Gynecologic Oncology 175 (2023) 45–52

Contents lists available at ScienceDirect

Gynecologic Oncology

journal homepage: www.elsevier.com/locate/ygyno

Harmonized molecular classification; assessment of a single-test ProMisE NGS tool

Amy Jamieson [a,1], Melissa K. McConechy [b,1], Amy Lum [c], Samuel Leung [c], Emily F. Thompson [c], Janine Senz [c], Aline Talhouk [c], David G. Huntsman [b,c], Ali Bashashati [d,e], C. Blake Gilks [e], Jessica N. McAlpine [a,*]

[a] Department of Gynecology and Obstetrics, Division of Gynecologic Oncology, University of British Columbia, Vancouver, Canada
[b] Imagia Canexia Health, Inc., Vancouver, Canada
[c] Department of Molecular Oncology, University of British Columbia, Vancouver, Canada
[d] School of Biomedical Engineering, University of British Columbia, Vancouver, BC, Canada
[e] Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, Canada

HIGHLIGHTS

- ProMisE is a multi-test platform; next generation sequencing (NGS) and immunohistochemistry for EC molecular classification.
- ProMisE NGS uses microsatellite instability assay and NGS for *TP53* and *POLE* mutations for a single-test DNA-based alternative.
- ProMisE NGS is easily achieved and highly concordant with the original ProMisE classifier.
- The prognostic value of this single-test ProMisE NGS EC classifier was maintained for the 4 molecular subtypes.
- Reliable molecular subtyping can be obtained from first diagnostic biopsy using a single-test NGS targeted assay.

ARTICLE INFO

Article history:
Received 21 April 2023
Received in revised form 30 May 2023
Accepted 31 May 2023
Available online xxxx

Keywords:
Endometrial cancer
Molecular classification
ProMisE
Next generation sequencing
Immunohistochemistry

ABSTRACT

Objectives. Despite recommendations for integrating molecular classification of endometrial cancers (EC) into pathology reporting and clinical management, uptake is inconsistent. To assign ProMisE subtype, all molecular components must be available (*POLE* mutation status, mismatch repair (MMR) and p53 immunohistochemistry (IHC)) and often these are assessed at different stages of care and/or at different centres resulting in delays in treatment. We assessed a single-test DNA-based targeted next generation sequencing (NGS) molecular classifier (ProMisE NGS), comparing concordance and prognostic value to the original ProMisE classifier.

Methods. DNA was extracted from formalin-fixed paraffin embedded (FFPE) ECs that had previously undergone ProMisE molecular classification (*POLE* sequencing, IHC for p53 and MMR). DNA was sequenced using the clinically validated Imagia Canexia Health Find It™ amplicon-based NGS gene panel assay to assess for pathogenic *POLE* mutations (unchanged from original ProMisE), *TP53* mutations (in lieu of p53 IHC), and microsatellite instability (MSI) (in lieu of MMR IHC),with the same order of segregation as original ProMisE used for subtype assignment. Molecular subtype assignment of both classifiers was compared by concordance metrics and Kaplan-Meier survival statistics.

Results. The new DNA-based NGS molecular classifier (ProMisE NGS) was used to determine the molecular subtype in 164 ECs previously classified with ProMisE. 159/164 cases were concordant with a kappa statistic of 0.96 and an overall accuracy of 0.97. Prognostic differences in progression-free, disease-specific and overall survival between the four molecular subtypes were observed for the new NGS classifier, recapitulating the survival curves of the original ProMisE classifier. ProMisE NGS was 100% concordant between matched biopsy and hysterectomy samples.

Conclusion. ProMisE NGS is feasible on standard FFPE material, demonstrates high concordance with the original ProMisE classifier and maintains prognostic value in EC. This test has the potential to facilitate implementation of molecular classification of EC at the time of first diagnosis.

* Corresponding author at: Department of Gynecology and Obstetrics, Division of Gynecologic Oncology, University of British Columbia, 2775 Laurel St, Vancouver, BC V5Z1M9, Canada.
E-mail address: jessica.mcalpine@vch.ca (J.N. McAlpine).
[1] Shared authorship.

https://doi.org/10.1016/j.ygyno.2023.05.073
0090-8258/

1. Introduction

Following the discovery of the four molecular subtypes of endometrial cancer (EC) by The Cancer Genome Atlas (TCGA) in 2013 [1], two research teams working independently identified lower cost and easy to interpret methods that could consistently classify ECs into four molecular subtypes. These subtypes recapitulate the prognostic significance of the TCGA molecular classification based on genomic architecture [2–6]. Both the Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) and the TransPORTEC team's classifier use a combination of targeted next generation sequencing (NGS) assays for the detection of pathogenic *POLE* mutations and immunohistochemistry (IHC) to assess mismatch repair proteins and p53 status to assign ECs to one of four molecular subtypes: *POLE*mut, mismatch repair deficient (MMRd), p53abn, and no specific molecular profile (NSMP). ProMisE was developed following the Institute of Medicine guidelines for developing 'omics-based' tests with discovery, confirmation and validation phases [2–4]. Despite the abundance of data on the value of molecular classification in ECs [1–7], the World Health Organization (WHO) recommendation for the integration of molecular features into pathological reporting [8], and incorporation into clinical guidelines [9–11], the uptake of molecular testing in clinical practice has been highly variable.

One challenge identified with the original molecular classification system has been that results from all three molecular components must be available to assign the molecular subtype, including: *POLE* mutation status, presence or absence of two or four MMR proteins by IHC, and complete loss, normal (1+) or overexpression of p53 on IHC [8]. Approximately 3% of ECs present with more than one molecular feature, and molecular subtype cannot be assigned unless all ProMisE components are known [12]. In standard clinical practice it is not uncommon to have each molecular component of ProMisE performed at different stages of patient care and at different centres, potentially resulting in delays in management. A common scenario we have experienced is when reflex MMR IHC is performed on an EC biopsy at a community hospital, p53 IHC is then requested after referral to our tertiary cancer centre, and NGS for *POLE* is performed on the final hysterectomy specimen (at a third site). By the time all these results are compiled the patient may have already been referred for adjuvant therapy and missed an opportunity to use this molecular information to guide care. An additional challenge to the implementation of molecular classification in some centers has been reimbursement, as the different components of ProMisE (IHC and NGS) may be paid for from different resource allocations.

