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Evaluation of endometrial carcinoma prognostic immunohistochemistry markers in the context of molecular classification

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Abstract

Molecular subclassification of endometrial carcinoma (EC) with Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) identifies four subtypes [DNA polymerase epsilon (*POLE*) mutant, mismatch repair-deficient, p53 wild-type (wt), and p53 abnormal]. The aim of this study was to evaluate additional EC biomarkers in the context of these subtypes. Tissue microarrays encompassing 460 previously characterized ECs were assessed for L1-cell adhesion molecule (L1CAM), progesterone receptor (PR), estrogen receptor (ER) alpha, stathmin, and phosphatase and tensin homolog (PTEN), by immunohistochemistry (IHC). Associations with clinicopathological parameters, molecular subtype, and outcomes were determined. About 413 ECs (75% endometrioid, >15% serous) had complete data. L1CAM overexpression was found in 16%, associated with older age, lower body mass index (BMI), advanced stage, grade 3 (97%), non-endometrioid histology (84%), deep myometrial invasion, lymphovascular space invasion (LVSI), and ER-negative, PR-negative status. Tumours overexpressing L1CAM were associated with poor outcomes {hazard ratio (HR) [95% confidence interval (CI)] 3.35 [2.10–5.23] for disease-specific survival [DSS], $p < 0.0001$ }. PR positivity was associated with younger women, higher BMI, early stage (77% stage I), low grade (61%), endometrioid histology (90%) without LVSI or nodal disease, ER positivity (90%), p53wt tumours (55%), and favourable outcomes [HR (CI) 0.39 (0.25–0.62) for DSS, $p < 0.0001$]. ER positive tumours were early stage (73%), low grade, endometrioid histology, with improved DSS. Stathmin and PTEN IHC were not associated with outcomes. There was minimal agreement between IHC and mutation status for *PTEN*. L1CAM overexpression was significantly associated with the p53 abnormal molecular subtype, which accounted for more than 70% of the tumours overexpressing L1CAM. PR expression also correlated with molecular subtype, with most PR negative tumours being p53 abnormal. Multivariable analysis demonstrated that only ProMisE subtype [overall survival (OS), DSS, and progression-free survival] and age (OS only) maintained an association with outcomes. The prognostic significance of the single biomarkers tested could be explained based on their being covariable with the ProMisE molecular subtype.

Keywords: endometrial cancer; prognosis; biomarker; TCGA; ProMisE; L1CAM; progesterone receptor; estrogen receptor

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[†]Conflict of interest statement: Authors JM, DH, and AT have filed a provisional patent for ProMisE (US patent# 62192230) for which the BC Cancer Agency, not the individuals, are the named beneficiaries. All other authors have no conflict of interest to declare.

Introduction

Endometrial cancer (EC) is the most common gynaecological cancer and fourth most common cancer in women overall in the developed world. There were 320 000 new cases of EC diagnosed worldwide in 2012 [1]. Advances in clinical management and research have been hampered by an inability to reproducibly categorize endometrial tumours based on histomorphological features [2,3]. Risk stratification systems used to guide treatment rely on these parameters [4–6], in the absence of more robust markers. A push to more biologically informative molecular tools to classify tumours and stratify according to risk of metastases and recurrence has led to a multitude of studies interrogating the prognostic and predictive implications of one or more biomarkers or molecular features.

The most comprehensive of these investigations was the collaborative Cancer Genome Atlas project (TCGA) that characterized 373 endometrioid and serous endometrial carcinomas (ECs) through genomic, proteomic, and gene expression assays, identifying four prognostic subtypes with distinct prognostic outcomes [7]. Subsequently, two research teams have pared down and simplified the molecular methods in TCGA to identify similar but not identical prognostic subtypes from formalin-fixed paraffin embedded material [8–12]. Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) identifies these four TCGA-based molecular subtypes using immunohistochemistry and sequencing of the *POLE* exonuclease domains (Figure 1), and has been developed according to the Institute of Medicine (IOM) guidelines for the development of ‘omics’ based tests, following a strict pathway of ‘discovery’ [9], ‘confirmation’ [10], and ‘validation’

(manuscript herein) steps. ProMisE is ready to cross the ‘bright line’ for application in the clinic [13], at which point all historical biomarkers (clinical, pathological, molecular) need to be re-evaluated in the context of these molecular subtypes. ProMisE has also proven to work on diagnostic endometrial biopsy/curettage specimens with high concordance to hysterectomy specimens, enabling earlier biologically relevant information for patients and clinicians that may guide management from the earliest time point [8].

Our objective in this study was to re-evaluate select molecular markers of purported prognostic significance: L1-cell adhesion molecule (L1CAM) [14–16], progesterone receptor (PR) [17–19], estrogen receptor alpha (ER) [18–20], stathmin (STMN) [21,22], and phosphatase and tensin homolog (PTEN) [23,24], assessing their value in the framework of modern molecular classification (by ProMisE).

Methods

Cohort

Following ethical review board approval, 460 cases of EC, encompassing the ‘discovery’ [9] and ‘confirmation’ [10] cohorts for the development of the ProMisE molecular classifier were represented in duplicate cores over six tissue microarrays (TMAs) (supplementary material, Figure S1). This cohort has been previously characterized, with demographic, clinical, pathological, and outcomes reported [9,10]. As performed previously [9,10], to ensure random censoring and minimize ascertainment bias, observations were censored on December 31 on the fifth year of follow-up.

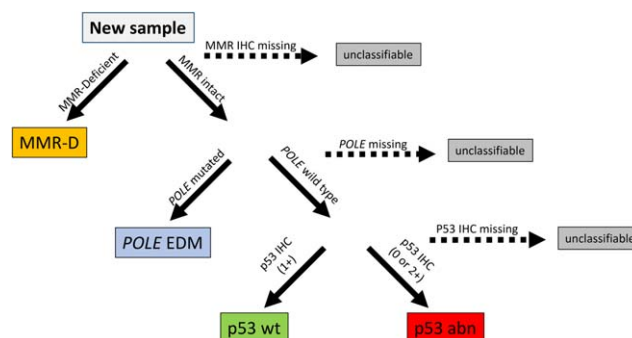


Figure 1. Algorithm for ProMisE molecular classifier. A sample is tested for MMR deficiency by IHC for the presence or absence of MSH6 and PMS2 proteins, sequencing is performed for exons 9–14 of the *POLE* exonuclease domain (EDM), and IHC for p53 performed to identify complete loss/null (IHC score 0), accumulation/misense (IHC score 2), or normal expression/wt (IHC score 1) yielding four molecular subtypes with distinct clinical outcomes. Reproduced with permission from [10].

