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INTEGRATING B-CATENIN AND AKT INTO THE
SURROGATE MOLECULAR CLASSIFICATION OF
ENDOMETRIAL CARCINOMA:
AN IMMUNOHISTOCHEMICAL APPROACH FOR
PROGNOSTIC RE-STRATIFICATION

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Abstract

Background: The molecular classification of endometrial cancer (EC) has revolutionized risk stratification; however, the impact of novel biomarkers across subgroups remains to be fully elucidated. This study evaluates the clinical and molecular implications of activation of the PI3K/AKT and Wnt/ β -catenin pathways.

Methods: We analyzed p-AKT and nuclear β -catenin expression via immunohistochemistry (IHC) in 60 EC cases. Findings were correlated with surrogate molecular subgroups (MMRd, NSMP, p53abn), clinicopathological features, and oncological outcomes. Cases were categorized by protein expression into three groups: double-positive (both proteins), single-positive, and double-negative.

Results: β -catenin expression was observed in 33 (55%) cases, p-AKT overexpression in 46 (76.6%) cases and a dual overexpression in 26 patients (43.3%). β -catenin expression and the double-positive profile were almost exclusively associated with endometrioid histology (97%, $p<0.001$ and $p=0.011$, respectively). These proteins and their co-occurrence were equally distributed across molecular classes ($p=0.4$). p-AKT hyperexpression was significantly associated with a shorter median Disease-Free Survival (DFS) (36 vs. 40 months, $p=0.013$) and a trend toward deeper myometrial invasion ($p=0.080$).

Conclusions: Proteomic activation of AKT and β -catenin may be helpful to identifies tumors with a more rapid recurrence independently of standard molecular subclasses. Combined IHC profiling could offer a functional and cost-effective supplement to genomic classification, potentially refining post-operative surveillance strategies. Further studies with larger cohorts and longer follow-up are warranted.

Summary

Clinical and pathological heterogeneity of endometrial carcinoma is not fully investigated. The integration of proteomic markers into the molecular framework of endometrial cancer may represent a useful and cost-saving approach. Our study revealed that p-AKT and β -catenin expression are common events that may occur independently of primary genomic drivers, as evidenced by their equal distribution among MMRd, NSMP, and p53abn subgroups. On the other hand, the co-expression of β -catenin and p-AKT, is strictly linked to the endometrioid phenotype. Rather than acting as independent risk factors for absolute relapse, these biomarkers could function as "drivers" that can accelerate the clinical course and shorten the Disease-Free Survival interval, especially when co-occurring. This proteomic synergy may potentially provide prognostic information, particularly within the biologically heterogeneous NSMP category. Consequently, combined IHC profiling may represent a functional and cost-effective supplement to genomic classification, helping clinicians refine post-operative surveillance and personalize follow-up intensity. This pilot exploration involving 60 cases investigates how proteomic re-stratification can enhance the precision of endometrial cancer management, offering a model for further studies.

CHAPTER 1

Background and Objectives

1.1 Background

Epidemiology and Global Health Burden

Endometrial cancer (EC) is the most common gynecological malignancy in high-income countries and is rapidly becoming a global health priority^{1,2}. According to the latest GLOBOCAN 2022 report, EC ranks as the 6th most common cancer in women globally, with approximately 417,367 new cases diagnosed annually and nearly 97,370 deaths recorded³.

The distribution of EC reflects a significant socioeconomic gradient. The highest age-standardized incidence rates (ASIR) are observed in North America (21.1 per 100,000) and Western/Northern Europe (15-19 per 100,000). In Italy, the trend mirrors the European average, with EC representing the 4th most frequent cancer in the female population, accounting for about 5-6% of all female malignancies.

While the 5-year survival rate for early-stage disease is high (approx. 95%), the global mortality rate is rising by roughly 1.9% per year. This is particularly concerning because EC is one of the few cancers where mortality is not declining. GLOBOCAN projections suggest that by 2040, the number of new cases will surge to 608,000 annually, a 46% increase compared to current figures. This trend is attributed to the aging population and the escalating prevalence of metabolic risk factors.

The Histopathological Landscape: From the Bokhman Dualistic Model to Morphological Complexity

The diagnostic framework for Endometrial Cancer (EC) has undergone a profound transformation. For decades, clinical management was guided by the Bokhman dualistic model (1983), which simplified EC into two distinct clinico-pathological types based on endocrine and metabolic profiles⁴.

The Bokhman Classification

- Type I (Endometrioid): Historically representing 80% of cases, Type I tumors were characterized as estrogen-dependent, often arising in the setting of obesity, hyperlipidemia, and hyperestrogenism (frequently following atypical endometrial hyperplasia). These tumors were generally low-grade (G1-G2), diagnosed at an early stage, and associated with a favorable prognosis.
- Type II (Non-Endometrioid): Accounting for approximately 10-20% of cases, Type II tumors were described as estrogen-independent, arising in atrophic endometrium of older, non-obese women. This group included serous and clear cell histotypes, inherently high-grade, with a high risk of deep myometrial invasion and distant metastasis.

Beyond the Binary: The Current Morphological Spectrum

While the Bokhman model provided a useful clinical shorthand, it failed to account for the significant biological overlap between types—particularly the aggressive behavior of high-grade endometrioid carcinomas (G3) and the molecular heterogeneity within the "Type I" group⁵. Today, the WHO and FIGO classifications recognize a more nuanced morphological spectrum^{6,7} (**Figure 1**):

- Endometrioid Endometrial Carcinoma (EEC): This remains the most prevalent subtype. It is characterized by the formation of glandular structures that recapitulate the normal secretory or proliferative endometrium. The FIGO Grading System (G1–G3) is the primary tool for architectural assessment:
 - o G1: <5% solid growth pattern.
 - o G2: 6–50% solid growth pattern.
 - o G3: >50% solid growth pattern.
- High-Grade Non-Endometrioid Subtypes:

o Serous Carcinoma: It is almost universally driven by TP53 mutations and exhibits high-grade cytologic features—such as "hobnail" cells, marked nuclear pleomorphism, and prominent nucleoli—even in the absence of solid growth.

o Clear Cell Carcinoma: A rare and highly aggressive entity characterized by "hobnail" cells, clear cytoplasm, and a prominent hyaline stroma.

o Carcinosarcoma (Malignant Mixed Müllerian Tumor): Once classified as a sarcoma, it is now recognized as a biphasic "metaplastic" carcinoma. It contains both a high-grade epithelial component and a mesenchymal component (which may be homologous or heterologous).

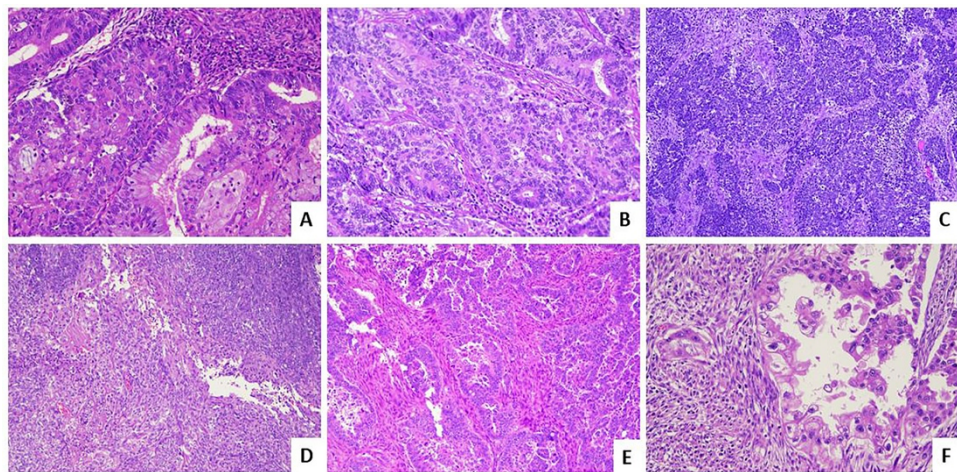


Figure 1. Histological subtypes of endometrial carcinoma: an overview. (A) An endometrioid carcinoma G1 FIGO with mucinous features (LSAB, 10×). (B) An endometrioid carcinoma G2 FIGO (LSAB, 10×). (C) An endometrioid carcinoma G3 FIGO with basaloid features (LSAB, 4×). (D) An endometrioid carcinoma G3 FIGO with spindle cell features (LSAB, 4×). (E) A serous carcinoma (LSAB, 10×). (F) A clear cell carcinoma (LSAB, 20×). From Zannoni GF et al. (2022) Front. Oncol. 12:805613

The Molecular Revolution: From TCGA Genomics to ProMisE Clinical Application

The landscape of EC was fundamentally redefined in 2013 when The Cancer Genome Atlas (TCGA) network published its comprehensive genomic characterization. Using advanced Next-Generation Sequencing (NGS) platforms, including whole-exome sequencing (WES), lead researchers identified four distinct molecular subgroups based on somatic mutation burden and copy-number alterations (CNA)⁸.

The Original TCGA Classification (2013): The NGS Era

The TCGA study was a landmark in precision oncology, utilizing high-throughput technology to move beyond microscopic evaluation. The four original classes were defined by their genomic "signature" (**Figure 2**):

1. POLE-ultramutated: Defined by an exceptionally high mutation burden (>100 mutations per Megabase). These tumors were characterized by mutations in the exonuclease domain of the POLE gene, leading to a massive accumulation of C>A transversions.

2. Microsatellite Instability (MSI)-hypermuted: Characterized by a high mutation rate (approx. 18 mutations/Mb) caused by defects in the DNA mismatch repair system, leading to widespread errors in repetitive DNA sequences (microsatellites).

3. Copy-Number Low (Endometrioid): Tumors with a low mutation burden and very few large-scale chromosomal alterations. This group was predominantly composed of low-grade endometrioid carcinomas.

4. Copy-Number High (Serous-like): Characterized by extreme chromosomal instability, numerous gains and losses of DNA segments, and a low mutation rate. This group, almost universally driven by TP53 mutations, showed the most aggressive clinical course.

While revolutionary, the TCGA's reliance on NGS for all cases was clinically and economically unsustainable for routine diagnostic labs worldwide.



