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## Integration of clinical sequencing and immunohistochemistry for the molecular classification of endometrial carcinoma

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### Abstract

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B. Weigelt, N.R. Abu-Rustum and C. Aghajanian conceived the study. B. Weigelt, L.H. Ellenson and J.J. Mueller supervised the study. E. Rios-Doria, B. Weigelt, L.H. Ellenson, A. Momeni-Boroujeni, C. Friedman and Carol Aghajanian developed methodology. L.H. Ellenson and A. Momeni-Boroujeni performed histologic review. E. Rios-Doria and M. Wu collected clinical data. A. Momeni-Boroujeni, P. Selenica and A. Marra performed bioinformatics analyses. Q.C. Zhou and A. Iasonos performed statistics analyses. E. Rios-Doria, L.H. Ellenson, B. Weigelt, P. Selenica and A. Marra performed data curation. M.M. Leitao Jr, K.M. Alektiar, Y. Sonoda, V. Makker, E. Jewell, Y. Liu, D. Chi, D. Zamarin, C.F. Friedman, N.R. Abu-Rustum, C. Aghajanian, and J.J. Mueller provided patients. E. Rios-Doria, A. Momeni-Boroujeni, P. Selenica, A. Marra, C.F. Friedman, N.R. Abu-Rustum, C. Aghajanian, J.J. Mueller, L.H. Ellenson and Britta Weigelt interpreted results. E. Rios-Doria, B. Weigelt, L.H. Ellenson, J.J. Mueller and C.F. Friedman drafted the manuscript. All authors reviewed and approved the final version of the manuscript.

#### CONFLICTS OF INTEREST

The remaining authors have no conflicts of interest to declare.

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**Purpose:** Using next generation sequencing (NGS), The Cancer Genome Atlas (TCGA) found that endometrial carcinomas (ECs) fall under one of four molecular subtypes, and a *POLE* mutation status, mismatch repair (MMR) and p53 immunohistochemistry (IHC)-based surrogate has been developed. We sought to retrospectively classify and characterize a large series of unselected ECs that were prospectively subjected to clinical sequencing by utilizing clinical molecular and IHC data.

**Experimental Design:** All patients with EC with clinical tumor-normal MSK-IMPACT NGS from 2014–2020 (n=2,115) were classified by integrating molecular data (i.e., *POLE* mutation, *TP53* mutation, MSI sensor score) and MMR and p53 IHC results. Survival analysis was performed for primary EC patients with upfront surgery at our institution.

**Results:** Utilizing our integrated approach, significantly more ECs were molecularly classified (1,834/2,115, 87%) as compared to the surrogate (1,387/2,115, 66%, p<0.001), with an almost perfect agreement for classifiable cases (Kappa 0.962, 95% CI 0.949–0.975). Discrepancies were primarily due to *TP53* mutations in p53-IHC-normal ECs. Of the 1,834 ECs, most were of copy number (CN)-high molecular subtype (40%), followed by CN-low (32%), MSI-high (23%) and *POLE* (5%). Histologic and genomic variability was present among all molecular subtypes. Molecular classification was prognostic in early- and advanced-stage disease, including early-stage endometrioid EC.

**Conclusions:** The integration of clinical NGS and IHC data allows for an algorithmic approach to molecularly classifying newly diagnosed EC, while overcoming issues of IHC-based genetic alteration detection. Such integrated approach will be important moving forward given the prognostic and potentially predictive information afforded by this classification.

## Keywords

molecular classification; endometrial carcinoma; clinical sequencing; mutation; immunohistochemistry

## 1. INTRODUCTION

Endometrial cancer (EC) is the sixth most common cancer worldwide with an increasing incidence and disease-associated mortality in 2020, estimated at 417,000 and 97,000, respectively [1]. Similar to other solid tumors, EC is a clinically, histologically and molecularly heterogeneous disease [2]. Through comprehensive molecular analysis, The Cancer Genome Atlas (TCGA) identified four molecular EC subtypes: 1) *POLE* (ultramutated) harboring *POLE* exonuclease domain hotspot mutations, 2) microsatellite instability (MSI)-high (hypermutated, MSI-H, DNA mismatch repair [MMR]-deficient), 3) copy number-high (CN-H) harboring high levels of CN alterations and recurrent *TP53* mutations, and 4) copy number-low (CN-L) lacking defining features of the other subtypes, also referred to as ECs of ‘non-specific molecular profile’ [3]. For implementation of molecular classification into the clinic, a surrogate, the Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE) algorithm, was developed [4]. This surrogate involves the mutational assessment of the *POLE* exonuclease domain and immunohistochemical (IHC) analysis of the MMR proteins (i.e., MLH1, MSH2, MSH6 and PMS2) and p53 [5], and has now been included in the NCCN guidelines and World Health Organization (WHO)

classification of Female Genital Tumors [6, 7]. ProMisE identifies four molecular subtypes that are analogous but not identical to the genomic subtypes described in TCGA [8]. For example, in the original TCGA study [3], only 92% of ECs classified as CN-H molecular subtype harbored *TP53* mutations, suggesting that the surrogate classifier misses a subset of non-*TP53*-altered CN-H ECs.

Integration of clinical next-generation sequencing (NGS) assays of tumor and germline DNA holds expanding diagnostic, prognostic, and therapeutic implications. The use of NGS also provides an orthogonal approach for the identification of MSI or *TP53* mutations not captured by IHC. Since 2014, the FDA-authorized tumor-normal MSK-IMPACT (Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets) targeted sequencing assay has been utilized at our institution [9, 10], and is offered to all newly diagnosed EC patients presenting for care. In addition to a high-depth NGS-based detection of mutations and CN alterations, MSK-IMPACT provides information on MSI and tumor mutational burden [10]. In this study, we sought to retrospectively classify a large series of unselected ECs of all histologic types by integrating clinical molecular data with IHC results. We further aimed to demonstrate the utility of relevant alternative parameters for molecular classification, including MSI sensor score and *TP53* mutation, define the concordance with the conventional molecular subtyping approach (ProMisE), and assess mutational profiles and oncologic outcomes of the defined molecular subtypes and specific histologic subsets of the disease.

## 2. MATERIALS AND METHODS

### 2.1. Case selection

This retrospective study was approved by the Institutional Review Board at MSK, and all patients provided written consent for genomic profiling. ECs subjected to clinical tumor-normal MSK-IMPACT sequencing from test inception in 2014 through 12/31/2020 were included (n=2,121; Fig. 1A). MSK-IMPACT sequencing was performed on tumor tissue from the primary diagnostic procedure (hysterectomy or pre-operative biopsy) or from recurrent tumor sample. For patients with multiple specimens that underwent MSK-IMPACT, sequencing data from the earliest occurrence (i.e., primary tumor or first recurrence specimen) were evaluated. Demographic and clinical data were extracted by review of electronic medical records. Cases were eligible for clinical outcomes analyses if MSK-IMPACT was performed prior to a documented recurrence with resultant unambiguous molecular classification, and if treatment planning with upfront surgical staging was performed at our institution (Supplementary Fig. S1).