As a way to overcome some of these challenges associated with a multi-test molecular classifier, we assessed a single-test, single-cost, DNA-based targeted amplicon panel with next generation sequencing (NGS) to determine *POLE* and *TP53* mutations and microsatellite instability (MSI). This NGS assay replaces p53 IHC with *TP53* mutation calls and MMR IHC with the identification of microsatellite instability within specific MSI genomic loci (no change in assessment of *POLE* mutations). This test would simplify financial reimbursement as well as avoiding the current challenge of results coming in at different times; with an opportunity to improve implementation. We describe the assessment of this single-test DNA-based NGS molecular classifier (ProMisE NGS), comparing concordance to the original ProMisE classifier, as well as the ability to provide equivalent prognostic information.

2. Methods

2.1. Case selection

With Institutional Review Board approval, we selected a recent cohort of patients diagnosed and treated at Vancouver General Hospital over one calendar year (Jan 1 2016-Dec 31 2016) who had already been assigned molecular subtype by the original ProMisE and where clinical outcomes were known and previously reported [13,14]. We selected cases that had not undergone neoadjuvant chemotherapy or neoadjuvant radiotherapy and in whom tissue blocks from biopsy and/or hysterectomy were available.

2.2. Molecular testing and molecular subtype diagnosis

For the original ProMisE classification, representative formalin fixed paraffin embedded (FFPE) tumor sections had been cut as whole sections, at four microns. IHC for mismatch repair proteins and p53 was performed and scored as described previously, in an accredited diagnostic laboratory [13,14]. FFPE DNA extraction from tumor cells was performed, with NGS to assess for *POLE* mutations using Imagia Canexia Health's Find It™ V4.0 panel. The panel includes 146 hotspots and 23 exons in 30 cancer associated genes (**see** Supplemental Table 1), encompassing single nucleotide variants (SNVs), deletions and insertions (up to 24 bp). An assay threshold of ≥5% VAF was used, with all mutations present at a VAF of 1–5% reviewed on a case-by-case basis by a molecular pathologist. Coverage of *POLE* included the exonuclease domain exons 9–14 however *POLE*mut assignment was limited to a list of 11 well-characterized pathogenic mutations (P286R, V411L, S297F, S459F, A456P, F367S, L424I, M295R, P436R, M444K, D368Y) [15]. Molecular classification was achieved as per the WHO [8] by first assigning patients identified to have pathogenic *POLE* mutations (*POLE*mut), then categorizing the remaining ECs by MMR status, identifying patients whose tumor demonstrated loss of one or more MMR proteins (MMRd) and finally by p53 status (abnormal (p53abn) vs. normal/wild type IHC protein staining patterns (NSMP)).

For the assessment of the ProMisE NGS molecular classifier, FFPE DNA was sequenced using the clinically validated Imagia Canexia Health Find It™ V5.0 amplicon-based NGS gene panel assay for the detection of somatic mutations in *POLE*, *TP53* and MSI-High or MS-Stable . For this specific study, the samples were sequenced on an Illumina MiSeq™, using a 2X150bp v2 chemistry kit. The panel is an updated version of the Find It™ gene panel including targeted exons in 37 genes for SNVs and indels, gene amplification in 9 genes, and 21 loci for the detection of microsatellite instability (MSI-high, Indeterminate or Possible evidence of MSI, or MS-Stable) (Supplemental Table 1). Similar to the Find It V4.0 assay, the VAF% threshold for calling SNVs and indels is ≥1%, however in clinical practice ≥5% is generally considered as a true positive and ≥ 1–5% is reviewed on a case-by-case basis. The microsatellite instability algorithm is a machine learning (ML) random forest classifier that was trained on clinical FFPE samples of known MSI-High and MS-Stable samples. The classifier uses the distribution of repeats and relationship with MSI status in 21 MSI loci. An apparently healthy normal sample (NA01953, Coriell Institute) is included in every sequencing run which is used to normalize the ML features to reduce artifacts caused by PCR and sequencing instrument error. A resulting MSI score is given in one of the following three categories: MSI-High >0.6, Possible or Indeterminate evidence of MSI (requiring orthogonal MMR IHC) > 0.4 and ≤ 0.6, and MS-Stable ≤0.4. The molecular classification for ProMisE NGS was achieved by first assigning patients identified to harbour one of the 11 (listed above) pathogenic *POLE* mutations (*POLE*mut), then categorizing the remaining ECs by MSI status (MSI-high, Indeterminate or Possible evidence of MSI, MS-Stable) identifying patients whose tumor demonstrated a high MSI score (MSI/MMRd) and finally segregating by *TP53* mutations status (*TP53*mut/p53abn vs *TP53* wild type/NSMP) (Fig. 1). Samples that do not pass quality control amplicon coverage thresholds are repeated by sequencing, or additional material obtained from the biopsy/hysterectomy for repeat DNA extraction and sequencing.

2.3. Statistical analyses

Concordance statistics comparing molecular subtypes classified by the original ProMisE and ProMisE NGS were evaluated using Cohen's kappa statistic. Kaplan-Meier curves and the log-rank test were used

