CTNNB1* mutation status was sufficiently strong to emerge in multivariable analysis, stressing its independent prognostic significance. Using this combined approach, an improved risk assessment resulted in which *POLE*, L1CAM, MSI and *CTNNB1* are integrated with histopathological factors.

Previous studies have shown improved risk stratification obtained by central pathology review.[29,30] The reviewed pathology in our analyses had the advantage to exclude prototypical non-endometrioid cancers. With regard to grading, lack of prognostic relevance of grade 2 was shown, advocating the use of a two-tiered grading system, as was also proposed by others.[31-33] The increased AUC of the model based on central pathology review as compared to the original inclusion pathology confirms these findings. The molecular integrated risk model showed an even higher increase in AUC; however, central pathology review did not add any additional value to the molecular integrated risk model. The molecular integrated risk model has three major advantages. Firstly, it is based on more objective variables, such as mutational status of *POLE*. Secondly, the molecular integrated risk model identifies significantly more patients with favorable features that would otherwise be classified as high-intermediate risk with central pathology review alone. Finally, this approach has also the advantage to facilitate pre-screening for Lynch syndrome.

Despite the strength of a randomized trial population, mature long-term follow-up, large group of early-stage EEC, and straightforward molecular analysis, this study has some limitations. Our focused and practical approach provides analyses that can easily be implemented in prospective studies and clinical practice. Most common hotspot mutations were analyzed but this does not rule out the possibility that other clinical relevant alterations may have

been missed. Although, molecular alterations were highly concordant between curettage and hysterectomy specimen,[15,17,18] intratumor heterogeneity may interfere with prediction of the patient's prognosis and requires further study. LVSI and the classic-histopathology, included in the integrated risk model, cannot be evaluated on preoperative specimen, therefore, it is recommended not to rely on preoperative specimens. No automated immunohisto-chemical protocols were used, while it is likely that robust, standardized automated staining procedures are the preferred method in diagnostic pathology. Molecular alterations in our integrated risk model have been proven in single biomarker studies; however, this integrated risk model needs to be validated or prospectively analyzed. Since the majority of our patient cohort has received adjuvant radiotherapy, the decision to omit adjuvant radiotherapy especially in the favorable subgroup remains to be elucidated in a prospective study. There is also need to further investigate whether certain molecular defined subgroups of EC may be more sensitive to radiotherapy. Nevertheless, we believe our data is unique and informative for patient's outcome, and may guide molecular-based trials and therapies for EC.

The proposed molecular integrated risk model outperforms the current clinicopathologic approach; therefore, the question arises whether this integrated model can be used for new clinical studies and guide treatment decisions. Especially in high-intermediate EC, this risk model may substantially reduce overtreatment of favorable cases, and select unfavorable cases who might need more intensive treatment. The clinical utility for tailoring adjuvant therapy, the feasibility of determining the molecular integrated profile within tight time limits and the cost-effectiveness aspects of this approach (e.g. costs of molecular testing vs. saving costs of adjuvant radiotherapy) will be prospectively established in a planned prospective trial PORTEC-4. Within ~10% of low-risk patients, p53 and L1CAM seem prognostic indicators for high recurrence rate and impaired survival, which is in line with Talhouk *et al.*[12] However, the small number of events in this subgroup limits these findings. Factors that are associated with favorable outcome or predict chemotherapy response in high-risk EC remain to be elucidated in future studies.

In conclusion, integration of molecular risk factors with clinicopathological factors in early-stage EC leads to improved risk stratification with potential clinical utility. This molecular integrated risk prediction holds promise to reduce both over- and undertreatment and should form the basis for future prospective clinical studies.

