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Research Article

Discordant Staining Patterns and Microsatellite Results in Tumors of *MSH6* Pathogenic Variant Carriers

Anne-Sophie van der Werf-'t Lam[a], Diantha Terlouw[b], Carli M. Tops[a], Merel S. van Kan[a], Liselotte P. van Hest[c], Hans J.P. Gille[c], Floor A.M. Duijkers[d], Anja Wagner[e], Ellis L. Eikenboom[e,f], Tom G.W. Letteboer[g], Mirjam M. de Jong[h], Sanne W. Bajwa-ten Broeke[h], Fonnet E. Bleeker[i], Encarna B. Gomez Garcia[j], Niels de Wind[k], J. Tom van Wezel[b], Hans Morreau[b], Manon Suerink[a], Maartje Nielsen[a,*]

[a] *Department of Clinical Genetics, Leiden University Medical Center, Leiden, The Netherlands;* [b] *Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands;* [c] *Department of Clinical Genetics, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands;* [d] *Department of Clinical Genetics, Amsterdam Medical Center, Amsterdam, The Netherlands;* [e] *Department of Clinical Genetics, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands;* [f] *Department of Gastroenterology and Hepatology, Erasmus MC Cancer Institute, University Medical Center Rotterdam, Rotterdam, The Netherlands;* [g] *Department of Genetics, University Medical Center Utrecht, Utrecht, The Netherlands;* [h] *Department of Genetics, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands;* [i] *Department of Clinical Genetics, Netherlands Cancer Institute, Amsterdam, The Netherlands;* [j] *Department of Clinical Genetics, Maastricht University Medical Center, Maastricht, The Netherlands;* [k] *Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands*

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ABSTRACT

Diagnosis of Lynch syndrome (LS) caused by a pathogenic germline *MSH6* variant may be complicated by discordant immunohistochemistry (IHC) and/or by a microsatellite stable (MSS) phenotype. This study aimed to identify the various causes of the discordant phenotypes of colorectal cancer (CRC) and endometrial cancer (EC) in *MSH6*-associated LS. Data were collected from Dutch family cancer clinics. Carriers of a (likely) pathogenic *MSH6* variant diagnosed with CRC or EC were categorized based on an microsatellite instability (MSI)/IHC test outcome that might fail to result in a diagnosis of LS (eg, retained staining of all 4 mismatch repair proteins, with or without an MSS phenotype, and other staining patterns). When tumor tissue was available, MSI and/or IHC were repeated. Next-generation sequencing (NGS) was performed in cases with discordant staining patterns. Data were obtained from 360 families with 1763 (obligate) carriers. *MSH6* variant carriers with CRC or EC (n = 590) were included, consisting of 418 CRCs and 232 ECs. Discordant staining was reported in 77 cases (36% of MSI/IHC results). Twelve patients gave informed consent for further analysis of tumor material. Upon revision, 2 out of 3 MSI/IHC cases were found to be concordant with the *MSH6* variant, and NGS showed that 4 discordant IHC results were sporadic rather than LS-associated tumors. In 1 case, somatic events explained the discordant phenotype. The use of reflex IHC mismatch repair testing, the current standard in most Western countries, may lead to the misdiagnosis of germline *MSH6* variant carriers. The pathologist should point out that further diagnostics for inheritable colon cancer, including LS, should be considered in case of a strong positive family history. Germline DNA analysis of the mismatch repair genes, preferably as part of a larger gene panel, should therefore be considered in potential LS patients.

* Corresponding author.
E-mail address: m.nielsen@lumc.nl (M. Nielsen).

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Introduction

Lynch syndrome (LS; MIM 120435) is an autosomal-dominant inherited disorder caused by a pathogenic germline variant in one of the mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, or *EPCAM*). LS is characterized by a clustering of colorectal cancer (CRC) and endometrial cancer (EC) cases within affected families. Carriers are also at an increased risk of developing other cancers such as ovarian, small bowel, and urothelial cell cancer.[1]

The cardinal features of LS tumors are microsatellite instability (MSI) and mismatch repair deficiency (MMRd), which can be detected by MSI analysis and immunohistochemistry (IHC) for corresponding MMR proteins, respectively.[2] These techniques are often used as pre-screens for CRC and EC to help detect LS families.[3] Importantly, up to 80% of MMRd tumors are attributable to somatic events and are, therefore, unrelated to LS.[4] The frequency of normal IHC staining patterns (ie, positive MMR staining) in EC and CRC occurring in *MSH6* carriers is unknown, and consequently, how often LS families are missed due to the use of IHC as a prescreening method is also poorly understood.

