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MLH1 Promotor Hypermethylation in Colorectal and Endometrial Carcinomas from Patients with Lynch Syndrome

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Screening for Lynch syndrome (LS) in colorectal cancer (CRC) and endometrial cancer patients generally involves immunohistochemical staining of the mismatch repair (MMR) proteins. In case of MLH1 protein loss, *MLH1* promotor hypermethylation (*MLH1*-PM) testing is performed to indirectly distinguish the constitutional *MLH1* variants from somatic epimutations. Recently, multiple studies have reported that *MLH1*-PM and pathogenic constitutional MMR variants are not mutually exclusive. This study describes 6 new and 86 previously reported *MLH1*-PM CRCs or endometrial cancers in LS patients. Of these, methylation of the *MLH1* gene promotor C region was reported in 30 *MLH1*, 6 *MSH2*, 6 *MSH6*, and 3 *PMS2* variant carriers at a median age at diagnosis of 48.5 years [interquartile range (IQR), 39—56.75 years], 39 years (IQR, 29—51 years), 58 years (IQR, 53.5—67 years), and 68 years (IQR, 65.6—68.5 years), respectively. For 31 *MLH1*-PM CRCs in LS patients from the literature, only the B region of the *MLH1* gene promotor was tested, whereas for 13 cases in the literature the tested region was not specified. Collectively, these data indicate that a diagnosis of LS should not be excluded when *MLH1*-PM is detected. Clinicians should carefully consider whether follow-up genetic MMR gene testing should be offered, with age <60 to 70 years and/or a positive family history among other factors being suggestive for a potential constitutional MMR gene defect. *(J Mol Diagn 2024, 26: 106—114; https://doi.org/10.1016/j.jmoldx.2023.10.005)*

Lynch syndrome (LS) is caused by constitutional pathogenic variants of one of the mismatch repair (MMR) genes [*MLH1*, *MSH2* (*EPCAM*), *MSH6*, or *PMS2*].[1] MMR deficiency occurs when a somatic hit compromises the remaining wild-type MMR allele, preventing proper correction of mismatches during DNA replication. This results in an increased tumor mutational burden and slippage at repetitive sequence stretches termed microsatellites, known as microsatellite instability (MSI).[2] As a consequence, MMR variant carriers have an increased risk of developing cancer, frequently manifesting as colorectal cancer (CRC) and endometrial cancer (EC) in particular.[1,2]

Because this increased risk of cancer requires intensified monitoring by regular colonoscopy surveillance,[3—6] establishing a LS diagnosis is of major importance. Previously used LS screening tools, such as the Amsterdam[7] and Bethesda[8] criteria, have low sensitivity because of their reliance on family and medical histories.[9] Consequently, they were replaced by universal immunohistochemical staining of MMR proteins and/or MSI testing, an LS

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screening approach currently recommended for all CRC and EC patients.[10–13]

Loss of MLH1 protein expression may be explained by either (i) a constitutional *MLH1* variant with a somatic hit comprising the remaining wild-type *MLH1* allele, (ii) double somatic variants of *MLH1*, or (iii) somatic *MLH1* silencing through (biallelic) hypermethylation of the CpG-rich *MLH1* promotor sequence.[9,14] Of these, the latter is most prevalent, especially in older age groups (eg, ages ≥60 to 70 years).[9,15–20] Methylation of the C region (−248 to 178 bp relative to the transcription start site) of the *MLH1* gene promotor correlates most strongly with loss of MLH1 expression.[21] Methylation of the B region (−552 to −266) and/or the D region (−109 to +15) also may be important in MLH1 silencing, yet correlates to a lesser extent with MLH1 expression, whereas methylation of the A region (-711 to -577) is linked strongly to aging and does not seem critical in MLH1 silencing.[21–24]

Testing for *MLH1* promotor hypermethylation (*MLH1*-PM) is recommended to distinguish indirectly between (constitutional) *MLH1* variants and somatic epigenetic silencing of *MLH1*.[13] However, several recent studies have shown that *MLH1*-PM and constitutional MMR variants are not always mutually exclusive,[25] and *MLH1*-PM occasionally can be found in the germline of (affected) individuals.[26–32] These findings suggest that the presence of *MLH1*-PM does not exclude LS, and genetic MMR testing and/or constitutional *MLH1*-PM testing may be necessary.

