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Assessment of DNA Ploidy in the ProMisE molecular subgroups of endometrial cancer

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HIGHLIGHTS

- Abnormal DNA ploidy was significantly higher in the p53 abn molecular group of EC, compared to the other molecular groups.
- Abnormal DNA ploidy correlated with worse PFS, lower BMI, higher grade and non-endometrioid histotypes.
- In the MMR-D group, DNA ploidy provided additional prognostic value, which merits further study in a larger series of EC.

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ABSTRACT

Objective. We sought to determine whether DNA ploidy correlates with the four molecular subgroups of endometrial carcinoma (EC) as determined using ProMisE (Proactive Molecular Risk Classifier for Endometrial Cancer).

Methods. 90 cases of EC previously characterized by clinicopathological parameters, outcomes, and ProMisE molecular subgroup (*POLE* EDM, MMR-D, p53 wt or p53 abn) were assessed for DNA ploidy using image cytometry. Associations of ploidy with traditional clinicopathological parameters were also tested.

Results. Abnormal DNA ploidy status differed amongst the ProMisE groups ($p < 0.001$) and was found in 80.9% (17/21) of p53 abn, 37.0% (10/27) of p53 wt, 28.6% (4/14) of *POLE* EDM and 14.3% (4/28) of MMR-D. Abnormal DNA content was significantly associated with lower BMI ($p = 0.034$) and grade 3 tumors ($p = 0.001$). In the entire cohort, abnormal DNA content was significantly associated with worse progression free survival ($p = 0.0094$) but not disease specific survival ($p = 0.249$) or overall survival ($p = 0.187$). When examining ploidy within each of the ProMisE groups, abnormal DNA content correlated with worse overall survival ($p = 0.041$) and progression free survival ($p = 0.011$) in the MMR-D group. No statistically significant relationship was seen in the remaining 3 groups.

Conclusion. Abnormal DNA ploidy status did correlate with the molecular subgroups of EC; abnormal DNA content was seen in the large majority of p53 abn cases. Abnormal ploidy however was also seen in smaller numbers in the p53 wt, *POLE* EDM and MMR-D groups; therefore abnormal DNA content was not a specific marker for any one molecular group. The addition of ploidy to the ProMisE molecular categories conferred additional prognostic value within the MMR-D group, which merits further study.

1. Introduction

Endometrial cancer (EC) is the fourth most common cause of cancer in women, and the most prevalent gynaecologic malignancy in the developed world [1]. Paralleling a global trend, in the last decade the

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incidence rate of EC in Canada has increased by 2.5% a year [2], likely attributable to an aging population and the obesity epidemic [3]. Although the majority of women are diagnosed at an early stage and have good outcomes, even within clinical stage 1 disease, the 5-year survival ranges from 42% to 90% [4]. This highlights the heterogeneity of the disease and the need to accurately predict high-risk cases.

Currently, pathological factors used to guide the need for adjuvant treatment include surgical FIGO stage (International Federation of Gynaecology and Obstetrics), histotype, tumor grade and the presence or absence of lymphovascular invasion [3,4]. Using these clinical parameters, multiple systems of risk prediction have been developed with the intention of guiding appropriate surgical and adjuvant treatment [5–9]. It is becoming increasingly evident however, that these pathological markers are not always reproducible and can be subject to marked interobserver variability [10–13]. This knowledge has served as a catalyst for the identification of objective, molecular-based prognostic determinants.

Using integrated genomic, transcriptomic and proteomic data, the Cancer Genome Atlas (TCGA) Research Network classified endometrial cancers into four prognostic molecular categories [14]. Since then two groups, including our own, have developed more pragmatic and cost-effective methods to replicate these groups [15,16]. ProMisE (Proactive Molecular Risk Classifier for Endometrial Cancer) uses an iterative combination of protein expression (immunohistochemistry) and focused sequencing to assign EC patients to one of four risk groups: MMR-D (Mismatch Repair Deficient), *POLE* EDM (Polymerase Epsilon Exonuclease Domain Mutated), p53 abn (p53 abnormal) and p53 wt (p53 wild-type), which are analogous albeit not identical to the TCGA MSI (microsatellite instability) hypermutated, *POLE* ultramutated, copy-number high and copy-number low categories.