Acknowledgements

We acknowledge all members of the PORTEC study group and colleagues N.T. ter Haar, E.J. Dreef, and H.E.D. Suchiman from the Leiden University Medical Center for excellent technical support. The many patients who participated in the trials are gratefully acknowledged.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. CA Cancer J.Clin. 2015;65(1):5-29.
2. Creasman WT, Odicino F, Maisonneuve P, et al. Carcinoma of the corpus uteri. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics. 2006;95 Suppl 1:S105-143.
3. Kong A, Johnson N, Kitchener HC, Lawrie TA. Adjuvant radiotherapy for stage I endometrial cancer: an updated Cochrane systematic review and meta-analysis. Journal of the National Cancer Institute. 2012;104(21):1625-1634.
4. Creutzberg CL, van Putten WL, Koper PC, et al. Surgery and postoperative radiotherapy versus surgery alone for patients with stage-1 endometrial carcinoma: multicentre randomised trial. PORTEC Study Group. Post Operative Radiation Therapy in Endometrial Carcinoma. Lancet. 2000;355(9213):1404-1411.
5. Keys HM, Roberts JA, Brunetto VL, et al. A phase III trial of surgery with or without adjunctive external pelvic radiation therapy in intermediate risk endometrial adenocarcinoma: a Gynecologic Oncology Group study. Gynecologic oncology. 2004;92(3):744-751.
6. Colombo N, Preti E, Landoni F, et al. Endometrial cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2013;24 Suppl 6:vi33-38.
7. Nout RA, Smit VT, Putter H, et al. Vaginal brachytherapy versus pelvic external beam radiotherapy for patients with endometrial cancer of high-intermediate risk (PORTEC-2): an open-label, non-inferiority, randomised trial. Lancet. 2010;375(9717):816-823.
8. Salvesen HB, Haldorsen IS, Trovik J. Markers for individualised therapy in endometrial carcinoma. The Lancet. Oncology. 2012;13(8):e353-361.
9. Murali R, Soslow RA, Weigelt B. Classification of endometrial carcinoma: more than two types. Lancet oncology. 2014;15(7):e268-e278.
10. Cancer Genome Atlas Research N, Kandoth C, Schultz N, et al. Integrated genomic characterization of endometrial carcinoma. Nature. 2013;497(7447):67-73.
11. Stelloo E, Bosse T, Nout RA, et al. Refining prognosis and identifying targetable pathways for high-risk endometrial cancer; a TransPORTEC initiative. Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc. 2015;28(6):836-844.
12. Talhouk A, McConechy MK, Leung S, et al. A clinically applicable molecular-based classification for endometrial cancers. Br.J.Cancer. 2015;113(2):299-310.
13. Bosse T, Peters EE, Creutzberg CL, et al. Substantial lymph-vascular space invasion (LVSI) is a significant risk factor for recurrence in endometrial cancer-A pooled analysis of PORTEC 1 and 2 trials. European journal of cancer. 2015;51(13):1742-1750.
14. McShane LM, Altman DG, Sauerbrei W, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). Journal of the National Cancer Institute. 2005;97(16):1180-1184.
15. Stelloo E, Nout RA, Naves LC, et al. High concordance of molecular tumor alterations between pre-operative curettage and hysterectomy specimens in patients with endometrial carcinoma. Gynecologic oncology. 2014;133(2):197-204.
16. McConechy MK, Talhouk A, Li-Chang HH, et al. Detection of DNA mismatch repair (MMR) deficiencies by immunohistochemistry can effectively diagnose the microsatellite instability (MSI) phenotype in endometrial carcinomas. Gynecologic oncology. 2015;137(2):306-310.
17. Church DN, Stelloo E, Nout RA, et al. Prognostic significance of POLE proofreading mutations in endometrial cancer. Journal of the National Cancer Institute. 2015;107(1):402.
18. Bosse T, Nout RA, Stelloo E, et al. L1 cell adhesion molecule is a strong predictor for distant recurrence and overall survival in early stage endometrial cancer: pooled PORTEC trial results. European journal of cancer. 2014;50(15):2602-2610.
19. Zeimet AG, Reimer D, Huszar M, et al. L1CAM in early-stage type I endometrial cancer: results of a large multicenter evaluation. Journal of the National Cancer Institute. 2013;105(15):1142-1150.
20. van Gool IC, Eggink FA, Freeman-Mills L, et al. POLE Proofreading Mutations Elicit an Antitumor Immune Response in Endometrial Cancer. Clinical cancer research: an official journal of the American Association for Cancer Research. 2015;21(14):3347-3355.
21. Howitt BE, Shukla SA, Sholl LM, et al. Association of Polymerase e-Mutated and Microsatellite-Instable Endometrial Cancers With Neoantigen Load, Number of Tumor-Infiltrating Lymphocytes, and Expression of PD-1 and PD-L1. JAMA oncology. 2015;1(9):1319-1323.
22. Liu Y, Patel L, Mills GB, et al. Clinical significance of CTNNB1 mutation and Wnt pathway activation in endometrioid endometrial carcinoma. Journal of the National Cancer Institute. 2014;106(9).