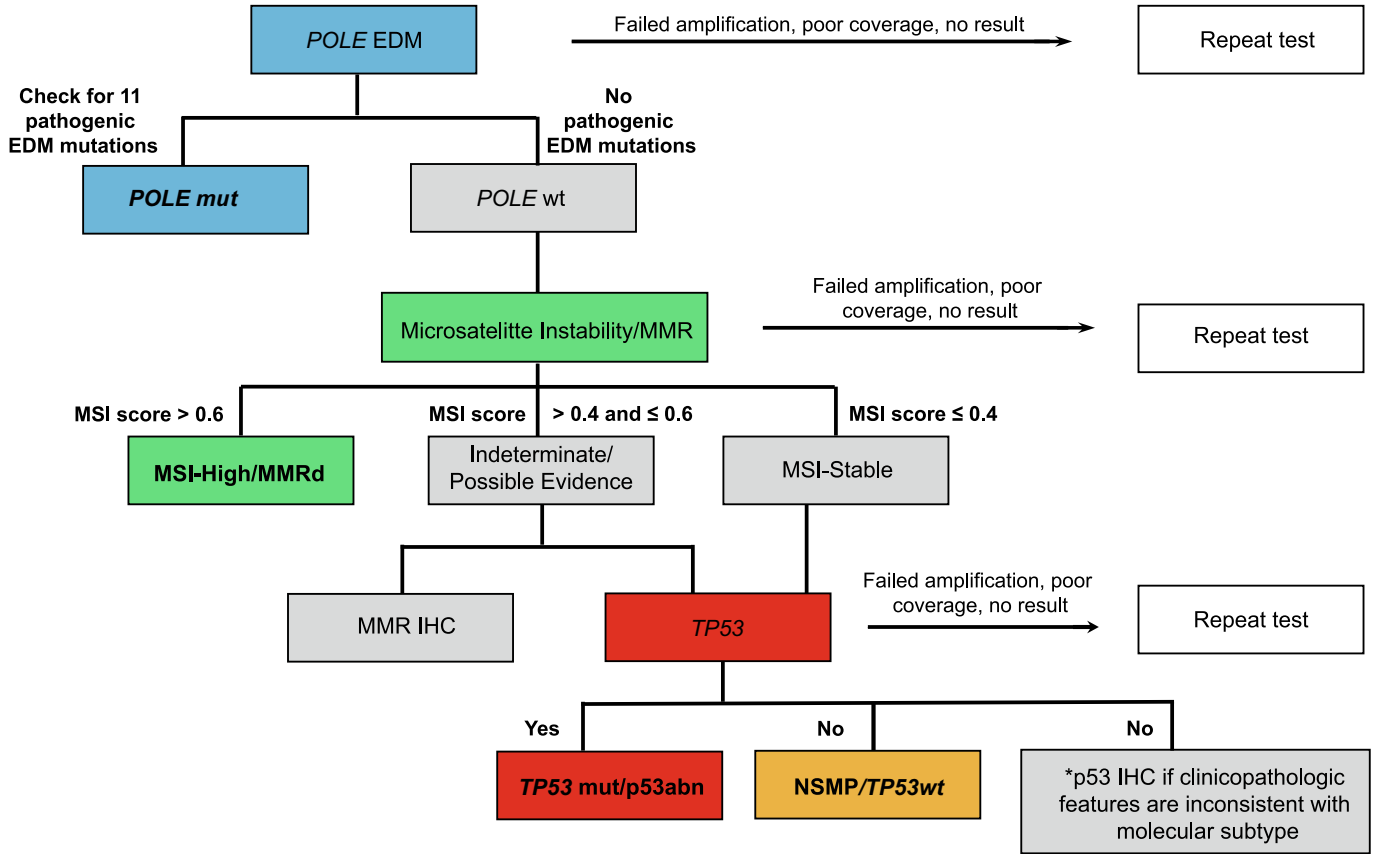

Fig. 1. DNA is extracted from formalin-fixed paraffin embedded tumor and sequenced using the clinically validated Imagia Canexia Health Find It™ V 5.0 amplicon-based NGS gene panel assay. The molecular classification for ProMisE NGS is achieved by first assigning patients identified to harbour one of the 11 pathogenic *POLE* mutations (*POLE*mut), then categorizing the remaining ECs by microsatellite instability status (MSI-high, Indeterminate or Possible evidence of MSI, MS-Stable) identifying patients whose tumor demonstrated a high MSI score (MSI/MMRd) and finally segregating by *TP53* mutation status (*TP53*mut/p53abn vs *TP53* wild type/NSMP). * While disagreement between p53 IHC and *TP53* NGS is uncommon, consideration should be given to performing p53 IHC in a tumor with wild type *TP53* when there are histopathological features associated with p53abn subtype (e.g. serous carcinoma or carcinosarcoma), as these histotypes are rarely NSMP.

to illustrate and test the association between outcomes (progression-free survival (PFS), disease-specific survival (DSS) and overall survival (OS)) and molecular subtypes assigned by original ProMisE and ProMisE NGS (enabling comparison of the prognostic value of these two tools). All statistical analyses were done using R project for statistical computing (https://www.r-project.org/) version 4.2.0 with significance set at $\alpha = 0.05$.

3. Results

3.1. Cohort

The single-test ProMisE NGS molecular classifier was performed on 164 ECs, including 15 cases with matched biopsy and hysterectomy specimens, and compared to the original ProMisE molecular subtype assignment. There were two cases that initially did not pass quality control sequencing amplicon coverage thresholds, however repeat sequencing was successful. Table 1 shows the clinicopathologic characteristics of the total cohort. Based on ProMisE NGS results, there were 10 (6.1%) *POLE*mut, 51 (31.1%) MSI/MMRd, 69 (42.1%) NSMP and 34 (20.7%) *TP53*mut/p53abn ECs.

3.2. Concordance metrics

The comparison of molecular subtype assignment for the original ProMisE and ProMisE NGS, resulted in 159/164 (97%) of cases that were concordant with a kappa statistic of 0.96 and an overall accuracy of 0.97. The five discordant cases (3%) were reviewed by an expert gynecologic pathologist (CBG). In one discordant case (Case 1, Table 2) the original ProMisE was classified as NSMP, however the ProMisE NGS test identified the case as MSI-High. Pathology review of the MMR IHC confirmed intact staining, whereas independent orthogonal MSI testing performed in a clinically accredited diagnostic lab using the Idylla MSI assay showed an MSI-high result. This indicated a false negative result by IHC and confirmed true positive MSI-High/MMRd status by NGS. There were two discordant cases (Case 2 and 3, Table 2) where the original ProMisE result classified both cases as p53abn but no *TP53* mutations were identified on ProMisE NGS. Pathology review of the p53 IHC for these cases showed complete absence of staining (null mutation pattern). This discordant *TP53* status is likely due to limitations in targeted NGS for the detection of large deletions. In another discordant case (Case 4, Table 2) the original ProMisE molecular assignment was NSMP, whereas the ProMisE NGS classification identified a *TP53* (p.E204D) mutation at 49% VAF (variant allele frequency) indicating a possible rare germline single nucleotide polymorphism (SNP). Pathology review of the p53 IHC confirmed wild type staining, therefore we obtained an adjacent normal FFPE tissue block and tested by NGS to confirm the germline or somatic status. The *TP53* mutation was confirmed germline, therefore the final molecular assignment by ProMisE NGS would be altered to NSMP as the final diagnosis. Finally, in one case (Case 5, Table 2) assigned as *POLE*mut on the

Table 1
Clinicopathologic characteristics of the total cohort ($n = 164$) and shown within the four ProMisE NGS molecular subtypes.