Table 1. Methodological details for the five immunohistochemical marker

Biomarker	Company	Catalogue	Titration	Staining protocol	Scoring criteria
L1CAM	Covance	SIG-3911	1:25	Ventana Discovery Ultra - 64 min CC1 - 32 min primary at 37°C - UltraMap DAB anti-Ms kit	Low = up to 50% High = >50%
PR	Ventana	790-2223 (clone 1E2)	Undiluted	Ventana Discovery Ultra - 64 min CC1 - 16 min primary at 36°C - DABMap kit	Negative = up to 1% Positive = >1%
ER	Thermo	RM-9101 (clone SP1)	1:25	Ventana Discovery Ultra - 64 min CC1 - 1 h primary at 37°C - DABMap kit	Negative = up to 5% Positive = >5%
STMN	Cell Signaling	3352	1:50	Ventana Discovery XT - Standard CC1 - 1 h primary with heat - DABMap kit	Low = negative, weak, moderate (scores 0–4) High = strong (scores 6, 9)
PTEN	Cell Signaling	9559	1:25	Ventana Discovery Ultra - 64 min CC1 - 1 h primary at room temp - UltraMap DAB anti-Rb kit	Negative = no staining Positive = any staining

Methods for ProMisE, including details on TMA construction, DNA extraction, sequencing, and interpretation have been described previously [9,10,25]; these studies yielded four prognostic subtypes; ECs with mismatch repair deficiency based on immunohistochemical (IHC) testing for the presence/absence of MSH6 and PMS2 [mismatch repair-deficient (MMR-D)], ECs with DNA polymerase epsilon (*POLE*) exonuclease domain mutations detected through focused sequencing, or ECs without the previously described molecular changes who have normal versus aberrant expression of p53 on IHC; p53 wild-type (p53wt) and p53 abnormal (p53abn), respectively. These four molecular EC subtypes (MMR-D, *POLE*, p53wt, p53abn) correspond to the hypermutated/microsatellite instability, ultramutated, copy number low, and copy number high subtypes of TCGA, respectively.

Immunohistochemistry: Additional biomarker evaluation

Additional IHC was performed on previously constructed TMAs for each marker as outlined in Table 1. Scoring was performed by gynaecological pathologists (BG, AK, CL, FK, MK) who were all blinded to clinical outcome. For tumours with only one core present for evaluation, the final score for the tumour was the score given to that individual core. For tumours with two cores, the final score was determined as if both cores were a single larger core.

L1 Cell Adhesion Molecule (L1CAM) scoring was based on Zeimet *et al* [16], with 0 = no epithelial staining, 1 = up to 10% staining, 2 = 11–50%

staining, 3 = >50% of epithelial cells staining. A predefined cut point was then used to divide tumours into two groups: low (0,1+) versus high (2+, 3+, also referred to as ‘overexpressors’) (see supplementary material, Figure S2).

Progesterone Receptor (PR) positivity was per the cutoff used in breast cancer, with a predefined cut point of 1% of tumour cell nuclei showing staining (<1% versus ≥1%) [17,26].

Estrogen Receptor (ER) scoring was determined with a predefined cut point of 5% of tumour cell nuclei staining (<5% ‘negative’, versus >5% encompassing weak intermediate or strongly positive), disregarding squamous metaplasia, based on a previous study on EC [27].

Stathmin (STMN) staining index (SI) was based on Trovik *et al* [21], which was the product of staining intensity (0, 1+, 2+, 3+) × percent of cells positive (0, 1 = <10%, 2 = 10–50%, 3 = >50%), resulting in a range of 0–9. This SI score was reduced to four categories: SI 0 = negative, SI 1,2 = weak, SI 3,4 = moderate, SI 6,9 = strong. A cut point was then applied [based on optimal separation of Kaplan Meier curves with disease-specific survival (DSS) on the whole cohort], designating STMN low (negative, weak, and moderate) versus high (strong).

Phosphatase and tensin homolog (PTEN) was scored as positive (any staining) versus negative (complete absence of staining suggestive of *PTEN* mutation or silencing) [24].

Statistical analysis

We tested for associations of previously identified prognostic parameters in this cohort [e.g. age, stage,

lymphovascular space invasion (LVSI)] with IHC biomarker. Chi-squared test was used for binary and categorical variables, and Welch's *t*-test (one way analysis of variance) was used for continuous variables [body mass index (BMI) and age]. Univariable cox regression models and Kaplan-Meier survival curves for each biomarker were assessed both across the full cohort, and within MMR-D, POLE, p53wt, and p53abn ProMisE molecular subtypes. Multivariable cox regression analysis was performed to look for parameters associated with outcomes after correction for adjuvant treatment and (1) prognostic parameters available at time of diagnosis (i.e. pre-surgical variables: age, BMI, histotype, grade but not stage, each IHC biomarker) or (2) pre-surgical plus post-surgical parameters (stage, myometrial invasion, LVSI, nodal status).

Concordance (Cohen's kappa coefficient) between IHC and mutation status of PTEN/*PTEN* was compared in ~400 of cases where both parameters were known.

In addition, we tested for any association between PTEN IHC expression or *PTEN* mutation and outcomes according to BMI category of obese (BMI \geq 30) or non-obese (BMI < 30) [23]. Welch's *t*-test (one-way analysis of variance with no assumption on homogeneity of variances) was used to assess significance of BMI distribution between PTEN status. Statistical significance was set at $p = 0.05$ and no attempts were made to adjust for multiple comparisons. The proportional hazard assumption for Cox models was assessed by visual examination of Kaplan-Meier plots and Schoenfeld residual plots. Proportional hazard assumption failed for L1CAM and ER. Therefore, Cox models were built separately for follow-up time between 0 and 2 years and after 2 years for these two markers based on visual examination of Kaplan-Meier plots (Figure 2A,B,F). All statistical analyses were performed using R project for statistical computing. The statistical analysis was carried out according to REMARK criteria (supplementary material, Table S1).

Results

Of the 460 cases evaluated, the proportion of uninterpretable cases (due to, e.g., no tumour or insufficient tumour in the tissue cores) varied by IHC marker; 10.2% for L1CAM, 16.7% for PR, 14.1% for ER, 10.8% for STMN, and 14.3% for PTEN. About 100% of cases had ProMisE molecular subtype previously assigned [9,10], but a proportion of these cases

had required whole sections to determine IHC for MMR status and/or p53, consistent with the known challenges of using TMAs. For the current investigation of additional IHC biomarkers, additional testing of whole sections was not performed, and we proceeded to evaluate only the subset interpretable on TMA.

A summary of the clinical and pathological features of the cohort in which these additional IHC parameters were tested is provided in Table 2. High L1CAM expression was seen in 16.2% of the cohort, PR positivity in 71.5%, ER positivity in 80.8%, high STMN in 42.2%, and PTEN loss in 46.2%. Univariable associations demonstrated L1CAM overexpression was associated with: higher age, lower BMI, more advanced stage, non-endometrioid histotype, presence of LVSI, deep (>50%) myometrial invasion, positive nodal status, and requiring additional treatment. Of tumours that exhibited overexpression of L1CAM, 70% were categorized by ProMisE as p53abn, with 11.9% MMR-D, 14.9% p53wt, and very rarely (2 cases, 3%) *POLE* subtype. However, approximately half the p53abn subtype (45/92, 49%) did not exhibit L1CAM overexpression indicating that this biomarker is not a consistent feature of this aggressive subgroup. Similarly, PR negativity was significantly associated with poor prognostic features listed for L1CAM. PR was negative in 29.1% of MMR-D, 25% of *POLE*, 16.1% of p53wt, and 55.3% of p53abn subtypes. Of the PR negative ECs, the highest proportion resided within the p53abn subtype.