Copy number alterations in tumor cells can be due to aneuploidies (whole or segmental) or smaller intragenic copy number variations. Large-scale copy number alterations are most often due to aneuploidy, which is also the most common genetic abnormality seen in malignant cells [17]. The prognostic value of DNA ploidy in endometrial cancer was first published by Atkin in 1959 [18]. Since then, multiple studies have evaluated the significance of aneuploidy or non-diploid DNA content in endometrial cancer [19]. DNA ploidy has been correlated with lymphovascular invasion [20] as well as lymph node involvement [21]. Ploidy has been shown to be an adverse prognosticator in microsatellite stable tumors [22] and is an independent prognostic marker in both early stage endometrioid and serous EC's [23,24]. Moreover, the methods used to test tumor ploidy have evolved from using traditionally flow cytometry (requiring the suspension of individual cells and thus making the use of archival formalin-fixed paraffin embedded tissues challenging) to automated image-based cytometry. As a result, ploidy has gained popularity over the last decade, and has been shown to be a valuable prognosticator in a wide spectrum of tumor types [25].

The primary goal of our study was to determine if DNA ploidy could be used as a surrogate marker for any of the ProMisE molecular subgroups, particularly if DNA ploidy could be used to distinguish between the p53 abn and p53 wt groups. Secondary goals of our study were to determine if abnormal DNA content (aneuploidy or tetraploidy) correlated with any traditional clinicopathologic variables and if ploidy could add additional prognostic information to any one of the four ProMisE molecular groups of endometrial cancer.

2. Methods

2.1. Selection and classification of EC cases

From a prior series of over 400 endometrial carcinomas from the Vancouver General Hospital OvCaRe Tissue Bank Repository, sequencing for the exonuclease domain (EDM) of polymerase epsilon (*POLE*) and immunohistochemistry (IHC) for DNA mismatch repair (MMR) proteins and p53 were performed as previously described [16]. Using ProMisE, cases were assigned to one of four molecular groups designated as: 1. MMR-D, 2. *POLE* EDM, 3. p53 abn or 4. p53 wt, as previously described by Talhouk et al. [16]. From this cohort, 90 of the most recent cases of EC were analyzed for DNA ploidy: 14 *POLE* EDM, 28 MMR-D, 27 p53 wt, and 21 p53 abn, with an attempt to acquire fair representation from all 4 groups. IHC for estrogen receptor (ER) and progesterone receptor (PR) were also performed on tissue microarrays. Any intensity of staining in >1% of tumor cells was considered positive.

Clinical and pathological parameters collected included age, body mass index (kg/m^2), stage (updated according to FIGO 2009 classification), grade, histological subtype, lymphovascular space invasion, nodal status, and adjuvant therapy. Clinical risk groups were assigned according to the European Society of Medical Oncologists (ESMO) criteria [26] by two clinicians. Discordant results were discussed and a consensus was reached.

Research ethics approval for the Tissue/Biospecimen Bank and this project was granted from the University of British Columbia Institutional Review Board and all patients underwent informed written consent for the use of their biospecimens for research purposes.

2.2. Image cytometric DNA ploidy analysis

DNA ploidy analyses were performed at the Institute for Cancer Genetics and Informatics, Radiumhospitalet, Oslo University Hospital. For DNA ploidy analyses, monolayers were prepared from 50 μm thick sections of tumor tissue obtained from paraffin-embedded tissue blocks as detailed [27]. Briefly, the sections were deparaffinized, rehydrated, treated with protease (Sigma P5380) and stirred with magnetic stirring bars to disaggregate the cells. After filtering and centrifuging the cell suspensions, monolayers were made on poly L-lysin coated slides using the pellets. Subsequently, the monolayers were air-dried, fixed in 4% formaldehyde, hydrolysed in 5 M HCl and stained with Schiff's solution.

