

Development and validation of a RNAseq signature for prognostic stratification in endometrial cancer

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Key words

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Abstract

Background. Despite recent advances in endometrial carcinoma (EC) molecular characterization, its prognostication remains challenging. We aimed to assess whether RNAseq could stratify EC patient prognosis beyond current classification systems.

Methods. A prognostic signature was identified using a LASSO-penalized Cox model trained on TCGA (N=543 patients). A clinically applicable polyA-RNAseq-based work-flow was developed for validation of the signature in a cohort of stage I-IV patients treated in two Hospitals [2010-2017]. Model performances were evaluated using time-dependent ROC curves (prediction of disease-specific-survival (DSS)). The additional value of the RNAseq signature was evaluated by multivariable Cox model, adjusted on high-risk prognostic group (2021 ESGO-ESTRO-ESP guidelines: non-endometrioid histology or stage III-IVA or *TP53*-mutated molecular subgroup).

Results. Among 209 patients included in the external validation cohort, 61 (30%), 10 (5%), 52 (25%), and 82 (40%), had mismatch repair-deficient, *POLE*-mutated, *TP53*-mutated tumors, and tumors with no specific molecular profile, respectively. The 38-genes signature accurately predicted DSS (AUC=0.80). Most disease-related deaths occurred in high-risk patients (5-years DSS=78% (95%CI=[68%-89%]) versus 99% [97%-100%] in patients without high-risk). A composite classifier accounting for the *TP53*-mutated subgroup and the RNAseq signature identified three classes independently associated with DSS: RNAseq-good prognosis (reference, 5-years DSS=99%), non-*TP53* tumors but with RNAseq-poor prognosis (adjusted-hazard ratio (aHR)=5.75, 95%CI[1.14-29.0]), and *TP53*-mutated subgroup (aHR=5.64 [1.12-28.3]). The model accounting for the high-risk group and the composite classifier predicted DSS with AUC=0.84, versus AUC=0.76 without ($p=0.01$).

Conclusion. RNA-seq profiling can provide an additional prognostic information to established classification systems, and warrants validation for potential RNAseq-based therapeutic strategies in EC.

INTRODUCTION

Endometrial carcinoma (EC) is the most frequent pelvic gynecological cancer in Western countries [1]. A higher risk of relapse has been associated with specific pathologic features (high grade or non-endometrioid histological subtype) or extra-uterine spread (stage III-IVA)[2]. In these patients with adverse prognostic features, adjuvant chemotherapy modestly improved 5-years relapse-free (RFS) and overall survival (OS) (from 69 to 77% and from 76 to 81%, respectively) in the PORTEC3 trial [3], leaving room for improvement.

Four molecular subgroups of EC were identified by the Cancer Genome Atlas (TCGA) [4]: ultra-mutated tumors, related to pathogenic variants in the DNA polymerase ϵ (*POLE*) catalytic domain (5% of tumors); tumors with a high number of gene copy-number alterations (high-CNA) and *TP53* mutations (20-30%); microsatellite instable tumors (MSI), related to a deficient mismatch repair machinery (dMMR), mainly due to *MLH1* promoter hypermethylation, or mutation in MMR genes (20-30%); and a group of heterogenous tumors with no specific molecular profile (NSMP), referred as low copy-number alteration (low-CNA) (40%). Classification methods based on surrogates of molecular subgroups (*POLE* sequencing, TP53 and MMR immunohistochemistry analysis) have been validated [5], and subclassified PORTEC3 patients (patients with adverse clinical or pathological features) with 5-years RFS of 98%, 72%, 74%, and 48% for *POLE*-mutated, dMMR, NSMP, and *TP53*-mutated tumors, respectively [6]. In 2021, ESGO/ESTRO/ESP jointly proposed a new classification in which patients with myometrial invasive *TP53*-mutated tumors, FIGO stage III-IVA, and non-endometrioid tumors are defined at high-risk of recurrence, and recommend adjuvant chemotherapy in this subgroup [7].

However, this high-risk group is heterogenous in terms of histological type, grade and stage, and mutational background [8,9]. Refining their prognostication is of importance to identify patients for whom new adjuvant therapeutic strategies should be developed [4].

Gene expression signatures emerged as major tools for breast cancer patient prognostic assessment [10–13]. Besides, new technologies such as polyA-RNA-sequencing (RNAseq) emerged and presently allow to perform clinically-applicable/cost-efficient RNAseq [14,15].

In the present study, we developed and validated a polyA-RNAseq prognostic signature to improve prognostication in EC-patients, particularly for these affected by high-risk tumors.

MATERIAL AND METHODS

Patient & data

Training. Clinical, RNAseq, and molecular data from TCGA/*PanCanAtlas* (<https://portal.gdc.cancer.gov>; <https://gdc.cancer.gov/about-data/publications/PanCan-DDR-2018>) (inclusion: 1995-2013) were used to train a prognostic model based on RNAseq data.

Validation. The prognostic performances of the RNAseq model were evaluated in a cohort of patients treated in a routine setting in two University Hospital (Paris, France). The validation was performed in two steps: (i) technical validation of a polyA-RNAseq bioinformatical pipeline on a pilot cohort, and (ii) prognostic validation in an extended cohort that gathered patients from the pilot cohort and from an extension cohort (all data analyzed using the same bioinformatical pipeline). **Pilot cohort.** The pilot cohort included samples/data from a previously reported cohort of chemo-naïve stage I-IV EC consecutive patients treated at Cochin Hospital (2010-2017)[16,17]. Patients for whom (i) molecular characterization was successfully performed, (ii) FFPE sample showed high quality histological and cellular architecture conservation and fixation, and (iii) RNA amount was sufficient for optimal RNAseq, were included. **Extension cohort.** All consecutive patients treated for chemo-naïve stage I-IV EC in the gynecological surgery department of the Georges Pompidou University Hospital (HEGP) (2011-2017) were considered for inclusion. FFPE samples were selected on the basis of fixation quality and tumor cell content >50%.