	Total	ProMisE NGS *POLE*mut	ProMisE NGS MSI/MMRd	ProMisE NGS NSMP/*TP53* wt	ProMisE NGS *TP53*mut/p53abn
Total	164	10 (6.1%)	51 (31.1%)	69 (42.1%)	34 (20.7%)
Age at diagnosis					
<40	6 (3.6%)	0 (0.0%)	1 (2.0%)	2 (2.9%)	3 (8.8%)
40–60	50 (30.3%)	7 (63.6%)	16 (32.0%)	21 (30.0%)	6 (17.6%)
>60	109 (66.1%)	4 (36.4%)	33 (66.0%)	47 (67.1%)	25 (73.5%)
BMI					
<30	63 (41.7%)	6 (60.0%)	16 (37.2%)	25 (38.5%)	16 (48.5%)
≥30	88 (58.3%)	4 (40.0%)	27 (62.8%)	40 (61.5%)	17 (51.5%)
Histological subtype					
Endometrioid (EM)	128 (78.6%)	8 (81.8%)	47 (96.0%)	61 (89.9%)	9 (27.3%)
Serous	21 (12.9%)	1 (9.1%)	0 (0.0%)	2 (2.9%)	18 (54.5%)
Clear cell	3 (1.8%)	0 (0.0%)	0 (0.0%)	3 (4.3%)	0 (0.0%)
Carcinosarcoma	1 (0.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (3.0%)
Dedifferentiated	2 (1.2%)	0 (0.0%)	1 (2.0%)	0 (0.0%)	1 (3.0%)
Undifferentiated	1 (0.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (3.0%)
Mixed EM and serous	5 (3.1%)	1 (9.1%)	1 (2.0%)	1 (1.4%)	2 (6.1%)
Tumor grade					
Grade 1	58 (35.6%)	4 (40.0%)	14 (28.0%)	38 (54.3%)	3 (8.8%)
Grade 2	48 (29.4%)	2 (20.0%)	21 (42.0%)	22 (31.4%)	3 (8.8%)
Grade 3	57 (35.0%)	4 (40.0%)	15 (30.0%)	10 (14.3%)	28 (82.4%)
FIGO stage					
IA	95 (58.6%)	5 (45.5%)	29 (59.2%)	46 (66.7%)	15 (45.5%)
IB	37 (22.8%)	2 (18.2%)	11 (22.4%)	14 (20.3%)	10 (30.3%)
II	4 (2.5%)	1 (9.1%)	0 (0.0%)	3 (4.3%)	0 (0.0%)
IIIA	6 (3.7%)	0 (0.0%)	2 (4.1%)	2 (2.9%)	2 (6.1%)
IIIB	5 (3.1%)	1 (9.1%)	3 (6.1%)	0 (0.0%)	1 (3.0%)
IIIC	10 (5.6%)	1 (9.1%)	3 (6.1%)	3 (4.3%)	3 (9.1%)
IVB	5 (3.1%)	1 (9.1%)	1 (2.0%)	1 (1.4%)	2 (6.1%)
LVI					
Negative	106 (65.8%)	5 (45.5%)	26 (54.2%)	56 (81.2%)	19 (57.6%)
Positive-unspecified	3 (1.9%)	1 (9.1%)	1 (2.1%)	1 (1.4%)	0 (0.0%)
Positive-focal	14 (8.7%)	1 (9.1%)	5 (10.4%)	4 (5.8%)	4 (12.1%)
Positive-extensive	38 (23.6%)	4 (36.4%)	16 (33.3%)	8 (11.6%)	10 (30.3%)
Original ProMisE					
NSMP	68 (41.2%)	0 (0.0%)	1 (2.0%)	66 (92.9%)	1 (5.9%)
p53abn	35 (20.0%)	0 (0.0%)	0 (0.0%)	2 (2.9%)	33 (97.1%)
MMRd	50 (30.9%)	0 (0.0%)	50 (100.0%)	0 (0%)	0 (0.0%)
*POLE*mut	11 (7.9%)	10 (90.9%)	0 (0.0%)	1 (9.1%)	0 (0%)

NOTE: Total cases within each parameter measured may be less than total numbers due to missing data.
BMI- body mass index. LVI- lymphovascular space invasion, NGS – next generation sequencing, MSI – microsatellite instable, MMRd – mismatch repair deficient, mut-mutated.

original ProMisE, however the same rare *POLE* (p.P436R) mutation was not identified by ProMisE NGS, likely due to the primer and amplicon design of the updated Find It assay. This false negative result will be addressed in an update to the assay. Further details of these five discordant cases including likely explanations for the discordances are shown in Table 2.

For a subset of patients, we were able to compare the new ProMisE NGS classifier assignment between diagnostic biopsy specimens and hysterectomy specimens within the same individual, with results concordant in 15 of 15 cases (100%).

Table 2
Five cases that were discordant with the original ProMisE and ProMisE NGS.

Case	Original ProMisE	ProMisE NGS	Possible explanations for discordance
1	NSMP	MSI/MMRd	MMR intact on review of MMR IHC. MSI high score on NGS. Independent orthogonal MSI testing performed in a clinically accredited diagnostic lab using the Idylla MSI assay showed a MSI-high result. This indicates a false negative result by IHC and confirmed true positive MSI-High by NGS. Protein can be present but is non -functional (this is uncommon).
2	p53abn	NSMP	Review of p53 IHC showed complete absence of staining (null mutation pattern). There are known difficulties in the detection of stop gained/splicing mutations and large deletions or insertions using NGS which could explain the discordance.
3	p53abn	NSMP	Review of p53 IHC showed complete absence of staining (null mutation pattern). There are known difficulties in the detection of stop gained/splicing mutations and large deletions or insertions using NGS which could explain the discordance.
4	NSMP	*TP53*mut/p53abn	Review of p53 IHC confirmed p53 wildtype. *TP53* mutation p.E204D 49% VAF We obtained an adjacent normal FFPE tissue block and tested by NGS to confirm the germline or somatic status. The *TP53* mutation was confirmed germline, therefore the final molecular assignment for ProMisE NGS would be altered to NSMP as the final diagnosis.
5	*POLE*mut	NSMP	The *POLE* mutation for this case in original ProMisE was p.P436R 18.31%. Confirmed by Sanger sequencing. The same *POLE* mutation was not identified by ProMisE NGS, likely due to the primer and amplicon design of the updated Find It assay. This false negative result will be addressed in an update to the assay. A variant of unknown significance *POLE* p.I296M at 15% VAF was identified in this sample.