The current study describes six new cases of LS patients with *MLH1*-PM CRCs or ECs and provides an overview of previously reported *MLH1*-PM CRCs and ECs from patients with LS. Collectively, these cases highlight the current inadequacy of *MLH1*-PM testing as a method to rule out LS.

Materials and Methods

Ethical Statement

This study was approved by the Medical Ethical Committee of Leiden The Hague Delft (protocol P17.098). Patient samples were handled according to the medical ethical guidelines described in the Helsinki Declaration and the Code of Conduct for responsible use of human tissue in the context of health research (Federation of Dutch Medical Scientific Societies). Samples were anonymized and patients provided informed consent for the use of tissue and clinical data.

Patient and Tissue Evaluation

Clinical and pathologic data on six cases of LS patients with *MLH1*-PM CRCs and ECs (study ID T1-6), originating from five confirmed MMR gene variant carriers, were retrieved from electronic health records of the Leiden University Medical Center, University Medical Center Groningen, and Amsterdam University Medical Center in the Netherlands. Immunohistochemical staining, MSI analysis (Idylla MSI test, Biocartis NV, Mechelen, Belgium), and *MLH1*-PM testing of these six tumors were performed in the context of standard clinical care. *MLH1*-PM testing was achieved by methylation-specific PCR according to a similar protocol described previously,[16] or by the SALSA MS-MLPA probemix ME011 Mismatch Repair Genes kit (MRC Holland, Amsterdam, the Netherlands).

Literature Review

To identify previously reported LS patients affected by *MLH1*-PM CRCs and/or ECs, a survey of the literature was performed in December 2022 in NCBI PubMed. Patients were included when all three of the following criteria were met: i) pathogenic MMR gene variant carrier; ii) CRC(s) and/or EC(s) diagnosis, and iii) somatic *MLH1*-PM in the tumor(s). Exclusion criteria involved the following: i) studies not written in English, ii) (systematic) reviews to avoid double inclusion of patients, and iii) reports analyzing just the A region of the *MLH1* gene promotor were excluded, based on the weak correlation between methylation of this region and MLH1 silencing.[21,22]

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics v25 (IBM Corp., New York, NY) and RStudio v2022.02.3 + 492 (Team R; Integrated Development for R, Boston, MA; *http://www.rstudio.com*). Continuous outcomes are presented as medians [interquartile range (IQR)], whereas categoric outcomes are presented as proportions. Age at cancer diagnosis was compared between the different *MLH1* gene promotor region groups and the different MMR mutation groups using the Kruskal-Wallis test, and raw P values were corrected for the number of pairwise comparisons using Benjamini and Hochberg correction. P values were two-tailed and considered statistically significant when $P < 0.05$.

Results

Identified Cases per *MLH1* Gene Promotor Region

In total, 92 *MLH1*-PM CRCs and ECs from LS patients were identified, including the 6 new cases described here and the 86 previously reported cases from the literature. A full description of all available histologic and molecular characteristics (including the constitutional MMR variant) for each individual tumor is presented in Supplemental Table S1.[18,23–25,33–55]

Of the 92 *MLH1*-PM CRCs and ECs, 48 (52%) tumors were known to constitute methylation in the C region of the *MLH1* gene promotor (Figure 1, A and B, and Supplemental Table S1).[21,22] For 31 *MLH1*-PM CRCs from the literature, only methylation of the B region of the *MLH1* gene promotor was tested/detected, whereas for 13 cases from the

literature, the tested *MLH1* gene promotor region was not specified (only A region methylation was not ruled out).

Clinical and Molecular Characteristics

A summary of the clinical characteristics of all tumors (stratified by affected *MLH1* gene promotor region) is presented in Table 1. *MLH1*-PM CRCs and ECs with C region methylation were reported in 30 *MLH1*, 6 *MSH2*, 6 *MSH6*, and 3 *PMS2* variant carriers at a median age at diagnosis of 48.5 years (IQR, 39 to 56.75 years), 39 years (IQR, 29 to 51 years), 58 years (IQR, 53.5 to 67 years), and 68 years (IQR, 65.6 to 68.5 years), respectively (Figure 1, C and D). All *MLH1*-PM CRCs tested for the presence of MSI ($n = 31$) were indeed MSI, whereas only 2 (study ID T1-2) of 18 (11%) *MLH1*-PM CRCs with a known *BRAF* V600E mutational status were positive for the *BRAF* V600E variant.