Concordant staining patterns leading to suspicion of LS consist of (1) absence of staining of MLH1 due to an *MLH1* germline variant (combined with the loss of PMS2 staining due to the lack of the MLH1-PMS2 heterodimer), (2) absence of staining of MSH2 (combined with MSH6) leads to clinical suspicion of a germline *MSH2* variant, and (3) and (4) solitary loss of MSH6 or PMS2 leads to suspicion of a germline *MSH6* or *PMS2* variant, respectively.[4] Atypical staining pattern, defined as a staining pattern other than that expected based on the above-described MMR complex heterodimers, are found in up to 1% of cases.[5-11] Discordant MSI/IHC results are seen in up to 10% of CRC universal tumor screening (UTS) cases.[12] Atypical staining patterns in EC have been reported in 0% to 15% of cases.[13-22] However, the frequency of discordant MSI/IHC results in *MSH6* variant carriers remains unclear. Therefore, this study was aimed to identify the various causes of discordant MSI/IHC results, as well as the causes of atypical staining patterns in *MSH6* variant carriers, leading to increased awareness and better detection of these patients.

Methods

Data Collection

Pedigrees of families with a (likely) pathogenic ((L)P) *MSH6* variant, who were counseled up to November 2020, were collected from the following genetic centers in The Netherlands: Amsterdam Medical Center, Amsterdam UMC, Vrije Universiteit, Netherlands Cancer Institute, Erasmus Medical Center, Leiden University Medical Center, Maastricht University Medical Center, University Medical Center Utrecht, and University Medical Center Groningen. Patients with a confirmed germline variant were approached by their counselor to obtain informed consent, to verify clinical information, and where applicable, to perform further analyses on tumor tissue.

Patients were eligible for this study when an LP or P *MSH6* germline variant was found, together with a CRC or EC diagnosis accompanied by a known MSI and/or IHC result. Discordant IHC results are defined as any other IHC staining besides solitary loss of MSH6. Discordant MSI results are defined as microsatellite stable (MSS) or MSI-low results.

Patients were classified into one of the following subgroups:

1. MSI and/or IHC results that may cause the clinician to miss an *MSH6*-associated Lynch syndrome diagnosis (MSS/MSI-low and/or retained staining of MSH6).
2. Any other staining pattern, including loss of MSH6 expression accompanied by loss of staining of 1 or more additional MMR proteins.

Study Procedures

When tumor tissue was available, IHC analyses were repeated. As MSI analysis becomes increasingly obsolete in standard practice, MSI analysis was only repeated when the results of the repeated IHC analyses were insufficiently explanatory. When repeated IHC and optionally repeated MSI analysis did not clearly explain a discordant result, panel analyses using next-generation sequencing (NGS) were performed.

The procedures for IHC and NGS were as described by Suerink et al.[23] Briefly, formalin-fixed paraffin-embedded (FFPE) tissue from tumors of *MSH6* variant carriers was requested for repeated IHC analyses when an atypical staining pattern was noted. Sections were cut from FFPE blocks (4 μm) and subjected to hematoxylin staining and immunohistochemical staining for the 4 MMR proteins (MLH1, MSH2, MSH6, and PMS2). Sequencing was performed using the Ion Torrent platform.

MSI analysis of 10 μm paraffin coupes was conducted using the Idylla platform with 7 biomarkers (ACVR2A, BTBD7, DIDO1, MRE11, RYR3, SEC31A, and SULF2). Tissue handling and analysis were performed according to the manufacturer's protocol. A