Using the Ploidy Work Station (PWS) Grabber (Room4 Ltd., Crowborough, East Sussex, UK) and a Zeiss Axioplan microscope equipped with a 546-nm green filter and a monochrome high-resolution digital camera (Axiocam MRM, Zeiss, Jena, Germany), images of minimum 1500 Feulgen stained nuclei were captured automatically. The images were automatically sorted into galleries: nuclei-of-interest for measurement, lymphocytes, plasma cells and fibroblast as reference cells. The automatically sorted nuclei were manually verified and edited to discard cut, overlapped and pyknotic nuclei using PWS Classifier (Room4 Ltd., Crowborough, East Sussex, UK). Integrated optical density of each nucleus was calculated. Histograms, generated using integrated optical density, were classified as diploid, tetraploid or aneuploid. A histogram was classified as diploid if only one peak with DNA index between 0.95 and 1.05 was present, the number of nuclei with DNA index between 1.9 and 2.1 did not exceed 10% of the total number of nuclei and the number of nuclei with a DNA content more than 5c did not exceed 1%. A histogram was classified as tetraploid if a peak with DNA index between 1.9 and 2.1 contained >10% of the nuclei-of-interest. A histogram was classified as aneuploid when one or more non-euploid peaks were present (DNA index <0.95, 1.05–1.89 or >2.1) or the number of nuclei not representing euploid populations with a DNA content more than 5c exceeded 1%.

2.3. Statistical analysis

We analyzed the univariable association between DNA ploidy and each clinicopathological feature using a Chi-squared test for binary and categorical variables and a one-way analysis of variance (using Welch's *t*-test) for continuous variables. Statistical significance was set at = 0.05. Univariable survival analyses (overall survival [OS], disease-specific survival [DSS], and progression-free survival [PFS]) for DNA ploidy, ProMisE and other clinicopathologic features of interest were

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performed. Analyses of ploidy were done using 3 ploidy groups (diploid vs. aneuploid vs. tetraploid) as well as dichotomized data using 2 ploidy groups (normal DNA content [diploid] vs. abnormal DNA content [aneuploid or tetraploid]). In the analysis of ploidy and survival within the ProMisE groups, data was analyzed using only dichotomized ploidy status due to the small number of cases and events within each ProMisE group. In all Cox models, hazard ratios (HRs) with corresponding 95% confidence intervals (CI) and likelihood ratio test (LRT) P values were reported. All statistical analyses were done using R project for statistical computing (v3.3.2, 2016). Only patients who had complete clinicopathologic features were considered in the analyses. A missing value comparison was undertaken to ensure that missing values were not associated with subgroups.

3. Results

All 90 cases were successfully analyzed for ploidy status. The mean number of nuclei analyzed was 1603 per case (range 1052–1807). The mean coefficient of variation of the diploid peaks was 2.5 (range 1.01–4.62).

Out of 90 cases of EC analyzed for DNA ploidy, 55 were diploid, 28 were aneuploid and 7 were tetraploid. The clinicopathologic findings for each of the 2 ploidy groups is shown in Table 1. Abnormal DNA content (aneuploid or tetraploid) status was associated with lower BMI ($p = 0.034$), higher grade ($p = 0.001$). and negative PR status ($p = 0.033$). When DNA ploidy was stratified based on ESMO risk categories, abnormal DNA content was seen more commonly in the high risk group, although this difference did not reach statistical significance ($p = 0.176$) (Table 2). In terms of traditional histotype classification, abnormal DNA content was more frequent in non-endometrioid histotypes ($p = 0.027$). Abnormal DNA content was present in most serous carcinomas (10/12, 83.3%) and one third of endometrioid carcinomas (22/69, 31.8%). Ploidy did not clearly correlate in rare histotypes. In our series, there were 2 carcinosarcomas (one aneuploid, one diploid), 2 undifferentiated carcinomas (one aneuploid, one diploid), one clear cell carcinoma (tetraploid) and three mixed endometrioid and serous carcinomas (all diploid).

Table 1
Clinicopathologic features of endometrial carcinomas stratified by DNA ploidy.