23. Diaz-Padilla I, Romero N, Amir E, et al. Mismatch repair status and clinical outcome in endometrial cancer: a systematic review and meta-analysis. Critical Reviews in Oncology/Hematology. 2013;88(1):154-167.
24. Supek F, Lehner B. Differential DNA mismatch repair underlies mutation rate variation across the human genome. Nature. 2015;521(7550):81-84.
25. Shinbrot E, Henninger EE, Weinhold N, et al. Exonuclease mutations in DNA polymerase epsilon reveal replication strand specific mutation patterns and human origins of replication. Genome research. 2014;24(11):1740-1750.
26. Byron SA, Gartside M, Powell MA, et al. FGFR2 point mutations in 466 endometrioid endometrial tumors: relationship with MSI, KRAS, PIK3CA, CTNNB1 mutations and clinicopathological features. PloS one. 2012;7(2):e30801.
27. Trovik J, Wik E, Werner HM, et al. Hormone receptor loss in endometrial carcinoma curettage predicts lymph node metastasis and poor outcome in prospective multicentre trial. European journal of cancer. 2013;49(16):3431-3441.
28. Huszar M, Pfeifer M, Schirmer U, et al. Up-regulation of L1CAM is linked to loss of hormone receptors and E-cadherin in aggressive subtypes of endometrial carcinomas. The Journal of pathology. 2010;220(5):551-561.
29. Scholten AN, Smit VT, Beerman H, van Putten WL, Creutzberg CL. Prognostic significance and interobserver variability of histologic grading systems for endometrial carcinoma. Cancer. 2004;100(4):764-772.
30. Khalifa MA, Dodge J, Covens A, Osborne R, Ackerman I. Slide review in gynecologic oncology ensures completeness of reporting and diagnostic accuracy. Gynecologic oncology. 2003;90(2):425-430.
31. Lax SF, Kurman RJ, Pizer ES, Wu L, Ronnett BM. A binary architectural grading system for uterine endometrial endometrioid carcinoma has superior reproducibility compared with FIGO grading and identifies subsets of advance-stage tumors with favorable and unfavorable prognosis. The American journal of surgical pathology. 2000;24(9):1201-1208.
32. Conlon N, Leitao MM, Jr., Abu-Rustum NR, Soslow RA. Grading uterine endometrioid carcinoma: a proposal that binary is best. The American journal of surgical pathology. 2014;38(12):1583-1587.
33. Alkushi A, Abdul-Rahman ZH, Lim P, et al. Description of a novel system for grading of endometrial carcinoma and comparison with existing grading systems. The American journal of surgical pathology. 2005;29(3):295-304.

Supplementary files

Supplementary Table 1. Clinicopathological characteristics of the PORTEC-1 and -2 trial populations: comparison of cases included in the current analysis and those excluded for lack of material (n=164), non-endometrioid histology (n=30) or failed molecular analysis (n=86).

