

## Supplemental Online Content

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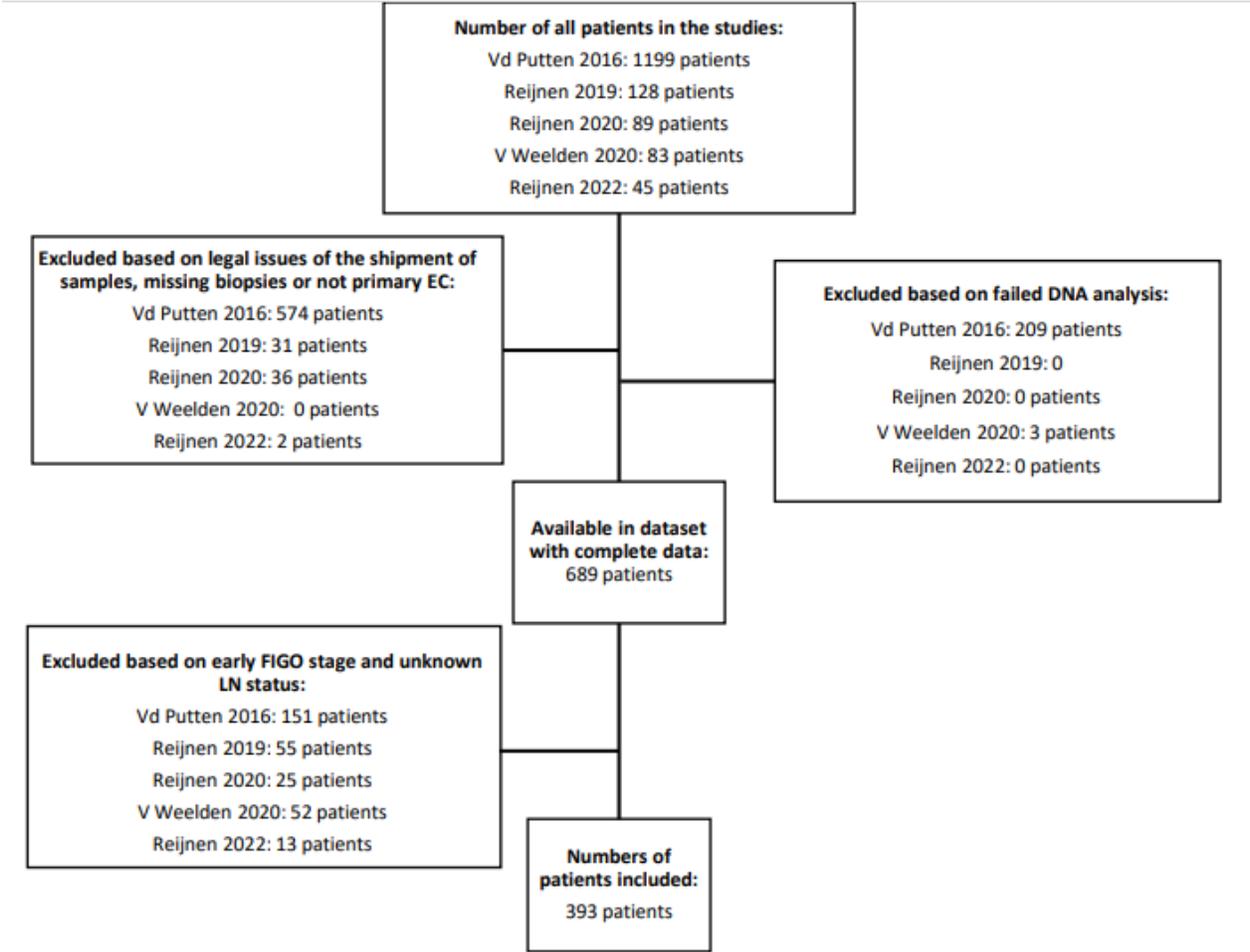
**eReferences**

This supplemental material has been provided by the authors to give readers additional information about their work.