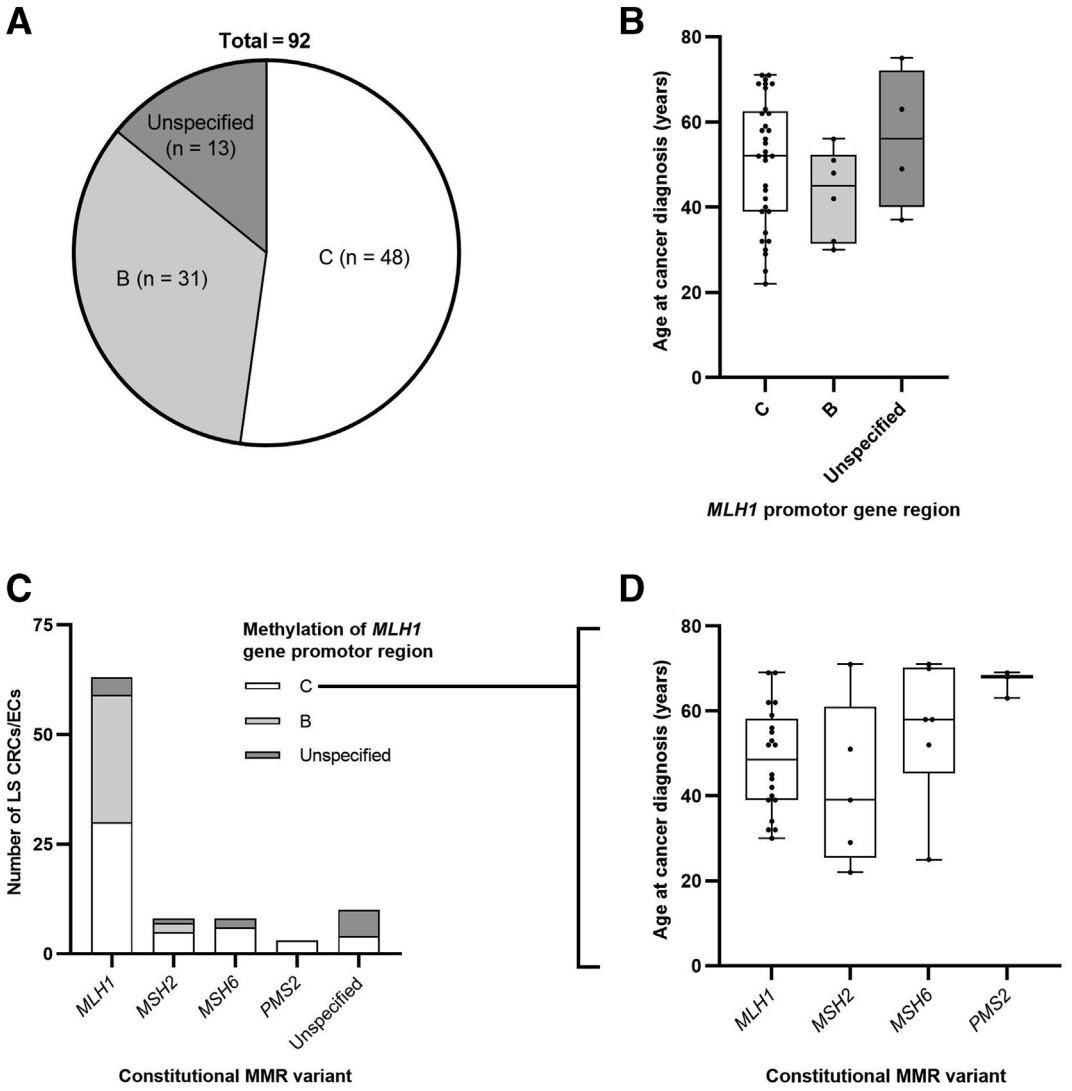

Figure 1 Age at diagnosis of *MLH1*-PM colorectal cancers (CRCs) and endometrial cancers (ECs) from Lynch syndrome (LS) patients per *MLH1* gene methylation region and the mismatch repair (MMR) mutation group. **A:** *MLH1* promotor hypermethylation (*MLH1*-PM) CRCs and ECs from LS patients stratified by methylated *MLH1* gene promotor region. **B:** The median age at diagnosis of patients with tumors with C region methylation was 52 years [interquartile range (IQR), 39 to 62 years], whereas the median age of tumors with B region methylation was 45 years (IQR, 34.5 to 50.25 years). None of the pairwise comparisons (C versus B versus unspecified region groups) were statistically significant. There were 15 missing values from the C region, 25 missing values from the B region, and 9 missing values from an unspecified region. **C:** *MLH1*-PM CRCs and ECs from LS patients stratified by MMR mutation group, including annotation for the methylated *MLH1* gene promotor region. **D:** *MLH1*-PM CRCs and ECs with C region methylation were reported in 30 *MLH1*, 6 *MSH2*, 6 *MSH6*, and 3 *PMS2* variant carriers at a median age at diagnosis of 48.5 years (IQR, 39 to 56.75 years), 39 years (IQR, 29 to 51 years), 58 years (IQR, 53.5 to 67 years), and 68 years (IQR, 65.6 to 68.5 years), respectively. One patient (study ID: T85) carried constitutional *MLH1* and *MSH2* variants and was included in both respective MMR mutation groups. None of the pairwise comparisons (*MLH1* versus *MSH2* versus *MSH6* versus *PMS2* groups) were statistically significant. There were 10 missing values from *MLH1*, 1 missing value from *MSH2*, and 4 missing