



Universiteit  
Leiden  
The Netherlands

## **Molecular alterations in endometrial cancer: implications for clinical management**

Stelloo, E.

### **Citation**

Stelloo, E. (2017, April 20). *Molecular alterations in endometrial cancer: implications for clinical management*. Retrieved from <https://hdl.handle.net/1887/48189>

Version: Not Applicable (or Unknown)

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/48189>

**Note:** To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/48189> holds various files of this Leiden University dissertation.

**Author:** Stelloo, E.

**Title:** Molecular alterations in endometrial cancer: implications for clinical management

**Issue Date:** 2017-04-20

# Chapter 6

## Practical guidance for mismatch repair-deficiency testing in endometrial cancer

Stelloo E, Jansen AM, Osse EM, Nout RA, Creutzberg CL, Ruano D, Church DN, Morreau H, Smit VT, van Wezel T, Bosse T

*Annals of Oncology*. 2016

## Abstract

**Background:** Mismatch repair (MMR)-deficiency analysis is increasingly recommended for all endometrial cancers, as it identifies Lynch syndrome-patients, and is emerging as a prognostic classifier to guide adjuvant treatment. The aim of this study was to define the optimal approach for MMR-deficiency testing and to clarify discrepancies between microsatellite instability (MSI) analysis and immunohistochemical (IHC) analysis of MMR protein expression.

**Patients and Methods:** 696 endometrial cancers were analyzed for MSI (pentaplex panel) and MMR protein expression (IHC). Agreement between methodologies was calculated using Cohen's Kappa. *MLH1* promoter hypermethylation, dinucleotide microsatellite markers and somatic MMR and *POLE* exonuclease domain (EDM) gene variants (using next-generation/Sanger sequencing) were analyzed in discordant cases.

**Results:** MSI was found in 180 patients. Complete loss of expression of one or more MMR proteins was observed in 196 cases. A PMS2- and MSH6-antibody panel detected all cases with loss of MMR protein expression. The results of MSI and MMR protein expression were concordant in 655/696 cases ( $\kappa=0.854$ ,  $P<0.001$ ). Ambiguous cases ( $n=41$ , 6%) included: subclonal loss of MMR protein expression ( $n=18$ ), microsatellite stable or MSI-low cases with loss of MMR protein expression ( $n=20$ ), and MSI-low or MSI-high cases with retained MMR protein expression ( $n=3$ ). Most of these cases could be explained by *MLH1* promoter hypermethylation. Five of seven cases with solitary loss of PMS2 or MSH6 protein expression carried somatic gene variants. Two MSI-high cases with retained MMR protein expression carried a *POLE*-EDM variant.

**Conclusion:** MSI and IHC analysis are highly concordant in endometrial cancer. This holds true for cases with subclonal loss of MMR protein expression. Discordant MMR-proficient/MSI-high cases (<1%), may be explained by *POLE*-EDM variants.

## Introduction

A defect in DNA mismatch repair (MMR) leads to the accumulation of mismatches, insertions and deletions in repeated sequences - a phenomenon named microsatellite instability (MSI). Approximately 20-30% of sporadic endometrial cancers (ECs) display MSI as a consequence of somatic promoter hypermethylation and silencing of *MLH1*.<sup>1</sup> Defective MMR due to pathogenic germline variants in MMR genes causes Lynch syndrome (LS), a tumor predisposition syndrome that accounts for 2% of ECs.<sup>2</sup>

Determination of MMR-deficiency in EC may be important for several reasons. First, recent studies have suggested that tumor molecular features, including MMR-deficiency, may improve prognostication and help guide adjuvant therapy for EC patients.<sup>3,4</sup> Second, accurate assessment of MMR-deficiency is essential to identify patients with EC caused by LS. However in contrast to colorectal cancer, where consensus guidelines for MMR-deficiency testing have been published,<sup>5</sup> there is no general agreement on screening EC patients for LS.<sup>6,7</sup> Finally, recent studies have shown that MMR-deficiency in colorectal and urothelial cancer is predictive of response to immunotherapy,<sup>8,9</sup> suggesting that MMR-deficient ECs may also benefit from these therapeutics.

MMR-deficiency can be detected by either MSI analysis and/or immunohistochemical (IHC) staining, typically for four MMR proteins. The National Cancer Institute microsatellite panel was optimized and correlated with IHC analysis (~95%) to detect MMR-deficiency in colorectal cancer.<sup>10,11</sup> IHC alone has become standard practice in multiple institutions. Experience in this setting is that while some tumors show uniform and widespread loss of MMR protein expression, cases with subclonal loss of MMR protein expression are also observed.<sup>11,12</sup> Such cases present with two populations of tumor cells; one with retained expression, and another with abrupt and complete regional loss of MMR protein expression.<sup>12</sup> Small studies have shown high agreement between MSI and loss of MMR protein expression in EC,<sup>13-15</sup> while others have described subclonal loss of MMR protein expression.<sup>16-20</sup> However, studies identifying the frequency of such staining patterns in large patient series are sparse.

In this study, we sought to establish the optimum method for MMR-deficiency testing by comparison of MSI with IHC analysis in a large series of ECs. We also investigated the frequency of subclonal loss of MMR protein expression and the number of potential LS cases. Cases showing disagreement between methodologies and those with subclonal loss of MMR protein expression were further characterized.