| <b>eTable 1. Baseline Characteristics of the 4 Previous Published Studies and 1 Unpublished Study</b> |                        |   |  |  |  |  |
|---|------------------------|---|--|--|--|--|
|   |                        | <b>Van der Putten et al. 2016<sup>1</sup></b> | <b>Reijnen et al. 2019<sup>2</sup></b> | <b>Reijnen et al. 2020<sup>3</sup></b> | <b>Van Weelden et al. 2020<sup>4</sup></b> | <b>Reijnen et al. 2022<sup>5</sup></b> |
| <b>Study characteristics</b>  |                        |   |  |  |  |  |
| N included  |                        | 265   | 42                                     | 28                                     | 28   | 30                                     |
| Diagnostic classification based on  |                        | Molecular profiling                           | ProMisE                                | ProMisE                                | Molecular profiling                        | Molecular profiling                    |
| Median follow-up (months)   |                        | 76.0 (0.0-197.0)                              | 41.0 (14.0-87.0)                       | 35.5 (0.0-197.0)                       | 37.5 (3.0-21.0)                            | 34.5 (3.0-168.0)                       |
| Demographics  |                        | European                                      | European                               | Dutch                                  | European                                   | Dutch                                  |
| <b>Patient characteristic</b>   |                        |   |  |  |  |  |
| Age (years)   |                        | 63.0 (34.0-86.0)                              | 66.0 (50.0-82.0)                       | 57.0 (31.0-81.0)                       | 66.0 (45.0-82.0)                           | 67.0 (49.0-77.0)                       |
| <b>Primary treatment</b>  |                        |   |  |  |  |  |
| Lymph node dissection   | No                     | 11 (4.2)                                      | 0 (0.0)                                | 0 (0.0)                                | 0 (0.0)                                    | 1 (3.3)                                |
|   | Yes                    | 254 (95.8)                                    | 42 (100.0)                             | 28 (100.0)                             | 24 (85.7)                                  | 28 (93.3)                              |
|   | Pelvic                 | 190 (74.8)                                    | 0 (0.0)                                | 0 (0.0)                                | 6 (25.0)                                   | 18 (64.3)                              |
|   | Para-aortic            | 4 (1.6)                                       | 0 (0.0)                                | 0 (0.0)                                | 2 (8.3)                                    | 7 (25.0)                               |
|   | Pelvic and para-aortic | 45 (17.7)                                     | 0 (0.0)                                | 0 (0.0)                                | 9 (37.5)                                   | 3 (10.7)                               |
|   | Unknown which nodes    | 15 (5.9)                                      | 42 (100.0)                             | 28 (100.0)                             | 7 (29.2)                                   | 0 (0.0)                                |
|   | Unknown                | 0 (0.0)                                       | 0 (0.0)                                | 0 (0.0)                                | 4 (14.3)                                   | 1 (3.3)                                |
| <b>Final pathologic characteristics</b>   |                        |   |  |  |  |  |
| Histology   | EEC                    | 26 (9.8)                                      | 42 (100.0)                             | 20 (71.4)                              | 17 (60.7)                                  | 0 (0.0)                                |
|   | NEEC                   | 239 (90.2)                                    | 0 (0.0)                                | 8 (28.6)                               | 11 (39.3)                                  | 30 (100.0)                             |
| Grade   | 1-2                    | 183 (69.1)                                    | 0 (0.0)                                | 18 (64.3)                              | 8 (28.6)                                   | 0 (0.0)                                |
|   | 3                      | 82 (30.9)                                     | 42.0 (100.0)                           | 10 (35.7)                              | 20 (71.4)                                  | 30 (100.0)                             |
| Molecular   | POLE-mut               | 26 (9.8)                                      | 3 (7.1)                                | 3 (10.7)                               | 0  | 1 (3.3)                                |
|   | MSI                    | 51 (19.2)                                     | 15 (35.7)                              | 2 (7.1)                                | 4 (14.3)                                   | 6 (20.0)                               |
|   | TP53-mut               | 31 (11.7)                                     | 9 (21.4)                               | 8 (28.6)                               | 16 (57.1)                                  | 8 (26.7)                               |
|   | NSMP                   | 157 (59.2)                                    | 15 (35.7)                              | 15 (53.6)                              | 8 (28.6)                                   | 15 (50.0)                              |
| MI  | <50%                   | 154 (58.1)                                    | 4 (9.5)                                | 16 (57.1)                              | 11 (39.3)                                  | 12 (40.0)                              |
|   | >50%                   | 111 (41.9)                                    | 36 (85.7)                              | 12 (42.9)                              | 17 (60.7)                                  | 18 (60.0)                              |
|   | Missing                | 0 (0.0)                                       | 2 (4.8)                                | 0 (0.0)                                | 0 (0.0)                                    | 0 (0.0)                                |
| LVSI  | No                     | 219 (82.6)                                    | 27 (64.3)                              | 26 (92.9)                              | 14 (50.0)                                  | 18 (60.0)                              |
|   | Yes                    | 46 (17.4)                                     | 15 (35.7)                              | 2 (7.1)                                | 14 (50.0)                                  | 12 (40.0)                              |
| Lymph nodes   | N0                     | 231 (87.2)                                    | 42 (100.0)                             | 0 (0.0)                                | 16 (57.1)                                  | 16 (53.3)                              |
|   | N1                     | 23 (8.7)                                      | 0 (0.0)                                | 0 (0.0)                                | 8 (28.6)                                   | 12 (40.0)                              |