Figure 2. TCGA molecular classification, from Levine DA, et al. Nature 2013; 497: 67–73.

The Evolution toward Clinical Surrogates: The ProMisE Algorithm and Clinical Impact

To translate these findings into daily practice, the Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) was developed and validated between 2015 and 2017^{9,10}. ProMisE introduced the use of immunohistochemistry (IHC) as a reliable and cost-effective surrogate for the complex NGS data, maintaining high prognostic accuracy¹¹ (**Figure 3-4**). The four validated classes are defined as follows:

1. POLE-ultramutated (POLEmut) [7–10%]

Characterized by pathogenic mutations in the exonuclease domain (exons 9, 13, 14) of DNA Polymerase Epsilon (POLE). This enzyme is responsible for proofreading during DNA replication. When mutated, the "proofreader" fails, leading to an astronomical mutation frequency (>100 mutations/Mb). This "ultramutational burden" generates an abundance of neoantigens, triggering a massive infiltration of tumor-infiltrating lymphocytes (TILs). Clinically, these patients have an excellent prognosis (near 100% survival in early stages), often allowing for treatment de-escalation, even when the tumor appears high-grade (G3).

2. Mismatch Repair Deficient (MMRd) [25–30%]

These tumors exhibit Microsatellite Instability (MSI) due to the loss of function of one or more Mismatch Repair proteins: MLH1, MSH2, MSH6, or PMS2. In most cases, this is due to epigenetic silencing (hypermethylation) of the MLH1 promoter, but in 10% of this group, it signals Lynch Syndrome. ProMisE identifies this group through IHC for the four MMR proteins. Loss of nuclear expression in any of these proteins confirms MMRd status. These tumors are highly immunogenic. Patients are prime candidates for Immune Checkpoint Inhibitors (ICIs) like Dostarlimab or Pembrolizumab, which "unleash" the immune system against the MSI-H cells.

3. p53-aberrant (p53abn) [8–15%]

Corresponds to the TCGA "Copy-Number High" group. These tumors are driven by TP53 mutations that lead to extreme chromosomal instability and widespread genomic rearrangements. ProMisE uses p53 IHC as a surrogate. The "aberrant" pattern can manifest as a total "null" phenotype (complete absence of staining), strong/diffuse nuclear overexpression ("mutation-type"), or cytoplasmic staining. This group carries the worst prognosis. It includes most

serous carcinomas and high-grade endometrioid cases. These patients require aggressive adjuvant therapy, typically involving platinum-based chemotherapy and radiotherapy.

4. No Specific Molecular Profile (NSMP) [50–60%]

Known in TCGA as "Copy-Number Low." These tumors lack POLE mutations, MMR deficiency, or p53 abnormalities. They are genetically "stable" but biologically heterogeneous.

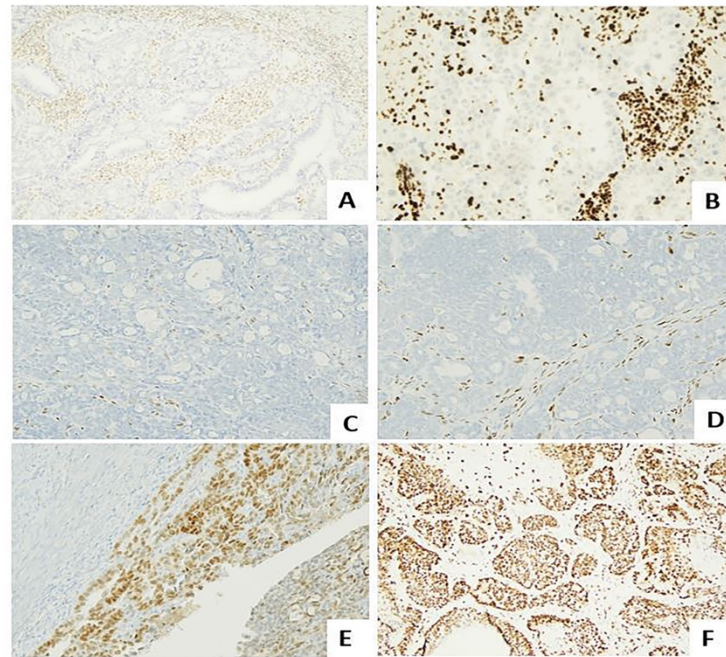


Figure 3. Molecular landscape of endometrial carcinoma: the role of the immunohistochemical surrogates. (A) An endometrioid carcinoma G2 FIGO with loss of MLH1 expression (LSAB, 10×). (B) An endometrioid carcinoma G2 FIGO with loss of MSH2 expression (LSAB, 10×). (C, D) An endometrioid carcinoma G3 FIGO with loss of ER (LSAB, 10×) and PR (LSAB, 10×). (E) An endometrioid carcinoma G2 FIGO with expression of ER in 80% of neoplastic cells, with a 2+ score intensity (LSAB, 10×). (F) A serous carcinoma with overexpressed aberrant pattern of p53 (LSAB, 4×). From Zannoni GF et al. (2022) Front. Oncol. 12:805613

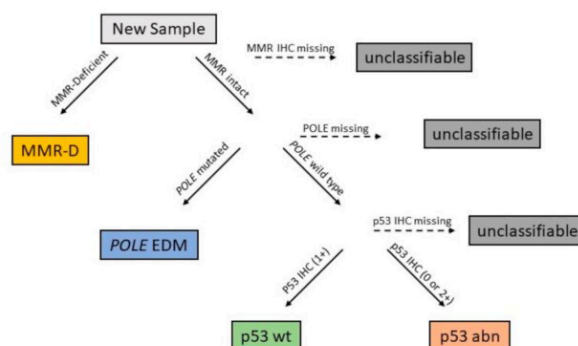


Figure 4. The Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) Algorithm to assess a new endometrial cancer sample. From Alexa, M. et al. Cancers 2021, 13,1478.

ESGO/ESTRO/ESP Risk Classification and the Molecular transformation

The integration of the ProMisE molecular classification into the ESGO/ESTRO/ESP guidelines (refined in the 2021 and 2025 updates)^{12,13} marks a definitive shift from anatomical staging to biological precision. This framework achieves the "Holy Grail" of modern oncology: matching treatment intensity to the tumor's genomic signature rather than its appearance alone.

The most transformative impact is seen in the de-escalation of care for POLEmut cases. Despite often presenting with "ugly" high-grade features that would traditionally mandate intensive therapy, POLEmut tumors in Stage I–II are now classified as Low Risk. This allows clinicians to safely omit toxic radiation and chemotherapy, sparing patients from unnecessary side effects. Conversely, the model demands the escalation of treatment for p53abn tumors. These aggressive, "serous-like" cancers are now categorized as High Risk even at Stage IA, ensuring that patients receive early systemic chemotherapy they might have previously been denied under traditional staging.

The Biological Complexity of Endometrial Cancer: Are Four Classes Enough?

While the four TCGA-inspired molecular subgroups—POLEmut, MMRd, p53abn, and NSMP—have provided a robust framework for risk stratification, they are increasingly viewed as a starting point for a complete understanding of the disease. The clinical reality of EC is characterized by a biological "grey zone" that the current four-class model struggles to fully encompass.¹⁴

One of the most significant complexities arises from cases that do not fit neatly into a single silo. Multiple-classifier tumors (e.g., cases harboring both a POLE mutation and a p53 abnormality, or MMRd combined with p53abn) present a unique diagnostic and prognostic challenge¹⁵. Current guidelines suggest a hierarchical approach—where POLE mutations generally trump p53abn status—but the biological rationale for this dominance is still being elucidated. This hierarchy suggests that the mere presence of a molecular marker is insufficient; we must understand the "evolutionary weight" of these mutations. A tumor with a "double hit" may behave differently than a "pure" classifier, suggesting that the molecular background influences the phenotypic expression of the dominant mutation.

The NSMP (Nonspecific Molecular Profile) group, which accounts for nearly half of all EC cases, represents perhaps the greatest argument against the "four classes are enough" sentiment. In fact, NSMP is not a single entity but a collection of tumors with diverse genetic drivers¹⁶. Unsupervised clustering has revealed that while most NSMP tumors have a stable genome, a significant subset exhibits "soft" genomic instability^{17,18}. The lack of a defining TCGA marker (like POLE or p53) means that clinicians must rely on secondary markers to predict which NSMP patients will recur¹⁴. The heterogeneity of this group explains why trials like PORTEC-3 showed only a marginal benefit of combined chemo-radiotherapy for the group as a whole, masking the benefit for high-risk sub-populations¹⁹.

Furthermore, the traditional assessment of Estrogen Receptor (ER) and Progesterone Receptor (PR) status—while long established for low-grade, endometrioid histotypes—is now gaining renewed attention as a critical marker for refined risk stratification²⁰⁻²². While strong, diffuse expression typically correlates with a favorable prognosis and lower-grade tumors, the loss of these receptors often serves as a surrogate marker for epithelial-mesenchymal transition (EMT) and increased aggressiveness²³.

Ultimately, the goal is to move past a rigid four-class system toward a multidimensional molecular-pathological profile. Only by identifying these sub-layers of heterogeneity can we refine our therapeutic strategies to avoid the twin pitfalls of over-treatment in low-risk patients and under-treatment in those with occult high-risk features²⁴⁻²⁶.

Refining the Landscape: The Role of β -catenin and AKT

In the effort to resolve the molecular ambiguity of the current classification, the search for "secondary" drivers that dictate clinical behavior within the established classes has become paramount. Among the signaling nodes identified as critical to EC progression, β -catenin and AKT stand out as pivotal regulators that potentially bridge the gap between genomic profile and phenotypic outcome.