## Methods

### *Study population*

The population comprised, 854 ECs from the PORTEC-1 and -2 clinical trials based on availability of formalin-fixed paraffin-embedded slides and sufficient tumor material for DNA isolation.<sup>21,22</sup> Further details are summarized in Supplementary Table S1 and Supplementary methods.

### *MSI assay*

DNA was isolated as previously described.<sup>23</sup> In cases with subclonal loss of MMR protein expression, tissue sections were used to microdissect the differentially expressed tumor areas. Tumor MSI status was determined as previously reported (Supplementary methods).<sup>4</sup> Tumors initially classified as MSS or MSI-L with concomitant loss of MMR protein expression underwent evaluation of three dinucleotide repeat markers,<sup>24</sup> and reclassified as MSI-H if instability was detected at two dinucleotide markers.

### *IHC analysis*

IHC staining for MLH1, PMS2, MSH2, and MSH6 was performed on all tumors in which MSI status was successfully determined (Supplementary methods). The slides were evaluated in three categories as retained, loss and subclonal loss of protein expression with stromal- and/or lymphocytic cells as internal controls.<sup>16</sup> The cases with subclonal loss of protein expression were re-evaluated to determine the percentage of tumor cells with loss of MMR expression.

### *Methylation-specific PCR for MLH1*

Tumors with loss of MLH1 protein expression underwent testing for hypermethylation status of the *MLH1* 5' regulatory region by methylation-specific PCR, as previously described.<sup>25</sup>

### *Somatic variant screening*

Subject to DNA availability and quality, tumors in which the results of MSI analysis and MMR protein expression were discordant underwent targeted next generation sequencing (NGS) of MMR and *POLE* genes using the Ion Proton™ System (ThermoFisher, MA, USA) as previously described (Supplementary methods).<sup>26</sup> Three additional cases were similarly analyzed using the Ion AmpliSeq Comprehensive Cancer Panel (ThermoFisher) at The Wellcome Trust Center for Human Genetics. Frameshift variants in the polycytosine tract in exon 5 of *MSH6* were analyzed using Sanger sequencing.<sup>27</sup>

## Results

Combined analysis of MMR protein expression and MSI was possible in 696 (81%) ECs (Supplementary Table S1). The frequencies of MSS, MSI-H and MSI-L were 74%, 24% and 2%, respectively. Among the 516 tumors assessed as MSS, 496 (96%) showed retained expression of all four MMR proteins (Table 1). The remaining twenty MSS cases showed loss of MMR protein expression as follows: combined MLH1 and PMS2 loss (n=14), combined MSH2 and MSH6 loss (n=3), solitary MSH6 loss (n=3, Figure 1A-D). Of the 11 cases assessed as MSI-L, six displayed combined loss of MLH1 and PMS2 expression (complete in four cases, subclonal in two cases), two cases showed solitary loss of PMS2, a further two cases had solitary MSH6 loss and one case retained expression of all four MMR proteins.

Table 1. Details on the MSI status and MMR protein expression in early-stage EC (n=696).

MSI status	MMR protein expression				protein expression	Count
	MLH1	PMS2	MSH6	MSH2		
MSS	1	1	1	1	Retained	496
MSS	2	2	1	1	Subclonal loss	6
MSS	1	1	2	2	Subclonal loss	2
MSS	0	0	1	1	Loss	8
MSS	1	1	0	1	Loss	3
MSS	1	1	0	0	Loss	1
MSI-L	1	1	1	1	Retained	1
MSI-L	2	2	1	1	Subclonal loss	2
MSI-L	0	0	1	1	Loss	4
MSI-L	1	0	1	1	Loss	2
MSI-L	1	1	0	1	Loss	2
MSI-H	1	1	1	1	Retained	2
MSI-H	2	2	1	1	Subclonal loss	8
MSI-H	0	0	2	1	Loss/Subclonal loss	6
MSI-H	0	0	1	1	Loss	130
MSI-H	1	1	0	0	Loss	10
MSI-H	1	0	1	1	Loss	8
MSI-H	1	1	0	1	Loss	5

Mismatch repair protein expression was scored as following: 0 – Complete loss; 1 – Retained; 2 -Subclonal loss. MMR=mismatch repair, MSS=microsatellite stable, MSI-L/H=microsatellite unstable with low- or high-frequency.

The majority of MSI-H cases (130 of 169, 77%) showed complete loss of MLH1 and PMS2 expression (Table 1). Sporadic MSI due to *MLH1* hypermethylation was observed in 97% of these 130 MSI-H cases. Eight MSI-H cases showed areas of subclonal loss of MLH1 and PMS2 (Figure 1E-H), and six cases displayed subclonal loss of MSH6 in addition to complete loss of MLH1 and PMS2 protein expression (Figure 1I-L). In ten cases (6%), combined loss of MSH2 and MSH6 protein expression was observed. The remaining MSI-H tumors showed solitary loss of PMS2 (n=8) or MSH6 (n=5) protein expression, or retained expression of all MMR proteins (n=2).

Table 2. Details on ECs with subclonal loss of MMR protein expression.

