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Original contribution

# Molecular subtype diagnosis of endometrial carcinoma: comparison of the next-generation sequencing panel and Proactive Molecular Risk Classifier for Endometrial Cancer classifier☆,☆☆,☆☆☆

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**Summary** The Cancer Genome Atlas–based molecular classification of endometrial carcinoma (EC) has the potential to better identify those patients whose disease is likely to behave differently than predicted when using traditional risk stratification; however, the optimal approach to molecular subtype assignment in routine practice remains undetermined. The aim of this study was to compare the results of two different widely available approaches to diagnosis the EC molecular subtype. EC specimens from 60 patients were molecularly subclassified using two different methods, by using the FoundationOne CDx next-generation sequencing (NGS) panel and using the Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) classifier and performing immunostaining for mismatch repair proteins and p53. *POLE* mutation status was derived from FoundationOne results in both settings. Molecular classification based on ProMisE was successful for all 60 tumors. Microsatellite instability status could be determined based on the NGS panel results in 53 of 60 tumors, so ProMisE and NGS molecular subtype assignment could be directly compared for these 53 tumors. Molecular subtype diagnosis based on NGS and ProMisE was in agreement for 52 of 53 tumors. One tumor was microsatellite stable but showed loss of MLH1 and PMS2 expression. Molecular subtype diagnosis of EC based on the NGS panel of formalin-fixed paraffin-embedded ECs and based primarily on immunostaining (ProMisE)

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yields identical results in 98.1% (52/53, kappa = 0.97) of cases. Although results obtained using these two approaches are comparable, each has advantages and disadvantages that will influence the choice of the method to be used in clinical practice.


## 1. Introduction

Endometrial cancer (EC) is the seventh most common cancer in women worldwide [1], and in the Western world, it is the fourth most common malignancy and the seventh most common cause of death. The incidence of EC is on the rise [2].

The classification of endometrial carcinomas was revolutionized with the identification of four molecular subtypes of EC, based on genomic architecture, by The Cancer Genome Atlas (TCGA) Research Network in 2013 [3]. Based on integrated genomic characterization, EC was classified as ultramutated, hypermutated, and copy-number low and high subtypes. The four TCGA subtypes were shown to have significant prognostic differences; the ultramutated subtype had the most favorable progression-free survival despite presenting with high-risk features such as high-grade and/or lymphovascular invasion.

These molecular subtypes were subsequently verified, and surrogate markers were identified to allow diagnosis of these molecular subtypes in routine clinical practice [4–9]. In addition, the nomenclature of these subtypes has been established as follows: *POLE*-mutated (*POLE*mut), mismatch repair–deficient (MMRd), no specific molecular profile (NSMP), and p53 abnormal (p53abn). It has become increasingly evident that these molecular subtypes have a significantly different prognosis, they are likely to benefit from different treatment modalities, and they have different clinicopathological risk factors [3–6,10–18].

The molecular subtype cannot be reliably inferred from clinicopathological factors. The majority, but not all, serous ECs are p53abn and low-grade endometrioid carcinomas are NSMPs, respectively, whereas high-grade endometrioid carcinomas can represent any of the four molecular subtypes, adding to the importance of implementing molecular subclassification [19]. These high-grade carcinomas have been shown to be especially challenging to histotype as interobserver agreement is only moderately reproducible in high-grade ECs ($\kappa \sim 0.5$–$0.65$) and diagnostic consensus, even among expert pathologists, was observed in less than 2 of 3 cases [20–22], thus limiting the value of histotype diagnosis.

The rationale for this study was to molecularly characterize a preselected group of patients using the ProMisE classifier and using a commercially available next-generation sequencing (NGS) panel to compare these methodologies and assess their usefulness in a clinical setting. We studied a representative cohort consisting of all four molecular subtypes to enable the comparison of these methodologies.

## 2. Materials and methods

### 2.1. Patient selection and characteristics

Our study consists of a retrospective cohort of patients from Turku University Hospital treated for EC between 2008 and 2018. Only patients with material present in at least two blocks of paraffin-embedded tissue material were enrolled in this study. Of 64 cases identified, 4 were excluded owing to insufficient tissue for further analysis, leaving a final study cohort of 60 cases. Patients were previously characterized by clinicopathological parameters as a part of the clinical pipeline and treated as per European Society of Gynaecological Oncology (ESGO) guidelines. Clinically challenging cases, ie, patients whose disease had either a more aggressive or more benign course than anticipated based on tumor histotype, grade, and stage, were chosen for this study together with histomorphologically corresponding comparison cases, which had expected clinical outcomes. The study was approved by Auria Biobank's Scientific Steering Committee and the Institutional Review Board of Turku University Hospital (Auria Biobank permission number: 20151221).

### 2.2. Tissue microarrays

Generation of tissue microarrays (TMAs) was performed at Auria Biobank (Turku, Finland). In brief, formalin-fixed paraffin-embedded (FFPE) tumor samples were selected, and the corresponding hematoxylin and eosin (H&E)–stained histologic slides were scanned and reviewed to choose areas for production of TMAs representing the 60 EC specimens. To construct the TMAs, two 1.6-mm-diameter cores from each donor block, one from the invasion front and one from the tumor center, were taken and assembled in an array format in a recipient TMA block. Additional cores from the endometrial stroma, myometrium, and adjacent atrophic and hyperplastic endometrium were taken for control tissue.