ER positive ECs were more likely to be stage I, low grade (95% of ER negative tumours were grade 3), endometrioid histology, without LVSI or nodal disease (thus not requiring adjuvant therapy). Although over 92% of p53wt tumours were ER positive, ER expression was common across all ECs subtypes [73.9% MMR-Ds, 75.7% of *POLE* and even within the majority (67.4%) of p53abn tumours]. Stathmin overexpression was statistically significantly associated with lower BMI, higher grade, non-endometrioid histotype, presence of LVSI (trend towards association with nodal disease), and requiring adjuvant treatment. Most p53wt tumours showed minimal stathmin expression (72.7% STMN negative), with overexpression in just over half of the MMR-D, *POLE*, and p53abn tumours (53.5%, 51.4%, 56%, respectively) PTEN loss was associated with younger age, higher BMI, low grade, endometrioid histotype but the differences between ECs with PTEN loss versus expression within each of these clinicopathological parameters was small (e.g. median age difference 2.4 years). Although PTEN expression was associated with ProMisE subtypes

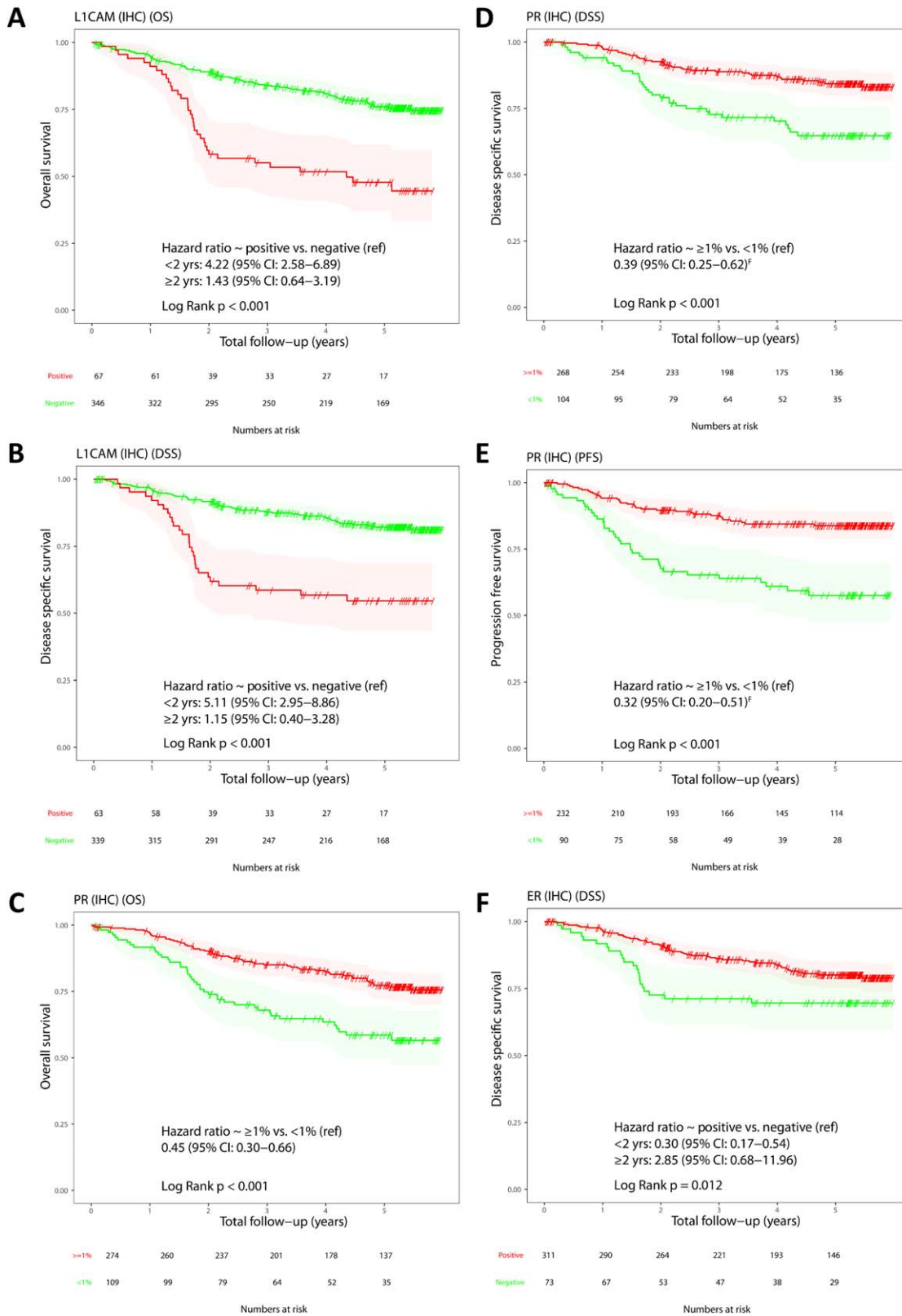


Figure 2. Kaplan-Meier plots of IHC markers on whole cohort. (A) L1CAM OS. (B) L1CAM disease specific survival. (C) PR OS. (D) PR disease specific survival. (E) PR PFS. (F) ER disease specific survival. For L1CAM and ER, HRs are shown for follow-up period 0–2 years and >2 years.

Table 2. Descriptive statistics of cohort ($n = 460$); demographic, clinicopathological, and molecular parameters within the four ProMisE molecular subtypes