### 2.2. Histologic typing, MMR and p53 IHC

Pathology reports authored by departmental gynecologic pathologists throughout the study timeframe were reviewed. These contain histopathologic data evaluated through a uniform diagnostic approach with biweekly diagnostic consensus conferences, as previously described [11]. Histologic type, FIGO 2009 stage, and endometrioid tumor grade were recorded based on the patient's initial pathologic diagnosis. All histologic subtypes were included (i.e., endometrioid, serous, clear cell, carcinosarcoma, undifferentiated/

dedifferentiated, and mixed/high-grade not otherwise specified (NOS)). ECs of unclassified histology were re-reviewed by gynecologic pathologists (L.H.E. or A.M.-B.) and, if unresolved, remained in 'other'. Similarly, ECs of endometrioid histology without defined FIGO grading were re-reviewed and designated 'N/A' if unresolved. The highest histologic grade for endometrioid type ECs was recorded from either the pre-operative biopsy or hysterectomy specimen. MMR and p53 IHC were generally performed on the same specimen as DNA extraction for MSK-IMPACT sequencing. If either IHC staining was missing, it was obtained from the pre-operative or recurrence specimen, if available. ECs lacking MLH1 or PMS2 protein expression were typically subjected to *MLH1* promoter methylation analysis [12].

### 2.3. Genomic data extraction

The genomic data extracted from the MSK-IMPACT assay, which targets between 341 (2014) and 505 (2020) cancer-related genes, included somatic mutation count, *POLE* mutational status, *TP53* mutation status, MSI sensor score, tumor purity, fraction of genome altered (FGA), and tumor mutational burden (TMB) [10, 11]. *POLE* exonuclease domain hotspot mutations were defined based on Leon-Castillo et al. [13]. MSI sensor scores of  $\geq 10$  were considered MSI-H, between 3 and  $<10$  as MSI-indeterminate, and  $<3$  as stable (MSS), as described [14].

### 2.4. Molecular classification of EC integrating clinical NGS and IHC data

Given that mutation and CN alteration detection as well as MSI sensor score are affected by the tumor cell content of a given sample, we included only high-quality samples with a minimum tumor purity of 20%. In addition, samples lacking any somatic mutations were excluded (Fig. 1A). Molecular subtype assignment was performed hierarchically (Fig. 1B): i) *POLE* molecular subtype was defined by the presence of a known *POLE* hotspot exonuclease domain mutation [13]. If a somatic *POLE* mutation was present at a known hotspot position/residue with a different amino acid change (e.g., V411M rather than known hotspot V411L), it was deemed unclassifiable. ii) MSI-H molecular subtype was assigned if the MSI sensor score was  $\geq 10$  [14, 15] and/or if MMR-deficient (MMRd) based on IHC irrespective of MSI sensor score. MMRd by IHC was defined as absence of MLH1, MSH2, and/or PMS2 and MSH6 protein expression from all tumor cell nuclei, in the presence of an internal control [16]. IHC results with equivocal or inconclusive staining results were not considered MMRd if the MSI sensor score was  $<10$ . iii) CN-H molecular subtype was assigned based on the presence of a *TP53* homozygous deletion or a pathogenic driver mutation defined by OncoKB, CIViC, and/or Cancer Hotspots in cBioPortal [17–19]. When available, p53 IHC results were used as a reference. We also assigned *TP53* wild-type ECs to the CN-H molecular subtype if they harbored an *MDM2* amplification paired with aberrant p53 IHC expression [20]. iv) CN-L molecular subtype was assigned if a tumor sample did not harbor any of the defining features of the other subtypes [21, 22]. ECs with ambiguous results were subjected to a central re-review of histologic type (see above), IHC results and/or molecular findings (E.R-D., A.M.-B., L.H.E and B.W.); if unresolved, ECs were deemed 'unclassifiable' and excluded from classification and downstream analyses. In addition, ECs were classified into the molecular subtypes according to the modified

ProMisE algorithm as reported in the NCCN/ WHO guidelines [5–7], utilizing i) *POLE* hotspot mutation, ii) MMRd by IHC, and iii) p53 abnormal by IHC.

## 2.5. Statistical analysis

Continuous variables were assessed by Kruskal-Wallis rank sum test and categorical variables by Fisher's exact test. Cohen's  $\kappa$  coefficients and corresponding 95% confidence intervals were used to measure agreement between molecular subtype assignments [23]. Progression-free survival (PFS) was measured from the date of surgery to the date of last gynecologic assessment or recurrence or death, whichever first. Disease status and disease progression were extracted from medical oncology notes, in combination with follow-up imaging studies according to the PRISSMM data model approach [24]. Overall survival (OS) was measured from date of surgery to date of last known follow-up or date of death. PFS and OS related analyses were only performed including patients who were treated at MSK for their primary disease and using their primary tumor sample for MSK-IMPACT. Kaplan-Meier method was applied to estimate the median survival time and survival rate at a specific time. The associations between variables and survival outcomes were tested by using Log-rank test for categorical variables and Wald test based on Cox-proportional hazard (CoxPH) model for continuous variables. Landmark analyses, with one month landmark time point, was applied to examine time-dependent variable of adjuvant treatment [25]. Multivariate CoxPH models were built to determine the independent prognostic factors for PFS and OS. All analyses were performed using R 4.1.2 (<https://www.R-project.org/>). All p-values were two-sided. A p-value less than 0.05 was considered significant.

## 3. RESULTS

### 3.1. Molecular subtypes of ECs subjected to clinical NGS sequencing

Of the 2,115 ECs subjected to MSK-IMPACT sequencing between 1/1/2014 and 12/31/2020, 281 ECs (13%) were excluded due to low tumor purity, lack of somatic mutations, ambiguous results, and/or duplicates, leaving a final cohort of 1,834 ECs of all histologic subtypes for molecular classification (Fig. 1A). For most ECs (82%, 1,501/1,834), clinical sequencing was performed on the initial diagnosis specimen, 323 (18%) on the recurrence tumor specimen, and 10 (0.5%) on non-descriptive tumor specimens. In the primary diagnosis group, MSK-IMPACT sequencing was performed primarily on hysterectomy specimens (1,312/1,501, 87%) rather than pre-operative biopsies (189/1,501, 13%). In the recurrence tumor group, MSK-IMPACT sequencing was mostly performed on the recurrence biopsy itself (318/323, 98.5%), and rarely on the initial diagnostic tumor. MMR and p53 IHC were performed clinically for 1,470 (80%) and 1,143 (62%) ECs, respectively.

Integrating the clinical NGS and IHC data for molecular classification (Fig. 1B) revealed that the majority of ECs at our tertiary cancer center were of CN-H molecular subtype (734/1,834, 40%), followed by CN-L (575/1,834, 31.3%), MSI-H (428/1,834, 23.3%) and *POLE* (97/1,834, 5.3%) molecular subtypes (Fig. 1C). The CN-H ECs either harbored pathogenic driver *TP53* mutations (n=729), *TP53* homozygous deletions (n=4) or *MDM2* amplification coupled with aberrant p53 IHC expression (n=1). For 63% (459/734) of CN-H

ECs, p53 IHC was available; of the 733 CN-H ECs with *TP53* alterations, 94% (431/458) had aberrant p53 protein expression (Fig. 2A). Pathogenic *TP53* missense mutations were the most commonly identified in CN-H ECs (512/734); followed by frameshift deletions (64/734) and nonsense mutations (58/734; Fig. 3A). The 27 *TP53*-mutant CN-H ECs with p53 wild-type expression by IHC harbored hotspot missense mutations (15/27), frameshift deletions (5/27), nonsense mutations (4/27), hotspot splice-site mutations (2/27) or a homozygous deletion (1/27). As expected, the majority of CN-H ECs were of high-grade histologic subtypes (Table 1). In contrast, ECs of CN-L molecular subtype (575/1,834, 31%) were primarily of endometrioid histology (482/575, 84%), but also included unclassified (28/575, 5%), clear cell (20/575, 3%) and mixed/ high-grade NOS ECs (17/575, 3%; Fig. 1C, Table 1).