values from unspecified constitutional MMR variants. Statistical differences between the different *MLH1* gene promotor region groups and the different MMR mutation groups were evaluated using the Kruskal-Wallis test. Raw *P* values from the pairwise comparisons were corrected for the number of comparisons using Benjamini-Hochberg correction.

Table 1 Clinical Characteristics of *MLH1*-PM CRCs and ECs from LS Patients

Description	*MLH1*-PM CRCs and ECs from LS patients, *n* (%)		
	C region (*n* = 48)	B region (*n* = 31)	Unspecified region (*n* = 13)
Cancer type			
CRC	45 of 48 (94)	31 of 31 (100)	11 of 13 (85)
EC	3 of 48 (6)	0	2 of 13 (15)
Constitutional MMR variant			
MLH1	30 of 44 (68)*	29 of 31 (94)	4 of 7 (57)
MSH2	6 of 44 (14)*	2 of 31 (6)	1 of 7 (14)
MSH6	6 of 44 (14)	0	2 of 7 (29)
PMS2	3 of 44 (7)	0	0
Yes, but undefined	4 of 48 (8)	0	6 of 13 (46)
Sex			
Male	7 of 23 (30)	N/A	1 of 4 (25)
Female	16 of 23 (70)	N/A	3 of 4 (75)
Not reported	25 of 48 (52)	31 of 31 (100%)	9 of 13 (69)
Age at diagnosis, median (IQR), years	52 (39 to 62)	45 (34.5 to 50.25)	56 (46 to 66)
MSI status			
MSI	19 of 19 (100)	10 of 10 (100)	2 of 2 (100)
Unknown/not tested	29 of 48 (60)	21 of 31 (68)	11 of 13 (85)
BRAF V600E status			
Mutant	2 of 16 (13)	N/A	0 of 2 (0)
Unknown/not tested	32 of 48 (67)	31 of 31 (100)	11 of 13 (85)

Data are presented as *n* (%) unless otherwise noted.

*One patient (study ID: T85) carried constitutional *MLH1* and *MSH2* variants.

CRC, colorectal cancer; EC, endometrial cancer; IQR, interquartile range; LS, Lynch syndrome; MLH1-PM, MLH1 promotor hypermethylation; MMR, mismatch repair; MSI, microsatellite instability; N/A, not applicable/not available.