	Total	MMR-D	POLE EDM	p53wt	p53 abn
Age					
Mean (\pm SD)	65.6 (\pm 12)	65.6 \pm 11.3	59.8 \pm 11.8	63.6 \pm 12.8	71.5 \pm 8.8
Median (IQR)	66.1(57.4–74.4)	66.1(56.6–73.0)	58.4(51.8–65.9)	63.3(54.9–72.9)	71.5(65.8–78.0)
Missing	3	1	0	2	0
BMI					
Mean (\pm SD)	31.8 (\pm 17.7)	33 \pm 31.1	26.6 \pm 4.9	33.7 \pm 12.5	29.4 \pm 7.8
Median (IQR)	28.5(23.6–36.7)	28.5(23.2–34.1)	27.2(22.3–29.2)	30.5(24.1–41.2)	28.0(23.4–33.5)
Missing	42	7	6	25	4
Stage					
IA	237 (52.0%)	47 (45.2%)	24 (58.5%)	129 (64.5%)	37 (33.3%)
IB	85 (18.6%)	24 (23.1%)	15 (36.6%)	31 (15.5%)	15 (13.5%)
II	31 (6.8%)	11 (10.6%)	0 (0.0%)	15 (7.5%)	5 (4.5%)
III	71 (15.6%)	16 (15.4%)	2 (4.9%)	20 (10.0%)	33 (29.7%)
IV	32 (7.0%)	6 (5.8%)	0 (0.0%)	5 (2.5%)	21 (18.9%)
Missing	4	1	1	2	0
Grade					
Grade 1	136 (29.6%)	19 (18.1%)	7 (16.7%)	107 (53.0%)	3 (2.7%)
Grade 2	74 (16.1%)	17 (16.2%)	7 (16.7%)	45 (22.3%)	5 (4.5%)
Grade 3	250 (54.3%)	69 (65.7%)	28 (66.7%)	50 (24.8%)	103 (92.8%)
Missing	0	0	0	0	0
Histological Subtype					
Endometrioid	332 (72.2%)	83 (79.0%)	35 (83.3%)	187 (92.6%)	27 (24.3%)
Serous	104 (22.6%)	11 (10.5%)	3 (7.1%)	11 (5.4%)	79 (71.2%)
Undifferentiated	6 (1.3%)	4 (3.8%)	1 (2.4%)	1 (0.5%)	0 (0.0%)
Clear Cell	5 (1.1%)	1 (1.0%)	1 (2.4%)	1 (0.5%)	2 (1.8%)
Mixed + small cell	13 (2.9%)	6 (5.9%)	2 (4.8%)	2 (1.0%)	3 (2.7%)
Missing	0	0	0	0	0
LVI					
No	266 (60.9%)	48 (47.5%)	22 (55.0%)	155 (80.7%)	41 (39.4%)
Yes	171 (39.1%)	53 (52.5%)	18 (45.0%)	37 (19.3%)	63 (60.6%)
Missing	23	4	2	10	7
Myometrial Invasion					
None	75 (16.6%)	4 (3.8%)	4 (9.8%)	49 (24.5%)	18 (16.8%)
<50%	205 (45.3%)	56 (53.3%)	20 (48.8%)	93 (46.5%)	36 (33.6%)
>50%	173 (38.2%)	45 (42.9%)	17 (41.5%)	58 (29.0%)	53 (49.5%)
Missing	7	0	1	2	4
Nodal Status					
Not Tested	150 (32.9%)	22 (21.0%)	11 (26.2%)	101 (50.2%)	16 (14.8%)
Tested Negative	269 (59.0%)	72 (68.6%)	31 (73.8%)	92 (45.8%)	74 (68.5%)
Tested Positive	37 (8.1%)	11 (10.5%)	0 (0.0%)	8 (4.0%)	18 (16.7%)
Missing	4	0	0	1	3
Treatment					
No Treatment	241 (53.6%)	52 (50.0%)	18 (43.9%)	140 (70.0%)	31 (29.5%)
Chemotherapy only	45 (10.0%)	10 (9.6%)	3 (7.3%)	6 (3.0%)	26 (24.8%)
Radiation therapy only	75 (16.7%)	18 (17.3%)	8 (19.5%)	31 (15.5%)	18 (17.1%)
Both chemo and radiation	81 (18.0%)	23 (22.1%)	10 (24.4%)	19 (9.5%)	29 (27.6%)
Vag.brachy.only	8 (1.8%)	1 (1.0%)	2 (4.9%)	4 (2.0%)	1 (1.0%)
Missing	10	1	1	2	6
ESMO					
Low	151 (33.0%)	23 (21.9%)	12 (29.3%)	112 (55.7%)	4 (3.6%)
Intermediate	71 (15.5%)	18 (17.1%)	12 (29.3%)	29 (14.4%)	12 (10.8%)
High	236 (51.5%)	64 (61.0%)	17 (41.5%)	60 (29.9%)	95 (85.6%)
Missing	2	0	1	1	0
L1CAM overexpr	67 (16.2%)	8 (11.9%)	2 (3.0%)	10 (14.9%)	47 (70.1%)
PR positive	274 (71.5%)	61 (22.3%)	24 (8.8%)	151 (55.1%)	38 (13.9%)
ER positive	319 (80.8%)	68 (21.3%)	28 (8.8%)	163 (51.1%)	60 (18.8%)
STMN overexpr	173 (42.2%)	53 (30.6%)	19 (11.0%)	50 (28.9%)	51 (29.5%)
PTEN loss	182 (46.2%)	63 (34.6%)	12 (6.6%)	91 (50.0%)	16 (8.8%)
Total	460 (100%)	105 (22.8%)	42 (9.1%)	202 (43.9%)	111 (24.1%)

ESMO, European Society for Medical Oncology; IQR, interquartile range.

this mainly reflects the very low proportion of ECs with PTEN loss that were p53abn. Even within the subgroup demonstrated to have *POLE* exonuclease domain mutations, where we would expect from the TCGA data that PTEN loss would be nearly ubiquitous, we saw PTEN loss of expression in only 12 of 35 tumours. Summary statistics for each marker and univariable associations with other clinicopathological parameters are found in supplementary material, Tables S2–S6.

Univariable survival analysis revealed statistically significant associations with outcome for L1CAM, PR, and ER. Women with tumours with L1CAM overexpression had a higher number of events (recurrence, death from disease). Hazard ratios (HRs) and 95% confidence intervals (CIs) for overall survival (OS), DSS, and progression free survival (PFS), for overexpression of L1CAM (0–2 years) were 4.22 (2.58–6.89), 5.11 (2.95–8.86), and 4.51 (2.64–7.73), respectively, for L1CAM (>2 years) were 1.43 (0.64–3.19), 1.15 (0.40–3.28), and 4.59 (1.75–12.09), respectively. In contrast, women with PR or ER positive tumours were much less likely to recur or die from their tumours. For PR positive tumours, OS, DSS, and PFS were 0.45 (0.3–0.66), 0.39 (0.25–0.62), and 0.32 (0.20–0.51), respectively. For ER positive tumours, OS, DSS, and PFS from 0 to 2 years were 0.40 (0.24–0.68), 0.30 (0.17–0.54), and 0.52 (0.29–0.93), respectively. However, for follow-up period >2 years, the opposite association was observed; OS, DSS, and PFS for this follow-up period were 2.90 (0.90–9.37), 2.85 (0.68–11.96), and 1.14 (0.33–3.93), respectively. PTEN loss was associated with PFS only (HR 0.60, 95% CI: 0.37–0.96). There was a trend towards worse DSS in tumours with STMN expression, but it did not reach statistical significance. As previously demonstrated, ProMisE molecular subtype was strongly associated with clinical outcomes, as were traditional prognostic parameters of age, stage, grade, histotype, LVSI, nodal disease, and treatment and degree of myometrial invasion. Full details, including HRs and log rank test (LRT) values are given in Table 3.

Kaplan-Meier survival analysis demonstrated statistically significant associations with outcomes (OS, DSS, and PFS) for both L1CAM and PR expression across the whole cohort ($p < 0.001$) with DSS curves shown in Figure 2.