Of the 428 ECs of MSI-H molecular subtype, MMR IHC was available for 402 cases (94%). Of the 289 ECs with an MSI sensor score  $\geq 10$ , MMR IHC results were concordant in 95% of ECs (250/289). In 39 ECs classified as MSI-H based on MSI sensor, 67% (26/39) did not have MMR IHC performed and 33% (13/39) had intact MMR protein expression (Fig. 2B). The ECs with intact MMR expression did not have methylation studies performed. Of note, the median tumor mutation burden (TMB) did not differ between MSI-H ECs with an MSI sensor score  $\geq 10$  and loss of MMR protein expression as compared to those with an MSI sensor score  $\geq 10$  and intact MMR protein expression (34.5 mt/Mb vs 36.0 mt/Mb,  $p=0.98$ ). The remaining 139 ECs had an MSI sensor  $<10$  and were classified as MSI-H molecular subtype based on loss MMR protein expression by IHC. The predominant histology of MSI-H ECs was endometrioid (345/428, 81%), and un-/dedifferentiated ECs were commonly of MSI-H molecular subtype (25/39, 64%).

Of the ECs subjected to clinical sequencing, 5% were of *POLE* molecular subtype (97/1,834). V411L and P286R were the most common *POLE* hotspot mutation identified (38/97, 39% and 33/97, 34%, respectively; Supplementary Fig. S2A). Like MSI-H or CN-L ECs, *POLE* ECs were primarily of endometrioid histology (75/97, 77%). Of note, mixed/ high-grade NOS was found in 10 *POLE* ECs (10%) and another 9 (9.3%) *POLE* ECs were of unclassified/other histology (Figs. 1 and 3).

### 3.2. Comparison of integrated vs conventional surrogate molecular subtyping approach

We next sought to compare our integrated molecular and IHC-based with the conventional ProMisE molecular subtyping. As noted above, MMR IHC (80%) and p53 IHC (62%) were clinically performed only for a subset of ECs. With the addition of tumor-normal clinical sequencing data to IHC results we classified a significantly larger set of ECs from our initial 2,115 patient cohort into the molecular subtypes (1,834/2115, 86.7%) as compared to the ProMisE algorithm (1,387/2115, 65.5%,  $p<0.001$ ), the latter of which requires both MMR and p53 IHC for all non-*POLE* cases.

Using the final study cohort ( $n=1,834$ ), we next ascertained agreement between molecular subtype assignments by our integrated and the ProMisE classification. Overall, we found only a moderate agreement between our integrated molecular/ IHC and the ProMisE classification due to the large number of unclassified cases (Kappa 0.555, 95% confidence interval (CI) 0.531–0.579); Fig. 2C, Supplementary Table S1). When focusing on the ECs

classifiable by the ProMisE algorithm (n=1,208), we recorded an almost perfect agreement (Kappa 0.962, 95% CI 0.949–0.975). Misclassifications were due to normal MMR or p53 expression patterns by IHC in the presence of high MSIsensor scores or *TP53* mutations, respectively. A subset of CN-L ECs (n=25) by ProMisE harbored *TP53* alterations but had normal p53 IHC-based expression, which were classified as CN-H using our integrated approach. In addition, a subset of CN-L (n=6) and CN-H (n=2) ECs by ProMisE had high MSIsensor scores but were MMR-proficient by IHC (Fig. 2C, Supplementary Table S1).

### 3.3. Clinicopathologic and somatic genomic features of EC molecular subtypes

There was a significant difference amongst the four molecular subtypes assigned by our integrated classification when comparing BMI, age at diagnosis, histology, stage, and adjuvant treatment type (p<0.001 each; Table 1). Surgical staging data were available for 90% of the ECs with molecular classification (1,657/1,834); the remaining either had clinical staging (advanced/metastatic disease) or no regional lymph node assessment at time of surgery. Patients with CN-H ECs had a greater association with stage III/IV disease (57% vs. 12–30%, p<0.001) and adjuvant chemotherapy with or without radiation treatment (77% vs 23–40%, p<0.001; Table 1). There was no difference in histology (p=0.24) or FIGO stage (p=0.09) between primary EC (n=1,501) and recurrent EC (n=323) tissue subjected to MSK-IMPACT sequencing (Supplementary Table S2).

Consistent with previous observations [3, 26], endometrioid cancers were preferentially of CN-L and MSI-H molecular subtypes (Supplementary Fig. S2B). In our series, 4% (30/760) of grade 1/2 and 20% (37/183) of grade 3 endometrioid ECs were of CN-H molecular subtype, as were almost all the serous ECs (265/274, 97%) and carcinosarcomas (195/223, 87%; Supplementary Fig. S2B).

Assessment of the somatic pathogenic mutations, amplifications and homozygous deletions affecting the cancer-related genes tested revealed that *PIK3CA*, *PTEN*, *TP53* and *ARID1A* alterations were the most common across all molecular subtypes (Fig. 3A). *ERBB2* and *CCNE1* amplification were primarily restricted to CN-H ECs (*ERBB2* amplification CN-H 13.4%, CN-L 1.7%; *CCNE1* amplification CN-H 17.4%, CN-L 0.9%) and absent in MSI-H and *POLE* ECs (Fig. 3A). Consistent with the whole-exome sequencing-based data from TCGA [3], the tumor mutational burden (TMB) was highest for *POLE* ECs (median 159.4 mut/Mb, range 19.3–667.9) compared to ECs of MSI-H molecular subtype (median 30.1 mut/Mb, range 0.0–397.9) or CN-H (4.4 mut/Mb, range, 0.8–74.6) and CN-L ECs (median 6.1, range 0.0–228.2, p<0.001; Fig. 3B). Chromosomal instability was highest in ECs of CN-H molecular subtype (median FGA 24.7, range 0.0–95.7) compared to CN-L ECs (median FGA 3.5, range 0.0–79.7) or the hypermutated MSI-H (median FGA 2.2, range 0.0–62.2) and *POLE* ECs (median FGA 0.1, range 0.0–16.8; p<0.001; Fig. 3C).

### 3.4. Association with oncologic outcome

The median follow-up time for the 925 primary EC patients that met criteria for survival analysis was 22.3 months (range 0.5–214 months; CONSORT diagram, Supplementary Fig. S1). In this subset of patients there was a significant difference between ECs of

the different molecular subtypes when comparing clinical characteristics ( $p < 0.001$  each; Supplementary Table S3).

When assessing the one-year progression-free (PFS) and overall survival (OS), we found that patients with ECs of *POLE* molecular subtype had the best outcomes, and those with ECs of CN-H molecular subtypes had the worst outcomes (one-year PFS: *POLE* 98.7% (95% CI 91.1–99.8%), CN-L 94.6% (95% CI 91.2–96.6%), MSI-H 91.3% (95% CI 86.7–94.4%), and CN-H 73.0% (95% CI 67.2–78%,  $p < 0.001$ ; one-year OS *POLE* 100%, MSI-H 98.1% (95% CI 95–99.3%), CN-L 99.3% (95% CI 97.3–99.8%), and CN-H 94.9% (95% CI 91.4–97%),  $p < 0.001$ ; Fig. 4A). Multivariate analysis revealed that age at diagnosis (i.e.,  $\geq 60$  years; HR 1.75, 95% CI 1.22–2.50), FIGO stage (i.e., stage III HR 4.23, 95% CI 2.77–6.44; and stage IV HR 7.13, 95% CI 4.63–11.0) and non-endometrioid histology (HR 1.78, 95% CI 1.12–2.82) remained statistically significantly associated with poor PFS (Supplementary Table S4). The multivariate analysis for OS had significant associations with age at diagnosis ( $\geq 60$  years; HR 2.57, 95% CI 1.32–5.02) and FIGO stage (i.e., stage III HR 4.25, 95% CI 1.99–9.08; and stage IV 9.43, 95% CI 4.45–20.0).