Ethical/regulatory considerations. All patients were included in the CAncer REsearch for PErsonalized Medicine (CARPEM) biobank cohort (ethical approvals from National Ethical Committee CPP Ile-de-France I & II). The study was approved by appropriate Institutional Ethical & Regulatory Committee (Approval ID APHP-2020-1109121347).

Clinical/follow-up data and risk stratification. Baseline data were *post-hoc* extracted from standardized electronic medical records (age, histological type/grade, lymphatic/vascular space involvement (LVSI), international federation of gynecology and obstetrics (FIGO) 2010 stage, and immunohistochemistry for MMR and TP53). The risk of recurrence was *post-hoc* evaluated according to 2021 guidelines for the management of EC [7]: patients were considered at high-risk of recurrence when affected by myometrial-invasive tumors belonging to the *TP53*-mutated molecular subgroup and/or stage III-IVA tumors and/or non-endometrioid carcinoma. Patients with stage IVB tumors or stage III-IVA with residual diseases were categorized as advanced/metastatic [7]. Patients were followed as per routine practice from surgery to database lock (December 2020). Survival was assessed by disease-specific-survival (DSS) (survival until EC-related death), and event-free-survival (EFS) (survival until any EC-related event: relapse, progression, EC-related death).

Because of the retrospective collection of follow-up data, DSS was considered as a more robust survival parameter and as the primary outcome of interest.

Molecular characterization (Supp. Method 1, Figure S1).

Tumor DNA was extracted from macrodissected FFPE tissue block sections using the Maxwell® 16 FFPE Plus LEV DNA Purification Kit (Promega™, France). The high-CNA/*TP53*-mutated molecular group was identified according to a validated method using sequential surrogates of the TCGA classification system [5]: dMMR (surrogate of MSI), *POLE* mutation (ultra-mutated tumors), and *TP53*-mutation (high-CNA tumors). Remaining tumors were classified as NSMP (low-CNA tumors). Therefore, all tumors without dMMR or pathogenic *POLE* mutation, and with a *TP53* mutation were classified as *TP53*-mutated tumors [4,5,18].

Gene expression analyses (Supp. Method 2-3).

Tumor RNA was extracted from macrodissected FFPE tissue block sections using the Maxwell® 16 LEV RNA FFPE Kit. qPCR data were previously generated for genes involved in oxidative stress response, and for housekeeping genes [17]. PolyA-RNAseq libraries were prepared using the QuantSeq 3'mRNA-Seq Kit FWD for Illumina (Lexogen™) according to the manufacturer's instructions. Libraries were sequenced on NovaSeq6000. Fastq RNA-seq files were analyzed using a standard bioinformatical pipeline, with adaptations related to polyA sequencing and expected fragmented RNA from FFPE-sample (Figure S2). Briefly, reads were trimmed by cutadapt [19] and mapped by STAR [20]. Reads-per-gene count was performed with HTseq [21] (GRCh38.p13 human genome). Count files were normalized (*edgeR R* package) to get log2-counts-per-million (logCPM) gene expression data [22,23]. A batch-effect correction was used because of technical variations between TCGA and CARPEM data (frozen versus FFPE samples, whole transcriptome versus polyA-RNAseq) data.

Prognostic signature: model training and validation (Supp. Method 4).

TCGA batch-corrected logCPM data were used to identify genes associated with poor outcome. Because of the limited follow-up available in TCGA [24], EFS was used as a surrogate of DSS. A cross-validated LASSO-penalized Cox model was trained to identify most important genes predictive of EFS [25–27], and to compute hazards (continuous score). A classification threshold was identified on area under time-dependent ROC curve (time-dependent AUC) analyses in the training set to categorize patients in good versus poor prognosis [28,29]. The model was applied on CARPEM data to evaluate predictive performances using time-dependent AUC (with and without consideration of competing events – death not related to EC in older patients)[30].

Control of sample eligibility for validation of prediction (Supp. Method 5).

A cross-validated LASSO-penalized binomial regression model was trained on TCGA to confirm each CARPEM samples as primary tumor or adjacent tissue (control of potential macrodissection failure resulting in low tumor cell content).

Statistical analyses.

Associations between qualitative/continuous variables were analyzed using Student t-test/Wilcoxon test. Associations between continuous variables were analyzed using linear regression. Associations between categorical/binary variables were assessed using chi-square/Fisher's exact test. Median [interquartile range (IQR)] follow-up was estimated using the 1-Kaplan-Meier method. Survivals were analyzed by Kaplan-Meier curves with t-time [95% confidence interval (95%CI)] survival rates, and compared using log-rank test.

Associations between patient/cancer characteristics and DSS/EFS were assessed by Cox models to estimate hazard-ratio (HR) with [95%CI]. Proportional hazard assumption was checked. Survival analyses were controlled using Fine-Gray model to account for potential competing death (validation cohort)[31]. The independent prognostic value of the RNAseq signature was evaluated by adjustment on the 2021 high-risk prognostic group (as composite criterion; described above)[7]. The additional prognostic value was evaluated by comparing time-dependent AUC of two Cox models, including the 2021 high-risk group, with or without RNAseq predictions (*timeROC R* package). All test were two-sided. Significance was defined by $p < 0.05$. Analyses were performed using *R* v4.