Case	Subclonal loss of protein expression	% tumor with loss of expression	MSI status <sup>a</sup>	MSI status <sup>b</sup>			No. of affected dinucleotides <sup>c</sup>	MLH1 hypermethylation <sup>d</sup>	gene variant
				Area with retained expression	Area with focal loss of expression	Area with retained expression			
1	MLH1, PMS2	90%	MSI-H	-	MSI-H	-	yes	-	
2	MLH1, PMS2	90%	MSI-H	-	MSI-H	-	yes	-	
3	MLH1, PMS2	75%	MSI-H	MSS	MSI-H	-	yes	-	
4	MLH1, PMS2	75%	MSI-H	MSS	MSI-H	-	yes	-	
5	MLH1, PMS2	75%	MSI-H	MSS	MSI-H	-	yes	-	
6	MLH1, PMS2	75%	MSI-H	MSS	MSI-H	-	yes	-	
7	MLH1, PMS2	75%	MSI-H	MSS	MSI-H	-	yes	-	
8	MLH1, PMS2	25-50%	MSI-H	MSS	MSI-H	-	yes	-	
9	MLH1, PMS2	75%	MSI-L	MSS	MSI-H	-	yes	-	
10	MLH1, PMS2	25-50%	MSI-L	MSS	MSI-H	-	yes	-	
11	MLH1, PMS2	>75%	MSS	MSS	MSI-H	-	yes	-	
12	MLH1, PMS2	25-50%	MSS	MSS	MSI-H	-	yes	-	
13	MLH1, PMS2	25-50%	MSS	MSS	MSS	0	yes	-	
14	MLH1, PMS2	25-50%	MSS	MSS	MSS	Failed	yes	-	
15	MLH1, PMS2	10%	MSS	MSS	MSS	0	Failed	None	
16	MLH1, PMS2	10%	MSS	MSS	MSI-H	-	Failed	None	
17	MSH2, MSH6	25-50%	MSS	MSS	MSS	-	-	-	
18	MSH2, MSH6	10%	MSS	MSS	MSS	-	-	-	

<sup>a</sup>first round of MSI analysis, <sup>b</sup>second round of MSI analysis in differently expressed areas (3 cases had too small areas) <sup>c</sup>'instability' in dinucleotide markers was only analyzed in discordant cases after the second round of MSI analysis <sup>d</sup>MLH1 promoter hypermethylation was only assessed in cases with loss of/subclonal loss of MLH1 protein expression. Grey colored lines are unexplained/failed cases. MSS=microsatellite stable, MSI-L/H=microsatellite unstable with low- or high-frequency.

Table 3. Details on ECs with discordant MSI status and MMR protein expression.

Case	Protein expression	MSI status	No. of affected dinucleotides <sup>a</sup>	MLH1 hypermethylation <sup>b</sup>	Somatic variants <sup>c</sup>	Variant allele frequency	Classification of pathogenicity <sup>d</sup>
19	retained	MSI-H	-	-	<i>POLE</i> p.(V411L), <i>PMS2</i> p.(R107W), p.(R563*)	0.36, 0.37, 0.42	-, class 3, class 5
20	retained	MSI-H	-	no	<i>POLE</i> p.(A428T)	0.46	-
21	retained	MSI-L	0	-	None	-	-
22	MLH1-PMS2 loss	MSI-L	0	yes	-	-	-
23	MLH1-PMS2 loss	MSI-L	0	yes	-	-	-
24	MLH1-PMS2 loss	MSI-L	2	yes	-	-	-
25	MLH1-PMS2 loss	MSI-L	Failed	yes	-	-	-
26	MLH1-PMS2 loss	MSS	Failed	no	<i>POLE</i> p.(P286R)	0.69	-
27	MLH1-PMS2 loss	MSS	0	Failed	None	-	-
28	MLH1-PMS2 loss	MSS	0	yes	-	-	-
29	MLH1-PMS2 loss	MSS	Failed	yes	-	-	-
30	MLH1-PMS2 loss	MSS	0	yes	-	-	-
31	MLH1-PMS2 loss	MSS	0	yes	-	-	-
32	MLH1-PMS2 loss	MSS	0	yes	-	-	-
33	MLH1-PMS2 loss	MSS	0	yes	-	-	-
34	PMS2 loss	MSI-L	3	-	<i>PMS2</i> p.(M1?), p.(V302I)(fs*4)	0.40, 0.27	class 3, class 5
35	PMS2 loss	MSI-L	Failed	-	-	-	-
36	MSH6 loss	MSI-L	0	-	<i>MSH6</i> p.(R495*), p.(N975K)(fs*10)	0.35, 0.41	class 5, class 5
37	MSH6 loss	MSI-L	0	-	<i>MSH6</i> p.(R922*)	0.47	class 5
38	MSH6 loss	MSS	0	-	<i>MSH6</i> p.(S445N)(fs*7)	0.44	class 5
39	MSH6 loss	MSS	0	-	<i>MSH6</i> p.(Q415H)	0.43	class 3
40	MSH6 loss	MSS	Failed	-	-	-	-
41	MSH2-MSH6 loss	MSS	Failed	-	-	-	-

<sup>a</sup>instability in dinucleotide markers was only analyzed in discordant MSI-L and MSS cases <sup>b</sup>MLH1 promoter hypermethylation was only assessed in cases with loss/focal loss of MLH1 protein expression <sup>c</sup>Mismatch repair gene variants were analyzed in those cases without MLH1 promoter hypermethylation <sup>d</sup>classification of pathogenicity was based upon InSiGHT guidelines. Class 3 variants are predicted to be pathogenic by at least 2 out of 3 protein prediction programs used: (Align GVGD, SIFT and MutationTaster). Grey colored lines are unexplained/failed cases. MSS=microsatellite stable, MSI-L/H=microsatellite unstable with low- or high-frequency.