Table 1
Overview of the cohort

		Total cohort (n = 1763)	Total cohort discordant results (n = 77)
Sex	Male (%)	44.1	40.3
Mean age (y) at cancer diagnosis	Age CRC (range)	55.6 (20-84)	50.8 (28-82)
	Age EC (range)	55.5 (31-86)	53.9 (34-74)
Total cancers	CRCs (no.)	420	63
	ECs (no.)	232	19
MSI analysis	Available in no. CRCs (%)	133 (31.6)	60 (95.2)
	No. of which discordant (%)	21 (15.8)	20 (33.3)
	Available in no. EC (%)	43 (18.5)	16 (84.2)
	No. of which discordant (%)	12 (5.2)	12 (75.0)
IHC analysis	Available in no. CRC (%)	145 (34.5)	58 (92.1)
	No. of which discordant, but MSH6− (%)	37 (25.5)	37 (63.8)
	No. of which discordant, but MSH6+ (%)	17 (11.7)	17 (29.3)
	Available in no. EC (%)	50 (34.7)	17 (89.5)
	No. of which discordant, but MSH6− (%)	5 (10.0)	5 (29.4)
	No. of which discordant, but MSH6+ (%)	5 (10.0)	5 (29.4)

CRC, clustering of colorectal; EC, endometrial cancer.

Table 2
Overview of patients with both MSS phenotype and retained staining

ID no.	Sex	Proband	*MSH6* variant			Tumor characteristics				Remarks
			DNA change	Protein change	NMD prediction	Tumor	Age of onset	MSI	IHC MMR	
10	M	N	c.3261dupC	p.(Phe1088Leufs*5)	Predicted	CRC	49	MSS	Retained staining all 4 proteins	Repeated analysis IHC MMR: results according to initial report. NGS showed possible LOH of mutated allele
						CRC	79	MSI-H	MLH1-/PMS2-	Hypermethylation of MLH1
430	F	Y	c.651dupT	p.(Lys218*)	Predicted	EC	59	MSI-L	Retained staining all 4 proteins	
678	M	N	c.651dupT	p.(Lys218*)	Predicted	CRC	63	MSS	Retained staining all 4 proteins	
1012	F	Y	c.2719_2720delGT	p.(Val907Argfs*10)	Predicted	EC	46	MSI-L	Retained staining MLH1/MSH2/MSH6	Repeated MSI showed MSI-H and repeated IHC showed MSH6-
1128	F	N	c.467C>G	p.(Ser156*)	Predicted	CRC	56	MSS	Retained staining all 4 proteins	Repeated analysis showed also retained staining of all 4 proteins. NGS showed an *APC* variant and LOH of *APC*
						CRC	56	MSI-H	MSH6-	NGS showed a second hit: missense variant (VUS) in MSH6
1138	F	Y	c.2087T>C and c.3163G>A	p.(Ile696Thr) and p.(Ala1055Thr)	Not predicted	CRC	30	MSI-L	Retained staining all 4 proteins	
1157	M	N	c.467C>G	p.(Ser156*)	Predicted	CRC	67	MSS	Retained staining all 4 proteins	Repeated analysis IHC MMR: results according to initial report. NGS: LOH of mutated allele
1195	F	N	c.1444C>T	p.(Arg482*)	Predicted	CRC	37	MSS	Retained staining all 4 proteins	Repeated analysis IHC MMR: results according to initial report. NGS: no second hit in MSH6. LOH cannot be judged
1217	M	N	c.3920_3924dupATCTC	p.(Pro1309Ilefs*20)	Not predicted	CRC	59	UNK	Retained staining all 4 proteins	
						CRC	76	MSI-H	MSH6-/PMS2-	Fixation defect
1408	F	N	c.1901_1902delTG	p.(Leu634*)	Predicted	CRC	71	MSI-L	Retained staining all 4 proteins	
						EC	74	MSI-H	MSH6-	
1678	M	N	c.2719_2720delGT	p.(Val907Argfs*10)	Predicted	CRC	47	MSS	Retained staining all 4 proteins	

CRC, colorectal carcinoma; EC, endometrial carcinoma; F, female; IHC MMR, immunohistochemistry of the mismatch repair proteins (ie, MLH1, MSH2, MSH6, and PMS2); M, male; MSI, microsatellite instability; MSI-L, MSI low; MSI-H, MSI high; MSS, microsatellite stable; N, no; NGS, next-generation sequencing; NMD, nonsense-mediated decay; Y, yes.