NGS – next generation sequencing, MSI – microsatellite instable, MMRd – mismatch repair deficient, mut-mutated, IHC- immunohistochemistry, VAF- variant allele frequency.

3.3. Clinical outcomes within the four molecular subtypes assigned by ProMisE NGS

Kaplan-Meier survival analysis of the four molecular subtype assignments from the new ProMisE NGS tool demonstrated statistically significant differences in PFS ($p = 0.0187$), DSS ($p = 0.0052$) and OS ($p = 0.0276$), and very closely reapproximated the survival curves of the original ProMisE classifier (Fig. 2). Patients with *POLE*mut ECs had no disease recurrences in five years compared to p53abn EC where 30% of patients had a disease recurrence in five years. Intermediate outcomes were observed for MMRd and NSMP subtypes.

4. Discussion

In this study we demonstrate excellent concordance of a single-test DNA-based NGS EC molecular classifier when compared to the original multi-test, multi-platform (NGS and IHC) ProMisE classifier. Perhaps more importantly, the prognostic value of this single-test ProMisE

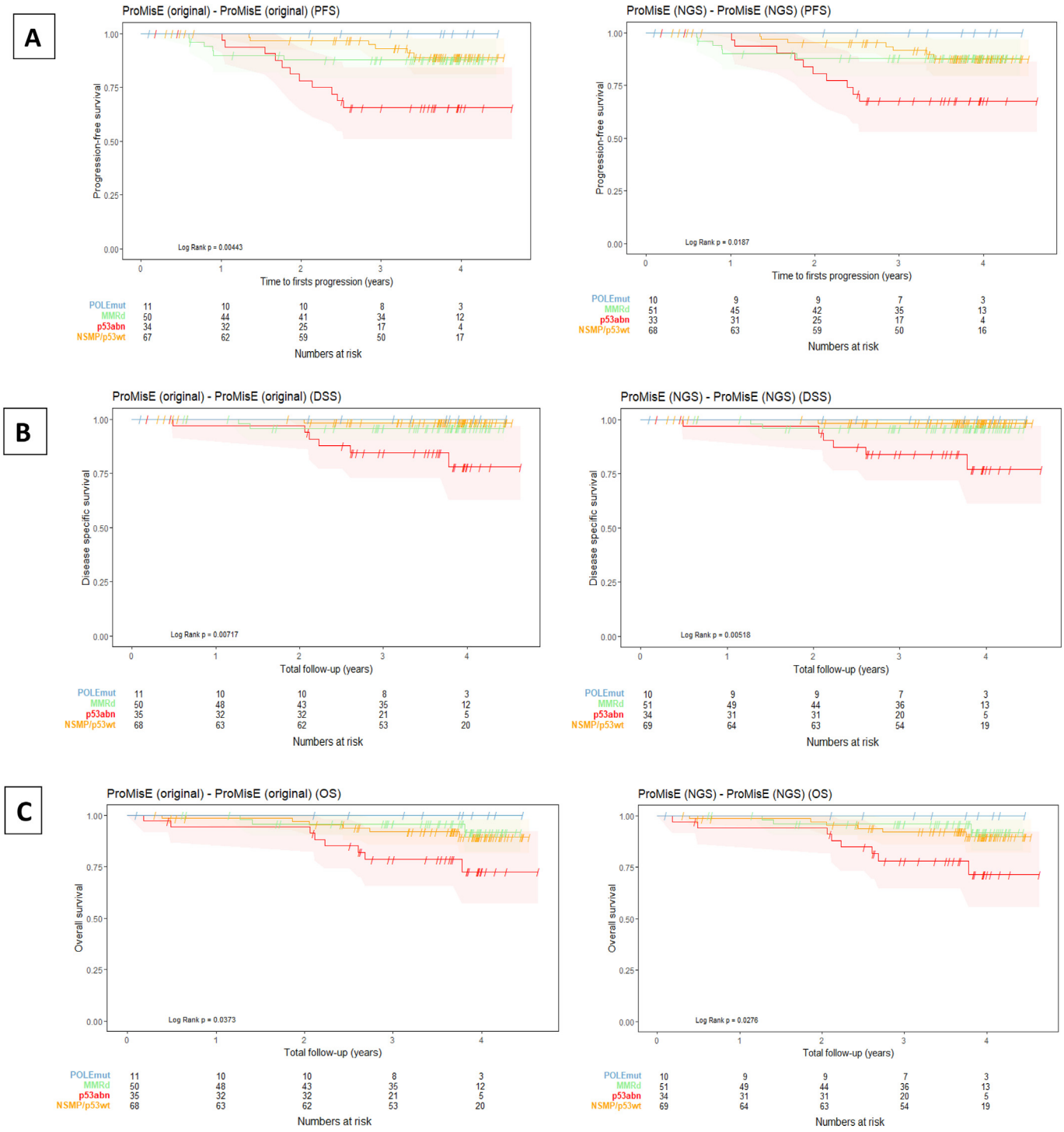

Fig. 2. Kaplan-Meier survival analyses demonstrating molecular subtype is significantly associated with outcomes across progression-free survival (A), disease-specific survival (B) and overall survival (C) in both the original ProMisE and ProMisE NGS.

NGS EC classifier was maintained, with recapitulation of the original ProMisE survival curves (Fig. 2). ProMisE NGS testing can be achieved from standard FFPE material with a low re-testing rate (2/164 (1.2%) cases), comparable to original ProMisE [2–4]. Furthermore, all cases where ProMisE NGS was assessed on matched biopsy and hysterectomy specimens were concordant, adding further evidence that reliable molecular testing can be obtained from time of first diagnosis.

Knowing the EC molecular subtype from time of first diagnosis provides an opportunity to direct what surgery is performed (e.g. lymph node assessment, omentectomy, etc) and/or where surgery is performed (e.g. community vs tertiary cancer center) with tremendous impact on costs to the health care system and patients [13]. Molecular subtype can also be used to guide adjuvant therapy decisions, e.g., MSI/MMR status qualifies for FDA-approved access to immune checkpoint blockade therapy [11,16], and the presence of *TP53* mutations identifies patients who may benefit from the addition of adjuvant chemotherapy [17,18]. Given the value that molecular classification provides, it is imperative that more than one option (with IHC, or single-test NGS) for testing is available for patients.

