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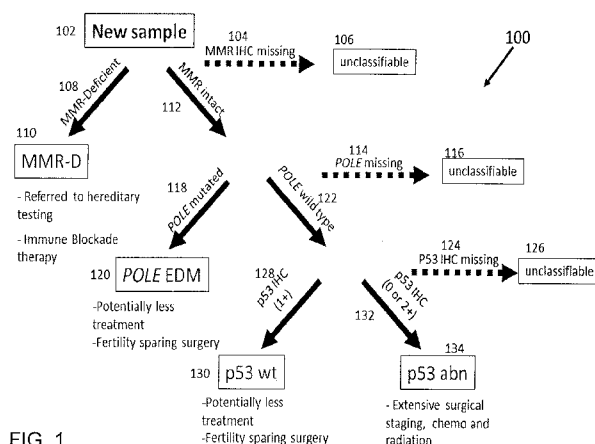


FIG. 1

(57) Abstract: Classification of endometrial cancers by morphologic features is inconsistent, and yields limited prognostic and predictive information. A method of classifying endometrial cancers and treating based on classification comprises obtaining a sample from a subject having endometrial cancer; evaluating the sample for: (a) a hypermutated mismatch repair protein comprising high microsatellite instability phenotype to classify the cancer as: (i) mismatch repair abnormal if present or mismatched repair microsatellite stable if absent; (b) subsequently evaluating the mismatch repair microsatellite stable sample from (a) for a POLE exonuclease domain mutation to classify the cancer as (ii) POLE mutated if present or POLE wild type if absent; and (c) subsequently evaluating the POLE wildtype sample from (b) for a p53 copy number abnormality comprising a high copy number or a low copy number to classify the cancer as (iii) p53 wild type, absent abnormality, and as (iv) p53 abnormal if present.

**CLASSIFICATION METHOD AND TREATMENT FOR ENDOMETRIAL CANCERS****CROSS REFERENCE TO RELATED APPLICATIONS**

**[0001]** This application claims the benefit of priority of U.S. Provisional Patent Application No. 62/192,230 filed July 14, 2015, which is hereby incorporated by reference.

**FIELD**

**[0002]** The present disclosure relates generally to a method for classifying endometrial cancers. More particularly, the molecular-based method can classify endometrial cancers into distinct and clinically significant sub-groups.

**BACKGROUND**

**[0003]** Endometrial cancers are the most prevalent gynecologic malignancies in the developed world and are the fourth most common cancer in women overall (Siegel et al, 2015). Although the majority of women with endometrial cancer have good outcomes, women with advanced disease or more aggressive subtypes may not be curable with adjuvant therapy. Classification of endometrial cancers by morphologic features is inconsistent, and yields limited prognostic and predictive information.

**[0004]** Endometrial cancers face the challenge of irreproducible pathological categorization, particularly of high grade tumors, which can lead to inaccurate risk category assignment and imprecise estimation of disease metastases, recurrence and death. This can result in the over- and under-treatment of women. Diverse subsets of endometrial cancers may be lumped together in clinical trials, making interpretation of treatment efficacy difficult. It is desirable to provide a method of classification for endometrial cancers.

**[0005]** The Cancer Genome Atlas (TCGA) (Cancer Genome Atlas Research et al, 2013) has permitted identification of genomic subgroups of endometrial cancer. For new cases of endometrial cancers, categorization into subgroups has the potential to provide prognostic and predictive information for individuals. An ability to classify cases into such subgroups might offer an improvement over the current clinical/pathology-based risk group system. Methodologies used for genomic study to identify genomic subgroups, including genome sequencing, are costly, complex and currently unsuitable for wide clinical application.

**SUMMARY**

**[0006]** It is an object of the present disclosure to obviate or mitigate at least one disadvantage of previous methods of endometrial cancer classification. The method described herein addresses one or more obstacles currently faced by clinicians and patients in management of

endometrial cancer; such as the inability to consistently classify endometrial cancer tumors. In one embodiment, a method of classification is provided to determine meaningful molecular subgroups using data acquisition methods appropriate for use in routine clinical practice.

**[0007]** In one embodiment, the method permits risk stratifications to direct patient care.

**[0008]** Four molecular subgroups of endometrial cancer determined by the method described herein are: ultramutated POLE exonuclease domain mutation, hypermutated MMR microsatellite instability (MMR MSI), copy number abnormalities-high, and copy number abnormalities-low. There is a benefit to determining such subgroups based on testing in a clinical setting with methods that are easy to perform, and interpret. Some benefits may be economical due to relatively low priced testing assays, and which can be conducted at any cancer center.

**[0009]** In a first aspect, the present disclosure provides a method of classifying endometrial cancers comprising: obtaining a sample from a subject having endometrial cancer; evaluating the sample for: (a) a hypermutated mismatch repair (MMR) protein comprising high microsatellite instability (MSI) phenotype to classify the cancer as (i) MMR abnormal (MMR abn) if present or MMR microsatellite stable (MMR MSS) if absent; (b) subsequently evaluating the MMR MSS sample from (a) for a POLE exonuclease domain mutation (EDM) to classify the cancer as (ii) POLE mutated (POLE mut) if present or POLE wild type (POLE wt) if absent; and (c) subsequently evaluating the POLE wt sample from (b) for a p53 copy number abnormality comprising a high copy number or a low copy number to classify the cancer as (iii) p53 wild type (p53 wt) if the abnormality is absent and as (iv) p53 abnormal (p53 abn) if present.

**[0010]** In a further aspect, there is provided a kit for use in the method described above of classifying endometrial cancers, comprising: one or more immunohistochemical reagent for evaluating the sample for the hypermutated mismatch repair protein; one or more PCR reagent for evaluating the sample for POLE exonuclease domain mutation; and one or more immunohistochemical reagent for evaluating the sample for the p53 copy number abnormality.

**[0011]** In further aspect, the present disclosure provides a use of (a) a hypermutated mismatch repair (MMR) protein; (b) a POLE exonuclease domain mutation (EDM); and (c) a p53 copy number abnormality for classifying an endometrial cancer in a sample from a subject, wherein the endometrial cancer is classified as: (i) MMR abn; (ii) POLE mut; (iii) p53 wt; or (iv) p53 abn.

**[0012]** Additionally, there is described in another aspect, a method for treating endometrial cancer with a fertility-sparing therapy comprising: obtaining a sample from a subject having endometrial cancer; evaluating the sample for: (a) a hypermutated mismatch repair (MMR) protein comprising high microsatellite instability (MSI) phenotype to determine MMR abnormal (MMR abn) if present or MMR microsatellite stable (MMR MSS) if absent; (b) subsequently evaluating the MMR MSS sample from (a) for a POLE exonuclease domain mutation (EDM) to determine POLE mutated (POLE mut) if present or POLE wild type (POLE wt) if absent; and (c) subsequently evaluating the

POLE wt sample from (b) for a p53 copy number abnormality comprising a high copy number or a low copy number to determine p53 wild type (p53 wt) if the copy number abnormality is absent and p53 abnormal (p53 abn) if present; and classifying the endometrial cancer as: (i) MMR abn; (ii) POLE mut; (iii) p53 wt; or (iv) p53 abn; wherein if the endometrial cancer can be classified as (ii) POLE mut or (iii) p53 wt tumor, the fertility-sparing therapy is used.

**[0013]** Further, there is described herein a system for classifying an endometrial cancer comprising: (a) an immunohistological test for detecting a hypermutated mismatch repair (MMR) protein comprising high microsatellite instability (MSI) phenotype to determine MMR abnormal (MMR abn) if present or MMR microsatellite stable (MMR MSS) if absent; (b) a test for subsequently evaluating the MMR MSS sample from (a) for a POLE exonuclease domain mutation (EDM) to determine POLE mutated (POLE mut) if present or POLE wild type (POLE wt) if absent; and (c) an immunohistological test for subsequently evaluating the POLE wt sample from (b) for a p53 copy number abnormality comprising a high copy number or a low copy number to determine p53 wild type (p53 wt) if absent and p53 abnormal (p53 abn) if present; and wherein results obtained from the tests in the system classifies the endometrial cancer as: (i) MMR abn; (ii) POLE mut; (iii) p53 wt; or (iv) p53 abn; and wherein (i) MMR abn and (ii) POLE mut are associated with intermediate clinical risk; (iii) p53 wt is associated with low clinical risk; and (iv) p53 abn is associated with high risk, wherein risk is evaluated by the European Society for Medical Oncology (ESMO) risk groupings.

**[0014]** Other aspects and features of the present disclosure will become apparent to those ordinarily skilled in the art upon review of the following description of specific embodiments in conjunction with the accompanying figures.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

**[0015]** Embodiments of the present disclosure will now be described, by way of example only, with reference to the attached Figures.

**[0016]** **Figure 1** is a schematic depiction of a classification method as a decision tree of binary parameters.

**[0017]** **Figure 2A** shows Kaplan-Meier survival analyses and log-rank survival statistics for models 1, 2, and 3 for pragmatic molecular classification of endometrial cancers applied to the Vancouver cohort.

**[0018]** **Figure 2B** shows Kaplan-Meier survival analyses and log-rank survival statistics for models 4, 5, and 6 for pragmatic molecular classification of endometrial cancers applied to the Vancouver cohort.

**[0019]** **Figure 2C** shows Kaplan-Meier survival analyses and log-rank survival statistics for models 7 and 8 for pragmatic molecular classification of endometrial cancers applied to the Vancouver cohort.

**[0020]**        **Figure 3** depicts Harrell's C-Index for models 1 to 8, ESMO clinical risk group, and combined molecular and risk groups or pathologic parameters as applied to the Vancouver cohort.

**[0021]**        **Figure 4** is a schematic depiction of the favoured pragmatic model for molecular classification of endometrial cancers, based on MMR IHC / POLE mut / p53 IHC, which corresponds to model 8 of **Figure 2C**.

**[0022]**        **Figure 5** is a cross-tabulation of clinicopathologic risk groups (ESMO) with molecular classification by the model MMR IHC / POLE mut / p53 IHC, which corresponds to model 8 of **Figure 2C**.

**[0023]**        **Figure 6** is a schematic representation of the generalized method for testing and categorizing endometrial cancer sample according to the classification method described herein.

**[0024]**        **Figure 7** shows the histopathologic features of endometrial carcinoma with discrepant mismatch repair protein results on endometrial sampling and hysterectomy.

**[0025]**        **Figure 8A** shows a breakdown of cases evaluated in both the discovery and confirmation cohorts.

**[0026]**        **Figure 8B** shows the described molecular subgroup distribution within the full cohort for Example 3 (n=460).

**[0027]**        **Figure 9A** shows the molecular subgroup for the full cohort for overall survival time (OS) outcomes in Example 3.

**[0028]**        **Figure 9B** shows the molecular subgroup for the full cohort for disease specific survival (DSS) outcomes in Example 3.

**[0029]**        **Figure 9C** shows the molecular subgroup for the full cohort for progression-free survival (PFS) outcomes in Example 3.

**[0030]**        **Figure 10A** shows Kaplan-Meier survival analyses for the full cohort in Example 3 according to the European Society for Medical Oncology (ESMO) subtype for overall survival time (OS) outcomes.

**[0031]**        **Figure 10B** shows Kaplan-Meier survival analyses for the full cohort in Example 3 according to the European Society for Medical Oncology (ESMO) subtype for disease specific survival (DSS) outcomes.

**[0032]**        **Figure 10C** shows Kaplan-Meier survival analyses for the full cohort in Example 3 according to the European Society for Medical Oncology (ESMO) subtype for progression-free survival (PFS) outcomes.

**[0033]**        **Figure 11** shows C-index data for three end-points for the full cohort based on ESMO risk groups, classifications of the current classification method (alternatively referred to herein as the ProMisE™ method) and combined, as well as diagnostic model and post-surgical model.

**[0034]**           **Figure 12** shows the four molecular subgroups determined according to the classification method versus ESMO risk groups.

#### **DETAILED DESCRIPTION**

**[0035]**           Generally, the present disclosure provides a method for classifying endometrial cancers into four different categories associated with different outcomes and modes of treatment. Advantageously, formalin-fixed paraffin embedded samples can be assessed from diagnostic endometrial samples such as from biopsy or curetting specimens, or hysterectomy samples may be used. Techniques that can be done routinely using immunohistochemistry methods are employed, which makes the method economically advantageous. Information gained in the instant classification method can be used to guide treatment decisions, including referring an individual to hereditary testing, such as for Lynch syndrome testing for abnormal MMR IHC, immune blockade therapy, fertility sparing options, or an individual may be found to be a candidate for more extensive and aggressive treatment involving chemotherapy and radiation. Early classification of endometrial cancers can optimize patient treatment outcomes.

**[0036]**           **Figure 1** depicts a classification method as a decision tree (100) of binary parameters, with the result being determined by the presence or absence of a protein or of a mutation. The method helps to ensure consistent subgroup assignment for patients having endometrial cancer. Molecular classification appears to offer prognostic information superior to current standards. Using the ESMO 2013 classifier, purportedly the strongest of the available traditional systems, only two outcomes groups were discernible, with the low- and intermediate-risk group survival curves overlapping. In contrast, molecular classification yielded four distinct subgroups with significantly different survival curves. A new sample (102) which may be from a diagnostic endometrial biopsy is assessed for MMR using immunohistochemistry. Where MMR is found to be missing (104), the sample is deemed unclassifiable (106). Where the sample is MMR-deficient (108), the sample is classified into the group shown as MMR-D (110), indicating that the individual may best be referred to hereditary testing, and/or may be a good candidate for immune blockade therapy. For samples in which MMR is intact (112), an assessment of POLE is made, for example using molecular methods such as PCR. When POLE is missing (114) the sample is considered unclassifiable (116). Should POLE be mutated (118), the sample is classified as POLE EDM (120), and the patient from whom the sample is derived may be advised to consider less treatment, and/or a fertility-sparing surgery.

**[0037]**           For sample exhibiting wild type Pole (122), additional testing for p53 is then conducted. Should P53 IHC be missing (124), the sample is deemed unclassifiable (126). If p53, as determined by immunohistochemistry is found to be wild type, designated as 1+, (128) then the p53 wt classification is determined (130), and the individual may be a candidate for less treatment and a fertility sparing surgery could be considered. For those exhibiting abnormal p53, (132), deemed as

either 0 or 2+, then the category is p53 abn (134), and the individual should consider treatment with a more aggressive approach, such as with using extensive surgical staging, including chemotherapy and radiation.

**[0038]** A method of classifying endometrial cancers is described. The method comprises obtaining a sample from a subject having endometrial cancer; evaluating the sample for: (a) a hypermutated mismatch repair (MMR) protein comprising high microsatellite instability (MSI) phenotype to classify the cancer as (i) MMR abnormal (MMR abn) if present or MMR microsatellite stable (MMR MSS) if absent; (b) subsequently evaluating the MMR MSS sample from (a) for a POLE exonuclease domain mutation (EDM) to classify the cancer as (ii) POLE mutated (POLE mut) if present or POLE wild type (POLE wt) if absent; and (c) subsequently evaluating the POLE wt sample from (b) for a p53 copy number abnormality comprising a high copy number or a low copy number to classify the cancer as (iii) p53 wild type (p53 wt) if the abnormality is absent and as (iv) p53 abnormal (p53 abn) if present.

**[0039]** The method categorizes (i) MMR abn and (ii) POLE mut and as associated with intermediate clinical risk; (iii) p53 wt as associated with low clinical risk; and (iv) p53 abn as associated with high risk, wherein risk is evaluated by the European Society for Medical Oncology (ESMO) risk groupings.

**[0040]** The hypermutated MMR protein and/or the p53 copy number abnormality is evaluated by immunohistochemistry. The POLE exonuclease domain mutation is evaluated by sequencing, for example using digital PCR (dPCR) for focused evaluation in a clinical setting.

**[0041]** The sample may be a hysterectomy sample, or a diagnostic endometrial sample, such as derived from an endometrial biopsy or an endometrial curetting specimen. The sample can formalin-fixed and paraffin-embedded.

**[0042]** Patients having cancers classified as p53 wt are associated with a good prognosis for survival. It is possible that a decision to delay surgery may be the prudent treatment when the endometrial cancer is classified as (ii) POLE mut or (iii) p53 wt tumor. Pursuing surgery may be advisable treatment when the endometrial cancer is classified as (i) MMR abn or (iv) p53 abn.

**[0043]** A kit for use in the method of classifying endometrial cancers as described herein may be provided as having: one or more immunohistochemical reagent for evaluating the sample for the hypermutated mismatch repair protein; one or more PCR reagent for evaluating the sample for POLE exonuclease domain mutation; and one or more immunohistochemical reagent for evaluating the sample for the p53 copy number abnormality. In such a kit, one or more immunohistochemical reagent for evaluating the sample for the hypermutated mismatch repair protein may comprises MSH6 and PMS2 antibodies, for example with a 2-panel model. Alternatively the test may additionally comprise MLH1 or MSH2 antibodies, in a 4-panel model.

**[0044]** The one or more immunohistochemical reagent for evaluating the sample for the p53 copy number abnormality may comprises an anti-p53 monoclonal antibody.

**[0045]** The use of (a) a hypermutated mismatch repair (MMR) protein; (b) a POLE exonuclease domain mutation (EDM); and (c) a p53 copy number abnormality for classifying an endometrial cancer in a sample from a subject is described. For this use, the endometrial cancer is classified as: (i) MMR abn; (ii) POLE mut; (iii) p53 wt; or (iv) p53 abn. A surgery may be delayed when the endometrial cancer is classified as (ii) POLE mut or (iii) p53 wt tumor. Further, a surgery may be pursued when the endometrial cancer is classified as (i) MMR abn or (iv) p53 abn. A fertility-sparing therapy may be pursued when the endometrial cancer is classified as (ii) POLE mut or (iii) p53 wt.

**[0046]** A method for treating endometrial cancer with a fertility-sparing therapy is described. The method comprises: obtaining a sample from a subject having endometrial cancer; evaluating the sample for: (a) a hypermutated mismatch repair (MMR) protein comprising high microsatellite instability (MSI) phenotype to determine MMR abnormal (MMR abn) if present or MMR microsatellite stable (MMR MSS) if absent; (b) subsequently evaluating the MMR MSS sample from (a) for a POLE exonuclease domain mutation (EDM) to determine POLE mutated (POLE mut) if present or POLE wild type (POLE wt) if absent; and (c) subsequently evaluating the POLE wt sample from (b) for a p53 copy number abnormality comprising a high copy number or a low copy number to determine p53 wild type (p53 wt) if the copy number abnormality is absent and p53 abnormal (p53 abn) if present; and classifying the endometrial cancer as: (i) MMR abn; (ii) POLE mut; (iii) p53 wt; or (iv) p53 abn; wherein if the endometrial cancer can be classified as (ii) POLE mut or (iii) p53 wt tumor, the fertility-sparing therapy is used.

**[0047]** Additionally, a method of treating an endometrial cancer comprising: classifying the endometrial cancer according to the methods described above. When the cancer is classified as MMR MSS; the subject may be subsequently treated by referral to hereditary testing, or use of immune blockade therapy. When the cancer is classified as POLE MUT; the subject may be subsequently treated with less aggressive treatment, and/or fertility sparing surgery. When the cancer is classified as p53 wt; the subject may be subsequently treated with less aggressive treatment, and/or fertility sparing surgery; and when the cancer is classified as p53 abn; the subject may be subsequently treated with extensive surgical staging, chemotherapy and radiation.

**[0048]** A system is described for classifying an endometrial cancer comprising: (a) an immunohistological test for detecting a hypermutated mismatch repair (MMR) protein comprising high microsatellite instability (MSI) phenotype to determine MMR abnormal (MMR abn) if present or MMR microsatellite stable (MMR MSS) if absent; (b) a test for subsequently evaluating the MMR MSS sample from (a) for a POLE exonuclease domain mutation (EDM) to determine POLE mutated (POLE mut) if present or POLE wild type (POLE wt) if absent; and (c) an immunohistological test for subsequently evaluating the POLE wt sample from (b) for a p53 copy number abnormality comprising

a high copy number or a low copy number to determine p53 wild type (p53 wt) if absent and p53 abnormal (p53 abn) if present; and wherein results obtained from the tests in the system classifies the endometrial cancer as: (i) MMR abn; (ii) POLE mut; (iii) p53 wt; or (iv) p53 abn; and wherein (i) MMR abn and (ii) POLE mut are associated with intermediate clinical risk; (iii) p53 wt is associated with low clinical risk; and (iv) p53 abn is associated with high risk, wherein risk is evaluated by the European Society for Medical Oncology (ESMO) risk groupings. Whether the hypermutated MMR protein and/or the p53 copy number abnormality is present, is evaluated by immunohistochemistry. The POLE exonuclease domain mutation is evaluated by molecular evaluation, such as by sequencing, for example by PCR such as digital PCR.

**[0049]** The sample for use in the system may be a hysterectomy sample, or a diagnostic endometrial sample comprising an endometrial biopsy or an endometrial curetting specimen.

**[0050]** Outcomes of endometrial cancer are described below. The time of origin for patient survival is set as the date of surgery. This is the date from which follow-up begins. Follow-up practice at different institutions may vary. If a patient lives in a location remote from a research institution, all follow-up may be conducted with her local physician. If the patient lives in a location proximal to her institution, she may have follow up visits with the institution until completion of adjuvant therapy, up to 5 years from therapy, or longer if undergoing continued treatment for recurrent disease. For the data obtained in the Examples below, follow-up data for patients living remotely may be missing or unobtainable, but can be actively pursued by contacting the primary physician. Follow-up data can be obtained from cancer registry data (in the case of the Examples below, a provincial registry in British Columbia, Canada) where date of recurrence, date of death, date of last contact and status of last contact are periodically updated.

**[0051]** A two-year clinical follow-up period may be utilized as a minimal clinical follow-up time for patients with endometrial cancer. Patients with potential follow-up less than two years were not considered in the Examples below. A "cohort wise" follow-up approach is used in the Examples below, censoring observations on December 31st of the fifth year following the year in which a patient had her surgery (year of diagnosis). This ensures random censoring and minimize ascertainment bias.

**[0052]** Time to death or overall survival time (OS) from surgery until death of any cause is a parameter evaluated in the Examples below. Patients who are still alive prior to the censoring date are censored at the time of last follow up, or on December 31st of the fifth year post diagnosis, whichever was sooner. If the event status or the date of last follow-up is unknown, data is considered missing.

**[0053]** Time to death from disease or disease specific survival (DSS), time from surgery until death due to endometrial cancer is evaluated in the Examples below. Patients who are alive prior to the censoring date or if they died of an unrelated cause, are censored at the time of last follow-up, or on December 31st of the fifth year post diagnosis, whichever was sooner. If the event status or the date of last follow-up is unknown data is considered missing. The event status is considered unknown

if patients die of an intercurrent disease or if the reason for death is unspecified. These patients would not be considered in the disease specific survival analyses.

**[0054]** Time to progression or progression-free survival (PFS) is the time from surgery until there is evidence of recurrent or progressive disease (this is based on either clinical evidence of recurrence, or imaging confirmation of recurrence) or if the patient died of the disease prior to the censoring date. Patients who are alive and disease-free prior to the censoring date or if they died of an unrelated cause, are censored at the time of last follow-up, or on December 31st of the fifth year post diagnosis, whichever was sooner. If the event status or the date of last follow-up is unknown data is considered missing.

**[0055]** EXAMPLES

**[0056]** Example 1

**[0057]** Molecular-Based Classification Method

**[0058]** ABSTRACT

**[0059]** Classification of endometrial carcinomas (ECs) by morphologic features is inconsistent, and yields limited prognostic and predictive information. A system is described herein for classification based on the molecular categories identified in The Cancer Genome Atlas. Genomic data from The Cancer Genome Atlas (TCGA) supports classification of endometrial carcinomas into 4 prognostically significant subgroups. The TCGA data set was used to develop surrogate assays that replicate the TCGA classification, but without the need for the labor-intensive and cost-prohibitive genomic methodology. Combinations of the most relevant assays were carried forward and tested on an independent cohort of 152 endometrial carcinoma cases, and molecular versus clinical risk group stratification was compared.

**[0060]** Replication of TCGA survival curves was achieved with statistical significance using multiple different molecular classification models (16 total tested). A classifier based on the following components was validated: mismatch repair protein immunohistochemistry, POLE mutational analysis, and p53 immunohistochemistry as a surrogate for 'copy number' status. This molecular classifier was associated with clinical outcomes, as was stage, grade, lymph-vascular space invasion, nodal involvement and adjuvant treatment. In multivariable analysis both molecular classification and clinical risk groups were associated with outcomes, but differed greatly in composition of cases within each category, with half of POLE and mismatch repair loss subgroups residing within the clinically defined 'high-risk' group. Combining the molecular classifier with clinicopathologic features or risk groups provides the highest C-index for discrimination of outcome survival curves. Molecular classification of endometrial cancers can be achieved using clinically applicable methods on formalin-fixed paraffin-embedded samples, and provides independent prognostic information beyond established risk factors. This pragmatic molecular classification tool has potential to be used routinely in guiding treatment for individuals with endometrial carcinoma and in stratifying cases in future clinical trials.

**[0061]            *Introduction***

**[0062]**            Endometrial cancers are the most prevalent gynecologic malignancies in the developed world and are the fourth most common cancer in women overall (Siegel et al, 2015). Incidence rates have increased dramatically over the last decades attributable at least in part to the global epidemic of obesity. Although the majority of women with EC have good outcomes, women with advanced disease or more aggressive subtypes may not be curable with adjuvant therapy. In Canada over the last decade, the annual percentage increase in age-standardized mortality rate for EC is greater than any other cancer in women, and society is in desperate need of new approaches, including diagnostic tools, to manage this cancer.

**[0063]**            There are many unanswered questions in EC pertaining to diagnosis and optimal management. Management considerations include which surgery to perform by either a generalist or subspecialist, which if any adjuvant therapies to administer, surveillance strategies and fertility-sparing options in young women. Currently, there are multiple systems of risk-group stratification based on post-surgical staging pathologic examination (principally histotype, tumour grade and stage) that may help guide treatments choices (AlHilli et al, 2014; Bendifallah et al, 2015; Colombo et al, 2013; Kong et al, 2015; Kwon et al, 2009; Mariani et al, 2008). However, pathologists are unable to reproducibly diagnose histotype and grade of EC due to lack of consensus between expert pathologists has been demonstrated, even with the addition of immunohistochemistry (Gilks et al, 2013; Guan et al, 2011; Han et al, 2013; Hoang et al, 2013). This lack of reproducibility is a major barrier to improving care for women with this disease, and treatments vary within and between cancer centers globally. Assessment of treatment efficacy when there is variable histotype and grade assignment hinders the ability to determine optimal management.

**[0064]**            Molecular classification of endometrial cancers has shown great promise, proving to be reproducible, and demonstrating associations with clinical outcomes (Cancer Genome Atlas Research et al, 2013; Le Gallo et al, 2012). The Cancer Genome Atlas (TCGA) has identified four genomic subgroups of EC. The group with POLE mutations and corresponding "ultramutated" phenotype was a novel finding, and particularly interesting given the very favorable outcomes even with high-grade tumours. This has been validated in other series of cases with POLE mutations (Billingsley et al, 2015; Church et al, 2015). The other distinct subgroups identified from the TCGA data included microsatellite instability (MSI), copy number low (CN low) and copy number high (CN high), the latter consisting mostly of cases diagnosed by referring center pathologists as high-grade serous cancers. For new cases of endometrial cancers, categorization into one of these four subgroups could potentially provide prognostic and predictive information for individuals. Therefore, an ability to classify cases in this manner might offer an improvement on the current clinical/pathology-based risk group system (Murali et al, 2014). Unfortunately, methodologies used for the TCGA study to identify these four genomic subgroups, including genome sequencing, were costly, complex and

unsuitable for wider clinical application. The goal of the work conducted in this Example was to determine whether the same molecular subgroups could be identified and the survival curves reproduced with assays that could be used in routine clinical practice.

**[0065]** A simple, lower cost, molecular-based classification methodology was designed, which can recover the TCGA subtypes described above. This classification methodology was tested and compared both on the TCGA data and on a separate cohort of patients from British Columbia center (n=152). Additionally, this pragmatic molecular classification model is compared to contemporary clinical risk group stratification. Improvement in outcome prediction resulting from the addition of a molecular classifier will lead to better management for women with EC.

**[0066]        *Methods***

**[0067]        Samples and Clinical Data**

**[0068]        *Patient Cohort-Vancouver.*** A retrospective cohort of 152 patients with primary endometrial carcinoma was identified from the Vancouver General Hospital cases banked in the OVCARE Tissue Bank Repository, Vancouver BC, Canada (McConechy et al, 2012). These patients were diagnosed with endometrial cancer (herein referred to as "EC") between 2002 and 2009. Patients were excluded if they had a diagnosis of a concurrent cancer that was being treated at the same time as their EC or any previous treatment which may have influenced her outcome (e.g., prior radiotherapy). Exclusion criteria also included uterine pre-cancers, cancers metastatic to the uterus, or no definitive surgery performed (no hysterectomy). Patients had comprehensive data collected including details of pathology, surgery, chemotherapy, radiation and outcomes with a minimum of two years potential follow-up. Patient management was according to BC Cancer Agency Guidelines. Research ethics approval for the Tissue/Biospecimen Bank and this project were granted from the University of British Columbia Institutional Review Board and all patients underwent informed written consent for use of their biospecimens for research purposes.

**[0069]        *Development of a Molecular Classification Model.*** In the TCGA cohort of fully evaluable cases (n=232), roughly 7% of cases were grouped as POLE ultramutator phenotype, 28% were designated with microsatellite instability (MSI), 26% copy number high (CN high), and 39% copy number low (CN low) (Cancer Genome Atlas Research et al, 2013). When applying the new classifier tool to the TCGA cohort, the primary objective was to classify all patients into the TCGA clusters, but also to minimize the number of false negatives in the CN high poor prognosis group, in order to avoid under treating women who may have aggressive disease.

**[0070]** In reproducing the TCGA clusters considerations at each step included the following.

**[0071]** (a) POLE: In TCGA, the POLE ultramutated cluster was identified based on a POLE mutation, a high percent of C to A transversions and low percent of C to G transversions, as well as more than 500 SNVs. For the described classifier method, PTEN was initially included in the assessment of the models along with POLE mutations because in the TCGA mutational analysis,

although PTEN mutations were seen to some degree across MSI and CN low subgroups, PTEN and POLE mutations were noted to co-occur almost all of the "ultramutated" subgroup and seemed to better define this category. Hence, two methods were initially proposed to identify the ultramutated subgroup; one using the POLE mutation status alone, and one that uses both the POLE and PTEN mutation status.

**[0072]** (b) MSI: The MSI group in the TCGA analysis was based on results from the MSI assay using 7 markers (Cancer Genome Atlas Research et al, 2013). These models identified the MSI phenotype subsequent to POLE ultramutated, as done in TCGA and also considered switching the order to identify the MSI cluster first. In practice this is practical as it would be useful to have this information as early as possible to enable referral to hereditary cancer programs for Lynch syndrome testing. Using the MSI assay was changed to using MMR IHC testing (MLH1, MSH2, MSH6, PMS2), which is highly concordant with MSI assay (data not shown) and more cost effective and practical.

**[0073]** (c) Copy Number (CN): In TCGA, copy number was assessed with Affymetrix SNP 6.0 microarrays using DNA originating from frozen tissue. It is desirable to have a more cost effective method that could be achieved on formalin fixed paraffin embedded (herein referred to as "FFPE") material, thus the TCGA data was mined, to find that copy number status at three specific loci (FGFR (4p16.3), SOX17 (8q11.23), and MYC (8q24.12) were most predictive of copy number status e.g., using just these 3 loci permitted identification of all cases within the CN high cluster. These 3 loci were assessed by FISH. In addition, TP53 was noted to be mutated in most of the copy number high cases in the TCGA cohort and in silico analysis demonstrated that p53 status was able to reproduce the CN high/low survival curves. TP53 mutation status was not equivalent to CN high subgroup in TCGA but identified a subgroup of EC cases with distinctly worse outcomes. Therefore CN status was determined as follows: i) FISH determination of copy number status at 3 loci most associated with CN high subgroup in TCGA (FGFR (4p16.3), SOX17 (8q11.23), and MYC (8q24.12), scored on two thresholds, and ii) p53 status determined by IHC or TP53 sequencing, yielding four possible ways to classify CN high following determination of MSI and POLE groups.