|   |                      |                        |            |            |            |           |            |
|---|----------------------|------------------------|------------|------------|------------|-----------|------------|
|   |                      | Pelvic                 | 14 (60.9)  | 0 (0.0)    | 0 (0.0)    | 4 (50.0)  | 0 (0.0)    |
|   |                      | Para-aortic            | 4 (17.4)   |            |            | 3 (37.5)  | 0 (0.0)    |
|   |                      | Pelvic and para-aortic | 5 (21.7)   | 0 (0.0)    | 0 (0.0)    | 1 (12.5)  | 0 (0.0)    |
|   |                      | Unknown which nodes    | 0 (0.0)    | 0 (0.0)    | 0 (0.0)    | 0 (0.0)   | 12 (100.0) |
|   | Nx                   |                        | 6 (2.3)    | 0 (0.0)    | 28 (100.0) | 4 (14.3)  | 2 (6.7)    |
| FIGO stage  | Early (I-II)         |                        | 223 (84.2) | 42 (100.0) |            | 13 (46.4) | 12 (40.0)  |
|   | Advanced (III-IV)    |                        | 42 (15.8)  | 0 (0.0)    | 28 (100.0) | 15 (53.6) | 18 (60.0)  |
| <b>Adjuvant treatment</b>   |                      |                        |            |            |            |           |            |
|   | None                 |                        | 66 (24.9)  | 12 (28.6)  | 4 (14.3)   | 6 (21.4)  | 9 (30.0)   |
|   | Radiotherapy         |                        | 163 (61.5) | 25 (59.5)  | 7 (25.0)   | 15 (53.6) | 15 (50.0)  |
|   |                      | EBRT                   | 53 (32.5)  | 8 (32.0)   | 0 (0.0)    | 6 (40.0)  | 0 (0.0)    |
|   |                      | VBT                    | 72 (44.2)  | 12 (48.0)  | 0 (0.0)    | 5 (33.3)  | 0 (0.0)    |
|   |                      | ERBT+VBT               | 38 (23.3)  | 5 (20.0)   | 0 (0.0)    | 4 (26.7)  | 0 (0.0)    |
|   |                      | Unknown                | 0 (0.0)    | 0 (0.0)    | 7 (100.0)  | 0 (0.0)   | 15 (100.0) |
|   | Chemotherapy         |                        | 9 (3.4)    | 1 (2.4)    | 14 (50.0)  | 5 (17.9)  | 4 (13.3)   |
|   | Chemoradiation       |                        | 27 (10.2)  | 1 (2.4)    | 2 (7.1)    | 2 (7.1)   | 2 (6.7)    |
|   | Unknown              |                        | 0 (0.0)    | 3 (7.1)    | 1 (3.6)    | 0 (0.0)   | 0 (0.0)    |
| <b>Mortality</b>  |                      |                        |            |            |            |           |            |
|   | Recurrence           |                        | 34 (12.8)  | 13 (31.0)  | 9 (32.1)   | 12 (42.9) | 6 (20.0)   |
|   | Mortality            |                        | 40 (15.1)  | 11 (26.2)  | 12 (42.9)  | 14 (50.0) | 13 (43.3)  |
|   | EC-related mortality |                        | 29 (10.9)  | 9 (21.4)   | 9 (32.1)   | 13 (46.4) | 13 (43.3)  |
| Data is presented as No. (%), median (IQR)<br>Abbreviations: <i>POLE</i> , Polymerase epsilon; MSI, Microsatellite instability; <i>TP53</i> , Tumor protein 53; NSMP, No-specific molecular profile; EEC, endometrioid endometrial cancer; NEEC, non-endometrioid endometrial cancer; MI, myometrial invasion; LVSI, lymphovascular space invasion; N0, negative lymph nodes, N1, positive lymph nodes; Nx, no information about the lymph nodes; FIGO, Federation International of Gynecology and Obstetrics; EBRT, external beam radiation therapy; VBT, vaginal brachytherapy; EC, endometrial cancer. |                      |                        |            |            |            |           |            |