The four molecular subtypes of endometrial carcinoma differ with respect to genetic and environmental risk factors, precursor lesions, patterns of spread, response to treatment, and outcomes. Although molecular subtypes were first identified based on genomic architecture in the landmark TCGA study [1], the methods used for classification in that study were not designed for diagnostic use and have never been rigorously validated for clinical application. Diagnosis of the molecular subtypes based on the ProMisE diagnostic algorithm (*POLE*mut, MMRd, p53abn and NSMP) was validated according to Institute of Medicine guidelines [2–4] and subsequently used to interrogate diverse EC cohorts [13,14,17–21]. This classification was adopted by the WHO in 2020 and is the standard by which new diagnostic approaches should be validated. With regards to the names of the four molecular subtypes, we have adopted the naming conventions of the WHO fifth edition [8]. While p53abn EC is sometimes still referred to as “Copy-number high”, the subtype is specifically characterized by loss of normal p53 protein function in the context of retained *POLE* and MMR activity., thus the designation “p53abn”. It could be argued that when the subtype is diagnosed based on DNA sequencing of *TP53* the more appropriate diagnostic designation for this group would be “*TP53*mut”, however we believe that more than one name for a diagnostic entity is confusing and not conducive to best patient care. Therefore, we have continued to use and recommend the diagnostic terminology adopted by the WHO. Similarly, although hypermutated ECs can be diagnosed based on either immunostaining for MMR proteins or one of a number of assays for microsatellite instability, these are equivalent (though not identical) approaches and both are characterized by loss of MMR activity. We have retained “MMRd” as the diagnosis for this subset of EC, rather than MSI.

Previous studies comparing MMR IHC vs. MSI assay [22–24] and comparing *TP53* mutation sequencing vs. p53 IHC [23,25,26] in EC have demonstrated discordance rates between methods ranging from 5 to 7%. Given these previous data relating to the same methods, as we transitioned to the new ProMisE NGS assay we anticipated there would be discordances between the original ProMisE classifier assignment and ProMisE NGS test. We found only 5/164 (3%) of cases that were discordant between the original ProMisE and ProMisE NGS. This is similar to findings of Huvila et al., who compared ProMisE subtype assignment to the FoundationOne NGS panel [27]. Their study found molecular subtype diagnosis based on NGS and the original ProMisE was in agreement for 52 of 53 tumors, however, MSI could not be determined in 7/60 cases. In this study, we were able to assess the MSI status of all 164 cases using NGS. Li et al. also found high concordance (69 of 70 cases) comparing an 11-gene NGS panel with ProMisE molecular subtype assignment [28].

Comparing p53 IHC and *TP53* NGS, there are known explanations for discordant results. The detection of mutations involving exon splice sites or large scale deletions or insertions (indels) are often missed using targeted NGS assays [23,25,26]. We had two cases where *TP53* mutations were missed, likely due to large indels, with a difference in molecular subtype between NSMP and p53abn for the two assays (Table 2). When considering p53 IHC and *TP53* NGS, it is also critical to use the correct order of segregation as per the WHO endorsed algorithm because excluding *POLE*mut and MMRd EC significantly improves the agreement between p53 IHC and *TP53* NGS [23,25,26]. The presence of non-driver mutations in *TP53* associated with the ultramutated *POLE*mut and hypermutated MMRd molecular subtypes are not associated with aggressive behaviour, and these ECs behave as *POLE*mut or MMRd EC, respectively, despite the presence of *TP53* mutations [12]. Another scenario is the presence of wild type p53 staining on IHC with a *TP53* mutation identified on NGS, which is rare after excluding *POLE*mut and MMRd EC [23,25,26]. We had one such case in this study, however the mutation was identified at 49% VAF indicating a possible germline SNP. We therefore performed NGS testing of normal tissue and confirmed the *TP53* mutation was a germline SNP, and not present as somatic. While disagreement between p53 IHC and *TP53* NGS is uncommon, consideration should be given to performing p53 IHC in a *POLE* wild type and MMR proficient (MS-Stable) tumor with wild type *TP53* when there are histopathological features associated with p53abn subtype (e.g. serous carcinoma or carcinosarcoma), as these histotypes are rarely NSMP [14,29]. Further study is needed for these rare ECs with discordant p53 IHC and *TP53* NGS. These cases remain a clinical challenge, with the possibility of significant differences in treatment recommendations. With increasing use of NGS, this may be encountered more often and consensus on how to manage these discordant p53 IHC and *TP53* NGS cases will be important.

Discordant results for MMR status by MSI assay and MMR IHC are well described in EC [22–24]. *MSH6* mutations detected by IHC can result in weaker or no detectable microsatellite instability in the tumor [22]. MMR protein can also be retained and detectable by immunostaining in EC but harbour mutations (somatic or germline) that results in loss of function and MSI-high status [22–24]. We had one such case in this study where the targeted NGS panel and orthogonal Idylla MSI both showed MSI-High/MMRd results, however the IHC showed intact MMR protein expression. This highlights the importance of germline testing for Lynch Syndrome in EC patients with tumors that show intact MMR proteins or microsatellite stability in the presence of a significant personal and/or family history of Lynch Syndrome related cancers. We also observed two cases where the NGS MSI score fell into the category of indeterminate or possible evidence of MSI results (0.4–0.6), which in practice would trigger orthogonal testing with MMR IHC to confirm. In both cases, review of MMR IHC showed subclonal MLH1 loss due to promotor methylation, which likely explains the borderline MSI score. At this present time, it is not known what percent of tumor cells showing MLH1 loss in EC is clinically significant. Over time, the NGS MSI classifier training set could be updated to account for these borderline cases possibly caused by subclonal MLH1 loss. Recent data have highlighted the diversity within MMRd tumors, with worse outcomes observed in patients with MLH1 loss as compared to Lynch Syndrome associated MMRd EC [30] and lower response to immune checkpoint blockade (ICB) therapy [31]. Further studies are needed to evaluate the relationship with subclonal MLH1 loss and response to ICB therapy.