	PORTEC-1			PORTEC-2		
	Included n=477	Excluded n=237	*P*-value	Included n=384	Excluded n=43	*P*-value
Age, years						
Mean (range)	66 (41-90)	66 (43-88)	0.387	70 (52-89)	70 (46-85)	0.471
< 60	131 (27.5)	69 (29.1)		14 (3.6)	2 (4.7)	
60-70	179 (37.5)	92 (38.8)	0.731	190 (49.5)	18 (41.9)	0.585
> 70	167 (35.0)	76 (32.1)		180 (46.9)	23 (53.4)	
Grade						
1-2	400 (83.9)	201 (84.8)	0.743	345 (89.8)	29 (67.4)	0.000
3	77 (16.1)	36 (15.2)		39 (10.2)	13 (30.2)	0.212**
Myometrial invasion						
<50%	198 (41.5)	96 (40.5)	0.798	61 (15.9)	10 (23.3)	0.379
>50%	279 (58.5)	141 (59.5)		323 (84.1)	33 (76.7)	
LVSI*						
Absent/Focal	452 (95.6)	125 (95.4)	0.945	356 (94.9)	29 (96.7)	0.701
Substantial	21 (4.4)	6 (4.6)		19 (5.1)	1 (3.3)	
Risk group						
Low	216 (45.3)	106 (44.7)		36 (9.3)	2 (4.7)	0.000
High-intermediate	234 (49.1)	112 (47.3)	0.477	327 (85.2)	28 (65.1)	0.339**
High	27 (5.7)	19 (8.0)		21 (5.5)	13 (30.2)	
Treatment						
NAT	246 (51.6)	123 (51.9)		2 (0.5)	1 (2.3)	
EBRT	231 (48.4)	114 (48.1)	0.934	190 (49.5)	19 (44.2)	0.727
VBT	0	0		192 (50.0)	23 (53.5)	

LVSI=lymphovascular space invasion, NAT=no additional treatment, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy. *Degree of LVSI unknown for 13 included cases, and 119 excluded cases. **Endometrioid EC only.

Supplementary Table 2. Hotspot mutation frequency according to the four molecular subgroups in early-stage endometrial cancer (n=834).►

	p53-mutant n=74	MSI n=219	*POLE*-mutant n=49	NSMP n=492	Total n=834
PTEN[1] (%)	15 (20)	105 (48)	34 (69)	195 (40)	349 (43)
p.R130G	5	24	3	75	107/790
p.R130fs*4	6	18	19	45	88/801
p.R233*	3	14	0	22	39/799
p.L318fs*2	2	11	0	17	30/796
p.R130*	2	7	0	11	20/790
p.T321fs*3	0	10	0	7	17/786
p.N323fs*2	0	10	0	7	17/791
p.K267fs*9	0	13	0	1	14/827
p.R173C	0	1	8	5	14/805
p.E7*	0	0	8	2	10/802
p.R130P	0	2	0	8	10/800
p.K267fs*31	0	5	0	4	9/798
p.R130L	0	2	0	6	8/800
p.R173H	0	2	3	3	8/801
p.K6fs*4	0	1	1	2	4/801
p.Q214*	0	1	0	3	4/798
p.R234W	0	2	0	2	4/787
p.248fs*5	0	2	0	2	4/801
p.R355*	0	1	0	3	4/803
p.V290fs*1	0	3	0	0	3/800
p.T321fs*23	0	1	0	1	2/797
p.N323fs*21	0	1	0	1	2/826
PIK3CA[1] (%)	17 (23)	69 (32)	24 (49)	151 (31)	261 (32)
p.R88Q	6	24	13	30	73/789
p.H1047R	4	13	0	34	51/800
p.E545K	3	5	0	27	35/800
p.E542K	0	5	2	15	22/809
p.M1043I	0	1	5	11	17/794
p.Y1021C	1	4	4	4	13/825
p.H1047Y	1	9	0	2	12/807
p.Q546K	1	5	0	5	11/804
p.Q546R	0	2	0	9	11/782
p.E545A	1	2	1	6	10/791
p.T1025A	0	2	4	3	9/785
p.H1047L	0	1	0	7	8/800
p.M1043V	0	1	0	4	5/805
p.E545G	0	1	0	3	4/791
p.Q546L	0	0	0	2	2/782
p.Q546P	0	0	0	2	2/782
p.E545D	0	0	0	1	1/793
p.Q546E	0	1	0	0	1/804
CTNNB1[1] (%)	5 (7)	18 (8)	8 (16)	126 (26)	157 (20)
p.S37T	2	1	1	34	38/804
p.S45F	0	0	1	11	12/801
p.S33F	0	1	0	10	11/796
p.T41I	0	2	1	8	11/796
p.D32N	0	2	1	6	9/801
p.S33Y	0	1	1	7	9/796
p.G34R	1	4	0	4	9/828
p.T41A	1	1	0	7	9/814
p.D32Y	0	0	0	8	8/801
p.G34E	0	3	2	3	8/793
p.S45P	0	1	0	6	7/805
p.S37C	1	0	0	5	6/804
p.S33C	0	0	0	5	5/796