**eFigure 1. Study Flowchart**



**Figure legend:** Abbreviations: EC, Endometrial Cancer; LN, Lymph node

## **eMethods.** Detailed Information on DNA Analysis, smMIP Design and Library Preparation, Sequencing, and Immunochemistry Analysis

### **DNA analysis**

Representative areas of EC in the surgical specimen were marked and selected for formalin-fixed paraffin-embedded (FFPE) 20 µm thick sections. Slides were cut from these FFPE section and stained with hematoxylin and eosin (H&E). Tumor areas were marked on these slides and the tumor cell percentage was estimated. These specimens were digested overnight at 56°C in TET-lysis buffer (10mmol/L Tris/HCL pH 8.5, 1 mmol/L EDTA pH 8.0, 0.01% Tween-20) with 5% Chelex-100 (Bio-Rad, Hercules, CA) and 0.2% proteinase K, with subsequent inactivation at 95°C for 10 min. After this was centrifugated, the supernatant was transferred into a clean tube. DNA concentration was determined using the Qubit Broad Range Kit (Thermo Fisher Scientific, Waltham, MA).

### **smMIP design and library preparation**

The panel consisted of 10 genes important for EC oncogenesis (ARID1A, CTNNB1, ERBB2, KRAS, MTOR, NRAS, PIK3CA, PTEN, POLE, TP53). The smMIPs were designed in a tiling manner for hotspots in oncogenes and all coding as well as splice site consensus sequences of tumor suppressor genes (TSGs), with preferential targeting of both strand by two independent smMIPs. All the smMIP probes are constructed by an extension and ligation probe arm (40 bp long) with a 112 bp gap and a common backbone sequence for PCR-based library amplification. The backbone and ligation probe arm are connected by means of an 8 bp degenerate sequence (8xN) serving as a Unique Molecular Identifier (UMI, “single-molecule tag”). Following, the smMIP probes were mixed and phosphorylated with 1 µl of T4 polynucleotide kinase (M0201; New England Biolabs). The molecular ratio between gDNA and smMIPs was set at 1:3,200 for each individual smMIP and the standard genomic DNA input was set at 100ng. A capture mix was made (volume 25 µl) with the phosphorylated smMIP pool, 1 unit of Ampligase DNA ligase (A0110K; EpiBio, Madison, WI) and Ampligase Buffer (A1905B, DNA ligase buffer), 3.2 units of Hemo KlenTaq (M0332; New England Biolabs), 8 mmol of dNTPs (28-4065-20/-12/-22/-32; GE Healthcare, Little Chalfont, UK) and 100 ng of genomic DNA in a 20 µl volume. This capture mix was denatured at 95°C for 10 min and subsequently incubated for probe hybridization, extension and ligation for 18hr at 60°C. To perform the exonuclease treatment, Exonuclease 1 (10 units; M0293; New England Biolabs) and III (50 units; M0206; New England Biolabs) and Ampligase Buffer was added to the capture mix after cooling (total of 27 µl). This mix was incubated at 37°C for 45 min, with subsequent inactivation at 95°C for 2 min. From the 27 µl, 20 µl was used for PCR in at total volume of 50 µl including a common forward primer, bar-coded reverse primers, and iProof high fidelity master mix (1725310, Bio-Rad, Veenendaal, the Netherlands). The resulting PCR products were then pooled and purified with 0.8x volume of Agencourt Ampure XP Beads (a63881, Beckman Coulter, Woerden, the Netherlands).