	Diploid (N, %)	Non-diploid (N, %)
Total number of cases	55 (61.1%)	35 (38.1%)
Age mean in years (SD)	63.9 (±12.5)	62.4 (±2.3)
BMI mean (SD)	34.9 (±11.3)	33 (±1.1)
Histology		
Endometrioid	47 (52.2%)	22 (24.4%)
Non-endometrioid	8 (8.9%)	13 (14.4%)
Grade		
Grade 1/2	37 (41.1%)	10 (11.1%)
Grade 3	18 (20%)	25 (27.8%)
Stage		
Stage I	43 (47.8%)	21 (23.3%)
Stage II–IV	12 (13.3%)	14 (15.6%%)
LVSI +	26 (28.9%)	15 (16.7%)
LVSI −	26 (28.9%)	20 (22.2%)
LVSI not available	3 (0.3%)	
Nodal status		
Negative	49 (54.4%)	30 (33.3%)
Positive	5 (5.6%)	5 (5.6%)
Not available	1 (0.1%)	
Treatment		
No adjuvant treatment	29 (32.2%)	13 (14.4%)
Any adjuvant treatment	25 (27.8%)	22 (24.4%)
Not available	1 (0.1%)	
ER status		
Negative	3 (3.33%)	5 (5.6%)
Positive	50 (55.6%)	30 (33.3%)
Not available	2 (0.2%)	
PR status		
Negative	12 (13.3%)	16 (17.8%)
Positive	39 (43.3%)	17 (18.9%)
Not available	6 (0.7%)	

BMI: body mass index; LVSI: lymphovascular space invasion; ER: estrogen receptor; PR: progesterone receptor.

Table 2
DNA ploidy in ESMO risk groups.

	Diploid	Aneuploid	Tetraploid
Low (n = 31)	23 (74.2%)	8 (25.8%)	0
Intermediate (n = 16)	10 (62.5%)	3 (18.8%)	3 (18.8)
High (n = 17)	10 (58.8%)	4 (23.5%)	3 (17.6%)

ESMO: European Society for Medical Oncology.

In the entire cohort, univariate analysis demonstrated that abnormal DNA content was significantly associated with PFS ($p = 0.0094$; aneuploid vs. diploid HR:1.91 [95% CI: 0.78–4.64]; tetraploid vs. diploid HR:3.01 [95% CI: 0.76–9.33]) but not DSS or OS ($p = 0.249$ and $p = 0.187$, respectively). The tetraploid tumors had a slightly worse PFS than the aneuploid tumors (Fig. 1). ESMO risk category, stage, grade, LVSI and treatment status were all also associated with worse survival (OS, DSS, and PFS) with statistical significance ($p < 0.05$).

When DNA ploidy was analyzed in the context of ProMisE, 80.9% (17/21) of the p53 abn group had abnormal DNA content, as compared to 37.0% (10/27) of p53 wt, 28.6% (4/14) of *POLE* EDM and 14.3% (4/28) of MMR-D ($p < 0.001$)(Table 3). Within the ProMisE subgroups, aneuploid tumors were more common than tetraploid in all groups except for *POLE* EDM, where three out of four cases with abnormal DNA content were tetraploid and the remaining one case was aneuploid.

In the MMR-D EC's, abnormal DNA content (tetraploid/aneuploid) was associated with a significantly worse OS (HR: 3.87; 95% CI: 0.96–15.62, $p = 0.041$) and PFS (HR: 7.87; 95% CI: 1.30–40.8, $p = 0.011$) (Fig. 2). Four cases in the MMR-D exhibited abnormal DNA content. Interestingly, the four MMR-D cases with abnormal DNA content had normal p53 status (3 cases were assessed using mutation testing and immunohistochemistry, and 1 case by immunohistochemistry only). Three cases were endometrioid carcinoma FIGO grade 3/3 (stage IA and stage 2). One case was endometrioid carcinoma FIGO grade 2/3 and stage 2. The remaining case was an undifferentiated carcinoma and stage IA.