A consideration when utilizing any NGS assay is determination of VAF assay thresholds (1 vs 5%). There was one case in this study with a *POLE* mutation identified (p.P286R) with a VAF of only 1% and a *TP53* mutation with VAF of 67% (c.783-1G > T, splice). If set at 1% VAF assay thresholds this individual would have been considered *POLE*mut based on the order of segregation and potentially offered de-escalated therapy. In this protocol we set the threshold at 5% VAF and this patient was categorized as p53abn and treated accordingly. There is currently a lack of evidence to guide decision making in cases with mutations

present at very low VAF (e.g. 1–5% range). Taking into account other features in patient presentation such as patient age and histotype may help direct these decisions. On clinical review of this case, it was a 72 year old low BMI patient with a serous endometrial carcinoma; a clinical presentation much more fitting with p53abn EC. This patient died of disease within 2 years of receiving standard therapy, and although this outcome would not be known at time of classification, certainly her presentation can help reaffirm (or question) molecular subtype assignment.

The strengths of this study include using a well characterized modern (2016) unselected cohort where pathologic assignment and IHC was consistent with current WHO practice. DNA extraction had been performed for this cohort to assign the original ProMisE with stored DNA available from this first extraction that could be used for ProMisE NGS. The company running the analysis of ProMisE NGS was blinded to the IHC data and results of the original ProMisE. We acknowledge the numbers in this study are lower than our original ProMisE classifier confirmation series [3] supporting the need to validate these findings in another cohort.

A next important step in implementation of this single-test NGS targeted panel will be assessing cost effectiveness of ProMisE NGS compared to the original ProMisE. Both cost and access to NGS testing for *POLE* mutations are cited as challenges in obtaining original ProMisE results. However, for centers routinely using gene panels for other solid tumors the additional cost of an expanded assay (MSI and mutation panel) is not markedly higher than mutation panel alone (already required for *POLE* status), and cost can be at least partially offset by savings from not performing p53 and MMR IHC. The addition of the other ProMisE NGS components simply provides an extension of this NGS step that can be performed by any commercial or institutional DNA based assay. The cost comparison of ProMisE NGS compared to the original ProMisE is currently being assessed and will be reported in a separate study.

Finally, before clinical implementation it will be imperative to validate the prognostic value and concordance of molecular classification by ProMIsE NGS as compared to the original ProMisE in an independent cohort. The data presented herein have been sufficiently encouraging to warrant this next step in assessment of this assay, aiming to provide an alternative tool to obtain molecular subtype and expand access for patients with this disease.

5. Conclusion

The single-test DNA-based ProMisE NGS molecular classifier is feasible, demonstrates excellent concordance with the original ProMisE classifier and maintains prognostic value. Reliable molecular subtyping can be obtained at diagnosis using a single-test NGS targeted gene assay, providing an alternative means to obtain molecular classification for patients with EC.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygyno.2023.05.073.

Funding

This work was supported by the Michael Smith Foundation for Health Research, Innovation to Commercialization grant (Phase I and II) (JMc). This team has also been supported by the Canadian Institute for Health Research (JMc), the BC Cancer Foundation (Clinician Scientist Award (JMc)), Vancouver General Hospital Foundation, the Vancouver Coastal Health Research Institute, the Chew Wei Memorial Chair in Gynecologic Oncology (JMc), and the Miller-Mindell Fellowship (AJ).

Declaration of Competing Interest

Dr. McConechy is a former employee and Dr. Huntsman is a founder and previous Chief Medical Officer of Imagia Canexia Health.

Acknowledgements

We would like the following employees from Imagia Canexia Health that contributed to sequencing and processing data from this study: Adrian Kense, Ka Mun Nip, Ebru Baran, Sonal Brahmbhatt, Dilmi Perera, Kurt Yakimovich, Rosalia Aguirre-Hernandez, and Ruth Miller.