Results

Study population.

The population included 752 patients (TCGA: N=543 patients, median follow-up=35 months [19-62]; CARPEM: N=209 patients, median follow-up=55 months [41-69]) (Flow-chart: **Figure S3**). Patient/cancer characteristics are provided in **Table 1**. Within the CARPEM cohort, 83 patients (39%) were found at high-risk according to 2021 guidelines. Most disease-related deaths occurred in this subgroup of patients (5-years DSS rate 78% [68%;89%] versus 99% [97%;100%] in patients without adverse prognostic features). Overall, 5-years DSS rate was 57% [43%;76%] in patients with *TP53*-mutated tumors (**Figure S4**).

Table 1. Patient and cancer characteristics

Characteristic	CARPEM cohort		TCGA cohort	p-value†
	Cochin, N = 75	HEGP, N = 134	N = 543	
Age at diagnostic	66 [56-76]	68 [61-78]	64 [57-71]	<0.001
Unknown	0	0	3	
Histological type				<0.001
Endometrioid	62 (83%)	91 (68%)	407 (75%)	
Serous	8 (11%)	23 (17%)	136 (25%)	
Carcinosarcoma	4 (5%)	9 (7%)	0 (0%)	
Clear cell	1 (1.3%)	10 (8%)	0 (0%)	
Neuroendocrine	0 (0%)	1 (1%)	0 (0%)	
Histological grade*				<0.001
Low grade	55 (73%)	79 (59%)	218 (40%)	
High grade	20 (27%)	55 (41%)	325 (60%)	
Lymphatic or vascular space involvement	22 (31%)	60 (45%)	-	-
Unknown	3	1	543	
Tumor stage‡				<0.001
Stage I	49 (65%)	84 (63%)	339 (62%)	
Stage II	1 (1%)	13 (10%)	51 (9%)	
Stage III	18 (24%)	31 (23%)	124 (23%)	
Stage IV	7 (9.3%)	6 (4%)	29 (5%)	
Molecular group¶				<0.001
dMMR/MSI	27 (36%)	34 (25%)	124 (25%)	
POLE/ultra-mutated	3 (4%)	7 (5%)	79 (16%)	
TP53-mutated/high-CNA	18 (24%)	34 (26%)	159 (31%)	
NSMP/low-CNA	27 (36%)	55 (42%)	143 (28%)	
Unknown	0	4	38	
Risk of recurrence (ESGO/ESTRO/ESP 2021)§				0.6
High risk	27 (36%)	56 (43%)	218 (42%)	
Low/intermediate/high-intermediate risk	41 (55%)	69 (53%)	267 (52%)	
Unknown	0	3	29	
Radiation therapy	29 (39%)	58 (43%)	226 (42%)	0.8
Unknown	1	0	0	
Chemotherapy	27 (36%)	50 (37%)	37 (6.8%)	<0.001
Unknown	1	0	0	

HEGP: Georges Pompidou European Hospital. TCGA: the cancer genome atlas cohort. Statistics presented: median [interquartile range] or N (%), as appropriate. *Low grade: grade I-II tumors, versus high grade for grade III tumors. † Statistical tests performed: Kruskal-Wallis/ANOVA test; Fisher's exact test; or chi-square test of independence, as appropriate. ‡ Tumor stage as per International Federation of Gynecology and Obstetrics 2010 staging system for Cochin and HEGP cohorts, and as per American Joint Committee on Cancer TNM staging system for TCGA. ¶ Molecular groups were assessed in TCGA as per the seminal paper (Levine et al. Nature 497, 67–73 (2013), and are available at <https://gdc.cancer.gov/about-data/publications/PanCan-DDR-2018> and as per the ProMISE surrogate classification in the CARPEM cohort (see supplemental method 1). Acronyms are indicated for CARPEM/TCGA: dMMR/MSI: mismatch repair deficient / microsatellite instable tumors; POLE: POLE-mutated tumors; TP53-mutated/high-CNA: TP53-mutated / tumors with high copy-number alterations; NSMP/low-CNA: no specific molecular subtype / tumors with low copy-number alterations. § Patients were classified at high-risk of recurrence based on TP53 alteration and/or stage III-IVA tumors and/or non-endometrioid carcinoma according to ESGO/ESTRO/ESP 2021 guidelines, excluding patients with stage IVB tumors.

Validity and robustness of polyA-RNAseq profiling (CARPEM cohort).

PolyA-RNAseq transcriptome profiling showed strong technical reproducibility (**Figure S5**). Gene-level qPCR and logCPM expression data were strongly correlated (**Figure S6**). Unsupervised clustering analyses confirmed the successful batch-effect correction (**Figure S7**). Biological replicates analyses identified pre-

analytical (tissue fixation quality) and analytical (library quality, post-trimming read length) criteria associated with reproducibility (**Table S1, Figure S8**).

Prognostic signature training in the TCGA cohort.

A 38-genes prognostic signature was identified and predicted EFS with 5-years AUC=0.82 [0.76;0.88] in the TCGA dataset (**Figure S9-S11, Figure 1**). Most high-CNA tumors were found in the poor prognostic RNAseq group (N=137/159 (86%) versus 22/159 (14%) in the good prognostic group), leading to create a composite classifier associating RNAseq signature with high-CNA molecular subtype (**Table S2**). Therefore, patients were classified in three groups: good prognosis tumors based on RNAseq signature and without high-CNA (reference), poor prognosis tumors based on RNAseq signature and without high-CNA (HR: 8.84 [4.76; 16.4]), and high-CNA tumors whatever the RNAseq signature (HR: 7.65 [4.04; 14.4]) (HR adjusted on stage and histological type) (**Table S3, Figure 1E**).