The finding of abnormal DNA content did not have statistically significant prognostic significance (OS, DSS, PFS) within each of the *POLE* EDM (OS HR: 2.09, 95% CI: 0.17–26.04, $p = 0.60$; DSS HR: 0.83, 95% CI: 0.01–15.62, $p = 0.53$; PFS HR: 6.75, 95% CI: 0.36–984, $p = 0.13$), the p53 wt group (OS HR: 0.87, 95% CI: 0.08–6.60, $p = 0.80$; DSS HR: 0.87, 95% CI: 0.08–6.60, $p = 0.80$; PFS HR: 1.93, 95% CI: 0.30–12.56, $p = 0.50$) and the p53 abn groups (OS HR: 1.77, 95% CI: 0.22–14, $p = 0.59$; DSS HR: 3.18, 95% CI: 0.37–416, $p = 0.23$; PFS HR: 3.96, 95% CI: 0.48–514, $p = 0.18$) (see Supplemental Fig. S1). Within p53 abn EC, abnormal DNA content trended towards a poorer prognosis, although this finding did not reach statistical significance. Even though the p53 wt group had a substantial number of aneuploid/tetraploid cases (37%), DNA ploidy status did not further differentiate outcomes within this group.

4. Discussion

The ongoing challenge with the management of EC lies in the irreproducibility of the current classification system, which uses pathological features to guide risk stratification. To this extent, we have developed and validated ProMisE (Proactive Molecular Risk Classifier for Endometrial Cancer), an objective, reproducible and prognostically-driven molecular classifier that was constructed on the basis of The Cancer Genome Atlas' (TCGA) collaborative project [16,28]. In ProMisE, abnormal p53 immunohistochemistry is used to assign the p53 abn group, which serves as the corollary to the TCGA's copy number high (CN-H) or "serous-like" group. It is well-established that

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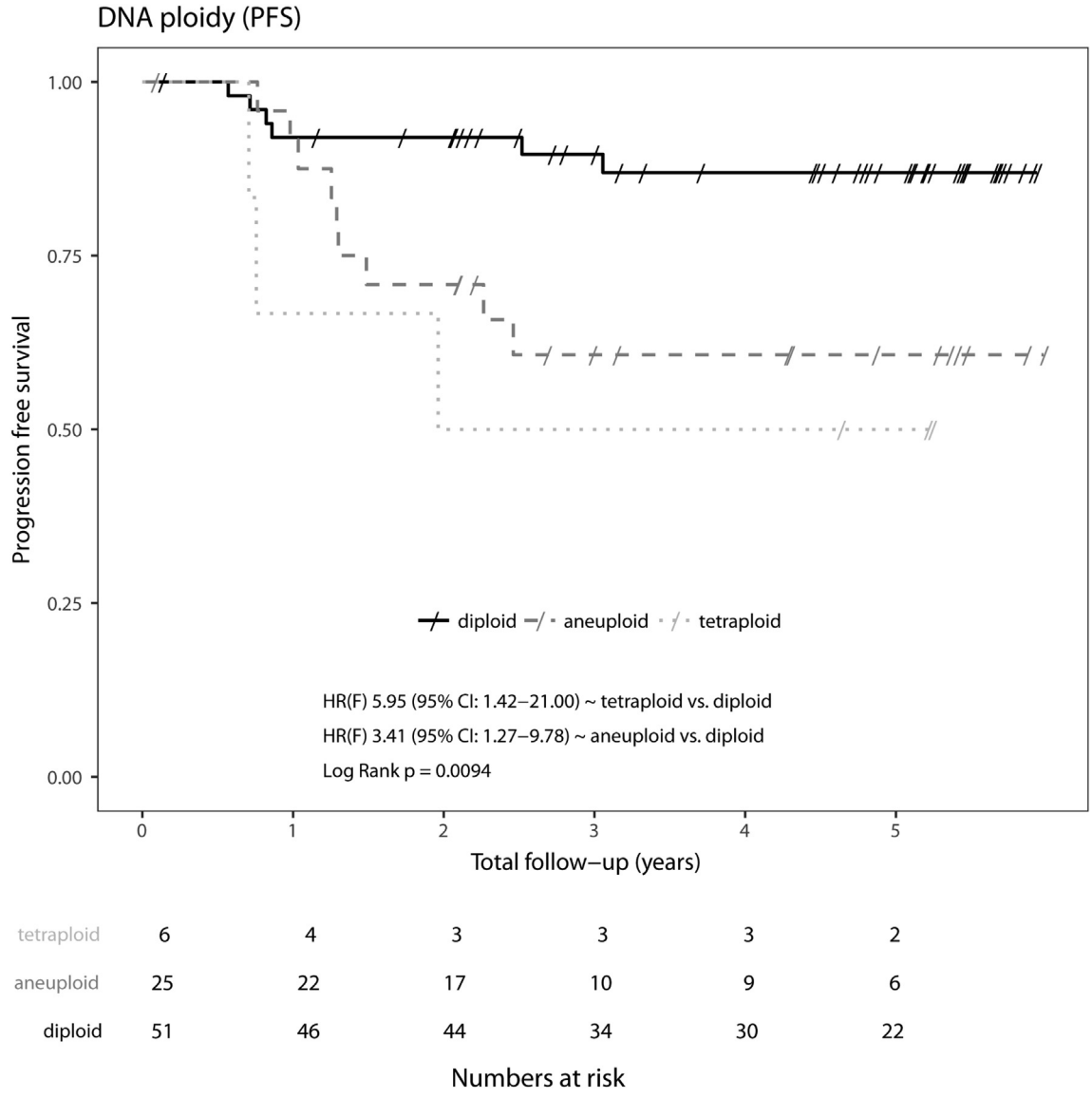