Discussion

Despite the fact that *MLH1*-PM testing is recommended to distinguish *MLH1* variants indirectly from epigenetic silencing of *MLH1* in the event of loss of MLH1 expression, an increasing number of studies have shown that *MLH1*-PM and constitutional MMR variants are not mutually exclusive. These previously reported patients were reviewed and six new LS patients with *MLH1*-PM CRCs or ECs were described.

This study provides further evidence for the occurrence of *MLH1*-PM in CRCs and ECs from patients with LS, most importantly by highlighting 48 CRCs and ECs from LS patients with methylation of the C region of the *MLH1* gene promotor, which is the focus of most diagnostics tests because of its strong correlation with MLH1 silencing.[21,22] Thirty-one CRCs and ECs from LS patients with methylation of the B region of the MLH1 gene promotor (in which C region methylation is either absent or not performed) additionally are described. These cases further illustrate the possibility of *MLH1*-PM in CRCs and ECs from LS patients, although it should be noted that B region methylation correlates less strongly with MLH1 silencing and is not consistently part of diagnostic testing,[21,22] and therefore likely poses a smaller clinical problem. Collectively, these data show that *MLH1*-PM does not always rule out a diagnosis of LS and may have important implications for LS research and management.

On a molecular level, the presence of *MLH1*-PM in CRCs and ECs from LS patients is intriguing because it suggests previously unknown routes by which MMR deficiency CRCs and ECs might develop in LS patients (Figure 2). The likelihood of these routes needs to be evaluated in further molecular studies. For example, the timing of *MLH1*-PM with regard to potential other (second) MMR hits remains to be studied, as well as the possibility/frequency of monoallelic *MLH1*-PM to occur. Moreover, it is not yet known whether the development of *MLH1*-PM in patients with LS is coincidental, or whether a constitutional MMR variant might impact/increase the chance of the occurrence of *MLH1*-PM. The latter situation already has been described for several *MLH1* variants, which by themselves are not pathogenic, yet segregate with or are linked to *MLH1*-PM.[56–58]

Similarly, further prospective studies are needed to determine the prevalence of *MLH1*-PM CRC/EC in LS patients and vice versa. The design of this study did not facilitate such prevalence calculations because case studies were included. However, a prior review by Parsons et al[59] estimated that *MLH1*-PM was present in 15% (58 of 388) of all CRC-affected MMR gene variant carriers and in 16% (39 of 250) of CRC-affected *MLH1* variant carriers. This underscores the fact that a considerable proportion of LS patients will be missed when the diagnosis of LS is excluded after detection of *MLH1*-PM.

When *MLH1*-PM is detected, multiple factors may guide clinicians in deciding whether to offer genetic MMR gene