Table 3
Overview of patients with retained staining with or without MSI-H phenotype

ID no.	Sex	Proband	*MSH6* variant			Tumor characteristics				Remarks
			DNA change	Protein change	NMD prediction	Tumor	Age of onset	MSI	IHC MMR	
314	F	N	c.3261delC	p.(Phe1088Serfs*2)	Predicted	CRC	39	MSI-H	Retained staining all 4 proteins	
349	M	Y	c.2672_2674delTCTinsC	p.(Ile891Thrfs*8)	Predicted	CRC	73	MSI-H	Retained staining all 4 proteins	
						CRC	73	MSI-H	UNK	
443	F	Y	c.1135-1139delAGAGA	p.(Arg379*)	Predicted	CRC	46	MSI-H	Retained staining all 4 proteins	
759	M	N	c.467C>G	p.(Ser156*)	Predicted	CRC	55	MSI-H	Heterogenous staining of MSH6	
807	F	N	c.3957dup	p.(Ala1320Serfs*5)	Not predicted	EC	64	MSI-H	Retained staining all 4 proteins	Repeated analysis MSI showed MSS, no second hit found in MSH6
877	M	Y	c.467C>G	p.(Ser156*)	Predicted	CRC	29	MSI-H	Retained staining all 4 proteins	Repeated IHC analysis showed absence of staining of MSH6-. Potential misinterpreted IHC result
1015	M	Y	c.3261dupC	p.(Phe1088Leufs*5)	Predicted	CRC	43	MSI-H	MSH6 weakly +	
1100	F	Y	c.4001G>A	p.(Arg1334Gln)	Not predicted	CRC	24	UNK	MSH6-	
						EC	52	MSI-H	MSH6 weakly +	
1168	M	Y	c.3202C>T	p.(Arg1068*)	Predicted	CRC	38	MSI-H	Retained staining all 4 proteins	
1338	F	Y	c.3261delC	p.(Phe1088Serfs*2)	Predicted	EC	52	UNK	Retained staining all 4 proteins	
						CRC	68	UNK	Retained staining MLH1/MSH2/MSH6	

CRC, colorectal carcinoma; EC, endometrial carcinoma; F, female; IHC MMR, immunohistochemistry of the mismatch repair proteins (ie, MLH1, MSH2, MSH6, and PMS2); M, male; MSI, microsatellite instability; MSI-L, MSI low; MSI-H, MSI high; MSS, microsatellite stable; N, no; NGS, next-generation sequencing; NMD, nonsense-mediated decay; UNK, unknown or not tested; Y, yes.

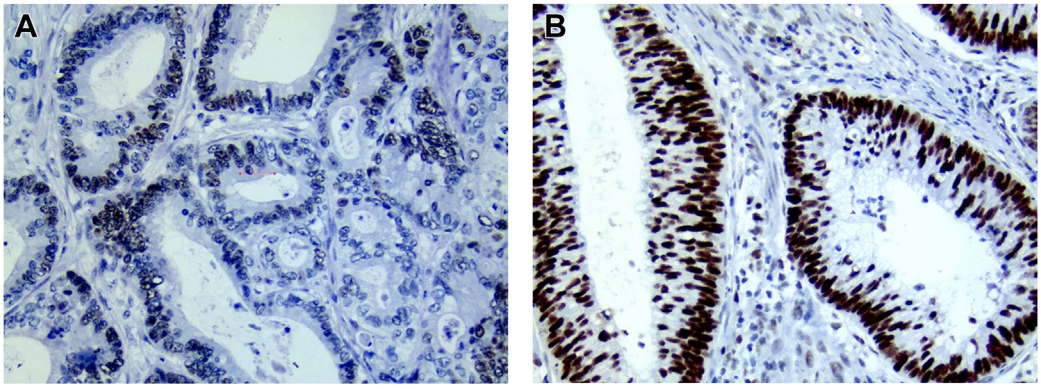

Figure 1.
Normal staining results of (A) MSH6 of ID no. 10 (colorectal carcinoma at 49 year) and normal staining results of (B) MSH6 of ID no. 1157 (colorectal carcinoma at 67 year).

tumor was considered unstable when 2 or more biomarkers were mutated, and stable when less than 2 markers were mutated.

Classification of RNA expression was carried out according to Inácio et al[24] and Shyu et al[25] or if data on RNA expression were available. Briefly, truncating mutations are predicted to trigger nonsense-mediated decay (NMD) when situated before or in the second to the last exon when the stop codon is 50 nucleotides before the splice donor site of the final exon junction. In the case of the *MSH6* gene, this means that a stop codon occurring after codon 1317 should not result in NMD.