### 2.3. Immunohistochemistry

Immunohistochemistry (IHC) was performed on FFPE tissue samples using 3.5 micros sections.

**Table 1** Details of pathology review.

| Pathology review | | | | | |
|---|---|---|---|---|---|
| **TMA consensus** | | | | | |
| **# p53** | **Reviewer 1** | **Reviewer 2** | **Consensus** | **Molecular group** | |
| 65 | WT | Overexpression | WT | *POLE*mut | |
| 84 | Overexpression? | Overexpression | Overexpression | p53abn | |
| 90 | WT | Null pattern | Repeat IHC | NSMP | |
| **# MMR** | **Reviewer 1** | **Reviewer 2** | **Consensus** | **MS status** | **Molecular group** |
| 33 PMS2 | Retained | Loss | Loss | MSI-I | MMRd |
| 46 MLH1, PMS2 | Retained | Weak | Repeat IHC | MSS | p53abn |
| 62 MLH1, PMS2 | Retained | Weak | Repeat IHC | MSI-I | MMRd |
| **Repeated on whole slides** | | | | | |
| **# p53** | **TMA** | **Whole slide** | **Consensus** | **Molecular group** | **p53 mutation on NGS** |
| 15 | WT | Mostly null, subclonal | abnormal | p53abn | yes |
| 30 | WT | Subclonal overexpression | abnormal | p53abn | yes |
| 62 | WT | Subclonal loss | abnormal | MMRd | yes |
| 82 | WT | Overexpression | overexpression | p53abn | yes |
| 90 | NULL | WT | WT | NSMP | no |
| **# MMR** | **TMA** | **Whole slide** | **Consensus** | **Molecular group** | **MSI status** |
| 11 MLH1, PMS2 | Lost | Lost | Loss | NSMP/MMRd | MSS |
| 46 MLH1, PMS2 | Repeat | Retained | Retained | p53abn | MSS |
| 62 MLH1, PMS2 | Repeat | Subclonal loss | Abnormal | MMRd | MSI-I |
| 87 MLH1, PMS2 | Retained | Abnormal, PMS2: very weak, MLH1: patchy | Abnormal | MMRd | MSI-I |

NOTE. Specifics of cases wherein immunohistochemistry on TMA cores was reviewed and those cases wherein IHC was repeated on whole-tissue slides. Abbreviations: MSS, microsatellite stable; TMA, tissue microarray; *POLE*mut, *POLE*-mutated; MMRd, mismatch repair−deficient; NSMP, no specific molecular profile; p53abn, p53 abnormal; MSI-I, microsatellite instability intermediate; MSI-H, microsatellite instability high; MMR, mismatch repair; mut, mutation; MS, microsatellite; IHC, immunohistochemistry; EEC, endometrioid endometrial carcinoma; G, grade; SEC, serous endometrial carcinoma; CCEC, clear cell endometrial carcinoma; WT, wild-type; null, complete loss of expression; NGS, next-generation sequencing.

Immunostaining were performed at the Department of Pathology of Turku University Hospital as part of the clinical pipeline using a BenchMark ULTRA ICH/ISH Staining Module (Roche Ventana, Tucson, Arizona, USA). The following antibodies and dilutions were used: p53 (clone Bp53-11, ready-to-use [RTU]; Roche), ER (clone SP1, RTU; Roche), PR (clone 1E2, RTU; Roche), MLH1 (clone M1, RTU; Roche), MSH2 (clone G219-1129, RTU; Roche), PMS2 (clone EPR3947, RTU; Roche Ventana, Tucson, Arizona, USA), and MSH6 (clone BSR100, 1:300; Optibodies, Nordic Biosite, Täby, Sweden).

After staining, immunostaining results were assessed by two authors (J.H. and P.V., who were blinded to clinical and NGS data) independently. Immunostaining for p53 was considered aberrant if completely negative or strong positive staining was observed in >75% of tumor cells (nuclear or cytoplasmic) and was considered wild-type (WT) if otherwise. Staining for MLH1, MSH2, MSH6, and PMS was interpreted based on guidelines for interpretation of mismatch repair (MMR) protein immunostaining in endometrial carcinoma [23], with loss of or markedly diminished expression relative to control tissues considered abnormal. For both p53 and MMR protein, subclonal abnormal expression was defined as an abnormal staining pattern present in a contiguous area of the tumor [24,25]. Nonmalignant areas and stromal tissue in tumor specimens served as controls. Cases with disagreement in immunohistochemical interpretation (n = 6) between the initial reviewers were reviewed together. A consensus was reached in all six cases (Table 1), in two cases after repeating immunohistochemical analysis on whole slides. In cases wherein there were discrepancies between the molecular classes assessed using the NGS panel and IHC, IHC was repeated on whole-section slides using the same staining protocol. p53 staining was repeated in 5 cases, staining in 4 cases was repeated owing to the presence of mutations on the NGS panel but a WT pattern on TMA IHC, and staining in 1 case was repeated owing to the null pattern on TMA IHC but no mutation identified with NGS. MLH1 and PMS2 staining was repeated in 2 cases, of which one was performed owing to microsatellite instability intermediate (MSI-I) status on molecular analysis but retained staining on TMA and one was performed owing to microsatellite stable (MSS) status based on molecular