β -catenin: a “new” biomarker

The Wnt/ β -catenin signaling pathway, primarily altered through mutations in the CTNNB1 gene, represents an innovative and potential prognostic variables in EC. Beta-catenin is a multifunctional protein expressed on the

surface of epithelial cells, acting as a structural component of the E-cadherin-related cell adhesion system. This molecule has a crucial role in maintenance of epithelial stability by regulating cell growth and intercellular adhesion. Beta-catenin also plays other important functional roles, including control of cell polarization, differentiation, “stemness,” and cell motility. At the cellular level, beta-catenin is the key mediator of the Wnt canonical pathway. Constitutive activation of the Wnt signaling is a major etiological factor for many cancers. In the resting state of Wnt signaling, beta-catenin is phosphorylated by glycogen synthase kinase 3 β (GSK3- β) within a protein complex including casein kinase 1, adenomatous polyposis coli (APC), and Axin. Phosphorylated beta-catenin is immediately degraded via the ubiquitin–proteasome pathway. In the active state, WNT binds to Frizzled (Fz), activating (Dsh), which inhibits the activity of GSK3- β , resulting in dephosphorylation and stabilization of beta-catenin, in this way enabling accumulation within the nucleus. A stable beta-catenin translates WNT signal into the transient transcription of a TCF/LEF (T-cell factor/lymphocyte enhancer factor) gene program, governing cell fate, proliferation, and other processes in several types of cancer. Stabilized molecule could be considered a cause of genomic instability, promoting tumor development and aggressiveness. Numerous studies have shown that CTNNB1, the gene of beta-catenin, together with APC and Axin are frequently mutated in different types of human cancers and that the nuclear accumulation of beta-catenin could be considered the final step of constitutive activation of WNT signaling²⁷. In particular, in EC, there are emerging evidence that a subset of low-grade, ECs can harbor CTNNB1 exon 3 mutations, and when this happens, prognosis is worse, overall intermediate, although their frequent clinic–pathological characteristics are commonly associated with lower risk of recurrence (younger age, squamous differentiation, low TILs, less incidence of deep myometrial invasion, and less incidence of LVSI, with a low number of other concurrent mutations, such as KRAS and FGFR2 mutation). Nuclear accumulation of beta-catenin, well detectable by immunohistochemistry, has been assessed as a possible surrogate for sequencing to identify CTNNB1-mutant cases, resulting in conflicting data, varying from an overall good agreement between protein nuclear staining and CTNNB1 status to high discordance. It seems that nuclear accumulation of beta-catenin in EC implies the presence of CTNNB1 mutation^{5,28,29}.

AKT: The Convergence Point of the PI3K Axis

Complementing the transcriptional shifts driven by β -catenin is the activation of the PI3K/AKT/mTOR pathway, which is arguably the most frequently disrupted signaling axis in endometrial cancer³⁰. While upstream mutations (such as in PIK3CA or PTEN) are ubiquitous across several molecular classes—most notably in MMRd and NSMP—the phosphorylation and activation of the AKT kinase represent the true functional convergence point for tumor survival. AKT serves as a metabolic "rheostat," governing glucose metabolism, protein synthesis, and the inhibition of apoptosis. Its hyperactivation provides a decisive survival advantage, potentially allowing tumors to bypass traditional growth constraints and maintain aggressive proliferation even in the absence of high-risk genomic "drivers" like TP53 mutations³¹⁻³⁴.

1.2 Objectives

The primary aim of this research is to improve the precision of prognostic stratification in endometrial cancer by expanding the surrogate molecular classification through an IHC-based approach.

Hypothesis: The integration of additional molecular alterations—detectable through surrogate biomarkers that are readily implementable in clinical practice via IHC—is expected to significantly improve prognostic stratification and the capacity to predict individual tumor behavior across all endometrial carcinoma subclasses.

The study aims to refine the current molecular classification framework (ProMisE), thereby enhancing oncological risk stratification and the prediction of both tumor behavior and treatment response across all molecular subclasses.

The specific objectives are as follows:

- To evaluate the prevalence of β -catenin and AKT expression within a cohort of EC patients.
- To correlate nuclear β -catenin and AKT expression with established clinicopathological parameters (FIGO stage, histology, grade, LVSI, myometrial invasion).
- To evaluate the prognostic role of nuclear β -catenin and AKT expressions within a cohort of EC patients.

CHAPTER 2

Materials and Methods

Study Design and Ethical Considerations

This study was designed as an observational prospective study. Patient recruitment and surgical procedures were conducted at the Unit of Gynecologic Oncology, ARNAS “Civico– Di Cristina– Benfratelli”, University of Palermo. The entire analysis of the specimen for the research purpose was performed at the laboratory of the Tumor Immunology Unit, Department of Sciences for Health Promotion and Mother-Child Care “G. D’Alessandro,” University of Palermo.

The conduction of the study received preliminary approval from the local Ethical Committee (“Comitato Etico Palermo 2”) (code n 146 CIVICO 2022). The study has been designed and conducted following the principles of the declaration of Helsinki. Before the inclusion in the study, each subject received extensive information and had the chance to ask questions regarding the study. All participants provided written informed consent for the use of their de-identified data for research purposes.

Study Population and Selection Criteria

Subjects’ inclusion has been conducted between November 2022 and November 2024 selecting a consecutive cohort of patients undergoing surgical treatment for primary endometrial cancer. Patients were eligible for inclusion if the following inclusion criteria were met.

Inclusion Criteria: Patients were eligible if they had histologically confirmed endometrial cancer, underwent surgical staging (including at least hysterectomy, bilateral salpingo-oophorectomy, and lymph node assessment via sentinel lymph node biopsy or lymphadenectomy), and provided written informed consent.

Exclusion Criteria: Exclusion criteria included age under 18 years, lack of

research authorization, diagnosis of non-epithelial uterine malignancies, or the presence of synchronous or metachronous second malignancies. Patients receiving conservative non-surgical treatments (e.g., primary hormone therapy, radiation, or chemotherapy) were also excluded.

Study Setting

Eligible patients undergoing surgical staging for endometrial cancer were prospectively enrolled in the study. Tumor sampling was performed on the final surgical specimens to ensure the acquisition of adequate biological material for the subsequent molecular analyses planned in the research project. To maintain confidentiality and facilitate the integration of clinical and laboratory results, each patient and their corresponding tumor sample were assigned a unique de-identified code. For optimal preservation, each tumor sample was partitioned and stored using two distinct methods: one portion was fixed in a closed-circuit, single-use container pre-filled with 4% formaldehyde (10% buffered formalin), while a second portion was immediately stabilized in a specific reagent (Allprotect Tissue Reagent) to preserve the integrity of DNA, RNA, and proteins for optional additional molecular tests.

Surgical management

All patients underwent surgical staging for endometrial cancer (including hysterectomy, bilateral salpingo-oophorectomy, and lymph node assessment: sentinel lymph node biopsy or lymphadenectomy. Sentinel lymph node (SLN) mapping was performed following National Comprehensive Cancer Network (NCCN)³⁵ SLN mapping guidelines, which requires the performance of a side-specific lymphadenectomy in cases of failed mapping and removal of any suspicious or enlarged lymph nodes in the pelvic or aortic regions regardless of mapping. When complete lymphadenectomy is performed, pelvic lymph nodes from the external iliac, internal iliac, obturator, and common iliac nodes are removed. Para-aortic nodal dissection includes inframesenteric and infrarenal regions. In patients with serous endometrial carcinoma, carcinosarcoma, and undifferentiated carcinoma, surgical staging includes omental sampling (omental biopsy or infra-colic omentectomy). SLNs were ultrastaged using the Memorial Sloan-Kettering Cancer Center approach or a modification of this protocol based on the anatomic pathology protocols^{36,37}.

Quantitative immunolocalization analyses

Formalin-fixed, paraffin-embedded (FFPE) endometrial cancer samples were collected, and four-micrometer-thick tissue sections were immunostained using the Bond-III automated staining system (Leica Microsystems). The following primary antibodies were used: p53 (clone DO-7, cat. no. PA007), MLH1 (clone ES05, cat. no. PA0988), MSH6 (clone EP49, cat. no. PA0990), MSH2 (clone 79H11, cat. no. PA0989), PMS2 (clone EP51, cat. no. PA0991), beta-Catenin (clone 17C2, cat. no. PA0083) and pAKT-1 (clone LP18, cat. no. AKT-PHOSP-L-CE).

Slide were digitalized using an Aperio CS2 digital scanner (Leica Biosystems), and images were acquired with ImageScope software (Aperio ImageScope version 12.3.2.8013, Leica Biosystems)³⁸.

For p53, quantitative analyses were performed by calculating the average percentage of positive nuclei across the entire tissue section using HALO image analysis software (v3.2.1851.229, Indica Labs). Results were reported as the "percentage of positive nuclei". In parallel, a semiquantitative assessment restricted to tumor cells was conducted by an experienced pathologist, focusing exclusively on nuclear staining in neoplastic cells. Mismatch repair (MMR) protein expression (MLH1, MSH2, MSH6, PMS2) was evaluated as retained or lost/subclonal loss of protein expression. Adjacent stromal, and inflammatory cells served as internal positive controls.

β-catenin expression was assessed based on subcellular localization in tumor cells. Membranous staining was considered the normal pattern, whereas nuclear localization in tumor cells was interpreted as aberrant and indicative of Wnt pathway activation.

Overexpression of phosphorylated AKT (pAKT) was evaluated by calculating the H-score across the entire section. Images were analyzed using Aperio software to quantify the percentage of nuclei exhibiting 1+ weak positivity (weak), 2+ moderate positivity (moderate); 3+ strong positivity (strong) pAKT staining intensity. H-scores were calculated according to the following formula:

$$H - score = (1 \times \% \text{ weak nuclei}) + (2 \times \% \text{ moderate nuclei}) + (3 \times \% \text{ strong nuclei})$$

H-score values range from 0 to 300. Samples with H-score ≥ 150 were considered positive for pAKT overexpression, as previously described³⁹.

Data collection - Clinical variables

The data collected were anonymized, taking into account the observational nature of the study, without personal data that could lead to formal identification of the patient. Clinical patient characteristics including age, BMI, clinical stage according to the International Federation of Gynaecology and Obstetrics classification, histopathologic results, tumor grade, and modalities of tumor treatments were recorded. Location of the recurrence was classified as: vaginal, peritoneal, hematogenous, lymphatic (pelvic, paraaortic, other distant lymphatic – inguinal, mediastinal, supraclavicular). Non-vaginal recurrence was defined as recurrence localized to the lymph nodes or peritoneum or was distant through hematogenous spread, with or without concomitant vaginal recurrence.