As a next step, we assessed the association of the molecular subtypes with outcomes separately for EC patients diagnosed with early-stage disease (stages I/II) and advanced-stage disease (stages III/IV). The molecular classification continued to be associated significantly with outcome, and *POLE* and CN-H ECs had the best and the worst outcomes in either early-stage or late-stage disease, respectively. We did observe, however, that there was a substantial decrease in advanced-stage survival outcomes for MSI-H (1-year PFS 82.8%, 95% CI 70.4–90.4%) and advanced-stage CN-L (1-year PFS 77.9%, 95% CI 61.8–87.8%) when compared to early-stage MSI-H (1-year PFS 94.2%, 95% CI 89.2–97%) and early stage CN-L (1-year PFS 97.2%, 95% CI 94.1–98.6%), respectively (Figs. 4B and 4C; Supplementary Fig. 3).

We further sought to assess whether the molecular subtype classification is prognostic in early-stage endometrioid ECs. While the majority of stage I/II endometrioid ECs were of CN-L ( $n=266$ , 53%) or MSI-H ( $n=152$ , 30%) molecular subtypes, stage III/IV ECs were most commonly MSI-H ( $n=48$ , 52%) or CN-L ( $n=29$ , 31%;  $p < 0.001$ ; Supplementary Table S5). The molecular subtypes were significantly associated with PFS in stage I/II endometrioid ECs, with *POLE* and CN-L ECs having the best (2-year PFS *POLE*: 96% (95% CI, 86–99%); CN-L: 98% (95% CI, 95–99%)), MSI-H intermediate (88%; 95% CI, 80–93%) and CN-H ECs having the worst outcomes (72%; 95% CI, 43–88%;  $p < 0.001$ ; Fig. 4D).

### 3.5. Serous ECs, endometrioid ECs and carcinosarcomas of CN-H molecular subtype

Analysis of the levels of chromosomal instability amongst CN-H serous ECs ( $n=265$ ), CN-H endometrioid ECs ( $n=74$ ) and CN-H carcinosarcomas ( $n=195$ ) revealed that carcinosarcomas of CN-H molecular subtype had significantly higher FGAs (median 34.5, range 0.0–90.4) compared to CN-H serous ECs (median 22.3, range 0.0–75.4) and CN-H endometrioid ECs (median 12.9, range 0.0–95.7;  $p < 0.001$ ; Fig. 5A). Notably, PFS and OS was not statistically significantly different between CN-H serous, endometrioid and carcinosarcomas (survival cohort;  $n=192$ ;  $p > 0.1$ ; Fig. 5B).

### 3.6. CN-L and MSI-H ECs with high chromosomal instability

Together with the observation that the majority but not all (92%) CN-H ECs in the TCGA study harbored a *TP53* mutations [3], we hypothesized that *TP53* mutation/ p53 IHC may be an incomplete surrogate of high levels of CN alterations and that analysis of chromosomal instability may be additive. In this exploratory analysis, in the survival cohort, 6% (21/324) of unselected primary CN-L ECs and 0.8% (2/249) of unselected primary MSI-H ECs had an FGA above the median of 20% found in CN-H ECs (Supplementary Fig. S4). In the CN-L group, these patients had worse outcomes than CN-L ECs with lower levels of chromosomal instability (PFS: CN-L FGA  $\geq 20$  HR 7.52, 95% CI 3.51–16.09 vs. FGA  $< 20$ ;  $p < 0.001$ ; OS FGA  $\geq 20$  HR 17.33, 95% CI 4.87–61.71;  $p < 0.001$ ; Figures 5C-D).

## 4. DISCUSSION

Here we demonstrate that through the utilization of prospective clinical tumor-normal sequencing data, we expand the number of ECs that can be classified into the molecular subtypes as compared to the ProMisE algorithm, which relies on the availability of clinical MMR and p53 IHC. Our integrated classification is mostly concordant with that of the ProMisE classification but also provides a refinement via the incorporation of the MSIsensor score as a validated proxy for MMRd/MSI status and *TP53* driver mutations for p53 IHC [14, 27, 28]. More specifically, clinical sequencing provides *TP53* alteration status, which in the absence of MMR/*POLE* mutations is the defining feature of CN-H ECs. By integrating NGS data, we found ECs that would have had a different or missing TCGA classification if based only on *POLE* mutation sequencing plus MMR/p53 IHC results.

Our data on CN-H ECs confirm that this group of tumors is associated with higher-risk, non-endometrioid histology and poor prognostic outcomes relative to other molecular subtypes. In the original description of the molecular classification by TCGA [3], a combination of TMB, MSI and somatic CN alterations was utilized for the molecular classification; all (60/60, 100%) ECs of CN-H molecular subtype harbored the highest levels of CN alterations (cluster 4), whereas none of the *POLE* (0/17) or CN-L ECs (0/90) and only 3% (2/65) of MSI-H ECs were of somatic CN alteration cluster 4 [3]. CN-L ECs are heterogeneous tumors with varying outcomes [22, 29], and we observed that CN-L tumors with levels of chromosomal instability similar to those seen on average in CN-H ECs are associated with significantly worse prognosis. Additional studies are warranted to further refine the CN-L group and to validate approaches for the identification of CN-L ECs with high levels of CN alterations associated with poor outcomes.

Analysis of this large population of ECs allowed for the assessment of the molecular subtype distribution in the less common histologic types. We found that each molecular subtype group had varying percentages of non-endometrioid histology ECs, particularly carcinosarcoma, clear cell, un-/de-differentiated and mixed/ high-grade NOS. Our study confirms recent data evaluating 1,336 EC patients from 29 Canadian institutions [30], where the majority of the 83 *POLE* tumors were of endometrioid histology (88% Jamieson et al [30] vs 77% this study), but had also representation of other histologies including carcinosarcoma, serous, and dedifferentiated carcinoma.

In this study, we found our patient outcomes were consistent with the existing literature, including TCGA [3, 4, 8, 31–33]. Of note, however, our survival subgroup only included patients who underwent MSK-IMPACT sequencing prior to a documented recurrence requiring escalation of care, underwent upfront surgery at our institution, and had surgical staging including regional lymph node analysis to reduce further bias in the analysis. Multivariate analysis identified MSI-H molecular subtype, CN-H molecular subtype, age 60 years/ diagnosis, FIGO stages III/IV and non-endometrioid histology to be associated with worse PFS outcomes. Our survival analyses further demonstrate that molecular classification is associated with outcome when assessing patients with advanced stage disease (i.e., stages III/IV) at diagnosis, especially patients with MSI-H and CN-L ECs having worse PFS and OS compared to their early-stage (i.e., stages I/II) complement.

Other than *POLE* exonuclease domain hotspot mutations and *TP53* mutations, genomic heterogeneity can be observed within each molecular subtype group. We further confirm that *PTEN* and *PIK3CA* are highly recurrently altered across subtypes, whereas *FBXW7*, *PPP2R1A*, *ERBB2* and *CCNE1* alterations are enriched in CN-H ECs. The addition of trastuzumab to carboplatin and paclitaxel is NCCN listed for patients with stage III-IV or recurrent HER2-positive EC based on a randomized phase 2 study [34]. This also remains an area of active clinical development with the recent FDA approval of trastuzumab deruxtecan for the treatment of HER2-positive breast [35] and gastric cancer [36], and *ERBB2*-mutant lung cancer [37]. Further refinement of the molecular subtypes in clinically meaningful ways is required, however, particularly for CN-L and MSI-H ECs, as these groups comprise patients with tumors that may behave poorly relative to their umbrella subtype group and harbor different targetable alterations. Recent studies also investigated the additional use of estrogen receptivity within the CN-L subgroup, which may lead to further stratify and identify higher-risk, subtype-specific ECs [38, 39].

