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## **Towards personalized treatment for high risk endometrial cancer**

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### **Citation**

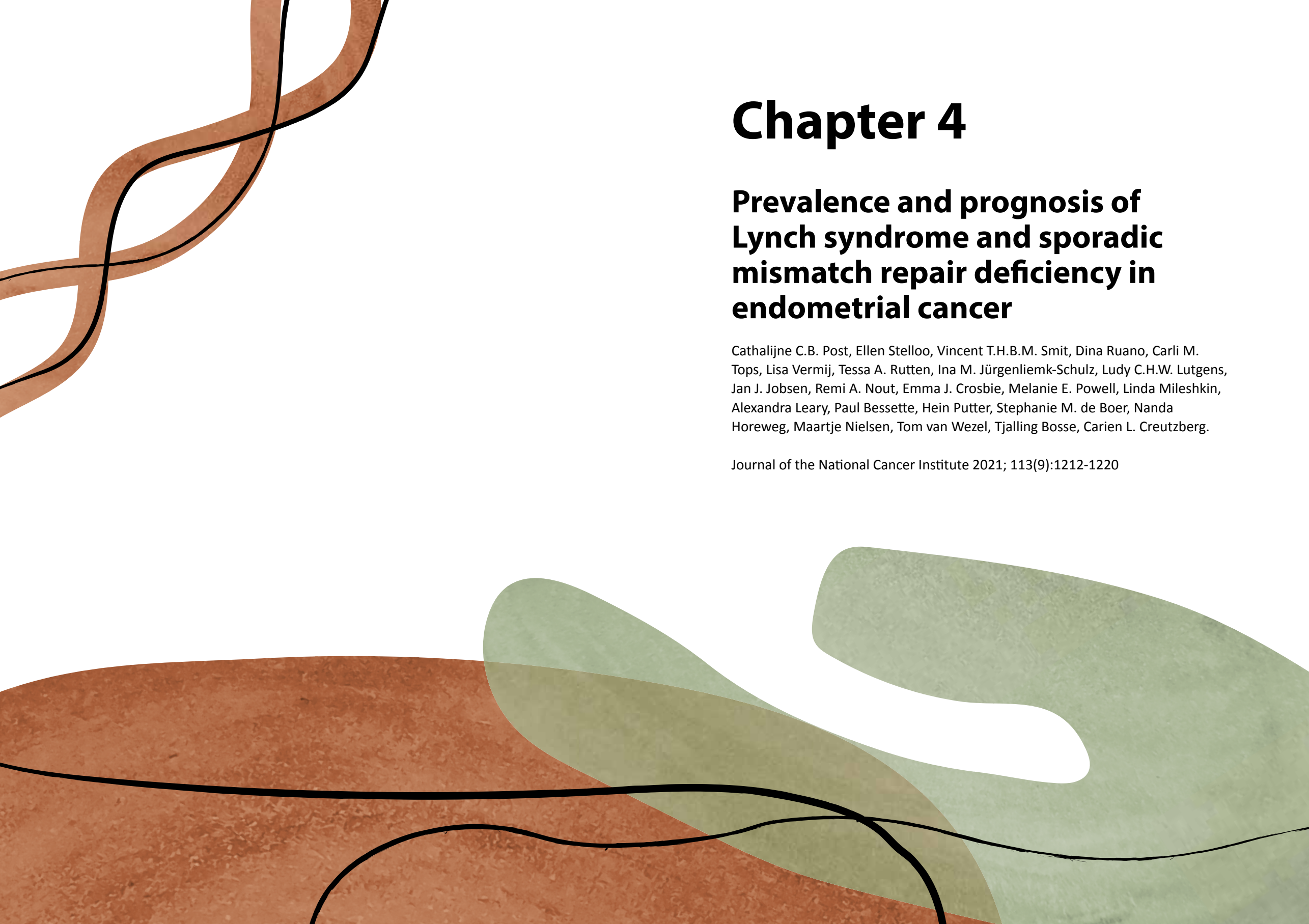
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# Chapter 4

## **Prevalence and prognosis of Lynch syndrome and sporadic mismatch repair deficiency in endometrial cancer**

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## ABSTRACT

### Background

Standard screening of endometrial cancer (EC) for Lynch syndrome (LS) is gaining traction; however, the prognostic impact of an underlying hereditary etiology is unknown. We established the prevalence, prognosis, and subsequent primary cancer incidence of patients with LS-associated EC in relation to sporadic mismatch repair deficient (MMRd)-EC in the large combined Post Operative Radiation Therapy in Endometrial Carcinoma-1, -2, and -3 trial cohort.

### Methods

After MMR-immunohistochemistry, *MLH1* promoter methylation testing, and next-generation sequencing, tumors were classified into 3 groups according to the molecular cause of their MMRd-EC. Kaplan-Meier method, log-rank test, and Cox model were used for survival analysis. Competing risk analysis was used to estimate the subsequent cancer probability. All statistical tests were 2-sided.

### Results

Among the 1336 ECs, 410 (30.7%) were MMRd. A total of 380 (92.7%) were fully triaged: 275 (72.4%) were *MLH1* hypermethylated MMRd-ECs; 36 (9.5%) LS MMRd-ECs, and 69 (18.2%) MMRd-ECs due to other causes. Limiting screening of EC patients to 60 years or younger or to 70 years or younger would have resulted in missing 18 (50.0%) and 6 (16.7%) LS diagnoses, respectively. Five-year recurrence-free survival was 91.7% (95% confidence interval [CI] 83.1% to 100%; hazard ratio 0.45, 95% CI 0.16 to 1.24;  $p=.12$ ) for LS, 95.5% (95% CI 90.7% to 100%; hazard ratio = 0.17, 95% CI 0.05 to 0.55;  $p=.003$ ) for “other” vs 78.6% (95% CI 73.8% to 83.7%) for *MLH1* hypermethylated MMRd-EC. The probability of subsequent LS-associated cancer at 10 years was 11.6% (95% CI 0.0% to 24.7%), 1.5% (95% CI 0.0% to 4.3%), and 7.0% (95% CI 3.0% to 10.9%) within the LS, “other,” and *MLH1* hypermethylated MMRd-EC groups, respectively.

### Conclusions

The LS prevalence in the Post Operative Radiation Therapy in Endometrial Carcinoma trial population was 2.8% and among MMRd-ECs was 9.5%. Patients with LS-associated ECs showed a trend towards better recurrence-free survival and higher risk for second cancers compared with patients with *MLH1* hypermethylated MMRd-EC.

## Introduction

The diagnosis of Lynch syndrome (LS) in endometrial cancer (EC) is crucial for counseling and cancer surveillance of patients and their relatives. LS is a highly penetrant, hereditary, cancer-prone syndrome caused by germline variants in the DNA mismatch repair (MMR) genes: mutL homologue 1 (*MLH1*), mutS homologue 2 (*MSH2*), mutS homologue 6 (*MSH6*), or postmeiotic segregation increased 2 (*PMS2*). The cancer risk varies per gene and is substantially lower for *PMS2*.<sup>1,2</sup> EC is often the first malignancy affecting women with LS,<sup>3</sup> and their risk of metachronous cancer is approximately 24% at 10 years.<sup>4</sup>

LS-associated cancers arise following MMR deficiency (MMRd) due to the somatic inactivation of the remaining wild-type MMR allele. MMRd leads to the accumulation of mismatches, insertions, and deletions in repeated sequences also known as microsatellite instability (MSI). MMRd is not an exclusive feature of LS; the vast majority (about 70%) of MMRd-ECs present with somatic inactivation of the *MLH1* gene via hypermethylation of the promoter region.<sup>5,6</sup> Most of the cases that are neither *MLH1* hypermethylated nor harbor a MMR germline variant are considered sporadic due to biallelic somatic *MMR* gene inactivation; few are caused by an undetectable hereditary syndrome (frequently referred to as Lynch-like syndrome).<sup>7-9</sup> MMRd-ECs are known to have an intermediate prognosis within the molecular classification with a good response to immunotherapy.<sup>10-13</sup> The diagnosis of LS may allow clinicians to tailor treatment and patient information; LS-associated tumors may have a more favorable outcome,<sup>14</sup> although there are no previous studies available on the prognostic impact of LS among MMRd-ECs.