Statistical Analysis

Quantitative variables were summarized using mean and standard deviation for normally distributed data, or median and interquartile range (IQR) for non-normal distributions. Categorical data were expressed as absolute frequencies and percentages. The association between molecular subgroups and clinicopathological features was assessed using Student's t-test, Mann-Whitney U test, chi-squared test, or Fisher's exact test, as appropriate. Recurrence-free survival (RFS) and overall survival (OS) were estimated using the Kaplan-Meier method, with the log-rank test employed to compare survival curves between molecular subclasses. Time-to-event was calculated from the date of surgery to the date of death or last follow-up for overall survival and from the date of surgery to the date of recurrence or last relevant clinical follow-up for recurrence-free survival. Univariable and multivariable Cox proportional hazard models were used to identify predictors of recurrence and death and hazard ratios (HRs) with 95 % confidence intervals (CIs) were reported. A two-sided p-value <0.05 was considered statistically significant. All statistical analyses were carried out using R software (version 2024.12.0 + 467).

CHAPTER 3

Results

Study Population and Cohort Selection

During the study period, a total of 99 patients with EC were prospectively enrolled at the promoting center based on specific inclusion criteria.

The cohort underwent a rigorous quality control process to ensure the adequacy of the biological material for the analysis. Out of the initial 99 patients, 18 were excluded due to an insufficient quantity of biological material. Of the remaining 82 patients, another 20 were excluded because the tissue was found to be unsuitable for the specific pathological evaluation of the required biomarkers. Consequently, the final analytical cohort consists of 60 patients (Figure 5).

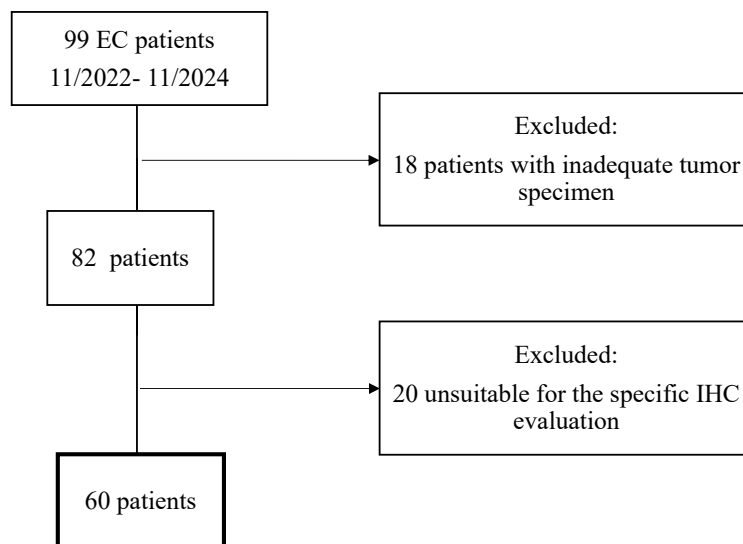


Figure 5: Flowchart of the study population.

Clinical and Pathological Characteristics

The median age of the final cohort was 64 years (Interquartile Range, IQR: 57–71 years) and the median body mass index (BMI) was 26.5 (13.6). Regarding histological distribution, the majority of the patients presented with endometrioid histology (n=48, 80%). Within this group, 33 patients (55%) had low-grade (G1) or moderate-grade (G2) tumors, while 15 (25%) were classified as high-grade (G3). The remaining 12 (20%) patients in the cohort exhibited more aggressive non-endometrioid histotypes, including serous carcinoma (n=10), clear cell carcinoma (n=1), and carcinosarcoma (n=1). LVSI (lymph vascular space invasion) was identified as diffuse in 12 (20%) patients, while myometrial infiltration $\geq 50\%$ was present in 36 (50%) patients (**Table 1**).

The final FIGO 2009 staging was as follows: IA in 17 (28.3%) patients, IB in 21 (35%) patients, II in 5 (8.3%) patients, IIIA in 1 (1.6%) patient, IIIB in 2 (3.4%) patients, IIIC1 in 5 (8.3%) patients, IIIC2 in 3 (5%) patients, and IVB in 6 (10%) patients. The final FIGO 2023 staging was as follows: IA1 in 3 (5%) patients, IA2 in 8 (13.3%) patients, IB in 14 (23.3%) patients, IIA in 1 (1.6%) patient, IIB in 1 (1.6%) patient, IIC in 16 (26.6%) patients, IIIA2 in 1 (1.6%) patient, IIIB1 in 2 (3.4%) patients, IIIC1 in 5 (8.3%) patients, IIIC2 in 3 (5%) patients, IVB in 5 (8.3%) patients, and IVC in 1 (1.6%) patient (**Table 2**).

Molecular Classification

Based on surrogate molecular classification using the IHC analysis, patients were categorized as follows: 9 (15%) p53-abnormal, 28 (46.6%) MMRd, and 23 (38.4%) no specific molecular profile (NSMP: p53wt/MMRp).

Of the 28 patients with MMR-d on IHC, patients considering the heterodimers involved in loss of function, patients were distributed as follows: 5 loss of MLH1/PMS2/MSH2/MSH6, 5 loss of MSH6 alone, 2 loss of MSH2/MSH6, 1 loss of MLH1 alone, 3 loss of MLH1/PMS2 and 12 loss of MLH1/PMS2/MSH6.

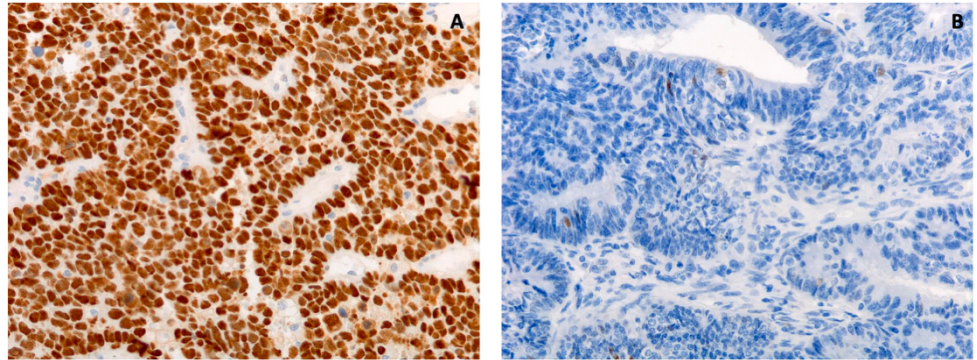


Figure 6: IHC images show over expression (A) and negative staining (B) of p53 in endometrial cancer tissue. Magnification 20X.

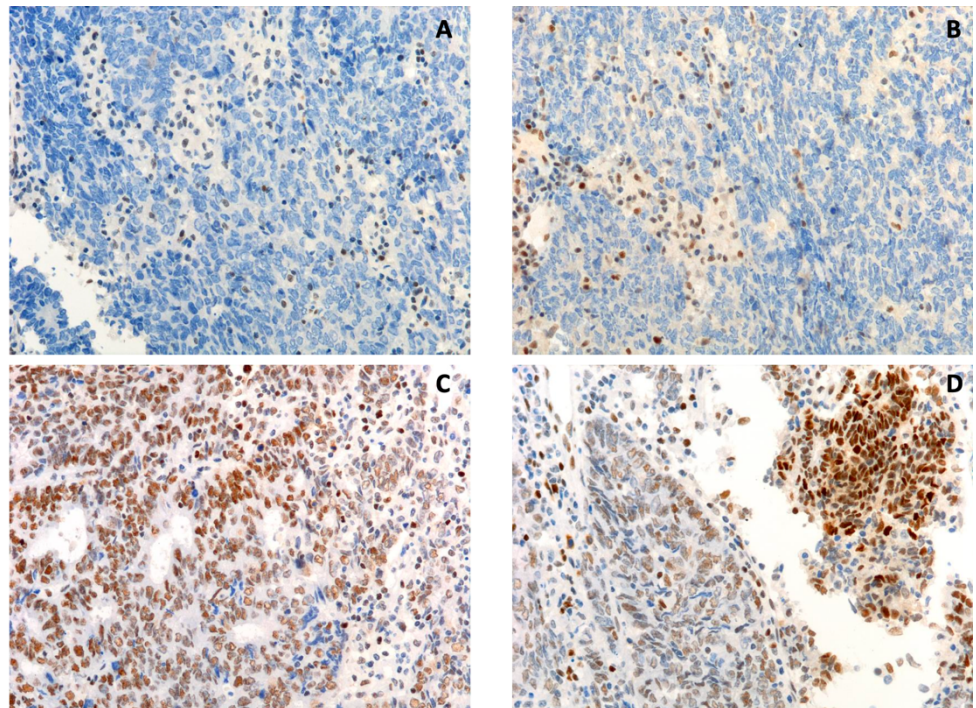


Figure 7: IHC images show loss of MLH1 (A) and PMS2(B) in endometrial cancer tissue. In the same sample MSH2 (C) was highly expressed, while an heterogenic expression was observed for MSH6 (D). Magnification 20X

β-catenin and AKT

Microscopic evaluation of β-catenin expressions with immunohistochemical staining showed positive expression of β-catenin in the nucleus in 33 (55%) cases (**Figure 8**). Within this group, 21 (63.6%) cases reported a strong intensity of stained cells with evaluation the percentage of the coloured area >10%. Microscope evaluation of the p-AKT overexpression was detected in 46 (76.6%) cases (**Figure 9**). A dual hyperexpression of both markers was identified in 26 patients (43.3%) (**Table 3**).