### **Sequencing**

The purified libraries were denatured and diluted to 1.2pmol/l, and then sequenced on a NexSeq500 device (Illumina, San Diego, CA) using the manufacturer’s instructions (300 cycles High Output sequencing kit, v2), resulting in 2x150bp paired-end reads. All Bcl files were converted to fastq files and bar-coded reads were then demultiplexed. Single-molecule-directed assembly of the duplicate reads was conducted generating consensus (‘unique’) reads with the software Sequence Pilot (version 4.4.0; JSI medical system, Ettenheim, Germany).

Variants were annotated as ‘malignant’, ‘likely malignant’, ‘unknown significance’, ‘likely benign’ and ‘benign’ using amongst others publicly available databases such as ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>), The Clinical Knowledgebase (CKB, <https://ckb.jax.org/>), Cancer Genome Interpreter (CGI, <https://www.cancergenomeinterpreter.org/home>), the Catalog of Somatic Mutations in Cancer (COSMIC, <https://cancer.sanger.ac.uk/cosmic>), OncoKB (<https://www.oncokb.org/>), Varsome (<https://varsome.com/>). The three first categories were taken into consideration and included known activating hotspot mutations for the oncogenes, and missense, nonsense, frameshift and splice site mutations for the included TSGs. Intronic mutations were excluded with exception of splice site sequences. To determine whether sufficient DNA molecules were sequenced to reliably exclude mutation, a cumulative binomial distribution was used for calculating the required unique read depths, above a certain mutant allele frequency with a certainty of >95%.<sup>6</sup> These required read depths were assessed in the context of estimated tumor percentage cells by microscopy.

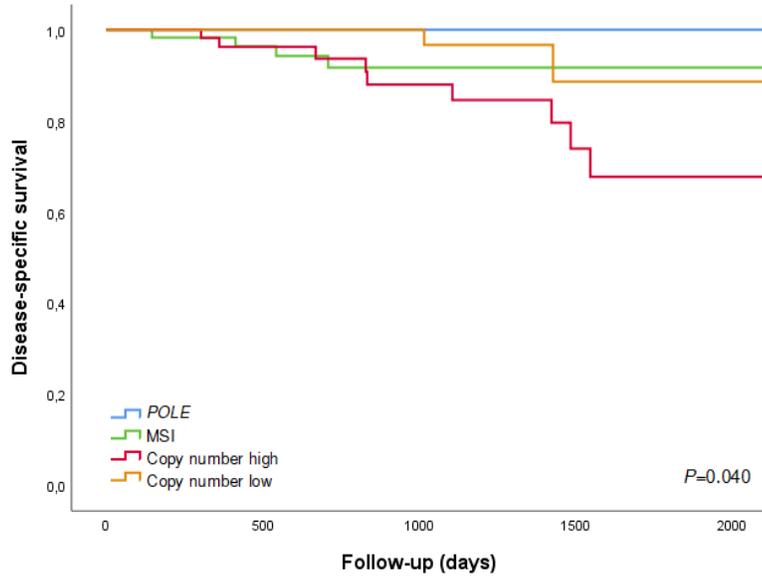
### **Immunohistochemical staining**

For p53 staining, antigen retrieval (30 minutes, pH 6-7) and blocking of endogenous peroxidase with hydrogen peroxide was performed. Subsequently, slides were incubated with p53 antibody (clone DO-7 + BP53-12, dilution 1:600). Powervision+ Poly-HRP was used and visualization was accomplished by using PowerVision DAB substrate solution (Leica Biosystems, Buffalo Grove, IL, US). Counterstaining was performed with hematoxylin, slides were dehydrated and mounted.