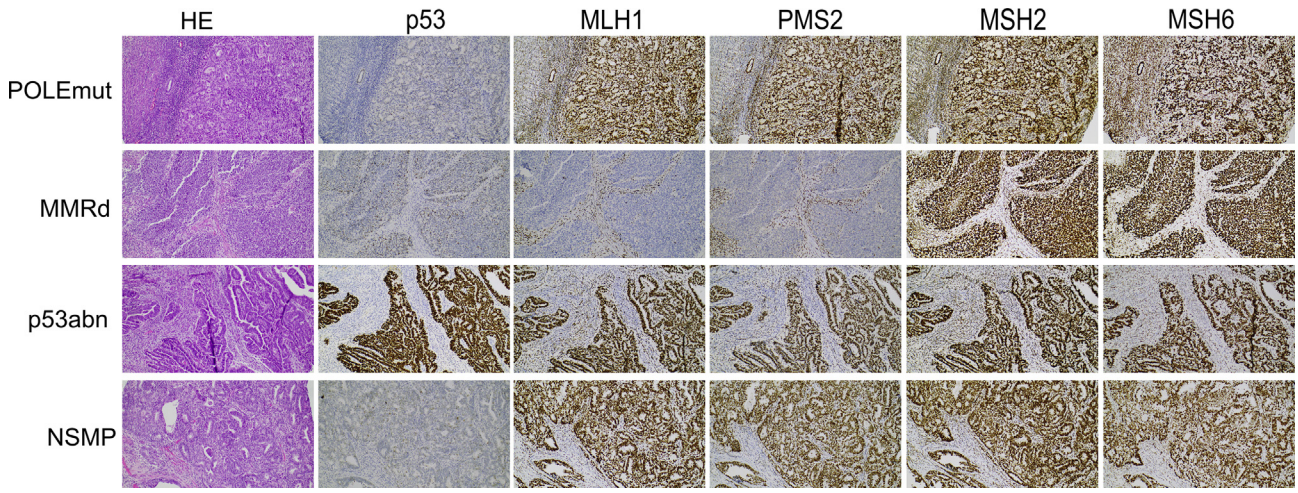


**Fig. 1** Typical immunostaining for p53, MLH1, PMS2, MSH2, and MSH6 within the different molecular subtypes (×10 original magnification). NSMP, no specific molecular profile; MMRd, mismatch repair−deficient; *POLE*mut, *POLE*-mutated.

analysis but loss of MLH1 and PMS2 expression on IHC (Table 1, Fig. 1).

## 2.4. NGS and MSI testing

All the study patients' samples were subjected to NGS using the FoundationOne CDx™, provided by Foundation Medicine (Cambridge, MA, USA). Ten unstained slides with 4-micron sections and one H&E-stained section were sent for analysis. When tumor cell percentage was <20%, the sections were trimmed to meet the test criteria. In seven of the 60 samples, MSI status could not be determined. This was likely due to insufficient tissue material as in six of these seven cases, the paraffin block had to be trimmed to achieve high enough tumor percentage. Samples were subjected to NGS during adaptor ligation and hybrid-capture NGS (FoundationOne CDx™, Penzberg, Germany), covering all exomes from 324 cancer-related genes and intron sequences of 28 genes frequently rearranged in cancer. The gene alterations discovered were considered biologically significant if they were also identified in the Catalog of Somatic Mutations in Cancer (Cosmic v89) and Seshat mutation database for TP53 mutation annotation. Variants of unknown significance were excluded. To determine the MSI status, 114 intronic homopolymer repeat loci with adequate coverage were analyzed for length variability and compiled into an overall MSI score via principal component analysis. Principal component analysis (PCA) ranges of the MSI score were manually assigned to MSI-high (MSI-H, >12), MSI-I (7.5−-12), or MSS (<7.5 [26].

## 2.5. Molecular classification

The patients were classified into one of the four molecular subtypes based on the ProMisE algorithm [9]. First cases with pathogenic *POLE* exonuclease domain mutations, as defined by León-Castillo et al. [27] and identified by NGS, were classified as *POLE*mut. Among the remaining cases, cases with loss of MMR protein expression on IHC were classified as MMRd. The rest were classified as NSMP if p53 IHC was WT and p53abn if p53 IHC showed an abnormal staining pattern.

Cases were similarly classified based on the NGS panel results; tumors with pathogenic *POLE* mutations, as defined by León-Castillo et al. [27], identified by NGS were classified as *POLE*mut. For the remaining cases, those with MSI-H status based on NGS or MSI-I status were classified as MSI-H. Those remaining cases with no deleterious *TP53* mutations were considered NSMP, or if *TP53* mutation was present, they were considered p53abn. The results of molecular subtype assignment by these two methods were compared.

## 2.6. Statistical analyses

Categorical variables were characterized using frequency and percentages, and kappa values were calculated. Continuous variables were characterized using the median and range. Statistical analyses were performed using IBM SPSS, version 26.0 (IBM Corp., Armonk, NY, USA).