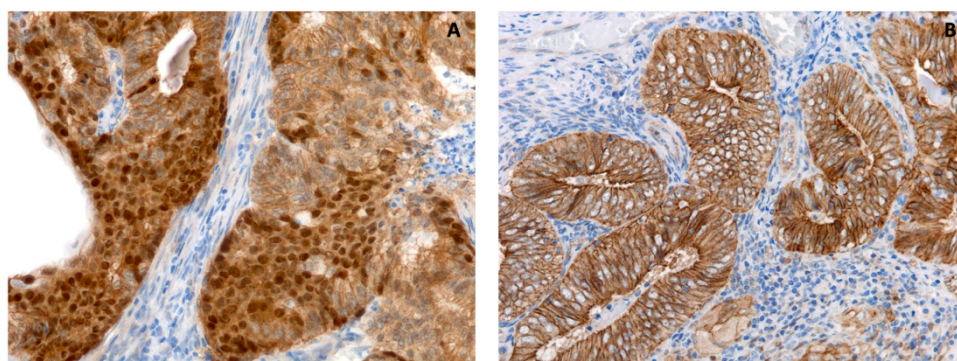


Figure 8: IHC images show nuclear (A) and membrane (B) expression of β-catenin in endometrial cancer tissue. Magnification 20X

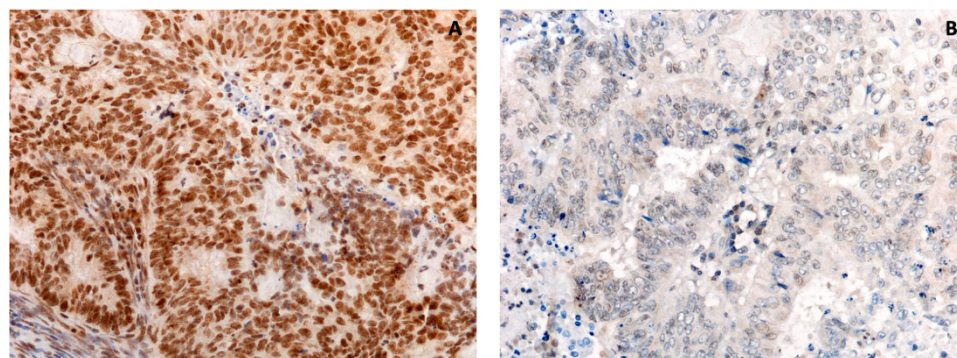


Figure 9: IHC images show over expression (A) and negative staining (B) of pAKT in endometrial cancer tissue. Magnification 20X

The distribution of IHC biomarkers varied across the molecular subgroups. In the MMRd group (n=28), the most frequent profile was the dual expression of nuclear β-catenin and p-AKT (15/28, 53.5%), followed by isolated p-AKT positivity (9/28, 32.1%), while isolated β-catenin and double-negative cases were rare (1/28 and 3/28, respectively). The NSMP group (n=23) showed a more heterogeneous distribution, with 8 cases of dual expression (34.8%), 7

cases of isolated p-AKT (30.4%), and the highest prevalence of isolated nuclear β -catenin expression (6/23, 26.1%) compared to other groups. In the p53-abnormal cohort (n=9), p-AKT expression predominated, either as dual expression (3/9) or isolated positivity (4/9), whereas no cases of isolated β -catenin were detected, and 2 cases (22.2%) were double-negative for both markers (**Figure 10**).

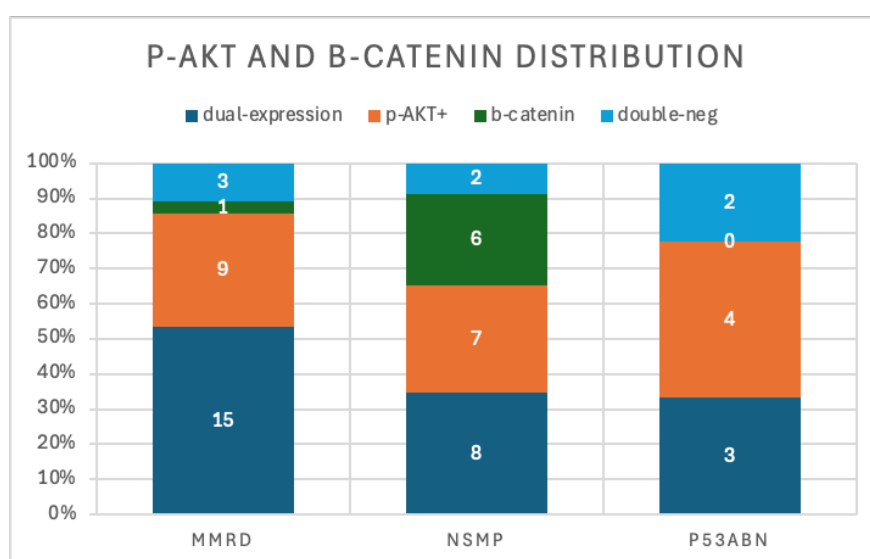


Figure 10: p-Akt and beta-catenin distribution among the molecular classes

The distribution of immunohistochemical markers was further statistically analyzed across molecular subgroups. Regarding nuclear β -catenin expression was equally distributed among MMRd, NSMP, and p53abn subgroups ($p=0.4$), with no significant difference observed in relation to MMR status ($p>0.9$) or p53 mutational profile ($p=0.3$). Notably, the presence of one marker did not significantly influence the expression of the other, as p-AKT and β -catenin remained equally distributed regardless of their respective positivity ($p=0.7$ for both comparisons). Similarly, p-AKT hyperexpression, no significant association was found with p53 status ($p>0.9$), MMR status ($p=0.091$), or the overall molecular classification ($p=0.2$), although a higher prevalence of p-AKT positivity was noted in the MMRd group (52% vs 29% in the negative group) (**Table 4-5**).

Clinical significance of β -catenin and AKT

Statistical comparison between β -catenin positive (n=33) and negative (n=27) cases revealed a highly significant association with tumor histology ($p<0.001$), as 97% of positive cases exhibited endometrioid morphology compared to only 59% in the negative group. Furthermore, patients with nuclear β -catenin expression showed a significantly different follow-up distribution (median 36 vs 38 months, $p=0.034$). No significant differences were observed regarding age, BMI, tumor grade, LVSI, or FIGO stage, although β -catenin positivity showed a trend toward low-grade (G1–G2) tumors (64% vs 44%, $p=0.14$) (**Table 6**). Regarding p-AKT status, hyperexpression was significantly associated with a shorter follow-up period (median 37 vs 42 months, $p=0.046$). While p-AKT positivity was more frequent in high-grade (G3) tumors (48% vs 36%) and cases with substantial LVSI (24% vs 7.1%), these associations did not reach statistical significance ($p=0.4$ and $p=0.3$, respectively). Similarly, age, BMI, histology, and FIGO stage were equally distributed between p-AKT positive and negative groups (**Table 7**).

Prognostic role of β -catenin and AKT

Overall, the median follow-up was 37 months (standard deviation [SD] 8.7; mean 35). Recurrence was documented in 10 patients (16.6%), with a median recurrence-free survival of 16 months (IQR; 9.5-25.75 months). The pathways of recurrences were as follow: 2 peritoneal, 4 lymphatics (2 lymphatic pelvic, 2 lymphatic para-aortic), 3 hematogenous (2 lung, 1 hepatic), and 1 isolated vaginal. In terms of overall survival, death was documented in 5 (8.3%) patients. Three patients died of disease, while 2 died of other causes. The median time to death was 19 months (IQR; 3-40 months).

In terms of negative prognostic pathological factors, p-AKT hyperexpression was associated with a trend toward deeper myometrial invasion, although it did not reach full statistical significance ($p=0.080$); specifically, 52% of p-AKT positive cases showed $\geq 50\%$ invasion compared to 86% in the negative group. Tumor size was slightly larger in the p-AKT positive cohort (median 43 mm vs 30 mm, $p=0.3$). The rate of lymph node metastasis was nearly identical between the two groups (20% vs 21%, $p>0.9$), with a higher prevalence of combined pelvic and aortic involvement in p-AKT positive patients (56% vs 33%).

Regarding oncological outcomes, p-AKT hyperexpression was significantly associated with a shorter Disease-Free Survival (DFS) interval, with a median of 36 months compared to 40 months in negative cases ($p=0.013$). Although p-AKT positive patients showed a higher absolute number of recurrences (20% vs 7.1%), this difference did not reach statistical significance ($p=0.4$), nor did the distribution of recurrence patterns or overall survival ($p>0.9$) (**Table 8**). However, the overall recurrence probability over time did not differ significantly in the Kaplan-Meier analysis ($p=0.17$) (**Figure 11A**). Regarding nuclear β -catenin expression, no significant correlations were found with tumor size (median 40 mm vs 35 mm, $p=0.2$) or myometrial invasion ($p>0.9$), which remained equally distributed regardless of marker positivity. The rate of lymph node metastasis was lower in β -catenin positive cases compared to negative ones (15% vs 26%, $p=0.3$), with a shift in distribution toward pelvic nodes (60% vs 29%) rather than combined pelvic and aortic involvement (20% vs 71%). About the oncological outcomes, nuclear β -catenin expression did not significantly impact oncological survival. No statistical differences were observed in recurrence rates (9.1% for positive vs 26% for negative cases, $p=0.10$), median DFS ($p=0.4$), or cancer-related death ($p=0.4$) (**Table 9**). Kaplan-Meier analysis confirmed these findings, showing that β -catenin ($p=0.14$) did not significantly predict the probability of recurrence-free over time in this patient cohort (**Figure 11B**).

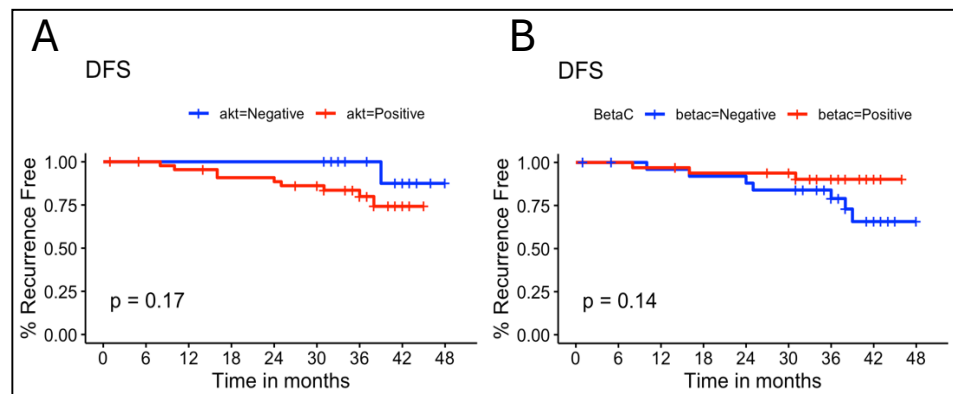


Figure 11: Kaplan-Meier curves showing disease-free survival (DFS) from the timepoint of primary surgery stratified by IHC analysis. A) K-M curves based on p-AKT hyperexpression. B) K-M curves based on β -catenin expression.