Univariable and Kaplan-Meier survival analyses were also performed *within* ProMisE subtypes for each IHC marker. In ECs classified as MMR-D with L1CAM overexpression (~7%), worse PFS was observed (Log Rank $p = 0.005$; Figure 3D). Within the p53wt subtype of ECs with L1CAM

overexpression (~6%), worse DSS and PFS were observed (Log Rank $p = 0.006$ and 0.004 , respectively; Figure 3B,C). Within p53abn, of which half the tumours exhibited L1CAM positivity, no association with outcomes was demonstrated. Low number of events are apparent across all subgroups, particularly within *POLE*, precluding meaningful assessment. PR overexpression was associated with good outcomes only within the p53wt group (Log Rank $p \leq 0.01$ for OS, DSS, and PFS; Figure 3E–G). ER positivity was also shown to be associated with OS, DSS, and PFS within the p53wt subtype (Log Rank $p \leq 0.04$ for OS, DSS, and PFS; Figure 3H–J), although the majority of p53wt tumours were ER positive (91%). No association of outcomes within ProMisE subtype was seen for STMN or PTEN.

On multivariable survival analysis, ProMisE molecular subtype maintained prognostic significance for DSS and PFS when correcting for each IHC biomarker (except for L1CAM where ProMisE remained significant for PFS only) and for clinicopathological parameters available at time of diagnosis (age, grade, histotype, and BMI). Age maintained an association with OS only. Among all the biomarkers tested, only ER was significantly associated with outcome when corrected for other prognostic parameters. In particular, between 0 and 2 years after surgery, ER positivity was associated with better DSS (trend towards significance: $p = 0.057$), while after 2 years, high ER expression correlated with worse overall and disease specific survival ($p \leq 0.002$). Age remained associated with OS when corrected for each additional IHC biomarker (L1CAM, PR, ER, STMN, or PTEN) and other clinicopathological parameters available at time of diagnosis (age, grade, histotype, ProMisE subtype, and BMI).

In testing for associations between IHC biomarkers tested, L1CAM expression was associated with STMN expression and ER/PR negative carcinomas. PR positive tumours were most commonly ER positive (>90%) with rare (7%) L1CAM overexpression and low STMN staining indices. PTEN expression revealed few correlations with other markers; PTEN was associated with PR expression (79% of ECs with PTEN loss were PR positive). One hundred forty cases had both IHC and mutational data for PTEN for concordance testing. PTEN IHC expression was demonstrated to be only in 'slight agreement' with *PTEN* mutation status, kappa statistic 0.19.

Testing for the prognostic impact of PTEN loss, by IHC or mutation status, in the context of BMI revealed no improved discernment of outcomes. As mentioned above, PTEN loss is associated with

Table 3. Univariable survival analysis with LRT values for each biomarker tested (IHC) and ProMisE molecular subgroups

	# of events/n		Hazard ratio (95% CI)	LRT P value
L1CAM (0–2 years)				
(Ref: negative)				
OS	65/413		4.22 (2.58–6.89)	<0.001
DSS	51/402		5.11 (2.95–8.86)	<0.001
PFS	56/344		4.51 (2.64–7.73)	<0.001
L1CAM (>2 years)				
(Ref: negative)				
OS	46/334		1.43 (0.64–3.19)	0.41
DSS	31/330		1.15 (0.40–3.28)	0.80
PFS	19/268		4.59 (1.75–12.09)	0.006
STMN				
(Ref: negative)				
OS	110/410		1.23 (0.85–1.79)	0.28
DSS	82/399		1.54 (1.00–2.37)*	0.051
PFS	73/339		1.44 (0.91–2.27)*	0.12
ER (0–2 years)				
(Ref: negative)				
OS	61/395		0.40 (0.24–0.68)	0.001
DSS	47/384		0.30 (0.17–0.54)	<0.001
PFS	54/329		0.52 (0.29–0.93)	0.035
ER (>2 years)				
(Ref: negative)				
OS	45/321		2.90 (0.90–9.37)	0.037
DSS	30/317		2.85 (0.68–11.96)	0.095
PFS	19/256		1.14 (0.33–3.93)	0.83
PR				
(Ref: <1%)				
OS	100/383		0.45 (0.30–0.66)	<0.001
DSS	71/372		0.39 (0.25–0.62)*	<0.001
PFS	69/322		0.32 (0.20–0.51)*	<0.001
PTEN				
(Ref: positive)				
OS	108/394		0.77 (0.52–1.13)	0.17
DSS	82/383		0.68 (0.43–1.06)*	0.087
PFS	73/325		0.60 (0.37–0.96)*	0.03
ProMisE molecular subgroup				
OS	120/460	MMR-D	2.04 (1.25–3.33)*	<0.001
		POLE EDM	0.59 (0.19–1.41)*	
		p53 abn	3.73 (2.44–5.77)*	
DSS	89/447	MMR-D	2.23 (1.23–4.01)*	<0.001
		POLE EDM	0.49 (0.10–1.51)*	
		p53 abn	4.68 (2.85–7.87)*	
PFS	89/387	MMR-D	1.90 (1.04–3.44)*	<0.001
		POLE EDM	0.26 (0.03–0.99)*	
		p53 abn	5.09 (3.14–8.47)*	
ESMO				
OS	120/458	Intermediate	1.23 (0.55–2.57)*	<0.001
		High	3.96 (2.46–6.73)*	
DSS	89/445	Intermediate	2.51 (0.87–7.45)*	<0.001
		High	9.53 (4.63–23.59)*	
PFS	89/386	Intermediate	3.91 (1.04–16.85)*	<0.001
		High	21.82 (8.66–78.69)*	
Age				
OS	118/457		1.04 (1.03–1.06)	<0.001
DSS	87/444		1.03 (1.01–1.05)	0.002
PFS	89/386		1.02 (1.00–1.04)	0.018
BMI				
OS	104/418		0.98 (0.96–1.00)	0.065
DSS	76/405		0.98 (0.96–1.00)	0.08