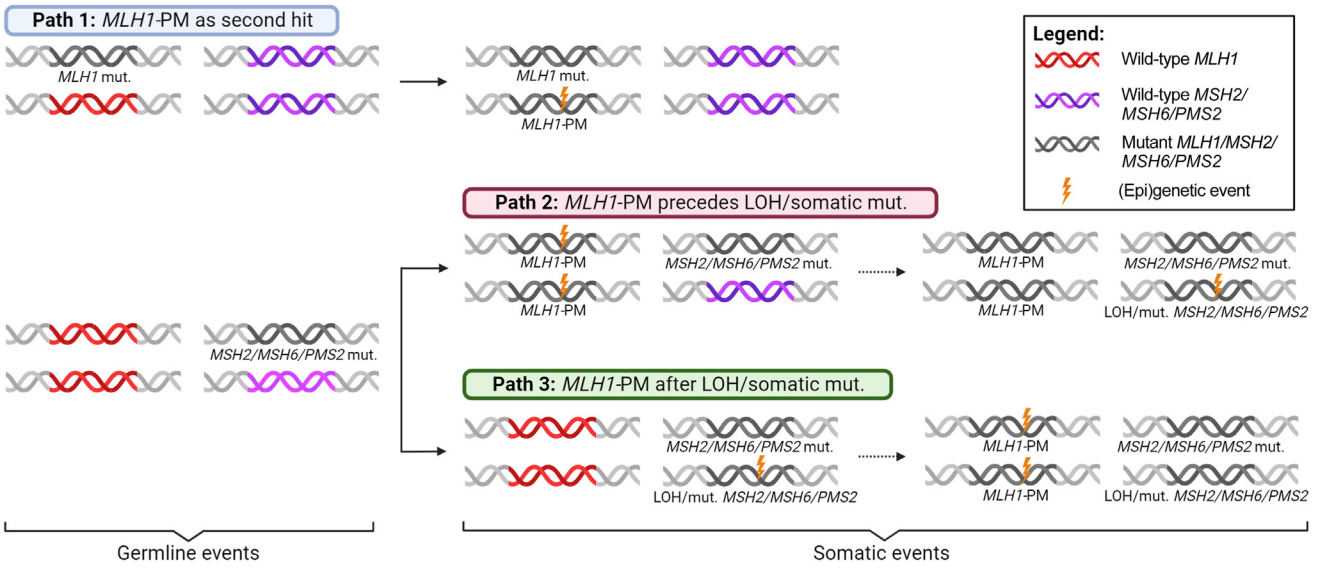

Figure 2 Paths to mismatch repair deficiency (dMMR) in *MLH1* promotor hypermethylation (*MLH1*-PM) colorectal cancers and endometrial cancers from Lynch syndrome patients. Somatic *MLH1*-PM may act as a second hit to the wild-type *MLH1* allele in *MLH1* variant carriers, leading to dMMR (Path 1). In carriers of *MSH2*, *MSH6*, or *PMS2* variants, *MLH1*-PM may cause dMMR through MLH1 deficiency independently of the constitutional MMR variant (Path 2). Alternatively, biallelic *MLH1*-PM may be preceded by a second hit in the *MSH2*, *MSH6*, or *PMS2* gene, leading to concurrent deficiencies of MLH1 and one of the other MMR proteins (Path 3). Hits represented by **dashed arrows** are redundant for the development of dMMR. *MLH1*-PM is considered to involve both alleles. Future studies are needed to test the possibility/frequency of monoallelic *MLH1*-PM, which would introduce multiple other paths not featured in this figure. LOH, loss of heterozygosity; mut., mutation. Figure generated with BioRender.com (Toronto, ON, Canada).

testing (Figure 3). First, patients with *MLH1*-PM CRC or EC who have loss of MLH1 expression, accompanied by complete loss of MSH2 and/or MSH6 expression, should be offered genetic MMR testing. This is based on the argument that *MLH1*-PM only explains the loss of MLH1 expression and not the complete loss of MSH2 and/or MSH6 expression. This scenario is unlikely to be missed in the current screening algorithm because genetic MMR testing already is recommended after complete loss of MSH2 and/or MSH6.[10–13] Of note, subclonal loss of expression of MSH6 may result from microsatellite variants in the *MSH6* gene and could be a direct consequence of *MLH1*-PM in sporadic CRCs and ECs as well.[60]

Second, follow-up genetic MMR gene testing may be considered in patients aged <60 to 70 years showing loss of MLH1 (and PMS2) expression. In the current study, the distribution of *MLH1*-PM CRCs and ECs from LS patients was skewed toward younger age groups, with a median age at diagnosis of 52 years for all patients and of 48.5 years for *MLH1* variant carriers specifically (C region methylation group only). These findings contrast with the distribution of *MLH1*-PM among sporadic (MSI) CRCs and ECs in general, in which *MLH1*-PM is much more prevalent in tumors from patients aged ≥60 to 70 years.[9,15–20,61] This suggests that genetic MMR testing of older age groups (eg, ≥60 to 70 years) therefore is less cost effective than genetic MMR testing of patients aged <60 to 70 years, although a comprehensive analysis of cost effectiveness, stratified per age group, will be needed to confirm this suspicion.

Third, follow-up genetic MMR gene testing might be considered for patients with *MLH1*-PM CRCs and ECs showing loss of MLH1 (and PMS2) staining, when family history is indicative of LS, as described by the revised Bethesda criteria.[8]