Supplementary Table 2 continued.

		p53-mutant n=74	MSI n=219	*POLE*-mutant n=49	NSMP n=492	Total n=834
	p.D32G	0	0	0	4	4/826
	p.S33P	0	1	0	3	4/814
	p.T41I	0	2	1	8	11/796
	p.D32N	0	2	1	6	9/801
	p.S33Y	0	1	1	7	9/796
	p.G34R	1	4	0	4	9/828
	p.T41A	1	1	0	7	9/814
	p.D32Y	0	0	0	8	8/801
	p.G34E	0	3	2	3	8/793
	p.S45P	0	1	0	6	7/805
	p.S37C	1	0	0	5	6/804
	p.S33C	0	0	0	5	5/796
	p.D32G	0	0	0	4	4/826
	p.S33P	0	1	0	3	4/814
	p.G34V	0	0	0	3	3/793
	p.D32H	0	0	0	2	2/801
	p.D32V	0	0	0	2	2/826
	p.S33A	0	1	0	1	2/814
	p.S37P	0	0	0	2	2/804
	p.S45Y	1	0	1	0	2/801
	p.S37A	0	0	0	1	1/804
	p.S37Y	0	0	0	1	1/804
	p.S45C	0	0	0	1	1/801
KRAS[1] (%)		7 (9)	43 (20)	3 (6)	86 (17)	139 (17)
	p.G12D	2	14	1	28	45/795
	p.G12V	3	10	1	23	37/795
	p.G13D	1	13	1	12	27/801
	p.G12A	0	4	0	10	14/795
	p.G12C	1	1	0	8	10/795
	p.G12S	0	0	0	3	3/795
	p.G13S	0	0	0	2	2/775
	p.Q61H(G)	0	1	0	1	2/791
	p.G13C	0	0	0	1	1/775
	p.G13R	1	0	0	0	1/775
	p.Q61L	0	0	0	0	1/784
FGFR2[1] (%)		2 (3)	19 (9)	0 (0)	59 (12)	80 (10)
	p.S252W	1	12	0	34	47/798
	p.N549K	1	1	0	15	17/795
	p.K659E	0	2	0	7	9/803
	p.C382R	0	4	0	2	6/805
	p.Y375C	0	2	0	1	3/806
POLE (%)		0 (0)	0 (0)	49 (100)	0 (0)	49 (6)
	p.P286R	0	0	32	0	32/834
	p.V411L	0	0	14	0	14/834
	p.S297F	0	0	3	0	3/834
FBXW7[1] (%)		8 (11)	13 (6)	1 (2)	18 (4)	40 (5)
	p.R465H	2	6	0	9	17/825
	p.R505C	4	3	0	5	12/799
	p.R479Q	2	3	1	1	7/803
	p.R465C	1	1	0	3	5/813
	p.R479L	0	1	0	0	1/803

Supplementary Table 2 continued.