Tumor triage by MMR-immunohistochemistry (IHC) and/or MSI analysis in combination with targeted *MLH1* methylation testing can identify patients with LS. The Proportion of Endometrial Tumours Associated Lynch Syndrome (PETALS) study showed that IHC-based triage is most accurate, whereas clinical selection based on age and family history were imprecise predictors.<sup>15</sup> Overall, an estimated 3% of EC cases are associated with LS,<sup>15-17</sup> which is similar in colorectal cancer (CRC).<sup>18</sup> However, these estimations were mostly based on small trials with methodological heterogeneity, often selecting their test population by age and/or family history, and incomplete testing.<sup>16</sup>

Given its relative rarity, the prevalence and prognosis of LS should be investigated in a large population, such as the well-documented combined cohort of the Post Operative Radiation Therapy in Endometrial Carcinoma (PORTEC)-1, -2, and -3 trials. These randomized controlled trials have had a major impact on guidelines for treatment in ECs.<sup>19-21</sup> Together they included 1336 evaluable patients comprising all risk groups with long and complete follow-up information and collected tumor blocks. The aim of our

study was to investigate the prevalence and prognosis of LS-associated EC in relation to *MLH1* hypermethylated MMRd-EC. Secondary objectives were to evaluate currently used age criteria for IHC-based tumor triage and the probability of developing a subsequent primary LS-associated cancer.

## Methods

### Study population

In total, 1336 of 1801 ECs from the PORTEC-1, -2, and -3 clinical trials were eligible for analysis based on availability of formalin-fixed paraffin-embedded (FFPE) slides. In the PORTEC-1 trial (1990-1997), 714 patients with stage I low-intermediate and high-intermediate risk EC were randomly assigned to receive pelvic radiotherapy or no additional treatment.<sup>19</sup> In the PORTEC-2 trial (2002-2006), 427 endometrioid EC patients with high-intermediate risk features were randomly assigned to receive pelvic radiotherapy or vaginal brachytherapy (if stage I:  $\geq 60$  years).<sup>20</sup> In the international PORTEC-3 trial (2006-2013), 660 EC patients with high-risk features were randomly assigned to receive pelvic radiotherapy or chemoradiotherapy.<sup>21</sup> In all trials, patients with a history of invasive cancer (for PORTEC-3 within the last 10 years), except for nonmelanoma skin cancer, were excluded. Full details and results of these trials have been published previously.<sup>19-21</sup> The study protocols were approved by the Dutch Cancer Society and the medical ethics committees at participating centers. All patients provided informed consent for participation in the trial, and for use of their tumor block for subsequent translational research. Clinicopathological data including p53-IHC and *POLE*-mutation status were obtained from the trial databases. Specific ethics approval was obtained for variant analysis on normal tissue among those suspected of LS. Cases from PORTEC-1 and -2 were analyzed anonymized in view of the long interval since recruitment. Cases from PORTEC-3 who were found to have LS were informed by their own physicians if LS had not been already diagnosed clinically. PORTEC-1 was conducted before time of trial registries. PORTEC-2 is registered with ISRCTN number ISRCTN16228756, and ClinicalTrials.gov number NCT00376844. PORTEC-3 is registered with ISRCTN number ISRCTN14387080, and ClinicalTrials.gov number NCT00411138.

### IHC, MSI, methylation analysis, and next-generation sequencing

Patients were included in the current analysis if they showed loss of expression of at least 1 of the 4 MMR proteins with positive internal control (including subclonal loss defined as abrupt and complete regional loss with intervening stromal positivity) or MSI-high status when MMR-IHC failed. Details on MMR-IHC and MSI testing and scoring were described previously.<sup>5, 11, 12, 22</sup> Cases with MMRd phenotype are referred to as MMRd-EC in this study irrespective of *POLE* mutation status.

*MLH1* methylation testing was performed on *MLH1*-deficient and/or MSI-high tumors as described previously.<sup>23</sup> All cases with loss of *MLH1* or MSI-high status without *MLH1* hypermethylation; loss of *MSH2* and/or *MSH6*; or isolated loss of *PMS2* were triaged as potential LS-associated MMRd-EC. DNA isolated from matched normal/tumor FFPE tissues of these cases was amplified using long-range polymerase chain reaction followed by targeted next-NGS for variants in the exonic regions of *MLH1*, *MSH2*, *MSH6*, *PMS2*, *POLE*, and *POLD1* using the Ion Proton System or Ion S5 System (Thermo Fisher Scientific, MA, USA).<sup>24, 25</sup> Variants were annotated according to the following GenBank reference sequences: NM\_000249.3 (*MLH1*), NM\_000251.2 (*MSH2*), NM\_000179.2 (*MSH6*), NM\_000535.5 (*PMS2*), NM\_006231.2 (*POLE*), and NM\_001256849.1 (*POLD1*). All patients with germline variants (likely) affecting function (*path\_MMR*) were verified by a clinical laboratory geneticist (C.M.T.) and considered to have LS.

### Statistical analysis

Following complete triage, cases were classified into 3 groups according to the molecular cause of their MMRd-EC: LS, methylated (including cases with *MLH1* hypermethylation and subclonal *MLH1* loss), and other causes (a mixed group having alternative causes of MMRd; see the Appendix Methods and Appendix Figure A1 for full definitions).  $\chi^2$  Statistics or Fisher's exact test for categorical variables and 1-way analysis of variance or Kruskal-Wallis test for continuous variables were used to compare characteristics. The sample size ensured sufficient power to detect an LS prevalence of 3.0% with a precision of 0.009 (95% confidence interval [CI] 2.1% to 3.9%) within the whole population and a prevalence of 12.0% with a precision of 0.03 (95% CI 9.0% to 15.0%) within the MMRd group.<sup>26</sup> Recurrence-free survival (RFS) was defined as time from random assignment to date of first relapse or death of any cause, whichever occurred first. Overall survival (OS) was defined as time from random assignment to date of death of any cause. Patients without an RFS or OS event were censored at the date of last contact. Five-year survival rates were estimated using the Kaplan-Meier method and compared with log-rank test. Cox proportional hazard models were used to estimate hazard ratios (HRs) over time; for adjusted analysis, age was included as covariate. The proportional hazard assumption was verified using Schoenfeld residuals. A competing-risk model with death as a competing event was used to estimate the cumulative incidence of developing a LS-associated second primary cancer (ie, colorectal, gallbladder, kidney, pancreas, small intestine, stomach, urinary bladder, and ureter cancer) in the different groups. A cause-specific Cox proportional hazard model was used to assess the statistical difference between the estimated probabilities. Time at risk started at random assignment and ended at date of occurrence of the first second cancer, death, or last date of study follow-up. *P* values less than .05 (2-tailed) were considered statistically significant. Statistical analyses were performed using R version 3.6.1.

Results

Study population

Among the 1336 evaluable ECs, 410 (30.7%) were MMRd and eligible for further analysis. Median age of MMRd-EC patients was 65 years (interquartile range = 59-73 years). Most MMRd-ECs were early-stage tumors (74.2%) of low-grade endometrioid subtype (66.8%) and were treated with pelvic radiotherapy (51.7%). All characteristics of MMRd-ECs differed between the 3 PORTEC trials, in line with the inclusion criteria (Table 1).