In scenarios two and three, an additional factor that could influence the decision to offer/perform follow-up genetic MMR gene testing is the *BRAF* V600E mutational status. Although *BRAF* V600E variants are rare and not associated with MMR status in ECs,[62] they are associated strongly with *MLH1*-PM in CRC patients older than age 50 years.[16,63–66] However, in contrast to sporadic *MLH1*-PM CRCs, *BRAF* V600E variants generally are absent in LS CRCs and the presence of *BRAF* V600E therefore appears to decrease, but not exclude, the probability of detecting constitutional MMR variants.[47,64,67–69] That *BRAF* V600E does not exclude LS is illustrated among others by T1, T2, and T6 (tumor IDs from Supplemental Table S1) from the current study. Moreover, *BRAF* V600E variants occasionally are found in sessile serrated lesions from LS patients, which through the serrated neoplasia pathway may progress to CRC and perhaps in part account for (sporadic) *MLH1*-PM CRCs in LS patients.[70,71]

There are other factors that may increase the likelihood of identifying LS, such as the presence of MMR deficiency crypt/gland foci in (normal) mucosa of affected patients, which are hypothesized to be suggestive for LS and may increase awareness of a possible underlying MMR gene defect.[72] Moreover, the affected *MLH1* gene promotor region may provide information regarding the likelihood of a