	p53-mutant n=74	MSI n=219	*POLE*-mutant n=49	NSMP n=492	Total n=834
PPP2R1A[1] (%)	12 (16)	6 (3)	1 (2)	20 (4)	39 (5)
p.R183W	1	1	0	14	16/783
p.S256F	3	0	0	3	6/778
p.P179L	4	0	0	1	5/807
p.R183Q	0	2	1	2	5/779
p.R258H	0	3	0	1	4/784
p.S256Y	3	0	0	0	3/778
p.P179R	1	0	0	0	1/807
NRAS (%)	1 (1)	8 (4)	0 (0)	16 (3)	25 (3)
p.Q61L	1	3	0	2	6/800
p.Q61R	0	0	0	5	5/800
p.G12D	0	2	0	2	4/828
p.G12S	0	1	0	3	4/806
p.Q61K	0	0	0	2	2/811
p.G12A	0	0	0	1	1/828
p.G12C	0	1	0	0	1/806
p.G12V	0	1	0	0	1/828
p.G13R	0	0	0	1	1/788
CDKN2A (%)	0 (0)	0 (0)	0 (0)	2 (<1)	2 (<1)
p.R80*	0	0	0	1	1/805
p.D108A	0	0	0	1	1/799
BRAF (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
FGFR3 (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
FOXL2 (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
HRAS (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[1] Some tumors had multiple mutations in one gene. Frequencies presented as n (%), where n represents the number of samples showing the mutation. Analyzed hot spot mutations which were not detected are not shown.

Supplementary Table 3. Clinicopathological characteristics, additional mutations and protein expression alterations in tumors with multiple classifying alterations.

	p53 & MSI n=13	p53 & *POLE* n=7	MSI & *POLE* n=6	p53 & MSI & *POLE* n=1
Age, years				
Mean (range)	64 (52-73)	62 (49-76)	70 (61-79)	74 (-)
< 60	3 (23.1)	4 (57.1)	0	0
60-70	6 (46.1)	0	3 (50.0)	0
70	4 (30.8)	3 (42.9)	3 (50.0)	1
Grade				
1-2	10 (76.9)	5 (71.4)	5 (83.3)	1
3	3 (23.1)	2 (28.6)	1 (16.7)	0
Myometrial invasion				
<50%	6 (46.1)	2 (28.6)	0	0
>50%	7 (53.9)	5 (71.4)	6 (100)	1
LVSI				
Absent	12 (92.3)	7 (100)	4 (66.7)	1
Substantial	1 (7.7)	0	2 (33.3)	0
Risk group				
Low	6 (46.1)	4 (57.1)	0	0
High-intermediate	6 (46.1)	3 (42.9)	5 (83.3)	1
High	1 (7.7)	0	1 (16.7)	0
Treatment				
NAT	3 (23.1)	3 (42.9)	0	1
EBRT	5 (38.4)	4 (57.1)	3 (50.0)	0
VBT	5 (38.4)	0	3 (50.0)	0
Mutations				
CDKN2A	1 (7.7)	0	0	0
FBXW7	3 (23.1)	3 (42.9)	1 (16.7)	0
KRAS	1 (7.7)	0	0	0
PIK3CA	3 (23.1)	4 (57.1)	2 (33.3)	1
PPP2R1a	1 (7.7)	0	1 (16.7)	0
PTEN	5 (38.4)	6 (85.7)	4 (30.8)	1
Altered protein expression				
>10% L1CAM	1 (7.7)	2 (28.6)	1 (16.7)	0
<10% ER	2 (18.2)	2 (33.3)	1 (16.7)	0
<10% PR	3 (27.3)	2 (28.6)	1 (20.0)	0
loss/clonal ARID1a	4 (30.8)	1 (14.3)	3 (50.0)	0
loss/ heterogeneous PTEN	6 (46.1)	3 (42.9)	5 (83.3)	0
nuclear β-catenin	2 (15.4)	1 (14.3)	2 (33.3)	0
Survival				
Alive	10 (76.9)	7 (100)	4 (66.7)	0
Dead	3 (23.1)	0	2 (33.3)	1
Recurrence				
Locoregional	0	0	1 (16.7)	0
Distant	2 (15.4)	0	1 (16.7)	1

LVSI=lymphovascular space invasion, NAT=no additional treatment, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy

Supplementary Table 4. Multivariable analysis on the prognostic role of the clinicopathological characteristics, molecular subgroups, and potential other classifiers in all cases of early-stage endometrial cancer (n=834) and in the subset of EC without substantial LVSI, >10% L1CAM, p53 and *POLE* mutation (n=620).