Fig. 1. Kaplan-Meier Curve showing progression free survival (PFS) of the endometrial carcinoma patients with diploid, tetraploid and aneuploid tumors.

p53 serves as an excellent diagnostic marker for serous carcinomas and is a predictor of poorer outcomes in EC [29,30], but we acknowledge that p53 is not directly related to copy-number.

In our development of ProMisE [16] we had explored different methods for identifying copy number status and the 'CN-high' TCGA subgroup, using fluorescence in-situ hybridization (FISH) for FGFR, SOX17 and MYC (3 loci which were most predictive of overall copy number status in the TCGA) but found poor correlation with outcome parameters. Particularly, more than half of cases that harboured TP53 mutations were not identified through FISH in contrast to TCGA findings that over 80% of tumors with TP53 mutations were captured in the CN-H group. Thus FISH did not prove to be a good surrogate for the CN-H group and was work intensive with a high level of subjectivity [16]. As a consequence of these prior findings, we decided to explore whether aneuploidy, a finding more reflective of copy-number alterations, was able to better distinguish the CN-H group from the other molecular groups of endometrial cancer.

Table 3
DNA ploidy in ProMisE subgroups.

	Diploid	Aneuploid	Tetraploid
MMR-D (n = 28)	24 (85.7%)	3 (10.7%)	1 (3.6%)
POLE EDM (n = 14)	10 (71.4%)	1 (7.1%)	3 (21.4%)
p53 wt (n = 27)	17 (63.0%)	10 (37.0%)	0 (0%)
p53 abn (n = 21)	4 (19.0%)	14 (66.7%)	3 (14.3%)

ProMisE: Proactive Molecular Risk Classifier for Endometrial Cancer; MMR-D: Mismatch Repair Deficient; *POLE* EDM: *POLE* ultramutated; p53 wt: p53 wild type; p53 abn: p53 abnormal.

In our study we saw that 38.9% of EC had abnormal ploidy, a proportion similar to prior studies using image cytometry, which ranged from 38 to 48% [31,32]. We found that DNA ploidy did correlate with the ProMisE subgroups ($p < 0.001$), with abnormal DNA content (aneuploid or tetraploid) being highest in the most aggressive of the four ProMisE categories (80.9% of p53 abn). There were smaller proportions of tumors in the p53 wt, *POLE* EDM and MMR-D groups that also had abnormal ploidy; therefore ploidy was not entirely specific for the p53 abn category. In particular, ploidy did not distinguish between the p53 abn/copy-number high and p53/copy-number low groups, which we initially hypothesized, and is inferior to p53 as a surrogate marker in making this distinction.