# 3. Results

For the 60 patients, the age ranged from 43 to 85 years (median = 67), and forty-four (73%) of the tumors were of endometrioid histotype and 16 (27%) were of non-endometrioid histotype, 10 being serous and 6 being clear cell carcinomas. FIGO stages were as follows: FIGO stage 1A, n = 35 (58.3%); stage 1B, n = 13 (21.8%); stage 2, n = 2 (3.3%); and stage 3, n = 10 (16.7%). All patients underwent surgical treatment in the form of transabdominal hysterectomy (n = 8, 13.3%), laparoscopy (n = 13,

**Table 2** The correlation of molecular classification based on the NGS panel (Foundation Medicine) and ProMisE classifier.

| | ProMisE classified | Foundation Medicine | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | | MMRd | | | | |
| | | *POLE*mut | MSI-I | MSI-H | NSMP | p53abn | |
| ProMisE classifier | *POLE*mut | 7 | 0 | 0 | 0 | 0 | 7 |
| | MMRd | 0 | 8 | 12 | 1[a] | 0 | 21 |
| | NSMP | 0 | 0 | 0 | 9 | 0 | 0 |
| | p53abn | 0 | 0 | 0 | 0 | 16 | 17 |
| Total | | 7 | 8 | 12 | 10 | 16 | 53 |

Abbreviations: ProMisE, Proactive Molecular Risk Classifier for Endometrial Cancer; *POLE*mut, *POLE*-mutated; MMRd, mismatch repair–deficient; NSMP, no specific molecular profile; p53abn, p53 abnormal; MSI-I, microsatellite instability intermediate; MSI-H, microsatellite instability high; IHC, immunohistochemistry; NGS, next-generation sequencing; MSS, microsatellite stable.

[a] MSS based on NGS; MLH1 and PMS2 loss based on IHC.

22.7%), or robot-assisted laparoscopy (n = 39, 65.0%). Lymphadenectomy was performed as part of surgical staging in 58% (n = 35) of the patients. Postoperative chemotherapy was the only adjuvant therapy in 15.0% (n = 9) cases, followed by external radiotherapy in 3.3% (n = 2) and vaginal brachytherapy in 13.0% (n = 8); 21.6% received both chemotherapy and radiotherapy (n = 13). The median follow-up time was 2.9 years. Of all patients, 31.7% (n = 19) had recurrence during the follow-up, and the median time until recurrence from diagnosis was 1.5 years.

In 60 cases, the molecular classification by ProMisE was successful. In 53 cases, MSI status could be determined based on the NGS panel. The correlation between the molecular classification using the Foundation Medicine NGS panel and using ProMisE (IHC together with *POLE* mutation status from the NGS panel) for these 53 tumors is presented in Table 2. The methods show excellent agreement: in 98.1% of cases, tumors were assigned to the same molecular group by both platforms (kappa value = 0.973).

In the 53 tumors for which both ProMisE and NGS classification was available, a pathogenic *POLE* mutation was identified in 7 (13%) cases (Table 3), and these cases were therefore classified as *POLE*mut.

MMR protein IHC showed loss of MMR protein expression in 21 of 46 cases that were not *POLE*mut (Table 3). Based on the NGS MSI assessment, 12 of 46 cases were classified as MSI-H, and all showed loss of protein expression on IHC. Eight of 46 tumors were classified as MSI-I, and all of these tumors also showed abnormal MMR protein immunostaining results. Seven of 21 tumors with abnormal MMR protein staining showed a mutation in one of the MMR genes as per NGS; in the remaining 14 tumors, there was loss of MLH1 and PMS2 expression, a pattern commonly seen in association with MLH1 promoter methylation, ie, epigenetic silencing. In summary, there were 20 MMRd cases identified by both ProMisE and NGS, whereas a single tumor was MSS based on NGS but showed loss of MLH1 and PMS2 expression as per IHC (Table 3 and Fig. 2). This case was reviewed and showed both normal endometrium in addition to cancerous tissue and high numbers of tumor-infiltrating lymphocytes.

A pathogenic *TP53* mutation was identified by NGS in 16 of 26 cases that were not *POLE*mut or MMRd based on NGS. IHC showed abnormal staining in the same 16 of 25 tumors that were not *POLE*mut or MMRd based on ProMisE. In summary, there were 16 p53abn EC cases based on NGS and ProMisE. There were 10 NSMP EC cases based on NGS and 9 NSMP EC cases based on ProMisE.

Of the 7 cases with no NGS-based MSI status available, two had a pathogenic *POLE* mutation. None of the remaining 5 cases showed loss of MMR proteins on IHC; one showed a *TP53* mutation with NGS and abnormal p53 immunostaining on IHC and was considered p53abn. The remaining four were considered NSMP.

For further analysis, a consensus molecular subtype was determined for all 60 tumors. The case that was MSS based on NGS but showed loss of MLH1 and PMS2 based on IHC was deemed MMRd. While we acknowledge uncertainty about the correct molecular subtype assignment for this tumor, the morphology (including increased levels of tumor-infiltrating lymphocytes) does support this being an MMRd EC. The distribution of the original diagnosis and molecular subtype together with the cases that relapsed during follow-up is presented in Table 4. Although the groups are small, some observations can be made. All of the *POLE*mut EC cases were associated with favorable outcomes, as expected. In addition, of all the non-endometrioid tumors, relapses occurred only in the p53abn subtype. Thirteen of 30 low-grade endometrioid tumors had a recurrence, and these were more frequent in MMRd and p53abn subtypes (Table 4).