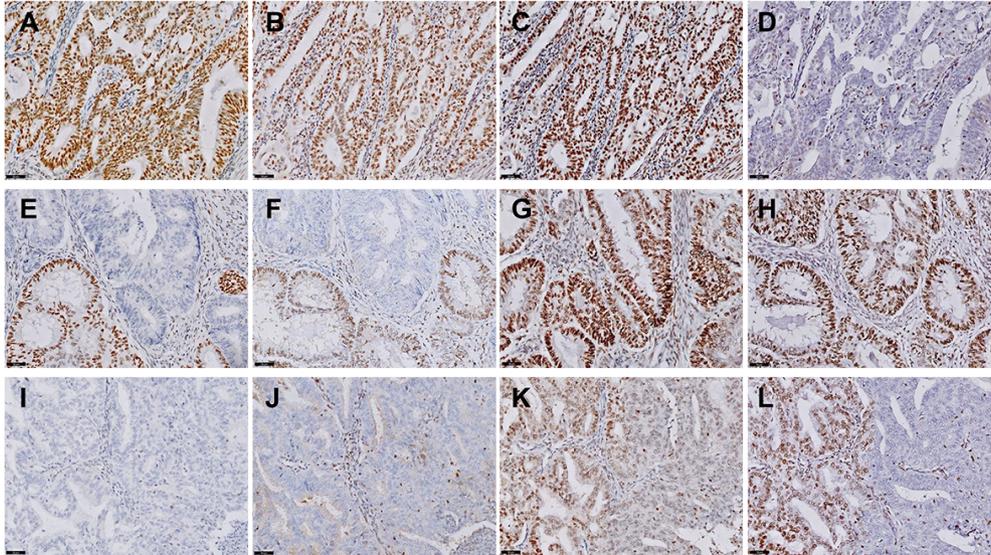


Figure 1. Representative images of MMR protein expression in EC. MMR protein expression of a MSS case with subclonal loss of *MLH1* and *PMS2* protein expression (A-D, case 13), a MSI-H case with complete loss of *MLH1* and *PMS2* and subclonal loss of *MSH6* protein expression (E-H), a MSS case with loss of *MSH6* protein expression (I-L, case 38). A-E-I) *MLH1* protein expression, B-F-J) *PMS2* protein expression, C-G-K) *MSH2* protein expression and D-H-L) *MSH6* protein expression. Scale bar represents 50  $\mu$ m.

Overall, concordance between MSI and IHC analysis was observed in 655 of 696 cases (94%,  $\kappa=0.854$ ; 95%CI 0.811-0.897,  $P<0.001$ ). A *PMS2*- and *MSH6*-antibody panel was as effective as the four-antibody panel in detecting MMR protein abnormalities. Twenty-seven concordant cases without *MLH1* promoter hypermethylation were identified as potential LS, but the underlying defect was not further tested. Discordant cases ( $n=41$ , 6%) included: subclonal loss of MMR expression ( $n=18$ ), MSS or MSI-low cases with loss of MMR expression ( $n=20$ ), and MSI-low or MSI-high cases with retained MMR protein expression ( $n=3$ ). Details on the sample analysis of discordant cases are shown in Supplementary Figure S1.

All cases with subclonal loss of MMR protein expression ( $n=18$ ) were evaluated in more detail by analyzing MSI in mono- and dinucleotide markers, *MLH1* promoter hypermethylation and somatic MMR- and *POLE*-exonuclease domain (EDM) variants in microdissected tumor areas (Table 2). Among 16 tumors with subclonal *MLH1* and *PMS2* loss, 14 had areas of differential expression that were sufficiently large to permit microdissection. Among these, MSI testing of microdissected areas was concordant with IHC analysis in 11 cases; tumor areas with retained MMR expression were MSS, whereas areas with loss of MMR expression showed MSI-H. A further three tumors showed microsatellite stability of markers in microdissected areas regardless of MMR protein expression (case 13-15, Table 2). All fourteen cases were found to have somatic promoter hypermethylation of *MLH1*. One case with subclonal loss of *MSH2* and *MSH6* protein expression showed microsatellite stability in the differently expressed areas

(case 17, Table 2). Unfortunately, both cases with subclonal loss of MSH2 and MSH6 protein expression had limited DNA available, and could not be analyzed in more detail.

Analysis of microdissected material from the six MSI-H cases with subclonal loss of MSH6 in addition to complete MLH1 and PMS2 protein loss demonstrated frameshift variants in the polycytosine tract of *MSH6* in areas with MSH6 loss and stable polycytosine tracts in areas with retained MSH6. Five of these cases displayed *MLH1* promoter hypermethylation.