It is estimated that approximately 20% of EC will recur despite having low-risk histopathologic features [19]. Susini et al. performed a 10-year prospective study on EC and found that aneuploidy was able to predict high-risk cases amongst patients thought to be low-risk based on

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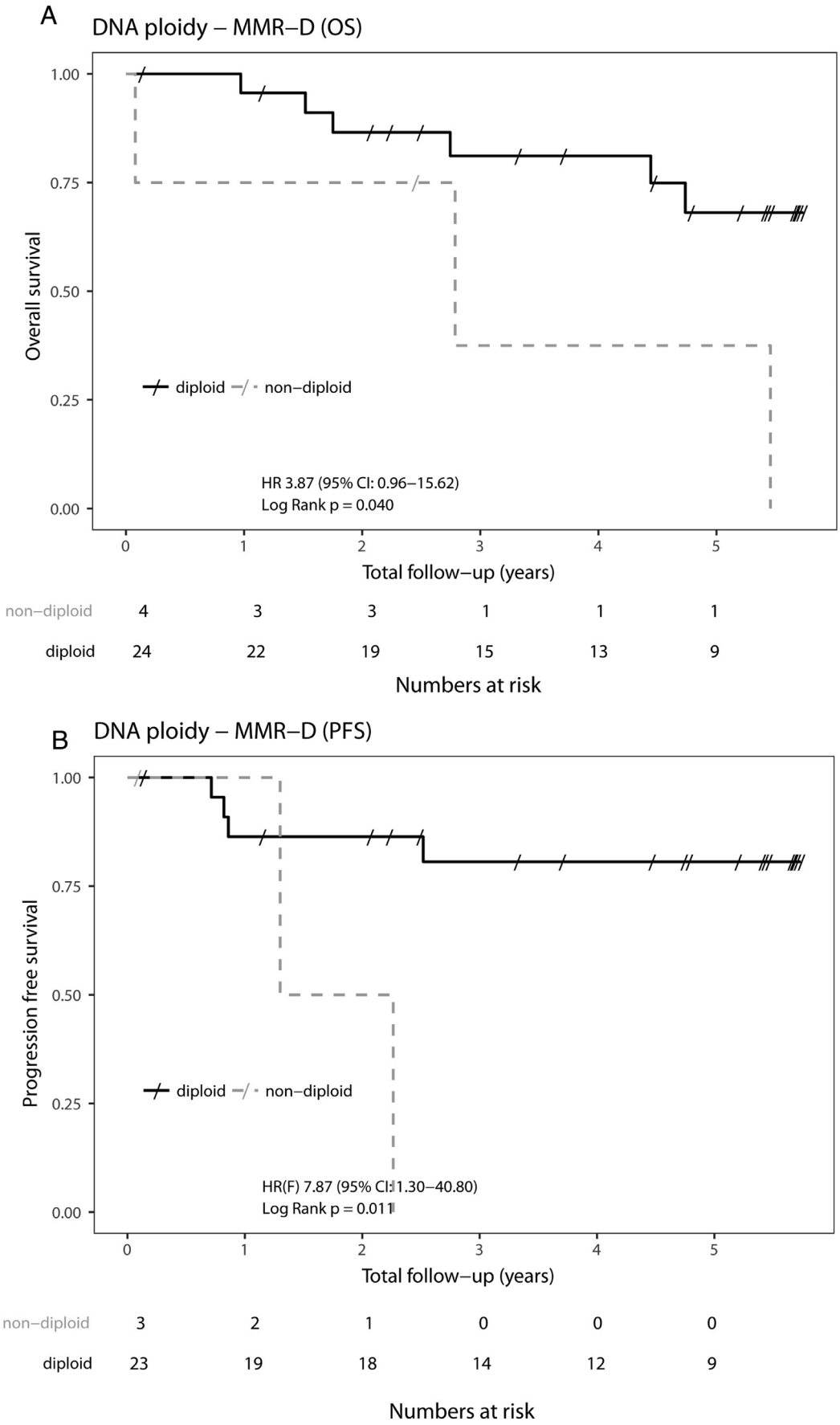

Fig. 2. Kaplan Meier Curves showing A) Overall Survival (OS) and B) progression free survival (PFS) for the patients with MMR-deficient endometrial cancers classified according to DNA ploidy. Patients with a non-diploid tumor had a worse prognosis compared to the patients with a diploid tumor.