Table 3. Continued

	# of events/n		Hazard ratio (95% CI)	LRT <i>P</i> value
PFS	84/357		0.98 (0.96–1.01)	0.11
Stage (Ref stage I)				
OS	118/456		3.90 (2.72–5.63)*	<0.001
DSS	87/443		5.21 (3.40–8.09)*	<0.001
PFS	89/386		6.71 (4.38–10.49)*	<0.001
Grade (Ref grade 1/2)				
OS	120/460		2.85 (1.92–4.32)*	<0.001
DSS	89/447		5.41 (3.18–9.89)*	<0.001
PFS	89/387		7.09 (4.05–13.53)*	<0.001
Histology (Ref: Endometrioid)				
OS	120/460		2.62 (1.82–3.75)*	<0.001
DSS	89/447		3.43 (2.26–5.21)*	<0.001
PFS	89/387		4.20 (2.77–6.39)*	<0.001
LVI				
OS	111/437		3.36 (2.30–4.97)*	<0.001
DSS	82/425		4.44 (2.82–7.21)*	<0.001
PFS	83/370		4.73 (3.00–7.66)*	<0.001
Any Nodes (Ref: Negative)				
OS	118/456	Not Tested	0.81 (0.54–1.22)	0.030
		Positive	1.90 (1.10–3.29)	
DSS	87/443	Not Tested	0.82 (0.50–1.32)*	0.003
		Positive	2.68 (1.48–4.61)*	
PFS	89/385	Not Tested	0.23 (0.10–0.44)*	<0.001
		Positive	2.72 (1.63–4.39)*	
Any Treatment (No treatment)				
OS	117/450		1.96 (1.36–2.86)*	<0.001
DSS	86/437		2.98 (1.90–4.81)*	<0.001
PFS	87/380		3.29 (2.10–5.30)*	<0.001
Myometrial invasion				
OS	117/453	<50	1.28 (0.67–2.68)*	<0.001
		>50	3.89 (2.13–7.90)*	
DSS	86/440	<50	1.13 (0.49–2.97)*	<0.001
		>50	5.34 (2.56–13.28)*	
PFS	89/385	<50	0.94 (0.46–2.11)*	<0.001
		>50	4.02 (2.13–8.54)*	

improved PFS across all ECs and there was a similarly reduced HR 0.47 (95% CI 0.21–0.97) (LRT-*P* value 0.03) within women with BMI < 30 but not associated with outcome in women with BMI ≥ 30. When restricted to endometrioid histology only, all associations of PTEN and outcomes were lost in both BMI categories (univariable and Kaplan-Meier survival analysis, *p* = ns).

Discussion

Molecular classification of EC enables reproducible categorization of tumours, identification of women

who may have hereditary cancer syndromes, provides prognostic information, and has the potential to guide therapy from first diagnostic specimen [7–10,12]. Optimal utilization of molecular classification, as through ProMisE, needs to be tested in prospective clinical trials. However, it is clear that the diagnostic inconsistency, and lack of biologically relevant information to inform EC risk stratification [6,28], make the current histopathological classification system suboptimal for this disease site.

We anticipate that ProMisE, although now validated as a standalone prognostic test [9,10,29], may be further strengthened by the addition of select demographic, pathological, or molecular parameters.

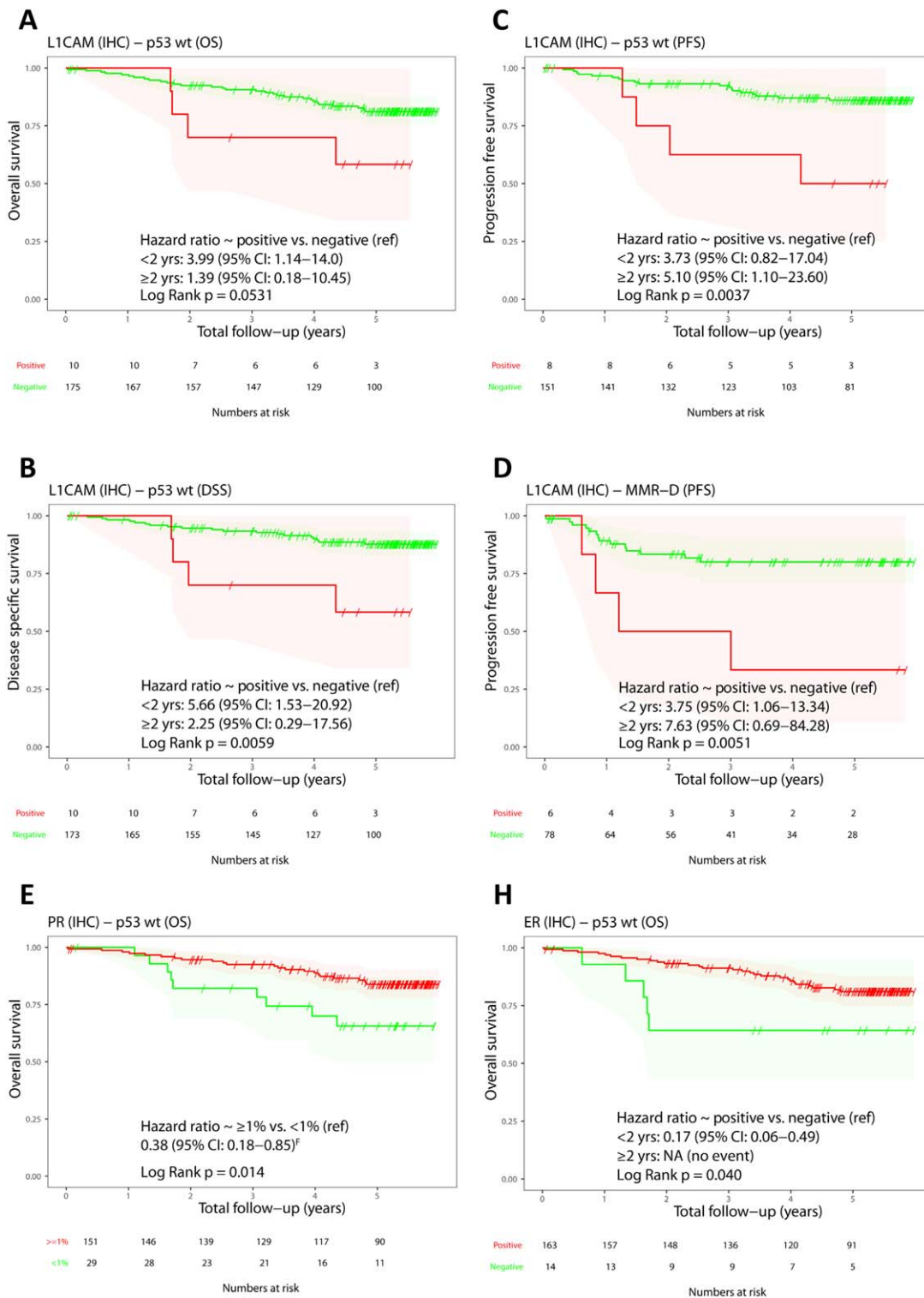


Figure 3. Kaplan-Meier plots of IHC markers within selected ProMisE subtypes. (A) L1CAM OS within p53wt. (B) L1CAM DSS within p53wt. (C) L1CAM PFS within p53wt. (D) L1CAM PFS within MMR-D. (E) PR OS within p53wt. (F) PR DSS within p53wt. (G) PR PFS within p53wt. (H) ER OS within p53wt. (I) ER DSS within p53wt. (J) ER PFS within p53wt. For L1CAM and ER, HRs are shown for follow-up period 0–2 years and >2 years.