The NGS approach identified 13 cases with more than one molecular feature: all 9 *POLE*mut tumors had *TP53* or MMR gene mutations (although all cases wherein MSI status was available were MSS), and 4 MMRd tumors had a *TP53* mutation. The ProMisE approach identified four multiple classifiers in the *POLE*mut group and one in the MMRd group (Table 3). In addition to the pathogenic *POLE* mutations, in 11 tumors, there were one to six

**Table 3** Characteristics of *POLE*-mutated and mismatch repair–deficient/microsatellite unstable cases.

| ID | Diagnosis | ProMisE | NGS panel | MS status | p53 IHC | TP53 mutation | MMR IHC abnormal | MMR mut known | MMR mut likely | *POLE* mut | npgm POLE (n) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 85 | EEC G3 | *POLE*mut | *POLE*mut | unknown | WT | | R | *MSH2, MLH1* | | P286R C > G | 0 |
| 39 | SEC | *POLE*mut | *POLE*mut | unknown | OE | 3 mutations | MLH1, PMS2 | | | V411L C > T | 1 |
| 25 | EEC G3 | *POLE*mut | *POLE*mut | MSS | WT | R213* | R | *MSH6* | | P286R C > G | 0 |
| 32 | EEC G3 | *POLE*mut | *POLE*mut | MSS | WT | 2 mutations | R | | | P286R C > G | 1 |
| 45 | CCEC | *POLE*mut | *POLE*mut | MSS | WT | C141R | R | *MSH2* | *MLH1* | P286R C > G | 1 |
| 74 | EEC G1-2 | *POLE*mut | *POLE*mut | MSS | WT | 2 mutations | R | | | M444K T > A | 2 |
| 65 | EEC G3 | *POLE*mut | *POLE*mut | MSS | WT | | MSH6 | *MSH6* | *MSH6* | S459F C > T | 6 |
| 50 | CCEC | *POLE*mut | *POLE*mut | MSS | NULL | R213* | MSH6 | | | P286R C > G | 0 |
| 58 | EEC G1–2 | *POLE*mut | *POLE*mut | MSS | OE | 4 mutations | R | *MSH6* | *MSH2* | A456P G > C | 2 |
| 11 | EEC G1–2 | MMRd | NSMP | MSS | WT | | MLH1, PMS2 | | | | |
| 62 | EEC G1–2 | MMRd | MMRd | MSI-I | WT (sc) | T102fs*47 (l) | MLH1, PMS2* | | | | |
| 87 | EEC G1–2 | MMRd | MMRd | MSI-I | WT | | MLH1, PMS2* | | | | |
| 5 | EEC G1–2 | MMRd | MMRd | MSI-I | WT | | MLH1, PMS2 | | | | 1 |
| 19 | EEC G1–2 | MMRd | MMRd | MSI-I | WT | | MLH1, PMS2 | | | | |
| 33 | EEC G3 | MMRd | MMRd | MSI-I | WT | | MLH1, PMS2 | | | | |
| 81 | EEC G1–2 | MMRd | MMRd | MSI-I | WT | | MLH1, PMS2 | | *MSH6* | | |
| 37 | SEC | MMRd | MMRd | MSI-I | OE | 3 mutations | MSH6 | *MSH6* | *MSH6* | | |
| 13 | EEC G1–2 | MMRd | MMRd | MSI-I | | | MLH1, PMS2 | | | | |
| 89 | EEC G3 | MMRd | MMRd | MSI-H | WT | R283C (wt) | MLH1, PMS2 | | *PMS2* | | |
| 92 | EEC G1–2 | MMRd | MMRd | MSI-H | WT | R273C | MLH1, PMS2 | | | | |
| 4 | EEC G1–2 | MMRd | MMRd | MSI-H | WT | T170M (wt) | MSH2, MSH6 | | *MSH2* | | |
| 21 | EEC G1–2 | MMRd | MMRd | MSI-H | WT | | MLH1, PMS2 | | | | |
| 22 | EEC G1–2 | MMRd | MMRd | MSI-H | WT | | MLH1, PMS2 | | | | 2 |
| 68 | CCEC | MMRd | MMRd | MSI-H | WT | | MLH1, PMS2 | | | | |
| 71 | EEC G3 | MMRd | MMRd | MSI-H | WT | | MLH1, PMS2 | | | | |
| 80 | EEC G1–2 | MMRd | MMRd | MSI-H | WT | | MLH1, PMS2 | | | | 2 |
| 88 | EEC G1–2 | MMRd | MMRd | MSI-H | WT | | MLH1, PMS2 | | | | |
| 3 | EEC G1–2 | MMRd | MMRd | MSI-H | WT | | MSH2, MSH6 | | *MSH2* | | |

(*continued on next page*)

**Table 3** (*continued*)

| ID | Diagnosis | ProMisE | NGS panel | MS status | p53 IHC | TP53 mutation | MMR IHC abnormal | MMR mut known | MMR mut likely | *POLE* mut | npgm POLE (n) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 42 | SEC | MMRd | MMRd | MSI-H | WT | | MSH2, MSH6 | | *MSH2* | | |
| 66 | EEC G3 | MMRd | MMRd | MSI-H | WT | | MSH2, MSH6 | *MSH2* | *MSH2* | | |
| 84 | CCEC | p53abn | p53abn | MSS | OE | R273H | | | | | 1 |
| 61 | EEC G1−2 | NSMP | NSMP | MSS | WT | | | | | | 1 |

Abbreviations: MSS, microsatellite stable; NGS, next-generation sequencing; *POLE*mut, *POLE*-mutated; MMRd, mismatch repair−deficient; NSMP, no specific molecular profile; p53abn, p53 abnormal; MSI-I, microsatellite instability intermediate; MSI-H, microsatellite instability high; MMR, mismatch repair; mut, mutation; MS, microsatellite; IHC, immunohistochemistry; npgm, nonpathogenic mutation; R, retained; EEC, endometrioid endometrial carcinoma; G, grade; SEC, serous endometrial carcinoma; CCEC, clear cell endometrial carcinoma; WT, wild-type; OE, overexpression; NULL, complete loss of expression; sc, subclonal; ProMisE, Proactive Molecular Risk Classifier for Endometrial Cancer.
*Fig. 2.

nonpathogenic *POLE* mutations identified; 6 of these 11 tumors also harbored a pathogenic *POLE* mutation (Table 3). Of the remaining 5 cases, in which there was a nonpathogenic *POLE* mutation, three were MMRd, and the two remaining tumors were p53abn and NSMP, respectively (Table 3).