**[0074]** Varying the combinations of the features described above resulted in 8 different ways to classify patients in the TCGA cohort, and a total of 16 in the Vancouver cohort. The performance of these different scenarios could be directly compared within the TCGA cohort, because the genomic-based data labels and outcome details were available to us (with the exception of immunohistochemistry and FISH). Since the TCGA equivalent genomics data was not available on the Vancouver cohort, performance measures of the more selective molecular components were based on survival outcomes. In this Example the four genomic subgroups and survival curves were able to be reproduced in both the TCGA (detailed data not shown) and Vancouver cohorts (see **Figures 2A, 2B, and 2C**, described below).

**[0075]**        **Statistical methods.** Univariable analyses of molecular classifier categories against overall survival (OS), disease specific survival (DSS) and recurrence free survival (RFS) were examined both using Kaplan-Meier plots with log-rank significance testing and Cox proportional hazard regression models. Multivariable cox proportional hazard regression model analysis was performed to assess any additional prognostic information that would be added by this classifier beyond the clinical risk group classification and the standard prognostic factors (age, BMI, grade, stage, histology, LVSI, and treatment). To assess the additional prognostic information added by the classifier model to clinicopathological parameters, two sets of multivariable analyses were performed: (i) multivariable analyses with ESMO clinical risk groups, (ii) multivariable analyses with individual clinicopathological parameters: age, BMI, grade, stage, histology, LVSI.

**[0076]**        Where the percent censoring exceeded 80%, a Firth bias reducing correction was applied to obtain estimates. P-values from omnibus likelihood ratio test in all Cox models were reported. Smoothed plots of weighted Schoenfeld residuals were used to assess proportional hazard assumptions. Only complete observations were used for model fitting. A missing value analysis was done to explore the distribution of missing values and to ensure they are missing at random.

**[0077]**        The performance of the models was first assessed visually based on their ability to reproduce a similar pattern as the TCGA-identified (integrated genomic data-based) groups. Furthermore, the performance was quantified by computing accuracy measures (in the TCGA cohort where true labels are available) and Harrell's C-index in the TCGA and in the Vancouver cohort when considering survival outcome. The C-index is a measure of discriminative ability of the model. A C-index of 0.5 indicates that the model has no discriminative ability and a C-index of 1 indicates that a model perfectly distinguishes between those who have an event and those who do not.

**[0078]**        Bootstrapping techniques were used for internal validation in both the TCGA data and the this cohort. Validation using bootstrap re-sampling would estimate the likely performance of the model on a new sample of patients from a same clinical setting. One thousand bootstrap samples are used; in each bootstrap iteration, a sample of size equal to the original cohort is drawn with replacement from the original cohort. Models assessed with the C-index were developed in the bootstrap samples were tested in those subjects not included in the bootstrap sample. In comparing the TCGA predicted subtypes to the actual labels, the sensitivity and the specificity is obtained on the bootstrap samples alone.

**[0079]**        Clinical risk groups were assigned according to the European Society of Medical Oncologists (ESMO) criteria (Colombo et al, 2013) and compared to molecular subgroups in both the TCGA (data not shown) and new endometrial carcinoma cohorts .

**[0080]**        The association of TCGA-inspired endometrial subtypes (POLE/MMR IHC abn/p53 wt/p53 abn) with other variables such as demographic (Age), clinical (Treatment), pathological (Stage), Grade, Histology, LVSI) were tested with non-parametric tests. Kruskal-Wallis rank sum test

was used for continuous variables (age and BMI) and Fisher's exact test was used for all other variables/categorical (stage, grade, histology, LVSI, any positive nodes and initial adjuvant treatment and clinical risk groups).

**[0081]** Statistical significance level was set to 0.05. P-values reported were not corrected for multiple comparisons. All statistical analyses were performed using the statistical software R v3.1.0 (from R Foundation for Statistical Computing, Vienna Austria).

**[0082]** Results

**[0083]** ***Application of the Molecular Classification Tool to a New Cohort of Endometrial Carcinomas.*** For the Vancouver cohort, beginning with 152 patients, one patient was excluded for having undergone neoadjuvant chemotherapy, 7 cases were excluded who failed sequencing or had no DNA available for POLE or TP53 sequencing, and one case had insufficient tumour tissue remaining to enable MMR IHC status to be determined, leaving 143 fully evaluable cases. 'MSI' status in the Vancouver cohort was determined by MMR IHC, and as highly concordant with MSI assay in endometrial cancers (data not shown). In total, 41 of 143 fully evaluable cases had abnormal MMR IHC (29%) ('MMR IHC abn'), consistent with the TCGA data (**Table 1**).

**Table 1 – Demographic Characteristics and Traditional Prognostic Variables for the Total Cohort (n=143) and within Molecular Subgroups According to the Model - MMR IHC/POLE/p53 IHC**

|                           | Total      | MMR IHC abn | POLE EDM | p53 wt   | p53 abn  | Assoc. test |
|---------------------------|------------|-------------|----------|----------|----------|-------------|
| Total (%)                 | 143 (100%) | 41 (29%)    | 12 (9%)  | 63 (45%) | 25 (18%) |             |
| Age (years)               |            |             |          |          |          |             |
| Mean                      | 63 ± 1     | 65 ± 2      | 54 ± 3   | 60 ± 1   | 71 ± 2   | P=0.000     |
| Range                     | 55-70      | 56-71       | 47-59    | 54-68    | 65-77    |             |
| BMI <sup>a</sup>          |            |             |          |          |          |             |
| Mean                      | 33 ± 1     | 32 ± 2      | 28 ± 2   | 36 ± 2   | 29 ± 2   | P=0.038     |
| Range                     | 24-40      | 26-36       | 24-33    | 26-43    | 23-33    |             |
| Stage                     |            |             |          |          |          |             |
| Stage I                   | 102 (71%)  | 24          | 12       | 51       | 14       | P=0.002     |
| Stage II/III/IV           | 41 (29%)   | 17          | 0        | 12       | 11       |             |
| Grade                     |            |             |          |          |          |             |
| Grade 1                   | 51 (36%)   | 10          | 3        | 35       | 2        | P=0.000     |
| Grade 2                   | 39 (27%)   | 14          | 4        | 18       | 2        |             |
| Grade 3                   | 53 (37%)   | 17          | 5        | 10       | 21       |             |
| Histology                 |            |             |          |          |          |             |
| Endometrioid              | 119 (83%)  | 35          | 11       | 61       | 10       | P=0.000     |
| Serous/Mixed <sup>b</sup> | 24 (17%)   | 6           | 1        | 2        | 15       |             |
| LSVI <sup>a</sup>         |            |             |          |          |          |             |
| No                        | 79 (58%)   | 14          | 5        | 48       | 10       | P=0.000     |
| Yes                       | 58 (42%)   | 25          | 7        | 12       | 14       |             |
| Any + Node <sup>a</sup>   |            |             |          |          |          |             |
| No                        | 120 (86%)  | 31          | 12       | 58       | 18       | P=0.001     |
| Yes                       | 19 (14%)   | 10          | 0        | 2        | 6        |             |
| Adjuvant Rx <sup>a</sup>  |            |             |          |          |          |             |
| No Rx                     | 79 (56%)   | 17          | 6        | 46       | 9        | P=0.001     |
| Any Rx                    | 63 (44%)   | 24          | 5        | 17       | 16       |             |
| Risk Group                |            |             |          |          |          |             |
| Low                       | 56 (39%)   | 13          | 4        | 35       | 3        | P=0.002     |
| Intermediate              | 23 (16%)   | 6           | 2        | 11       | 4        |             |
| High                      | 64 (45%)   | 22          | 6        | 17       | 18       |             |

**[0084]** Table 1 Abbreviations: BMI = Body mass index; SVSI = lymphovascular space invasion; range = interquartile range. Associations between given parameter and molecular subgroups are calculated using the Kruskal-Wallis rank sum test for continuous variables (age and BMI for this analysis) and Fisher's exact for categorical variables. Two cases were 'unclassifiable' by

p53 IHC. "a" – Missing data for 22 cases for BMI, 6 cases for LSVI, 4 for notal disease and 1 for treatment; "b" – Serous/mixed cases included 15 serous carcinomas (10% of total cohort), 7 mixed and 1 undifferentiated.

**[0085]** POLE exonuclease domain (EDM) mutations were found in 13 cases in the total cohort. In one case, (VOA 843, data not shown) a low level (5%) validated POLE mutation was found in exon 12 that is not a known hot spot mutation, and this tumour also demonstrated isolated MSH6 loss with IHC. As the first step in the classifier model was to assess MMR IHC this case was classified as MMR IHC abnormal (not grouped with 'POLE'). Of the remaining cases classified as 'POLE' mutant (12 of 143 (8.4%) cases), they were exclusively stage I, with 5 of 12 (42%) grade 3, and all but one case (92%) showing endometrioid histology (**Table 1**). In this small cohort all tumours with POLE EDM mutations had normal MMR IHC. TP53 mutations were identified in 3 of 12 POLE mutated cases by sequencing and 1 of 12 cases by abnormal p53 IHC (score 0 or 2+). Full details of the subset of cases in the Vancouver cohort with POLE mutations, including chromosome, genomic position, and amino acid change as well as TP53 and PTEN mutation details and the status of MMR IHC (MLH1, MSH2, MSH6, PMS2) and p53 IHC for these cases were obtained, but are not shown here. There were no recurrences or deaths in the cases with POLE EDM mutations, with an observation time of over five years for this subgroup.

**[0086]** Using p53 IHC status as a surrogate for 'copy number high' to identify p53 abnormal ('p53 abn') subgroup, 25 cases had aberrant p53 equalling 17.5% of the total cohort of 143, or 28% of cases following the exclusion of those classified as 'MMR IHC abn' and 'POLE' positive (**Table 1**). Using TP53 sequencing for determination of 'p53 abn' revealed mutations in 27 cases in the total cohort or 19 of the 88 (22%) cases remaining after the exclusion of those classified as 'MMR IHC abn' and 'POLE' EDM mutation positive. Other data, including the specifics on POLE, TP53, PTEN mutations and MMR and p53 IHC for the full cohort (n=153) were obtained but are not shown here.

**[0087]** FISH testing was interpretable in 121 cases. Results for threshold 1 (T1) suggest copy number high status in 12 cases, 11 of which also had TP53 mutations but an additional 15 cases had TP53 mutations and were not designated 'CN high' by FISH. Similarly, for Threshold 2 (T2) only 9 cases met criteria of 'CN high' designation, 8 of which also had TP53 mutations but 18 other cases had TP53 mutations and did not qualify for 'CN high' status based on FISH T2. Within the non-MMR IHC abn, non-POLE cohort, and using TP53 mutation status for comparison the kappa statistic for level of agreement between testing methods for T1 is 0.66(95%CI 0.42-0.84) with a sensitivity, specificity, positive (PPV) and negative predictive value (NPV) of 1, 0.56, 0.88, and 1 respectively. For T2 the kappa statistic is 0.49 (95%CI 0.24-0.72) with corresponding accuracy calculations 1, 0.39, 0.85, and 1. A comparison of p53 IHC to TP53 mutation status was made in the whole cohort and in the cohort remaining after removal of 'MMR IHC abn' and 'POLE' mutated cases (n=88) and a kappa

statistic of 0.77(95%CI 0.59-0.92) was found, with sensitivity, specificity, PPV and NPV of 0.9, 0.94, 0.98 and 0.74, respectively.

**[0088]** The Vancouver cohort included the major histological subtypes (83% endometrioid histotype, the remainder being of serous or mixed histotypes, with the exception of a single undifferentiated carcinoma), all stages, and grades (**Table 1**) similar in distribution to both TCGA (data not shown) and the general population. The estimated median follow-up time in the Vancouver cohort, as calculated by the reverse Kaplan-Meier method is 5 years. The median observation time is 4.67 years. A total of 27 recurrences and 28 deaths were observed. A comparison of patient demographics and clinicopathologic details for the full cohort and within 'MMR IHC abn', 'POLE', 'p53 wt' and 'p53 abn' categories based on a pragmatic classification is given in **Table 1**. The distribution of multiple parameters differs across the molecular subgroups, notably an increased presence of LVSI in the 'MMR IHC abn' and 'p53 abn' subgroups, and one third of both 'MMR IHC abn' and 'p53 abn' cases having node positive disease. Stage was also more advanced in the 'MMR IHC abn' and 'p53 abn', with 71% and 79% of cases with disease beyond the uterus respectively. Not surprisingly, average age was highest in the 'p53 abn' group with the highest proportion of serous/non endometrioid cases. Women with MMR IHC abn cases were also older, likely secondary to the higher proportion of MMR IHC loss at MLH1 in this cohort, with a known increased frequency of methylation in older individuals.

**[0089]** There were 16 different possible combinations with which to analyze outcomes for a molecular classifier, based on defining MMR IHC as normal or abnormal first or after classification of POLE cases (one decision), POLE mutations or POLE and PTEN mutations together (two ways to categorize this step) and copy number which could be determined by four different options in surrogate testing (four ways to categorize: p53 IHC, TP53 mutations, FISH for 3 loci T1, FISH T2). As the cases with POLE EDM mutations all had normal MMR IHC, changing the order of these two tests in the model for this cohort (e.g. stepwise analysis of MMR IHC first then POLE status vs. POLE first then MMR IHC) made no difference, thus Kaplan-Meier analyses and the log-rank statistic for 8 models (not 16) are shown in **Figures 2A, 2B and 2C**.

**[0090]** **Figures 2A, 2B, and 2C** show Kaplan–Meier survival analyses and log-rank statistics of eight possible models for pragmatic molecular classification of endometrial cancers applied to the Vancouver cohort (n=143). Overall survival (OS), disease-specific survival (DSS) and recurrence-free survival (RFS) are shown for each model and molecular subgroups are POLE (upper-most data line of each plot), MMR IHC abn, p53 wt, and p53 abn (lower-most-line of each plot). Model numbers 1 through 8 are shown in each row, with models 1 to 3 on **Figure 2A**, models 4 to 6 shown on **Figure 2B**, and models 7 and 8 shown on **Figure 2C**. Model 8 is outlined in **Figure 2C**, as it is the model that was used for subsequent univariate and multivariate analysis. Model 8 was combined with either European Society of Medical Oncologists clinical risk groups or pathological parameters.

**[0091]** Statistical significance of the log-rank statistic is noted in the majority of these models, with the exception of FISH (T2 or threshold 2), shown in Models 3 and 6 illustrated in **Figures 2A and 2B**, respectively. Although FISH testing of three loci (MYC, SOX17, FGFR3) segregated by the first threshold (T1) may act as a surrogate test for copy number, it is suboptimal for clinical use because 1) results were only achieved in subset of cases, 2) there was a high level of subjectivity in scoring, and 3) the log-rank test for T1 did not reach statistical significance for all outcome parameters in Models 2 and 5 of **Figures 2A and 2B**, respectively. Addition of PTEN mutation status to POLE mutation categorization although seemingly helpful in the initial discovery phase with the TCGA data (not shown) did not add apparent benefit to the models based on the Vancouver data set (Models 4, 5, 6, 7 of **Figures 2B and 2C**, respectively). Of note, 11 of 12 cases with POLE mutations also harbour PTEN mutations in this data set.

**[0092]** ***Assessment of the Molecular Classification Tool Compared to Traditional Clinical/Pathological Risk groups.*** ESMO clinical risk group stratification assigned based on complete clinicopathologic data from staging was also demonstrated to be associated with OS, DSS and PFS in the Vancouver data set ( $p < .005$  for all) (data not shown).

**[0093]** Harrell's C-Index measuring the discriminative ability of a model to predict an event (e.g., outcomes; OS, DSS and RFS) is shown for each of 8 models in **Figure 3**.

**[0094]** **Figure 3** shows Harrell's C-Index for Models 1 to 8, ESMO clinical risk group, and combined molecular and risk groups or pathologic parameters as applied to the Vancouver cohort ( $n=143$ ). A C-index of 0.5 (dotted horizontal line) indicates that the model has no discriminative ability and a C-index of 1 indicates that a model perfectly distinguishes between those who have an event and those who do not. The model parameters used in the pragmatic model chosen to move forward (column 8) is outlined in **Figure 3**. Also outlined are the indices for the molecular classifier combined with clinical risk groups (column 10) or pathological parameters (column 11), suggesting an improved ability to discriminate outcomes when taken together.

**[0095]** It was also shown that the C-index for ESMO clinical risk group stratification, clinical risk group stratification combined with molecular classification, or pathologic parameters (each component of grade, stage, LVSI etc added to the model) combined with molecular classification demonstrating the improved ability to discriminate EC outcomes when both traditional and molecular tools are used, with confidence intervals no longer crossing the threshold of 0.5 (**Figure 3**). Kaplan-Meier analyses for the 8 models, C-indices, sensitivity and specificity for the molecular classifier models with and without ESMO clinical risk group stratification were also applied to the TCGA cohort but appear less able to discern outcomes (data not shown).

**[0096]** **Figure 4** shows the favoured pragmatic model for molecular classification of endometrial cancers (Model 8 of **Figure 2C**). Selection was based on survival analyses, C-index, anticipated clinical benefit in order of testing, and cost and accessibility of methods. In this model

option (Model 8 of **Figure 2C**, and see column 8 in **Figure 3**), based on pragmatic surrogate molecular assays inclusive of: 1) MMR IHC abnormalities ('MMR IHC abn'), 2) 'POLE' EDM mutations, and 3) p53 status determined by IHC, as a surrogate to delineate 'p53 wt' and 'p53 abn' groups. Subsequent comparisons of the molecular classifier in univariate and multivariable analysis are used this model specifically for classification.

**[0097]** From the entire cohort (n=143), MMR MSS status was evaluated for abn (MMR MSI-high) was first determined, and found in 41 subjects. Of the remaining subjects (MMR-MSS) (n=102), POLE mutation was assessed, and found in 12 subjects. Of the remaining subjects with POLE wild type (n=88), p53 was evaluated with IHC, and determined either as p53 wt (n=63) or p53 abn (n=25). For any with p53 missing (n=2), these subjects were deemed unclassifiable.

**[0098]** Univariable analysis was performed to test for associations of known prognostic impact with outcomes (OS, DSS and RFS). These include the molecular subtypes resulting from the chosen model (Model 8), demographic, and clinicopathologic parameters as well as the clinical risk groups (ESMO) (**Table 2**). Increased hazard ratios (HR) were demonstrated for p53 abn molecular subgroup, stage indicative of disease beyond the uterine corpus (e.g., > stage I), grade, presence of lymphatic or vascular space invasion, positive lymph nodes or receiving adjuvant treatment (**Table 2**).

**Table 2 – Univariable Analysis Showing the Individual Association Between The Molecular Classifier and Standard Demographic and Pathological Variables with Outcomes**

|                                               | Outcome | No. of events/n |              | Hazard Ratio (95% CI)           | LRT P-value |
|-----------------------------------------------|---------|-----------------|--------------|---------------------------------|-------------|
| Molecular classifier Subgroups<br>Ref: p53 wt | OS      | 28/141          | MMR IHC abn  | 1.80 (0.72–4.49) <sup>(F)</sup> | 0.0044      |
|                                               |         |                 | POLE mut     | 0.23 (0.00–1.77) <sup>(F)</sup> |             |
|                                               |         |                 | p53 abn      | 3.29 (1.36–8.09) <sup>(F)</sup> |             |
|                                               | DSS     | 22/139          | MMR IHC abn  | 0.36 (0.47–3.78) <sup>(F)</sup> | 0.024       |
|                                               |         |                 | POLE mut     | 0.25 (0.00–2.03) <sup>(F)</sup> |             |
|                                               |         |                 | p53 abn      | 2.89 (1.10–7.63) <sup>(F)</sup> |             |
|                                               | RFS     | 27/133          | MMR IHC abn  | 0.85 (0.31–2.12) <sup>(F)</sup> | 0.016       |
|                                               |         |                 | POLE mut     | 0.16 (0.00–1.25) <sup>(F)</sup> |             |
|                                               |         |                 | p53 abn      | 2.19 (0.91–5.08) <sup>(F)</sup> |             |
| Age* at Surgery                               | OS      | 28/143          |              | 1.03 (0.99–1.07)                | 0.093       |
|                                               | DSS     | 22/141          |              | 1.01 (0.97–1.05)                | 0.49        |
|                                               | RFS     | 27/135          |              | 1.01 (0.98–1.05)                | 0.43        |
| BMI* (kg/m <sup>2</sup> )                     | OS      | 26/121          |              | 0.99 (0.96–1.02)                | 0.55        |
|                                               | DSS     | 20/119          |              | 0.99 (0.95–1.03)                | 0.49        |
|                                               | RFS     | 25/114          |              | 0.98 (0.94–1.02)                | 0.31        |
| Stage<br>Ref: Stage I                         | OS      | 28/143          | Stage II–IV  | 3.41 (1.61–7.21)                | 0.001       |
|                                               | DSS     | 22/141          | Stage II–IV  | 3.80 (1.62–8.89)                | 0.002       |
|                                               | RFS     | 27/135          | Stage II–IV  | 5.17 (2.36–11.31)               | 0.000       |
| Grade*                                        | OS      | 28/143          |              | 2.02 (1.24–3.30)                | 0.003       |
|                                               | DSS     | 22/141          |              | 2.49 (1.37–4.53)                | 0.001       |
|                                               | RFS     | 27/135          |              | 2.03 (1.22–3.36)                | 0.004       |
| Histology<br>Ref: Endometrioid                | OS      | 28/143          | Serous/mixed | 1.94 (0.85–4.40)                | 0.13        |
|                                               | DSS     | 22/141          | Serous/mixed | 2.24 (0.91–5.51)                | 0.097       |
|                                               | RFS     | 27/135          | Serous/mixed | 2.16 (0.94–4.93)                | 0.086       |
| Presence of LVSI<br>Ref: absence of LVSI      | OS      | 27/137          | LVSI present | 3.75 (1.64–8.59)                | 0.001       |
|                                               | DSS     | 21/135          | LVSI present | 5.05 (1.84–13.83)               | 0.001       |
|                                               | RFS     | 26/130          | LVSI present | 2.89 (1.29–6.48)                | 0.008       |
| Any Positive Node<br>Ref: no positive nodes   | OS      | 26/139          | Nodes +      | 2.69 (1.13–6.41)                | 0.040       |
|                                               | DSS     | 21/137          | Nodes +      | 3.57 (1.44–8.85)                | 0.012       |
|                                               | RFS     | 24/131          | Nodes +      | 3.09 (1.28–7.46)                | 0.022       |
| Any Adjuvant Rx<br>Ref: no Rx                 | OS      | 28/142          | Adjuvant Rx  | 3.07 (1.35–6.97)                | 0.005       |
|                                               | DSS     | 22/140          | Adjuvant Rx  | 4.21 (1.55–11.42)               | 0.002       |
|                                               | RFS     | 27/134          | Adjuvant Rx  | 3.79 (1.60–8.96)                | 0.001       |
| Clinical Risk Group*                          | OS      | 28/143          |              | 2.24 (1.33–3.76)                | 0.001       |
|                                               | DSS     | 22/141          |              | 2.98 (1.50–5.93)                | 0.000       |
|                                               | RFS     | 27/135          |              | 2.67 (1.52–4.70)                | 0.000       |

**[0099]** **Table 2** Abbreviations: CI = Confidence Interval; DSS = Disease Specific Survival; LRT = likelihood ratio test; OS = Overall Survival; RSF = Recurrence-Free Survival. Hazard Ratios (HR) are given with 95% confidence intervals and P=values are from LRT. Molecular classifier

subgroups MMR IHC abn, POLE EDM mutated and p53 abn are considered. For continuous variables in this analysis (\*) the HRs reflect a relative increase in risk associated with unit change, for example, HR for each additional year of age. For the categorical variables the reference for comparison is indicated. (F) indicates that the Firth's penalized maximum likelihood bias reduction method was used.

**[00100]** Multivariable analysis was performed to determine whether the molecular classifier adds any additional prognostic information to the "traditional" clinicopathologic risk-group categorization, defined here by ESMO criteria (encompassing stage, grade, histology), and suggests that the molecular tool remains prognostic independently of the ESMO risk groups (**Table 3**) e.g., both predictors are significant in the model, and the hazard ratio for p53 abn group (1.94) is on the same scale as for ESMO risk group (1.98). It was also determined whether the molecular classifier was of additional prognostic benefit to demographic or pathology risk factors (as were tested in univariable analysis). Comparing molecular vs. clinical models (summative of age, BMI, stage, grade, histology, LVSI, and nodal status), the molecular classifier was prognostic for OS, as well as DSS and RFS (data not shown) after accounting for the additional demographic and pathology parameters. However, given the low number of events and high number of parameters assessed these results must be interpreted with caution. Visual examination of the Schoenfeld residual plots indicate no evidence of classifier model (MMR/POLE mut/p53 IHC) violating the proportional hazard assumption (data not shown).

| <b>Table 3 – Multivariable Analyses Comparing Molecular Classifier Model (MMR IHC/POLE mut/p53 IHC) With Clinical Risk Group (ESMO)</b> |                 |                                 |             |
|-----------------------------------------------------------------------------------------------------------------------------------------|-----------------|---------------------------------|-------------|
|                                                                                                                                         | No. of events/n | Hazard ratio (95% CI)           | LRT P-value |
| <b>OS</b>                                                                                                                               |                 |                                 |             |
| MMR IHC abn/POLE mut/p53 abn: MMR IHC abn POLE p53 abn Ref: p53 wt                                                                      | 28/141          | 1.30 (0.51–3.33) <sup>(F)</sup> | 0.031       |
|                                                                                                                                         |                 | 0.17 (0.00–1.36) <sup>(F)</sup> |             |
|                                                                                                                                         |                 | 1.94 (0.77–5.02) <sup>(F)</sup> |             |
| Clinical risk group (ESMO)                                                                                                              |                 | 1.98 (1.20–3.55) <sup>(F)</sup> | 0.0055      |
| <b>DSS</b>                                                                                                                              |                 |                                 |             |
| MMR IHC abn/POLE mut/p53 abn: MMR IHC abn POLE p53 abn Ref: p53 wt                                                                      | 22/139          | 0.84 (0.28–2.40) <sup>(F)</sup> | 0.089       |
|                                                                                                                                         |                 | 0.17 (0.00–1.38) <sup>(F)</sup> |             |
|                                                                                                                                         |                 | 1.42 (0.52–3.91) <sup>(F)</sup> |             |
| Clinical risk group (ESMO)                                                                                                              |                 | 2.74 (1.48–5.98) <sup>(F)</sup> | 0.0006      |
| <b>RFS</b>                                                                                                                              |                 |                                 |             |
| MMR IHC abn/POLE mut/p53 abn: MMR IHC abn POLE p53 abn Ref: p53 wt                                                                      | 27/133          | 0.50 (0.18–1.29) <sup>(F)</sup> | 0.015       |
|                                                                                                                                         |                 | 0.10 (0.00–0.77) <sup>(F)</sup> |             |
|                                                                                                                                         |                 | 1.10 (0.45–2.64) <sup>(F)</sup> |             |
| Clinical risk group (ESMO)                                                                                                              |                 | 2.78 (1.64–5.23) <sup>(F)</sup> | 0.0001      |

**[00101]** **Table 3** Abbreviations: CI = Confidence Interval. For clinical risk group (ESMO) the HR reflects an increase in hazard for each jump in ESMO risk group category. (F) indicates that the Firth's penalized maximum likelihood bias reduction method was used.

**[00102]** **Figure 4** shows the design of the study and the number of individuals classified into MMR IHC abn (41 of 143, or 28.7%); POLE EDM (12 of 143, or 8.4%); p53 wt (63 of 143; or 44%), and p53 abn (25 or 143, or 17.5%). Two subjects were in the unclassifiable categories.

**[00103]** Cross tabulation of the four molecular subgroups generated from MMR IHC/POLE/p53 IHC with ESMO risk groups in the Vancouver data set are shown in **Figure 5** (data obtained for TCGA cohort not shown).

**[00104]** **Figure 5** shows the cross-tabulation of clinicopathologic risk groups (ESMO) with molecular classification by proposed model: MMR IHC / POLE / p53 IHC. Approximately half of the POLE and MMR IHC abn molecular subgroups are noted to include cases that would be designated as 'high risk' by traditional clinical risk group stratification. The p53 abn molecular subgroup includes about 25% 'low' and 'intermediate' risk cases who would usually be designated to receive minimal

(e.g., vaginal brachytherapy) or no therapy. Although both molecular subgroups and clinical risk groups were associated with outcomes, they may identify different women with endometrial cancers.

**[00105]** It is apparent these classification systems are identifying different subgroups of women in both cohorts but more profoundly in the new Vancouver series where more precise ESMO classification was achievable. Focusing on the Vancouver cohort the 'low risk' clinical risk group can be seen across all four molecular subgroups, including over 30% of the POLE mutated cases but also almost 10% of p53 abn tumours. Greater diversity in outcomes in the 'low risk' group is also noted as 3 recurrences and 4 deaths were observed in this assigned cohort, exceeding the POLE molecular group (0 events). Not surprisingly most of the p53 abn cases were 'high risk', however, approximately half of the cases with POLE mutation and MMR IHC abn phenotype are also 'high risk' patients who under standard clinical care would go on to receive chemotherapy and radiation (**Figure 5**).

**[00106] Discussion**

**[00107]** Endometrial carcinomas, in particular high-grade cancers, cannot be reliably classified by histomorphologic criteria, even by expert pathologists and with the addition of immunohistochemistry (Gilks et al, 2013; Han et al, 2013; Hoang et al, 2013). Interobserver agreement among pathologists for morphologic risk factors such as grade and LVSI is poor ( $K = 0.35$  and  $0.23$ , respectively) (Guan et al, 2011), and histotype shows only a moderate degree of interobserver agreement ( $K = 0.58$ ) (Han et al, 2013). In order to move towards precision medicine, more reliable systems of categorizing endometrial cancers are needed in order to determine efficacy and appropriateness of treatments. Mutational profiling of endometrial cancers has shown promise (Cancer Genome Atlas Research et al, 2013; Le Gallo et al, 2012; McConechy et al, 2012; Stelloo et al, 2015) but methodologies to assign genomic subgroups can be expensive and complex, and consequently may not be achievable at all centers. In this Example, there is presented a molecular classifier for endometrial cancers that is based in part on the discoveries of the TCGA, but pared down to key components evaluable by relatively simple molecular methods. These methods were applied to a new training set of cases having detailed clinicopathologic data and outcomes.

**[00108]** The method was able to reproduce the four subgroups with distinct survival curves as identified in TCGA, with significant p-values achieved in survival analyses. Although these data suggest that p53 immunohistochemistry (IHC) and TP53 mutation status results are not completely equivalent, both methods of assessment were successful in identifying the 'p53 abn' molecular subgroup. Lower cost and wide availability of p53 immunohistochemistry in all pathology departments support immunohistochemistry as the preferred tool. Removing the FISH assessment, both for practical reasons (work intensive, subjective, results achievable in lower number of cases, and higher cost vs. p53 IHC) and due to lower performance compared to other models, is prudent. No surrogate for the POLE mutation detection is needed, but next generation sequencing may reveal such a

surrogate in the confirmation cohort. Characterization of POLE mutated cases in terms of immunophenotype will be informative.