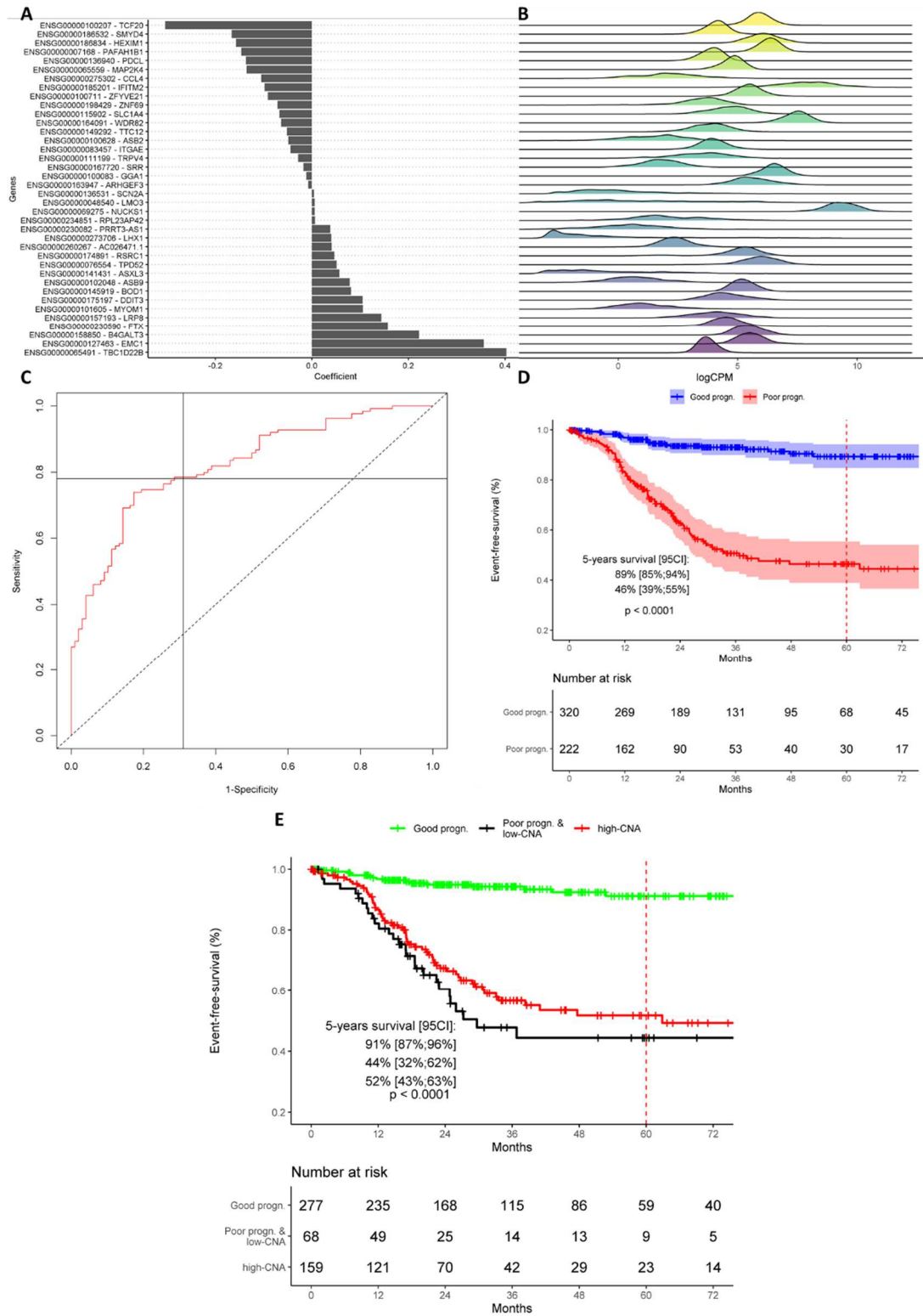


Figure 1. LASSO penalized Cox regression model and predictions in the TCGA training dataset. A. Genes automatically selected in the LASSO penalized Cox regression model training. ENSG: Ensembl gene identifiers, provided with gene name. Coefficient refers to the Beta coefficient computed by the LASSO penalized regression model. **B. Distribution of gene expression across the TCGA dataset.** Density plots, by gene (gene expression expressed in normalized log2 copy-per-million reads). Each density line refers to gene as an extension of plot A. **C. Time dependent ROC curve at 5 years for event-free-survival.** ROC curve estimated by marginal inverse probability of censoring weighting estimation (Kaplan-Meier estimator). AUC=0.82. Vertical and horizontal lines figure the threshold finally selected (5-years predictive positive and negative values of 51% and 89%). **D. Event-free-survival according to the RNASeq prognostic group.** p: log-rank derived p-value. Kaplan-Meier curve censored at 6 years. **E. Event-free survival according to the composite RNAseq-molecular classifier.** Patient are classified as: *Good progn.*: tumors with good prognosis based on RNAseq signature and without high-CNA tumor; *Poor progn. & low-CNA*: tumors with poor prognosis based on RNAseq signature but without high-CNA tumor; *High-CNA*: high-CNA tumors, whatever the RNAseq prognostic group. p: log-rank derived p-value. Kaplan-Meier curve censored at 6 years.