The immunohistochemical analysis was primarily carried out on TMAs but repeated on whole tissues when needed to interpret immunohistochemical results or to assess discrepancies between ProMisE and NGS classification results (Table 1).

## 4. Discussion

The diagnosis and classification of endometrial carcinomas are evolving. The rapidly accumulating evidence of the value of the integrative molecular diagnosis of EC has led to the need to integrate molecular subtype diagnosis into clinical practice. Molecular subtype is included in the new 5th edition of the World Health Organization classification of tumors of the female genital tract, and ancillary studies for POLE mutations, MMR/MSI, and aberrant p53 expression are encouraged to complement morphologic assessment of the histologic tumor type in the National Comprehensive Cancer Network guidelines [28]. It has recently become evident that the molecular subtypes also have predictive importance. Based on preliminary data, which require independent confirmation, patients with *POLE*mut EC could be spared the adverse effects of adjuvant therapy, whereas patients with MMRd EC benefit from radiotherapy but not from chemotherapy, while patients with p53abn EC stand to benefit from chemotherapy [10,18]. Our aim was to access molecular subtype diagnosis using the ProMisE classifier and an NGS-based approach, allowing comparison between these methods. In both settings, the *POLE* mutation status was derived from NGS.

The two methods show excellent agreement, with 98.1% (52/53) of cases assigned to the same molecular group. The only discrepant result was a tumor that was MSS based on NGS but showed loss of MLH1 and PMS2 expression as per IHC. This phenomenon of MSS with loss of MLH1 and PMS2 expression has previously been described in association with MLH1 promoter hypermethylation [29]. Another possible explanation for the different results by IHC and NGS is that the normal nuclei present (normal endometrium and high numbers of tumor-infiltrating lymphocytes) were too many to allow the microsatellite unstable mutant alleles to be detected by NGS.

The advantages of the IHC-based approach to molecular subtype diagnosis are that the methodology is readily available, is inexpensive, has rapid turnaround time, and can be performed even on small biopsy specimens. The interpretation of p53 and MMR protein IHC results is mostly straightforward and is carried out as per the established guidelines [23−25], but some cases proved to be difficult to interpret owing to subclonal or weak patchy staining patterns. The latter problem is particularly noticeable in hysterectomy specimens wherein there may be delayed fixation and can be avoided by staining biopsy specimens. No IHC-based surrogate marker has been identified for POLE mutation, so *POLE* sequencing will still be needed as a part of the ProMisE classifier, which is one of the main limitations of this approach and an obstacle in the integration of ProMisE into clinical diagnostics. Targeted sequencing just for POLE exonuclease domain hot spot mutations of established pathogenicity (n = 11 at the time of writing), however, can be carried out more quickly and at less expense, using less input DNA, than using a large hybrid-capture NGS panel. Without *POLE* testing, of the nine *POLE*mut cases in our study, one would have been misclassified as MMRd, two would have been misclassified as p53abn, and the remaining six would have been misclassified as NSMP. Although *POLE*mut EC cases have a very favorable prognosis, it is not established whether this is because they are inherently less aggressive or because they respond exceptionally well to adjuvant therapy. Until