**[00109]** Thus three major components in the model are maintained; MMR IHC for MSI phenotype, POLE mutation status, and p53 status. The order of the determination of these three major components is also worth consideration. Initially it was considered to pull out MMR IHC abnormal cases first, prompting hereditary cancer referral and yielding information that could be important for both patients and physicians to learn of early. A young woman diagnosed with EC may be considering conservative management (e.g. oral or local progesterone therapy), but if she carries a germline MMR gene mutation with increased associated lifetime risk of colon, uterine and ovarian carcinoma this would likely change her course with a recommendation made to pursue definitive surgical management, or may alter her decision to preserve her ovaries (as well as prompting colonoscopy screening) (Lu et al, 2005). Surgery with a specialist (gynecologic oncologist) for comprehensive staging rather than general gynecologist might be favored secondary to a higher likelihood of advanced stage, higher grade, and LVSI in these patients. Finally, identification of MMR IHC abnormal tumors may prove to have predictive implications in EC, as observed in colorectal cancers, that would influence choice of treatment. At present, MMR IHC results can be available at the same time as initial pathologic diagnosis of malignancy; POLE sequencing is not widely available and thus takes time to receive results. However improved turnaround time for POLE testing is expected in the future. POLE mutation status identifies women with the most favourable outcomes (Cancer Genome Atlas Research et al, 2013; Church et al, 2015), seeming to supersede other prognostic factors such as high grade disease. In a cohort of the size used in this Example, and given that all cases classified as 'POLE' had normal MMR IHC, it was not determined whether changing the order of molecular assessments such that POLE EDM mutations were detected first, would be more informative.

**[00110]** Ultimately, the classifier model chosen in this Example (**Figure 4**) is based on performance (survival analyses, Harrell's C-index), practicality of methods, and clinical utility.

**[00111]** In addition to testing the classifier model in hysterectomy specimens, assessment in cases of matched endometrial biopsy or dilatation and curettage (D&C) were commenced. Data from such assessments suggest that endometrial samplings (pipelle or D&C) are highly accurate (>97% sensitivity) at detecting cancer (Stovall et al, 1991), but grade and histotype may be discrepant with the final diagnoses based on examination of the hysterectomy specimens in up to one half of cases (Francis et al, 2009; Karateke et al, 2011); in contrast, molecular parameters are highly concordant between biopsy and hysterectomy (Stelloo et al, 2014). If equivalence can be demonstrated of a molecular classification system in diagnostic endometrial samples and prognostic significance of a classifier, then women and their physicians could have valuable information that would help them guide decision making at the earliest time point in their cancer journey, preferably at diagnosis. Decisions could be made prior to surgical staging regarding the urgency and extent of surgery,

anticipated adjuvant therapy, and follow-up plans. This information would be particularly helpful in guiding young women, with 14% of endometrial cancers arising in women < 50 years of age and in younger women who are consideration of fertility-sparing options or conservation of ovaries/hormonal function can be weighed against the risk of metastatic, or concurrent ovarian disease or worsened prognosis with deferred surgery.

**[00112]** The described method of classification can improve upon the current system of clinicopathologic risk group stratification that is based on stage and the irreproducible variables of grade and histotype assignment (Colombo et al, 2013; Kong et al, 2015; Kwon et al, 2009; Mariani et al, 2008), which is not highly predictive of outcomes (Bendifallah et al, 2015). Using the ESMO criteria it was demonstrated both in the TCGA data set and in the training set of cases, that the clinicopathologic risk groups are not equivalent to the molecular subgroups identified. What is evident is the number of cases that would be considered 'undertreated' or 'overtreated' depending on categorization. For example, over one quarter of the CN high molecular subgroups were designated as low or intermediate risk and may have been undertreated, with subsequent recurrence and death. Half of the POLE molecular subgroup and MSI subgroups were identified as 'high-risk' based on grade, stage and/or histotype. These women would have received chemotherapy and radiation based on the clinical centers' and consensus guidelines. The prognosis for women with POLE mutations is excellent. Whether that is because they received aggressive treatment or is independent of this remains to be determined. It may be that the POLE ultramutated phenotype is exquisitely sensitive to therapy or, as has been suggested previously, has a higher immune infiltrate (Hussein et al, 2015) that may be further stimulated by the introduction of treatment(s). However it may be that these women had an excellent prognosis, independent of treatment, and received toxic therapies with long term treatment side effects with no survival benefit.

**[00113]** Clinical risk groups were associated with outcomes in both TCGA and the cohorts of this Example, and should not be abandoned. But in terms of managing an individual the inconsistency of histotype and grade classification, it means that the same woman may be recommended to receive vastly different treatments depending on where her pathology is read. For example a woman with a pathology report indicating an endometrial high-grade serous cancer invading less than half her myometrial wall, with all other sites negative for disease (stage IA) receives systemic chemotherapy and pelvic radiation based on being HIGH risk. That same woman whose pathology is interpreted at another center as high-grade endometrioid EC would receive vaginal vault radiation only (associated with intermediate risk). Molecular classification adds prognostic information for these women and can directly impact care (e.g. referral for hereditary testing). It may prove to be more reproducible than histopathological assessment, when evaluated. The combination of both clinical/pathologic parameters (either summarized as ESMO risk groups or taken separately e.g. LVSI, grade) and molecular parameters results in an improvement upon either system alone, as it yields a higher C-index.

**[00114]** Limitations to this study include a relatively small sample size, and no definitive determination of the optimal order of molecular testing. In addition, the distribution of mismatch repair deficient cases that also harbour POLE mutations varies in the literature (Billingsley et al, 2015; Church et al, 2015) and were rare in small series such as these, therefore it remains uncertain how best to classify cases with both POLE EDM mutations and MMR IHC abn. The training set of cases reported herein was a retrospective cohort with potential selection bias related to being drawn from a tertiary cancer treatment center. These limitations are acknowledged.

**[00115]** In summary, it was demonstrated that a set of simple assays, applicable to formalin-fixed paraffin-embedded samples, can reproduce the four TCGA genomically-defined prognostic subgroups. These subgroups are associated with clinical outcomes, and identify women who may have a risk of recurrence of their EC that is very different than what is designated by traditional clinical risk group assessment. This classification method can be used across cancer centers and on preoperative endometrial samplings thus influencing management from time of diagnosis. Independent of any prognostic ability, molecular classification has the ability to direct clinical care, such as by referral to hereditary cancer programs for Lynch syndrome testing for abnormal MMR IHC. Molecular classification in ECs will allow stratification of cases for clinical trials and assessment of treatment efficacy within specific molecular subgroups. This has been a game-changing approach in ovarian cancers (Despierre et al, 2014) and has the potential to greatly advance progress in EC research.

**[00116]**        **Example 2**

**[00117]**        **Molecular Classification Of Endometrial Carcinoma On Diagnostic Specimens Is Highly Concordant With Final Hysterectomy: Earlier Prognostic Information To Guide Treatment**

**[00118]**        *ABSTRACT*

**[00119]**        Categorization and risk stratification of endometrial carcinomas is inadequate; histomorphologic assessment shows considerable interobserver variability, and risk of metastases and recurrence can only be derived after surgical staging. There is described herein a proactive molecular risk classification tool for Endometrial cancers (herein referred to as a method or system) that identifies four distinct prognostic subgroups. The objective of this Example was to assess whether molecular classification could be performed on diagnostic endometrial specimens obtained prior to surgical staging and its concordance with molecular classification performed on the subsequent hysterectomy specimen. Sequencing of tumors for exonuclease domain mutations (EDMs) in POLE and immunohistochemistry for mismatch repair (MMR) proteins and p53 were applied to both pre- and post-staging archival specimens from 60 individuals to identify four molecular subgroups: MMR-D, POLE EDM, p53 wild type, p53 abn(abnormal). Three gynecologic subspecialty

pathologists assigned histotype and grade to a subset of samples. Concordance of molecular and clinicopathologic subgroup assignments were determined, comparing biopsy/curetting to hysterectomy specimens.

**[00120]** Complete molecular and pathologic categorization was achieved in 57 cases. Concordance metrics for pre- vs. post-staging endometrial samples categorized by the described method were highly favorable; average per each class sensitivity (0.9), specificity(0.96), PPV(0.9), NPV(0.96) and kappa statistic 0.86(95%CI, 0.72-0.93), indicating excellent agreement. The highest level of concordance was observed for 'p53 abn' tumours, the group associated with the worst prognosis. In contrast, grade and histotype assignment from original pathology reports pre- vs. post-staging showed only moderate levels of agreement (kappa=0.55 and 0.44 respectively); even with subspecialty pathology review only moderate levels of agreement were observed. Molecular classification can be achieved on diagnostic endometrial samples and accurately predicts the molecular features in the final hysterectomy specimens, demonstrating concordance superior to grade and histotype. This biologically relevant information, available at initial diagnosis, has the potential to inform management (surgery, adjuvant therapy) from the earliest time point in cancer care.

**[00121]** *Introduction*

**[00122]** In this Example, the classifier method described in Example 1, and referred to as the proactive molecular risk classification tool for endometrial cancers (or as the instant "method" or "system") was evaluated to determine if it could be applied to endometrial biopsy or curetting specimens containing EC that were obtained for diagnostic purposes (e.g., to evaluate post-menopausal uterine bleeding), and if classification of these samples was concordant with final hysterectomy endometrial samples obtained at definitive surgical staging.

**[00123]** *Methods*

**[00124]** Cohort selection

**[00125]** To determine an appropriate cohort selection, an a priori power calculation was performed using the distribution of molecular subgroups in the TCGA (~7% POLE (ultramutated), 28% MSI-high, 39% CN-low and 26% CN-high), to reveal that a sample of size n=47 would be sufficiently large to detect concordance between pre- and post-staging endometrial samples greater than 0.65 (Power = 0.8,  $\alpha=0.05$ ). Previous studies (Karateke et al., 2011) have demonstrated that it is common for grade assignment to change between diagnostic (pre-) and final (post- surgical staging) endometrial specimens ( $\kappa=0.65$ ); therefore, it was considered that the molecular classification tool (also referred to as a method or system) to be clinically useful if it improved upon this figure. In order to account for a potential loss of cases due to molecular test failure, 60 women with EC were selected where both diagnostic (pre-) and hysterectomy (post-staging) endometrial specimens were available. With Institutional Review Board approval, 40 cases were identified from the previously described EC hysterectomy cohort noted in Example 1, that had undergone molecular classification with the

described method, based on the hysterectomy specimen, for whom there were available pre-surgical staging samples (endometrial biopsies or curettage specimens) that had not undergone molecular classification. These initial 40 cases were selected to ensure representation from all four molecular subgroups. Additionally, 20 recent cases of EC were identified where both diagnostic and final endometrial specimens were available; for these cases there was no prior knowledge of molecular subgroup. Hysterectomies performed after neo-adjuvant treatment were excluded from the study to ensure that there was not disagreement between samples secondary to treatment-induced molecular changes.

**[00126]** The molecular classification method described herein was used to assign EC specimens (both diagnostic and final hysterectomy within the same individual) to one of four molecular subgroups using methodologies described in Example 1. Testing involved sequential assessment of i) IHC for MMR proteins MLH1, MSH2, MSH6 and PMS2 ii) sequencing for polymerase epsilon (POLE) exonuclease domain mutations (EDMs), and iii) p53 IHC (**Figure 6**). Agreement of the molecular classification method was then compared between pre- and post-surgical staging specimens.

**[00127]** **Figure 6** shows how new EC samples are tested and categorized according to the above steps. First, immunohistochemistry (IHC) for the presence of mismatch repair proteins (MLH1, MSH2, MSH6, PMS2) where cases with loss of protein expression classified as MMR deficient (MMR-D). Second, sequencing for the presence of POLE exonuclease domain mutations (POLE EDM). Third, IHC for p53 to distinguish normal expression (IHC score 1) associated with wild type (p53 wt) from null/loss of function mutations (IHC score 0) or missense/gain of function mutations (IHC 2) grouped together as p53 abn.

**[00128]** **TMA construction.** For all diagnostic endometrial samples (endometrial biopsy, endometrial curettage specimens), a tissue microarray was constructed using 0.6 mm cores in duplicate.

**[00129]** **Immunohistochemistry.** Methodological details regarding IHC for mismatch repair proteins (MLH1, MSH2, MSH6, PMS2) and for p53 have previously been described in Example 1. In cases with equivocal or uninterpretable immunohistochemical results based on the TMA slides, immunohistochemistry was repeated on full sections. Scoring was performed by one of three pathologists designated as CBG, QN, or JL. MMR status was interpreted as lost if there was complete absence of staining in the tumor cells with adequate positive staining of internal controls (inflammatory cells or stroma). p53 was interpreted as abnormal if there was complete negative staining (null-pattern) or strong/diffuse staining in >70% of tumor cells (aberrant positive pattern). All other patterns were interpreted as wild-type.

**[00130]** **DNA extraction.** Methods have previously been described in Example 1. Briefly, DNA from formalin fixed paraffin embedded (FFPE) tumour blocks were extracted using the Qiagen

FFPE tissue kit, and all DNA was quantified using the Qubit™ fluorometer kit (Life Technologies™). To determine somatic status normal DNA was either extracted from available buffy coat or representative normal FFPE blocks.

**[00131]        *Sequencing.*** Targeted primers were designed to cover the POLE EDM exons 9-14. PCR products (150-200bp) were amplified using the Fluidigm 48X48 Access Arrays, as per manufacturer's protocol, with input of 100ng FFPE derived DNA, and 50ng high-quality DNA from buffy coat or frozen tumour DNA. DNA barcodes (10bp) with Illumina cluster-generating adapters were added to the libraries, and 96 samples pooled. The library pools were sequenced using the Illumina MiSeq for ultra-deep sequencing. All validated POLE mutations were bi-directionally sequenced twice at minimum using tumor DNA, and once in the normal to validate somatic or germline status using either ultra-deep MiSeq sequencing or Sanger sequencing.

**[00132]        *Histotype and Grade assignment.*** Original diagnoses from the host institutions were obtained on both diagnostic (biopsy/curettage specimens) and final hysterectomy specimens. In addition, three gynecologic subspecialty pathologists from three independent tertiary care institutions (designated as: RS, MK, CHL) reviewed 1-2 representative haematoxylin and eosin stained slides of diagnostic and final hysterectomy specimens with the goal of assigning histotype and grade. For grade, three choices (grade 1, 2, or 3) were considered. For histotype, pathologists were asked to render a diagnosis in one of the following categories: endometrioid, mucinous, serous, clear cell, dedifferentiated, carcinosarcoma, mixed and other. These pathologists were blinded to the original pathology reports.

**[00133]        *Statistics.*** Descriptive statistics were used to characterize the demographic, clinical and pathological data for evaluable cases according to molecular subgroups assigned in both the diagnostic and final hysterectomy specimens. To compare the diagnostic (biopsy/curettage) and final hysterectomy specimens using the original diagnoses assigned at the institution, overall accuracy and Cohen's kappa ( $\kappa$ ) statistic were calculated for the described molecular classification method, grade and histotype. In addition, the average per class sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were computed. To account for the ordinal nature of grade a weighted kappa with squared weights was additionally computed. Histotype was ultimately grouped into 4 more encompassing categories: endometrioid, serous, mixed, and other. Interobserver agreement between three subspecialty pathologists was calculated using Fleiss' generalized Kappa coefficient for multiple raters and Krippendorff's alpha for ordinal data for within sample type assessment. Cohen's kappa and a weighted Cohen's kappa was used to compare across sample type and the result was averaged. 95% confidence intervals were computed using a bootstrap approach with 1000 bootstrap samples.

**[00134]**      Results

**[00135]**      **Cohort.** Of the 60 cases considered, two were excluded as they were found to have received neoadjuvant therapy before hysterectomy, and one case was excluded secondary to insufficient tumor volume for DNA extraction and POLE sequencing. A total of 57 cases were compared for grade, histotype, and molecular subgroup assignment by the described method in both diagnostic (pre-staging) and final hysterectomy (post-staging) specimens. Surgical staging had occurred between 1987 and 2013. Histotype, grade, and pathological details assigned to diagnostic specimens and final hysterectomy specimens were taken from original pathology reports from this center. Details such as patient demographics, tumour grade, and histotype for the diagnostic specimens were recorded.

**[00136]**      The descriptive statistics of the cohort were provided according to the molecular subgroups described herein, as defined by diagnostic (biopsy or curettage) specimen. Further, the stage, LVSI, myometrial invasion, nodal status, adjuvant therapy, and ESMO risk group as designated on final hysterectomy/post-surgical staging specimens were evaluated and recorded for the cohort.

**[00137]**      The distribution of cases shows good representation of all four molecular subgroups. This cohort was enriched for p53 abn and POLE EDM cases to ensure that these lower frequency subgroups could be adequately evaluated.

**[00138]**      **Concordance of molecular classifier.** Table 4A shows the overall concordance and the concordance metrics are shown in Table 4B, including average per molecular subgroup sensitivity, specificity, PPV, NPV and kappa statistic for the method of molecular classification described herein comparing diagnostic and final surgical samples, with the latter held to be the "gold standard". Kappa statistic of 0.86 (95%CI) was consistent with a "near perfect" level of agreement, fulfilling the goal of improvement over previously published data showing poor concordance between pre- and post-operative samples, when assessed for the conventional histopathological parameters of grade and histotype.

| <b>Table 4A - Comparison of the described method of molecular classification of diagnostic samples (rows) and post-staging hysterectomy samples (columns)</b> |          |                             |                 |               |                |              |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|-----------------------------|-----------------|---------------|----------------|--------------|
|                                                                                                                                                               |          | <b>Post-staging Samples</b> |                 |               |                | <b>Total</b> |
|                                                                                                                                                               |          | <b>MMR-D</b>                | <b>POLE EDM</b> | <b>p53 wt</b> | <b>p53 abn</b> |              |
| <b>Diagnostic Samples</b>                                                                                                                                     | MMR-D    | 11                          | 1               | 2             | 0              | 14           |
|                                                                                                                                                               | POLE EDM | 0                           | 10              | 1             | 0              | 11           |
|                                                                                                                                                               | p53 wt   | 1                           | 0               | 19            | 0              | 20           |
|                                                                                                                                                               | p53 abn  | 0                           | 1               | 0             | 11             | 12           |
| <b>Total</b>                                                                                                                                                  |          | 12                          | 12              | 22            | 11             | 57           |

**Table 4B** - Comparison of overall concordance statistics (5% confidence intervals) based on the described molecular classification of diagnostic samples and post-staging samples

|                           | <u>Overall Concordance Statistics</u> |
|---------------------------|---------------------------------------|
| Overall Accuracy          | 0.89 (0.78-0.96)                      |
| Cohen's kappa             | 0.86 (0.72-0.93)                      |
| No Information Rate (NIR) | 0.39                                  |
| P-Value (Accuracy > NIR)  | 0                                     |

**[00139]** Also shown are the concordance metrics within each molecular subgroup (**Table 4C**), which are highly favorable for all parameters (>.9 except sensitivity for p53 wt subgroup; 0.84) with perfect or near perfect metrics within the p53 abn subgroup.

**Table 4C** - Comparison of concordance statistics (95% confidence intervals) for each of the described molecular subgroups.

|                | Average | MMR-D            | POLE EDM         | p53 wt           | p53 abn          |
|----------------|---------|------------------|------------------|------------------|------------------|
| Sensitivity    | 0.9     | 0.94 (0.72-1)    | 0.82 (0.52-0.95) | 0.84 (0.62-0.94) | 1 (0.74-1)       |
| Specificity    | 0.96    | 0.93 (0.81-0.97) | 0.98 (0.89-1)    | 0.97 (0.87-1)    | 0.98 (0.89-1)    |
| Pos Pred Value | 0.9     | 0.83 (0.61-0.94) | 0.9 (0.6-0.99)   | 0.94 (0.73-1)    | 0.92 (0.65-1)    |
| Neg Pred Value | 0.96    | 0.97 (0.87-1)    | 0.96 (0.86-0.99) | 0.92 (0.8-0.97)  | 1 (0.92-1)       |
| Prevalence     |         | 0.28 (0.18-0.41) | 0.19 (0.11-0.31) | 0.33 (0.22-0.46) | 0.19 (0.11-0.31) |
| Detection Rate |         | 0.26 (0.17-0.39) | 0.16 (0.09-0.27) | 0.28 (0.18-0.41) | 0.19 (0.11-0.31) |
| Detection Prev |         | 0.32 (0.21-0.44) | 0.18 (0.1-0.29)  | 0.3 (0.2-0.43)   | 0.21 (0.12-0.33) |
| Accuracy       | 0.95    | 0.93 (0.83-0.97) | 0.95 (0.86-0.98) | 0.93 (0.83-0.97) | 0.98 (0.91-1)    |
| Balanced Acc   | 0.93    | 0.93             | 0.9              | 0.91             | 0.99             |

**[00140]** *Interrogation of cases discordant on molecular classification.* In total there were 6 of 57 cases with discordant results between diagnostic and final surgical sample as assessed using the described method, only one of which was within the p53 abn subgroup. There were six discordant cases according to the described subclassifications, which are presented in more detail, case by case, below. A summary is found in **Table 5**.

| <b>Table 5 - Discordant Cases according to Subclassification Using the Classification Method</b> |                   |                            |                          |                                |                       |               |
|--------------------------------------------------------------------------------------------------|-------------------|----------------------------|--------------------------|--------------------------------|-----------------------|---------------|
| <b><u>Case/Specimen</u></b>                                                                      | <b>Pathology</b>  | <b>MMR IHC; POLE;p53</b>   | <b>Initial Subclasss</b> | <b>Reassessment</b>            | <b>Final Subclass</b> | <b>Retest</b> |
| <b><u>1/</u></b> Diagnostic                                                                      | CAH+gr 1 EM       | Intact; no mut; abn        | p53 abn                  | Retest: no POLE mut found      | p53 abn               | 1/<br>Discord |
| Hysterectomy                                                                                     | gr 1 EM, stage 1B | Intact; P286S; abn         | POLE EDM                 | Retest validates               | POLE EDM              |               |
| <b><u>2/</u></b> Diagnostic                                                                      | gr 2 EM           | MSH6 loss; no mut; wt      | MMR-D                    | Retest: Δ to intact            | p53 wt                | 2/<br>Concord |
| Hysterectomy                                                                                     | gr 2 EM, stage 1B | Intact; no mut; wt         | p53wt                    | Retest confirms intact         | p53 wt                |               |
| <b><u>3/</u></b> Diagnostic                                                                      | gr 1 EM           | MSH2/MSH6 loss; no mut; wt | MMR-D                    | Retest: changed to intact      | p53 wt                | 3/<br>Concord |
| Hysterectomy                                                                                     | gr 2 EM, stage 1A | Intact; no mut; wt         | p53 wt                   | Retest confirms intact         | p53 wt                |               |
| <b><u>4/</u></b> Diagnostic                                                                      | gr 3 undiff       | MSH2/MSH6 loss; P441L; abn | MMR-D                    | Retest confirms MSH2/MSH6 loss | MMR-D                 | 4/<br>Concord |
| Hysterectomy                                                                                     | gr 3 SC, stage 1B | Intact; P441L; abn         | POLE EDM                 | Retest: Δ to MSH2/MSH6 loss    | MMR-D                 |               |
| <b><u>5/</u></b> Diagnostic                                                                      | gr 1 EM           | Intact; no mut; wt         | p53 wt                   | Retest confirms intact         | p53 wt                | 5/<br>Discord |
| Hysterectomy                                                                                     | gr 3 EM, Stage 1B | MLH1/PMS2 loss; no mut; wt | MMR-D                    | Retest confirms loss of MLH1   | MMR-D                 |               |
| <b><u>6/</u></b> Diagnostic                                                                      | gr 1 EM           | Intact; P286R; wt          | POLE EDM                 | Retest validates               | POLE EDM              | 6/<br>Discord |
| Hysterectomy                                                                                     | gr 1 EM, stage 1A | Intact; no mut; wt         | p53 wt                   | Retest; No POLE mut found      | p53 wt                |               |

**[00141]** In case 1 a POLE EDM was detected at low allelic frequency (8% then 1% on retest) in the final hysterectomy sample (grade 1 endometrioid, early stage), but could not be detected in the diagnostic endometrial biopsy (grade 1 endometrioid). This patient had a very favorable outcome, and was a long term survivor. Sequencing results from a Fluidigm panel (3 genes in addition to POLE) revealed mutations in TP53: G360R, and PPP2R1A:R183W, and it is suspected that her TP53 mutation is secondary; consistent with a POLE EDM/ultramutated tumour, and this EC is appropriately categorized as POLE EDM, although without whole genome or even exome sequencing 'ultramutator' phenotype cannot be determined accurately.

**[00142]** Cases 2 and 3 are similar, both demonstrating loss of MMR proteins on endometrial biopsy specimen leading to classification as MMR-D, however the final hysterectomy shows all proteins intact. The diagnostic samples were retested, using full sections rather than tissue microarrays, and were able to demonstrate presence of all four MMR proteins thus changing their classification to p53 wt and concordant with final hysterectomy. For Case 4, MSH2/MSH6 loss was noted on endometrial biopsy specimen and confirmed on re-testing. MMR IHC had been interpreted as intact originally on hysterectomy however on re-testing with whole sections revealed MSH2/MSH6 loss thus the described classification is also concordant in this case upon review of whole sections. These misclassifications are therefore attributable to the small samples present on tissue microarrays, with 0.6 mm cores.

**[00143]** Case 5 remained discordant after re-review and repeat whole section MMR testing, and the discordant results were due to tissue sampling. In the endometrial sampling, there was only a low-grade endometrioid adenocarcinoma which had retention of MLH1 and PMS2. The hysterectomy however, had a low-grade endometrioid adenocarcinoma as well as dedifferentiated carcinoma, and this latter component, which was not sampled in the endometrial biopsy, showed loss of MLH1 and PMS2, as is commonly seen in dedifferentiated carcinoma of the endometrium (**Figure 7**).

**[00144]** **Figure 7** shows the histopathologic features of endometrial carcinoma with discrepant mismatch repair protein results on endometrial sampling and hysterectomy. The endometrial sampling consists of only low-grade endometrioid adenocarcinoma (Panel A). On the hysterectomy, the superficial portion of the tumor contains the low-grade endometrioid adenocarcinoma while the deeper portion is higher grade with solid architecture (Panel B). In the solid areas, the nuclei are enlarged, irregular and the cells are mildly discohesive; peritumoral lymphocytes are also present at the leading edge of the tumor (Panel C). Immunohistochemical staining for MLH1 shows retained staining in the low-grade glandular component and loss of staining in the high-grade solid component (Panel D).

**[00145]** Case 6 shows discordance in POLE EDM results, with mutations found in the diagnostic biopsy sample at 18% frequency (23% on retesting) but no POLE EDMs found in the final hysterectomy sample. Both diagnostic and hysterectomy samples were grade 1 endometrioid tumors, with minimal myometrial invasion in the hysterectomy specimen.

**[00146]** In summary, 3 of the 6 discrepancies between diagnostic sample and hysterectomy are attributable to the use of TMAs with the small sample size and were easily resolved with the more use of whole section immunostaining, as would be done in clinical practice. In 2 cases there was failure to detect POLE EDM (in the diagnostic sample in one case and the hysterectomy specimen in the other) because of low tumor cellularity or frequency of the mutant allele, and again these discordant results do not reflect inferiority of the biopsy/curettage specimen compared to the hysterectomy specimen for molecular classification. Instead, they reflect as yet unsolved issues around detection and interpretation of low frequency POLE EDM. There was thus a single tumor

where the diagnostic specimen had failed to sample a high-grade (dedifferentiated) component of the tumor. This case (case 5) was a true sampling error as the biopsy was not reflective of the final tumor with respect to grade, histotype or molecular classification.

**[00147]** **Concordance of grade and histotype in diagnostic endometrial vs. final hysterectomy samples.** The overall concordance and concordance metrics for grade and histotype, based on the original pathology reports, comparing diagnostic pre-surgical staging samples to final hysterectomy samples, are shown in **Tables 6A – 6D**.

| <b>Table 6A - Comparison of histology assessment of diagnostic samples and post-staging hysterectomy samples from original pathology reports.</b> |              |                             |        |       |       |              |
|---------------------------------------------------------------------------------------------------------------------------------------------------|--------------|-----------------------------|--------|-------|-------|--------------|
|                                                                                                                                                   |              | <b>Post-staging Samples</b> |        |       |       | <b>Total</b> |
|                                                                                                                                                   |              | Endometrioid                | Serous | Mixed | Other |              |
| <b>Diagnostic Samples</b>                                                                                                                         | Endometrioid | 37                          | 0      | 2     | 1     | 40           |
|                                                                                                                                                   | Serous       | 0                           | 4      | 0     | 0     | 4            |
|                                                                                                                                                   | Mixed        | 3                           | 1      | 1     | 0     | 5            |
|                                                                                                                                                   | Other        | 4                           | 3      | 0     | 1     | 8            |
| <b>Total</b>                                                                                                                                      |              | 44                          | 8      | 3     | 2     | 57           |

**[00148]** **Table 6A** shows a comparison of histology assessment (using simplified categories) of diagnostic samples (rows) and post-staging hysterectomy samples (columns) from original pathology reports.

| <b>Table 6B - Comparison of overall concordance statistics based on histotype assessment of diagnostic samples and post-staging samples</b> |                                       |
|---------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|
|                                                                                                                                             | <b>Overall Concordance Statistics</b> |
| <b>Overall Accuracy</b>                                                                                                                     | 0.75 (0.62-0.86)                      |
| <b>Cohen's kappa <sup>†</sup></b>                                                                                                           | 0.44 (0.23-0.65)                      |
| <b>No Information Rate (NIR)</b>                                                                                                            | 0.77                                  |
| <b>P-Value (Accuracy &gt; NIR)</b>                                                                                                          | 0.69                                  |

**[00149]** **Table 6B** shows a Comparison of overall concordance statistics (with 95% confidence intervals) based on histotype assessment (using simplified categories) of diagnostic samples and post-staging samples from original pathology reports. Please note that “†” is used in the table to indicate that kappa must be interpreted with caution due to symmetrical imbalance of row and column marginals, in **Table 6A**.

| <b>Table 6C -Comparison of grade assessment of diagnostic samples and post-staging samples from original pathology reports</b> |                |                             |                |                |              |
|--------------------------------------------------------------------------------------------------------------------------------|----------------|-----------------------------|----------------|----------------|--------------|
|                                                                                                                                |                | <b>Post-staging Samples</b> |                |                | <b>Total</b> |
|                                                                                                                                |                | <b>Grade 1</b>              | <b>Grade 2</b> | <b>Grade 3</b> |              |
| <b>Diagnostic Samples</b>                                                                                                      | <b>Grade 1</b> | 12                          | 9              | 2              | 23           |
|                                                                                                                                | <b>Grade 2</b> | 3                           | 7              | 1              | 11           |
|                                                                                                                                | <b>Grade 3</b> | 1                           | 1              | 21             | 23           |
|                                                                                                                                | <b>Total</b>   | 16                          | 17             | 24             | 57           |

**[00150]** Table 6C shows a comparison of grade assessment of diagnostic samples (rows) and post-staging samples (columns) from original pathology reports.

| <b>Table 6D -Comparison of overall concordance based on assessment of grade of diagnostic samples and post-staging samples</b> |                                       |
|--------------------------------------------------------------------------------------------------------------------------------|---------------------------------------|
|                                                                                                                                | <b>Overall Concordance Statistics</b> |
| <b>Overall Accuracy</b>                                                                                                        | 0.7 (0.57-0.82)                       |
| <b>Weighted kappa</b>                                                                                                          | 0.7 (0.5-0.83)                        |
| <b>No Information Rate (NIR)</b>                                                                                               | 0.42                                  |
| <b>P-Value (Accuracy&gt; NIR)</b>                                                                                              | 0                                     |

**[00151]** Table 6D shows a comparison of overall concordance statistics (with 95% confidence intervals) based on assessment of grade of diagnostic samples and post-staging samples from original pathology reports.