Our study has several limitations, including bias inherent to any retrospective study. First, MMR and p53 IHC data were not available for all patients. For patients presenting for care at our institution at the time of a recurrence, MMR IHC analyses were often not repeated due to lack of sample availability and interpretation relied upon recorded documentation. In addition, IHC analyses performed on the biopsy were not typically repeated on the hysterectomy specimen given that high concordance has been reported in the literature [40]. For NGS data, we used a 20% tumor purity cut-off to ascertain high quality sequencing results; further studies would be required to further refine and define the optimal tumor cellularity cut-off from sequencing studies. We recognize the cost and availability of NGS may pose a barrier for obtaining *POLE*, MSI sensor, and *TP53* results within and across institutions. Though the use of targeted sequencing in EC remains investigational, the potential to stratify patients for a tailored adjuvant treatment may ultimately lead to cost-effective alternatives. Lastly, to minimize survival bias, we applied a very stringent criteria for the inclusion of cases in the survival analyses.

In this study, we demonstrate that in a large, unselected, prospectively clinically sequenced series of ECs the integration of molecular and IHC data leads to the classification of a larger set of ECs into the TCGA molecular subtypes as compared to the ProMisE classifier. In addition, orthogonal sequencing-based information on *TP53* mutation status and MSI

refines the classification of cases with discordant IHC and mutation/ MSI status capturing a greater number of classifiable tumors. Given the insights gained from the molecular studies over the past decade, the NCCN has included the consideration of comprehensive genomic profiling for the initial evaluation of uterine neoplasms [6]. With the more widespread access of NGS, the integration of mutational data into the molecular stratification may assist in the further subclassification/refinement of some of the molecular subtypes and will be essential for prospective clinical trials to identify the best possible adjuvant approach for this heterogeneous disease.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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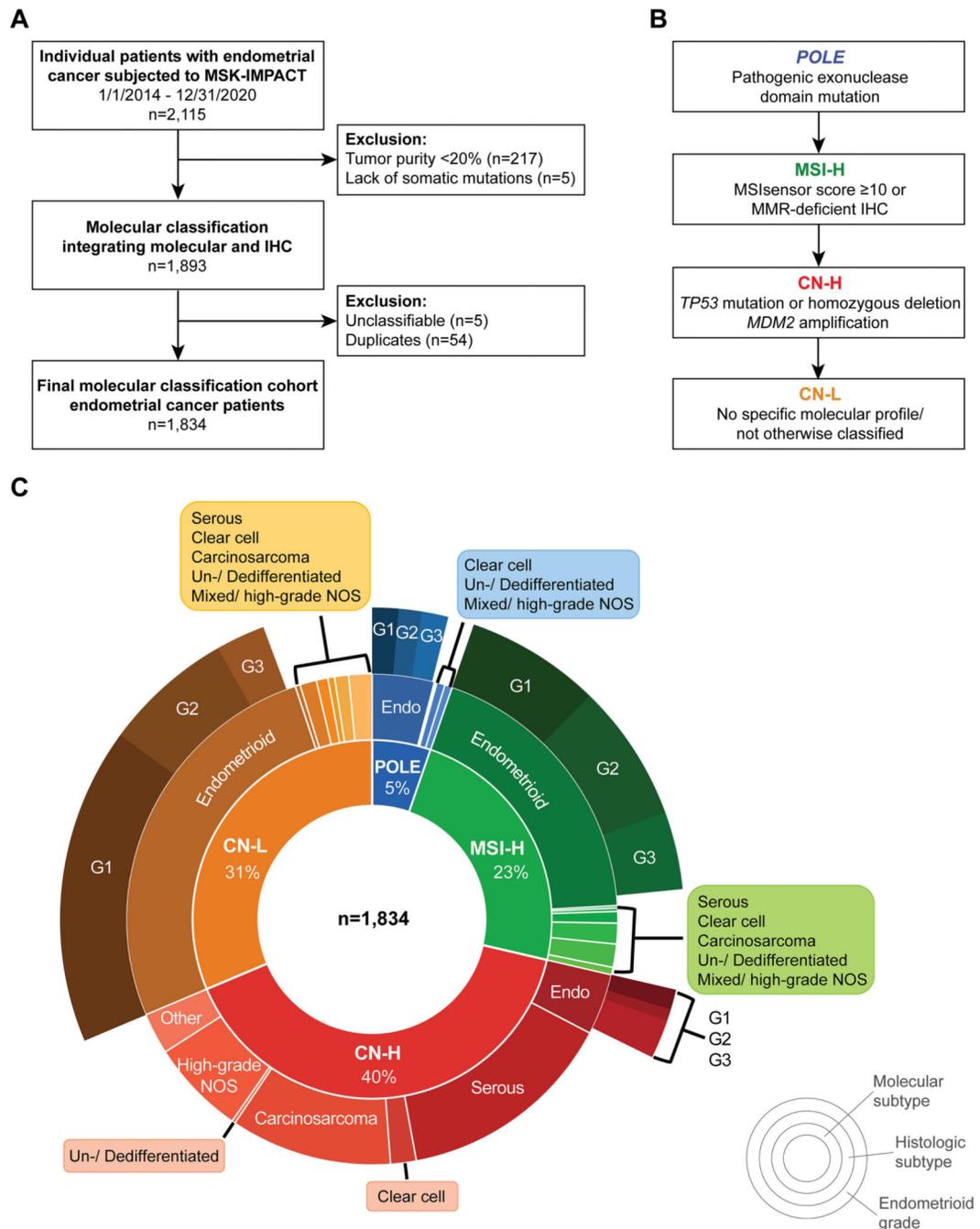
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### Highlights