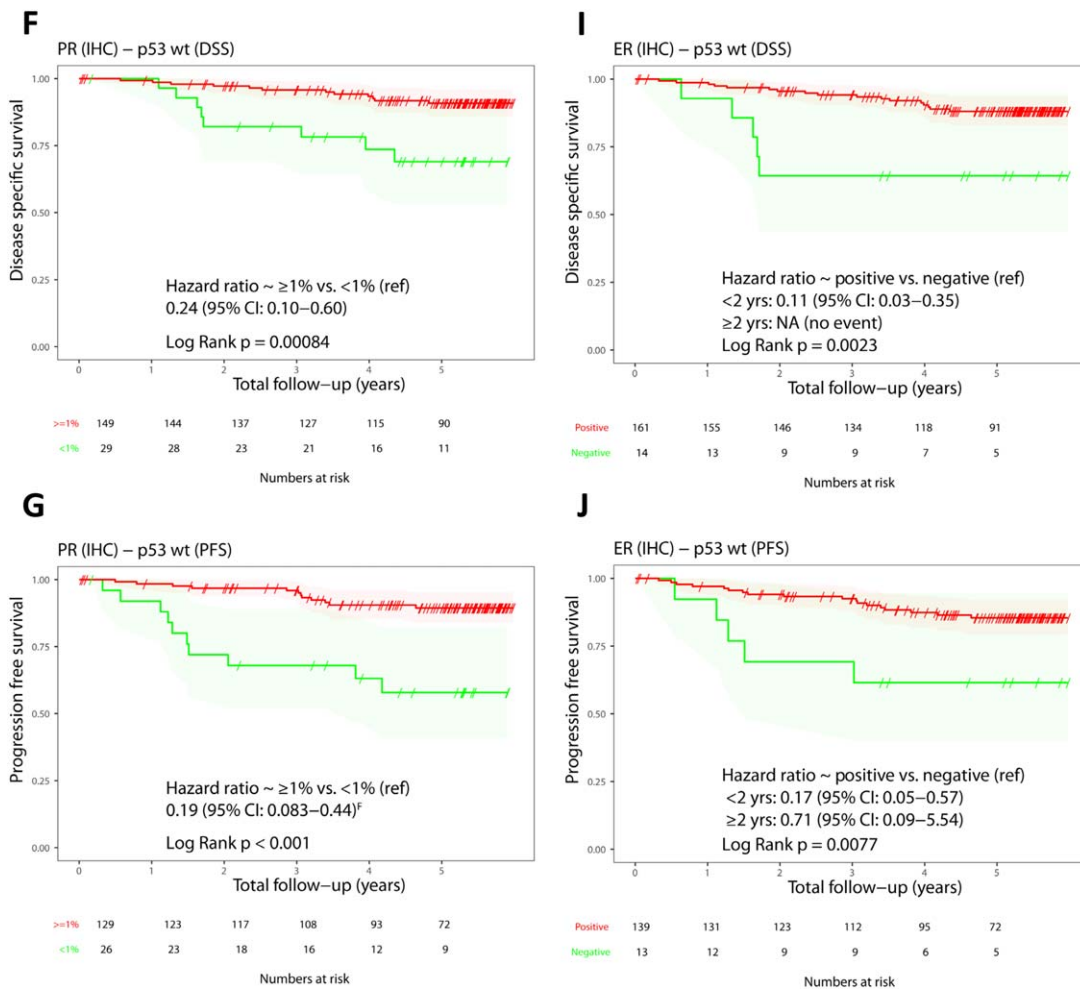


Figure 3. Continued.

Focusing on molecular parameters of purported prognostic significance in EC that can be performed at relatively low cost (IHC), we investigated five protein biomarkers for their prognostic value in the context of post-TCGA era of molecularly defined subtypes.

L1-cell adhesion molecule (L1CAM) is a transmembrane protein implicated in driving tumour invasion, cell motility, and angiogenesis [30,31]. Overexpression of L1CAM has been associated with aggressive tumour features in multiple cancers, including head and neck, vulvar, ovarian and ECs. L1CAM overexpression in ECs has been demonstrated to be associated with worse outcomes, even in low stage tumours [14–16,32]. Whether these worse outcomes are secondary to L1CAM itself or if L1CAM is simply a marker for ECs with poor prognostic features (non-endometrioid histology, LVSI, high grade) has not yet been definitively answered. Interestingly, RNA-seq data from the TCGA cohort

has been used to examine L1CAM overexpression but L1CAM status tested against traditional clinico-pathological parameters and outcomes, not TCGA subtypes. The authors concluded that L1CAM was associated with multiple poor prognostic factors. Worse outcomes were seen across all L1CAM tumours; however, again, these cases were more likely advanced stage, grade, non-endometrioid histology so whether or not the worse outcomes were secondary to L1CAM or these other features was unclear. On multivariable analysis, looking within stage I tumours L1CAM overexpression trended towards worse survival but did not reach statistical significance [33]. This is not the first study to look at this marker in the context of molecular subtypes; in the TRANSPORTEC series, L1CAM was the only molecular marker, of many tested, that was of prognostic significance independent of molecular subtypes [12]. Together with our finding that L1CAM overexpression is associated with a worse outcome in the

important MMR-D and p53wt groups suggests it may have value within those subtypes, but further validation is required. It should be noted that there are very few tumours with LICAM overexpression in the MMR-D and p53wt groups (7 and 6%, respectively), which limits our power to validate previous studies demonstrating the prognostic significance of LICAM among low-risk tumours [12,15,16,32].

Hormone receptor status has long been appreciated as prognostic in ECs; PR and estrogen receptor (ER) positivity are associated with low grade low-risk ECs and more favourable outcomes [18–20,34] in addition to providing therapeutic opportunities. A recent comprehensive review in over 200 ‘high-risk’ ECs showed that although the proportion of cases with PR expression was lower in their high-grade cohort, PR positive grade 3 endometrioid and serous carcinomas had improved OS [17]. The utility of PR and ER IHC in the context of molecular subtypes was recently evaluated in the postoperative radiation therapy for endometrial carcinoma (PORTEC) cohorts [12]; ER/PR status correlated with molecular subtype, and low ER and PR correlated with higher incidence of loco-regional and recurrence and lower OS.

We confirmed the association of both hormone receptors with outcomes; however, on multivariable analysis this association was not maintained except for a trend towards significance ($p = 0.057$) for improved DSS for ER-positive tumours (supplementary material, Tables S7 and S8). Furthermore, for patients surviving more than 2 years, we observed that ER-positive tumours were associated with *worse* prognosis (Figure 2F). However, due to the limitation of our relatively small and heterogeneous study cohort, we cannot explore this finding further. Our data show that, *within* the p53wt subtype, the presence of either hormone receptor may add value in discerning improved outcomes (Figure 3E–J). A recent meta-analysis showed that hormonal status is associated with a greater response to hormonal therapies in advanced ECs [35], so there may both a prognostic and predictive role of ER and PR in the appropriate clinical setting.