Table 1. Patient, tumor and treatment characteristics

|                                    | All MMRd-EC<br>n=410 (100.0) | PORTEC 1<br>n=145 (35.6) | PORTEC 2<br>n=114 (27.8) | PORTEC 3<br>n=151 (36.8) | p-value |
|------------------------------------|------------------------------|--------------------------|--------------------------|--------------------------|---------|
| <b>Age at randomization</b>        |                              |                          |                          |                          | <0.001  |
| <b>Median (IQR)</b>                | 65 (59-73)                   | 67 (61-73)               | 70 (65-77)               | 60 (56-66)               |         |
| <b>FIGO 2009 stage</b>             |                              |                          |                          |                          | <0.001  |
| IA                                 | 104 (25.4)                   | 62 (42.8)                | 25 (21.9)                | 17 (11.3)                |         |
| IB                                 | 200 (48.8)                   | 83 (57.2)                | 87 (76.3)                | 30 (19.9)                |         |
| II                                 | 36 (8.8)                     | 0 (0.0)                  | 1 (0.9)                  | 35 (23.2)                |         |
| III                                | 70 (17.1)                    | 0 (0.0)                  | 1 (0.9)                  | 69 (45.7)                |         |
| <b>Histological grade and type</b> |                              |                          |                          |                          | <0.001  |
| EEC grade 1/2                      | 274 (66.8)                   | 122 (84.1)               | 91 (79.8)                | 61 (40.4)                |         |
| EEC grade 3                        | 99 (24.1)                    | 22 (15.2)                | 21 (18.4)                | 56 (37.1)                |         |
| Serous                             | 11 (2.7)                     | 1 (0.7)                  | 2 (1.8)                  | 8 (5.3)                  |         |
| Clear Cell                         | 12 (2.9)                     | 0 (0.0)                  | 0 (0.0)                  | 12 (7.9)                 |         |
| Other                              | 14 (3.4)                     | 0 (0.0)                  | 0 (0.0)                  | 14 (9.3)                 |         |
| <b>Myometrial invasion</b>         |                              |                          |                          |                          | 0.001   |
| ≥50%                               | 274 (66.8)                   | 83 (57.2)                | 90 (78.9)                | 101 (66.9)               |         |
| <b>LVSI</b>                        |                              |                          |                          |                          | <0.001  |
| Present                            | 131 (32.0)                   | 13 (9.0)                 | 16 (14.0)                | 102 (67.5)               |         |
| <b>Received adjuvant treatment</b> |                              |                          |                          |                          | <0.001  |
| No treatment                       | 73 (17.8)                    | 71 (49.0)                | 2 (1.8)                  | 0 (0.0)                  |         |
| EBRT                               | 212 (51.7)                   | 74 (51.0)                | 58 (50.9)                | 80 (53.0)                |         |
| VBT                                | 54 (13.2)                    | 0 (0.0)                  | 54 (47.4)                | 0 (0.0)                  |         |
| CTRT                               | 71 (17.3)                    | 0 (0.0)                  | 0 (0.0)                  | 71 (47.0)                |         |

NOTE. Data reported as No. (%) unless otherwise indicated.

CTRT = combined adjuvant chemotherapy and radiotherapy; EBRT = external beam radiotherapy; EC = endometrial cancer; EEC = endometrioid endometrial cancer; FIGO = International Federation of Gynecology and Obstetrics; LVSI = lymphovascular space invasion; MMRd = mismatch repair deficient; PORTEC = Post Operative Radiation Therapy in Endometrial Carcinoma; VBT = vaginal brachytherapy.

MMR causes and variant analysis

Complete triage was accomplished for 380 (92.7%) of the MMRd-ECs (Figure 1; insufficient material in 27 cases for *MLH1* methylation assay and 3 for NGS). Thirty-six *path\_MMR* variant carriers were identified, giving a 2.8% LS prevalence in the overall population and a 9.5% LS prevalence within the MMRd group. There were 18 *path\_MSH6*, 10 *path\_PMS2*, 6 *path\_MSH2*, and 2 *path\_MLH1* variant carriers. An overview of the LS cases is displayed in Table 2. In total, 275 (72.4%) cases were classified as methylated. The remaining 69 (18.2%) MMRd cases were neither LS nor *MLH1* hypermethylated and were therefore classified as “other.”

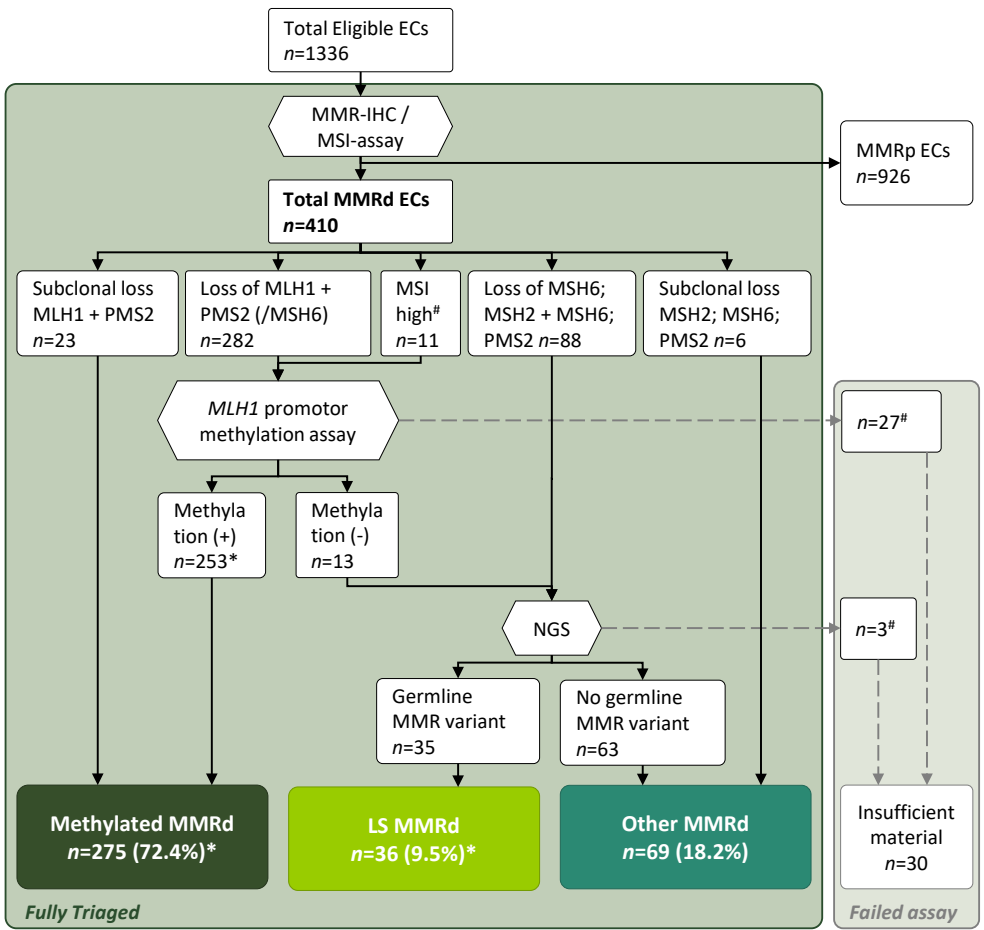


Figure 1. Flowchart

\*One case with *MLH1* promoter hypermethylation in the tumor carried a germline *MLH1* variant.  
# Insufficient material for assay; EC = endometrial cancer; LS = Lynch syndrome; Methylation (+) = *MLH1* promoter hypermethylation; Methylation (-) = no *MLH1* promoter hypermethylation; MMR = mismatch repair; MMRd = mismatch repair deficient; MMRp = mismatch repair proficient; MSI = microsatellite instability; NGS = next-generation sequencing.

Table 2. Patient and tumor characteristics of proven LS-associated endometrial cancers