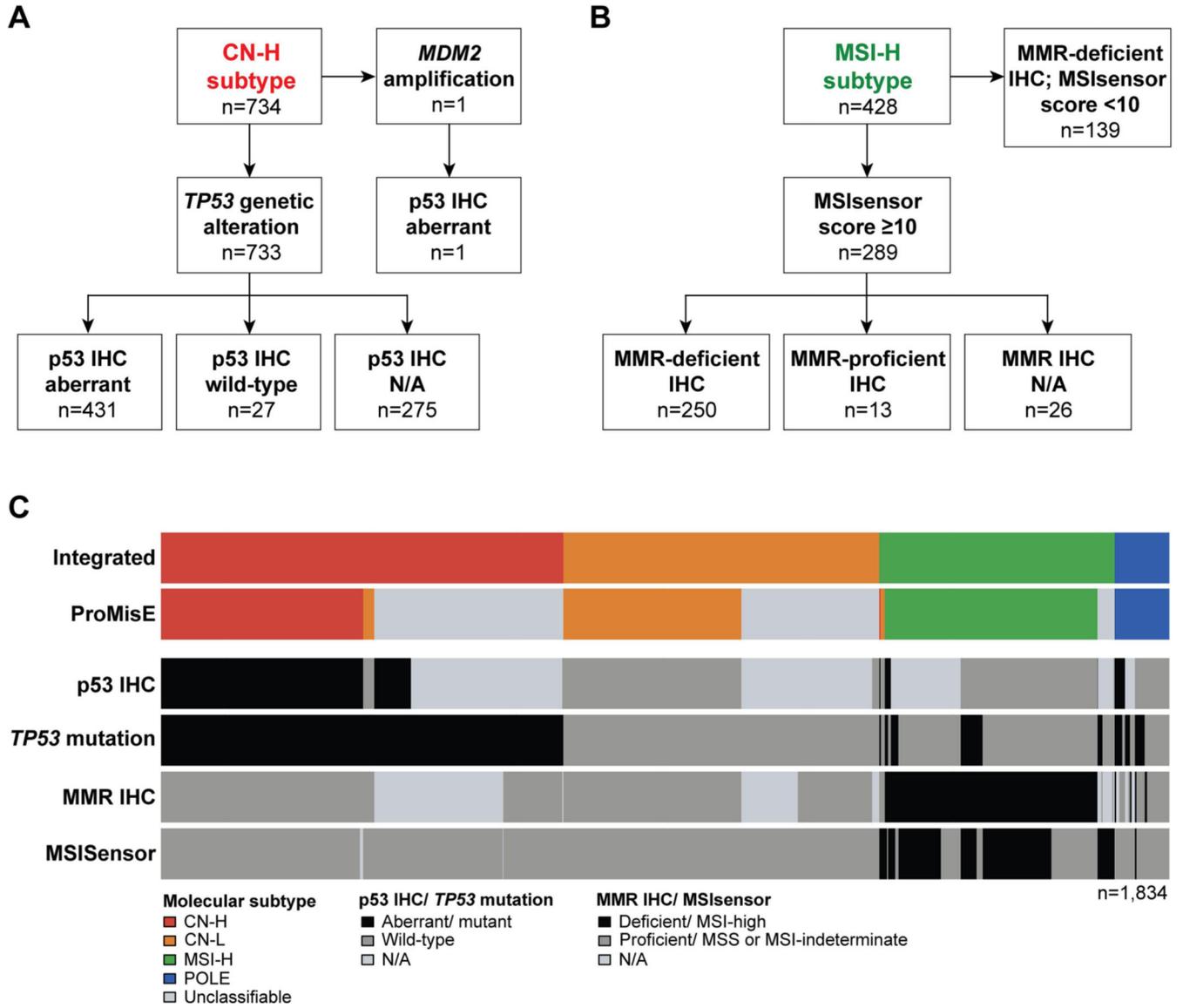
- Clinical sequencing and immunohistochemistry data integration allows for endometrial cancer (EC) molecular classification
- Integrated classification is highly concordant with existing surrogates while allowing more ECs to be classified
- Integrated classification overcomes issues of immunohistochemistry-based detection of genetic alterations
- Integrated molecular classification has prognostic value across all stages and histologic types of EC



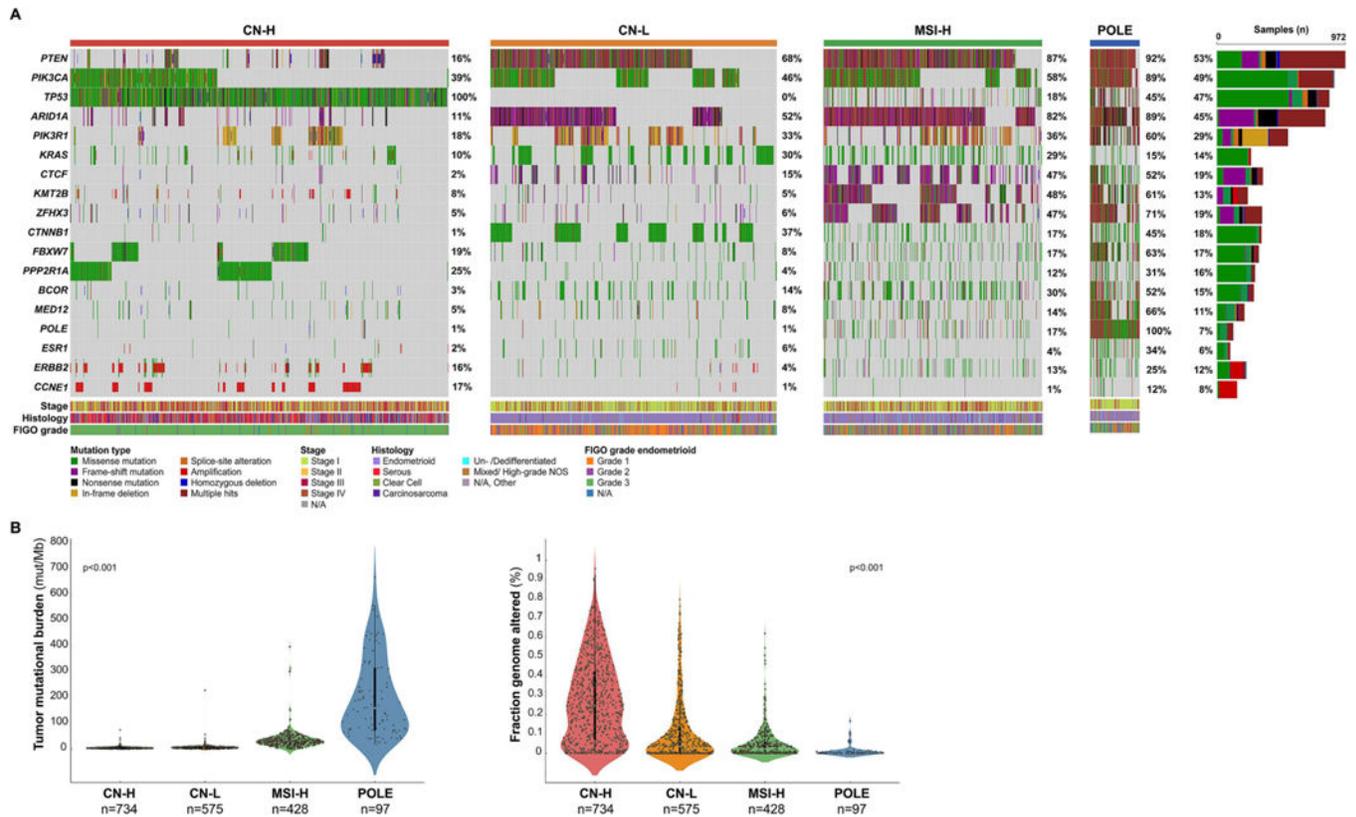
**Figure 1. Patient selection and molecular classification of endometrial cancers integrating clinical tumor-normal sequencing and immunohistochemistry data.**