References

[1] G. Getz, S.B. Gabriel, K. Cibulskis, et al., Integrated genomic characterization of endometrial carcinoma, Nature. 497 (7447) (2013) 67–73, https://doi.org/10.1038/nature12113.
[2] A. Talhouk, M.K. McConechy, S. Leung, et al., A clinically applicable molecular-based classification for endometrial cancers, Br. J. Cancer 113 (2) (2015) 299–310, https://doi.org/10.1038/bjc.2015.190.
[3] A. Talhouk, M.K. McConechy, S. Leung, et al., Confirmation of ProMisE: A simple, genomics-based clinical classifier for endometrial cancer, Cancer. 123 (5) (2017) 802–813, https://doi.org/10.1002/cncr.30496.
[4] S. Kommoss, M.K. McConechy, F. Kommoss, et al., Final validation of the ProMisE molecular classifier for endometrial carcinoma in a large population-based case series, Ann. Oncol. 29 (5) (2018) 1180–1188, https://doi.org/10.1093/annonc/mdy058.
[5] E. Stelloo, T. Bosse, R.A. Nout, et al., Refining prognosis and identifying targetable pathways for high-risk endometrial cancer; A TransPORTEC initiative, Mod. Pathol. 28 (6) (2015) 836–844, https://doi.org/10.1038/modpathol.2015.43.
[6] E. Stelloo, R.A. Nout, E.M. Osse, et al., Improved risk assessment by integrating molecular and clinicopathological factors in early-stage endometrial cancer-combined analysis of the PORTEC cohorts, Clin. Cancer Res. 22 (16) (2016) 4215–4224, https://doi.org/10.1158/1078-0432.CCR-15-2878.
[7] A. Jamieson, J.N. Mcalpine, Molecular profiling of endometrial cancer from TCGA to clinical practice, 21 (2) (2023) 210–216, https://doi.org/10.6004/jnccn.2022.7096.
[8] Organization WHO, WHO Classification of Tumours. Female Genital Tumours: International Agency for Research on Cancer Lyon (France), International Agency for Research on Cancer, 2020.
[9] A.N. Concin, X. Matias-guiu, I. Vergote, et al., ESGO / ESTRO / ESP guidelines for the management of patients with endometrial carcinoma, Int. J. Gynecol. Cancer (2020) 1–63, https://doi.org/10.1136/ijgc-2020-002230 Published online.
[10] A. Oaknin, T.J. Bosse, C.L. Creutzberg, et al., Endometrial cancer: ESMO clinical practice guideline for diagnosis, treatment and follow-up ☆, Ann. Oncol. 33 (9) (2022) 860–877, https://doi.org/10.1016/j.annonc.2022.05.009.
[11] NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines), Uterine Neoplasms Version 1, Retrieved Feb 5, 2022, from www.nccn.org 2022.
[12] A. León-Castillo, E. Gilvazquez, R. Nout, et al., Clinicopathological and molecular characterisation of 'multiple-classifier' endometrial carcinomas, J. Pathol. 250 (3) (2020) 312–322, https://doi.org/10.1002/path.5373.
[13] A. Jamieson, J. Huvila, E.F. Thompson, et al., Variation in practice in endometrial cancer and potential for improved care and equity through molecular classification, Gynecol. Oncol. xxxx (2022) https://doi.org/10.1016/j.ygyno.2022.02.001.
[14] E.F. Thompson, J. Huvila, A. Jamieson, et al., Variability in endometrial carcinoma pathology practice: opportunities for improvement with molecular classification, Mod. Pathol. (August) (2022) 1–9, https://doi.org/10.1038/s41379-022-01165-w.
[15] A. León-Castillo, H. Britton, M.K. McConechy, et al., Interpretation of somatic POLE mutations in endometrial carcinoma, J. Pathol. 250 (3) (2020) 323–335, https://doi.org/10.1002/path.5372.
[16] A.K. Green, J. Feinberg, V. Makker, Gynecologic Cancer a Review of Immune Checkpoint Blockade Therapy in Endometrial Cancer, Published online 2022.
[17] A. León-Castillo, S.M. de Boer, M.E. Powell, et al., Molecular classification of the PORTEC-3 trial for high-risk endometrial cancer: impact on prognosis and benefit from adjuvant therapy, J. Clin. Oncol. (2020) https://doi.org/10.1200/jco.20.00549 Published online. JCO.20.00549.
[18] A. Jamieson, J. Huvila, S. Leung, et al., Gynecologic oncology molecular subtype strati fi ed outcomes according to adjuvant therapy in endometrial cancer, Gynecol. Oncol. 170 (2023) 282–289, https://doi.org/10.1016/j.ygyno.2023.01.025.
[19] S.R. Kim, B.T. Cloutier, S. Leung, et al., Molecular subtypes of clear cell carcinoma of the endometrium: opportunities for prognostic and predictive stratification, Gynecol. Oncol. 158 (1) (2020) 3–11, https://doi.org/10.1016/j.ygyno.2020.04.043.
[20] Tjalling Bosse, Remi A. Nout, Jessica N. McAlpine, et al., Molecular classification of grade 3 endometrioid endometrial cancers identifies distinct prognostic subgroups, Am. J. Obstet. Gynecol. 42 (5) (2018) 561–568, doi:10.1097/PAS.0000000000001020.Molecular.
[21] A. Leon-Castillo, N. Horeweg, E.E.M. Peters, et al., Prognostic relevance of the molecular classification in high-grade endometrial cancer for patients staged by lymphadenectomy and without adjuvant treatment, Gynecol. Oncol. 164 (3) (2022) 577–586, https://doi.org/10.1016/j.ygyno.2022.01.007.
[22] M.K. McConechy, A. Talhouk, H.H. Li-Chang, et al., Detection of DNA mismatch repair (MMR) deficiencies by immunohistochemistry can effectively diagnose the microsatellite instability (MSI) phenotype in endometrial carcinomas, Gynecol. Oncol. 137 (2) (2015) 306–310, https://doi.org/10.1016/j.ygyno.2015.01.541.
[23] S. Streel, A. Salmon, A. Dheur, et al., Diagnostic performance of immunohistochemistry compared to molecular techniques for microsatellite instability and p53 mutation detection in endometrial cancer, Int. J. Mol. Sci. 24 (5) (2023) 4866, https://doi.org/10.3390/ijms24054866.
[24] F. Dedeurwaerdere, K.B. Claes, J. Van Dorpe, et al., Comparison of microsatellite instability detection by immunohistochemistry and molecular techniques in

colorectal and endometrial cancer, Sci. Rep. 11 (1) (2021) 1–15, https://doi.org/10.1038/s41598-021-91974-x.

[25] N. Singh, A.M. Piskorz, T. Bosse, et al., p53 immunohistochemistry is an accurate surrogate for TP53 mutational analysis in endometrial carcinoma biopsies, J. Pathol. 250 (3) (2020) 336–345, https://doi.org/10.1002/path.5375.

[26] L. Vermij, A. Léon-Castillo, N. Singh, et al., p53 immunohistochemistry in endometrial cancer: clinical and molecular correlates in the PORTEC-3 trial, Mod. Pathol. 35 (10) (2022) 1475–1483, https://doi.org/10.1038/s41379-022-01102-x.

[27] J. Huvila, K. Orte, P. Vainio, T. Mettälä, T. Joutsiniemi, S. Hietanen, Molecular subtype diagnosis of endometrial carcinoma: comparison of the next-generation sequencing panel and proactive molecular risk classifier for endometrial Cancer classifier, Hum. Pathol. 111 (2021) 98–109, https://doi.org/10.1016/j.humpath.2021.02.006.

[28] L. Li, F. Chen, J. Liu, et al., Molecular classification grade 3 endometrial endometrioid carcinoma using a next- generation sequencing – based gene panel, Front. Oncol. (August) (2022) 1–12, https://doi.org/10.3389/fonc.2022.935694.

[29] Jutta Huvila, Amy Jamieson, Emily Thompson, Jessica N. Mcalpine, CBG., Molecular classification of endometrial carcinosarcoma shows uniformly P53ABN subtype and provide opportunities for diagnostic improvement, Int. J. Gynecol. Cancer 32 (3) (2022) A1–A274.

[30] C.C.B. Post, E. Stelloo, V.T.H.B.M. Smit, D. Ruano, Prevalence and prognosis of lynch syndrome and sporadic mismatch repair deficiency in endometrial Cancer, J. Natl. Cancer Inst. 113 (9) (2021) 1212–1220, https://doi.org/10.1093/jnci/djab029.

[31] L. Borden, J. Dvorak, Z. Barrett, et al., MLH1 hypermethylation predicts poor outcomes with pembrolizumab in recurrent endometrial cancer, Gynecol. Oncol. (2022) (Abstract 8.) (2022) S59.