| No. | Study    | Age | FIGO 2009 | Histotype    | Grade | Molecular Class by TCGA surrogate | Affected MMR proteins |
|-----|----------|-----|-----------|--------------|-------|-----------------------------------|-----------------------|
| 1   | PORTEC-1 | 47  | IB        | Endometrioid | G2    | MMRd                              | MSH2 + MSH6           |
| 2   | PORTEC-1 | 67  | IB        | Endometrioid | G1    | MMRd                              | MSH2 + MSH6           |
| 3   | PORTEC-1 | 52  | IA        | Endometrioid | G3    | MMRd                              | MSH2 + MSH6           |
| 4   | PORTEC-1 | 54  | IA        | Endometrioid | G1    | MMRd                              | MSH2 + MSH6           |
| 5   | PORTEC-3 | 48  | IIIC      | Endometrioid | G2    | MMRd                              | MSH2 + MSH6           |
| 6   | PORTEC-3 | 37  | IIIC      | Clear cell   | G2    | MMRd                              | MSH2 + MSH6           |
| 7   | PORTEC-3 | 59  | IA        | Clear cell   | G3    | MMRd                              | MSH2 + MSH6           |
| 8   | PORTEC-1 | 56  | IA        | Serous       | G3    | MMRd-p53abn                       | MSH2 subclonal + MSH6 |
| 9   | PORTEC-1 | 67  | IB        | Endometrioid | G1    | MMRd                              | MSH6                  |
| 10  | PORTEC-1 | 58  | IA        | Endometrioid | G1    | MMRd                              | MSH6                  |
| 11  | PORTEC-2 | 67  | IB        | Endometrioid | G1    | MMRd                              | MSH6                  |
| 12  | PORTEC-2 | 66  | IB        | Endometrioid | G3    | MMRd                              | MSH6                  |
| 13  | PORTEC-2 | 73  | IB        | Endometrioid | G1    | MMRd-p53abn                       | MSH6                  |
| 14  | PORTEC-2 | 82  | IIIA      | Endometrioid | G1    | MMRd                              | MSH6                  |
| 15  | PORTEC-2 | 71  | IB        | Endometrioid | G2    | MMRd-p53abn                       | MSH6                  |
| 16  | PORTEC-3 | 51  | IIIA      | Endometrioid | G1    | MMRd                              | MSH6                  |
| 17  | PORTEC-3 | 55  | IIIC      | Endometrioid | G3    | MMRd-p53abn                       | MSH6                  |
| 18  | PORTEC-3 | 61  | IB        | Clear cell   | G3    | MMRd                              | MSH6                  |
| 19  | PORTEC-3 | 68  | IIIA      | Endometrioid | G1    | MMRd                              | MSH6                  |
| 20  | PORTEC-3 | 59  | IB        | Serous       | G3    | MMRd-p53abn                       | MSH6                  |
| 21  | PORTEC-3 | 60  | IA        | Serous       | G3    | POLEmut-MMRd                      | MSH6                  |
| 22  | PORTEC-3 | 59  | IB        | Clear cell   | G3    | MMRd                              | MSH6                  |
| 23  | PORTEC-3 | 76  | IB        | Serous       | G3    | MMRd                              | MSH6                  |
| 24  | PORTEC-3 | 74  | IA        | Serous       | G3    | MMRd-p53abn                       | MSH6                  |
| 25  | PORTEC-1 | 57  | IB        | Endometrioid | G3    | MMRd                              | PMS2                  |
| 26  | PORTEC-1 | 66  | IB        | Endometrioid | G1    | MMRd                              | PMS2                  |
| 27  | PORTEC-1 | 64  | IB        | Endometrioid | G3    | MMRd-p53abn                       | PMS2                  |
| 28  | PORTEC-1 | 65  | IB        | Endometrioid | G1    | MMRd                              | PMS2                  |
| 29  | PORTEC-2 | 61  | IB        | Endometrioid | G1    | MMRd                              | PMS2                  |
| 30  | PORTEC-2 | 61  | IB        | Endometrioid | G3    | MMRd                              | PMS2                  |
| 31  | PORTEC-2 | 78  | IB        | Endometrioid | G1    | POLEmut-MMRd                      | PMS2                  |
| 32  | PORTEC-2 | 62  | IB        | Endometrioid | G2    | MMRd                              | PMS2                  |
| 33  | PORTEC-3 | 54  | IB        | Endometrioid | G3    | MMRd                              | PMS2                  |
| 34  | PORTEC-3 | 48  | II        | Endometrioid | G3    | MMRd                              | PMS2                  |

| MLH1 promoter methylation | Germline Variant  |                                        |                      | Class |
|---------------------------|-------------------|----------------------------------------|----------------------|-------|
| NA                        | MSH2              | c.1351C>T                              | p.(Gln451*)          | 5     |
| NA                        | MSH2              | c.363T>G                               | p.(Tyr121*)          | 5     |
| NA                        | MSH2              | c.646-2A>G                             | p.(?)                | 4     |
| NA                        | MSH2              | c.2458+1G>A                            | p.(?)                | 4     |
| NA                        | MSH2              | c.1285C>T                              | p.(Gln429*)          | 5     |
| NA                        | MSH2 <sup>a</sup> | NA                                     | NA                   | 5     |
| NA                        | MSH6              | c.3188T>G                              | p.(Ileu1063Arg)      | 5     |
| NA                        | MSH6              | c.1784delT                             | p.(Leu595Tyrfs*15)   | 5     |
| NA                        | MSH6              | c.1189_1190insTT                       | p.(Tyr397Phefs*15)   | 5     |
| NA                        | MSH6              | c.642C>A                               | p.(Tyr214*)          | 5     |
| NA                        | MSH6              | c.2764C>T                              | p.(Arg922*)          | 5     |
| NA                        | MSH6              | c.1483C>T                              | p.(Arg495*)          | 5     |
| NA                        | MSH6              | c.1628_1629delAA                       | p.(Lys543Argfs*19)   | 5     |
| NA                        | MSH6              | c.3729_3732dupATTA                     | p.(Phe1245Ilefs*31)  | 5     |
| NA                        | MSH6              | c.2719_2720delGT                       | p.(Val907Argfs*10)   | 5     |
| NA                        | MSH6              | c.3477C>A                              | p.(Tyr1159*)         | 5     |
| NA                        | MSH6              | c.2906_2907delAT                       | p.(Tyr969Leufs*5)    | 5     |
| NA                        | MSH6              | c.3838C>T                              | p.(Gln1280*)         | 5     |
| NA                        | MSH6              | c.467C>G                               | p.(Ser156*)          | 5     |
| NA                        | MSH6              | c.3527_3549delGACTTG GTGCCTCAGACAGAATA | p.(Arg1176Asnfs*4)   | 5     |
| NA                        | MSH6              | c.2342dupC                             | p.(Leu782Thrfs*3)    | 5     |
| NA                        | MSH6              | c.3863_3865dupAAT                      | p.(Phe1289*)         | 5     |
| NA                        | MSH6              | c.3847_3850dupATTA                     | p.(Thr1284Asnfs*6)   | 5     |
| NA                        | MSH6              | c.10C>T                                | p.(Gln4*)            | 4     |
| NA                        | PMS2              | c.1882C>T                              | p.(Arg628*)          | 5     |
| NA                        | PMS2              | c.1882C>T                              | p.(Arg628*)          | 5     |
| NA                        | PMS2              | c.247_250dupTTAA                       | p.(Thr84Ilefs*9)     | 5     |
| NA                        | PMS2              | c.1261C>T                              | p.(Arg421*)          | 5     |
| NA                        | PMS2              | c.904_911delGTCTGCAG                   | p.(Val302Thrfs*4)    | 5     |
| NA                        | PMS2              | c.1831dupA                             | p.(Ile611Asnfs*2)    | 5     |
| NA                        | PMS2              | c.1882C>T                              | p.(Arg628*)          | 5     |
| NA                        | PMS2              | c.904_911delGTCTGCAG                   | p.(Val302Thrfs*4)    | 5     |
| NA                        | PMS2              | c.137G>T                               | p.(Ser46Ile)         | 5     |
| NA                        | PMS2              | c.989-2A>G                             | p.(Glu330_Glu381del) | 4     |

**Table 2.** Patient and tumor characteristics of proven LS-associated endometrial cancers (continued)

| No. | Study    | Age | FIGO 2009 | Histotype    | Grade | Molecular Class by TCGA surrogate | Affected MMR proteins |
|-----|----------|-----|-----------|--------------|-------|-----------------------------------|-----------------------|
| 35  | PORTEC-3 | 52  | IIIC      | Endometrioid | G2    | Not classified                    | MLH1 + PMS2           |
| 36  | PORTEC-1 | 48  | IB        | Endometrioid | G1    | MMRd                              | MSI-high <sup>b</sup> |

NOTE. Classification according to the 5-tiered InSiGHT rules: class 5 is pathogenic and class 4 is likely pathogenic.

<sup>a</sup>Loss-of-function variant in *MSH2* gene identified by genetic testing (clinical data) but insufficient material for normal tissue next-generation sequencing.

<sup>b</sup>No material for MLH1 and PMS2 IHC

G = grade; NA = not available; MMRd = mismatch repair deficient; p53abn = p53 abnormal; *POLE*mut = *POLE*-ultramutated; PORTEC = Post Operative Radiation Therapy in Endometrial Carcinoma; TCGA = The Cancer Genome Atlas.

LS patients were younger, with a median age of 60years (interquartile range = 54-67 years) and more often had p53 aberrant staining (20.0%) and serous (13.9%) or clear cell (8.3%) histology compared with the patients with methylated MMRd-EC (Table 3). Limiting screening of EC patients to age 50 years or younger, 60 years or younger, and 70 years or younger would have missed 31 (86.1%), 18 (50.0%), and 6 (16.7%) LS diagnoses, respectively. Figure 2 displays the distribution of the involved MMR proteins; all LS cases identified by the 4-panel approach would also have been identified by a 2-panel approach including only PMS2- and MSH6-IHC. No germline *POLE/POLD1* variants affecting function were identified. LS patients with *path\_MSH6* and *path\_PMS2* variants were older than those with *path\_MLH1* and *path\_MSH2* variants (median age = 63, 62, 50, and 50 years, respectively;  $p = .01$ ; Appendix Table A1).