**A.** CONSORT diagram summarizing the endometrial cancer patients included in this study.

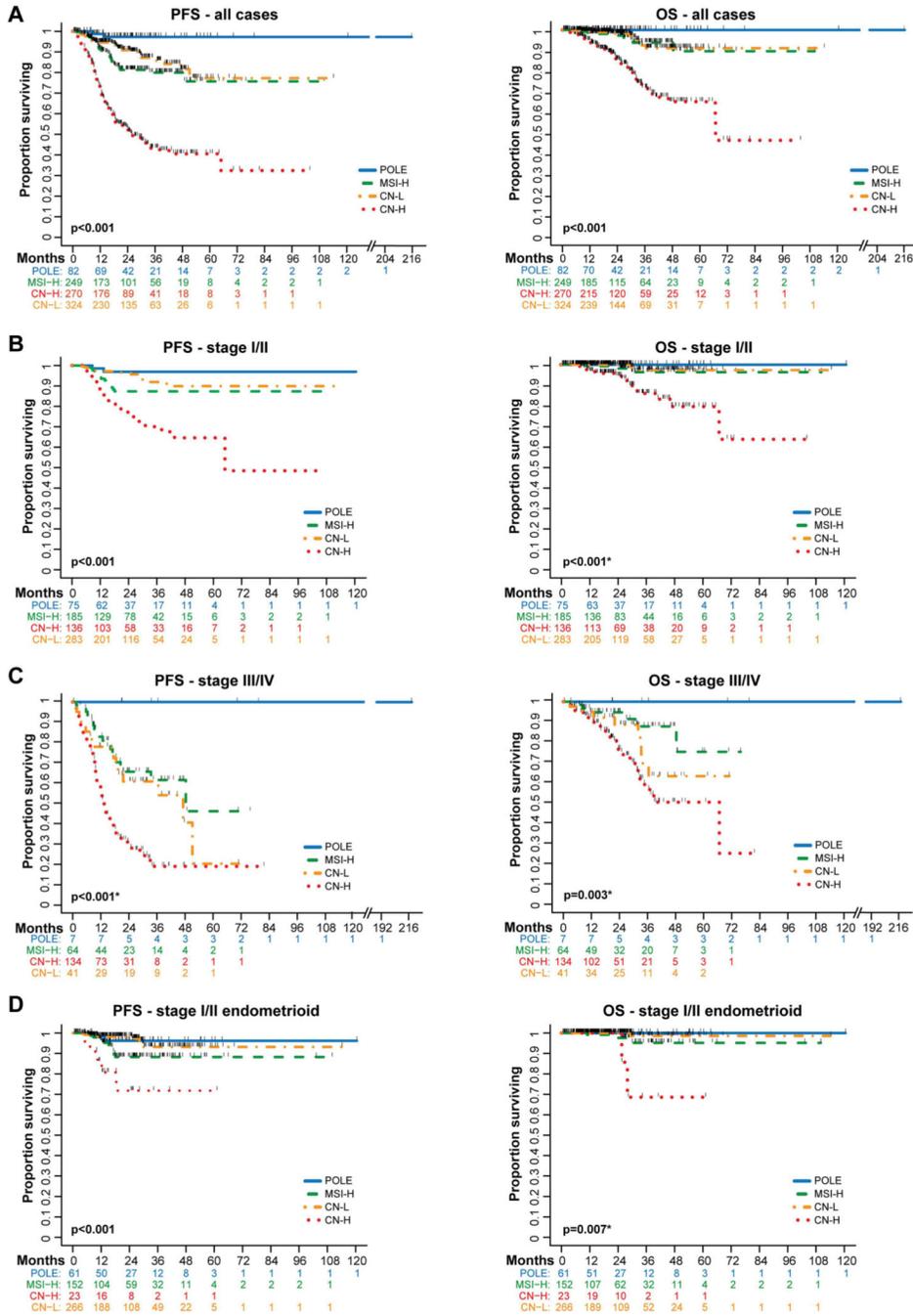
**B.** Method employed for classification of endometrial cancers into the molecular subtypes using clinical MSK-IMPACT sequencing results and immunohistochemistry. **C.** Distribution of histologic subtypes and molecular subtypes for 1,834 endometrial cancers subjected to clinical sequencing. CN-H, copy number-high; CN-L, copy number-low; G1, grade 1; G2, grade 2; G3, grade 3; MSI-H, microsatellite instability-high; NOS, not otherwise specified.



**Figure 2. Endometrial cancer molecular classification according to the integrated clinical tumor-normal sequencing/immunohistochemistry-based and the conventional ProMisE approaches.**  
**A.** *TP53* mutations and p53 expression patterns by immunohistochemistry in endometrial cancers classified as of copy number-high molecular subtype. **B.** Sequencing-derived MSI-sensor scores and DNA mismatch repair (MMR) deficiency/ proficiency by immunohistochemistry in endometrial cancers classified as of MSI-H molecular subtype. **C.** Agreement of molecular subtype assignments using our integrated molecular and immunohistochemistry-based approach (top) and the Proactive Molecular Risk Classifier for Endometrial Cancer (ProMisE; second row from top). Information on the *TP53* mutation status, p53 immunohistochemistry, MSISensor score and MMR immunohistochemistry are shown below.



**Figure 3. Landscape of somatic mutations and gene copy number alterations of endometrial cancers by molecular subtype integrating clinical sequencing and immunohistochemistry.** **A.** Oncoprints depicting the most recurrent somatic alterations in cancer-related genes in endometrial carcinomas by molecular subtype. Each column represents a tumor and the Oncoprint rows depict alterations for each gene. The bottom part of the graph shows the summary of histopathologic and clinical information for each case. The bar graph on the right of the panel shows the number and distribution of alterations for each gene across all subtypes. Mutation types and clinicopathologic features are color coded according to the legend. **B.** Tumor mutational burden in endometrial cancer by molecular subtype. **C.** Fraction of genome altered endometrial cancer by molecular subtype. P-value, Kruskal-Wallis rank sum test. CN-H, copy number-high; CN-L, copy number-low; MSI-H, microsatellite instability-high; N/A, not available; NOS, not otherwise specified.



**Figure 4. Survival outcomes of endometrial cancer patients by molecular subtype using an integrated clinical sequencing – immunohistochemistry-based classification approach.** Survival was assessed in 925 patients with endometrial cancer of all histologic types whose tumors were subjected to MSK-IMPACT prior to recurrence, had upfront surgery, were surgically staged, and had surgery performed at our institution (see Supplementary Fig. S1). **A.** Kaplan-Meier curve comparing progression-free survival (PFS) and overall survival (OS) in all 925 endometrial cancer patients by molecular subtype. **B.** PFS (n=679) and OS (n=502) of endometrial cancer patients with stage I/II disease. **C.** PFS and OS of