Dual over-expression of β -catenin and AKT

The same analysis was conducted by classifying the cases by the presence of only one-protein expression (β -catenin expression or p-AKT hyperexpression), vs. both-protein expression (β -catenin expression and p-AKT hyperexpression) vs. no-protein expression. The comparative analysis revealed that patients with concurrent both-protein expression are almost exclusively associated with endometrioid histology (96% vs 57% in no-protein cases, $p=0.011$). While grading and stage remained equally distributed across the groups. Moreover, the both-protein group exhibited a significantly shorter follow-up period (median 34 months, $p=0.016$)(**Table 10**). Regarding molecular stratification, the distribution of MMRd, NSMP, and p53abn subgroups did not differ significantly between one-protein, no-protein, and both-protein subgroups ($p=0.4$), confirming that these proteomic activations may occur independently of the genomic class. Specifically, within the both-protein group, 58% of cases were MMRd, 31% were NSMP, and 12% were p53abn (**Table 11**). In terms of oncological outcomes, the both-protein group showed a trend toward a shorter DFS (median 34 months compared to 39 months in no-protein cases, $p=0.082$), while there was no difference in terms of recurrence rate ($p=0.7$), pattern of recurrence ($p=0.9$) or death ($p=0.3$). However, 2 out of the 3 cancer-related death occurred in the both-protein group (**Table 12**). Kaplan-Meier analysis did not show a significant difference by protein expression among the three subgroups ($p=0.71$) (**Figure 12**). However, the curves illustrate a faster decline in the recurrence-free percentage for cases with protein expression (one or both) compared to no-protein expression ones. Furthermore, Cox regression analysis showed that the both-proteins expression (HR 0.61, $p=0.5$) did not act as independent significant predictors of recurrence compared to the single-protein expression group (**Table 13**).

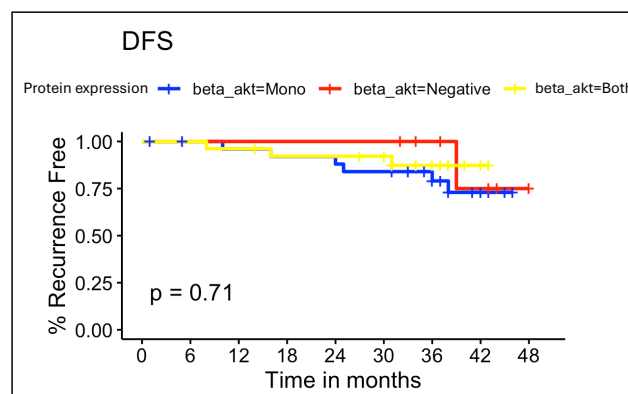


Figure 11: Kaplan–Meier curves showing disease-free survival (DFS) from the timepoint of primary surgery stratified by protein expression

CHAPTER 4

Discussion

The molecular revolution initiated by the TCGA in 2013 has fundamentally changed the management of EC⁸. However, the "one-size-fits-all" approach within the four standard molecular classes remains a clinical challenge. Our research aimed to increase the prognostic granularity of this classification by integrating two pivotal biomarkers: b-catenin and AKT-1.

In our study cohort of 60 patients, we observed a high prevalence of both nuclear β -catenin expression (55%) and p-AKT hyperexpression (76.6%), while a concomitant expression of these proteins was identified in 26 cases (43.3%). Moreover, a primary finding is the strong association of nuclear β -catenin with endometrioid histology, conversely, the lack of statistical significance regarding other pathological features may be attributed to the limited sample size and the heterogeneity of the cohort.

Looking at the molecular classification, our analysis reveals that both p-AKT and β -catenin expression are distributed across the molecular groups (p53abn, MMRd, NSMP) without a statistically significant preference ($p=0.2$ and $p=0.4$, respectively). This suggests that the activation of these pathways might represent a common oncogenic 'hit' that transcends the primary genomic driver (MMR deficiency, p53 mutation). Moreover, when analyzing cases with concomitant overexpression of both β -catenin and p-AKT (both-protein), we confirmed that this co-occurrence is also equally distributed across molecular subgroups ($p=0.4$). Interestingly, the NSMP group remains the primary reservoir for isolated nuclear β -catenin expression, consistent with its known association with CTNNB1 mutations²⁹.

In this regard, the NSMP group remains biologically heterogeneous, and nuclear β -catenin expression could serve as a potential 'red flag' for aggressive behaviour. Although this trend was not statistically confirmed in our analysis, this could be attributed to the limited sample size within this specific category ($n=23$). On the other hand, β -catenin expression in our overall cohort did not show a detrimental impact on oncologic outcomes. Specifically, it did not significantly affect

prognosis regarding recurrence or death, with Kaplan-Meier analysis showing no statistical difference between patients with and without β -catenin expression ($p=0.14$). While our findings regarding the lack of impact of β -catenin on the clinical course of the disease may appear inconsistent with existing literature, these results should be interpreted with caution. The relatively small sample size, low event rate, and limited follow-up period may affect these outcomes; therefore, our findings should be re-evaluated in a larger, more robust patient cohort.

In this sense, the potential role of the β -catenin pathway in the identification of “more-aggressive” EC cases is strongly supported by the latest data from the PORTEC-4a trial, that included 564 patients⁴⁰. The PORTEC-4a results have demonstrated that a molecular-integrated risk profile is not only feasible but superior to traditional pathological staging in guiding adjuvant treatment. Crucially, PORTEC-4a identified CTNNB1 mutations as a key unfavorable prognostic factor within the intermediate-risk group. In fact, the study showed that approximately 46% of patients with a “favorable” molecular profile could safely avoid adjuvant radiotherapy (VBT) without compromising cancer control, significantly improving their quality of life. Conversely, patients with an “unfavorable” molecular profile (including those with CTNNB1 mutations or L1CAM expression) who received pelvic radiotherapy (EBRT) instead of standard VBT showed a significantly lower locoregional recurrence rate (8.4% vs 30.5% in historical or standard-care cohorts). By using IHC for β -catenin as a surrogate for CTNNB1 mutations, our study aligns with the PORTEC-4a strategy, demonstrating that identifying these “unfavorable” features within the NSMP group is critical to avoid undertreating patients who appear clinically low-risk but are biologically predisposed to recurrence. Moreover, always more evidence suggests the potential role of additional biomarker in refining the prognosis of EC, especially those belonging to the NSMP group. In fact, a recent study on 72 patients with EC of NSMP subtype showed ARID1A mutation in 24 cases (33.3%). ARID1A mutations were significantly associated with a higher risk of recurrence (37.5% vs 12.5%, OR 4.20, 95% CI 1.28 to 13.80, $p=0.018$) and impaired progression-free survival (HR 3.96, 95% CI 1.41 to 11.15, $p=0.009$). Nevertheless, progression-free survival appears not to translate into reduced disease-specific survival¹⁶.

While this report supports the fact that ARID1A assessment could be used as a future prognostic biomarker, it is important to note that its evaluation has been performed through a DNA sequencing. In contrast, our report reinforces the use of IHC-surrogate biomarkers as a widely applicable alternative, especially given the high costs, complexity, and significant time required for molecular genomic assessments.

On the other hand, the high frequency of p-AKT across the entire cohort supports the hypothesis that PI3K/AKT pathway activation is a near-ubiquitous driver in EC, often resulting from PTEN loss or PIK3CA mutations. The trend observed between p-AKT hyperexpression and myometrial invasion ($p=0.080$), coupled with the significantly shorter DFS interval ($p=0.013$), suggests that AKT activation may favour a more infiltrative and rapidly progressing phenotype. However, it is important to note that the association of p-AKT hyperexpression with the negative pathologic features, such as the deep myometrial invasion, was not statistically significant limiting the interpretation of our results about a potential aggressive behaviour of tumour with this AKT hyperexpression. Moreover, it is noteworthy that the presence of p-AKT did not appear to be detrimental to oncologic outcomes. In fact, p-AKT hyperexpression did not significantly impact prognosis regarding recurrence or death; Kaplan-Meier analysis showed no statistical difference between patients with and without hyperexpression ($p=0.17$). However, the relatively small sample size, low event rate, and limited follow-up period may affect the correlation with the clinical outcomes; therefore, our findings should be re-evaluated in a larger, more robust patient cohort. In fact, a recent study of 107 EC patients showed that higher expression of AKT1, along with other genes, is significantly associated with a poor prognosis (overall survival: HR=1.863, 95% CI 1.050–3.218, $p=0.034$; progression-free survival: HR=1.789, 95% CI 1.014–3.107, $p=0.047$)⁴¹. Nevertheless, that study primarily included patients with advanced-stage disease (100 out of 107), limiting the generalizability of this detrimental association; in contrast, our study also included several patients with early-stage disease.

In this sense, our study may be interpreted as a hypothesis generating research that highlight the potential role of AKT in driving the behaviour of EC. In fact, the identification of high-risk patients with AKT activation may open the possibility of future targeted PI3K/AKT inhibitors, aligning with the direction suggested by the most recent trials and upcoming immunotherapy-combination protocols⁴²⁻⁴⁴.

PI3K/AKT/mTOR pathway inhibitors fall into 4 main categories: mTOR inhibitors, PI3K inhibitors, dual mTOR/PI3K inhibitors, and AKT inhibitors³². For example, everolimus and temsirolimus (mTOR inhibitors), showed antitumor activity in endometrial cancer cell lines, with greatest sensitivity in cells with PIK3CA and/or PTEN mutations⁴⁵. However, a limitation to the use of PI3K/AKT/mTOR pathway inhibitors in EC will likely be the presence of numerous signaling feedback loops and cross-talk between signaling pathways. Combining PI3K/AKT/mTOR pathway inhibitors with other therapies might improve efficacy. For example, the aromatase inhibitor letrozole in combination with everolimus, showed enhanced activity in a phase II clinical trial in advanced

EC⁴⁶. Moreover, activation of the PI3K/AKT/mTOR pathway has also been implicated as a mechanism of resistance to standard cytotoxic agents in EC. In a phase I trial of temsirolimus in combination with carbo-platin and paclitaxel, 9 of 11 patients (82%) with EC achieved objective partial responses⁴⁷. A randomized, 3-arm, phase II trial (NCT00977574) is now evaluating this combination in patients with advanced or recurrent EC who have received no prior chemotherapy or targeted therapy.