**Survival**

The estimated RFS for the MMRd population at 5 years was 83.7% (95% CI 80.1% to 87.4%): 91.7% (95% CI 83.1% to 100%) for patients with LS-associated MMRd-EC, 78.6% (95% CI 73.8% to 83.7%) for patients with methylated MMRd-EC, and 95.5% (95% CI 90.7% to 100%) for patients with other causes of MMRd-EC ( $p = .001$ ; Figure 3A; LS vs methylated: HR 0.45, 95% CI 0.16 to 1.24,  $p = .12$ ; other vs methylated: HR 0.17, 95% CI 0.05 to 0.55,  $p = .003$ ).

| MLH1 promoter methylation | Germline Variant            | Class |
|---------------------------|-----------------------------|-------|
| Methylated                | MLH1 c.794G>C p.(Arg265Pro) | 4     |
| Unmethylated              | MLH1 c.806C>G p.(Ser269*)   | 5     |

| MMR protein expression |                   |                   |      | Triaged MMRd-EC              |     |                |           |        |
|------------------------|-------------------|-------------------|------|------------------------------|-----|----------------|-----------|--------|
| MLH1                   | PMS2 <sup>a</sup> | MSH6 <sup>a</sup> | MSH2 | All MMRd-EC No. <sup>b</sup> | %   | Methylated No. | Other No. | LS No. |
| R                      | R                 | CL                | R    | 32                           | 8%  | 0              | 16        | 16     |
| R                      | CL                | R                 | R    | 15                           | 4%  | 0              | 5         | 10     |
| R                      | R                 | CL                | CL   | 33                           | 8%  | 0              | 25        | 7      |
| R                      | R                 | CL                | SL   | 3                            | 1%  | 0              | 1         | 1      |
| CL                     | CL                | R                 | R    | 254                          | 62% | 226            | 8         | 1      |
| SL                     | CL                | R                 | R    | 6                            | 2%  | 5              | 1         | 0      |
| CL                     | CL                | SL                | R    | 11                           | 3%  | 9              | 1         | 0      |
| UK                     | CL                | R                 | R    | 5                            | 1%  | 2              | 0         | 0      |
| CL                     | R                 | R                 | R    | 4                            | 1%  | 1              | 0         | 0      |
| CL                     | SL                | R                 | R    | 1                            | 0%  | 1              | 0         | 0      |
| CL                     | CL                | CL                | R    | 1                            | 0%  | 1              | 0         | 0      |
| SL                     | SL                | CL                | CL   | 2                            | 1%  | 0              | 2         | 0      |
| R                      | R                 | R                 | CL   | 1                            | 0%  | 0              | 1         | 0      |
| SL                     | CL                | SL                | R    | 1                            | 0%  | 0              | 1         | 0      |
| R                      | CL                | CL                | CL   | 1                            | 0%  | 0              | 0         | 0      |
| SL                     | SL                | R                 | R    | 22                           | 5%  | 22             | 0         | 0      |
| R                      | R                 | SL                | SL   | 4                            | 1%  | 0              | 4         | 0      |
| R                      | SL                | R                 | R    | 1                            | 0%  | 0              | 1         | 0      |
| R                      | R                 | R                 | SL   | 1                            | 0%  | 0              | 1         | 0      |
| SL                     | R                 | R                 | R    | 1                            | 0%  | 1              | 0         | 0      |
| UK                     | UK                | UK                | UK   | 11                           | 3%  | 7              | 2         | 1      |

CL

 Complete loss

SL

 Subclonal loss

R

 Retained

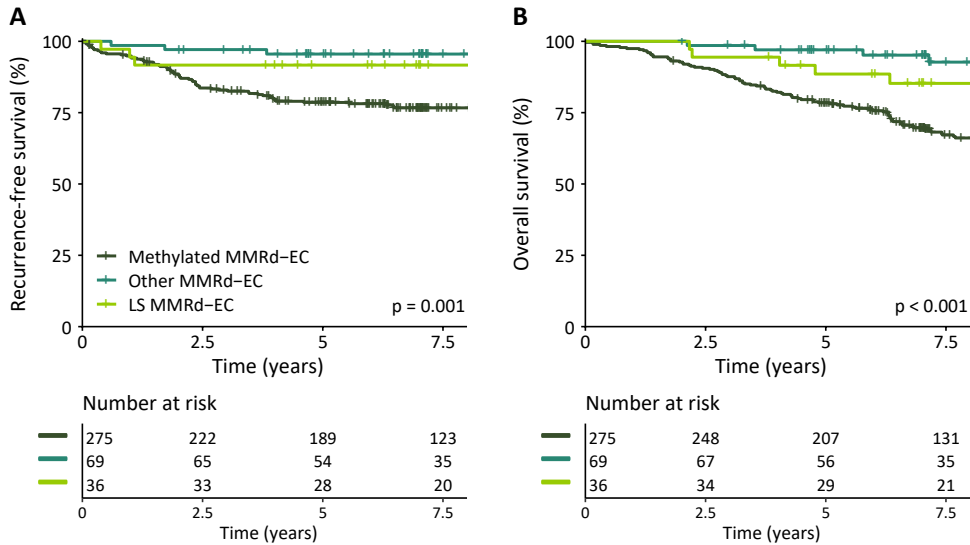
UK

 Unknown

**Figure 2.** Details on the mismatch repair (MMR) protein expression according to the molecular cause of their MMR-deficient endometrial cancer (MMRd-EC).

MMR protein expression was scored as following: complete loss (CL), retained (R), subclonal loss (SL), unknown/failed (UK). <sup>a</sup>The concordance of these 2 columns shows that a 2-antibody (MSH6 and PMS2) panel is as sensitive as the full panel to detect Lynch syndrome (LS). <sup>b</sup>All MMRd-ECs including those with insufficient material for *MLH1* methylation assay ( $n = 27$ ) and next-generation sequencing ( $n = 3$ ).

The estimated OS for the MMRd population at 5 years was 82.8% (95% CI 79.2% to 86.5%): 88.5% (95% CI 78.5% to 99.8%) for patients with LS-associated MMRd-EC, 78.5% (95% CI 73.7% to 83.5%) for patients with methylated MMRd-EC, and 97.0% (95% CI 93.0% to 100%) for patients with other causes of MMRd-EC ( $p < .001$ ; Figure 3B; LS vs methylated: HR 0.50, 95% CI 0.24 to 1.02,  $p = .06$ ; other vs methylated: HR 0.27, 95% CI 0.13 to 0.55,  $p < .001$ ). After adjustment for age, the trend for better OS in the LS group was no longer observed (vs methylated MMRd-EC: HR 0.73, 95% CI 0.35 to 1.52,  $p = .40$ ), whereas age and having another cause of MMRd were statistically significant prognostic factors (HR 1.07, 95% CI 1.04 to 1.09,  $p < .001$ ; other vs methylated MMRd-EC: HR 0.41, 95% CI 0.20 to 0.85,  $p = .02$ ).



**Figure 3.** Kaplan-Meier survival curves for recurrence-free survival (A) and overall survival (B) for patients with methylated mismatch repair deficient (MMRd), other MMRd and Lynch syndrome (LS) associated MMRd endometrial cancer (EC) including cases with a concurrent *POLE* variant affecting function (*POLE*mut-MMRd-EC). P values reflect 2-sided log-rank test.

### Second primary cancers

At 10 years, the cumulative incidence of developing a second LS-associated tumor was 11.6% (95% CI 0.0% to 24.7%) among EC patients with LS, 1.5% (95% CI 0.0% to 4.3%) among patients with other MMRd-EC, and 7.0% (95% CI 3.0% to 10.9%) among patients with methylated MMRd-EC (Appendix Figure A2). Three of the 4 LS-patients who developed a second primary LS-associated cancer had colon cancer (after 3.8, 4.8, and 14.9 years) and 1 had ureteral cancer (after 8.0 years; Appendix Table A2, shows cancer type distribution). The cause-specific hazard ratio for developing an LS-associated second cancer was 1.9 (95% CI 0.63 to 5.7;  $p = .26$ ) for patients with LS vs patients with methylated MMRd-EC.