For PMS2 and MSH6 staining, antigen retrieval with EnVision FLEX High pH Target Retrieval Solution, and blocking of endogenous peroxidase with hydrogen peroxide was performed. After, slides were incubated with anti-MSH6 (clone EPR3945 1:400, Abcam, Cambridge, UK) or anti-PMS2 (clone A16-4 dilution 1:20, BD Biosciences, San Jose, CA). Incubation was performed with EnVision FLEX and visualized with High pH visualization system. Counterstaining was performed with hematoxylin, slides were dehydrated and mounted.

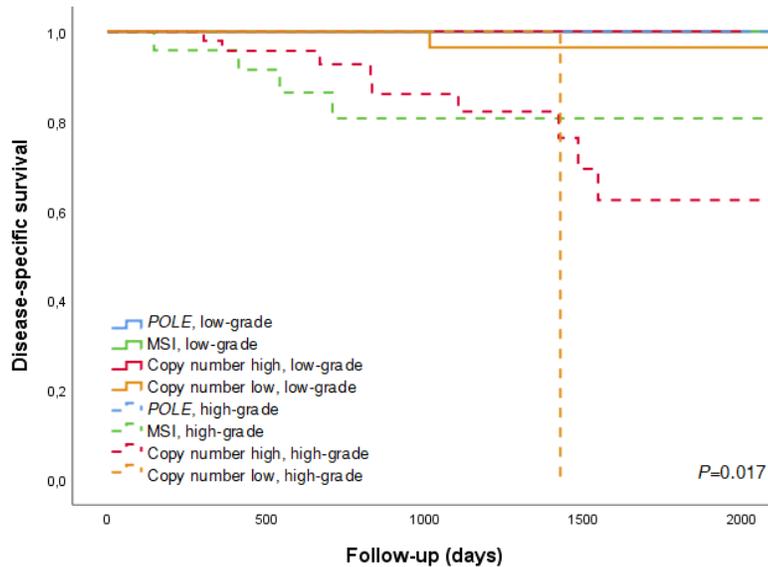
| <b>eTable 2. Baseline Characteristics of the Included vs Excluded Patients</b>  |         |                           |                           |          |
|---|---------|---------------------------|---------------------------|----------|
|   |         | <b>Included<br/>N=393</b> | <b>Excluded<br/>N=296</b> | <b>P</b> |
| <b>Patient characteristic</b>   |         |                           |                           |          |
| Age (years)   |         | 63.0 (31.0-82.0)          | 64.5 (35.0-93.0)          | .09      |
| <b>Pathologic characteristics</b>   |         |                           |                           |          |
| <i>POLE</i> -mutant   |         | 33 (8.4)                  | 14 (4.7)                  | .001     |
| MSI   |         | 78 (19.8)                 | 79 (26.7)                 |          |
| <i>TP53</i> -mutant   |         | 72 (18.3)                 | 29 (9.8)                  |          |
| NSMP  |         | 210 (53.4)                | 174 (58.8)                |          |
| Histology   | EEC     | 318 (80.9)                | 275 (92.9)                | <.001    |
|   | NEEC    | 75 (19.1)                 | 21 (7.1)                  |          |
| Grade   | 1-2     | 209 (53.2)                | 217 (73.3)                | <.001    |
|   | 3       | 184 (46.8)                | 79 (26.7)                 |          |
| MI  | <50%    | 197 (50.1)                | 178 (61.0)                | .006     |
|   | >50%    | 194 (49.4)                | 114 (39.0)                |          |
|   | Unknown | 2 (0.5)                   |                           |          |
| LVSI  | No      | 304 (77.4)                | 238 (80.4)                | .33      |
|   | Yes     | 89 (22.6)                 | 58 (19.6)                 |          |
| <b>Adjuvant treatment</b>   |         |                           |                           |          |
| None  |         | 97 (24.7)                 | 148 (50.3)                | <.001    |
| Radiotherapy  |         | 225 (57.3)                | 124 (42.2)                |          |
| Chemotherapy  |         | 33 (8.4)                  | 17 (5.8)                  |          |
| Chemoradiation  |         | 34 (8.7)                  | 5 (1.7)                   |          |
| Unknown   |         | 4 (1.0)                   |                           |          |
| <b>Mortality</b>  |         |                           |                           |          |
| Recurrence  |         | 74 (18.8)                 | 38 (12.8)                 | .013     |
| Mortality   |         | 90 (22.9)                 | 55 (18.6)                 | .17      |
| EC-related mortality  |         | 73 (18.6)                 | 26 (8.8)                  | <.001    |
| Data is presented as No. (%), median (IQR)<br>Abbreviations: <i>POLE</i> , Polymerase epsilon; MSI, Microsatellite instability; <i>TP53</i> , Tumor protein 53; NSMP, No-specific molecular profile; EEC, endometrioid endometrial cancer; NEEC, non-endometrioid endometrial cancer; MI, myometrial invasion; LVSI, lymphovascular space invasion; EC, endometrial cancer. |         |                           |                           |          |