We proceeded to perform detailed analysis of the 23 cases with discordant MSI status and MMR protein expression by examination of dinucleotide markers, *MLH1* promoter hypermethylation and/or NGS of the MMR- and *POLE* genes (Table 3). The two MSI-H cases (case 19-20) with retained MMR protein expression had a *POLE*-EDM variant, p.(V411L), and p.(A428T). The *POLE*-EDM p.(V411L) mutant case also harbored a truncating (p.(R563\*)) and missense p.(R107W) variant in *PMS2*. The solitary MSI-L case with retained MMR protein expression (case 21) showed stability in dinucleotide markers and no somatic MMR or *POLE*-EDM gene variants.

One of 12 cases classified as MSS or MSI-L despite combined loss of MLH1 and PMS2 protein expression, showed mobility shifts in the dinucleotide markers (case 24, Table 3). Analysis of the *MLH1* promoter was successful in 11 of these cases, and revealed promoter hypermethylation in ten cases, while the single case lacking *MLH1* promoter hypermethylation was found to harbor a pathogenic *POLE*-EDM variant, p.(P286R).

Of the two MSI-L tumors with solitary PMS2 loss (case 34-35, Table 3), only one had sufficient DNA quality for further analysis. This confirmed MSI in the dinucleotide markers and revealed two likely pathogenic somatic *PMS2* variants, a start loss (p.(Met1?)) and a frameshift variant (p.(Val302Thrfs\*4)).

Four of five cases classified as MSS/MSI-L with solitary MSH6 loss were informative for further analysis. All four showed microsatellite stable dinucleotide markers. Three tumors carried two (n=1) or one (n=2) pathogenic *MSH6* variants, while one tumor carried one somatic VUS predicted to affect function by two out of three protein prediction software used (Table 3). Case 41 with loss of MSH2 and MSH6 protein expression and a MSS phenotype had limited DNA, and was therefore excluded for further analysis.

## Discussion

Accurate identification of MMR-deficiency in EC may be important to identify patients with a higher risk of recurrence,<sup>3,4,28</sup> and those whose tumors may be a consequence of LS. Similarly

to two small studies, we demonstrated high agreement (94%) between MSI and IHC analysis in 696 ECs.<sup>13,14</sup> Most discordant cases involved loss of MMR protein expression and a MSS/MSI-L phenotype and could be explained by *MLH1* promoter hypermethylation or MMR variants. In addition, subclonal loss of MMR protein expression generally corresponded to *MLH1* promoter hypermethylation and subclonal MSI within microdissected area of the tumor.

Importantly, the present study demonstrated that <3% of cases displayed subclonal loss of MMR protein expression. The fact that MSI and *MLH1* promoter hypermethylation were commonly found in areas with subclonal loss of *MLH1* and *PMS2* protein expression indicates sporadic intratumor heterogeneity.<sup>16,18,19</sup> However, *MLH1* germline epimutations cannot be totally excluded.<sup>2</sup> Subclonal loss of *MSH6* expression, either in conjunction with or without *MSH2* protein expression was also previously observed in EC but the underlying molecular mechanisms remain unclear.<sup>12,20</sup> In accordance with our findings, subclonal loss of *MSH6* in cases with complete loss of *MLH1* and *PMS2* protein expression has been related to secondary MSI events in *MSH6*.<sup>20,29</sup> Although numbers are limited, subclonal loss of MMR protein expression is not associated with LS.

Our data suggest that cases with subclonal loss of MMR protein expression are best classified as MMR-deficient, even though the areas with retained expression are MSS. With regard to MMR-deficiency as a prognostic or predictive marker, it remains to be determined whether subclonal loss of MMR protein expression has the same biological behavior as tumors with MMR-proficiency. In view of the limited numbers of cases with subclonal loss of MMR protein expression (~3%), (inter)national collaborations are essential to obtain sufficient cases for such an analysis. Pending these future studies, we suggest for uniformity to classify tumors with 10% subclonal loss of MMR protein expression, as being MMR-deficient.

The interpretation of MSI-L cases remains controversial in EC and it is uncertain whether such cases are best considered as MSS or MSI. Similar numbers of DNA slippage events were observed in MSS and MSI-L ECs.<sup>30</sup> To date, no extensive research on the clinical implications of MSI-L in ECs has been performed, and the number of MSI-L cases in our study (n=11) was too low to permit such an analysis as well. However, most of these showed loss of MMR protein expression and would generally be regarded as abnormal by strategies that rely on IHC alone. Noteworthy, several studies have also shown MSI-L and MSS in association with loss of MMR expression and/or pathogenic germline MMR variants.<sup>13,14,31</sup>

Our study shows high agreement between IHC and MSI analysis, but not 100%. Of note, other assessments of DNA defects by IHC analysis e.g. *HER2* gene amplification only reaches 69-98% agreement.<sup>32</sup> Assessment of MMR protein expression is preferred over MSI analysis for the following reasons: lower costs, widely available, and determination of affected MMR gene.

Our findings confirm the utility of testing MMR-deficiency using a PMS2- and MSH6-IHC approach,<sup>33</sup> which can be followed by MLH1- and MSH2-IHC in case loss of PMS2 or MSH6 was observed. To overcome suboptimal fixation, drawback of IHC analysis, pathologists can rely on IHC analysis in pre-operative EC specimen.<sup>23</sup> IHC with standard well accepted techniques would appear adequate to identify EC patients with LS and to serve as a biomarker for trials of EC patients harboring MMR-deficiency.