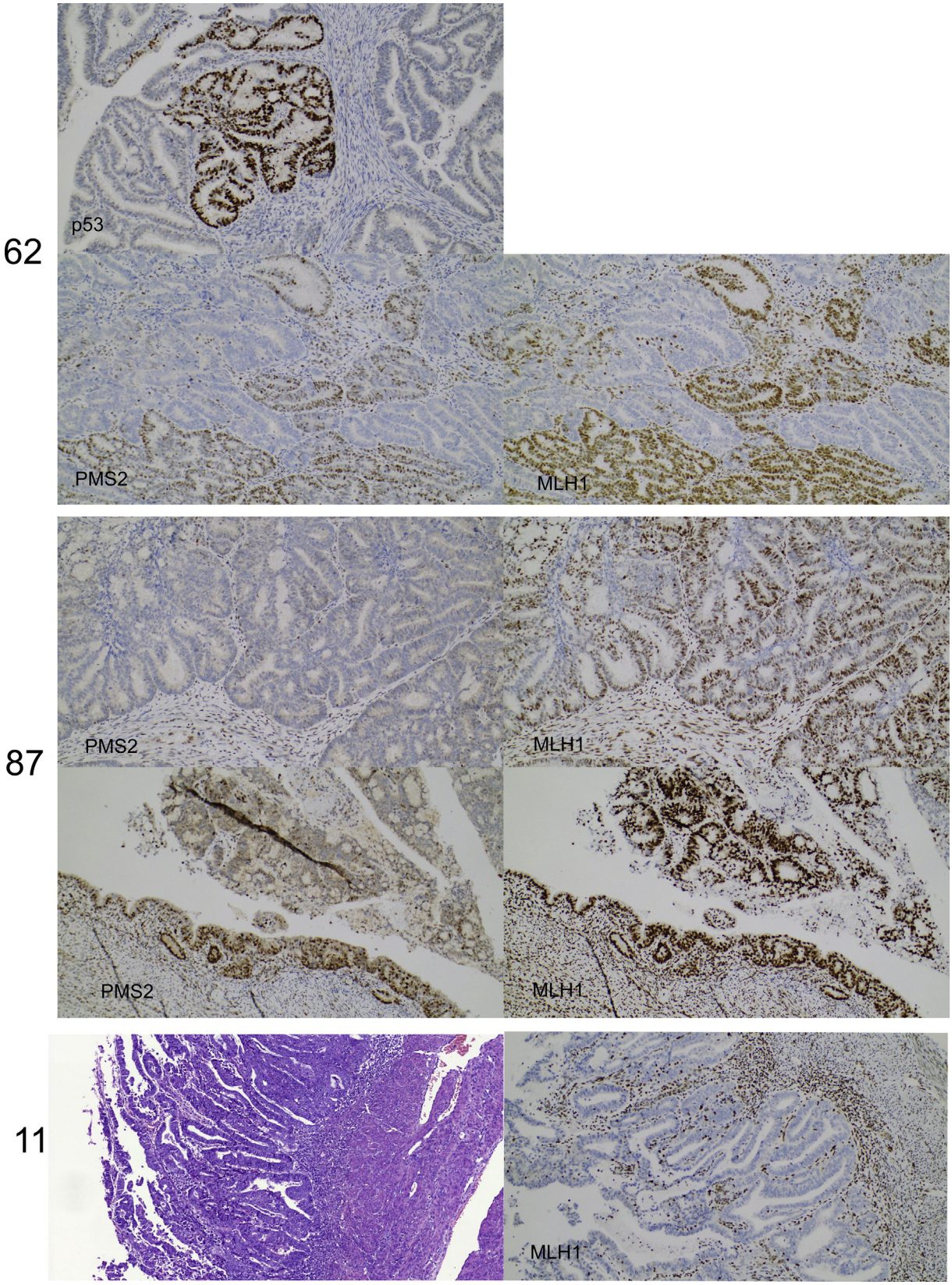


**Fig. 2** Photomicrographs of cases that had discrepancies between the molecular classes. Case 62: p53, MLH1, and PMS2 immunostaining from case 62 illustrates the subclonal staining pattern in one MMRd, MSI-I case. Case 87: Two different foci from PMS2 and MLH1 immunostaining show weak and patchy staining in an MSI-I case. Case 11: Images illustrate the H&E and MLH1 staining from the single case with NGS and ProMisE classification produced discrepant results, ie, MSS and MLH1 and PMS2 loss, respectively. NGS, next-generation sequencing; H&E, hematoxylin and eosin; ProMisE, Proactive Molecular Risk Classifier for Endometrial Cancer; MMRd, mismatch repair−deficient; MSI-I, microsatellite instability intermediate; MSS, microsatellite stable.

**Table 4** Comparison of original diagnosis and the molecular subtype.

| Molecular class | Original histological diagnosis | | | | Total | % |
|---|---|---|---|---|---|---|
| | Grade 1–2 EEC | Grade 3 EEC | Serous EC | Clear cell EC | | |
| POLEmut | 2 | 4 | 1 | 2 | 9 | 15% |
| Relapsed | – | – | – | – | 0 | 0% |
| MMRd | 14 | 4 | 2 | 1 | 21 | 35% |
| Relapsed | 7 | 1 | – | – | 8 | 38% |
| NSMP | 12 | 1 | – | – | 13 | 22% |
| Relapsed | 5 | – | – | – | 5 | 38% |
| p53abn | 2 | 5 | 7 | 3 | 17 | 28% |
| Relapsed | 1 | 1 | 3 | 1 | 6 | 35% |
| Total | 30 | 14 | 10 | 6 | 60 | |
| Relapsed | 13 | 2 | 3 | 1 | 19 | |

Abbreviations: EEC, endometrioid endometrial carcinoma; EC, endometrial carcinoma; *POLE*mut, *POLE*-mutated; MMRd, mismatch repair–deficient; NSMP, no specific molecular profile; p53abn, p53 abnormal.

this is resolved through clinical trials assessing decreased treatment for *POLE*mut EC, the *POLE* mutation status arguably will not have an impact on management, and it is not necessary to sequence *POLE* in every EC case at this time as the p53 and MMR protein IHC can provide sufficient information to guide treatment decisions in most patients.

The advantage of an NGS panel–based approach to molecular subtype diagnosis is that it generates all data needed for molecular subtyping and additional information beyond the molecular subtype that may be helpful in guiding treatment, eg, beta-catenin mutation status, tumor mutation burden, targetable gene alterations, and potential detection of pathogenic germ line mutation and so on. However, as a disadvantage, it needs more sample material for input, which may be problematic with small biopsy specimens and is more expensive (~3500 USD, compared with ~300 USD for ProMisE [30,31]. However, NGS panels generate also irrelevant and even misleading data if not interpreted carefully, eg, nonpathogenic *POLE* mutations and functionally insignificant *TP53* passenger mutations. In our series, we found 20 nonpathogenic *POLE* mutations; these are not associated with favorable outcomes, unlike the pathogenic *POLE* mutations. There were also mutations in MMR genes identified in the *POLE*mut tumors; none were MSI-I or MSI-H, and they are presumably also passenger rather than pathogenic mutations in these ultramutated tumors.