Validation in the CARPEM cohort.

One sample was not identified as a tumor sample and was excluded (**Figure S12, Table S1**). The RNAseq model accurately predicted 5-years DSS (AUC=0.80 [0.69;0.91]) and EFS (AUC=0.74 [0.61;0.86]) (**Figure 2.A-D**). Patients were classified using the composite RNAseq-molecular classifier described above (considering the *TP53*-mutated molecular subgroup as surrogate of high-CNA [5]) in three groups with significantly different DSS, EFS (**Figure 2.E-F, Table 2**) and post-relapse/progression DSS (**Figure 2.G**).

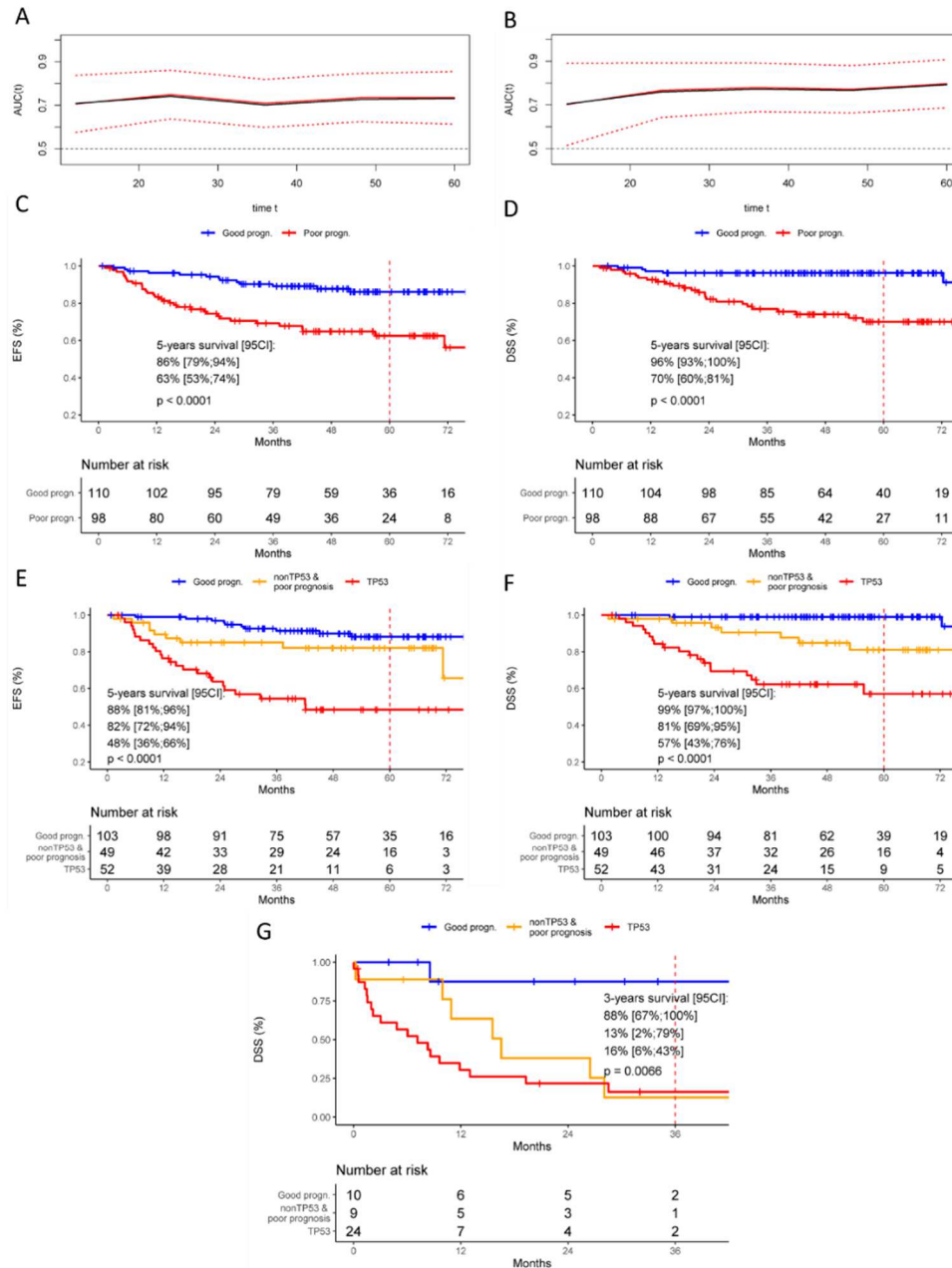


Figure 2. Validation in the CARPEM cohort. CARPEM: Cochin Hospital and HEGP cohorts. p: log-rank derived p -value. DSS: disease-specific survival. EFS: event-free-survival. **A-B. time-dependent AUC of the RNAseq signature for EFS (A) and DSS (B) prediction at follow-up time t (AUC(t)).** time t: time (months). Red line: AUC at time t estimated using marginal inverse probability of censoring weighting estimation (Kaplan-Meier estimator) (dashed red line: 95% confidence interval). Black line: AUC at time t estimated using the inverse probability of censoring weighting estimation accounting for competing death (additive Aalen model). **C-D. EFS and DSS according to the RNAseq signature.** Kaplan-Meier curves censored at 6 years (<10% of patients). *Good* and *Poor progn.* as per the raw RNAseq signature, without consideration of the *TP53*-mutated molecular subgroup. **E-F. EFS and DSS according to the composite RNAseq-molecular group classifier.** *Good progn.*: tumors with good prognosis based on RNAseq signature and without *TP53*-mutated tumor; *nonTP53 & poor prognosis*: tumors with poor prognosis based on RNAseq signature and without *TP53*-mutated tumor; *TP53*: *TP53*-mutated tumors. **G. DSS after first relapse/progression according to the composite RNAseq-molecular group classifier.** Kaplan-Meier curves censored at 3.5 years.