Furthermore, our analysis investigated the potential synergy of β -catenin and AKT co-expression. We observed an absolute association of the both-proteins (p-AKT and β -catenin expression) group with endometrioid histology ($p=0.011$), suggesting a synergistic role in maintaining this specific lineage. From a clinical perspective, the co-overexpression status could potentially act as a 'tempo driver' in the clinical course. Indeed, the both-proteins group was significantly associated with a shorter clinical follow-up ($p=0.016$) and exhibited a trend toward earlier recurrence, with a reduced DFS interval: median 34 months in double-positive cases compared to 37 months in single-positive and 39 months in double-negative cases. However, this association with a reduced DFS did not reach a statistical significance ($p=0.082$) and so, the clinical impact may need further investigation to validate this preliminary finding. Moreover, also the Kaplan-Meier analysis did not reach statistical significance in terms of DFS probability, but the curves appear to show an acceleration of the clinical course when comparing cases with protein overexpression (single or both) against those with no expression. Yet these findings need to be interpreted with caution while representing an area of future research for a further understanding on the biological perspective.

This underscores the potential of dual IHC profiling as a functional supplement to genomic classification for refining the intensity of post-operative surveillance. However, while we recorded clinical outcomes, it must be emphasized that the lack of POLE status limits the precision of our surrogate molecular grouping. The inadvertent inclusion of POLE-ultramutated cases into other categories likely influences the observed survival rates and warrants caution when generalizing these results.

On the other hand, in an era where Next-Generation Sequencing (NGS) remains economically or logistically inaccessible for many centres, our IHC-based approach for beta-catenin and AKT may offer a cost-effective additional tool. The potential future integration of these markers into the ESGO/ESTRO/ESP framework allows for a more personalized "precision medicine" approach, identifying true low-risk NSMP (negative for both beta-catenin and AKT) to avoid unnecessary adjuvant treatment.

The clinical necessity of refining the molecular categories is further underscored by the RAINBOW (Refining Adjuvant treatment IN endometrial cancer Based On molecular features) umbrella trial⁴⁸. This international research program represents a definitive shift from purely prognostic stratification to a "personalized-by-molecular-class" therapeutic approach. Unlike previous protocols, the RAINBOW trial assigns patients to four different treatment arms based on their specific molecular signature, effectively moving away from the "one-size-fits-all" model of adjuvant therapy. In this context, the trial explores de-escalation for the POLEmut subgroup through simple observation (Blue arm) and evaluates the addition of immunotherapy to radiotherapy for MMRd tumors (Green arm). Yet, the NSMP-specific "Orange" arm, which investigates whether hormonal maintenance with progestins for two years can provide a more effective and less toxic alternative to standard chemoradiation in estrogen-receptor-positive cases. At the other end of the spectrum, the p53abn "Red" arm tests the synergy between chemoradiation and PARP inhibitors like Olaparib.

The study has several strengths. First, its prospective nature ensured high-quality specimen preservation and robust clinical follow-up. Second, the research introduces significant novelty by investigating specific biomarkers; in particular, the study of AKT represents an innovative approach to evaluating the PI3K/AKT/mTOR pathway in this context. Last, the adoption of digital pathology represents a major technical strength of our study. As a novel technique in the field of endometrial cancer research, it offers objective insights that will have important implications for the future of automated clinical diagnostics. However, we acknowledge several limitations. First, the relatively small cohort size may limit the statistical power required to detect complex interactions between beta-catenin and AKT across all molecular subgroups. Second, the heterogeneity of our cohort in terms of histology and FIGO stage limits the generalizability of our findings. Furthermore, the limited follow-up duration and the low frequency of clinical events, such as recurrence or mortality, mean that our survival analyses must be interpreted as hypothesis-generating rather than definitive. Another significant limitation was the unavailability of POLE mutational testing due to the lack of access to NGS facilities during the study period. Consequently, we cannot exclude the possibility that a small number of 'ultra-favorable' POLE-mutated cases were inadvertently included within our cohort.

Conclusion

In conclusion, our study demonstrates that an 'expanded IHC' approach—integrating beta-catenin and AKT-1 into the standard molecular framework—is a feasible and cost-effective method, especially in resource-limited settings. Considering the limited sample size and the relatively short follow-up, this research should be interpreted as a pilot study regarding the clinical significance of these proteins' overexpression in the prognosis of EC patients.

Looking ahead, we envision the expansion of this cohort into a larger, multicentric study to confirm these preliminary findings over a longer clinical period. Moreover, the natural evolution of this research will involve integrating these IHC findings with comprehensive genomic analysis. Moving beyond surrogate markers to include targeted NGS panels—covering not only POLE sequencing but also a broader exploration of the PI3K/AKT/mTOR and CTNNB1 landscapes—will be essential to provide a comprehensive view of the tumor's biology, eventually bridging the gap between molecular diagnosis and personalized patient care.

Tables and Figures

Table 1: Population characteristics.

Characteristic	Overall (n=60)
Age at surgery (y), median (SD)	64 (10.4)
BMI, median (SD)	26.5 (13.6)
Tumor diameter (mm), median (IQR)	38.5 (25.2-59.2)
FIGO grade, N (%)	
1	13 (21.7)
2	20 (33.3)
3	27 (45)
Histology, N (%)	
Non-endometrioid	12* (20)
Endometrioid	48 (80)
LVSI, N (%)	
Absent	37 (61.7)
Focal	11 (18.3)
Diffuse	12 (20)
Myometrial invasion, N (%)	
None	3 (5)
<50%	21 (35)
≥50%	36 (60)
Type of ATx received	
None	22 (36.6)
VB	12 (20)
EBRT ± VB	8 (13.4)
Chemotherapy ± VB	2 (3.4)
Chemotherapy and EBRT ± VB	16 (26.6)

*non-endometrioid: 1 carcinosarcoma, 1 clear cell, 10 serous. Abbreviations: BMI, body mass index; chemotherapy; EBRT, external beam radiotherapy; FIGO, International Federation of Gynecology and Obstetrics; SD, standard deviation; LVSI, lymphovascular space invasion; VB, vaginal brachytherapy; ATx, adjuvant therapy

Table 2: Distribution according to FIGO 2009 and FIGO 2023 staging systems

Stage FIGO 2009	Patients n (%)	Stage FIGO 2023	Patients n (%)
IA	17 (28.3)	IA1	3 (5%)
IB	21 (35%)	IA2	8 (13.3%)
II	5 (8.3%)	IA3	0 (0%)
IIIA	1 (1.6%)	IB	14 (23.3%)
IIIB	2 (3.4%)	IC	0 (0%)
IIIC1	5 (8.3%)	IIA	1 (1.6%)
IIIC2	3 (5%)	IIB	1 (1.6%)
IVA	0 (0%)	IIC	16 (26.6%)
IVB	6 (10%)	IIIA1	0 (0%)
—	—	IIIA2	1 (1.6%)
—	—	IIIB1	2 (3.4%)
—	—	IIIB2	0 (0%)
—	—	IIIC1	5 (8.3%)
—	—	IIIC2	3 (5%)
—	—	IVA	0 (0%)
—	—	IVB	5 (8.3%)
—	—	IVC	1 (1.6%)

Abbreviations: FIGO, International Federation of Gynecology and Obstetrics.

Table 3: β -catenin and AKT

Immunohistochemical analysis	N	%
β -catenin (+)	33	55,0%
p-AKT Hyperexpression (+)	46	76,6%
Dual Expression (beta-cat/p-AKT)	26	43,3%
Single Expression (only p-AKT)	20	33,3%
Single Expression (only beta-cat)	7	11,7%
Double Negative	7	11,7%

Table 4: β -catenin expression distribution across molecular

Characteristic	β -catenin expression Negative, N = 27 ¹	β -catenin expression Positive, N = 33 ¹	p-value ²
p53			0.3
abn	6, (22%)	3, (9.1%)	
wt	21, (78%)	30, (91%)	
MMR			>0.9
deficient	13, (48%)	16, (48%)	
proficient	14, (52%)	17, (52%)	
Molecular subgroup			0.4
MMRd	12, (44%)	16, (48%)	
NSMP (p53wt/MMRp)	9, (33%)	14, (42%)	
p53abn	6, (22%)	3, (9.1%)	
p-AKT			0.7
Negative	7, (26%)	7, (21%)	
Positive	20, (74%)	26, (79%)	

¹n, (%)²Fisher's exact test; Pearson's Chi-squared test**Table 5:** p-AKT hyperexpression distribution across molecular

Characteristic	p-AKT hyperexpression Negative, N = 14 ¹	p-AKT hyperexpression Positive, N = 46 ¹	p-value ²
p53			>0.9
abn	2, (14%)	7, (15%)	
wt	12, (86%)	39, (85%)	
MMR			0.091
deficient	4, (29%)	25, (54%)	
proficient	10, (71%)	21, (46%)	
Molecular subgroup			0.2
MMRd	4, (29%)	24, (52%)	
NSMP (p53wt/MMRp)	8, (57%)	15, (33%)	
p53abn	2, (14%)	7, (15%)	
β -catenin expression			0.7
Negative	7, (50%)	20, (43%)	
Positive	7, (50%)	26, (57%)	

¹n, (%)²Fisher's exact test; Pearson's Chi-squared test

Table 6: Descriptive statistics of patients with EC based on β -catenin expression