Microsatellite instability or MMR protein expression testing is important for two reasons: to identify MMRd cases that potentially could benefit from immune checkpoint inhibitor treatment and/or radiation therapy and also to identify patients who have a genetic susceptibility to develop EC, ie, Lynch syndrome, and who should therefore be referred for genetic counseling and enter a surveillance program that has been demonstrated to reduce mortality. In our work, we show that MSI-H status correlated in all cases with loss of one or two MMR proteins. MSI-I was associated with loss of staining, very weak staining, or subclonal loss in all cases, as described previously. According to Trabucco et al. [32], MSI-I, identified using an NGS panel, has not been established as a biomarker for response to immune checkpoint inhibitors, and the use of this status in identifying MMRd is not clinically established. In our study, the correlation between MSI/MMRd as determined by NGS and by IHC was excellent. Classification of MSI-I should be verified with IHC. Currently, neither of these approaches (ProMisE or NGS) is adequate to diagnose Lynch syndrome, and abnormal results (MSI-H, MMRd, mutation in an MMR gene) could be a somatic mutation, epigenetic event, or germ line mutation. An NGS-based approach can identify MSI cases and cases with MMR gene mutations; MLH1 methylation analysis can be directed at those cases that are MSI but do not have MMR gene mutations. An immunohistochemical approach identifies which MMR protein(s) are absent, with methylation analysis needed to identify those tumors with loss of MLH1 expression attributable to hypermethylation. Thereafter, additional testing will be needed for diagnosis of Lynch syndrome, using either approach.

Both approaches identified the so-called multiple classifier ECs, which by definition have more than one of the three molecular classifying features [9]. These include *POLE*mut tumors with MSI or loss of MMR protein expression and/or aberrant pattern of p53 expression/*TP53* mutations and MMRd EC that also show aberrant p53 expression or *TP53* mutations, and they behave similar to *POLE*mut and MMRd EC, respectively. In our work, NGS identified 13 multiple classifiers: all nine *POLE*mut tumors had *TP53* or MMR gene mutations (all were MSS), and four MMRd tumors had a *TP53* mutation. In contrast, IHC identified only four cases: three *POLE*mut tumors had

aberrant p53 expression or MMR protein immunostaining results, and one MMRd tumor had aberrant p53 immunostaining results. This finding highlights the extraordinary mutational burden in the ultramutated *POLE*mut and hypermutated MMRd groups and the tendency for these tumors to accumulate passenger mutations (that do not have an impact on prognosis) [9]. A similar phenomenon is likely to be the underlining cause of the discrepancy between MMR protein mutations and MMR protein loss. In two cases, the loss of MLH1 and PMS2 staining was paradoxically associated with MSH6 or PMS2 mutation. The most likely explanation is that the primary event is MLH1 promoter hypermethylation, with the identified MMR gene mutation being merely passenger mutations secondary to the hypermutable state [9].

There are some limitations to this study. First, the study consisted of a limited sample size and a selection bias toward cases that had an unexpected clinical behavior, and hence, this study does not necessarily reflect the distributions of molecular classes in EC in general. IHC was performed primarily on TMAs, but repeated in cases wherein the immunostaining result of TMA cores was ambiguous, bringing the findings closer to a clinical practice wherein IHC would always be performed on whole-tissue slides. The NSG-based MSI analysis failed in seven of 60 cases, which was possibly owing to insufficient tissue material. As reflex testing of EC for MMR/MSI is becoming a widespread routine, and p53 and *POLE* testing are becoming increasingly common, it is important to ensure that tissue material optimal for molecular testing is collected.

In this study, 15% of cases were *POLE*mut, which is more than what would be expected in a population-based case series and most probably reflects the selection criteria, eg, including patients with favorable outcomes despite high-risk histopathological features in their tumor. Previous work has shown that the *POLE*mut group is histologically heterogeneous and may present with serous-like or clear cell−like features [21,33−37], but the high-grade features are associated with an excellent prognosis [18,38,39]. This appears to be true also in our current work; none of the *POLE*mut tumors, even those with serous or clear cell features, recurred. Should sufficient evidence warrant treatment of *POLE*mut EC differently, eg, deescalation of therapy emerge, would this highlight the potential impact of *POLE* testing, as they would be overtreated based on conventional risk assessment tools.

Both approaches to molecular subtype diagnosis show subclonal/low allelic frequency changes, and more work is needed to understand their clinical significance. A subclonal staining pattern was identified in a subset of p53 and MLH1/PMS2 staining. The latter is most likely to be caused by promotor hypermethylation [29,40] and was associated with MSI-I status. Subclonal p53 mutations have previously been described in 46.7% and 60% of multiple classifier tumors of *POLE*mut and MMRd subtypes, respectively [9]. In the p53abn subtype, the same *TP53* mutation has been recognized both in the WT appearing and aberrant staining foci [25].

Overall, NGS and IHC provide equivalent information for molecular subtype diagnosis. Both methods have unique advantages and challenges in their application that are manageable in clinical practice.

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