**Table 3.** Characteristics according to the molecular cause of their MMRd-EC.

|                                    | All MMRd-EC<br>n=410* | Methylated<br>n=275 (72.4) | Other<br>n=69 (18.2) | LS<br>n=36 (9.5) | p-value |
|------------------------------------|-----------------------|----------------------------|----------------------|------------------|---------|
| <b>Age at randomization</b>        |                       |                            |                      |                  | <0.001  |
| Median (IQR)                       | 65 (59-73)            | 67 (62-74)                 | 59 (55-66)           | 60 (54-67)       |         |
| <b>Trial</b>                       |                       |                            |                      |                  | 0.002   |
| PORTEC-1                           | 145 (35.4)            | 99 (36.0)                  | 22 (31.9)            | 12 (33.3)        |         |
| PORTEC-2                           | 114 (27.8)            | 87 (31.6)                  | 8 (11.6)             | 9 (25.0)         |         |
| PORTEC-3                           | 151 (36.8)            | 89 (32.4)                  | 39 (56.5)            | 15 (41.7)        |         |
| <b>FIGO 2009 stage</b>             |                       |                            |                      |                  | 0.199   |
| IA                                 | 104 (25.4)            | 70 (25.5)                  | 17 (24.6)            | 7 (19.4)         |         |
| IB                                 | 200 (48.8)            | 137 (49.8)                 | 27 (39.1)            | 21 (58.3)        |         |
| II                                 | 36 (8.8)              | 22 (8.0)                   | 11 (15.9)            | 1 (2.8)          |         |
| III                                | 70 (17.1)             | 46 (16.7)                  | 14 (20.3)            | 7 (19.4)         |         |
| <b>Histological grade and type</b> |                       |                            |                      |                  | <0.001  |
| EEC grade 1/2                      | 274 (66.8)            | 197 (71.6)                 | 40 (58.0)            | 19 (52.8)        |         |
| EEC grade 3                        | 99 (24.1)             | 64 (23.3)                  | 18 (26.1)            | 8 (22.2)         |         |
| Serous                             | 11 (2.7)              | 2 (0.7)                    | 4 (5.8)              | 5 (13.9)         |         |
| Clear Cell                         | 12 (2.9)              | 2 (0.7)                    | 6 (8.7)              | 3 (8.3)          |         |
| Other                              | 14 (3.4)              | 10 (3.6)                   | 1 (1.4)              | 1 (2.8)          |         |
| <b>Myometrial invasion</b>         |                       |                            |                      |                  | 0.407   |
| >50%                               | 274 (66.8)            | 187 (68.0)                 | 43 (62.3)            | 27 (75.0)        |         |
| <b>LVSI</b>                        |                       |                            |                      |                  | 0.957   |
| Present                            | 131 (32.0)            | 90 (32.7)                  | 23 (33.3)            | 11 (30.6)        |         |
| <b>POLEmut in tumour</b>           |                       |                            |                      |                  | 0.002   |
| EDM                                | 19 (4.7)              | 8 (2.9)                    | 9 (13.4)             | 2 (5.7)          |         |
| <b>p53 IHC</b>                     |                       |                            |                      |                  | <0.001  |
| Aberrant                           | 31 (7.7)              | 7 (2.6)                    | 14 (20.9)            | 7 (20.0)         |         |
| <b>Received adjuvant treatment</b> |                       |                            |                      |                  | 0.104   |
| No treatment                       | 73 (17.8)             | 47 (17.1)                  | 10 (14.5)            | 9 (25.0)         |         |
| EBRT                               | 212 (51.7)            | 145 (52.7)                 | 40 (58.0)            | 13 (36.1)        |         |
| VBT                                | 54 (13.2)             | 39 (14.2)                  | 3 (4.3)              | 6 (16.7)         |         |
| CTRT                               | 71 (17.3)             | 44 (16.0)                  | 16 (23.2)            | 8 (22.2)         |         |

NOTE. Data reported as No. (%) unless otherwise indicated.

CTRT = combined adjuvant chemotherapy and radiotherapy; EBRT = external beam radiotherapy; EC = endometrial cancer; EDM = exonuclease domain mutations; EEC = endometrioid endometrial cancer; FIGO = International Federation of Gynecology and Obstetrics; IHC = immunohistochemistry; LS = Lynch syndrome; LVSI = lymphovascular space invasion; MMRd = mismatch repair deficient; *POLE*mut = *POLE*-ultramutated; PORTEC = Post Operative Radiation Therapy in Endometrial Carcinoma; VBT = vaginal brachytherapy.

\*All MMRd-ECs including those with insufficient material for *MLH1* methylation assay ( $n = 27$ ) and normal tissue NGS ( $n = 3$ ).

## Discussion

After complete IHC-based tumor triage, we found a 2.8% prevalence of LS in 1 of the largest EC trial populations worldwide, comprising all risk groups with long and complete follow-up. The prevalence of LS among patients with MMRd-EC was 9.5%. Patients with LS were relatively young, but restricted testing to women who are 60 years or younger would have missed one-half of the cases. Patients with LS tend to have a better RFS and a higher risk of developing second primary cancers compared with patients with methylated MMRd-ECs. No trend for more favorable OS was found after adjustment for age.

This is the first study to our knowledge investigating the prognostic value of LS within the MMRd-EC subgroup. Most of the recent research showed that MMRd-ECs, predominantly driven by the large number of *MLH1* hypermethylated cases, have an intermediate prognosis within the molecular classification introduced by The Cancer Genome Atlas.<sup>10-12</sup> Our survival analysis showed that EC patients with LS tend to have a better RFS than patients with methylated MMRd-EC (HR 0.45;  $p = .12$ ), whereas LS had no statistically significant prognostic value for OS after adjustment for age (age-adjusted HR 0.73;  $p = .40$ ). The favorable prognosis has been assumed to be induced by the active local immune response.<sup>14, 27</sup> Comparable survival analysis in CRC has been published. One study showing a better OS for 85 CRC patients with LS compared with 67 sporadic MMRd patients after adjustment for age, stage, and *BRAF* status (HR 0.29, 95% CI 0.09 to 0.95;  $p = .04$ ).<sup>28</sup> The other study also showing better OS in 37 CRC patients with LS compared with 106 methylated MMRd patients, although the difference was minimal after adjusting for age and stage.<sup>29</sup>

The cumulative incidence for developing a second LS-associated cancer at 10 years was 11.6% (95% CI 0.0% to 24.7%) for patients with LS vs 7.0% (95% CI 3.0% to 10.9%) for patients with methylated MMRd-EC (HR 1.90, 95% CI 0.63 to 5.7;  $p = .26$ ). Our analysis was underpowered due to the small number of events in the LS group. Nevertheless, the elevated risk strengthens previous reports on subsequent cancers in EC or non-CRC LS patients (15%-24%)<sup>4, 30</sup> and is of importance for surveillance strategies.

The 2.8% prevalence of LS-EC is consistent with previous publications in which prevalences of 2.8% to 3.2% were reported.<sup>15-17</sup> This prevalence is likely a slight underestimation. Firstly, our NGS panel did not include *EPCAM* and could not detect large rearrangements. To detect large rearrangement in *EPCAM* or the MMR genes, Multiplex Ligation-dependent Probe Amplification is most commonly used but performs poorly on FFPE tissue. Secondly, the patient selection in our trial design may have affected the prevalence. Patients younger than 60 years with stage I ECs were excluded from the PORTEC-2 trial. Nevertheless, the

total PORTEC population deviates minimally from the general EC population as suggested by the similar age in the PETALS study, an unselected, prospective, cross-sectional study in the United Kingdom among 500 EC patients.<sup>15</sup> Moreover, patients with a history of cancer were excluded from the PORTEC trials. The PORTEC population represents women with EC as their sentinel LS-associated malignancy, which is the case in more than one-half of those women with LS who develop cancer.<sup>3</sup> Although this selection has potentially led to a slight underestimation of the prevalence of LS in EC, it does represent the patients in which LS could be detected by IHC-based tumor triage. The recently published meta-analysis by Ryan et al.<sup>16</sup> included mostly small trials with methodological heterogeneity, often selecting their test population by age and/or family history, and incomplete testing; only 1 publication included over 1000 ECs, but germline testing was limited to the minority of the triaged potential LS cases.<sup>6</sup> Our study is the first with LS testing in an EC population consisting of more than 1000 women with almost complete MMR-IHC, targeted *MLH1* methylation testing, and MMR germline testing, making our estimates more reliable.