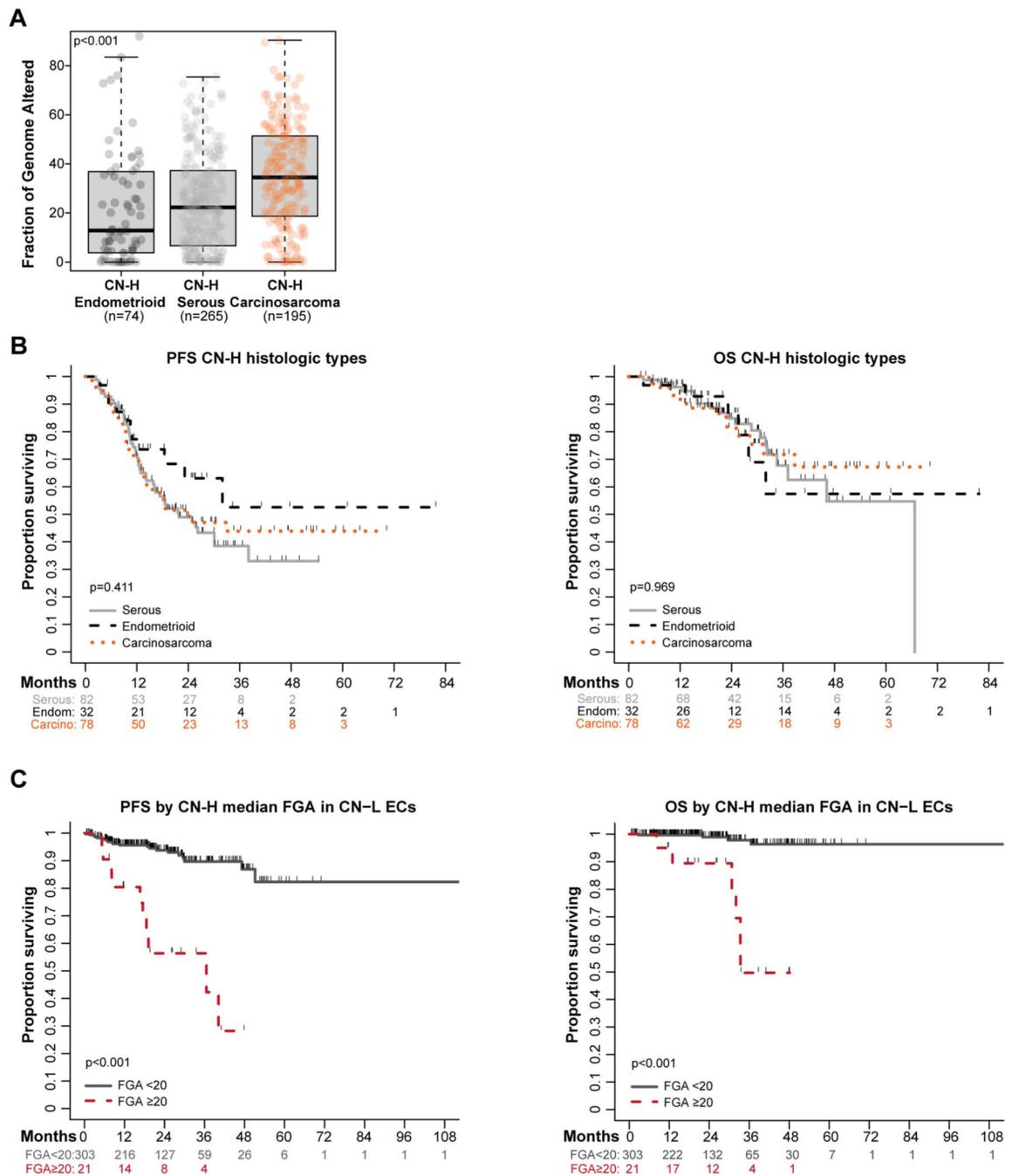
endometrial cancer patients with stage III/IV disease (n=246). For stage III and stage IV analysis separately, see Supplementary Fig. S3. **D.** PFS and OS of patients with stage I/II endometrioid endometrial cancer (n=502). Survival compared with log-rank test. \*p-value defined excluding the *POLE* molecular subtype due to the lack of events. CN-H, copy number-high; CN-L, copy number-low; MSI-H, microsatellite instability-high.

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**Figure 5. Survival outcomes in high-risk histologic types and in copy number-low endometrial cancer patients by chromosomal instability.**

**A.** Fraction of genome altered (FGA, %) among copy number-high (CN-H) endometrial cancers of serous histology (n=265), endometrioid histology (n=74), and carcinosarcoma (n=195). **B.** Progression-free survival (PFS) and overall survival (OS) of patients meeting survival criteria with endometrial carcinomas of CN-H serous (n=82), CN-H endometrioid (n=32), and CN-H carcinosarcoma (n=78) histologic types. **C.** PFS and OS of patients with CN-low (CN-L) endometrial cancer (n=249; survival cohort) dichotomized by the

median FGA of CN-H endometrial cancer (median 20%; Supplementary Fig. S4). Survival compared with log-rank test.

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Clinicopathologic and molecular characteristics of 1,834 endometrial cancers according to molecular subtype defined by integrating clinical tumor-normal sequencing and immunohistochemistry data.

Table 1.

	Total n=1,834	POLE n=97	MSI-H n=428	CN-H n=734	CN-L n=575	p value*
<b>Age at diagnosis years, median (range)</b>	29.6 (14.9–67.6)	26.3 (17.7–43.0)	30.5 (16.6–59.3)	29.1 (17.5–66.2)	30.7 (14.9–67.6)	<0.001
<b>BMI kg/m<sup>2</sup>, median (range)</b>						<0.001
<b>Histology, n (%)</b>						
Endometrioid	976 (53)	75 (77)	345 (81)	74 (10)	482 (84)	
Serous	274 (15)	0	3 (0.7)	265 (36)	6 (1)	
Clear Cell	56 (3.1)	1 (1)	4 (0.9)	31 (4.2)	20 (3.5)	
Carcinosarcoma	223 (12)	0	14 (3.3)	195 (27)	14 (2.4)	
De-/ Undifferentiated	39 (2.1)	2 (2.1)	25 (5.8)	4 (0.5)	8 (1.4)	
Mixed/ high-grade NOS	170 (9.3)	10 (10)	28 (6.5)	115 (16)	17 (3)	
Other	96 (5.2)	9 (9.3)	9 (2.1)	50 (6.8)	28 (4.9)	
<b>Endometrioid grade, n (%)</b>						<0.001
1	473 (50)	25 (35)	128 (38)	19 (28)	301 (64)	
2	287 (30)	22 (31)	131 (39)	11 (16)	123 (26)	
3	183 (19)	25 (35)	74 (22)	37 (55)	47 (10)	
<b>Stage, n (%)</b>						<0.001
I	956 (58)	79 (85)	256 (66)	256 (39)	365 (71)	
II	72 (4.3)	3 (3.2)	18 (4.6)	29 (4.4)	22 (4.3)	
III	341 (21)	7 (7.5)	82 (21)	182 (28)	70 (14)	
IV	288 (17)	4 (4.3)	34 (8.7)	190 (29)	60 (12)	
<b>Adjuvant treatment<sup>†</sup>, n (%)</b>						<0.001
None	371 (36)	30 (36)	95 (35)	43 (14)	203 (58)	
Chemo +/- RT	452 (44)	19 (23)	108 (40)	245 (77)	80 (23)	
RT	199 (19)	35 (42)	67 (25)	30 (9.4)	67 (19)	
<b>Tissue type sequenced, n (%)</b>						<0.001
Primary	1501 (82)	93 (97)	355 (83)	590 (81)	463 (81)	
Recurrence/ metastasis	323 (18)	3 (3)	73 (17)	139 (19)	108 (19)	
<b>Tumor purity (%)</b> , median (range)	40 (20–90)	40 (20–85)	40 (20–90)	50 (20–90)	40 (20–90)	<0.001

	<b>Total n=1,834</b>	<b>POLE n=97</b>	<b>MSI-H n=428</b>	<b>CN-H n=734</b>	<b>CN-L n=575</b>	<b>p value*</b>
<b>TMB</b> (mutations/Mb), median (range)	6.1 (0.0–667.9)	159.4 (19.3–667.9)	30.1 (0.0–397.9)	4.4 (0.8–74.6)	6.1 (0.0–228.2)	<0.001
<b>Somatic mutations</b> (n), median (range)	7 (1–758)	171 (22–758)	34 (1–483)	5 (1–85)	7 (1–258)	<0.001
<b>Fraction genome altered</b> (%), median (range)	5.6 (0.0–95.7)	0.1 (0.0–16.8)	2.2 (0.0–62.2)	24.7 (0.0–95.7)	3.5 (0.0–79.7)	<0.001
<b>MSIsensor score</b> , median (range)	0.5 (0.0–47.2)	0.2 (0.0–22.5)	16.6 (0.0–47.2)	0.6 (0.0–8.0)	0.1 (0.0–8.5)	<0.001

\* Kruskal-Wallis rank sum test; Fisher's exact test; Fisher's Exact Test for Count Data with simulated p-value (based on 2000 replicates)

<sup>†</sup> Adjuvant treatment: none, chemotherapy with or without radiation therapy, radiation therapy alone

BMI, body mass index; Chemo, chemotherapy; CN-H, copy number-high; CN-L, copy number-low; MSI-H, microsatellite instability-high; RT, radiotherapy; TMB, tumor mutational burden.