It is debatable whether not screening for germline MMR variants is a limitation of this study. Of note, 5% of all cases in this study can be classified as potential LS (no *MLH1* promoter hypermethylation). Somatic screening of the discordant cases did show somatic variants but not in all cases. However, we cannot exclude the possibility of missed large genomic rearrangements within the tested genes, which is a limitation of NGS. Further analysis would improve understanding the molecular basis of the discordant cases, however, this study did not aim to determine the sensitivity and specificity of the two methodologies to identify LS. MSI and IHC analysis are highly concordant therefore germline testing is not needed to conclude which approach is best suitable for identifying patients with LS.

In conclusion, MSI and IHC analysis are highly concordant, also in cases with subclonal loss of MMR expression, therefore, an IHC approach is sufficient for determining MMR-deficiency in EC. Pathologists should be aware of the MMR protein expression patterns, including subclonal loss, to ensure correct classification in daily diagnostic pathology.

## Funding

This study was supported by the Dutch Cancer Society (KWF-UL2012-5719 and KWF-UL2012-5542).

## Acknowledgements

We acknowledge all members of the PORTEC study group and the many patients who participated in the trials, and M. Ventayol Garcia from the Leiden University Medical Center for excellent technical support.

## References

1. Cancer Genome Atlas Research N, Kandoth C, Schultz N et al. Integrated genomic characterization of endometrial carcinoma. *Nature*. 2013; 497: 67-73.
2. Lynch HT, Snyder CL, Shaw TG et al. Milestones of Lynch syndrome: 1895-2015. *Nature Reviews Cancer*. 2015; 15: 181-194.
3. Talhouk A, McConechy MK, Leung S et al. A clinically applicable molecular-based classification for endometrial cancers. *British Journal of Cancer*. 2015; 113: 299-310.

4. Stelloo E, Nout RA, Osse EM et al. Improved risk assessment by integrating molecular and clinicopathological factors in early-stage endometrial cancer - combined analysis of PORTEC cohorts. *Clinical cancer research: an official journal of the American Association for Cancer Research*. 2016.
5. Umar A, Boland CR, Terdiman JP et al. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. *Journal of the National Cancer Institute*. 2004; 96: 261-268.
6. Oncoline. Guidelines for hereditary nonpolyposis colorectal cancer.
7. American College of Obstetricians and Gynecologists. Lynch Syndrome Practice Bulletin. 2014; 147.
8. Le DT, Uram JN, Wang H et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. *The New England journal of medicine*. 2015; 372: 2509-2520.
9. Castro MP, Goldstein N. Mismatch repair deficiency associated with complete remission to combination programmed cell death ligand immune therapy in a patient with sporadic urothelial carcinoma: immunotherapeutic considerations. *Journal for immunotherapy of cancer*. 2015; 3: 58.
10. Shia J. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part I. The utility of immunohistochemistry. *Journal of Molecular Diagnostics*. 2008; 10: 293-300.
11. Chapusot C, Martin L, Puig PL et al. What is the best way to assess microsatellite instability status in colorectal cancer? Study on a population base of 462 colorectal cancers. *The American journal of surgical pathology*. 2004; 28: 1553-1559.
12. Joost P, Veurink N, Holck S et al. Heterogenous mismatch-repair status in colorectal cancer. *Diagnostic pathology*. 2014; 9: 126.
13. Bartley AN, Luthra R, Saraiya DS et al. Identification of cancer patients with Lynch syndrome: clinically significant discordances and problems in tissue-based mismatch repair testing. *Cancer Prevention Research*. 2012; 5: 320-327.
14. McConechy MK, Talhouk A, Li-Chang HH et al. Detection of DNA mismatch repair (MMR) deficiencies by immunohistochemistry can effectively diagnose the microsatellite instability (MSI) phenotype in endometrial carcinomas. *Gynecologic oncology* 2015; 137: 306-310.
15. de Leeuw WJ, Dierssen J, Vasen HF et al. Prediction of a mismatch repair gene defect by microsatellite instability and immunohistochemical analysis in endometrial tumours from HNPCC patients. *The Journal of pathology*. 2000; 192: 328-335.
16. Watkins JC, Nucci MR, Ritterhouse LL et al. Unusual Mismatch Repair Immunohistochemical Patterns in Endometrial Carcinoma. *The American journal of surgical pathology*. 2016.
17. Lassen Ring K, Bruegl AS, Batte BAL et al. A prospective evaluation of universal tumor testing strategies for Lynch syndrome in endometrial cancer. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*; 32: Abstract 5512.
18. Kato A, Sato N, Sugawara T et al. Isolated Loss of PMS2 Immunohistochemical Expression is Frequently Caused by Heterogenous MLH1 Promoter Hypermethylation in Lynch Syndrome Screening for Endometrial Cancer Patients. *The American journal of surgical pathology*. 2016.
19. Pai RK, Plessec TP, Abdul-Karim FW et al. Abrupt loss of MLH1 and PMS2 expression in endometrial carcinoma: molecular and morphologic analysis of 6 cases. *The American journal of surgical pathology*. 2015; 39: 993-999.
20. Graham RP, Kerr SE, Butz ML et al. Heterogenous MSH6 loss is a result of microsatellite instability within MSH6 and occurs in sporadic and hereditary colorectal and endometrial carcinomas. *The American journal of surgical pathology*. 2015; 39: 1370-1376.
21. Creutzberg CL, van Putten WL, Koper PC et al. Surgery and postoperative radiotherapy versus surgery alone for patients with stage-1 endometrial carcinoma: multicentre randomised trial. PORTEC Study Group. *Post Operative Radiation Therapy in Endometrial Carcinoma*. *Lancet*. 2000; 355: 1404-1411.
22. Nout RA, Smit VT, Putter H et al. Vaginal brachytherapy versus pelvic external beam radiotherapy for patients with endometrial cancer of high-intermediate risk (PORTEC-2): an open-label, non-inferiority, randomised trial. *Lancet*. 2010; 375: 816-823.
23. Stelloo E, Nout RA, Naves LC et al. High concordance of molecular tumor alterations between pre-operative curettage and hysterectomy specimens in patients with endometrial carcinoma. *Gynecologic oncology*. 2014; 133: 197-204.
24. Loukola A, Eklin K, Laiho P et al. Microsatellite Marker Analysis in Screening for Hereditary Nonpolyposis Colorectal Cancer (HNPCC). *Cancer Research*. 2001; 61: 4545-4549.
25. Bosse T, ter Haar NT, Seeber LM et al. Loss of ARID1A expression and its relationship with PI3K-Akt pathway alterations, TP53 and microsatellite instability in endometrial cancer. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc*. 2013; 26: 1525-1535.
26. Jansen AML, van Wezel T, van den Akker BEWM et al. Combined mismatch repair and POLE/POLD1 defects explain unresolved suspected Lynch syndrome cancers. *European Journal of Human Genetics*. 2015.
27. Planck M, Halvarsson B, Palsson E et al. Cytogenetic aberrations and heterogeneity of mutations in repeat-containing genes in a colon carcinoma from a patient with hereditary nonpolyposis colorectal cancer. *Cancer Genet*