The *path\_MSH6* carrier rate of 50.0% among the PORTEC patients with LS is consistent with LS testing results in other unselected EC populations,<sup>15, 17</sup> but it is remarkably high compared with LS registry data. Only 13% of *path\_MMR* carriers in the clinically selected Prospective Lynch Syndrome Database bear *path\_MSH6*.<sup>1</sup> As mentioned above, our cohort represents patients with EC as their sentinel cancer likely to induce a lower frequency of *path\_MLH1* and *path\_MSH2*. Moreover, it must be considered that most of our participants were Dutch, and the *path\_MSH6* rate of 30% among the Dutch LS registry patients was relatively high compared with the overall Prospective Lynch Syndrome Database.<sup>31</sup> Lastly, *path\_MSH6* families are not identified efficiently by current clinical criteria for LS<sup>32</sup> due to the later age of onset of CRC, incomplete penetrance, and a higher risk and later age of onset of EC.<sup>1, 33-35</sup> The same applies to *path\_PMS2* carriers with a substantially lower cancer risk.<sup>1, 2, 15, 16</sup> Correspondingly, the *path\_MSH6* and *path\_PMS2* carriers were older than the *path\_MLH1* and *path\_MSH2* carriers in our population.

Triage of incident ECs based on IHC with targeted *MLH1* methylation testing, as has been adopted widely for CRC, may be a more effective strategy to identify these LS families than age- and family history-based triage. An upper age screening limit would not be recommended, because limiting screening to EC patients who are aged 70 years or younger would have missed 6 (16.7%) LS diagnoses. We confirmed that a 2-antibody panel including MSH6- and PMS2-IHC, with MSH2- or MLH1-IHC only in case of inconclusive staining, is as sensitive as the full panel to detect LS, so this could be a reliable alternative to improve cost-effectiveness.<sup>5, 36</sup>

A limitation of our study was the lack of germline LS sequencing on the whole study population. Therefore, sensitivity of the IHC-based triage to identify LS patients could not be assessed. Some patients with LS might have been diagnosed before entering the trial, although many were diagnosed after inclusion and had no prior knowledge of the germline mutation.

The diagnosis of LS in EC is crucial for counseling and cancer surveillance even though these patients might be older than those presenting with CRC.<sup>18</sup> Moreover, LS screening in incident ECs will have consequences for the patient's family. Cascade testing of at-risk relatives can identify *path\_MMR* carriers who can benefit from cancer surveillance and risk-reducing treatment.<sup>37, 38</sup> The clinical impact depends on the gene-specific cancer risk, which is substantially lower for *path\_PMS2* carriers.<sup>1, 2</sup> Finally, LS identification may have consequences by allowing clinicians to better estimate and explain prognosis, and to potentially tailor treatment in the upcoming immunotherapy era.<sup>14, 27, 39</sup>

Further research into the causes of the 63 cases with neither *MLH1* hypermethylation nor a *MMR* germline variant is ongoing. It is hypothesized that the majority will be explained by a sporadic origin through biallelic somatic MMR inactivation.<sup>15, 40</sup> The determination of a sporadic explanation excludes potential undetectable LS (or 'Lynch-like' syndrome) and will avoid a clinical management dilemma in those cases.

In conclusion, Lynch syndrome was identified using MMR-IHC with targeted *MLH1* methylation-based triage in 2.8% of 1336 patients with EC from the combined PORTEC-1, -2, and -3 trials, corresponding to 9.5% of the MMRd tumors. LS was mainly caused by germline variants in the *MSH6* and *PMS2* genes. Patients with LS-associated ECs showed a trend towards better RFS and higher risk for second primary cancers compared with patients with ECs caused by *MLH1* hypermethylation. Besides a prognostic impact, screening all incident ECs without an upper age limit to identify LS using tumor-based triage may benefit counseling, affect treatment decisions, and facilitate prevention strategies for current and future patients and their families.

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## APPENDIX

### Supplementary Methods

#### Definitions

**MMRd-EC** = All EC with loss of expression of one or more MMR proteins and positive internal control irrespective of *POLE*- and MSI-status. In case MSI testing was performed, but no MMR-IHC, all EC with MSI-high status. In case not all four MMR proteins could be stained: All EC with loss of expression of at least one MMR protein or MSI-high status.

**MMRp-EC** = All EC with retained expression of all four MMR proteins. In case MSI testing is performed, but no MMR-IHC, all EC with MSI-low or MSS status.

**Suspected of Lynch syndrome/Potential LS-associated MMRd-EC** = All MMRd-EC with loss of MLH1 expression without hypermethylation of the *MLH1* promotor; loss of MSH2 and/or MSH6 expression or isolated loss of PMS2 expression. In case MSI testing was performed, but no MMR-IHC, all MMRd-EC with MSI-high status without hypermethylation of the *MLH1* promotor. In case not all four MMR proteins could be stained: MMRd-EC with loss of MSH2, MSH6 and/or PMS2 with retained MLH1 expression, or loss of MLH1 expression or MSI-high if MLH1-IHC is not available without *MLH1* promotor hypermethylation.

**LS-associated MMRd-EC** = MMRd-EC with a germline variant (likely) affecting function corresponding with MMR protein loss. Class 4 or 5 according to InSiGHT Variant Classification.

**MMRd caused by *MLH1* promotor hypermethylation** = All MMRd-EC with loss of MLH1 expression and proven *MLH1* promotor hypermethylation by methylation specific PCR. In case MSI testing was performed, but no MMR-IHC, all EC with MSI-high status and proven *MLH1* promotor hypermethylation. In case not all four MMR proteins could be stained: MMRd-EC with loss of PMS2 and/or MLH1 expression and proven *MLH1* promotor hypermethylation. Also including cases with subclonal loss of MLH1 and total loss of PMS2 expression with *MLH1* promotor hypermethylation.

**Subclonal loss of MMR expression** = Subclonal loss ( $\geq 10\%$ ) of one or more MMR proteins (NB excluding cases with complete loss of expression of another MMR protein, than the complete loss of another MMR protein is leading in group allocation).

**Methylated MMRd-EC** = All EC with MMRd caused by *MLH1* promotor hypermethylation and subclonal loss of MLH1 expression.

**MMRd-EC with other causes** = MMRd-EC with neither a MMR germline variant affecting function in DNA isolated from normal tissue nor promotor hypermethylation of *MLH1* in the tumor. A mixed group having alternative causes of MMRd. It is hypothesised that the majority will be explained by sporadic origin through biallelic somatic MMR inactivation (i.e. variants affecting function or loss of heterozygosity [LOH]), and few cases may have an undetectable hereditary syndrome (frequently referred to as 'Lynch-like syndrome' in literature).

**MMRd-EC with unknown *MLH1* methylation status** = All MMRd-EC with loss of MLH1 expression and insufficient material for *MLH1* promotor methylation assay.

**Complete triage/Fully triaged** = All identified MMRd-EC with successful *MLH1* promotor methylation assay and next-generation sequencing when indicated.

| Definition                                                                                                                  | Depending on performed MMR/MSI test(s)                                  |                    |                                                                                            |
|-----------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------|--------------------|--------------------------------------------------------------------------------------------|
|                                                                                                                             | MMR-IHC + MSI test<br>or only MMR-IHC                                   | Only MSI-test      | MMR-IHC of <4 proteins<br>performed + MSI-test                                             |
| MMRd                                                                                                                        | Loss of ≥1 MMR<br>proteins                                              | MSI-high           | Loss of ≥1 MMR proteins or MSI<br>high                                                     |
| MMRp                                                                                                                        | All retained<br>expression                                              | MSI low or MSS     | all IHC retained expression + MSI<br>low or MSS                                            |
| Suspected of LS /<br>Potential LS-associated                                                                                | MSH2 and/or MSH6<br>loss, PMS2 loss<br>MLH1 loss                        | MSI-high           | MSH2, MSH6 or PMS2 loss with<br>retained MLH1<br>MLH1 loss or MSI-high if MLH1-<br>IHC N/A |
| Methylated MMRd-EC<br>Included subgroups:<br>1. <i>MLH1</i> methylated;<br>2. Subclonal MLH1<br>loss;                       | 1 MLH1 loss<br>2 Subclonal MLH1 loss <sup>a</sup>                       | MSI-high           | PMS2 and/or MLH1 loss or MSI-<br>high if MLH1-IHC N/A                                      |
| LS-associated EC                                                                                                            | Suspected of LS;<br>Corresponding to<br>NGS                             |                    | Suspected of LS;<br>Corresponding to NGS                                                   |
| MMRd-EC with other<br>cause<br>Included subgroups:<br>1. Explained somatic<br>2 (a/b). Unexplained                          | Suspected of LS or<br>Subclonal MSH2,<br>MSH6 or PMS2 loss <sup>a</sup> | Suspected<br>of LS | Suspected of LS                                                                            |
| Failed cases<br>Included subgroups:<br>1. Unknown <i>MLH1</i><br>methylation status<br>2. Suspected of LS but<br>failed NGS | 1 MLH1 loss<br>2 Suspected of LS                                        |                    | PMS2 and/or MLH1 loss<br><br>Suspected of LS                                               |

**Figure A1.** Definitions

Definition depend on the combination of the results of the MMR/MSI test (choose one of the three green columns based on the available test results; MMR-IHC [dark green column] is preferable when available), *MLH1* methylation status (blue) and NGS (orange) in the corresponding row.