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standard tumor stage and grade assessment [33]. The p53 wt group remains the largest in size, constituting almost half of EC, in the ProMisE classifier. There remains a need to identify additional prognostic markers within this large p53 wt group, to detect the minority of cases that tend to behave poorly despite low-risk clinicopathologic features. In our series, p53 wt had the second highest number of cases with abnormal DNA content, but the addition of DNA ploidy unfortunately did not help to further prognosticate the p53 wt group.

As previously described, MMR deficient EC's often present with FIGO stage I disease (74.6%) however these tumors tend to have high risk clinicopathologic uterine factors (81.2% grade 3, 42% deep myometrial invasion and 45% LVSI) [28]. MMR-D EC's also have the second worst observed outcomes of the ProMisE groups, after p53 abn [28]. In analyzing the prognostic implications of DNA ploidy within the four ProMisE subgroups, we found that abnormal DNA ploidy was associated with a significantly worse OS and PFS in the MMR-D group, and this finding merits validation in a future study including a larger series of EC.

The newly described phenotype of women with *POLE* EDM ECs are younger, thinner women, who similar to MMR-D, have tumors with aggressive pathologic features (70% grade 3, 35% deep myometrial invasion, 40% LVSI). Unlike MMR-D however, *POLE* EDM has favorable outcomes comparable to p53 wt. No clear association between ploidy and survival was seen in the *POLE* EDM group.

Genomic instability is a major hallmark of cancer cells, and describes the perturbation of genetic material via mutations, chromosomal rearrangements or aneuploidies. Such genetic alterations lead to a genome that is more susceptible to acquiring driver mutations or losing tumor suppressor genes, ultimately leading to oncogenesis and tumor progression [19]. In the *POLE* EDM group, genomic instability is acquired through the loss of *POLE*, which encodes for a DNA polymerase with special proofreading function, resulting in ultramutated tumors (with $>200 \times 10^{-6}$ mutations/Mb) [14]. In the MMR-D group, genomic instability occurs through the loss of DNA mismatch repair proteins, through genetic or epigenetic mechanisms, causing frameshift mutations at multiple microsatellite loci [14]. As the underlying mechanisms of genomic instability in the *POLE* EDM and MMR-D groups are not primarily due to aneuploidies, or alterations changing large segments of DNA, it is therefore plausible that the addition of DNA ploidy information within these two particular groups may bear only minor prognostic significance.

There is still some uncertainty as to whether DNA ploidy can be exploited in the clinical setting. Njølstad et al. investigated 785 ECs and found that non-diploid status on endometrial curettage was associated with greater lymph node metastases, suggesting ploidy status may be used to guide surgical management [21]. However, Pradhan et al. found that ploidy status was discrepant between biopsy and hysterectomy in approximately one quarter of cases, with more robust results in the hysterectomy [34]. As ultimately we plan to apply ProMisE to diagnostic endometrial specimens to provide early biologically relevant information for patients and clinicians, the value/accuracy of ploidy in these specimens remains to be determined. There appeared to be at least trends towards worse prognosis in many of the ProMisE groups with abnormal DNA content, but were not sizable enough to reach a statistical significance; This trend does merit further investigation in a larger cohort of EC, particularly in the MMR-D group. We recognize that our study size may be underpowered in detecting subtle prognostic changes within each ProMisE category.

In summary, DNA ploidy correlated with high-grade tumors, non-endometrioid histotypes and poor PFS in our series of EC. DNA ploidy was highest in the p53 abn group, with smaller numbers in the p53 wt, *POLE* EDM and MMR-D groups. Although abnormal DNA content was highest in the p53 abn group, it was not an entirely specific marker for the p53 abn group and did not distinguish between the p53 abn and p53 wt groups. Abnormal DNA ploidy was associated with worse OS and PFS in the MMR-D group, with no statistically significant associations of DNA ploidy and survival in the remaining ProMisE molecular subgroups. The value of ploidy in the MMR-D group warrants further study.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2017.06.020.

Conflicts of interest

US patent pending for ProMisE #62192230. BC Cancer Agency is the named party.

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