**[00152]** Kappa statistics for simplified (4-category) histotype was 0.44(0.23-0.65) (Table 6A) and for grade a weighted kappa of 0.7 (0.5-0.83) (Table 6B) were comparable to what has been reported previously, and was worse than the high level of reproducibility seen with molecular subclassification.

**[00153]** *Concordance of grade and histotype between gynecologic subspecialty pathologists.* Table 7A shows interobserver agreement between three subspecialty pathologists, computed using Fleiss's kappa. Table 7A illustrates that the average concordance metrics for grade and histotype between the three gynecologic pathologists as evaluated within the 48 diagnostic (pre-surgical staging) and final hysterectomy (post-surgical staging) samples available for review.

Concordance remains low; kappa for grouped grade (grade 1/2 vs. grade 3) (0.74) and simplified histotype (0.51), even when assigned by experts.

| <b>Table 7A – Agreement for Grade and Histotype</b> |                           |                             |
|-----------------------------------------------------|---------------------------|-----------------------------|
|                                                     | <u>Diagnostic Samples</u> | <u>Post-Staging Samples</u> |
| <b>Simplified Histology</b>                         | 0.51 (n=48)               | 0.59 (n=48)                 |
| <b>Grouped Grade</b>                                | 0.74 (n=47)               | 0.73 (n=46)                 |

**[00154]** **Table 7B** shows concordance between diagnostic samples and post-staging hysterectomy specimen pathologic assignment computed using Cohen's kappa. Level of agreement for each pathologist is shown as well as averages. Finally comparing each subspecialty pathologists diagnoses for grade and histotype in diagnostic vs. final hysterectomy specimens i.e., WITHIN an individual patient there was on average kappa of 0.56 and 0.57 respectively (**Table 7B**).

| <b>Table 7B –Interobserver Agreement for Grade and Histotype</b> |                      |                      |                      |                |
|------------------------------------------------------------------|----------------------|----------------------|----------------------|----------------|
|                                                                  | <u>Pathologist 1</u> | <u>Pathologist 2</u> | <u>Pathologist 3</u> | <u>Average</u> |
| <b>Simplified Histology</b>                                      | 0.49                 | 0.56                 | 0.62                 | 0.56           |
| <b>Grouped Grade</b>                                             | 0.56                 | 0.56                 | 0.57                 | 0.57           |

**[00155]** Discussion

**[00156]** Inadequacies in the current system of EC classification and risk stratification have prompted a call to action to identify new biologically informative tools. This Example confirms the previously reported lack of reproducibility of conventional histopathological assessment, both between observers, and in comparing biopsy/curettage to hysterectomy specimens. Attempts at comprehensive treatment guidelines, interpretation of past and future clinical trials, and EC research are severely limited by the inability to consistently classify this disease. The tremendous advances made in research and treatment of other cancers have not been realized in EC.

**[00157]** Although TCGA represented a positive step towards informative classification, the methods used were impractical. Lower cost methods applicable to formalin-fixed paraffin-embedded (FFPE) specimens are desirable to identify four prognostically distinct molecular subgroups of EC. For the molecular classification tool described herein, the Institute of Medicine guidelines for the development of 'omics based testing are followed.

**[00158]** Reproducibility of any classifier is of critical importance, and one aspect of reproducibility is the potential to give a definitive classification based on diagnostic specimens e.g. biopsy or curetting. Almost all women ultimately diagnosed with endometrial carcinoma have some sort of assessment of their endometrium-either by office biopsy or dilatation and curettage, which both identifies an EC and informs proceeding to the next step of surgical staging. This specimen is usually abundant/high volume and is immediately fixed often resulting in superior quality immunohistochemistry compared to final hysterectomy specimens, which may sit at room temperature for a variable period of time before processing. MMR and p53 proteins have relatively short half-lives and their detection is therefore dependent on prompt fixation, as is also true for detection of estrogen receptor and HER2 in breast cancer. There have been few studies looking at traditional pathological parameters and molecular features in endometrial biopsies. These results provide indirect evidence that addresses one of the questions raised by the aforementioned studies demonstrating only moderate agreement of histotype and grade assignment in diagnostic versus hysterectomy specimens i.e. is the lack of reproducibility primarily due to inadequate tumor sampling or to the inter- and intraobserver variability of grade and histotype assignment. Only one case where there was clearly a sampling issue, with a high-grade dedifferentiated component not present in the biopsy specimen. This suggests that the observed problems with imperfect concordance between diagnostic and hysterectomy specimens reflects the inherent lack of reproducibility of grade and histotype rather than true sampling differences.

**[00159]** The obvious advantage to successful molecular classification with the method described herein, in diagnostic specimens is earlier availability of prognostic information. Knowledge about a woman's risk of having metastatic disease, recurrence, and/or death may impact the urgency and comprehensiveness of surgical staging, and/or adjuvant therapy. Globally, there is a wide range of surgical practice, ranging from delay of hysterectomy (progesterone treatment) to preserve fertility, maintaining ovaries to preserve endogenous hormonal production and avoid associated comorbidities, pelvic +/- para-aortic lymph node assessment (complete, sampling, sentinel, or none), washings, omental/upper abdominal assessment (complete, biopsy, none). Each component of these surgical procedures has a cost: to the patient (fertility, cardiovascular disease, perioperative risk of injury, lymphedema) or the health care system (pathology processing and interpretation, operating room time). Determining the 'best' surgical procedure would be a tremendous start in personalized medicine. In conventional methods for classification, risk stratification is achievable only after surgical staging (myometrial invasion and stage are major components). Although adjuvant therapy can be guided from the post-staging information and risk group assignment, these again are limited by the irremediable irreproducibility of histotype and grade. This classification method described herein would change this.

**[00160]** In summary, use of this molecular classifier is a pragmatic option for classifying all endometrial cancers at the time of initial diagnosis. The techniques described herein are practical and achievable at any cancer center. Endometrial biopsy or curettage specimens are routinely obtained during the work up and evaluation of endometrial cancers and if/when cancer is diagnosed the herein-described method can be applied. Processing of the sample is done with well-known methods, requiring no special handling, as steps can be performed on FFPE material. Experience with more than 3000 clinical cases supports the use of endometrial biopsies or curetting specimens for MMR assessment as they are promptly fixed, with better antigen preservation than the corresponding hysterectomy specimen.

**[00161]** Discordant cases will be encountered, as they were in this series. Although far more consistent than grade or histotype assignment, there remains the possibility that the molecular classifier tool could assign a woman with EC to the inappropriate subgroup, based on analysis of the biopsy. Importantly, incorrect assignment appears to be very unlikely in the subgroup with the worst outcomes (p53 abn), suggesting that the classification method is unlikely to miss someone who may need more comprehensive surgery and additional chemotherapy and/or radiotherapy. Three of six cases with discordant results in this study can be explained based on the use of a tissue microarray as a research tool; in these three cases this resulted in an error in MMR assessment in either the diagnostic or hysterectomy specimen and these errors were easily resolved through use of whole sections for immunostaining, as would be done in routine practice. In two cases there were discrepancies attributable to low frequency POLE EDM mutations or possibly low tumor cellularity, resulting in the diagnostic specimen in one case and the hysterectomy specimen in the other being considered to have intact POLE. In theory, mixed carcinomas may also pose a challenge to classify using the described method. However, truly biologically mixed tumors are exceptionally rare and most cases diagnosed as mixed carcinoma are actually due to morphologic ambiguity rather than admixtures of molecularly distinct clones.

**[00162]** With regards to the order of testing, it was ultimately decided that MMR testing first made sense as it was believed that this information would prompt early referral to the hereditary cancer program for testing for Lynch syndrome and information from that test may impact on the patient's decision regarding management e.g. foregoing a trial of medical therapy with high dose progesterone. An outstanding question is how to handle cases that are positive for more than one of the classifiers. This is particularly important for tumors with POLE EDM or MMR-D and p53 abn. Such 'double feature' cases only account for 3-4% of EC, and based on available information the POLE EDM or MMR-D appears to be more important than p53 abn when both are present.

**[00163]** Consistent molecular subclassification of EC is an important goal. There are advantages to stratifying clinical trials by molecular subgroups to provide earlier and more reliable prognostic information to patients and their physicians. The data provided herein clearly demonstrates

the utility of the described classification method for diagnostic endometrial samples obtained prior to surgical staging, expanding the potential clinical impact of this method.

**[00164] Example 3**

**[00165] Confirmation Of A Genomics-Based Clinical Classifier For Endometrial Cancer**

**[00166] ABSTRACT**

**[00167]** Classification of endometrial cancers by morphologic features is irreproducible and imperfectly reflects tumor biology. A molecular classification method and system are described herein based on the four genomic subgroups of endometrial carcinoma identified through The Cancer Genome Atlas (TCGA) project. The prognostic ability of this method is confirmed in a large cohort of ECs, comparing its prognostic ability to a widely used clinicopathological-based risk-stratification system (European Society for Medical Oncology (ESMO)). Immunohistochemistry (IHC) for presence or absence of mismatch repair (MMR) proteins (identifies MMR-deficient or 'MMR-D'), sequencing for POLE exonuclease domain mutations ('POLE EDM') and IHC for p53 (wild type vs. null or missense mutations; 'p53 wt' and 'p53 abn', respectively) were performed on 460 EC samples from patients in British Columbia. Molecular subgroups were derived according to a decision tree classification system and characterized by demographic and clinicopathologic features. Association between molecular risk groups and survival outcomes were examined and the prognostic ability was compared to current risk stratification methods.

**[00168]** The classification method identified four prognostic subgroups with distinct overall, disease specific and progression free survival ( $p < 0.001$ ). POLE EDM having the most favourable prognosis and p53 abn having the worst prognosis consistent with TCGA genomic subgroups and the initial discovery cohort with separation of the two middle survival curves (p53 wt and MMR-D) now observed. In contrast, there were no significant survival differences between the ESMO low- and intermediate-risk groups. The method improved the ability to discriminate outcomes (Harrell's C-index) compared to conventional (ESMO) risk assessment. There was substantial overlap (85%) between the p53 abn and 'high-risk' ESMO subgroups but the other molecular subgroups were distributed across the ESMO risk groups. Molecular classification of ECs can be achieved using clinically applicable methods and provides independent prognostic information beyond that of established clinicopathologic risk factors. If applied in practice, this will allow consistent biologically relevant categorization, stratification of ECs for clinical trials and/or targeted therapy, and identification of women who are at increased risk of having Lynch syndrome.

**[00169] Introduction**

**[00170]** There are over 60,000 new cases of endometrial cancer (EC) diagnosed in North America each year. The increased incidence and mortality of EC observed globally has been

described as epidemic. Still, EC is understudied and as yet, no progress has been made to address its greatest challenge: irreproducible pathological categorization, particularly of high grade tumors, which can lead to inaccurate risk category assignment and imprecise estimation of disease metastases, recurrence and death. This results in the over- and under-treatment of thousands of women. Diverse subsets of EC's are lumped together in clinical trials making interpretation of treatment efficacy impossible.

**[00171]** To address these shortfalls, there is described herein a molecular classification method (interchangeably referred to as a "classifier" or a "classification system" or "classification scheme", based on proactive molecular risk classification for EC that assigns EC patients to one of four risk groups based on a combination of mutation and protein expression analysis as shown in **Figure 6** and described above. This classification method was based on results from The Cancer Genome Atlas (TCGA) collaborative project in endometrial carcinomas that yielded four molecularly defined subgroups, but which utilized cost-prohibitive methods for group assignment in routine clinical practice, and was dependent on fresh frozen tumor samples, which requires special sample handling. As described in Example 1, above, of the 16 models tested using key molecular parameters, the current method is based on the best model to take forward for further evaluation.

**[00172]** Pragmatic molecular-based classification tools for endometrial carcinoma, that can reliably group biologically similar tumours and stratify EC patients based on risk of recurrence and death from disease, will result in better surgery, post-surgical treatment and surveillance programs and improve outcomes for women with this disease. The objectives of this Example were, first, to test the described classification method in a new, larger 'confirmation' cohort of cases, to confirm that this classification scheme yields prognostic information consistent with previous findings. Second, a comparison was made between the prognostic ability of the described classification method against a traditional risk stratification system commonly used to direct treatment (European Society for Medical Oncology (ESMO): Low-Intermediate- and High-risk).

**[00173]** **Methods**

**[00174]** **Cohort selection, clinicopathological data and outcomes.** All EC specimens evaluated are from departments associated with the University of British Columbia and Vancouver General Hospital, where gynecologic oncology surgical care is centralized in the region. The hospital offers subspecialty gynecologic oncology services, resulting in a referral bias in the case series, as higher grade and advanced stage tumors are more likely to be referred to this hospital.

**[00175]** Following requisite approvals, patients were identified retrospectively by searching for all EC cases with desired clinical data and adequate specimen volume for molecular analysis. Some cases were from a hospital-associated tumor bank where patient consent was obtained prior to banking and some cases were archival. Clinical, pathology (original diagnoses), and outcome data was collected on all patients by one of two individuals (JM, JP) until Dec 31, 2015. Surgical staging

and adjuvant therapy were directed by British Columbia Cancer Agency guidelines, which have changed multiple times over the study period. Clinical and pathology parameters collected included age, body mass index ( $\text{kg/m}^2$ ), stage (updated according to FIGO 2009 classification), grade, histology, lymphovascular space invasion (LVSI, yes/no), myometrial invasion (none/<50%/>50%), positive nodes (not tested/tested positive/tested negative), and adjuvant treatment (any, including vaginal brachytherapy vs. none). Definitions of endpoints (overall (OS), disease specific (DSS), progression-free survival (PFS)) and specifics on clinicopathologic parameters are as described in Examples 1 and 2. Two clinicians (referred to as: JM and JK), blinded to outcomes and molecular profiles, independently assigned ESMO risk group (low-, intermediate, or high-risk) to each case and the consensus was used for analysis

**[00176]** *Tissue Microarray (TMA) construction and Immunohistochemistry.* To construct the endometrial TMA, tumours were annotated by a pathologist (referred to as: BG) and two 0.6mm cores of FFPE endometrial carcinoma per case were arrayed. TMAs were cut at 4 $\mu\text{m}$  thickness onto Superfrost<sup>TM</sup>+ glass slides, and were processed using the Ventana<sup>TM</sup> Discovery XT, and the Ventana Benchmark XT automated system (Ventana Medical Systems, Tucson, AZ, USA) as per manufacturer's protocol with proprietary reagents. After slides were baked at 60°C for 1 h, they were deparaffinized on the automated system with EZ Prep solution (Ventana). Heat induced antigen retrieval method was used in Cell Conditioning solution (CC1-Tris based EDTA buffer, pH 8.0, Ventana). Anti- hMSH6 (1:600, PU29, Novacastra<sup>TM</sup>, Vector Labs) and-Anti hPMS2 (1:150, MOR4G, Novacastra, Vector Labs), with appropriate positive and negative controls according to the manufacturers' standardized staining protocols. Tumours were deemed to be mismatch repair intact with any degree of nuclear expression of the mismatch repair (MMR) proteins MSH6 and PMS2. Tumours were considered to demonstrate aberrant protein expression (mismatch repair deficient or MMR-D) if tumour cells showed complete absence of nuclear staining, in conjunction with preserved expression in non-neoplastic internal control cells. Staining for individual MMR proteins was repeated on corresponding whole sections whenever there was aberrant, equivocal or uninterpretable staining (e.g., no tumour tissue) on the TMA. Results were ultimately dichotomized as MMR-D (one or more than one of two MMR proteins missing and confirmed on whole section), or intact (both proteins present). Comparison of four protein (MLH1, MSH2, MSH6, PMS2) and two protein (MSH6, PMS2) panel immunohistochemistry (IHC) and comparison of MMR IHC to microsatellite instability (MSI) assay has previously been reported, with high concordance demonstrated.

**[00177]** For p53 immunostaining, the primary antibody was DO-7 mouse anti-p53 monoclonal antibody (Dako, Carpinteria CA), used at a dilution of 1:400. Antigen retrieval was done using "CC1" for 64 minutes on machine protocol. Tissue sections were incubated with the primary antibody for 60 minutes at 37 degrees followed by the pre-diluted Ventana Universal Secondary detection reagent, used according to the manufacturer's protocol. Appropriate on-slide positive and negative controls

were used. Immunostaining for p53 was considered abnormal when there was no staining of tumour cell nuclei strong and diffuse staining (absent p53 protein or aberrant increased protein accumulation, respectively), while intermediate levels of expression was considered to be wild-type. Specifically 0=negative, 1='1-80% of tumor cell nuclei showing weak to moderate expression', and 2= '>80% of tumor cell nuclei showing strong expression'.

**[00178]      *DNA extraction.*** Methods as described above were used, and were successful in both fresh frozen and FFPE samples. Briefly, DNA from frozen tumours and buffy coat were extracted using the Qiagen™ Gentra Puregene™ kit (Qiagen) as per manufacturer's protocols. FFPE tumour blocks were extracted using the Qiagen FFPE tissue kit, and all DNA was quantified using the Qubit™ fluorometer kit (Life Technologies). To determine somatic status normal DNA was either extracted from available buffy coat or representative normal FFPE blocks.

**[00179]      *Sequencing.*** Targeted primers were designed to cover the POLE exonuclease domain exons 9-14. PCR products (150-200bp) were amplified using the Fluidigm 48X48 Access Arrays, as per manufacturer's protocol, with input of 100ng FFPE derived DNA, and 50ng high-quality DNA from buffy coat or frozen tumour DNA. DNA barcodes (10bp) with Illumina cluster-generating adapters were added to the libraries, and 96 samples pooled. The library pools were sequenced using the Illumina™ MiSeq™ for ultra-deep sequencing. All validated POLE mutations were bi-directionally sequenced twice at minimum, and once in the normal (to validate somatic or germline status) using either ultra-deep MiSeq sequencing or Sanger sequencing.

**[00180]      *Classification by Molecular subgroup assignment.*** New EC samples were assigned in the following order: Mismatch repair (MMR) status by immunohistochemistry identifying MMR-Deficient ('MMR-D') and 'MMR-intact' based on absence of either MSH6 or PMS2 proteins. MMR-intact cases were then segregated by presence or absence of POLE exonuclease domain mutations ('POLE EDM'). Finally MMR intact, POLE wild type cases were segregated as either 'p53 wild type' (IHC score 1) or abnormal ('p53 abn')(IHC score 0 or 2)(**Figure 6**). The described molecular subgroups MMR-D, POLE EDM, p53 wild type and p53 abn approximate the TCGA genomic subgroups microsatellite instability, POLE ultramutated, copy-number low, and copy-number high, respectively.

**[00181]      *Figure 6*** illustrates the steps in molecular classification with the described method, interchangeably referred to herein as the Proactive Molecular Risk Classifier for Endometrial Cancer. The first assessment is immunohistochemistry for the presence of mismatch repair (MMR) deficiency proteins in order to identify women who may have Lynch syndrome to enable rapid referral to the hereditary cancer program and possibly direct surgical or therapeutic decisions. Tumors are next assessed for POLE EDM mutations, and finally p53 IHC yielding four subgroups: MMR-D, POLE, p53 wt and p53 abn.

**[00182]**        *Statistical Analysis.* Results of the interrogation of 16 molecular classification models, based on considerations to order of testing and methodologies applicable to clinical samples, i.e. requiring no special specimen handling and relatively low cost and yielded the described method of classification.

**[00183]**        Analytical methods were locked down and the prognostic ability of the described method was evaluated on an additional larger independent cohort of cases (termed 'confirmation' cohort).

**[00184]**        The initial discovery cohort and the subsequent larger confirmation cohort were compared using descriptive statistics on all parameters of interest. The prognostic signature of the described classification method in the confirmation cohort was compared to the one obtained in the previous Examples as well as in the TCGA study, by considering the survival pattern of the molecular subgroups, obtained from Kaplan Meier survival plots. Harell's C-index was used to evaluate the classification method's ability to discriminate between good and bad outcomes in new patients from the confirmation cohort, using the previously developed model parameters. Estimated hazard ratios associated with the described molecular subgroups, obtained using cox proportional hazard model adjusted for treatment, were compared across cohorts. Cases from the discovery cohort were combined with the new cases from the confirmation cohort to form the full cohort, which was used to establish the described classification method as an independent prognostic marker in EC.

**[00185]**        The molecular subgroups were characterized in the full cohort (discovery+confirmation) using descriptive statistics, as well as univariable and multivariable associations with clinicopathological parameters. Univariable associations of individual clinicopathological parameters with the described subgroups were compared using one-way analysis of variance for continuous data (age at surgery, BMI) and chi-squared test for categorical data (stage, grade, histological subtype at diagnosis, LVSI, myometrial invasion, and nodal status). Multivariable associations were modeled using a multinomial logistic regression, with subgroups as outcome and all clinicopathological parameters as additive covariates.

**[00186]**        To avoid sparse subcategories and to be consistent, in all statistical analyses, grade was recoded as low grade (grades 1 and 2) versus high grade, similarly stage was recoded as advanced stage (disease spread beyond the uterus encompassing stages II-IV) versus low stage, and histological subtype was recoded as endometrioid subtype versus non-endometrioid. Adjuvant treatment was additionally dichotomized into any treatment (chemotherapy, external beam radiotherapy, vaginal brachytherapy) versus no treatment. Only cases with complete clinicopathological parameters were considered in analyses. In order to ensure that missing values were not associated with cohort or subgroup, a missing value comparison was undertaken.

**[00187]**        Univariable and multivariable survival analysis of the classification method was considered, as well as other clinicopathologic parameters of interest in association with OS, DSS and

PFS. Those were performed using cox proportional hazard models and Kaplan-Meier analysis as applicable.

**[00188]** For multivariable survival analyses, a cox proportional hazard model was considered with classification subgroups and prognostic factors available from time of diagnosis as it is desirable to ultimately apply this classification method to specimens PRIOR to surgical staging. Thus groups were corrected for age, BMI, grade and histotype in addition to molecular subgroup as assigned by the classification method. Multivariable analysis was also performed using additional parameters available from post-surgical staging as several of these are known to be important prognostic factors (e.g., stage, nodal status, myometrial invasion and presence of LVSI). Adjuvant treatment status was included as a covariate in all multivariable models to account for the possible confounding effect of treatment, since all cases were not treated.

**[00189]** In all cox models, hazard ratios with corresponding 95% CI's and Likelihood Ratio Test (LRT) p-values were reported. The Firth's penalized maximum likelihood bias reduction method was used to estimate the hazard ratio (indicated by an (F)) when the number of censored cases exceeded 80%, the corresponding confidence intervals were obtained using the profile likelihood. Proportional hazard assumptions were verified by study of the graphs of the Schoenfeld's residuals.

**[00190]** Accounting for treatment effect, the discriminatory ability of all multivariable survival models, using the described molecular subgroups and ESMO risk group (assigned using pathological data from surgical staging) separately and together in a model, as well as the classification method described herein, and parameters available at time of diagnosis and with parameters available post-staging, was compared using Harell's C-index. These models were internally validated in the full cohort using bootstrap resampling techniques to adjust for potential 'optimism' in the performance of the model. One thousand bootstrap samples were used; in each bootstrap iteration, a sample of size equal to the full cohort was drawn with replacement from the full cohort of cases. Models were developed in the bootstrap samples and tested in those subjects excluded from the bootstrap sample.

**[00191]** Kaplan-Meier survival analysis was performed on the full cohort, plotting OS, DSS and RFS for each of the four described molecular subgroups as well as for ESMO clinical risk groups. The two classification systems were then compared using cross-tabulation.

**[00192]** All statistical hypothesis tests performed were two-sided. Statistical significance was set at  $\alpha=0.05$  and no attempts were made to correct for multiple comparisons. All statistical analyses were done using R project for statistical computing.

## **[00193] Results**

**[00194]** ***Confirmation of prognostic signature in a new cohort of cases.*** The 'confirmation cohort' consisted of 319 new cases and were drawn from 519 surgical specimens of EC. **Figure 8A** outlines the reasons for exclusion from analysis; including having received neoadjuvant chemotherapy, missing molecular data, or follow-up time of less than 2 years. The 'discovery cohort'

is as described in Example 1. Together, the 'confirmation' and 'discovery cohorts' encompass 460 cases and are termed the 'full cohort'.

**[00195]** **Figure 8A** shows a breakdown of cases evaluated in both the discovery and confirmation cohorts. Cases were excluded for reasons listed, ultimately yielding 460 fully evaluable EC's (full cohort). **Figure 8B** shows the described molecular subgroup distribution within the full cohort (n=460).

**[00196]** The confirmation cohort included cases from a longer time period (1983-2013) than the original discovery series (2002-2009). Overall follow up was 5.2 years (reverse Kaplan-Meier), and this was consistent in both cohorts. Full details on the cohorts including comparison of age, BMI, stage histotype, presence of LVSI, depth of myometrial invasion, nodal status, adjuvant treatment administered, ESMO risk group, and the molecular subgroup across the discovery, confirmation and full cohorts (discovery + confirmation) were assessed and revealed a slight enrichment for grade 3 and serous cases in the confirmation cohort. The described molecular subgroup distribution of the classification method is consistent with this observation with more p53 abn cases (27%) in the confirmation cohort than in the discovery set (~18%). The confirmation cohort had slightly less tumors displaying MMR-deficiency (MMR-D) (20% compared with 29% in the discovery cohort). Both cohorts are selected, in that they are drawn from a tertiary referral center (majority of low grade endometrioid ECs are managed in the community) and do not represent EC incidence/case distribution in the general population.

**[00197]** Of note, the discovery cohort consisted of mostly fresh frozen samples (87.9%) and the confirmation cohort mostly (85.3%) FFPE samples yielding a total of 289 (62.8%) FFPE and 171 (37.2%) frozen samples in the full cohort.

**[00198]** The described molecular classification method can discriminate outcomes (OS, DSS, PFS) in comparison with ESMO, another risk stratification system. Evaluate of the classification method alone, ESMO alone, and the described classification method and ESMO combined and after accounting for treatment was undertaken. To do so, model parameters estimated from the discovery cohort (n=141) to new cases (n=319, the confirmation set) and Harrell's C-index computed from the confirmation cohort was compared to the one previously derived in the discovery cohort. It was found that the resulting C-index ranged from 0.65-0.78 depending on the model and the end-point selected. The discriminatory ability of the classification method alone in the new cases was as good as ESMO alone, and when combining both together, performance was better than each separately. The discriminatory ability was higher for the DSS and PFS endpoints and slightly poorer for OS. Kaplan-Meier survival curves for the confirmation cohort were consistent with what was shown in the previous Examples for the Discovery cohort and TCGA. Because the number of cases in the discovery cohort was small, and with few events, a comparison was made between the estimated hazard ratios for each described molecular subgroup (p53 wt as reference) across cohorts, accounting for treatment.

Hazard ratio estimates are stabilizing with increased number of cases and events, and are consistent in direction and magnitude across cohorts. The remainder of analyses were done on the full cohort, combining discovery set and confirmation set.

**[00199]**      ***Characterizing subgroups in the full cohort.*** Figure 8B outlines the breakdown of cases in the full cohort with statistically significant univariable associations seen between molecular subgroups for all clinical and pathological parameters measured shown in **Table 9A** and **Table 9B**. Details were obtained on follow up times and number of events by subtypes, and further details on the clinicopathological parameters and molecular features were evaluated, including tissue type examined and a breakdown of IHC scores for the full cohort. Missing data analysis revealed no associations with clinicopathologic parameters or outcomes, with grade and histotype available in all cases and <10 cases with missing values for age, stage, myometrial invasion, nodal status, treatment and ESMO risk group.

**Table 8A:** Univariable association of endometrial subtypes with demographic, clinical and pathological variables

|                           | Total             | MMR-D             | POLE EDM          | p53 wt            | p53 abn           | P-Value |
|---------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|---------|
| <b>Number of Patients</b> | 460               | 105               | 42                | 202               | 111               |         |
| <b>Age</b>                |                   |                   |                   |                   |                   |         |
| Mean( $\pm$ se)           | 65.6 ( $\pm$ 0.6) | 65.6 ( $\pm$ 1.1) | 59.8 ( $\pm$ 1.8) | 63.6 ( $\pm$ 0.9) | 71.5 ( $\pm$ 0.8) | 0.000   |
| Median                    | 66.1              | 66.1              | 58.4              | 63.3              | 71.5              |         |
| missing                   | 3                 | 1                 | 0                 | 2                 | 0                 |         |
| <b>BMI</b>                |                   |                   |                   |                   |                   |         |
| Mean( $\pm$ se)           | 31.8 ( $\pm$ 0.9) | 33 ( $\pm$ 3.1)   | 26.6 ( $\pm$ 0.8) | 33.7 ( $\pm$ 0.9) | 29.4 ( $\pm$ 0.8) | 0.000   |
| Median                    | 28.5              | 28.5              | 27.2              | 30.5              | 28                |         |
| missing                   | 42                | 7                 | 6                 | 25                | 4                 |         |
| <b>Stage</b>              |                   |                   |                   |                   |                   |         |
| I                         | 322(70.6%)        | 71(68.3%)         | 39(95.1%)         | 160(80%)          | 52(46.8%)         | 0.000   |
| II-IV                     | 134(29.4%)        | 33(31.7%)         | 2(4.9%)           | 40(20%)           | 59(53.2%)         |         |
| missing                   | 4                 | 1                 | 1                 | 2                 | 0                 |         |
| <b>Grade</b>              |                   |                   |                   |                   |                   |         |
| Grade 1/2                 | 210(45.7%)        | 36(34.3%)         | 14(33.3%)         | 152(75.2%)        | 8(7.2%)           | 0.000   |
| Grade 3                   | 250(54.3%)        | 69(65.7%)         | 28(66.7%)         | 50(24.8%)         | 103(92.8%)        |         |

**[00200]** Several observations about molecular subgroup and phenotype that have been described, above, in the discovery cohort are noted again with the addition of more cases; including younger age and lower BMI of women who have POLE EDM tumors, older age and lower BMI associated with p53 abn tumors, and low stage (95% stage I)/high grade (66.7% grade 3) predominantly endometrioid (83%) tumors in the POLE EDM subgroup. The majority of p53 abn ECs had IHC scores of 2 (91/111 or 82%) indicative of TP53 missense mutations, with the remainder being p53 null/IHC score 0 (20/111 or 18%). p53 abn tumors had aggressive pathologic features (92% grade 3, 76% non-endometrioid histology, 61% LVSI and highest proportion of lymph node positivity (17%), advanced stage), but aggressive pathologic features were also noted (66% grade 3, 21% non-endometrioid histotype, 52.5% with LVSI, 32% advanced stage II-IV) associated with MMR-D ECs (**Table 8A** and **8B**) as compared to p53 wild type subgroup.

| Table 8B: Further univariable association of endometrial subtypes<br>with demographic, clinical and pathological variables |            |           |           |            |           |         |
|----------------------------------------------------------------------------------------------------------------------------|------------|-----------|-----------|------------|-----------|---------|
| Parameter                                                                                                                  | Total      | MMR-D     | POLE EDM  | p53 wt     | p53 abn   | P-Value |
| <b>Histology</b>                                                                                                           |            |           |           |            |           |         |
| Endometrioid                                                                                                               | 332(72.2%) | 83(79%)   | 35(83.3%) | 187(92.6%) | 27(24.3%) | 0.000   |
| Non-Endometrioid                                                                                                           | 128(27.8%) | 22(21%)   | 7(16.7%)  | 15(7.4%)   | 84(75.7%) |         |
| <b>LVSI</b>                                                                                                                |            |           |           |            |           |         |
| No                                                                                                                         | 266(60.9%) | 48(47.5%) | 22(55%)   | 155(80.7%) | 41(39.4%) | 0.000   |
| Yes                                                                                                                        | 171(39.1%) | 53(52.5%) | 18(45%)   | 37(19.3%)  | 63(60.6%) |         |
| missing                                                                                                                    | 23         | 4         | 2         | 10         | 7         |         |
| <b>Myo Invasion</b>                                                                                                        |            |           |           |            |           |         |
| None                                                                                                                       | 75(16.6%)  | 4(3.8%)   | 4(9.8%)   | 49(24.5%)  | 18(16.8%) | 0.000   |
| <50%                                                                                                                       | 205(45.3%) | 56(53.3%) | 20(48.8%) | 93(46.5%)  | 36(33.6%) |         |
| >50%                                                                                                                       | 173(38.2%) | 45(42.9%) | 17(41.5%) | 58(29%)    | 53(49.5%) |         |
| missing                                                                                                                    | 7          | 0         | 1         | 2          | 4         |         |
| <b>Nodal Status</b>                                                                                                        |            |           |           |            |           |         |
| Negative                                                                                                                   | 269(59%)   | 72(68.6%) | 31(73.8%) | 92(45.8%)  | 74(68.5%) | 0.000   |
| Not Tested                                                                                                                 | 150(32.9%) | 22(21%)   | 11(26.2%) | 101(50.2%) | 16(14.8%) |         |
| Positive                                                                                                                   | 37(8.1%)   | 11(10.5%) | 0(0%)     | 8(4%)      | 18(16.7%) |         |
| missing                                                                                                                    | 4          | 0         | 0         | 1          | 3         |         |
| <b>Treatment</b>                                                                                                           |            |           |           |            |           |         |
| None                                                                                                                       | 241(53.6%) | 52(50%)   | 18(43.9%) | 140(70%)   | 31(29.5%) | 0.000   |
| Any                                                                                                                        | 209(46.4%) | 52(50%)   | 23(56.1%) | 60(30%)    | 74(70.5%) |         |
| missing                                                                                                                    | 10         | 1         | 1         | 2          | 6         |         |
| <b>ESMO</b>                                                                                                                |            |           |           |            |           |         |
| Low                                                                                                                        | 151(33%)   | 23(21.9%) | 12(29.3%) | 112(55.7%) | 4(3.6%)   | 0.000   |
| Intermediate                                                                                                               | 71(15.5%)  | 18(17.1%) | 12(29.3%) | 29(14.4%)  | 12(10.8%) |         |
| High                                                                                                                       | 236(51.5%) | 64(61%)   | 17(41.5%) | 60(29.9%)  | 95(85.6%) |         |
| missing                                                                                                                    | 2          | 0         | 1         | 1          | 0         |         |

**[00201]** All four cases with POLE EDMs and recurrence or death were reviewed. Although one of these was an advanced stage (IIIC1) mixed serous and endometrioid carcinoma, the remaining three were endometrioid, early stage (1A or 1B) and grade 3 or, in one case, grade 2. Interestingly three of four were in older patients; 75 (Stage 1B, grade 2 endometrioid), 92 (Stage 1A, grade 3 endometrioid), and 89 years old (mixed histotype case, described above). Three of the four cases received both adjuvant chemotherapy and radiation, yet still experienced recurrence and died of their

disease. In the 92 year old patient, no adjuvant treatment or recurrence was documented and she died of intercurrent illness.