Table 2. Patient and cancer characteristics according to the composite RNAseq-molecular classifier in the CARPEM cohort.

Characteristic	TP53-wild type & RNA seq good prognosis, N = 103	TP53-wild type & RNA seq poor prognosis, N = 49	TP53-mutated tumors, N = 52	p-value†
Age at diagnostic	65 [56-76]	68 [59-78]	71 [66-78]	0.004
Histological type				<0.001
Endometrioid	95 (92%)	43 (88%)	12 (23%)	
Clear cell	5 (4.9%)	3 (6.1%)	2 (3.8%)	
Carcinosarcoma	0 (0%)	1 (2.0%)	12 (23%)	
Neuroendocrine	0 (0%)	1 (2.0%)	0 (0%)	
Serous	3 (2.9%)	1 (2.0%)	26 (50%)	
Histological grade*				<0.001
Low grade	85 (83%)	39 (80%)	7 (13%)	
High grade	18 (17%)	10 (20%)	45 (87%)	
Lymphatic or vascular space involvement	32 (32%)	15 (32%)	32 (62%)	<0.001
Unknown	2	2	0	
FIGO stage				<0.001
Stage I	80 (78%)	31 (63%)	19 (37%)	
Stage II	4 (3.9%)	3 (6.1%)	6 (12%)	
Stage III	19 (18%)	13 (27%)	16 (31%)	
Stage IV	0 (0%)	2 (4.1%)	11 (21%)	
Molecular subtype§				
dMMR	42 (41%)	19 (39%)	0 (0%)	
POLE-mutated	9 (8.7%)	1 (2.0%)	0 (0%)	
TP53-mutated	0 (0%)	0 (0%)	52 (100%)	
NSMP	52 (50%)	29 (59%)	0 (0%)	
Risk of recurrence (ESGO/ESTRO/ESP 2021)¶				
Adv./metastatic	0 (0%)	2 (3%)	11 (21%)	
High risk	24 (24%)	16 (33%)	41 (79%)	
Low/intermediate/high-intermediate risk	77 (76%)	31 (63%)	0 (0%)	
Unknown	2	0	0	
Adjuvant radiation therapy	37 (36%)	17 (35%)	29 (57%)	0.027
Unknown	0	0	1	
Adjuvant chemotherapy	24 (23%)	13 (27%)	38 (75%)	<0.001
Unknown	0	0	1	

Patient are classified as: “TP53-wild type & RNA seq good prognosis”: tumors with good prognosis based on RNAseq signature and without TP53-mutated tumor (molecular group); “TP53-wild type & RNA seq poor prognosis”: tumors with poor prognosis based on RNAseq signature and without TP53-mutated tumor; and “TP53-mutated tumors”: tumors belonging to the TP53-mutated molecular group. Statistics presented: Median (IQR); n (%). † Statistical tests performed: Kruskal-Wallis test; Fisher’s exact test; chi-square test of independence. *Low grade: grade I-II endometrioid tumors, versus high grade for non-endometrioid and grade III endometrioid tumors. FIGO: International Federation of Gynecology and Obstetrics 2010 staging system. § molecular subtypes as per the ProMisE classification (supplemental method 1): dMMR: mismatch repair deficient tumors; POLE-mutated: POLE-mutated tumors; TP53-mutated: TP53-mutated tumors; NSMP: no specific molecular subtype. ¶ Risk of recurrence group as per 2021 guidelines (Int J Gynecol Cancer. 2021 Jan;31(1):12-39). Note that 5 patients presented in the table 1 does not figure in this table (unavailable molecular group: N=4; one sample not identified as a tumor: N=1).

Tumors without TP53 mutations but classified at poor prognostic based on RNAseq were strongly associated with poor DSS (**Table 3, Table S4**), despite these tumors had similar histologic features compared to RNA seq good prognostic tumors (**Table 2**). When adjusted on the 2021 high-risk prognostic group, the composite RNAseq-molecular classifier was independently associated with DSS (**Table 3, Table S4**).

Table 3. Association between disease specific survival and the composite RNAseq-molecular classifier in the CARPEM cohort.

Categories	Number of patients	Number of death	HR [95%CI]	p*	Adjusted HR [95%CI]	p*
RNAseq-molecular risk group						
Good prognosis/TP53-wild type tumors	101	2	1 (reference)	-	1 (reference)	-
Poor prognosis/TP53-wild type tumors	47	6	7.15 [1.44; 35.6]	0.016	5.75 [1.14; 29.0]	0.034
TP53-mutated	41	10	14.4 [3.15; 66.0]	<0.001	5.64 [1.12; 28.3]	0.036
Risk of recurrence (ESGO/ESTRO/ESP 2021)†						
No high-risk features	108	2	1 (reference)	-	1 (reference)	-
High risk	82	16	10.3 [2.4; 44.7]	0.002	5.77 [1.15; 29.1]	0.034

DSS: disease-specific survival. HR [95%CI]: hazard ratio with 95% confidence interval, estimated using Cox regression model. *p-value computed using Cox regression model. RNAseq-molecular risk account for RNAseq risk and TP53 molecular group. Patient are classified as: “*TP53-wild type & RNA seq good prognosis*”: tumors with good prognosis based on RNAseq signature and without *TP53*-mutated tumor (molecular group); “*TP53-wild type & RNA seq poor prognosis*”: tumors with poor prognosis based on RNAseq signature and without *TP53*-mutated tumor; and “*TP53-mutated tumors*”: tumors belonging to the *TP53*-mutated molecular group. † Risk of recurrence group as per 2021 guidelines (Int J Gynecol Cancer. 2021 Jan;31(1):12-39). Patients with advanced/metastatic diseases are not included in this analysis.