Results

Total Cohort Description

As of November 2020, data were available on 360 Dutch *MSH6* families, which consisted of 1513 proven variant carriers and 250 obligate variant carriers (individuals who have not been tested but are presumed to carry a gene variant based on genetic testing in the family). One patient with constitutional mismatch repair deficiency was excluded. Among the 650 included patients, 418 were diagnosed with CRC and 232 were diagnosed with EC. Of these 650 patients, 69 were diagnosed with both CRC and EC. MSI and/or IHC results were available for 213 patients, including MSI results for 131 CRCs and 43 ECs, and IHC staining results for 144 CRCs and 50 ECs. Table 1 provides a summary of the cohort.

Cohort Description: Discordant Results

Of the included patients, 77 patients (36.1% of those with known MSI/IHC results) had discordant results, including 51 probands (the first person identified with a (likely) pathogenic variant in a family). In 11 patients, both the MSI and IHC results could potentially have caused the clinician to miss an *MSH6*-associated LS diagnosis. In another 10 patients, retained staining was observed together with an MSI-high (MSI-H) phenotype or unknown MSI results.

Twenty patients gave informed consent for further tissue analysis (27 tumors) and tumor material was (partially) available from 12 of these patients.

Subgroup 1 — MSS and/or Retained Staining of MSH6

Of the 77 discordant results, 21 patients (including 10 probands), could potentially have been misdiagnosed based on MSI results and/or staining results (see also Tables 2 and 3). Two of these patients developed later in life another tumor with an MSI-H phenotype and absence of MSH6 staining.

Results for 7 of the remaining 19 patients could be repeated. For ID no. 1012, repeated MSI analysis found an MSI-H tumor, rather than MSI-L. Therefore, the tumor phenotype, in this case, was concordant with an *MSH6* germline variant. In the case of repeated IHC analyses for ID no. 877, absence of MSH6 staining was found (see Supplementary Figure S1). In 5 additional cases, other (possible) explanations for the discordancy were found. In 2 carcinomas (ID no. 10 and 1157), (possible) loss of heterozygosity (LOH) of the mutated allele was found, indicating that tumor development was unrelated to the *MSH6* variant (staining results of ID no. 10 and 1157 are depicted in Figure 1). In another case, a previously reported MSI-H tumor (ID no. 807) proved to be MSS, which was more consistent with the reported and repeated normal staining patterns, suggesting that this case could be a sporadic endometrial cancer (phenocopy). In the next case (ID no. 1128), no second hit was found in *MSH6*. In combination with the MSS phenotype and normal staining patterns, this tumor is likely to be sporadic in nature (see Supplementary Figure S2). In the final tumor (ID no. 1195), no second hit in *MSH6* was detected, and LOH could not be determined due to insufficient representative single nucleotide polymorphisms (see Supplementary Figure S3 for MSH6 staining results).

Subgroup 2 — Any Other Staining Results

Other staining patterns were observed in 56 cases (Tables 4-6). Repeated analyses were possible for 8 tumors from 5 patients. In 1 case, a patient was diagnosed with CRC at 53 years and also at 61 years (ID no. 645). The first tumor showed weak MSH2 and absence of MSH6 staining, whereas the second tumor showed absence of staining of both MSH2 and MSH6 (Figure 2). Although LOH of *MSH2* was observed in the CRC in the latter case, no somatic variants were detected.

In the second case (ID no. 106) showing weak IHC staining of MSH2 and absence of staining of MSH6, 2 variants of unknown significance (VUS) in *MSH2* were observed. Furthermore, an *MSH3* class 3 variant was found together with the LOH of *MSH3*. In the third case (ID no. 515), with absence of IHC staining for both MSH2 and MSH6, no somatic events in *MSH2* were detected. In the fourth case (ID no. 706), the patient developed 2 synchronous tumors at the age of 58 years. Repeated IHC analyses showed absence of staining of MLH1 and PMS2 because of hypermethylation of MLH1 and absence of staining of MSH6

Table 4
Overview of patients with MSS/MSI-low phenotype with other staining than retained staining or MSH2−/MSH6−