All cases (n=834)

	Locoregional Recurrence 60 events			Distant Recurrence 65 events			Overall Survival 252 events		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (Cont.)	1.040	1.007-1.075	0.018	1.010	0.978-1.044	0.536	1.079	1.061-1.097	0.000
Grade									
1-2	1			1			1		
3	1.852	0.981-3.496	0.057	2.543	1.402-4.613	0.002	1.456	1.030-2.058	0.033
Myometrial invasion									
<50%	1			1			1		
>50%	1.315	0.727-2.381	0.365	1.681	0.913-3.094	0.096	1.077	0.798-1.455	0.627
LVSI									
Absent/mild	1			1			1		
Substantial	3.224	1.431-7.267	0.005	3.150	1.508-6.581	0.002	2.027	1.235-3.328	0.005
Treatment									
NAT	1			1			1		
EBRT	0.217	0.117-0.402	0.000	1.437	0.749-2.757	0.276	1.003	0.752-1.339	0.982
VBT	0.404	0.204-0.799	0.009	1.552	0.743-3.242	0.242	0.840	0.569-1.241	0.382
Molecular subgroup									
NSMP	1			1			1		
p53	4.089	2.060-8.116	0.000	4.422	2.221-8.803	0.000	2.475	1.682-3.642	0.000
MSI	1.425	0.797-2.645	0.224	1.622	0.876-3.004	0.124	1.444	1.071-1.948	0.016
POLE	-	-	0.964	1.060	0.245-4.592	0.938	1.247	0.625-2.488	0.531
L1CAM									
<10%				1			1		
>10%				3.028	1.540-5.953	0.001	2.098	1.366-3.221	0.001

Cases without substantial LVSI, >10% L1CAM, p53 and *POLE* mutation (n=620)

	Locoregional Recurrence 36 events			Distant Recurrence 30 events			Overall Survival 175 events		
	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (Cont.)	1.057	1.012-1.104	0.013	0.999	0.952-1.048	0.966	1.094	1.071-1.117	0.000
Grade									
1-2	1			1			1		
3	2.134	0.892-5.106	0.089	6.583	2.751-15.75	0.000	1.609	0.999-2.590	0.051
Myometrial invasion									
<50%	1			1			1		
>50%	1.199	0.549-2.622	0.649	1.539	0.639-3.709	0.336	0.914	0.635-1.315	0.628
Treatment									
NAT	1			1			1		
EBRT	0.256	0.118-0.555	0.001	1.456	0.610-3.476	0.397	1.050	0.747-1.476	0.777
VBT	0.218	0.071-0.663	0.007	1.065	0.333-3.402	0.916	0.807	0.486-1.339	0.407
Molecular subgroup									
NSMP	1			1			1		
MSI	1.181	0.579-2.409	0.647	2.181	0.997-.4770	0.051	1.431	1.036-1.976	0.030
CTNNB1									
No mutation				1					
Mutation				2.834	1.284-6.257	0.010			

Cont.=continuous, LVSI=lymphovascular space invasion, NAT=no additional treatment, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy

Supplementary Table 5. Univariable analysis of clinicopathological characteristics, molecular subgroups, and potential other classifiers in low-risk early-stage endometrial cancer (n=242).