Analysis 1: n= 189 patients in multivariate analysis, number of events= 18.

The Cox model accounting for the RNAseq-molecular classifier and the 2021 high-risk prognostic group predicted DSS with an AUC of 0.84 [0.75;0.94], versus 0.76 [0.69;0.85] when accounting only for the 2021 high-risk prognostic group ($p=0.013$). The RNAseq-molecular classifier allowed to significantly stratify patient prognostic within the high-risk prognostic group, while the *TP53*-mutated molecular group alone did not (**Figure 3**). No significant bias related to tumor stage, histological type/grade, and molecular subtype was found (**Table S5**). Robustness of predictions were confirmed when quality criteria previously identified were satisfied (**Figure S13-16**).

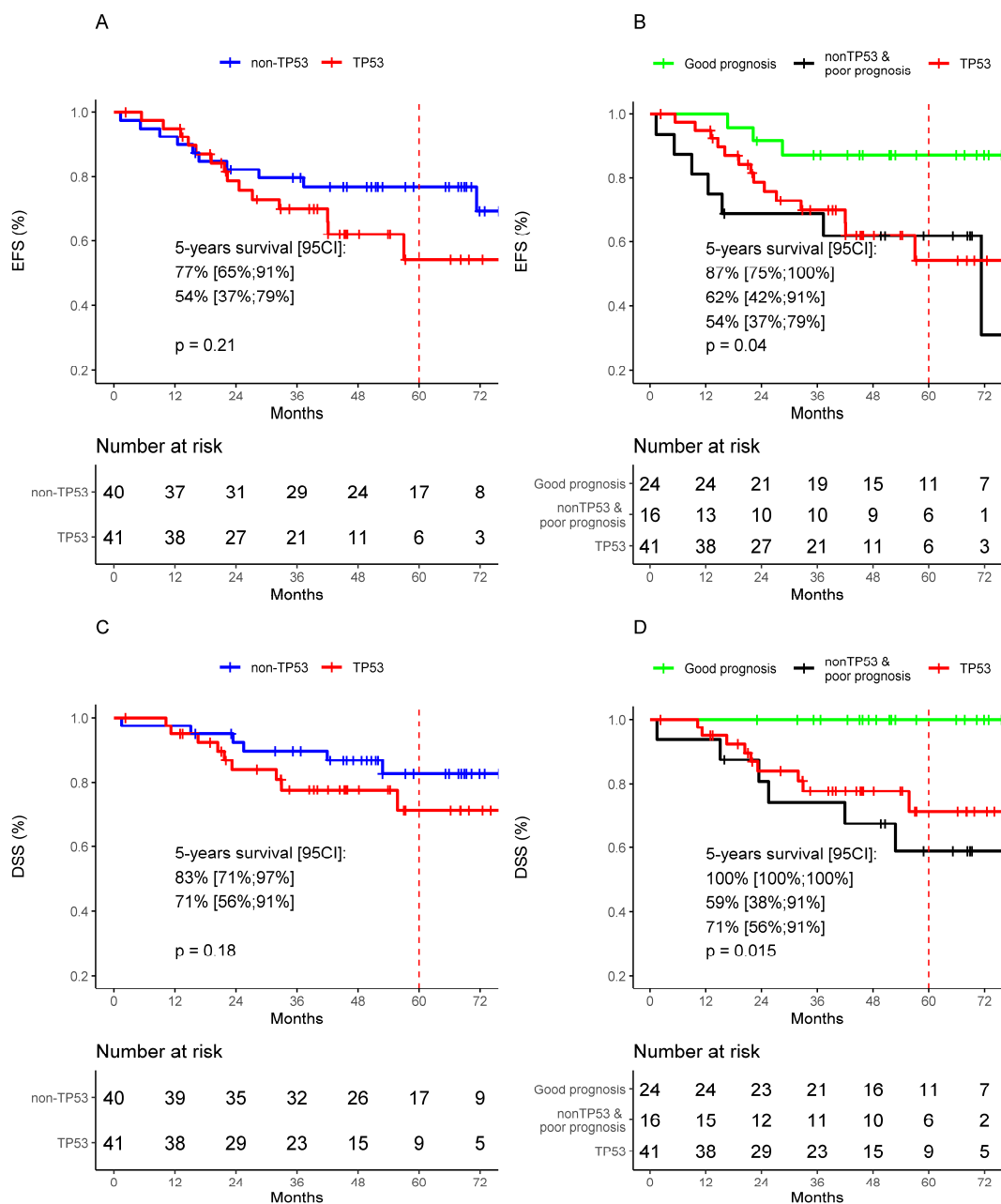


Figure 3. Survival according to *TP53*-mutated molecular group versus composite RNAseq-molecular classifier in patients with high-risk prognostic features according to ESGO/ESTRO/ESP 2021 guidelines. (*TP53*-mutated tumors or non-endometrioid histology or FIGO stage III-IVA). Patients are classified by the composite RNAseq-molecular classifier as: *Good progn*: tumors with good prognosis based on RNAseq signature and without *TP53*-mutated tumor; *nonTP53 & poor prognosis*: tumors with poor prognosis based on RNAseq signature and without *TP53*-mutated tumor; *TP53*: *TP53*-mutated tumors. EFS: event-free-survival. DSS: disease-specific survival. Kaplan-Meier curves censored at 6 years, considering 5 years as the primary criteria of interest in PORTEC3 trial. p: log-rank derived *p*-value. **A-B. EFS according to *TP53*-mutated molecular group (A) or composite RNAseq-molecular classifier (B).** **C-D. DSS according to *TP53*-mutated molecular group (C) or composite RNAseq-molecular classifier (D).** Note that one high-risk patient presented in the table 1 does not figure in this plot because of molecular group unavailable.

Biological understanding of the RNAseq-molecular risk classifier (Supp. Methods 6).

The 38-gene list and corresponding annotations are provided as **Table S6**. A differential gene expression analysis within NSMP/dMMR tumors was performed to explore whether the group identified at poor prognostic despite no high-CNA/*TP53* mutation was associated with specific biological processes (**Figure S17**). TCGA analyses suggested a downregulation in T cell activation, and an upregulation in processes related to cell cycle

regulation, nuclear processes, and metabolic changes. Tumors with poor prognosis showed lower lymphocyte infiltration scores and IFN-g response, while higher proliferation scores and an enrichment in wound healing processes. CARPEM cohort data confirmed the upregulation of cell cycle and nuclear organization genes, and the immune depletion in poor prognostic tumors (**Table S7-S9**).

DISCUSSION

Our results demonstrate the additional value of a polyA-RNAseq signature to stratify EC-patient prognostic beyond established classification systems. We propose a classifier combining the TP53-molecular subgroup and the RNAseq-prognostic group. This classifier identified, within high-risk patients according to 2021 guidelines, a subgroup of patients without *TP53*-mutated tumors but with a poor prognosis similar to patients with *TP53*-mutated tumors, and a group with a 5-years DSS rate of 100%, despite adverse histological subtypes and/or stage III-IVA EC.