ID no.	Sex	Proband	*MSH6* variant			Tumor characteristics				Remarks
			DNA change	Protein change	NMD prediction	Tumor	Age of onset	MSI	IHC MMR	
187	M	UNK	c.1614_1615delTCinsAG	p.(Tyr538*)	Predicted	CRC	42	MSI-L	MSH6-	
242	F	Y	c.2150_2153delTCAG	p.(Val717Alafs*18)	Predicted	CRC	46	MSI-L	MSH6-	
244	F	N	c.651dupT	p.(Lys218*)	Predicted	CRC	59	MSS	UNK	
247	F	Y	c.651dupT	p.(Lys218*)	Predicted	CRC	35	MSI-L	MLH1-/MSH6-/PMS2-	
						EC	35	UNK	UNK	
277	F	Y	c.651dupT	p.(Lys218*)	Predicted	CRC	60	MSI-L	UNK	
						EC	65	MSI-L	UNK	
307	M	Y	c.2672_2674delTCTinsC	p.(Ile891Thrfs*8)	Predicted	CRC	47	MSI-H	UNK	
						CRC	48	MSI-L	UNK	
435	F	Y	c.3984_3987dupGTCA	p.(Leu1330Valfs*12)	Not predicted	EC	36	MSS	MLH1-/MSH6-	No MLH1 hypermethylation and no germline *MLH1* (L)P variant
490	F	Y	c.3182delT	p.(Leu1061Argfs*18)	Predicted	EC	50	MSI-L	UNK	
632	M	N	c.2719_2720delGT	p.(Val907Argfs*10)	Predicted	CRC	42	MSS	UNK	
702	F	Y	c.2731C>T	p.(Arg911*)	Predicted	EC	55	MSS	MSH6-	
						CRC	65	MSS	MSH6-	
741	F	Y	c.1784delT	p.(Leu595Tyrfs*15)	Predicted	EC	48	MSI-L	MSH6-	
913	F	N	c.1784delT	p.(Leu595Tyrfs*15)	Predicted	EC	60	MSI-L	MSH6-	
915	F	N	c.1784delT	p.(Leu595Tyrfs*15)	Predicted	EC	57	MSI-L	MSH6-	
1177	F	Y	c.3920_3927dup	p.(Glu1310Ilefs*20)	Predicted	CRC	55	MSI-L	MSH6-	
						EC	57	UNK	UNK	
1218	F	Y	c.651dupT	p.(Lys218*)	Predicted	EC	49	MSI-L	MSH6-	
						CRC	59	UNK	UNK	
1234	F	Y	c.3801+1_3801+5delGTATG	p.(Arg1217Metfs*6)	Predicted	EC	59	MSI-L	MSH6-	
						CRC	64	MSI-H	MSH6-	

CRC, colorectal carcinoma; EC, endometrial carcinoma; F, female; IHC MMR, immunohistochemistry of the mismatch repair proteins (ie, MLH1, MSH2, MSH6, and PMS2); (L)P, (likely) pathogenic; M, male; MSI, microsatellite instability; MSI-L, MSI low; MSI-H, MSI high; MSS, microsatellite stable; N, no; NGS, next-generation sequencing; NMD, nonsense-mediated decay; UNK, unknown or not tested, Y, yes.

(Supplementary Figure S4). In the second tumor of this patient, the same IHC results were found. In both tumors, a second hit in *MSH6* was found. For the final case (ID no. 835) with a reported absence of MLH1 and MSH6 IHC staining, no archive material was available for repeated IHC analyses. However, in this case, a recently developed tumor was available, which showed a typical staining pattern (Supplementary Figure S5).

Discussion

Today, IHC staining and/or MSI analysis are the standard procedures in most countries for detecting MMR deficiency in CRC and EC patients under the age of 70 years, a procedure also known as UTS.[3,18,26] However, UTS for MMR deficiency does not detect all patients with LS.[27] The tumors found in *MSH6* variant carriers are particularly prone to discordant MSI and/or IHC phenotypes compared with other MMR genes,[28-30], and are, therefore, at risk of being overlooked during UTS.[28,31] To raise awareness of the risks of missing *MSH6* variant carriers when using these methods, this study aimed to identify possible causes of discordant MSI and IHC results in the CRCs and ECs of *MSH6* variant carriers.