**[00202]** A multinomial logistic regression model was used to assess the association between clinicopathological parameters that were most significantly associated with the described molecular subgroups, accounting for all other parameters: age, BMI, stage, grade, histotype, LVSI, myometrial invasion, nodal status and any adjuvant therapy. Older age at surgery was significantly associated with increased odds of having a p53 abn tumor. Increased BMI was associated with decreased odds of having a tumor with a POLE EDM. Women with grade 3 (vs. grade 1 or 2) tumours were more likely to have p53 abn or MMR-D tumors. Non-endometrioid histotype and > 50% myometrial invasion were associated with p53 abn subgroup whereas tumors that exhibited 0-50% myometrial invasion were more likely to be MMR-D. Patients with LVSI had higher odds of having tumors that were p53 abn or MMR-D.

**[00203]** *Interrogation of cases with more than one molecular feature of the classification method identified.* The order of molecular testing for the described classification method, as shown in **Figure 6**, means that some cases may have more than one molecular feature but get categorized based on the first feature identified in the diagnostic tree (e.g. MMR-D). In the full cohort of 460 cases, 16 cases (3.4%) had more than one molecular feature: 11 cases with MMR-D and p53 abn (classified as MMR-D), 4 cases with POLE EDM and p53 abn (classified as POLE EDM) and a single case with MMR-D and POLE mutations (classified as MMR-D). The molecular parameters as well as clinicopathologic features of these cases were evaluated. Results from additional genes tested as part of the select sequencing panel (TP53, PP2R1A and FBXW7) were available and interrogated for these 'double feature' cases.

**Table 9 - Clinicopathologic and molecular parameters of 'double feature' cases**

|   | Age | Stage | Hist +Gr | ESMO | Treatment | Recurrence Status | PFS  | OS    | MMR IHC   | POLE EDM  | p53 IHC | p53 mut    | Other mutations |
|---|-----|-------|----------|------|-----------|-------------------|------|-------|-----------|-----------|---------|------------|-----------------|
| 1 | 56  | IA    | CC3      | 3    | EBRT,VB   | No                |      | 19.96 | MSH6 loss | wild type | 2+      | R175H      | None            |
| 2 | 73  | IB    | EM3      | 3    | None      | No                |      | 11.12 | MSH6 loss | wild type | 2+      | R175H      | None            |
| 3 | 75  | IB    | EM3      | 3    | None      | Yes               | 0.75 | 0.75  | PMS2 loss | wild type | 2+      | N239D      | None            |
| 4 | 48  | IA    | Undiff3  | 3    | None      | Yes               | 0.29 | 0.29  | MSH6 loss | wild type | 0       | frameshift | None            |
| 5 | 71  | IV    | S3       | 3    | CP        | Yes               | 3.00 | 10.10 | MSH6 loss | wild type | 2+*     | wild type  | None            |
| 6 | 64  | IV    | S3       | 3    | CP        | Yes               | 0.60 | 1.11  | PMS2 loss | wild type | 2+      | D281H      | None            |

|    |    |       |         |   |                 |    |  |      |              |                |     |                 |                                                  |
|----|----|-------|---------|---|-----------------|----|--|------|--------------|----------------|-----|-----------------|--------------------------------------------------|
| 7  | 42 | IIIC1 | S/CC3   | 3 | CP, EBRT,<br>PA | No |  | 5.12 | PMS2<br>loss | wild<br>type   | 2+* | wild<br>type    | FBXW7: R505C                                     |
| 8  | 55 | IA    | Undiff3 | 3 | None            | No |  | 6.98 | PMS2<br>loss | wild<br>type   | 2+* | wild<br>type    | None                                             |
| 9  | 59 | IIIC1 | EM3     | 3 | CP, EBRT,<br>PA | No |  | 2.50 | PMS2<br>loss | wild<br>type   | 2+  | R273H,<br>R273C | None                                             |
| 10 | 65 | IV    | EM3     | 3 | CP, EBRT,<br>PA | No |  | 5.21 | PMS2<br>loss | wild<br>type   | 2+* | wild<br>type    | PP2R1A: R183W                                    |
| 11 | 65 | IA    | S3      | 3 | CP, EBRT        | No |  | 2.73 | MSH6<br>loss | wild<br>type   | 2+  | R248Q           | PP2R1A: S95L,<br>E216K; FBXW7:<br>R224X          |
| 12 | 59 | IIIC1 | EM3     | 3 | EBRT, VB        | No |  | 9.67 | Intact       | V411L<br>(59%) | 2+  | E286K<br>(43%)  | PP2R1A:<br>E297K(44%);<br>FBXW7:<br>R658X(45%)   |
| 13 | 61 | 1A    | EM3     | 2 | CP              | No |  | 5.24 | Intact       | P286S<br>(35%) | 0   | N200T<br>(25%)  | FBXW7:<br>R658X(42%)                             |
| 14 |    | 1A    | SC3     | 3 | CP              | No |  | 5.70 | Intact       | V411           | 2+  | wild<br>type    | PP2R1A:R183W,<br>E471D;<br>FBXW7:R479Q,<br>S438Y |
| 15 | 57 | 1B    | EM3     | 3 | CP, EBRT,<br>VB | No |  | 5.32 | Intact       | A456P          | 2   | R169X,<br>Y31C  | PP2R1A:R221W;<br>FBXW7: R689W                    |
| 16 | 55 | 1A    | S3      | 3 | None            | No |  | 7.03 | PMS2<br>loss | F367L          | 1+  | D41N            | None                                             |

**[00204]** Table 9 notes: Age at diagnosis, recurrence free survival (RFS) and overall survival (OS) are given in years. EM=endometrioid, CC-clear cell, S=serous, Undiff=undifferentiated; Treatment refers to primary adjuvant treatment and does not include treatment that may be given at recurrence. C=carboplatin, P=paclitaxel, EBRT= external beam radiotherapy to pelvis VB= vaginal brachytherapy, PA= para-aortic boost. Mutation frequencies are given in % where known. All mutations shown have been validated. MMR IHC results for two panel testing (MSH6 and PMS2) given. \* p53 staining mixed on older studies (1+ and 2+).

**[00205]** In Tables 10A, 10B and 10C the univariable survival analysis of molecular subgroup, risk stratification group, and clinicopathologic parameters is provided for the parameters of overall survival (OS), disease specific survival (DSS) and recurrence free survival (RFS) in the full cohort. As used in these tables, the superscript "F" indicates Firth confidence intervals were computed using the profile likelihood and may not match the LRT p-values.

**Table 10A** – (OS) Overall Survival Analysis of molecular subgroup, risk stratification group, and clinicopathologic parameters for full cohort.

| OS                         |                 |                               | LRT P-value |
|----------------------------|-----------------|-------------------------------|-------------|
|                            | # of events / n | Hazard Ratio (95% CI)         |             |
| <b>ProMisE™ subgroup</b>   |                 |                               |             |
| p53 wt (ref)               | 120 / 460       | -                             |             |
| MMR-D                      |                 | 2.04 (1.25-3.33) <sup>F</sup> | 0.0000      |
| POLE EDM                   |                 | 0.59 (0.19-1.41) <sup>F</sup> |             |
| p53 abn                    |                 | 3.73 (2.44-5.77) <sup>F</sup> |             |
| <b>ESMO</b>                |                 |                               |             |
| Low (ref)                  | 120 / 458       | -                             |             |
| Intermediate               |                 | 1.23 (0.55-2.57) <sup>F</sup> | 0.0000      |
| High                       |                 | 3.96 (2.46-6.73) <sup>F</sup> |             |
| <b>Age at Surgery</b>      | 118 / 457       | 1.04 (1.03-1.06)              | 0.0000      |
| <b>BMI</b>                 | 104 / 418       | 0.98 (0.96-1.00)              | 0.0517      |
| <b>Stage</b>               |                 |                               |             |
| Stage I (ref)              | 118 / 456       | -                             |             |
| Stage II-IV                |                 | 3.90 (2.72-5.63) <sup>F</sup> | 0.0000      |
| <b>Grade</b>               |                 |                               |             |
| Grade 1/2                  | 120 / 460       | -                             |             |
| Grade 3                    |                 | 2.85 (1.92-4.32) <sup>F</sup> | 0.0000      |
| <b>Histology</b>           |                 |                               |             |
| Endometrioid               | 120 / 460       | -                             |             |
| Non-Endometrioid           |                 | 2.62 (1.82-3.75) <sup>F</sup> | 0.0000      |
| <b>LVSI</b>                |                 |                               |             |
| No (ref)                   | 111 / 437       | -                             |             |
| Yes                        |                 | 3.36 (2.30-4.97) <sup>F</sup> | 0.0000      |
| <b>Nodes Tested</b>        |                 |                               |             |
| Negative (ref)             | 118 / 456       | -                             |             |
| Not Tested                 |                 | 0.81 (0.54-1.22)              | 0.0303      |
| Positive                   |                 | 1.90 (1.10-3.29)              |             |
| <b>Treatment</b>           |                 |                               |             |
| None                       | 117 / 450       | -                             |             |
| Any                        |                 | 1.96 (1.36-2.86) <sup>F</sup> | 0.0003      |
| <b>Myometrial Invasion</b> |                 |                               |             |
| None                       | 117 / 453       | -                             |             |
| <50%                       |                 | 1.28 (0.67-2.68) <sup>F</sup> | 0.0000      |
| ≥50%                       |                 | 3.89 (2.13-7.90) <sup>F</sup> |             |

**Table 10B** – Disease Specific Survival (DSS) Analysis of molecular subgroup, risk stratification group, and clinicopathologic parameters for full cohort.

| <b>DSS</b>                 |                        |                                |                    |
|----------------------------|------------------------|--------------------------------|--------------------|
| <b>ProMisE™ subgroup</b>   | <b># of events / n</b> | <b>Hazard Ratio (95% CI)</b>   | <b>LRT P-value</b> |
| p53 wt (ref)               |                        |                                |                    |
| MMR-D                      | 89 / 447               | -                              |                    |
| POLE EDM                   |                        | 2.23 (1.23-4.01) <sup>F</sup>  | 0.0000             |
| p53 abn                    |                        | 0.49 (0.10-1.51) <sup>F</sup>  |                    |
| <b>ESMO</b>                |                        | 4.68 (2.85-7.87) <sup>F</sup>  |                    |
| Low (ref)                  |                        |                                |                    |
| Intermediate               | 89 / 445               | -                              |                    |
| High                       |                        | 2.51 (0.87-7.45) <sup>F</sup>  | 0.0000             |
| <b>Age at Surgery</b>      |                        | 9.53 (4.63-23.59) <sup>F</sup> |                    |
| <b>BMI</b>                 | 87 / 444               | 1.03 (1.01-1.05)               | 0.0018             |
| <b>Stage</b>               | 76 / 405               | 0.98 (0.96-1.00)               | 0.0664             |
| Stage I (ref)              |                        |                                |                    |
| Stage II-IV                | 87 / 443               | -                              |                    |
| <b>Grade</b>               |                        | 5.21 (3.40-8.09) <sup>F</sup>  | 0.0000             |
| Grade 1/2                  |                        |                                |                    |
| Grade 3                    | 89 / 447               | -                              |                    |
| <b>Histology</b>           |                        | 5.41 (3.18-9.89) <sup>F</sup>  | 0.0000             |
| Endometrioid               |                        |                                |                    |
| Non-Endometrioid           | 89 / 447               | -                              |                    |
| <b>LVI</b>                 |                        | 3.43 (2.26-5.21) <sup>F</sup>  | 0.0000             |
| No (ref)                   |                        |                                |                    |
| Yes                        | 82 / 425               | -                              |                    |
| <b>Nodes Tested</b>        |                        | 4.44 (2.82-7.21) <sup>F</sup>  | 0.0000             |
| Negative (ref)             |                        |                                |                    |
| Not Tested                 | 87 / 443               | -                              |                    |
| Positive                   |                        | 0.82 (0.50-1.32) <sup>F</sup>  | 0.0026             |
| <b>Treatment</b>           |                        | 2.68 (1.48-4.61) <sup>F</sup>  |                    |
| None                       |                        |                                |                    |
| Any                        | 86 / 437               | -                              |                    |
| <b>Myometrial Invasion</b> |                        | 2.98 (1.90-4.81) <sup>F</sup>  | 0.0000             |
| None                       |                        |                                |                    |
| <50%                       | 86 / 440               | -                              |                    |
| ≥50%                       |                        | 1.13 (0.49-2.97) <sup>F</sup>  | 0.0000             |

**Table 10C** – Recurrence Free Survival (RFS) Analysis of molecular subgroup, risk stratification group, and clinicopathologic parameters for full cohort.

|                            |                 | <b>PFS</b>                      |             |
|----------------------------|-----------------|---------------------------------|-------------|
|                            | # of events / n | Hazard Ratio (95% CI)           | LRT P-value |
| <b>ProMisE™ subgroup</b>   |                 |                                 |             |
| p53 wt (ref)               | 89 / 387        | -                               | 0.0000      |
| MMR-D                      |                 | 1.90 (1.04-3.44) <sup>F</sup>   |             |
| POLE EDM                   |                 | 0.26 (0.03-0.99) <sup>F</sup>   |             |
| p53 abn                    |                 | 5.09 (3.14-8.47) <sup>F</sup>   |             |
| <b>ESMO</b>                |                 |                                 |             |
| Low (ref)                  | 89 / 386        | -                               | 0.0000      |
| Intermediate               |                 | 3.91 (1.04-16.85) <sup>F</sup>  |             |
| High                       |                 | 21.82 (8.66-78.69) <sup>F</sup> |             |
| <b>Age at Surgery</b>      | 89 / 386        | 1.02 (1.00-1.04)                | 0.0177      |
| <b>BMI</b>                 | 84 / 357        | 1.00 (0.99-1.01)                | 0.5893      |
| <b>Stage</b>               |                 |                                 |             |
| Stage I (ref)              | 89 / 386        | -                               | 0.0000      |
| Stage II-IV                |                 | 6.71 (4.38-10.49) <sup>F</sup>  |             |
| <b>Grade</b>               |                 |                                 |             |
| Grade 1/2                  | 89 / 387        | -                               | 0.0000      |
| Grade 3                    |                 | 7.09 (4.05-13.53) <sup>F</sup>  |             |
| <b>Histology</b>           |                 |                                 |             |
| Endometrioid               | 89 / 387        | -                               | 0.0000      |
| Non-Endometrioid           |                 | 4.20 (2.77-6.39) <sup>F</sup>   |             |
| <b>LVSI</b>                |                 |                                 |             |
| No (ref)                   | 83 / 370        | -                               | 0.0000      |
| Yes                        |                 | 4.73 (3.00-7.66) <sup>F</sup>   |             |
| <b>Nodes Tested</b>        |                 |                                 |             |
| Negative (ref)             | 89 / 385        | -                               | 0.0000      |
| Not Tested                 |                 | 0.23 (0.10-0.44) <sup>F</sup>   |             |
| Positive                   |                 | 2.72 (1.63-4.39) <sup>F</sup>   |             |
| <b>Treatment</b>           |                 |                                 |             |
| None                       | 87 / 380        | -                               | 0.0000      |
| Any                        |                 | 3.29 (2.10-5.30) <sup>F</sup>   |             |
| <b>Myometrial Invasion</b> |                 |                                 |             |
| None                       | 89 / 385        | -                               | 0.0000      |
| <50%                       |                 | 0.94 (0.46-2.11) <sup>F</sup>   |             |
| ≥50%                       |                 | 4.02 (2.13-8.54) <sup>F</sup>   |             |

**[00206]**      *Establishing the classification method as an independent prognostic marker in EC (full cohort).* Univariable survival analysis revealed all demographic, clinical and pathological parameters, known or suspected prognostic factors, were significantly associated with outcomes except BMI. Hazard ratios (95% CI) and Likelihood Ratio Test..

**[00207]**      Kaplan-Meier survival analysis in the discovery cohort had shown near overlap of the p53 wild type and MMR-D curves. With the larger number of cases in the full cohort, these two intermediate outcome survival curves clearly separate. **Figures 9A, 9B, and 9C** shows the K-M analyses for OS, DSS and PFS in the full cohort and confirmation cohorts respectively (log-rank  $p < 0.001$  for all). The number and type of events across each cohort were evaluated.

**[00208]**      Kaplan-Meier survival analyses for the full cohort ( $n=460$ ) was conducted according to each molecular subgroup: A. OS, B. DSS and C. PFS. Multivariable survival analysis using parameters available at diagnosis, e.g., correcting for age, BMI, grade, histology, any treatment, and molecular subgroup as assigned by the classification method demonstrated that only the molecular subgroup determined according to the method was associated with all measured outcomes (OS ( $p=0.004$ ), DSS ( $p=0.002$ ) and PFS ( $p=0.0000$ )). High grade (grade 3 vs. grade 1/2) was associated with DSS ( $p=0.02$ ) and PFS ( $p=0.0001$ ) only. Multivariable analysis accounting for parameters available post-surgical staging, e.g., age, BMI, stage, LVSI, lymph node involvement, myometrial invasion, grade, histotype, any treatment and molecular subgroup also demonstrated evidence that the molecular classifier continues to be associated with outcome (OS ( $p=0.028$ )), DSS ( $p=0.018$ ), PFS ( $p=0.005$ ), with significant probability. The proportional hazard assumptions were graphically evaluated by means of smoothed Schoenfeld residuals. The discriminatory ability of these models, as measured by Harell's C-index, were internally validated.

| <b>Table 11A - Multivariable Survival Analysis, Parameters available at diagnosis</b> |                          |                               |                    |                               |                    |                                |                |
|---------------------------------------------------------------------------------------|--------------------------|-------------------------------|--------------------|-------------------------------|--------------------|--------------------------------|----------------|
|                                                                                       |                          | <b>OS</b>                     |                    | <b>DSS</b>                    |                    | <b>PFS</b>                     |                |
| # of events / n                                                                       |                          | 104 / 412                     |                    | 76 / 399                      |                    | 82 / 351                       |                |
|                                                                                       |                          | Hazard Ratio<br>(95% CI)      | LRT<br>P-<br>value | Hazard Ratio<br>(95% CI)      | LRT<br>P-<br>value | Hazard Ratio<br>(95% CI)       | LRT<br>P-value |
| Molecular<br>subgroup<br>(ref: p53<br>wt)                                             | MMR-D                    | 1.54 (0.87-2.72) <sup>F</sup> | <b>0.0042</b>      | 1.11 (0.54-2.22) <sup>F</sup> | <b>0.0017</b>      | 0.71 (0.35-1.41) <sup>F</sup>  | <b>0.0000</b>  |
|                                                                                       | POLE<br>EDM              | 0.62 (0.16-1.73) <sup>F</sup> |                    | 0.23(0.03-0.95) <sup>F</sup>  |                    | 0.13 (0.01-0.52) <sup>F</sup>  |                |
|                                                                                       | p53 abn                  | 2.39 (1.33-4.38) <sup>F</sup> |                    | 2.06 (1.06-4.17) <sup>F</sup> |                    | 1.76 (0.94-3.42) <sup>F</sup>  |                |
| Age                                                                                   |                          | 1.03 (1.01-1.06) <sup>F</sup> | <b>0.0007</b>      | 1.01 (0.99-1.03) <sup>F</sup> | 0.4371             | 0.99 (0.97-1.02) <sup>F</sup>  | 0.5725         |
| BMI                                                                                   |                          | 1.00 (0.98-NA) <sup>F</sup>   | 0.5530             | 1.00 (0.97-NA) <sup>F</sup>   | 0.5235             | 1.01 (1.00-1.01) <sup>F</sup>  | 0.2145         |
| Grade<br>(ref Grade<br>1/2)                                                           | Grade 3                  | 1.32 (0.75-2.36) <sup>F</sup> | 0.3913             | 2.44 (1.17-5.25) <sup>F</sup> | <b>0.0225</b>      | 4.61 (2.18-10.22) <sup>F</sup> | <b>0.0001</b>  |
| Histology<br>(ref:<br>Endometri<br>oid)                                               | Non-<br>Endome<br>trioid | 1.08 (0.64-1.85) <sup>F</sup> | 0.8043             | 1.17 (0.65-2.15) <sup>F</sup> | 0.6428             | 1.08 (0.61-1.92) <sup>F</sup>  | 0.7853         |
| Treatment<br>(ref: None)                                                              | Any                      | 1.35 (0.86-2.15) <sup>F</sup> | 0.1850             | 1.48 (0.85-2.65) <sup>F</sup> | 0.1538             | 1.47 (0.86-2.60) <sup>F</sup>  | 0.1473         |

The superscript "F" refers to Firth confidence intervals were computed using the profile likelihood and may not match the LRT p-values.

| <b>Table 11B - Multivariable Survival Analysis, Parameters available at diagnosis</b> |               |                               |                 |                               |                 |                               |                 |
|---------------------------------------------------------------------------------------|---------------|-------------------------------|-----------------|-------------------------------|-----------------|-------------------------------|-----------------|
|                                                                                       |               | <b>OS</b>                     |                 | <b>DSS</b>                    |                 | <b>PFS</b>                    |                 |
| # of events / n                                                                       |               | 98 / 393                      |                 | 71 / 381                      |                 | 77 / 338                      |                 |
|                                                                                       |               | Hazard Ratio<br>(95% CI)      | LRT P-<br>value | Hazard Ratio (95%<br>CI)      | LRT P-<br>value | Hazard Ratio (95%<br>CI)      | LRT P-<br>value |
| Molecular<br>subgroup<br>(ref: p53<br>wt)                                             | MMR-D         | 1.62 (0.88-2.97) <sup>F</sup> | <b>0.0283</b>   | 1.29 (0.62-2.70) <sup>F</sup> | <b>0.0183</b>   | 0.77 (0.37-1.56) <sup>F</sup> | <b>0.0051</b>   |
|                                                                                       | POLE<br>EDM   | 0.76 (0.19-2.17) <sup>F</sup> |                 | 0.32 (0.03-1.37) <sup>F</sup> |                 | 0.16 (0.02-0.67) <sup>F</sup> |                 |
|                                                                                       | p53 abn       | 2.41 (1.22-4.85) <sup>F</sup> |                 | 2.12 (0.99-4.81) <sup>F</sup> |                 | 1.42 (0.69-3.04) <sup>F</sup> |                 |
| Age                                                                                   |               | 1.02 (1.00-1.05) <sup>F</sup> | <b>0.0278</b>   | 1.00 (0.97-1.02) <sup>F</sup> | 0.7400          | 0.98 (0.96-1.01) <sup>F</sup> | 0.1498          |
| BMI                                                                                   |               | 1.00 (0.97-NA) <sup>F</sup>   | 0.5562          | 1.00 (0.97-NA) <sup>F</sup>   | 0.5587          | 1.01 (1.00-1.02) <sup>F</sup> | 0.0700          |
| Stage                                                                                 | II-IV         | 2.43 (1.44-4.15) <sup>F</sup> | <b>0.0008</b>   | 2.69 (1.46-5.05) <sup>F</sup> | <b>0.0018</b>   | 3.71 (1.97-7.17) <sup>F</sup> | <b>0.0000</b>   |
| LVS1                                                                                  | Yes           | 1.71 (1.02-2.95) <sup>F</sup> | <b>0.0307</b>   | 1.65 (0.88-3.32) <sup>F</sup> | 0.0762          | 1.37 (0.73-2.66) <sup>F</sup> | 0.3004          |
| Nodal<br>Status                                                                       | Not<br>Tested | 1.44 (0.85-2.38) <sup>F</sup> | 0.0858          | 1.87 (1.00-3.41) <sup>F</sup> | 0.0645          | 0.47 (0.18-1.06) <sup>F</sup> | <b>0.0345</b>   |
|                                                                                       | Positive      | 0.61 (0.32-1.10) <sup>F</sup> |                 | 0.69 (0.35-1.30) <sup>F</sup> |                 | 0.56 (0.30-1.01) <sup>F</sup> |                 |
| Myometrial<br>Invasion<br>(ref: None)                                                 | <50%          | 0.94 (0.45-2.11) <sup>F</sup> | <b>0.0149</b>   | 0.82 (0.31-2.48) <sup>F</sup> | <b>0.0005</b>   | 0.76 (0.31-2.11) <sup>F</sup> | <b>0.0015</b>   |
|                                                                                       | >50%          | 1.90 (0.89-4.35) <sup>F</sup> |                 | 2.56(1.00-7.64) <sup>F</sup>  |                 | 2.15 (0.85-6.13) <sup>F</sup> |                 |

|                                         |                          |                               |        |                               |        |                               |               |
|-----------------------------------------|--------------------------|-------------------------------|--------|-------------------------------|--------|-------------------------------|---------------|
| Grade<br>(ref Grade<br>1/2)             | Grade 3                  | 1.18 (0.65-2.17) <sup>F</sup> | 0.6566 | 2.12 (0.96-4.75) <sup>F</sup> | 0.0773 | 3.72 (1.73-8.47) <sup>F</sup> | <b>0.0007</b> |
| Histology<br>(ref:<br>Endometrio<br>id) | Non-<br>Endomet<br>rioid | 1.08 (0.58-1.98) <sup>F</sup> | 0.8576 | 1.37 (0.68-2.70) <sup>F</sup> | 0.4339 | 1.24 (0.66-2.38) <sup>F</sup> | 0.5115        |
| Treatment<br>(ref: None)                | Any                      | 0.77 (0.45-1.31) <sup>F</sup> | 0.2923 | 0.73 (0.39-1.41) <sup>F</sup> | 0.2919 | 0.59 (0.31-1.13) <sup>F</sup> | 0.1085        |

**[00209]** In **Table 11B**, the superscript “F” refers to the Firth confidence intervals were computed using the profile likelihood and may not match the LRT p-values.

**[00210]** **Comparison and Internal validation of prognostic models.** Harrell's C-index was computed on the full cohort, comparing the ability of the described method of molecular classification, ESMO risk-group stratification, a combination of both the method of classification and ESMO as well as the classification method with the addition of select clinicopathologic parameters available from time of diagnosis or post-surgical staging to discern outcomes (OS, DSS and PFS). As expected, the greater numbers used in the full cohort improved the results and tightened confidence intervals. Strongest ability to discern outcomes was observed with the combination of the classification method and post-surgical parameters. The use of the classification method with parameters from time of diagnosis is also shown to be strong, or in combination with ESMO. Regardless, the described classification method alone appears to perform as well as ESMO (**Figures 10A, 10B, and 10C**).

**[00211]** **Figures 10A, 10B, and 10C** show Kaplan-Meier survival analyses for the full cohort (n=460) according to the European Society for Medical Oncology (ESMO) EC risk group for A. OS, B. DSS and C. PFS.

**[00212]** **Comparison of Described subgroups and ESMO risk groups.** Comparison of the ESMO risk group assignment (low-intermediate-high-risk) between the two clinicians revealed agreement in all but four cases of the total 460 cases in the full cohort included in analysis. These four cases were re-reviewed and a final risk group assigned with agreement of both parties.

**[00213]** Kaplan-Meier survival analyses plotting the three ESMO risk groups (low-, intermediate-, and high-risk) against outcomes of OS, DSS and PFS for each of the confirmation, discovery and full cohorts revealed essentially indistinguishable low- and intermediate-risk group outcomes, with no statistically significant difference measured between these two curves (wide ranging CIs covering 1 for all except PFS in the full cohort (1.04-16.85)). Statistically significant differences were demonstrated between high- and low-risk ESMO group outcomes. Results for the full cohort are shown in **Figure 11**.

**[00214]** **Figure 11** shows an internal validation of prognostic models in the full cohort (n=460). The ability to discern outcomes for ESMO risk group assignment alone, the described classification method alone, the classification method with ESMO, the classification method with select parameters

available at time of diagnosis, and the classification method with select parameters available post-surgical staging (highest C-index) were also assessed.

**[00215]** As performed for the discovery cohort described in Example 1, molecular subgroups were cross-tabulated with ESMO risk groups in the full cohort (**Figure 12**) to determine whether there were any associations between the molecular vs. clinical classification systems. Less diversity was seen within the p53 abn group; the majority (85.6%) of cases in the full cohort that were classified as p53 abn were also classified as high-risk by ESMO criteria (**Figure 12**). However, diversity amongst the other molecular subgroups was profound. Essentially all tumors classified as POLE EDM were early stage with excellent outcomes, however these cases were approximately equally distributed across ESMO low- and intermediate-risk groups (29.3%) with the greatest proportion of cases (41.5%) classified as high-risk. Based on the ESMO risk group classification, up to 70% (combining high- and intermediate risk groups) of these cases would receive some form of adjuvant therapy. MMR-D tumors mostly fell in the high-risk group and most p53 wt tumors were low risk, but again, they did range across ESMO subgroups.

**[00216]** **Figure 12** shows cross-tabulation of ESMO risk group assigned and the ProMisE™ method derived molecular subgroup across the full cohort (n=460).