ics and Cytogenetics. 2002; 134: 46-54.

28. Diaz-Padilla I, Romero N, Amir E et al. Mismatch repair status and clinical outcome in endometrial cancer: a systematic review and meta-analysis. *Critical Reviews in Oncology/Hematology*. 2013; 88: 154-167.

29. Goodfellow PJ, Buttin BM, Herzog TJ et al. Prevalence of defective DNA mismatch repair and MSH6 mutation in an unselected series of endometrial cancers. *Proceedings of the National Academy of Sciences of the United States of America*. 2003; 100: 5908-5913.

30. Kim TM, Laird PW, Park PJ. The landscape of microsatellite instability in colorectal and endometrial cancer genomes. *Cell*. 2013; 155: 858-868.

31. Goodfellow PJ, Billingsley CC, Lankes HA et al. Combined Microsatellite Instability, MLH1 Methylation Analysis, and Immunohistochemistry for Lynch Syndrome Screening in Endometrial Cancers From GOG210: An NRG Oncology and Gynecologic Oncology Group Study. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology*. 2015; 33: 4301-4308.

32. Yan M, Schwaederle M, Arguello D et al. HER2 expression status in diverse cancers: review of results from 37,992 patients. *Cancer and Metastasis Reviews*. 2015; 34: 157-164.

33. Mojtahed A, Schrijver I, Ford JM et al. A two-antibody mismatch repair protein immunohistochemistry screening approach for colorectal carcinomas, skin sebaceous tumors, and gynecologic tract carcinomas. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc*. 2011; 24: 1004-1014.

## Supplementary files

### Supplementary Methods

#### *Patients and study design*

In total, 854 early-stage ECs from the randomized PORTEC-1 and -2 clinical trials based on availability of formalin-fixed paraffin-embedded slides and sufficient tumor material for DNA isolation were included in this study. Full details and results of both studies have been published previously.<sup>21,22</sup> PORTEC-1 (1990-1997) included 714 patients with stage I endometrial cancer, grade 1 or 2 with deep myometrial invasion, or grade 2 or 3 with superficial invasion. The PORTEC-2 study included 427 endometrial cancer patients between 2002 and 2006 with high-intermediate risk features: stage I, age >60 years, grade 1-2 with deep invasion or grade 3 with superficial invasion and stage IIA disease (except grade 3 with deep invasion). The PORTEC study protocols were approved by the Dutch Cancer Society and the medical ethics committees at participating centers. All patients provided informed consent for collection of their data and somatic analysis of tumor alterations. During the clinical trial period, universal LS screening was not performed. Patient and tumor characteristics, including results of pathology review, were obtained from the trial databases.

#### *MSI assay*

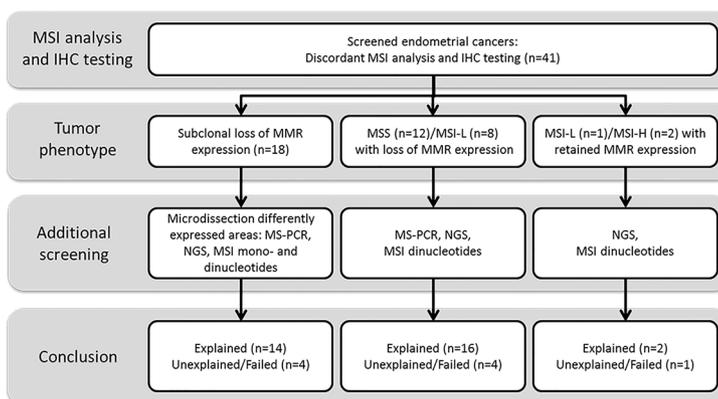
Tumor MSI status was determined as previously reported (Promega MSI analysis system (version 1.2)).<sup>4</sup> Tumors with instability in at least two of the five mononucleotide repeat markers were defined as being microsatellite instability-high (MSI-H), whereas those showing no instability were classified as being microsatellite stable (MSS). Tumors in which instability was detected at a single repeat were retested together with DNA extracted from unaffected myometrium to exclude biallelicity of the marker, and defined as microsatellite instability-low (MSI-L) if somatic instability was confirmed.