Despite expected routine-practice pathology constraints [32], we observed less than 5% of RNAseq technical failure in the extension cohort, suggesting the feasibility of RNAseq in clinical practice. Importantly, quality criteria associated with high reproducibility and robustness were identified (**Figure S18**). The performances of the RNAseq signature were consistent with previously reported models for cancer patient survival prediction (AUC~80%)[33–35]. Relying on RNAseq offers flexibility to adjust models/predictions based on futures observations. Reverseely, gene expression-based prognostic signatures used in breast cancer are based on custom microarray or RT-PCR (rather than on genome-wide analysis), and are therefore limited by gene panels included in these assays [10–13].

CARPEM cohort data were comparable with previously published cohorts [3,5], while TCGA patient accrual was controlled to include a planned number of endometrioid and serous carcinoma. Tumors without pathological/molecular adverse feature (i.e. endometrioid, *TP53*-wild-type, stage I-II tumors) were confirmed at low risk of EC-related death, supporting the high sensitivity of the 2021 ESGO/ESTRO/ESP classification. Reverseely, our results highlight the prognostic heterogeneity of patients with high-risk features: about one third of so-called high-risk patients were found at low risk of death by the composite RNAseq-molecular classifier, while 40% of non-*TP53* tumors were associated with a 5-years DSS rate similar to *TP53*-mutated tumors.

Beyond initial management, the composite RNAseq-molecular classifier correlated with tumor aggressiveness after relapse/progression. A better knowledge of post-relapse survival could help to select patients with relapsed/metastatic diseases most eligible for local/regional treatment. Our biological findings were consistent with previous TCGA results that identified tumors driven by immune deprivation/wound healing processes as of poor prognostic [36]. New therapeutic strategies using anti-PD1 monoclonal antibodies are under

development in advanced EC [37,38]. Studying whether the composite RNAseq-molecular classifier could be used as a stratification parameter in this setting would be of interest.

Several limitations should be taken into consideration. The statistical power precluded analyses integrating the wide diversity of EC. Hence, whether the RNAseq classifier is independent of histological type within each molecular group and tumor stage remains to be confirmed. The inclusion period of CARPEM patients could have introduced heterogeneity in the pathology analysis and in the therapeutic management. To ensure consistency between pathology data and therapeutic management, we did not perform a post-hoc pathology review. The retrospective inclusion of patients could also have introduced a selection bias. Finally, molecular characterization was based on surrogates of molecular groups [5]: high-CNA tumors and *TP53*-mutation are not fully equivalent, as are MMR immunochemistry analysis and MSI assessment by molecular analyses [4]. Despite these limitations, the distribution of patients/cancer characteristics and survival profiles were similar to previously published cohorts, suggesting that such bias would be limited [5].

In conclusion, we demonstrate that RNA-seq profiling could accurately stratify EC patient prognosis, and could provide an additional prognostic information to established classification systems. Subgroup analyses suggest that the RNAseq classifier could be of particular interest in patients with adverse histological features but without *TP53*-mutated tumors. Despite further validations are warranted, the presented method paves the way for the development of RNAseq-based molecular assessment and personalized treatment in routine clinical practice for EC patients.

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Materials, data, and protocols described in the manuscript will be made available upon reasonable request at the corresponding author.

Disclosure of potential conflicts of interest

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