**[00217]** Discussion

**[00218]** There is a need for biologically informative molecular tools to improve both i) reproducibility of EC categorization, and ii) risk stratification, guiding optimal surgery, adjuvant therapy, and cancer surveillance regimens for women with EC. Neither is adequately achieved in the current systems. Lack of consensus among expert pathologists in morphological assessment of histotype and grade is a long-standing problem, despite attempts to refine diagnostic criteria, and this implies that reproducibility in assignment of these subjective morphological variables cannot be significantly improved. The lack of diagnostic reproducibility in current classification methods exerts a pervasive negative effect; in clinical research it is impossible to compare cohorts diagnosed in different centers, which in turn makes it impossible to specifically study subgroups or move towards subgroup specific treatment. The lack of diagnostic reproducibility also means that the same patient will be offered different treatments in different centres. While there is clearly intent to offer the best treatment by all practitioners, the lack of uniformity in treatment reflects uncertainty about what is best, and this state of uncertainty is a result, at least in part, of the challenges that lack of diagnostic reproducibility poses for clinical research and management. The only way to break this impasse is to move towards a more robust molecular classifier, given the limitations of histomorphological classification, and a way forward in doing so was examined in this Example. Four molecular subgroups of EC (Ultramutated (POLE), Hypermutated (MSI), Copy number abnormalities- High, and Copy number abnormalities – Low) were previously established. There was a need for determining such subgroups based on testing in the clinical setting with methods that are easy to perform, interpret and relatively low cost, and thus can be

achieved at any cancer center. Integrating clinicopathological and molecular factors can improve risk assessment for early-stage EC. A classification method that is accurate and feasible to conduct in daily may reduce under- and over-treatment of patients.

**[00219]** The molecular classification method described herein can be stated as a decision tree of binary parameters, with the result being determined by the presence or absence of a protein or of a mutation. Consistent subgroup assignment should therefore be achievable with only limitations related to sample quality e.g. low cellularity of samples or low quality of DNA extracted from FFPE. A very small (3.4%) subset of patients was observed with more than one discerning molecular parameter identified. It will be important to know how to classify such uncommon cases in practice, but a multicenter study will likely be needed to gather enough cases to fully understand their natural history. Some comments are possible, however, based on this case series. Four women had both POLE EDM mutations and p53 abnormalities and are classified by the described method as part of the POLE EDM group. The clinical course and molecular landscape of these tumours (evidence of increased mutations across multiple genes) was more consistent with POLE ultramutated phenotype; this is in agreement with the findings of Hussein et al (2015), that such POLE EDM/p53 abn tumors are more appropriately classified as POLE EDM and therefore the classification method, involving pulling out POLE mutated cases as an early step and classifying these tumours as POLE EDM, would seem appropriate. POLE EDMs and MMR-D have been reported to be mutually exclusive. It was surprising to find one case with a validated POLE mutation (F3676) and PMS2 loss (classified as MMR-D) and would have cast doubt on MMR IHC results described herein, however a known family history of PMS2 mutation was confirmed in this individual and she truly appears to have had both POLE and PMS2 mutations present in her tumour. Her clinical course was favorable as far as she could be followed (lost to follow-up at 7 years) and the 'best' categorization of her tumour is uncertain. There were 11 cases of MMR-D tumours that also had p53 abnormalities. This is an important group to understand fully but more cases will be needed to allow adequate characterization.

**[00220]** Even given these challenges, molecular classification appears to offer prognostic information superior to current standards. Deficiencies across all risk stratification systems for EC have been highlighted in recent publications. Using the ESMO 2013 classifier, purportedly the strongest of the available traditional systems, the data of this Example reveals that essentially only two outcomes groups were discernible, with the low- and intermediate-risk group survival curves overlapping. In contrast, molecular classification yielded four distinct subgroups with significantly different survival curves. Cross-tabulation of the ESMO method and the classification method described herein revealed significant overlap of cases in the high-risk and p53 abn subgroups, but otherwise tremendous diversity of risk group assignment within the molecular subgroups. It is clear that there may be both over treatment and under treatment of women based solely on application of the ESMO risk assessment tool. Specifically, the POLE subgroup is known to be associated with

excellent outcomes has an almost equal distribution of low-intermediate, and high- assigned risk groups meaning treatment for these women would have ranged from observation only, to vaginal brachytherapy, to combined chemotherapy and radiation. The optimal treatment within each molecular subgroup can be affirmed, further, through collaborative trials but with a robust clinically applicable classifier in hand, such trials will become possible.

**[00221]** On multivariable analysis, accounting for age, BMI, tumour grade and histotype the herein described molecular classification method remained significantly associated with OS, DSS and PFS (LRT p-values 0.0042, 0.0017 and 0.000 respectively). The subgroups defined by the instant method can be successfully determined in diagnostic endometrial samples (i.e obtained prior to surgery) and thus have the potential to inform management from the earliest time point. This is particularly relevant for young women with EC interested in future fertility who wish to delay definitive hysterectomy, or for surgical candidates deemed vulnerable (e.g., elderly, multiple comorbidities), where less aggressive surgery may be favored if risk of metastatic disease was determined to be either low or sufficiently high that adjuvant treatment will be given, without needing the information generated by extended staging surgery to guide this decision. Future treatment considerations may, for example, encourage a patient only to consider progesterone therapy and delayed hysterectomy in POLE EDM (also referred to herein as POL mut), or p53 wt tumors are determined.

**[00222]** The herein described molecular classification method may be shown to be more accurate than traditional risk-group classification with regard to determining differences in clinical outcomes, patient reported outcomes/quality of life, and health economic impact.

**[00223]** In the short term, the classification method described herein offers benefits that can be immediately realized. Implementation of MMR IHC for all cases will help identify women who may have Lynch Syndrome, who should be referred for counselling and testing to distinguish somatic events from germline mutations. Identifying women with Lynch syndrome will direct screening for other Lynch-associated cancers, initiate referrals for family members to be tested, and allow subsequent risk reducing interventions to be undertaken, saving lives. 2) Stratification of clinical trials, both enabling the interpretation of completed trials, for example results interpreted within molecular subgroups, and enrollment of future trials according to the subgroups determined as a result of the herein described classification method. 3) Identifying opportunities for targeted therapy. Given the success demonstrated with immune blockade in cancers with MMR-D (including ECs), clinicians may consider directing therapy based on molecular subgroup with high neoantigen load and associated immune infiltrates (MMR-D or even POLE EDM in the rare cases of recurrence).

**[00224]** The molecular classification method described herein addresses the greatest obstacles currently faced by clinicians and patients in management of EC; namely the inability to consistently classify EC tumors, and deficient risk stratification systems to direct care. The described method provides biologically relevant information to patients and physicians; using molecular data to

group EC patients based on risk of recurrence and death. The method described herein is simple enough to be performed in any cancer center, on formalin-fixed paraffin embedded material, and at relatively low cost enabling easy translation into the clinic. The information provided by the classification method is particularly relevant for young women struggling with difficult decisions about their reproductive health. Molecular classification using the method described may advance both clinical management and research for endometrial cancers.

**[00225]** In the preceding description, for purposes of explanation, numerous details are set forth in order to provide a thorough understanding of the embodiments. However, it will be apparent to one skilled in the art that these specific details are not required. In other instances, well-known electrical structures and may be shown in block diagram form in order not to obscure the understanding. For example, specific details are not provided as to whether the embodiments described herein are implemented as a software routine, hardware circuit, firmware, or a combination thereof.

**[00226]** Embodiments of the disclosure can be represented as a computer program product stored in a machine-readable medium (also referred to as a computer-readable medium, a processor-readable medium, or a computer usable medium having a computer-readable program code embodied therein). The machine-readable medium can be any suitable tangible, non-transitory medium, including magnetic, optical, or electrical storage medium including a diskette, compact disk read only memory (CD-ROM), memory device (volatile or non-volatile), or similar storage mechanism. The machine-readable medium can contain various sets of instructions, code sequences, configuration information, or other data, which, when executed, cause a processor to perform steps in a method according to an embodiment of the disclosure. Other instructions and operations necessary to implement the described implementations can also be stored on the machine-readable medium. The instructions stored on the machine-readable medium can be executed by a processor or other suitable processing device, and can interface with circuitry to perform the described tasks.

**[00227]** The above-described embodiments are intended to be examples only. Alterations, modifications and variations can be effected to the particular embodiments by those of skill in the art. The scope of the claims should not be limited by the particular embodiments set forth herein, but should be construed in a manner consistent with the specification as a whole.

**[00228]** **References.**

**[00229]** AlHilli MM, Mariani A, Bakkum-Gamez JN, Dowdy SC, Weaver AL, Peethambaram PP, Keeney GL, Cliby WA, Podratz KC (2014) Risk-scoring models for individualized prediction of overall survival in low-grade and high-grade endometrial cancer. *Gynecologic oncology* 133(3): 485-93.

- [00230]** Bendifallah S, Canlorbe G, Collinet P, Arsene E, Huguet F, Coutant C, Hudry D, Graesslin O, Raimond E, Touboul C, Darai E, Ballester M (2015) Just how accurate are the major risk stratification systems for early-stage endometrial cancer? *British journal of cancer* (2015) 112, 793–801.
- [00231]** Billingsley CC, Cohn DE, Mutch DG, Stephens JA, Suarez AA, Goodfellow PJ (2015) Polymerase varepsilon (POLE) mutations in endometrial cancer: Clinical outcomes and implications for Lynch syndrome testing. *Cancer* 121(3): 386-94.
- [00232]** Burleigh A, Talhouk A, Gilks CB, McAlpine JN. *Gynecologic Oncology* 138 (2015):141-146. (April 12, 2015)
- [00233]** Cancer Genome Atlas Research N, Kandoth C, Schultz N, Cherniack AD, Akbani R, Liu Y, Shen H, Robertson AG, Pashtan I, Shen R, Benz CC, Yau C, Laird PW, Ding L, Zhang W, Mills GB, Kucherlapati R, Mardis ER, Levine DA (2013) Integrated genomic characterization of endometrial carcinoma. *Nature* 497(7447): 67-73.
- [00234]** Church DN, Stelloo E, Nout RA, Valtcheva N, Depreeuw J, ter Haar N, Noske A, Amant F, Tomlinson IP, Wild PJ, Lambrechts D, Jurgenliemk-Schulz IM, Jobsen JJ, Smit VT, Creutzberg CL, Bosse T (2015) Prognostic significance of POLE proofreading mutations in endometrial cancer. *Journal of the National Cancer Institute* 107(1): 402.
- [00235]** Colombo N, Preti E, Landoni F, Carinelli S, Colombo A, Marini C, Sessa C, Group EGW (2013) Endometrial cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* 24 Suppl 6: vi33-8.
- [00236]** Despierre E, Yesilyurt BT, Lambrechts S, Johnson N, Verheijen R, van der Burg M, Casado A, Rustin G, Berns E, Leunen K, Amant F, Moerman P, Lambrechts D, Vergote I, Eortc GCG, Group EGTR (2014) Epithelial ovarian cancer: rationale for changing the one-fits-all standard treatment regimen to subtype-specific treatment. *International journal of gynecological cancer: official journal of the International Gynecological Cancer Society* 24(3): 468-77.
- [00237]** Francis JA, Weir MM, Ettler HC, Qiu F, Kwon JS (2009) Should preoperative pathology be used to select patients for surgical staging in endometrial cancer? *International journal of gynecological cancer: official journal of the International Gynecological Cancer Society* 19(3): 380-4.
- [00238]** Gilks CB, Oliva E, Soslow RA (2013) Poor interobserver reproducibility in the diagnosis of high-grade endometrial carcinoma. *The American journal of surgical pathology* 37(6): 874-81.
- [00239]** Guan H, Semaan A, Bandyopadhyay S, Arabi H, Feng J, Fathallah L, Pansare V, Qazi A, Abdul-Karim F, Morris RT, Munkarah AR, Ali-Fehmi R (2011) Prognosis and reproducibility of new and existing binary grading systems for endometrial carcinoma compared to FIGO grading in

hysterectomy specimens. *International journal of gynecological cancer: official journal of the International Gynecological Cancer Society* 21(4): 654-60.

**[00240]** Han G, Sidhu D, Duggan MA, Arseneau J, Cesari M, Clement PB, Ewanowich CA, Kalloger SE, Kobel M (2013) Reproducibility of histological cell type in high-grade endometrial carcinoma. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc* 26(12): 1594-604.

**[00241]** Hoang LN, McConechy MK, Kobel M, Han G, Rouzbahman M, Davidson B, Irving J, Ali RH, Leung S, McAlpine JN, Oliva E, Nucci MR, Soslow RA, Huntsman DG, Gilks CB, Lee CH (2013) Histotype-genotype correlation in 36 high-grade endometrial carcinomas. *The American journal of surgical pathology* 37(9): 1421-32.

**[00242]** Hussein YR, Weigelt B, Levine DA, Schoolmeester JK, Dao LN, Balzer BL, Liles G, Karlan B, Kobel M, Lee CH, Soslow RA (2015) Clinicopathological analysis of endometrial carcinomas harboring somatic POLE exonuclease domain mutations. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc. Mod Pathol.* 2015 Apr; 28(4):505-14.

**[00243]** Karateke A, Tug N, Cam C, Selcuk S, Asoglu MR, Cakir S (2011) Discrepancy of pre- and postoperative grades of patients with endometrial carcinoma. *European journal of gynaecological oncology* 32(3): 283-5.

**[00244]** Kong TW, Chang SJ, Paek J, Lee Y, Chun M, Ryu HS (2015) Risk group criteria for tailoring adjuvant treatment in patients with endometrial cancer: a validation study of the Gynecologic Oncology Group criteria. *Journal of gynecologic oncology* 26(1): 32-9.

**[00245]** Kwon JS, Qiu F, Saskin R, Carey MS (2009) Are uterine risk factors more important than nodal status in predicting survival in endometrial cancer? *Obstetrics and gynecology* 114(4): 736-43.

**[00246]** Le Gallo M, O'Hara AJ, Rudd ML, Urick ME, Hansen NF, O'Neil NJ, Price JC, Zhang S, England BM, Godwin AK, Sgroi DC, Program NIHISCCS, Hieter P, Mullikin JC, Merino MJ, Bell DW (2012) Exome sequencing of serous endometrial tumors identifies recurrent somatic mutations in chromatin-remodeling and ubiquitin ligase complex genes. *Nature genetics* 44(12): 1310-5.

**[00247]** Lu KH, Dinh M, Kohlmann W, Watson P, Green J, Syngal S, Bandipalliam P, Chen LM, Allen B, Conrad P, Terdiman J, Sun C, Daniels M, Burke T, Gershenson DM, Lynch H, Lynch P, Broaddus RR (2005) Gynecologic cancer as a "sentinel cancer" for women with hereditary nonpolyposis colorectal cancer syndrome. *Obstetrics and gynecology* 105(3): 569-74.

**[00248]** Mariani A, Dowdy SC, Cliby WA, Gostout BS, Jones MB, Wilson TO, Podratz KC (2008) Prospective assessment of lymphatic dissemination in endometrial cancer: a paradigm shift in surgical staging. *Gynecologic oncology* 109(1): 11-8.

- [00249]** McConechy MK, Ding J, Cheang MC, Wiegand KC, Senz J, Tone AA, Yang W, Prentice LM, Tse K, Zeng T, McDonald H, Schmidt AP, Mutch DG, McAlpine JN, Hirst M, Shah SP, Lee CH, Goodfellow PJ, Gilks CB, Huntsman DG (2012) Use of mutation profiles to refine the classification of endometrial carcinomas. *The Journal of pathology* 228(1): 20-30.
- [00250]** McConechy MK, Talhouk A, Li-Chang HH, Leung S, Huntsman DG, Gilks CB, McAlpine JN. *Gynecologic Oncology* 137 (2015): 1306-310. (January 28, 2015).
- [00251]** Murali R, Soslow RA, Weigelt B (2014) Classification of endometrial carcinoma: more than two types. *The Lancet Oncology* 15(7): e268-78.
- [00252]** Nelson BH, McAlpine JN. *Gynecologic Oncology* 138 (2015): 1-2. (June 2, 2015).
- [00253]** Siegel RL, Miller KD, Jemal A (2015) Cancer statistics, 2015. *CA: a cancer journal for clinicians* 65(1): 5-29.
- [00254]** Stelloo E, Bosse T, Nout RA, MacKay HJ, Church DN, Nijman HW, Leary A, Edmondson RJ, Powell ME, Crosbie EJ, Kitchener HC, Mileskin L, Pollock PM, Smit VT, Creutzberg CL (2015) Refining prognosis and identifying targetable pathways for high-risk endometrial cancer; a TransPORTEC initiative.. *Modern Pathol.* 2015 Jun;28(6):836-44. doi: 10.1038/modpathol.2015.43. Epublished: Feb 27, 2015.
- [00255]** Stelloo E, Nout RA, Naves LC, Ter Haar NT, Creutzberg CL, Smit VT, Bosse T (2014) High concordance of molecular tumor alterations between pre-operative curettage and hysterectomy specimens in patients with endometrial carcinoma. *Gynecologic oncology* 133(2): 197-204.
- [00256]** Stovall TG, Photopulos GJ, Poston WM, Ling FW, Sandles LG (1991) Pipelle endometrial sampling in patients with known endometrial carcinoma. *Obstetrics and gynecology* 77(6): 954-6.

**CLAIMS**

1. A method of classifying endometrial cancers comprising:  
obtaining a sample from a subject having endometrial cancer;  
evaluating the sample for:  
(a) a hypermutated mismatch repair (MMR) protein comprising high microsatellite instability (MSI) phenotype to classify the cancer as (i) MMR abnormal (MMR abn) if present or MMR microsatellite stable (MMR MSS) if absent;  
(b) subsequently evaluating the MMR MSS sample from (a) for a POLE exonuclease domain mutation (EDM) to classify the cancer as (ii) POLE mutated (POLE mut) if present or POLE wild type (POLE wt) if absent; and  
(c) subsequently evaluating the POLE wt sample from (b) for a p53 copy number abnormality comprising a high copy number or a low copy number to classify the cancer as (iii) p53 wild type (p53 wt) if the abnormality is absent and as (iv) p53 abnormal (p53 abn) if present.
2. The method of claim 1 wherein:  
(i) MMR abn and (ii) POLE mut and are associated with intermediate clinical risk;  
(iii) p53 wt is associated with low clinical risk; and  
(iv) p53 abn is associated with high risk,  
wherein risk is evaluated by the European Society for Medical Oncology (ESMO) risk groupings.
3. The method of claim 1 or 2, wherein the hypermutated MMR protein and/or the p53 copy number abnormality is evaluated by immunohistochemistry.
4. The method of any one of claims 1 to 3, wherein the POLE exonuclease domain mutation is evaluated by sequencing.
5. The method of any one of claims 1 to 4, wherein the sample is a hysterectomy sample, or a diagnostic endometrial sample comprising an endometrial biopsy or an endometrial curetting specimen.
6. The method of any one of claims 1 to 5, wherein the sample is formalin-fixed and paraffin-embedded.

7. The method of claim 1, wherein patients having cancers classified as p53 wt are associated with a good prognosis for survival.
8. The method according to any one of claims 1 to 6, additionally comprising delaying surgery when the endometrial cancer is classified as (ii) POLE mut or (iii) p53 wt tumor.
9. The method according to any one of claims 1 to 6, additionally comprising pursuing surgery when the endometrial cancer is classified as (i) MMR abn or (iv) p53 abn.
10. A kit for use in the method of classifying endometrial cancers according to any one of claims 1 to 9, comprising:
  - one or more immunohistochemical reagent for evaluating the sample for the hypermutated mismatch repair protein;
  - one or more PCR reagent for evaluating the sample for POLE exonuclease domain mutation;
  - and
  - one or more immunohistochemical reagent for evaluating the sample for the p53 copy number abnormality.
11. The kit of claim 10, wherein the one or more immunohistochemical reagent for evaluating the sample for the hypermutated mismatch repair protein comprises MSH6 and PMS2 antibodies.
12. The kit of claim 11, wherein the one or more immunohistochemical reagent for evaluating the sample for the hypermutated mismatch repair protein additionally comprises MLH1 or MSH2 antibodies.
13. The kit of claim 10, wherein the one or more immunohistochemical reagent for evaluating the sample for the p53 copy number abnormality comprises an anti-p53 monoclonal antibody.
14. Use of (a) a hypermutated mismatch repair (MMR) protein; (b) a POLE exonuclease domain mutation (EDM); and (c) a p53 copy number abnormality for classifying an endometrial cancer in a sample from a subject,
  - wherein the endometrial cancer is classified as: (i) MMR abn; (ii) POLE mut; (iii) p53 wt; or (iv) p53 abn.
15. The use of claim 14, wherein a surgery is delayed when the endometrial cancer is classified as (ii) POLE mut or (iii) p53 wt tumor.

16. The use of claim 14, wherein a surgery is pursued when the endometrial cancer is classified as (i) MMR abn or (iv) p53 abn.
17. The use of claim 14, wherein a fertility-sparing therapy is pursued when the endometrial cancer is classified as (ii) POLE mut or (iii) p53 wt.
18. A method for treating endometrial cancer with a fertility-sparing therapy comprising:  
obtaining a sample from a subject having endometrial cancer;  
evaluating the sample for:  
(a) a hypermutated mismatch repair (MMR) protein comprising high microsatellite instability (MSI) phenotype to determine MMR abnormal (MMR abn) if present or MMR microsatellite stable (MMR MSS) if absent;  
(b) subsequently evaluating the MMR MSS sample from (a) for a POLE exonuclease domain mutation (EDM) to determine POLE mutated (POLE mut) if present or POLE wild type (POLE wt) if absent; and  
(c) subsequently evaluating the POLE wt sample from (b) for a p53 copy number abnormality comprising a high copy number or a low copy number to determine p53 wild type (p53 wt) if the copy number abnormality is absent and p53 abnormal (p53 abn) if present; and  
classifying the endometrial cancer as: (i) MMR abn; (ii) POLE mut; (iii) p53 wt; or (iv) p53 abn;  
wherein if the endometrial cancer can be classified as (ii) POLE mut or (iii) p53 wt tumor, the fertility-sparing therapy is used.
19. A method of treating an endometrial cancer comprising:  
classifying the endometrial cancer according to the method of any one of claims 1 to 9 as:  
MMR MSS; and subsequently treating by referral to hereditary testing, or use of immune blockade therapy;  
POLE MUT; and subsequently treating with less aggressive treatment, and/or fertility sparing surgery;  
p53 wt; and subsequently treating with less aggressive treatment, and/or fertility sparing surgery; or  
p53 abn; and subsequently treating with extensive surgical staging, chemotherapy and radiation.

20. A system for classifying an endometrial cancer comprising:
- (a) an immunohistological test for detecting a hypermutated mismatch repair (MMR) protein comprising high microsatellite instability (MSI) phenotype to determine MMR abnormal (MMR abn) if present or MMR microsatellite stable (MMR MSS) if absent;
  - (b) a test for subsequently evaluating the MMR MSS sample from (a) for a POLE exonuclease domain mutation (EDM) to determine POLE mutated (POLE mut) if present or POLE wild type (POLE wt) if absent; and
  - (c) an immunohistological test for subsequently evaluating the POLE wt sample from (b) for a p53 copy number abnormality comprising a high copy number or a low copy number to determine p53 wild type (p53 wt) if absent and p53 abnormal (p53 abn) if present; and
- wherein results obtained from the tests in the system classifies the endometrial cancer as: (i) MMR abn; (ii) POLE mut; (iii) p53 wt; or (iv) p53 abn; and
- wherein (i) MMR abn and (ii) POLE mut are associated with intermediate clinical risk; (iii) p53 wt is associated with low clinical risk; and (iv) p53 abn is associated with high risk, wherein risk is evaluated by the European Society for Medical Oncology (ESMO) risk groupings.
21. The system of claim 20, wherein the hypermutated MMR protein and/or the p53 copy number abnormality is evaluated by immunohistochemistry.
22. The system of claim 20, wherein the POLE exonuclease domain mutation is evaluated by sequencing.
23. The system of any one of claims 20 to 22, wherein the sample is a hysterectomy sample, or a diagnostic endometrial sample comprising an endometrial biopsy or an endometrial curetting specimen.

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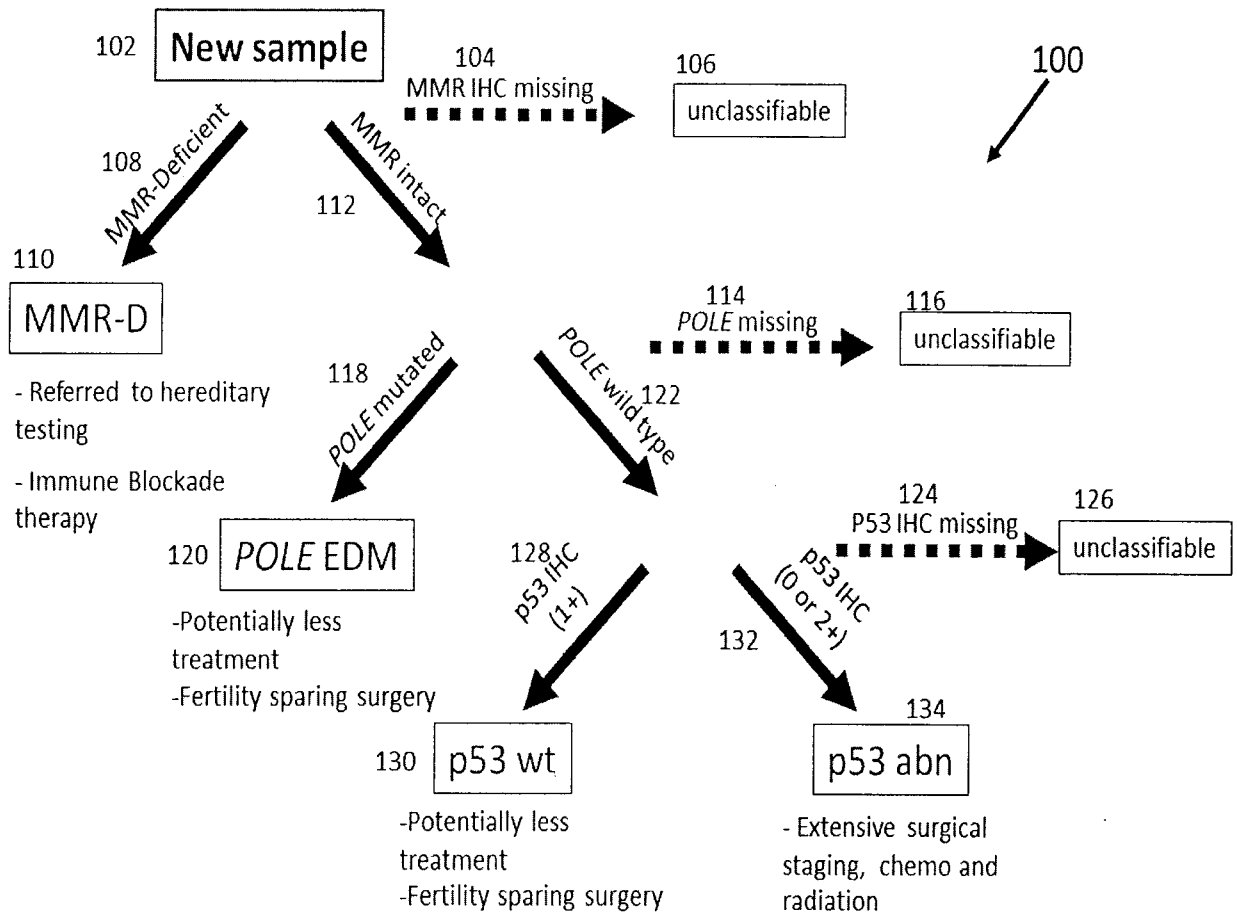


FIG. 1

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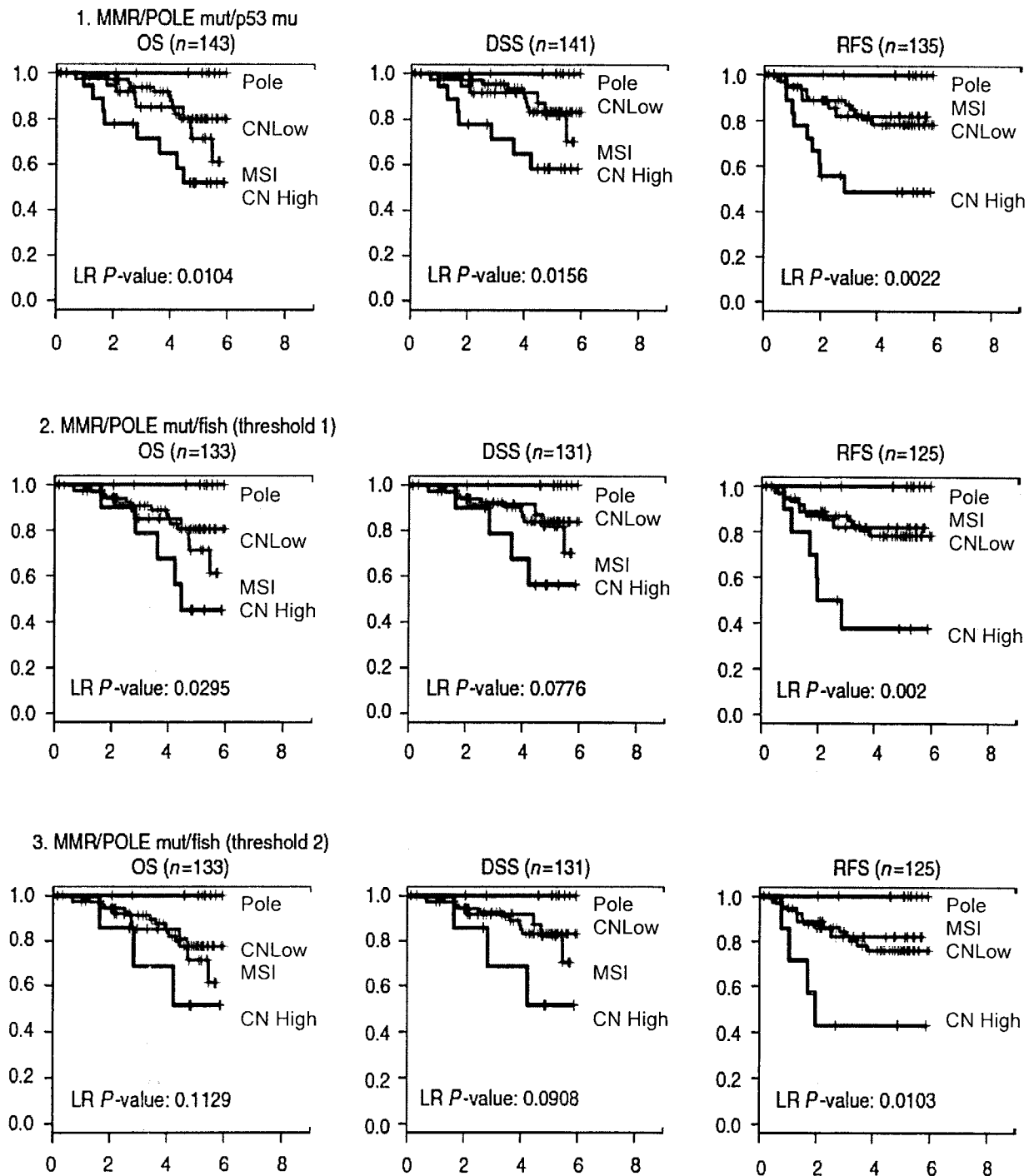