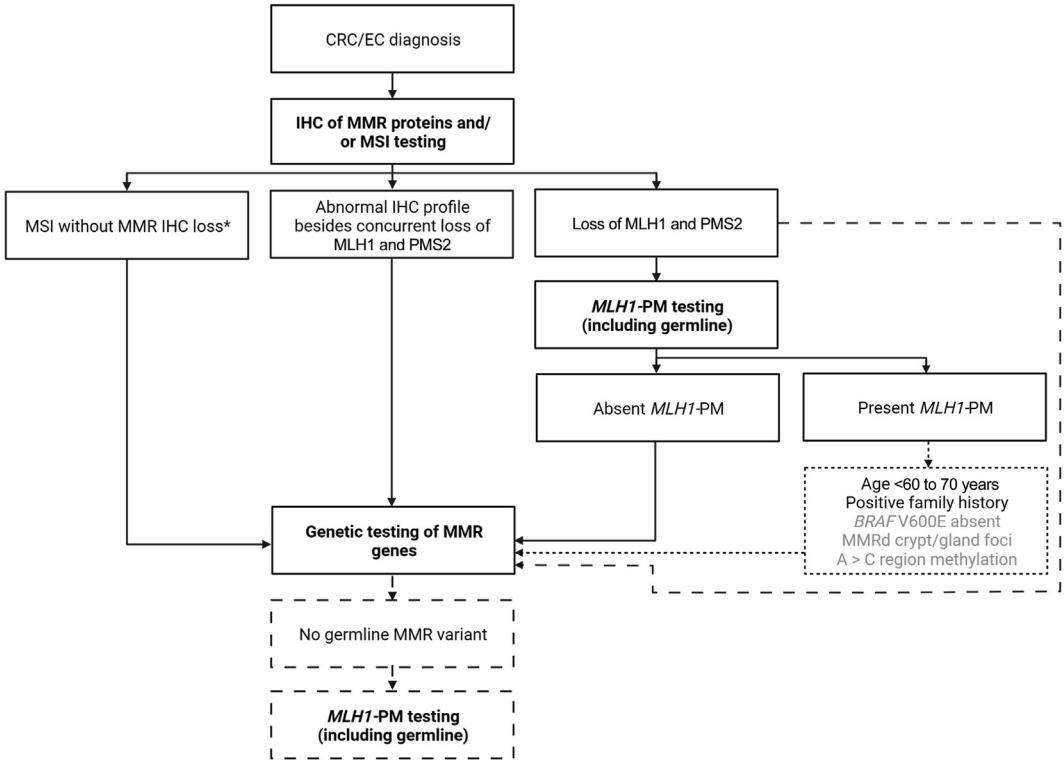

Figure 3 Proposed considerations regarding *MLH1* promotor hypermethylation (*MLH1*-PM) colorectal cancers (CRCs) and endometrial cancers (ECs) in the Lynch syndrome (LS) screening protocol. Upon diagnosis of CRC and/or EC, screening for loss of mismatch repair (MMR) protein expression using MMR immunohistochemistry (IHC) and/or microsatellite instability (MSI) testing is recommended in all CRC and EC patients. In case of an abnormal IHC profile besides concurrent loss of MLH1 and PMS2 (ie, MSH2/MSH6 loss), genetic testing of the corresponding gene(s) should be offered because loss of MSH2/MSH6 cannot be attributed to *MLH1*-PM but potentially could be caused by constitutional variants. In case of concurrent loss of MLH1 and PMS2 protein expression, *MLH1*-PM testing currently is recommended to distinguish *MLH1* variants indirectly from epigenetic silencing of MLH1. When *MLH1*-PM is absent, genetic testing of MMR genes should be continued. When *MLH1*-PM is present, current screening protocols generally consider a sporadic origin and recommend no further genetic investigations. These data, however, suggest that *MLH1*-PM does not exclude a diagnosis of LS. Upon detecting *MLH1*-PM, clinicians are encouraged to consider carefully whether follow-up genetic MMR gene testing should be offered (**short dashes**), with age <60 to 70 years and/or a positive family history among other factors being suggestive for a potential MMR gene defect. An alternative approach to diagnose LS patients with *MLH1*-PM CRCs and/or ECs may involve a broader implementation of genetic MMR testing in all CRCs and ECs with abnormal IHC staining and/or a positive MSI test result (**large dashes**). In this way, *MLH1*-PM testing may be limited to detect constitutional *MLH1*-PM epimutations in MLH1-negative CRCs and ECs from patients in whom no MMR variant is identified. *If no IHC profile is available and only MSI testing is performed, then all MSI CRCs should be triaged further with *MLH1*-PM testing or undergo direct genetic MMR testing. MMRd, MMR deficiency. Figure generated with BioRender.com (Toronto, ON, Canada).

MMR gene defect, with *MLH1*-PM of the A region being more frequent than the C region in *MLH1* variant carriers, but not in MMR variant-negative MSI CRCs.[59] Further research, however, is needed to investigative the discriminative value of these and other potential molecular factors in distinguishing sporadic from LS CRCs and ECs.

An alternative approach to diagnose LS patients with *MLH1*-PM CRCs and/or ECs may involve broader implementation of genetic MMR testing in all CRCs and ECs with abnormal immunohistochemical staining and/or a positive MSI test result (Figure 3), although cost-effectiveness analyses of this approach currently are lacking. In this case, testing for *MLH1*-PM may be limited to exclude constitutional *MLH1*-PM secondarily to genetic MMR testing in MLH1-deficient CRCs and ECs from patients in which no MMR variant was detected. The frequency of constitutional *MLH1*-PM in MLH1-negative CRCs without constitutional variants of *MLH1* is estimated to range between 0.6% and 2%.[58,73] Considering the increased risk of cancer, the correct diagnosis of this group of patients is of extreme importance to offer these patients appropriate care.

A limitation of this study was the retrospective design, including the dependence on data reported in the literature. Because not all identified studies had the specific aim to detect/describe *MLH1*-PM in LS patients, *MLH1*-PM detection may have been suboptimal (eg, not covering the C region of the *MLH1* gene promotor) and data-entry errors cannot be excluded. Moreover, the age of the combined cohort should be considered carefully because previously published cohorts might have had a testing bias, with genetic MMR gene testing more likely to be performed in younger populations.

In conclusion, this study provides further support for the argument that *MLH1*-PM does not always exclude a diagnosis of LS. Clinicians carefully should consider follow-up genetic MMR gene testing upon detecting *MLH1*-PM.

Among other factors, age <60 to 70 years and/or a family history indicative for LS should be part of these considerations. Further studies, including those investigating the prevalence and cost effectiveness, are needed to gain more insights into the molecular and clinical consequences of *MLH1*-PM in CRCs and ECs from patients with LS.

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Author Contributions

N.C.H. and M.N. conceptualized the study, curated, analyzed, and visualized the data, developed methodology, and wrote the original draft of the manuscript; K.D.A. analyzed and visualized the data; N.C.H., K.D.A., M.E.v.L., L.P.v.H., D.R.H., S.W.B.-t.B., E.M.J.v.d.L., F.I., T.v.W., H.M., and M.N. performed the investigations; M.N. acquired funding and supervised the study; and all authors reviewed and edited the manuscript.

Disclosure Statement

None declared.

Supplemental Data

Supplemental material for this article can be found at *http://doi.org/10.1016/j.jmoldx.2023.10.005*.

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