		Locoregional Recurrence 12 events			Distant Recurrence 10 events			Overall Survival 67 events		
	n	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (cont.)	242	1.064	1.001-1.131	0.045	1.005	0.935-1.079	0.900	1.087	1.057-1.117	0.000
Grade										
1-2	227	1			1			1		
3	15	1.511	0.195-11.706	0.693	1.775	0.225-14.015	0.586	1.356	0.544-3.379	0.513
Myometrial invasion										
<50	189	1			1			1		
>50	53	0.305	0.039-2.366	0.256	0.880	0.187-4.143	0.871	0.763	0.416-1.399	0.382
LVSI										
Absent/mild	234	1			1			1		
Substantial	6	-	-	0.705	-	-	0.729	-	-	0.428
Given treatment										
NAT	112	1			1			1		
EBRT	111	0.098	0.012-0.762	0.026	2.022	0.506-8.084	0.319	1.067	0.651-1.751	0.796
VBT	19	0.681	0.086-5.400	0.716	2.017	0.210-19.404	0.544	1.722	0.592-5.011	0.318
Molecular subgroup										
NSMP	134	1			1			1		
p53	22	0.849	0.106-6.787	0.877	3.939	0.941-16.487	0.061	1.989	0.977-4.048	0.058
MSI	62	0.819	0.217-3.089	0.769	1.154	0.135-9.877	0.896	1.231	0.694-2.182	0.478
POLE	24	-	-	0.983	0.439	0.051-3.760	0.453	0.716	0.279-1.836	0.487
CTNNB1										
No mutation	176	1			1			1		
Mutation	53	1.664	0.501-5.527	0.406	2.579	0.693-9.606	0.158	0.909	0.502-1.646	0.753
FBXW7										
No mutation	217	1			1			1		
Mutation	12	1.563	0.201-12.131	0.669	-	-	0.634	0.443	0.108-1.818	0.259
FGFR2										
No mutation	217	1			1			1		
Mutation	12	-	-	0.589	2.442	0.305-19.539	0.400	1.236	0.445-3.434	0.685
KRAS										
No mutation	183	1			1			1		
Mutation	46	2.009	0.605-6.672	0.255	3.216	0.863-11.978	0.082	0.746	0.379-1.466	0.395
NRAS										
No mutation	221	1			1			1		
Mutation	8	-	-	0.671	-	-	0.718	1.998	0.725-5.506	0.181
PIK3CA										
No mutation	157	1			1			1		
Mutation	72	0.440	0.096-2.009	0.289	0.271	0.034-2.164	0.218	1.007	0.592-1.713	0.979
PPP2R1A										
No mutation	223	1			1			1		
Mutation	6	-	-	0.713	4.841	0.605-38.721	0.137	0.528	0.073-3.813	0.527
PTEN										
No mutation	129	1			1			1		
Mutation	100	0.628	0.189-2.087	0.448	0.643	0.161-2.572	0.533	0.751	0.452-1.247	0.268

Supplementary Table 5 continued.

		Locoregional Recurrence 12 events			Distant Recurrence 10 events			Overall Survival 67 events		
	n	HR	95% CI	*P*-value	HR	95% CI	*P*-value	HR	95% CI	*P*-value
L1CAM										
<10%	208	1			1			1		
>10%	8	10.72	2.211-52.000	0.003	10.49	2.107-52.263	0.004	4.167	1.765-9.835	0.001
ER										
>10%	188	1			1			1		
<10%	11	2.280	0.285-18.254	0.437	6.752	1.360-33.532	0.020	1.270	0.456-3.540	0.648
PR										
>10%	184	1			1			1		
<10%	20	1.259	0.157-10.071	0.828	3.365	0.679-16.681	0.137	1.897	0.929-3.874	0.079
ARID1a										
Positive	122	1			1			1		
Loss/clonal	82	0.417	0.087-2.008	0.276	1.508	0.377-6.029	0.562	0.980	0.579-1.659	0.940
PTEN										
Positive	122	1			1			1		
Loss/hetero.	86	1.818	0.488-6.774	0.373	0.460	0.093-2.281	0.342	1.023	0.604-1.735	0.931
β-catenin										
Membrane	155	1			1			1		
Nuclear	55	1.380	0.345-5.526	0.649	0.905	0.183-4.485	0.903	0.892	0.489-1.628	0.710

Cont.=continuous, LVSI=lymphovascular space invasion, NAT=no additional treatment, EBRT= external beam radiotherapy, VBT=vaginal brachytherapy, hetero.=heterogeneous.