### IHC analysis

IHC staining for MLH1, PMS2, MSH2, and MSH6 was performed on all tumors in which MSI status was successfully determined. Antigen retrieval was achieved by microwave oven procedure in 10 mmol/L Tris-EDTA buffer, pH 9.0. Sections were incubated overnight with primary antibodies against MLH1 (clone ES05, 1:100; DAKO), MSH2 (clone FE11, 1:200, DAKO), MSH6 (clone EPR3945, 1:800, Genetex) at room temperature, and PMS2 (clone EP51, 1:75, DAKO) at 4°C. Sections were incubated at room temperature with Envision FLEX+ Linker (DAKO) for 15 minutes followed by 30 minutes incubation with secondary antibody (Poly-HRP-GAM/R/R; DPV0110HRP; ImmunoLogic). 3,3'-diaminobenzidine+ was used as a chromogen. The slides were counterstained with hematoxylin, dehydrated and mounted. Slides were evaluated by two observers blinded for patient and tumor characteristics, and discrepancies were discussed and reviewed at a multihead microscope until consensus was reached.

### Somatic variant screening

A custom panel was designed with the AmpliSeq™ Designer tool containing *MLH1*, *MSH2*, *MSH6*, *PMS2*, the *POLE*- and *POLD1* exonuclease domains (EDM) and exons 2, 6, 7, 12 and 13 of *MUTYH*.<sup>26</sup> The panel consisted of 201 amplicons (21378 bp), covering 99.3%, 99.3%, 100 and 76.8% of the coding regions of *MLH1*, *MSH2*, *MSH6* and *PMS2*, respectively. Variants were annotated 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*), NM\_001256849.1 (*POLD1*) and NM\_001128425.1 (*MUTYH*). Raw data analysis, alignments, variant calling and data analysis was performed as previously described.<sup>26</sup>



Supplementary Figure 1. Flowchart of sample analyses of cases with discordant MSI status and MMR protein expression. Explained cases are further described in the white colored lines and unexplained/failed cases in the grey colored lines in Table 2 and Table 3.

Supplementary Table S1. Clinicopathological characteristics according to the MSI status in early-stage EC (n=696).

	Total n=696 (100%)	MSS n=516 (74.1%)	MSI-L n=11 (1.6%)	MSI-H n=169 (24.3%)	P-value
Age, years					
Mean (range)	69 (41-88)	68 (41-88)	67 (61-79)	69 (43-88)	0.305
< 60	101 (14.5)	76 (14.7)	0	25 (14.8)	
60-70	300 (43.1)	224 (43.4)	9 (81.8)	67 (39.6)	0.096
> 70	295 (42.4)	216 (41.9)	2 (18.2)	77 (45.6)	
Tumor type					
EEC	679 (97.6)	500 (96.9)	11 (100)	168 (99.4)	
NEEC	17 (2.4)	16 (3.1)	0	1 (0.6)*	0.162
Grade					
1-2	587 (84.4)	442 (85.7)	7 (63.6)	138 (81.7)	
3	109 (15.6)	74 (14.3)	4 (36.4)	31 (18.3)	0.075
Myometrial invasion					
<50%	200 (28.7)	143 (27.7)	4 (36.4)	53 (31.4)	
>50%	496 (71.3)	373 (72.3)	7 (63.6)	116 (68.6)	0.564
Lymph vascular space invasion**					
Absent/Mild	643 (95.8)	493 (98.2)	9 (90.0)	141 (88.7)	
Substantial	28 (4.2)	9 (1.8)	1 (10.0)	18 (11.3)	0.000
Risk group					
Low	179 (25.7)	135 (26.2)	2 (18.2)	42 (24.8)	
High-intermediate	465 (66.8)	343 (66.5)	8 (72.7)	114 (67.4)	0.997
High	52 (7.5)	38 (7.3)	1 (0.9)	13 (0.8)	
Treatment					
NAT	181 (26.0)	133 (25.8)	2 (18.2)	46 (27.2)	
EBRT	336 (48.3)	248 (48.1)	5 (45.4)	83 (49.1)	0.876
VBT	179 (25.7)	135 (26.1)	4 (36.4)	40 (23.7)	

\* The MSI-H NEEC with serous histology showed complete loss of MSH2 and MSH6 expression and harbored a pathogenic *TP53* mutation. \*\*25 unknown. MSS=microsatellite stable, MSI-L/H= microsatellite unstable with low- or high-frequency, EEC=endometrioid endometrial cancer, NEEC= non-endometrioid, NAT=no additional treatment, EBRT=external beam radiotherapy, VBT=vaginal brachytherapy.