Current literature describes several possible causes of the atypical staining patterns that could potentially result in a missed LS diagnosis. One important factor, especially applicable to older staining results, is a misinterpretation of IHC due to lack of experience[32] and/or interobserver variability.[28,33-36] In practice, staining results cannot always be interpreted unambiguously,[35,37] with variation in the degree of staining observed in approximately 3% of IHC results[35] and retained staining in approximately 5% to 6% of MSI-H cases.[38-40] Heterogeneous staining or staining that is weaker than that observed in controls may be incorrectly interpreted as intact MMR expression,[41,42] and may, therefore, result in misdiagnosis of LS. Heterogenous staining of MSH6 is not associated with a germline *MSH6* variant but is associated with MMR deficiency secondary to another MMR abnormality.[43] In contrast, heterogenous loss of MSH6 in ECs indicates a possible underlying germline defect rather than solely somatic variants.[44]

MSI analysis also presents challenges when used to detect *MSH6* variant carriers. First, MSI analysis is more reliable in CRCs than in ECs.[45-49] Second, the sensitivity of MSI analysis is lower in *MSH6* variant carriers compared with *MLH1* and *MSH2* variant carriers.[50,51] Although the majority of *MSH6* variant carriers can be identified using mononucleotide markers,[52,53] adding another dinucleotide repeat marker to the pentaplex panel in MSI analysis increases the sensitivity of *MSH6* variant carrier detection.[54] MSH6-deficient tumors are, therefore, at risk of being misclassified as MSI-L or MSS, depending on the markers chosen.[29,55,56] Furthermore, MSI results can also be negatively affected by an inadequate proportion of tumor cells.[49] Importantly, MSS status and loss of MSH6 is the most frequently observed discordancy,[55] an outcome that may also be explained by the partial redundancy of MSH6 and MSH3 protein function. If MutSα is impaired, the MutSβ complex still functions and can partially correct DNA mismatch errors,[57] possibly explaining an MSS profile accompanying loss of MSH6 protein.

In this study, MSI and IHC results of 12 tumors were repeated to investigate the extent to which revision may yield different results. In 3 tumors, the repeated MSI and IHC analyses showed different results from the original report; in 2 cases the new result was concordant with an *MSH6* germline variant, underlining the