**eFigure 2. Disease-Specific Survival Curves of the Validation Cohort**



Numbers at risk

|                  |    |    |    |    |    |
|------------------|----|----|----|----|----|
| <i>POLE</i>      | 17 | 12 | 9  | 4  | 3  |
| MSI              | 65 | 46 | 23 | 16 | 11 |
| Copy number high | 60 | 43 | 28 | 13 | 5  |
| Copy number low  | 90 | 68 | 30 | 10 | 7  |



Numbers at risk

|                   |    |    |    |    |   |
|-------------------|----|----|----|----|---|
| <b>Low-grade</b>  |    |    |    |    |   |
| <i>POLE</i>       | 9  | 7  | 5  | 1  | 1 |
| MSI               | 40 | 27 | 14 | 10 | 7 |
| Copy number high  | 7  | 6  | 4  | 3  | 1 |
| Copy number low   | 84 | 64 | 28 | 10 | 7 |
| <b>High-grade</b> |    |    |    |    |   |
| <i>POLE</i>       | 8  | 5  | 4  | 3  | 2 |
| MSI               | 25 | 19 | 9  | 6  | 4 |
| Copy number high  | 53 | 37 | 24 | 10 | 4 |
| Copy number low   | 6  | 4  | 2  | 0  | 0 |

**Figure legend:** A. The 5-years disease-specific survival (DSS) of the molecular subgroups in the entire cohort. B. 5-years DSS of the molecular subgroups and low- versus high-grade endometrial cancer (EC).

Abbreviations: *POLE*, Polymerase epsilon; MSI, Microsatellite instability; *TP53*, Tumor protein 53; NSMP, No-specific molecular profile

| <b>eTable 3. Cox Regression Univariable and Multivariable Analysis of Disease-Specific Survival in Patients With High-Grade Disease</b>  |                        |                |                          |                |
|--|------------------------|----------------|--------------------------|----------------|
| <b>Variable</b>  | <b>Univariable DSS</b> |                | <b>Multivariable DSS</b> |                |
|  | 61 events              |                | 61 events                |                |
|  | HR (95% CI)            | <i>P</i> value | HR (95% CI)              | <i>P</i> value |
| <b>Molecular subgroup</b>  |                        |                |                          |                |
| <i>POLE</i> -mutant  | 0.15 (0.02-1.09)       | .06            | 0.19 (0.02-1.46)         | .12            |
| MSI  | 0.27 (0.09-0.77)       | .02            | 0.45 (0.16-1.24)         | .12            |
| <i>TP53</i> -mutant  | 1.93 (1.13-3.28)       | .02            | 1.70 (0.99-2.91)         | .05            |
| NSMP   | 1                      |                | 1                        |                |
| <b>LVSI</b>  |                        |                |                          |                |
| No   | 1                      | .002           | 1                        | .67            |
| Yes  | 2.23 (1.34-3.68)       |                | 1.12 (0.65-1.92)         |                |
| <b>FIGO</b>  |                        |                |                          |                |
| Stage I-II   | 1                      | <.001          | 1                        | <.001          |
| Stage III-IV   | 5.67 (3.30-9.73)       |                | 4.05 (2.24-7.29)         |                |
| Abbreviations: DSS, disease-specific survival; HR, hazard ratio; CI, confidence interval; <i>POLE</i> , Polymerase epsilon; MSI, Microsatellite instability; <i>TP53</i> , Tumor protein; NSMP, No-specific molecular profile, LVSI, lymphovascular space invasion; FIGO, Federation International of Gynecology and Obstetrics. |                        |                |                          |                |

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