Stathmin (STMN) is an oncoprotein that has been linked to phosphoinositide-4,5-bisphosphate 3-kinase (PI3K) pathway activation, aggressive clinicopathological features, and poor prognosis in several solid tumours including ECs [21,36–38]. Stathmin has also been suggested as a predictive biomarker, associated with resistance to antimicrotubule agents [39], and possibly identifying tumours that may benefit from PI3Kinase/mTOR (mammalian target of rapamycin) inhibitor therapy [40]. In our series, although we demonstrated STMN was associated with several

adverse prognostic features, no clear associations with outcomes were demonstrated. This may be a result of our simplified scoring system (9-category, ultimately binarized to overexpression versus not) but even upon testing at several different thresholds, the lack of profound differences in clinicopathological parameters between ECs with stathmin overexpression versus low expression cases, the lack association with outcomes, and the somewhat tedious and complex initial scoring system dampens our enthusiasm for widespread use of this marker.

Mutations in the tumour suppressor gene *PTEN* are common in ECs. Publications on the prognostic implications of PTEN loss in ECs, as measured by IHC or mutation status, are discordant [23,25,41]. The impact of functional PTEN loss on tumour behavior and clinical outcomes may need to be interpreted within the context of metabolic phenotype (BMI). Westin *et al* demonstrated that PTEN loss (by mutation status or IHC) was not associated with outcomes across a cohort of ~190 ECs but, after restricting evaluation to obese individuals ($BMI \geq 30$), PTEN loss was associated with improved PFS [23]. Functional PTEN loss can arise by somatic mutations, epigenetic/post-transcriptional events, or instability and degradation of the PTEN protein. Concordance between PTEN mutation status and IHC is poor and arguably PTEN IHC may better represent functional loss [24]. However, difficulties with staining, heterogeneity of staining patterns, and interpretation have all hindered its use in clinical practice [42,43]. In this study, correlation between PTEN IHC and mutation status was poor, differences between clinicopathological variables for ECs with PTEN loss versus expression were minimal, and prognostic value of PTEN status limited; PTEN loss was associated with PFS only ($p = 0.03$). Although PTEN loss by IHC was associated with obesity, no differences in our ability to predict outcomes were seen within obese ($BMI \geq 30$) individuals. That said, evaluation of PTEN loss might still be of use as a predictive biomarker for response to poly (ADP-ribose) polymerase (PARP) inhibitors [44,45].

In this series of over 400 ECs, survival analysis demonstrated that although these additional biomarkers evaluated may be associated with selected clinicopathological variables or outcome parameters, no single IHC biomarker outperformed ProMisE. The additional IHC parameters evaluated frequently segregated with specific molecular subtype (e.g. LICAM overexpression with the p53abn subtype), suggesting these proteins may simply be a feature of an overriding genotype/phenotype that classification tools such as ProMisE provide. Although both

L1CAM and PR were significantly associated with OS, DSS, and PFS (ER with DSS only, PTEN with PFS only), on multivariable survival analysis, only the ProMisE molecular classifier maintained a statistically significant association with OS, DSS, and PFS. In terms of clinicopathological parameters, only age maintained an association with outcomes and only for OS.

The polarity of outcomes in the p53abn subtype (poor) and POLE subtype (highly favourable) suggests that these subtypes will likely not benefit from further refinement through the application of additional prognostic parameters. Of greater clinical import would be discerning outcomes within the two 'intermediate' survival outcome molecular subtypes; both the p53wt and MMR-D subtypes exhibit diversity of outcomes within them, with little indication as yet as to how to discriminate between these tumours. The greatest potential to add prognostic value within these intermediate outcome ProMisE subtypes was shown for L1CAM and PR. We demonstrated an improved ability to discern outcomes through the addition of L1CAM testing *within* the MMR-D and p53wt subtypes but only a small proportion of these cases exhibited L1CAM overexpression. Nonetheless, for those MMR-D or p53wt ECs that did have L1CAM overexpression outcomes were clearly worse. Some of the prior publications on L1CAM and prognosis, which suggested it helps discern a worse outcome group within low or intermediate risk tumours may actually reflect that these tumours were incorrectly assigned by histopathological features and may represent 'missed' serous or high grade aggressive endometrioid adenocarcinomas incorrectly classified. With molecular classification, as through ProMisE, serous carcinomas would be identified as p53abn and thus clearly identified as high risk for recurrence and managed appropriately. An additional benefit of L1CAM status within the p53abn subtype was not seen in our series. PR status improved the ability to discern outcomes within the p53wt subtype only, and if this association is validated, could be considered as an additional prognostic test within this select cohort.

Strengths of this study include utilization of a previously described large, well-characterized EC cohort with mature outcome data. TMAs had proven successful for IHC in the ProMisE classifier and were again successfully utilized, with ~10–15% of uninterpretable cases. However, TMAs do not reflect what would be used in clinical practice (e.g. whole sections) and heterogeneity of these tumours means TMA may not always provide the best representation of the tumour. For example, L1CAM expression can be heterogeneous and is often only expressed at the

invasive tumour front, which is not typically represented on TMAs, so our data may underestimate L1CAM overexpression in our cohort. The usual challenges of IHC for PTEN were demonstrated again in this series, lessening its applicability in clinical practice. While the ProMisE classifier has been validated in a retrospective cohort [10], it awaits validation in a prospective setting. The need to sequence *POLE* could be considered a limitation to the widespread implementation of this molecular classifier in clinical practice.

In summary, testing L1CAM, PR, ER, STMN, and PTEN IHC biomarkers across all ECs does not appear to add prognostic value over ProMisE classification alone. Potential value added within molecular subtypes associated with intermediate outcomes may justify further studies on L1CAM and hormone receptor status specifically in the MMR-D and p53wt subtypes.

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Author contributions statement

JMc, BG: conceived of and designed study with DH and AK contributing; JMc: wrote the manuscript with BG and AK; AK, FK, MK, CHL, JM, BG: scored/interpreted the IHC on TMA, and contributed to writing and editing of paper; MMc, WY: did the molecular work including POLE testing and interpretation; SL, AT: designed and performed the statistical analyses; CC: created TMA and aided in all steps IHC with assistance JM.

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SUPPLEMENTARY MATERIAL ONLINE

Figure S1. Case selection

Figure S2. Immunohistochemistry for LICAM demonstrating LICAM low (up to 10%) versus high (>10%) expression

Table S1. REMARK criteria

Table S2. Clinicopathological Variables by IHC Marker Status. Univariable association of predictors with LICAM IHC

Table S3. Clinicopathological Variables by IHC Marker Status. Univariable association of predictors with STMN IHC

Table S4. Clinicopathological Variables by IHC Marker Status. Univariable association of predictors with PR IHC

Table S5. Clinicopathological Variables by IHC Marker Status. Univariable association of predictors with ER IHC

Table S6. Clinicopathological Variables by IHC Marker Status. Univariable association of predictors with PTEN IHC

These tables show summary statistics of clinicopathological variables of interest split by score of each IHC marker; LICAM, STMN, PR ER, and PTEN. For categorical and binary variables, counts and corresponding column percentages are shown. For continuous variables, the mean, median, IQR, and range are calculated. In addition, the number of missing cases is shown at the bottom of each subsection in the table. Note that the missing cases do not contribute to the percentages.

Table S7. Multivariable model with parameters available at diagnosis (adjusting for treatment)

Table S8. Multivariable model with parameters available after surgery (adjusting for treatment)