Characteristic	β -catenin expression	β -catenin expression	p-value ²
	Negative, N = 27 ¹	Positive, N = 33 ¹	
Age	66, (9)	63, (15)	0.083
BMI	28, (7)	30, (13)	0.8
Histology			<0.001
Endometrioid	16, (59%)	32, (97%)	
Non-endometrioid	11, (41%)	1, (3.0%)	
Grading			0.14
High Grade (G3)	15, (56%)	12, (36%)	
Low Grade (G1–G2)	12, (44%)	21, (64%)	
LVSI			0.7
Absent or focal	21, (78%)	27, (82%)	
Substantial	6, (22%)	6, (18%)	
Stage			0.8
I-II	19, (70%)	24, (73%)	
III-IV	8, (30%)	9, (27%)	
Adjuvant treatment			0.5
CT only	0, (0%)	2, (6.1%)	
CT+EBRT $\pm$ VBRT	8, (30%)	8, (24%)	
EBRT $\pm$ VBRT	5, (19%)	3, (9.1%)	
None or VB only	14, (52%)	20, (61%)	
FU	38, (7)	36, (8)	0.034

¹Median, (IQR); n, (%)²Wilcoxon rank sum test; Pearson's Chi-squared test; Fisher's exact test

Abbreviations: BMI, body mass index; chemotherapy; EBRT, external beam radiotherapy; LVSI, lymphovascular space invasion; VBRT, vaginal brachytherapy; CT, chemotherapy; FU, follow-up

Table 7: Descriptive statistics of patients with EC based on p-AKT hyperexpression

Characteristic	p-AKT hyperexpression Negative, N = 14 ¹	p-AKT hyperexpression Positive, N = 46 ¹	p-value ²
Age	66, (16)	63, (9)	0.7
BMI	31, (10)	29, (9)	0.064
Histology			>0.9
Endometrioid	11, (79%)	37, (80%)	
Non-endometrioid	3, (21%)	9, (20%)	
Grading			0.4
High Grade (G3)	5, (36%)	22, (48%)	
Low Grade (G1–G2)	9, (64%)	24, (52%)	
LVSI			0.3
Absent or focal	13, (93%)	35, (76%)	
Substantial	1, (7.1%)	11, (24%)	
Stage			0.7
I-II	11, (79%)	32, (70%)	
III-IV	3, (21%)	14, (30%)	
Adjuvant treatment			0.2
CT only	0, (0%)	2, (4.3%)	
CT+EBRT ± VBRT	2, (14%)	14, (30%)	
EBRT ± VBRT	4, (29%)	4, (8.7%)	
None or VB only	8, (57%)	26, (57%)	
FU	42, (9)	37, (7)	0.046

¹Median, (IQR); n, (%)²Wilcoxon rank sum test; Fisher's exact test; Pearson's Chi-squared test

Abbreviations: BMI, body mass index; chemotherapy; EBRT, external beam radiotherapy; LVSI, lymphovascular space invasion; VBRT, vaginal brachytherapy; CT, chemotherapy; FU, follow-up

Table 8: Prognostic role of p-AKT hyperexpression

Characteristic	p-AKT hyperexpression Negative, N = 14¹	P p-AKT hyperexpression Positive, N = 46¹	p-value²
Tumor size	30, (30)	43, (27)	0.3
Myometrial invasion			0.080
<50%	2, (14%)	19, (41%)	
≥50%	12, (86%)	24, (52%)	
None	0, (0%)	3, (6.5%)	
Lymph node metastasis	3, (21%)	9, (20%)	>0.9
Node metastasis distribution			0.7
aortic	0, (0%)	1, (11%)	
pelvic	2, (67%)	3, (33%)	
pelvic + aortic	1, (33%)	5, (56%)	
Missing	11	37	
Recurrence	1, (7.1%)	9, (20%)	0.4
Recurrence pattern			>0.9
hematogenous	0, (0%)	3, (33%)	
lymphatic	1, (100%)	3, (33%)	
peritoneal	0, (0%)	2, (22%)	
vaginal	0, (0%)	1, (11%)	
Missing	13	37	
DFS	40, (8)	36, (8)	0.013
Survival			>0.9
Alive	13, (93%)	41, (89%)	
Cancer-related death	1, (7.1%)	2, (4.3%)	
Death of other causes	0, (0%)	2, (4.3%)	
Lost at FUP	0, (0%)	1, (2.2%)	

¹Median, (IQR); n, (%)

Table 9: Prognostic role of β -catenin expression

Characteristic	β -catenin expression	β -catenin expression	p-value ²
	Negative, N = 27 ¹	Positive, N = 33 ¹	
Tumor size	35, (18)	40, (34)	0.2
Myometrial invasion			>0.9
<50%	9, (33%)	12, (36%)	
$\geq$ 50%	17, (63%)	19, (58%)	
None	1, (3.7%)	2, (6.1%)	
Lymph node metastasis	7, (26%)	5, (15%)	0.3
Node metastasis distribution			0.15
aortic	0, (0%)	1, (20%)	
pelvic	2, (29%)	3, (60%)	
pelvic + aortic	5, (71%)	1, (20%)	
Missing	20	28	
Recurrence	7, (26%)	3, (9.1%)	0.10
Recurrence pattern			>0.9
hematogenous	2, (29%)	1, (33%)	
lymphatic	3, (43%)	1, (33%)	
peritoneal	1, (14%)	1, (33%)	
vaginal	1, (14%)	0, (0%)	
Missing	20	30	
DFS	37, (10)	34, (7)	0.4
Survival			0.4
Alive	24, (89%)	30, (91%)	
Cancer-related death	1, (3.7%)	2, (6.1%)	
Death of other causes	2, (7.4%)	0, (0%)	
Lost at FUP	0, (0%)	1, (3.0%)	

¹Median, (IQR); n, (%)

Table 10: Descriptive statistics of patients with EC based on protein expression

Characteristic	β -catenin expression or p-AKT hyperexpression , N = 27 ¹	No proteins expression , N = 7 ¹	β -catenin expression and p-AKT hyperexpression , N = 26 ¹	p-value ²
Age	64, (7)	68, (13)	63, (14)	0.3
BMI	29, (13)	31, (4)	30, (10)	0.6
Histology				0.011
Endometrioid	19, (70%)	4, (57%)	25, (96%)	
Non-endometrioid	8, (30%)	3, (43%)	1, (3.8%)	
Grading				0.3
High Grade (G3)	10, (37%)	5, (71%)	12, (46%)	
Low Grade (G1–G2)	17, (63%)	2, (29%)	14, (54%)	
LVI				>0.9
Absent or focal	22, (81%)	6, (86%)	20, (77%)	
Substantial	5, (19%)	1, (14%)	6, (23%)	
Stage				0.3
I-II	22, (81%)	4, (57%)	17, (65%)	
III-IV	5, (19%)	3, (43%)	9, (35%)	
Adjuvant treatment				0.2
CT only	0, (0%)	0, (0%)	2, (7.7%)	
CT+EBRT $\pm$ VBRT	6, (22%)	2, (29%)	8, (31%)	
EBRT $\pm$ VBRT	3, (11%)	3, (43%)	2, (7.7%)	
None or VB only	18, (67%)	2, (29%)	14, (54%)	
FU	38, (6)	43, (10)	34, (6)	0.016

¹Median, (IQR); n, (%)²Kruskal-Wallis rank sum test; Fisher's exact test

Table 11: protein expression subgroups distribution across molecular

Characteristic	β -catenin expression or p-AKT hyperexpression , N = 27 ¹	No proteins expression , N = 7 ¹	β -catenin expression and p-AKT hyperexpression , N = 26 ¹	p-value ²
p53				0.5
abn	4, (15%)	2, (29%)	3, (12%)	
wt	23, (85%)	5, (71%)	23, (88%)	
MMR				0.5
deficient	11, (41%)	3, (43%)	15, (58%)	
proficient	16, (59%)	4, (57%)	11, (42%)	
Molecular subgroup				0.4
MMRd	10, (37%)	3, (43%)	15, (58%)	
NSMP (p53wt/MMRp)	13, (48%)	2, (29%)	8, (31%)	
p53abn	4, (15%)	2, (29%)	3, (12%)	

¹n, (%)²Fisher's exact test

Table 12: Prognostic role of dual protein expression β -catenin and p-AKT

Characteristic	β -catenin expression or p-AKT hyperexpressio n , N = 27 ¹	No proteins expression , N = 7 ¹	β -catenin expression and p-AKT hyperexpressio n , N = 26 ¹	p-value ²
Tumor size	37, (25)	30, (13)	43, (34)	0.3
Myometrial invasion				0.6
<50%	9, (33%)	1, (14%)	11, (42%)	
$\geq$ 50%	17, (63%)	6, (86%)	13, (50%)	
None	1, (3.7%)	0, (0%)	2, (7.7%)	
Lymph node metastasis	4, (15%)	3, (43%)	5, (19%)	0.3
Node metastasis distribution				0.14
aortic	0, (0%)	0, (0%)	1, (20%)	
pelvic	0, (0%)	2, (67%)	3, (60%)	
pelvic + aortic	4, (100%)	1, (33%)	1, (20%)	
Missing	23	4	21	
Recurrence	6, (22%)	1, (14%)	3, (12%)	0.7
Recurrence pattern				>0.9
hematogenous	2, (33%)	0, (0%)	1, (33%)	
lymphatic	2, (33%)	1, (100%)	1, (33%)	
peritoneal	1, (17%)	0, (0%)	1, (33%)	
vaginal	1, (17%)	0, (0%)	0, (0%)	
Missing	21	6	23	
DFS	37, (11)	39, (8)	34, (7)	0.082
survival				0.3
Alive	25, (93%)	6, (86%)	23, (88%)	
Cancer-related death	0, (0%)	1, (14%)	2, (7.7%)	
Death of other causes	2, (7.4%)	0, (0%)	0, (0%)	
Lost at FUP	0, (0%)	0, (0%)	1, (3.8%)	

¹Median, (IQR); n, (%)²Kruskal-Wallis rank sum test; Fisher's exact test

Table 13: Cox regression analysis of RFS

Characteristic	log(HR) ¹	95% CI ¹	p-value
Protein expression			
One-protein	—	—	
No-protein	-0.63	-2.7, 1.5	0.6
Both-protein	-0.49	-1.9, 0.91	0.5

¹HR = Hazard Ratio, CI = Confidence Interval

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