FIG. 2A

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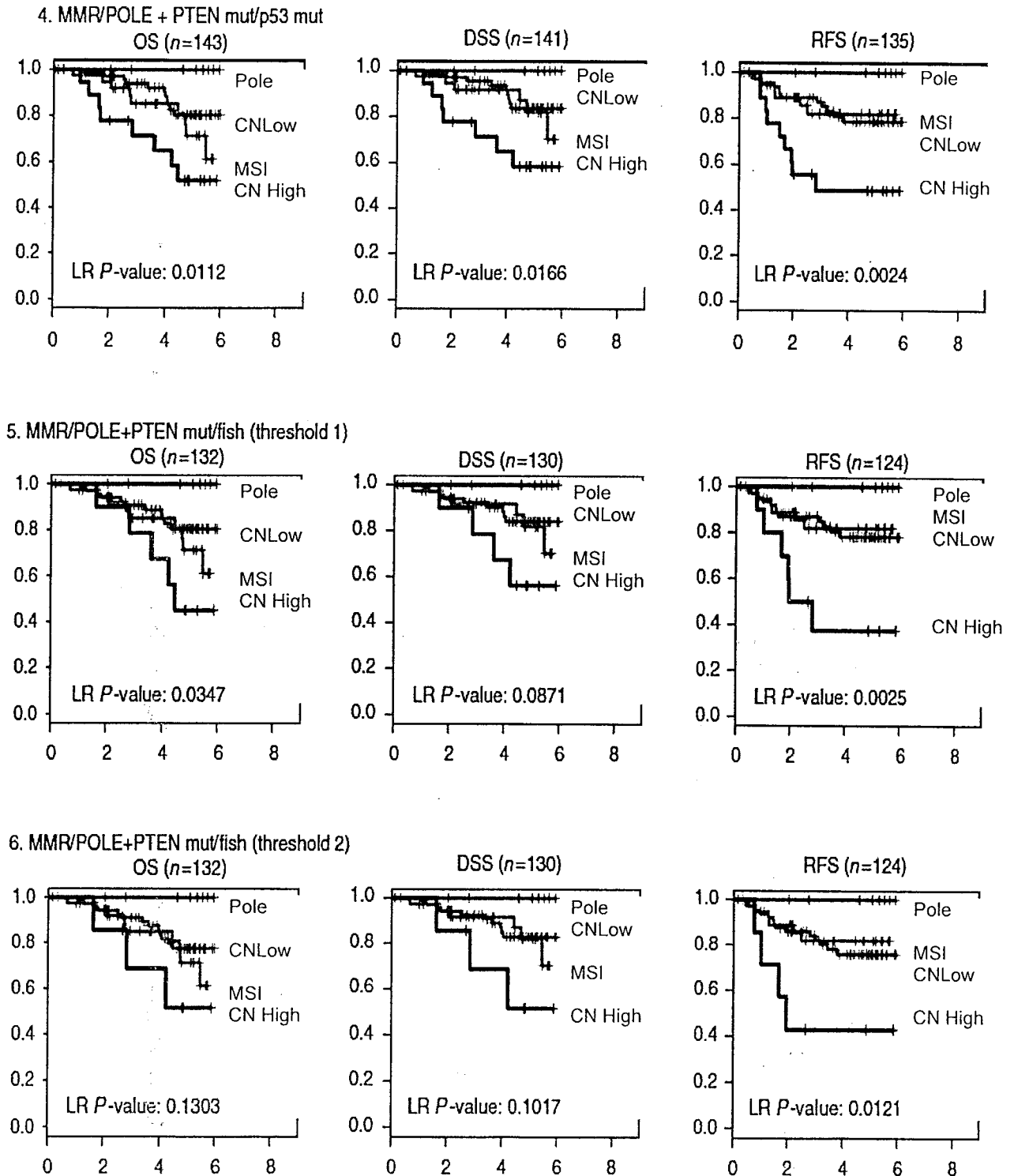


FIG. 2B

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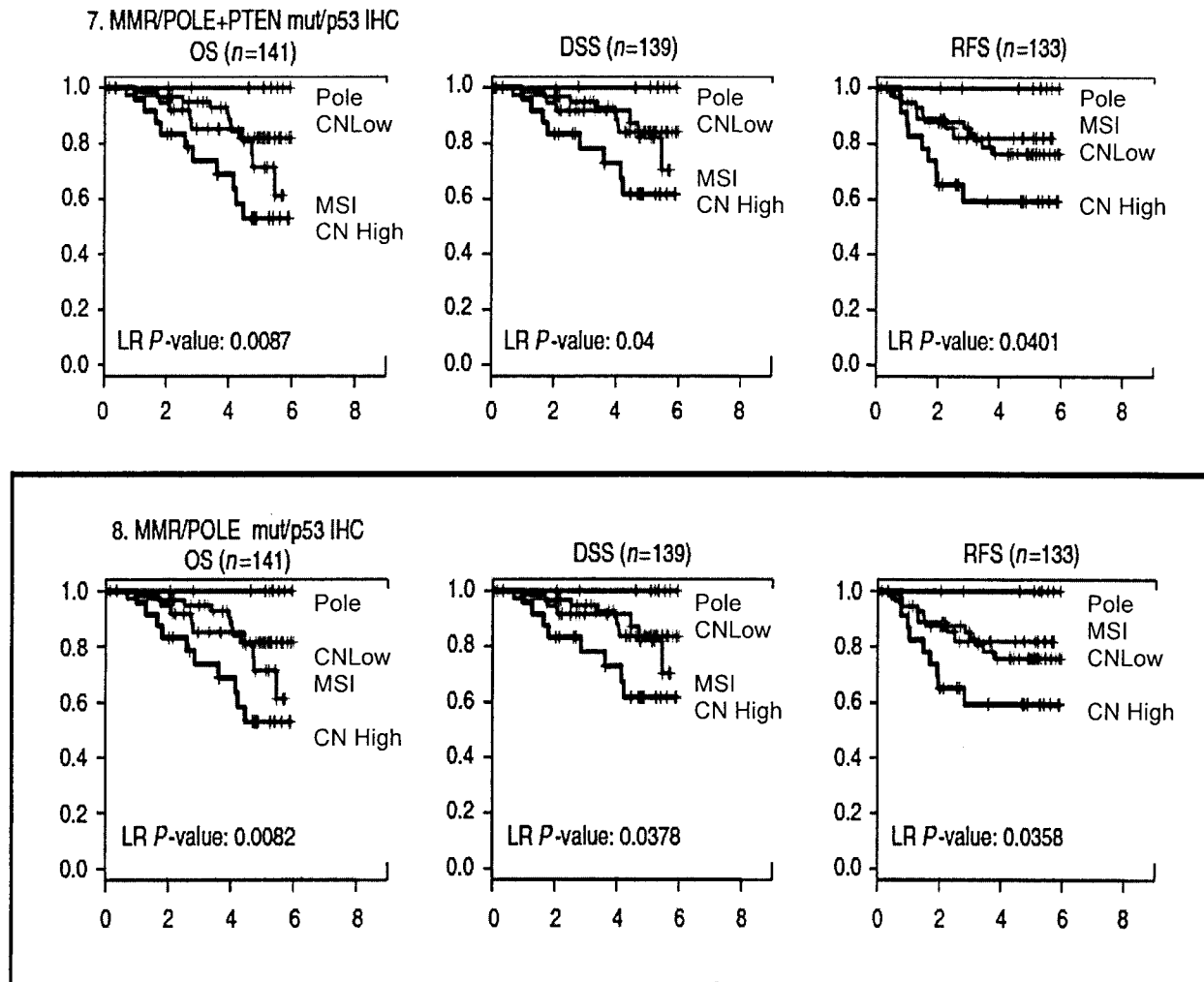


FIG. 2C

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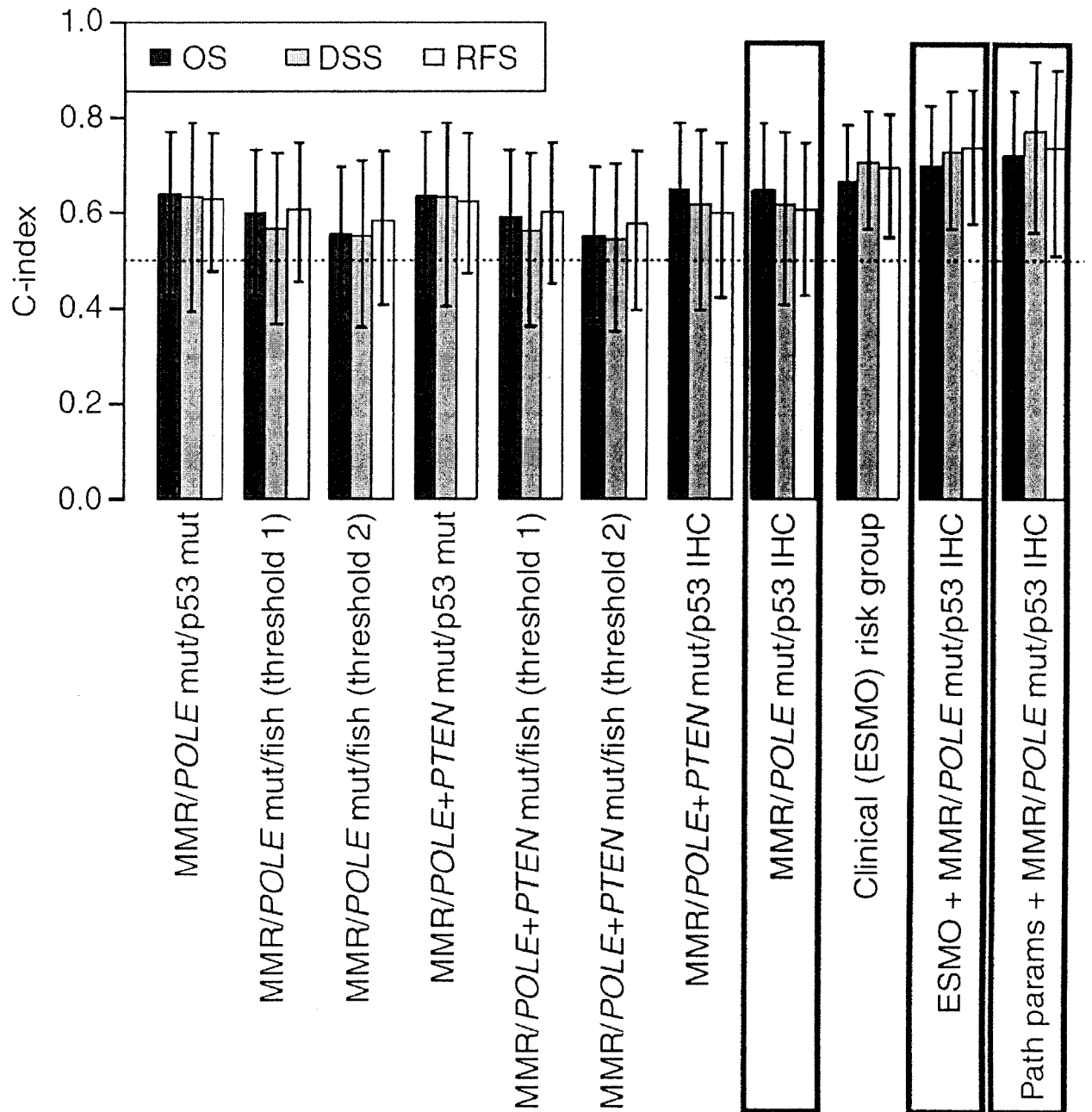


FIG. 3

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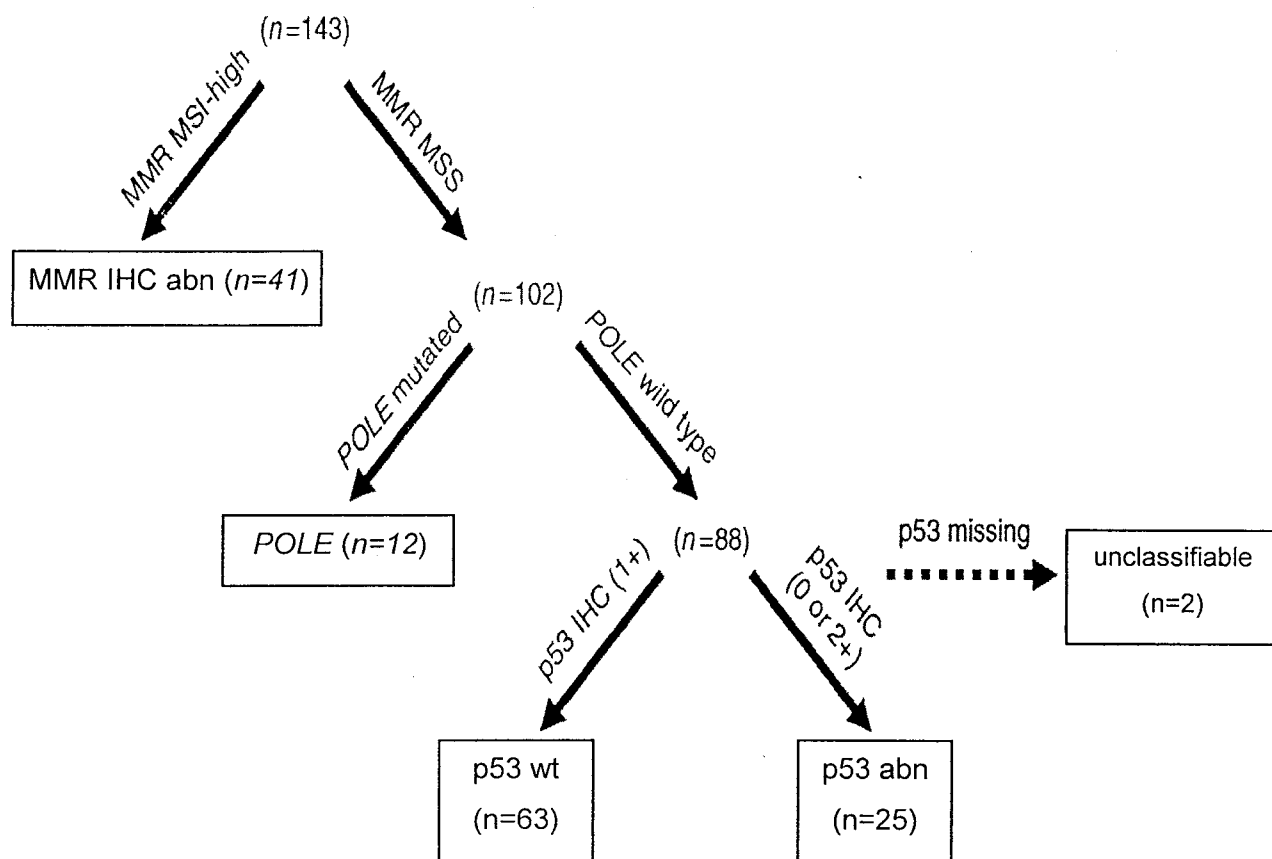
Model: MMR IHC/*POLE* mut/p53 IHC

FIG. 4

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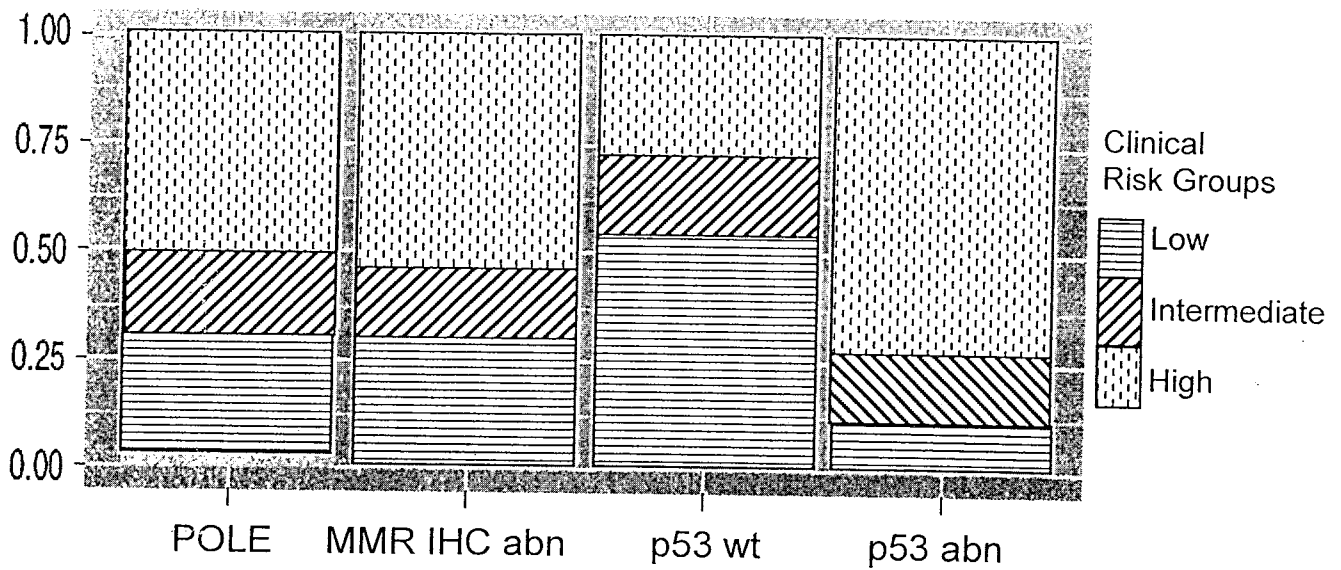


FIG. 5

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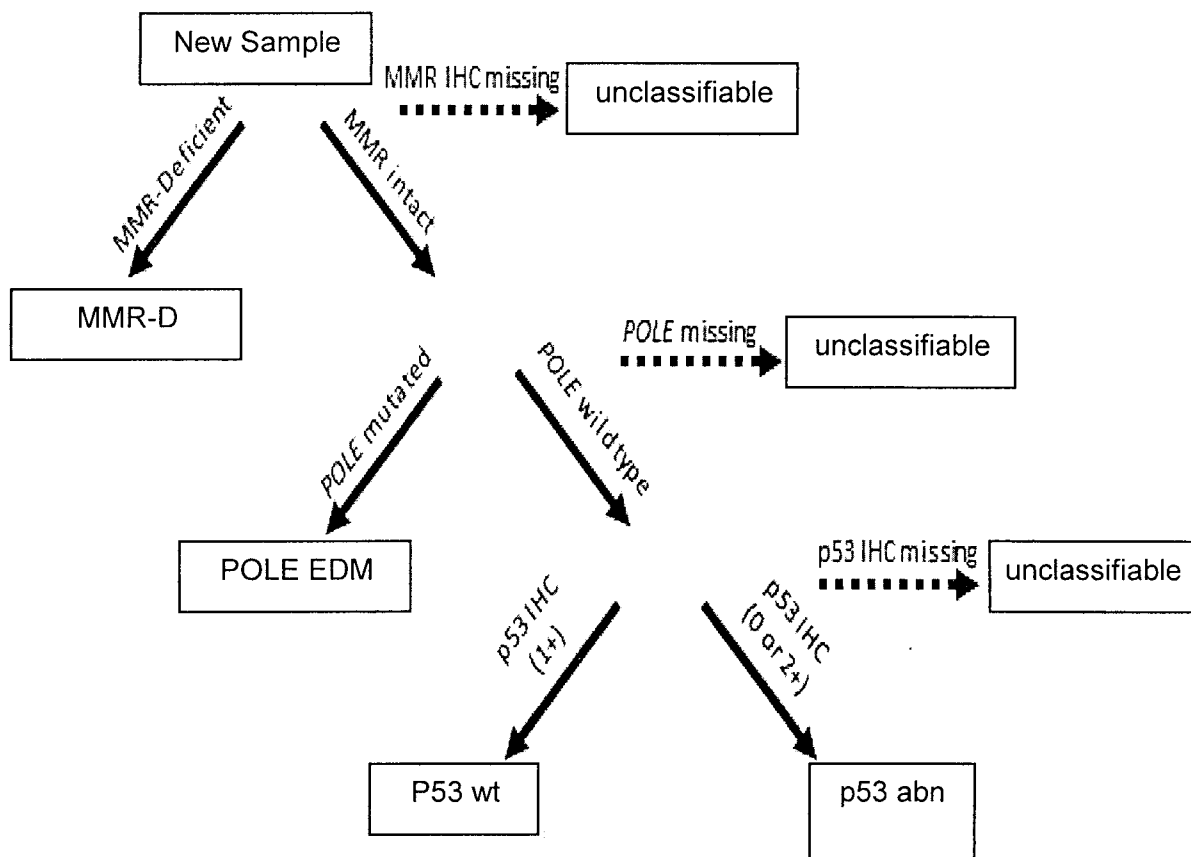


FIG. 6

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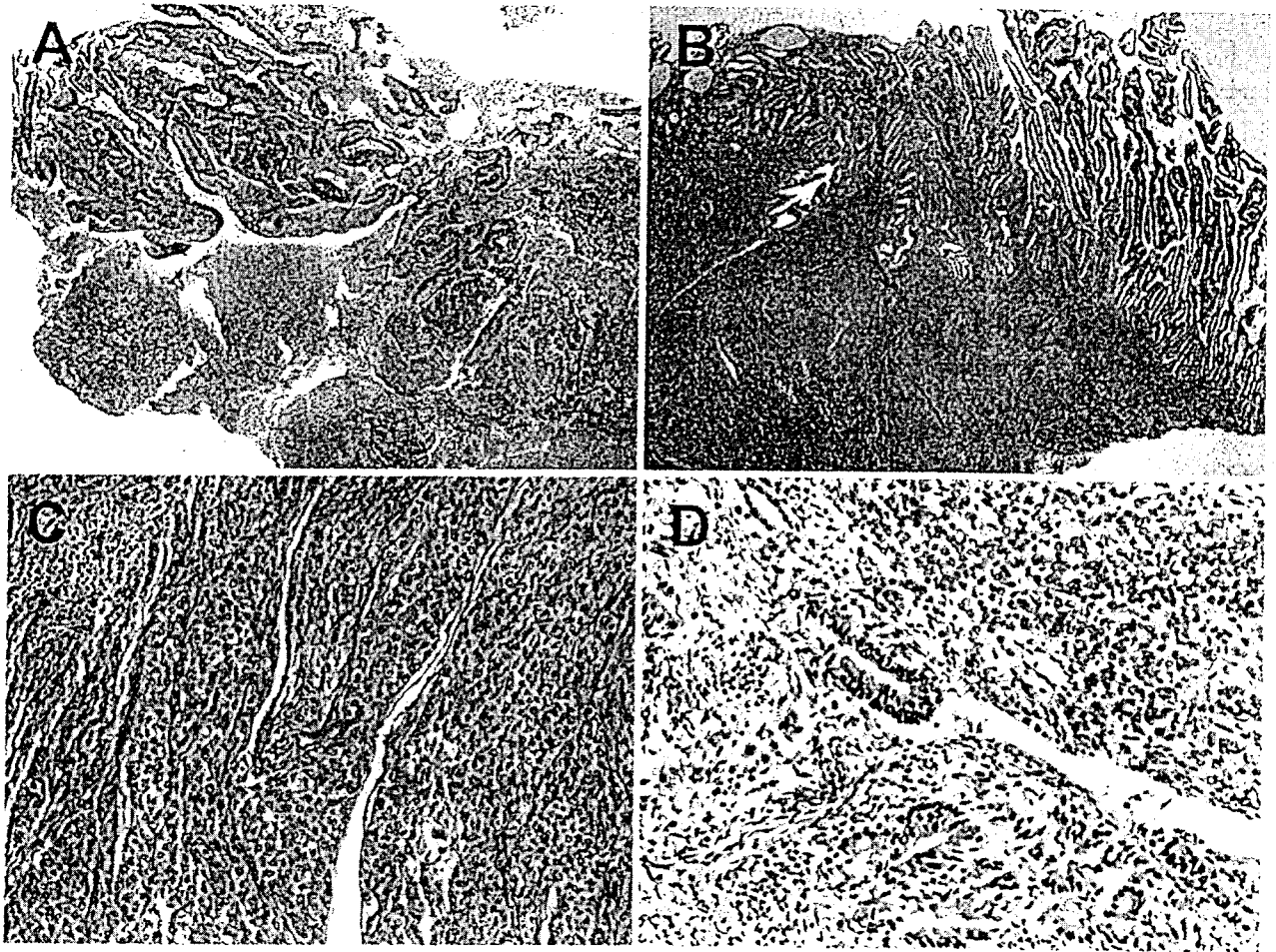


FIG. 7

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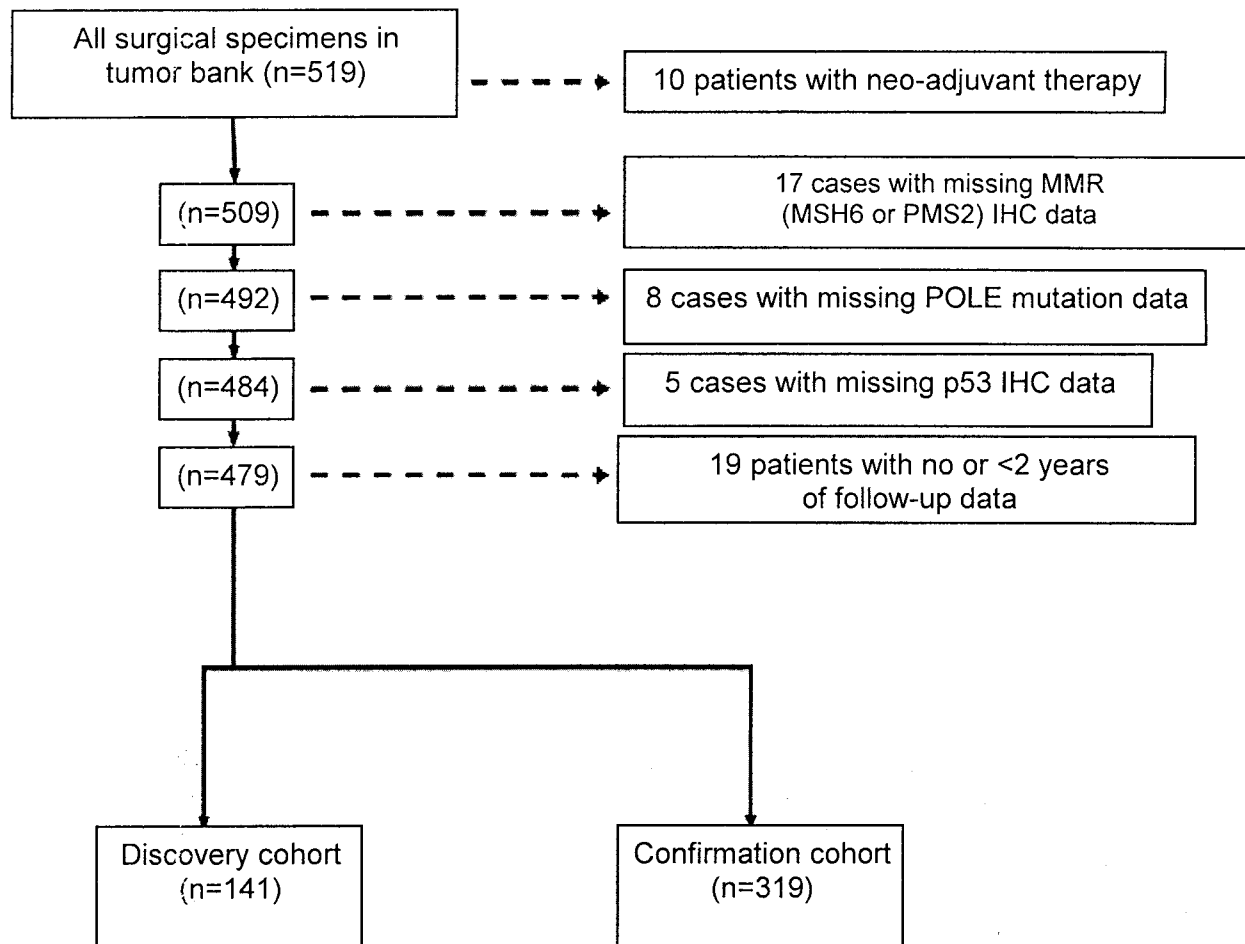


FIG. 8A

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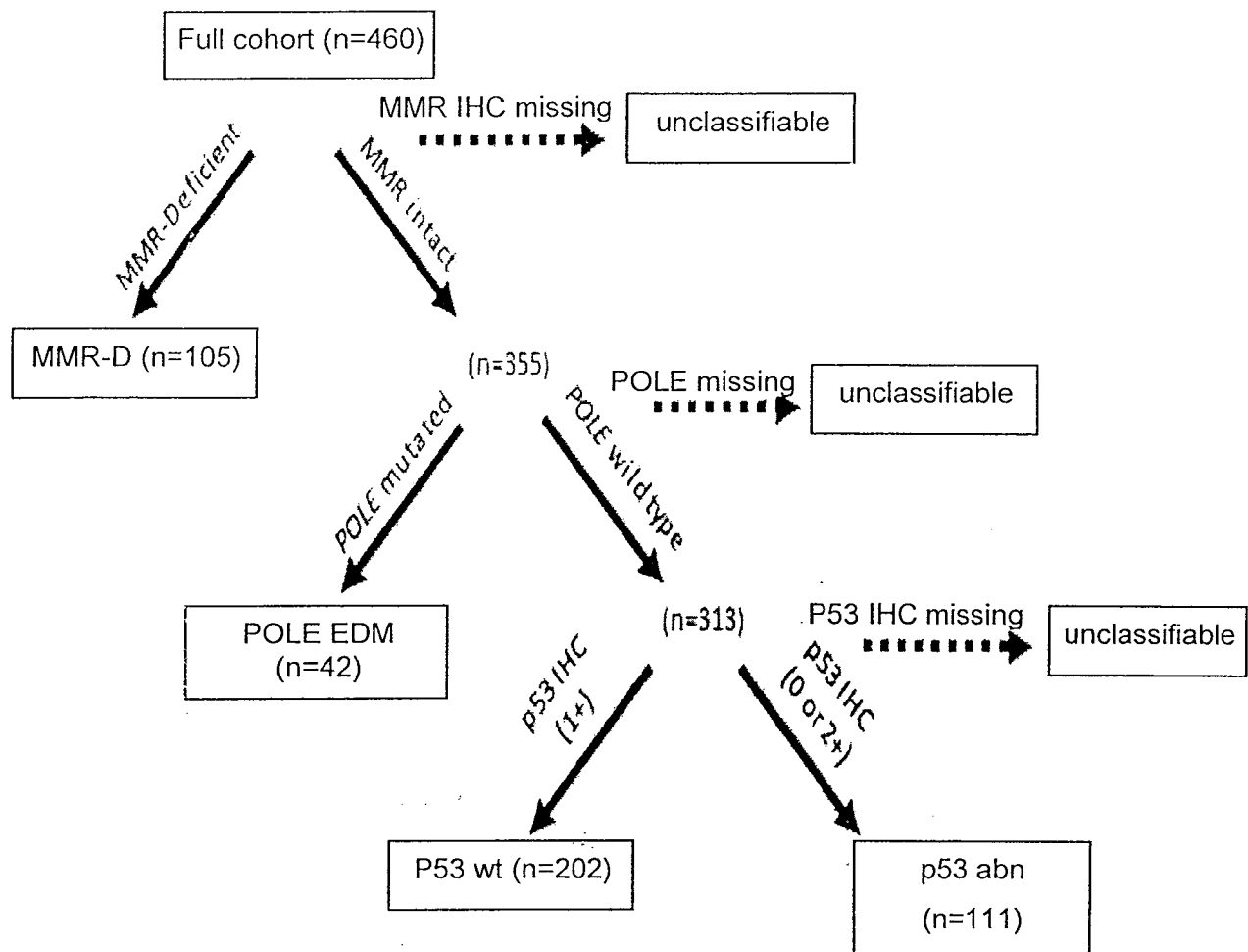