<sup>a</sup> NB excluding cases with complete loss of another MMR protein; the complete loss of another MMR protein is leading in group allocation.

EC = endometrial cancer; IHC = immunohistochemistry; LS = Lynch syndrome; MMRd = mismatch repair deficient; MMRp = mismatch repair proficient; MSI = microsatellite instability; N/A = not available; NGS = next-generation sequencing

| <i>MLH1</i> methylation status | NGS                                                                                                                                                                                                                                                                                                       |
|--------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|                                |                                                                                                                                                                                                                                                                                                           |
|                                |                                                                                                                                                                                                                                                                                                           |
|                                |                                                                                                                                                                                                                                                                                                           |
| Unmethylated                   |                                                                                                                                                                                                                                                                                                           |
| Hypermethylated                |                                                                                                                                                                                                                                                                                                           |
|                                | Pathogenic mutation in normal (and tumor) tissue                                                                                                                                                                                                                                                          |
|                                | 1. Double somatic mutations in tumor without pathogenic<br>mutation in normal tissue (Ongoing research)<br>2a. No pathogenic mutation in normal tissue found and<br>no double somatic alteration in tumor (Ongoing research)<br>2b. No pathogenic mutation in normal tissue found and<br>tumor NGS failed |
| Failed                         | Normal tissue NGS failed                                                                                                                                                                                                                                                                                  |

**Table A1.** Patient, tumor and treatment characteristics of patients with proven MMR germline variant according to affected gene

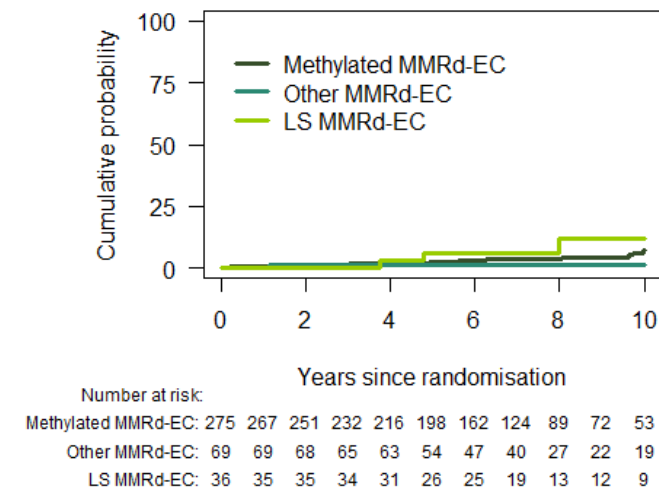
|                                    | MLH1<br>n=2 | PMS2<br>n=10 | MSH2<br>n=6 | MSH6<br>n=18 | p-value |
|------------------------------------|-------------|--------------|-------------|--------------|---------|
| <b>Age at randomization</b>        |             |              |             |              | 0.011   |
| Median (IQR), y                    | 50 (49-51)  | 62 (58-65)   | 50 (47-54)  | 63 (59-70)   |         |
| <b>Trial</b>                       |             |              |             |              | 0.182   |
| PORTEC-1                           | 1 (50.0)    | 4 (40.0)     | 4 (66.7)    | 3 (16.7)     |         |
| PORTEC-2                           | 0 (0.0)     | 4 (40.0)     | 0 (0.0)     | 5 (27.8)     |         |
| PORTEC-3                           | 1 (50.0)    | 2 (20.0)     | 2 (33.3)    | 10 (55.6)    |         |
| <b>FIGO 2009 stage</b>             |             |              |             |              | 0.195   |
| IA                                 | 0 (0.0)     | 0 (0.0)      | 2 (33.3)    | 5 (27.8)     |         |
| IB                                 | 1 (50.0)    | 9 (90.0)     | 2 (33.3)    | 9 (50.0)     |         |
| II                                 | 0 (0.0)     | 1 (10.0)     | 0 (0.0)     | 0 (0.0)      |         |
| III                                | 1 (50.0)    | 0 (0.0)      | 2 (33.3)    | 4 (22.2)     |         |
| <b>Histological grade and type</b> |             |              |             |              | 0.302   |
| EEC grade 1/2                      | 2 (100.0)   | 5 (50.0)     | 4 (66.7)    | 8 (44.4)     |         |
| EEC grade 3                        | 0 (0.0)     | 5 (50.0)     | 1 (16.7)    | 2 (11.1)     |         |
| Serous                             | 0 (0.0)     | 0 (0.0)      | 0 (0.0)     | 5 (27.8)     |         |
| Clear cell                         | 0 (0.0)     | 0 (0.0)      | 1 (16.7)    | 2 (11.1)     |         |
| Other                              | 0 (0.0)     | 0 (0.0)      | 0 (0.0)     | 1 (5.6)      |         |
| <b>Myometrial invasion</b>         |             |              |             |              | 0.18    |
| >50%                               | 1 (50.0)    | 10 (100.0)   | 4 (66.7)    | 12 (66.7)    |         |
| <b>LVSI</b>                        |             |              |             |              | 0.569   |
| Present                            | 0 (0.0)     | 2 (20.0)     | 2 (33.3)    | 7 (38.9)     |         |
| <b>POLEmut in tumour</b>           |             |              |             |              | 0.858   |
| EDM                                | 0 (0.0)     | 1 (10.0)     | 0 (0.0)     | 1 (5.6)      |         |
| <b>p53 IHC</b>                     |             |              |             |              | 0.224   |
| Aberrant                           | 0 (0.0)     | 1 (10.0)     | 0 (0.0)     | 6 (33.3)     |         |
| <b>Received Adjuvant Treatment</b> |             |              |             |              | 0.127   |
| No treatment                       | 1 (50.0)    | 3 (30.0)     | 4 (66.7)    | 1 (5.6)      |         |
| EBRT                               | 1 (50.0)    | 4 (40.0)     | 0 (0.0)     | 8 (44.4)     |         |
| VBT                                | 0 (0.0)     | 1 (10.0)     | 0 (0.0)     | 5 (27.8)     |         |
| CTRT                               | 0 (0.0)     | 2 (20.0)     | 2 (33.3)    | 4 (22.2)     |         |

NOTE. Data reported as No. (%) unless otherwise indicated.

CTRT = combined adjuvant chemotherapy and radiotherapy; EBRT = external beam radiotherapy; EC = endometrial cancer; EDM = exonuclease domain mutations; EEC = endometrioid endometrial cancer; FIGO = International Federation of Gynecology and Obstetrics; LVSI = lymphovascular space invasion; MMRd = mismatch repair; *POLE*mut = *POLE*-ultramutated; PORTEC = Post Operative Radiation Therapy in Endometrial Carcinoma; VBT = vaginal brachytherapy.

**Table A2.** Distribution of the Lynch syndrome associated second primary cancer types

|                                     | Methylated      | Other          | Lynch           |
|-------------------------------------|-----------------|----------------|-----------------|
| <b>2nd Primary cancers, No. (%)</b> | <b>15 (5.5)</b> | <b>2 (2.9)</b> | <b>4 (11.1)</b> |
| <b>Type, No. (%)</b>                | <b>N</b>        | <b>N</b>       | <b>N</b>        |
| Colon                               | 5 (33.3)        | 2 (100)        | 3 (75.0)        |
| Gallbladder                         | 1 (6.7)         | 0 (0)          | 0 (0)           |
| Kidney                              | 1 (6.7)         | 0 (0)          | 0 (0)           |
| Pancreas                            | 2 (13.3)        | 0 (0)          | 0 (0)           |
| Rectosigmoid                        | 1 (6.7)         | 0 (0)          | 0 (0)           |
| Rectum                              | 2 (13.3)        | 0 (0)          | 0 (0)           |
| Stomach, excl. cardia               | 2 (13.3)        | 0 (0)          | 0 (0)           |
| Urinary bladder                     | 1 (6.7)         | 0 (0)          | 0 (0)           |
| Ureter                              | 0 (0)           | 0 (0)          | 1 (25.0)        |

**Figure A2.** Cumulative incidence of developing a subsequent Lynch syndrome associated primary cancer after a primary endometrial cancer.