FIG. 8B

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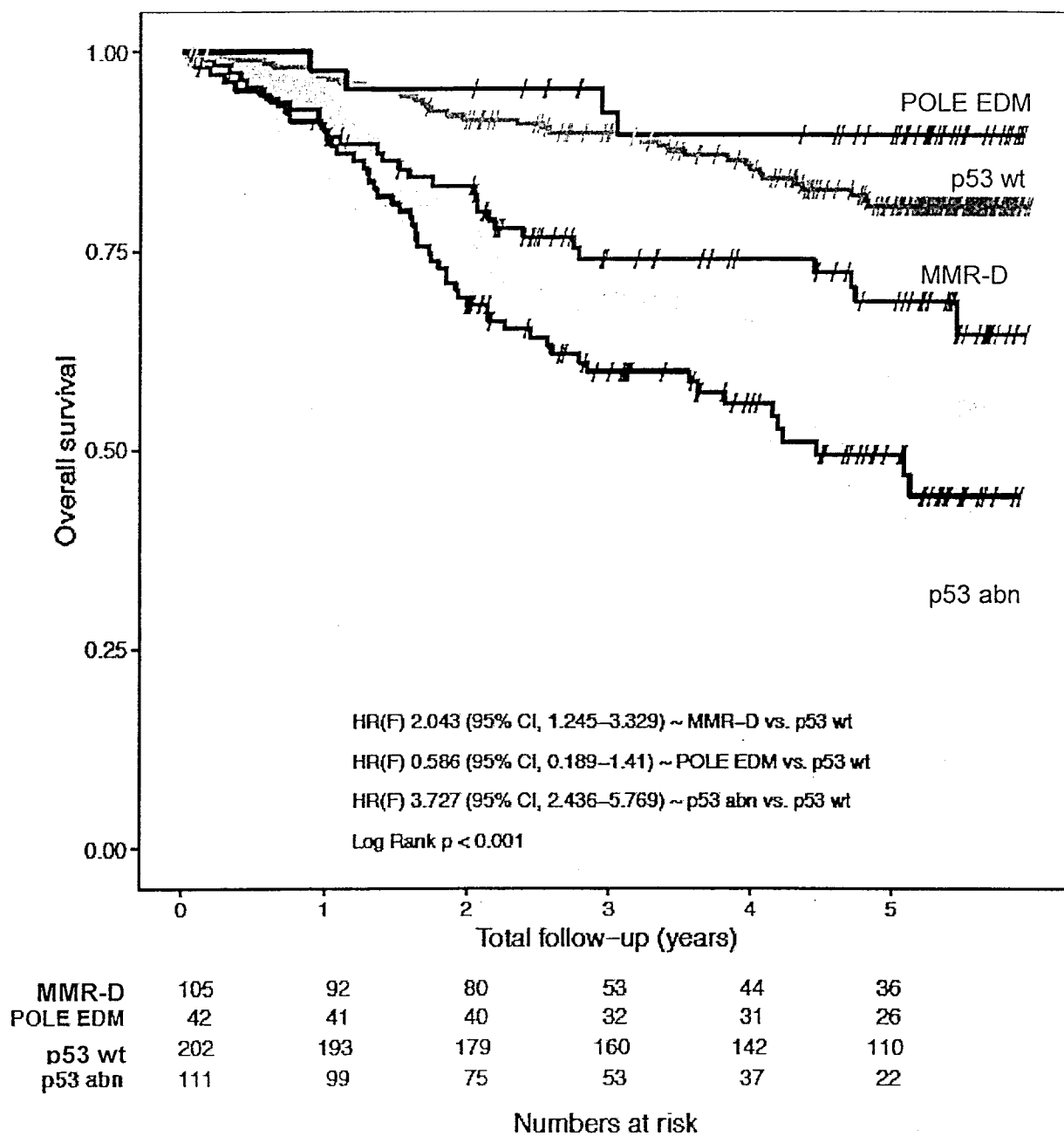


FIG. 9A

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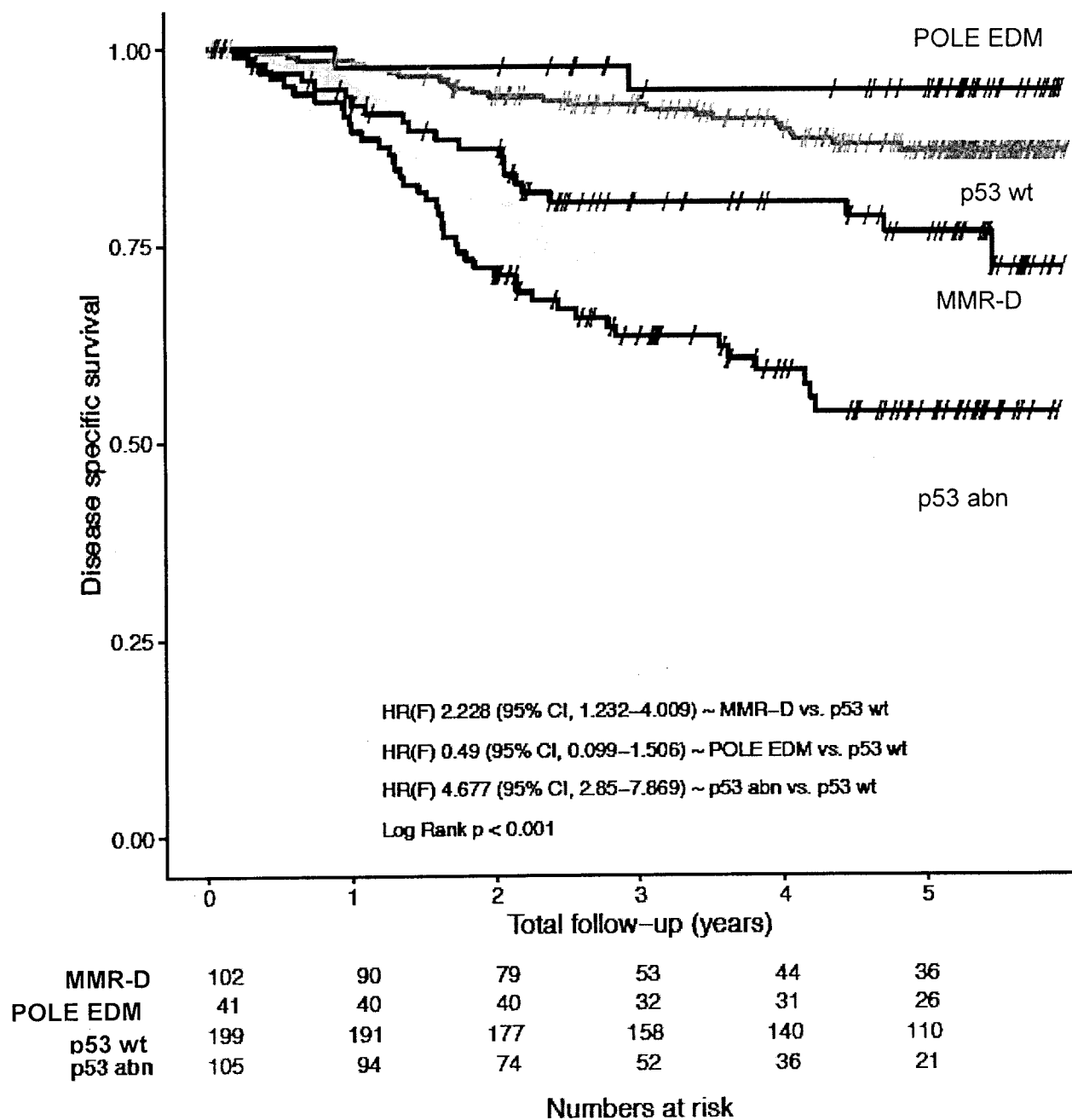


FIG. 9B

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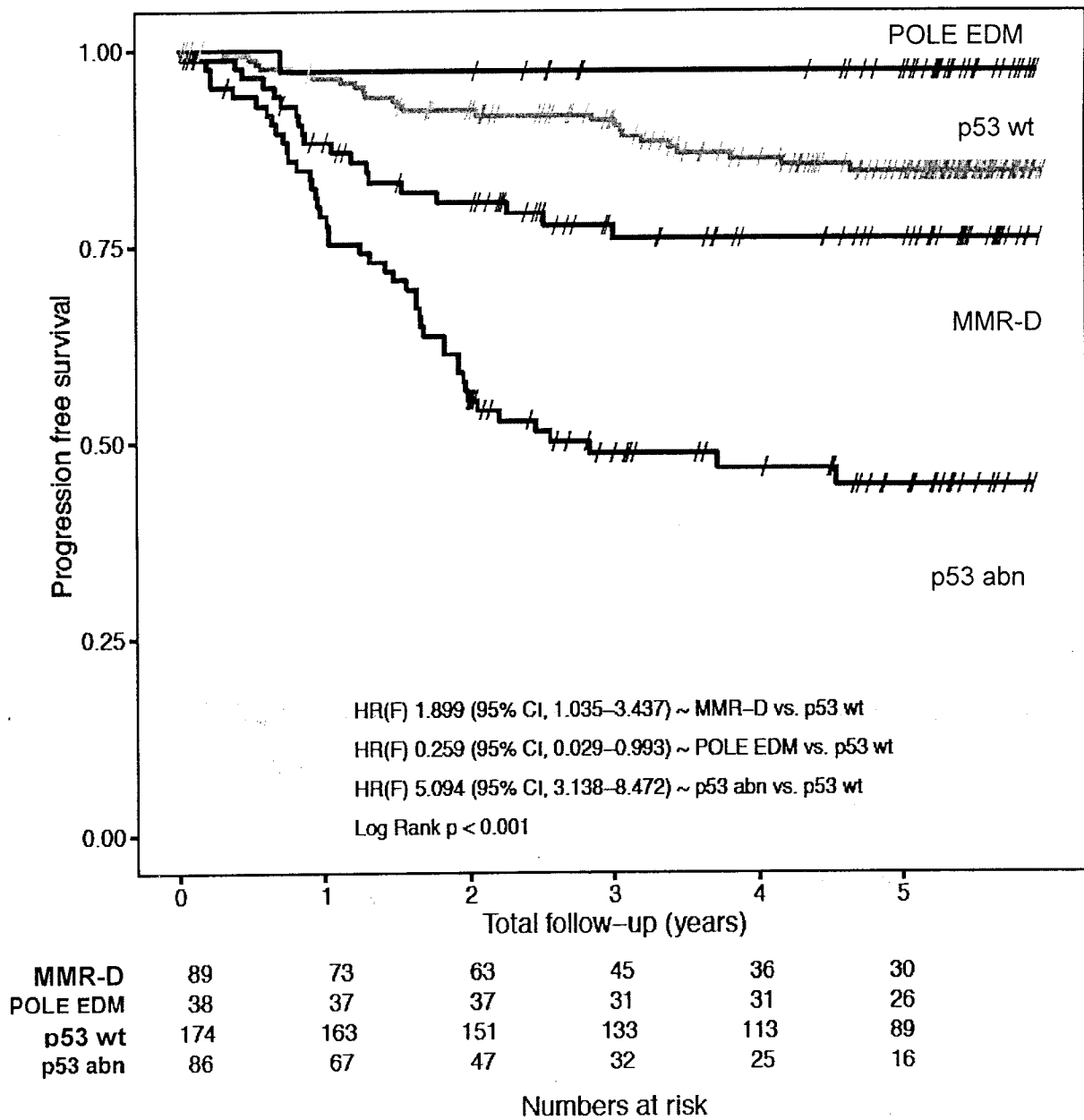


FIG. 9C

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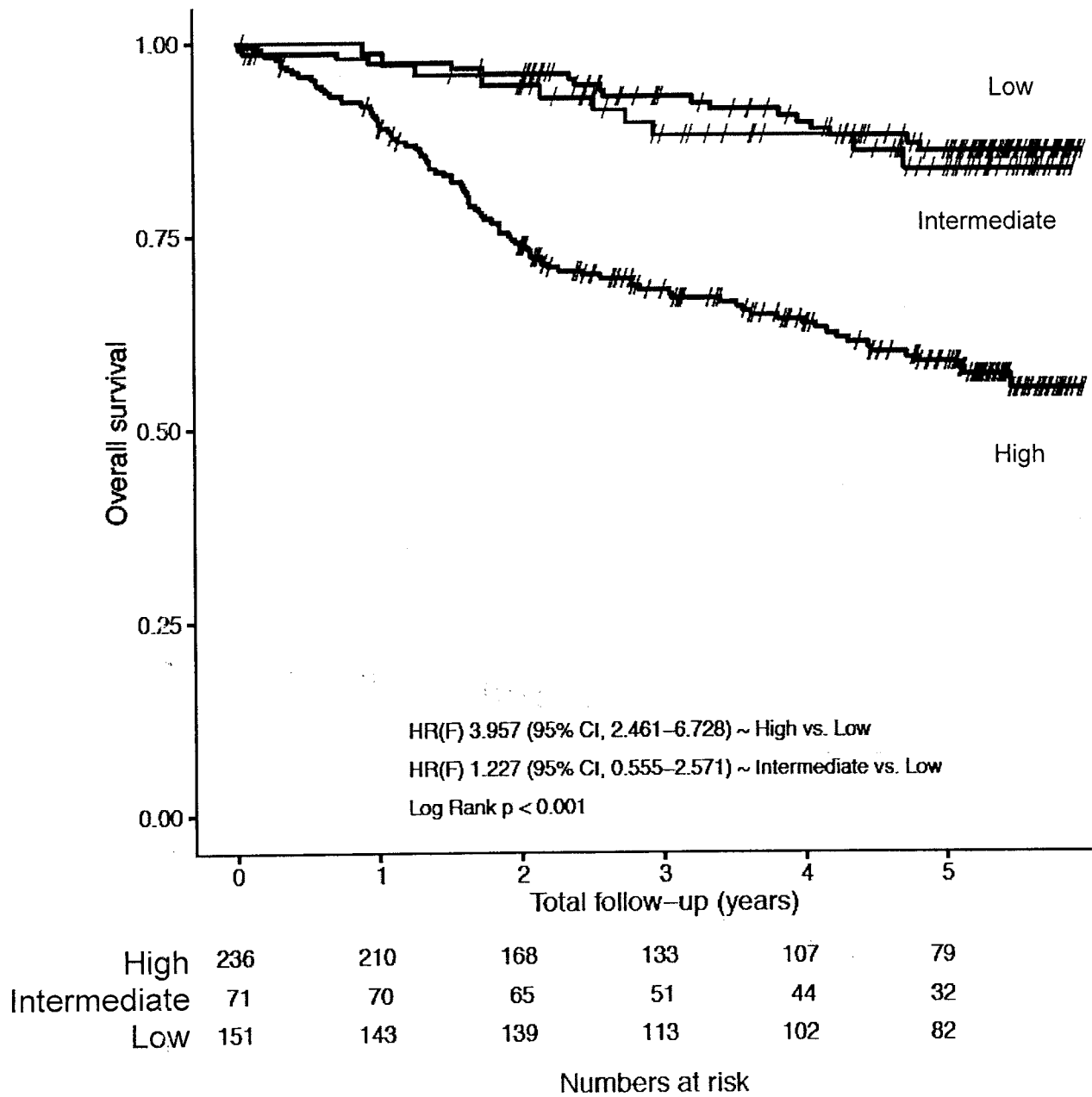


FIG. 10A

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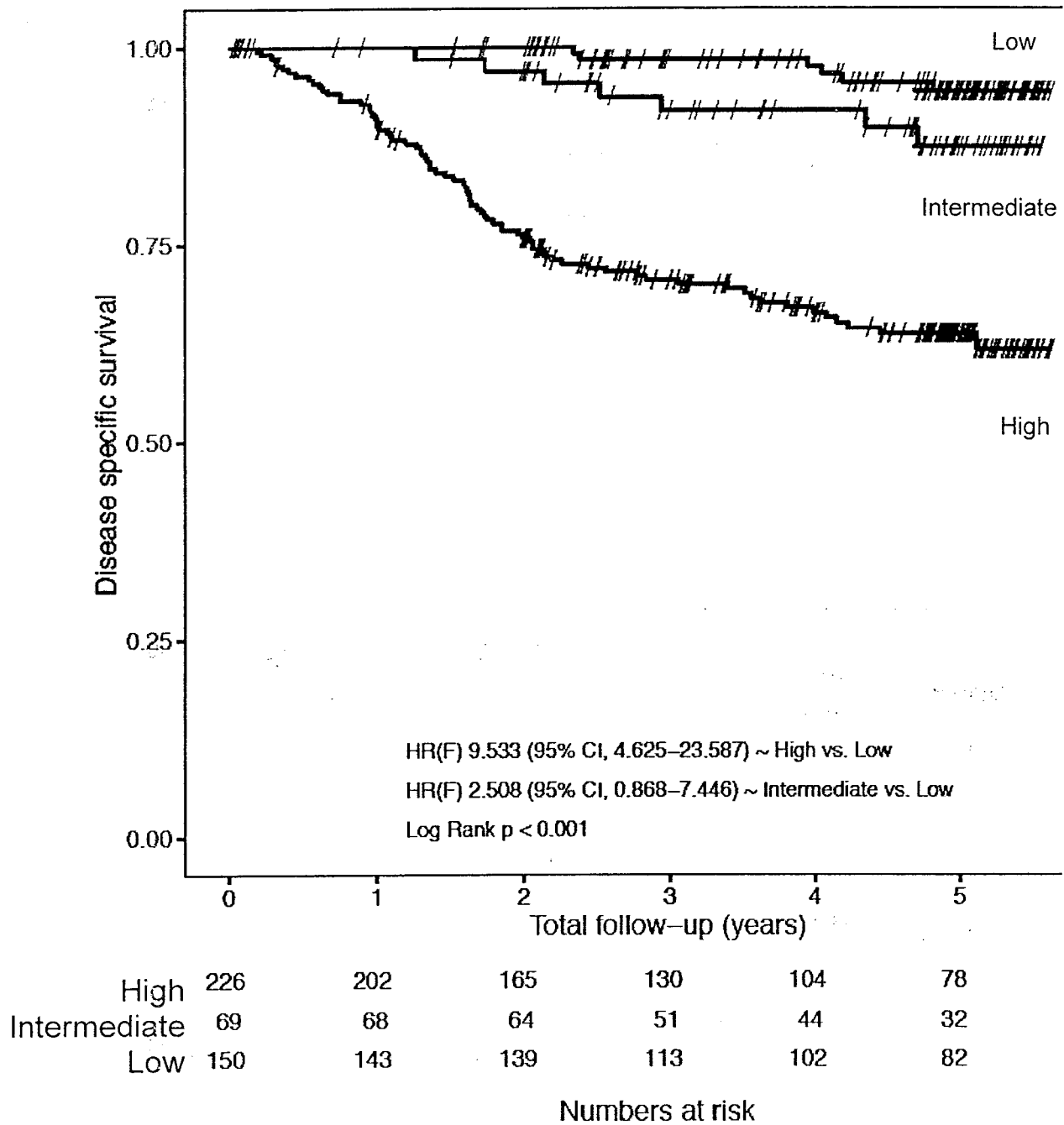


FIG. 10B

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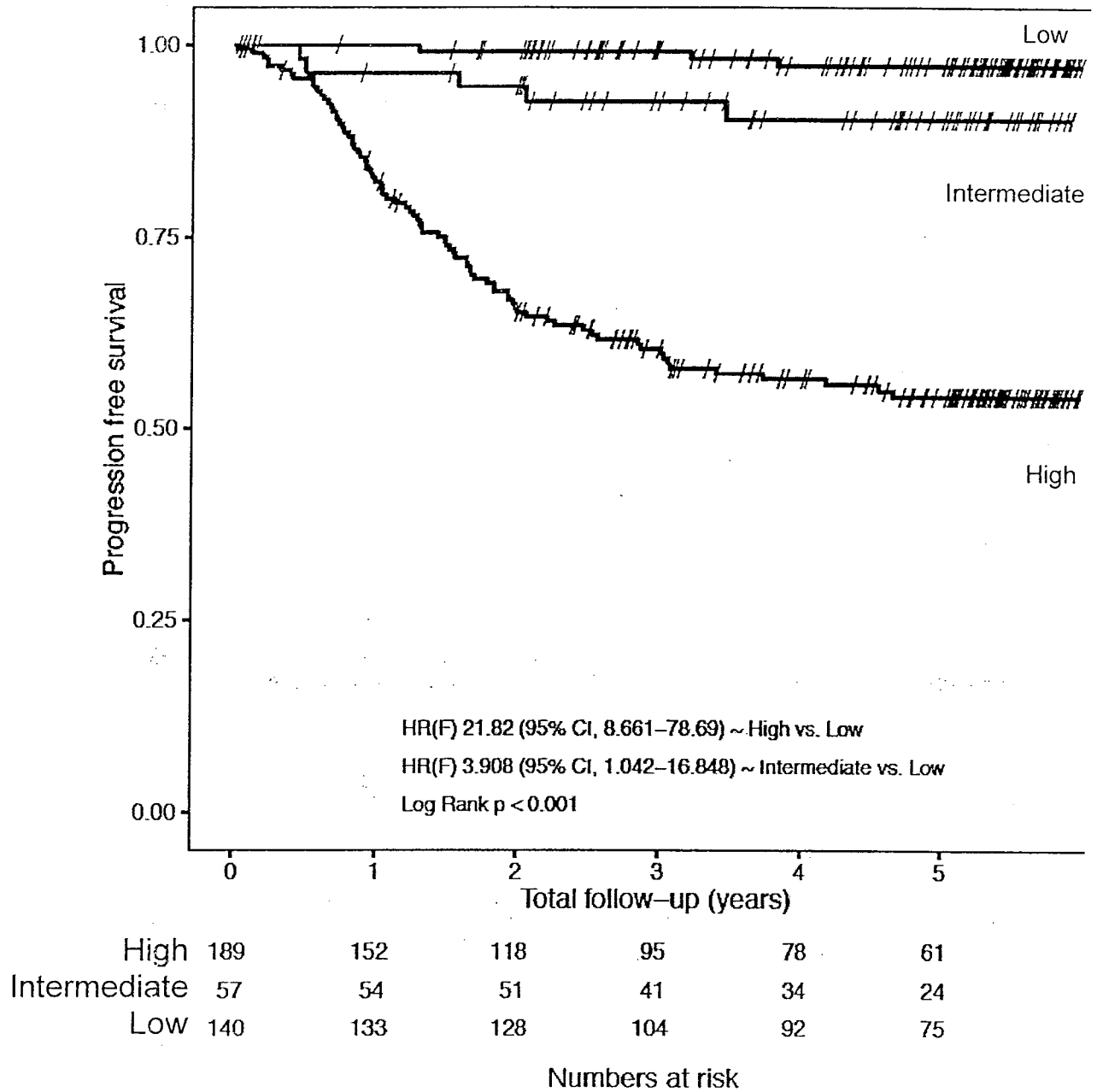


FIG. 10C

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Full Cohort

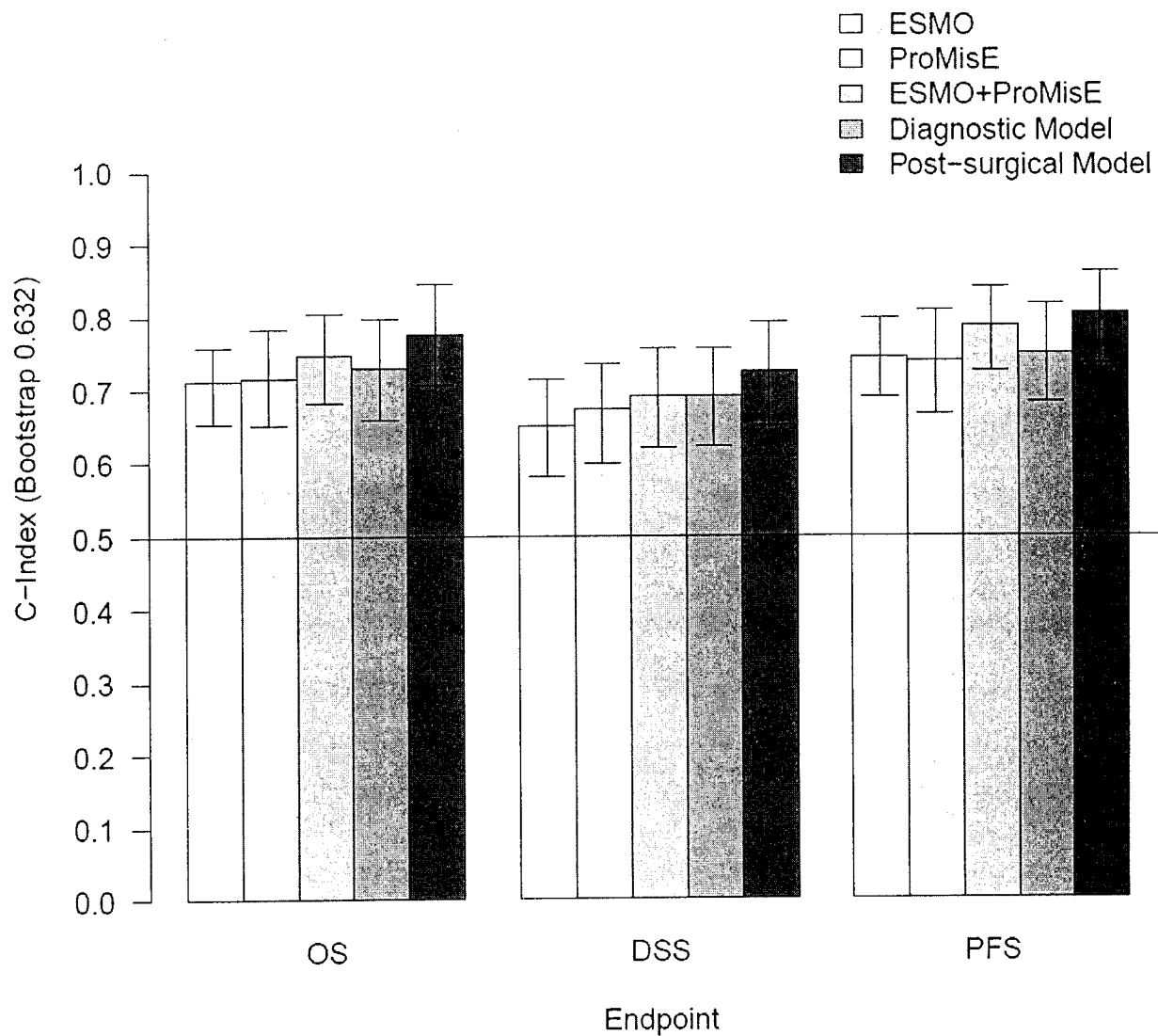


FIG. 11

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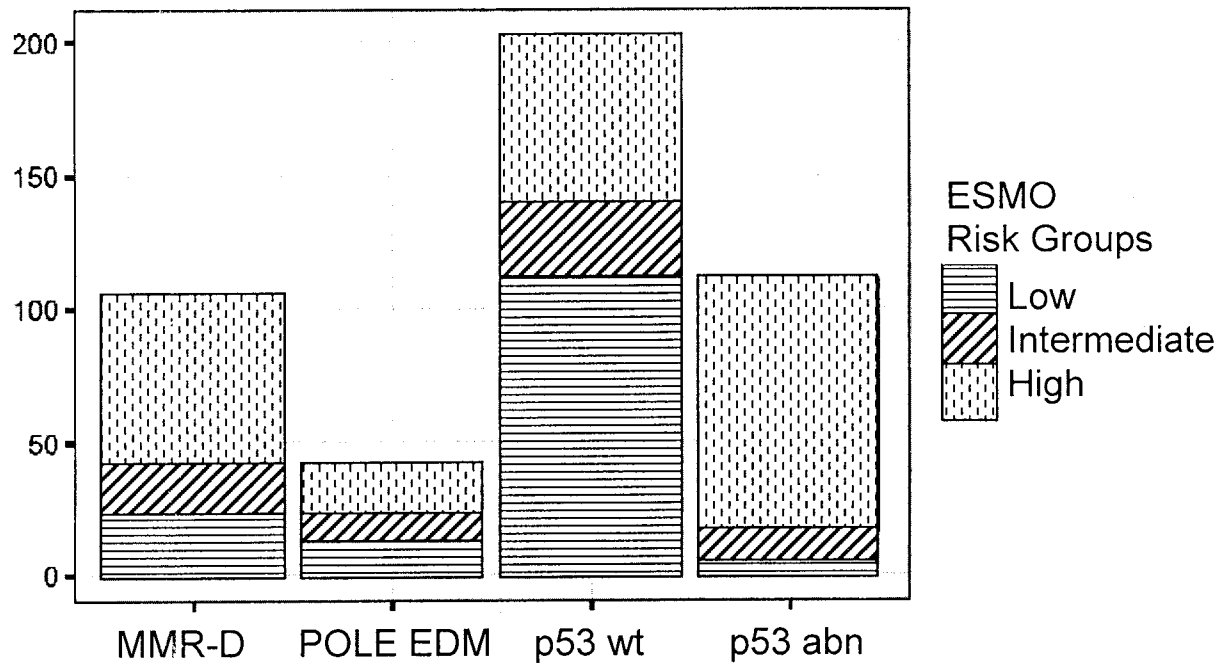


FIG. 12

## INTERNATIONAL SEARCH REPORT

International application No.

**PCT/CA2016/050829**

## A. CLASSIFICATION OF SUBJECT MATTER

IPC: **G01N 33/574** (2006.01), **A61B 10/00** (2006.01), **A61B 5/00** (2006.01), **A61K 45/00** (2006.01),  
**G01N 33/48** (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 G01N-033/574 OR A61B-010/00 OR A61B-005/00 OR A61K-045/00 OR G01N-033/48

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)

Databases Searched: Questel Orbit. CIPO NPL Discovery Tool (EBSCOHOST), Canadian Patent Database.

Search terms: endometrial, cancer, carcinoma, POLE, exonuclease domain, P53, TP53, MSI, microsatellite instability, MMR, mismatch repair,

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                   | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | The Cancer Genome Atlas Research Network. " <i>Integrated Genomic Characterization of Endometrial Carcinoma</i> ." 2 May 2013. Nature. Vol 497. Pp 67-73. Cited in the application.                                  | 1-23                  |
| A         | Nout, RA., et al. " <i>Improved risk assessment of endometrial cancer by combined analysis of MSI, PI3K-AKT, Wnt/<math>\beta</math>-catenin and P53 pathway activation</i> ." Gynecol Oncol. 2012 Sep;126(3):466-73. | 1-23                  |
| A         | Garcia-Dios, D., et al " <i>High-throughput interrogation of PIK3CA, PTEN, KRAS, FBXW7 and TP53 mutations in high primary endometrial carcinoma</i> ." Gynecol Oncol. 2012 December. 128. 327-334.                   | 1-23                  |
| A         | Church, D., et al " <i>Prognostic Significance of POLE proofreading mutations in endometrial cancer</i> ." J Nat Cancer Inst. 2015 December. 107(1).                                                                 | 1-23                  |

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

|                                      |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |                          |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |
|--------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| *<br>"A"<br>"E"<br>"L"<br>"O"<br>"P" | Special categories of cited documents:<br>document defining the general state of the art which is not considered to be of particular relevance<br>earlier application or patent but published on or after the international filing date<br>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)<br>document referring to an oral disclosure, use, exhibition or other means<br>document published prior to the international filing date but later than the priority date claimed | "T"<br>"X"<br>"Y"<br>"&" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention<br>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone<br>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art<br>document member of the same patent family |
|--------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

Date of the actual completion of the international search  
 27 September 2016 (27-09-2016)

Date of mailing of the international search report  
 29 September 2016 (29-09-2016)

Name and mailing address of the ISA/CA  
 Canadian Intellectual Property Office  
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 50 Victoria Street  
 Gatineau, Quebec K1A 0C9  
 Facsimile No.: 819-953-2476

Authorized officer

ADAM MACKENZIE (819) 639-8506

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claim Nos.: 1-23  
because they relate to subject matter not required to be searched by this Authority, namely:  
Claims 1-17 and 20-23 are directed to a diagnostic method, which the International Searching Authority is not required to search under PCT Rule 39.1(iv). However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in claims 1-23. Claims 18-19 are directed to a method for treatment of the human or animal body by surgery or therapy, which the International Searching Authority is not required to search under PCT Rule 39.1(iv). However, this Authority has carried out a search based on the alleged effect or purpose/use of the product defined in claims 18-19
2. ☐ Claim Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.