Table 5
Overview of patients with absence of or less staining of MSH2 and MSH6

ID no.	Sex	Proband	*MSH6* variant			Tumor characteristics				Remarks
			DNA change	Protein change	NMD prediction	Tumor	Age of onset	MSI	IHC MMR	
106	F	Y	c.1276delT	p.(Cys426Valfs*27)	Predicted	CRC	64	UNK	MSH2 weakly+/ MSH6−	Repeated analysis showed PMS2+.[a] NGS showed a second hit in MSH6, Class 3 variant (VUS) in MSH2 and MSH3 with LOH of MSH3
132	F	N	c.1614_1615delTCinsG	p.(Tyr538*)	Predicted	CRC	82	UNK	MSH2 weakly+/ MSH6−	
193	M	UNK	c.467C>G	p.(Ser156*)	Predicted	CRC	61	MSI-H	MSH2−/MSH6−	
196	F	Y	c.3261delC	p.(Phe1088Serfs*2)	Predicted	CRC	45	MSI-L	MSH2−/MSH6−	possibly splice mutation
220	F	Y	c.467C>G	p.(Ser156*)	Predicted	EC	51	UNK	UNK	
						CRC	54	MSI-H	MSH2 weakly+/ MSH6−	
227	M	Y	c.467C>G	p.(Ser156*)	Predicted	CRC	45	MSI-H	MSH2−/MSH6−	
245	M	N	c.651dupT	p.(Lys218*)	Predicted	CRC	37	MSI-H	MSH2−/MSH6−	
309	F	Y	c.1190_1191delAT	p.(Tyr397Cysfs*3)	Predicted	CRC	52	MSI-H	MSH2−/MSH6−	
318	M	Y	c.3934_3937dup	p.(Ile1313fs)	Not predicted	CRC	52	MSI-H	MSH2 weakly+/ MSH6−	
453	F	Y	c.651dupT	p.(Lys218*)	Predicted	EC	56	MSI-H	MSH2 weakly+/ MSH6−	
457	M	Y	c.3959_3962delCAAG	p.(Ala1320Glufs*6)	Not predicted	CRC	50	MSI-H	MSH2−/MSH6−	
515	F	Y	c.2926_2929dupCGTT	p.(Tyr977Serfs*8)	Predicted	EC	42	MSI-H	MSH2−/MSH6−	Repeated IHC analyses showed also MSH2−/MSH6−. NGS showed a second hit in MSH6 (class 5) and no somatic variants in MSH2 or MSH3 or LOH
555	M	Y	c.3261dupC	p.(Phe1088Leufs*5)	Predicted	CRC	38	MSI-L	MSH2−/MSH6−	IHC done after radiotherapy
573	F	Y	c.261-1G>A	p.?	Predicted	CRC	49	MSI-H	MSH2 weakly+/ MSH6−	
625	F	Y	c.3261dupC	p.(Phe1088Leufs*5)	Predicted	CRC	45	MSI-L	MSH2−/MSH6−	
645	M	N	c.1483C>T	p.(Arg495*)	Predicted	CRC	53	MSI-H	MSH2 weakly+/ MSH6−	Repeated IHC analysis showed also MSH2 weakly+/MSH6−, probably because of technical reasons. Second hit in MSH6 (VUS)
						CRC	61	UNK	MSH2−/MSH6−	NGS showed LOH of MSH2 and MSH6
653	M	Y	c.467C>G	p.(Ser156*)	Predicted	CRC	60	MSI-H	MSH2−/MSH6−	
729	M	Y	c.3261dupC	p.(Phe1088Leufs*5)	Predicted	CRC	47	MSI-H	MSH2−/MSH6−	
733	F	N	c.-13022_-1711del	p.0	Probably no RNA	CRC	45	MSI-H	MSH2−/MSH6−	
						CRC	45	UNK	UNK	
956	F	Y	c.1634_1635delAA	p.(Lys545Argfs*17)	Predicted	CRC	28	MSI-H	MSH2 weakly+/ MSH6−	
992	F	Y	c.1784delT	p.(Leu595Tyrfs*15)	Predicted	CRC	58	MSI-H	MSH2−/MSH6−	
						CRC	59	UNK	UNK	
1120	F	Y	c.1614_1615delTCinsG	p.(Tyr538*)	Predicted	CRC	49	MSI-H	MSH2−/MSH6−	
						EC	58	UNK	UNK	

CRC, colorectal carcinoma; EC, endometrial carcinoma; F, female; IHC MMR, immunohistochemistry of the mismatch repair proteins (ie, MLH1, MSH2, MSH6, and PMS2); LOH, loss of heterozygosity; M, male; MSI, microsatellite instability; MSI-L, MSI low; MSI-H, MSI high; MSS, microsatellite stable; N, no; NGS, next-generation sequencing; NMD, nonsense-mediated decay; UNK, unknown or not tested; VUS, variant of unknown significance; Y, yes.

[a] Quality of material was insufficient, probably because of old material. Therefore, no good internal controls were available for MLH1, MSH2, and MSH6, and results of these protein stainings cannot be shown.

important role of an experienced pathologist when interpreting IHC staining.

Besides the diagnostic pitfalls, the type of *MSH6* germline variant also plays a part in the effectiveness of UTS. One potential explanation is retained antigenicity of proteins harboring missense variants, resulting in a (false)-positive MSH6 staining of a dysfunctional protein.[36,38,58-60] However, this could not be confirmed in our cohort due to the inclusion of patients with primarily frameshift variants, which is a potential result of the current testing strategy for LS. Only those (index) patients with negative MMR staining and/or MSI-high testing, a very young age of onset, and/or a strongly affected family history are offered germline testing.

Somatic variants in one or more of the other MMR genes can also cause atypical staining patterns. The most frequent discordant staining pattern in our cohort was absence ofstaining of both MSH2 and MSH6. Knockout of both alleles of *MSH3* and *MSH6*, either by germline or somatic events, can lead to an absence of MSH2 staining, as a lack of both binding partners can result in protein degradation.[61] However, we did not identify any *MSH3* variants to substantiate this theory. In 1

Table 6
Overview of patients with other staining patterns

ID no.	Sex	Proband	*MSH6* variant			Tumor characteristics				Remarks
			DNA change	Protein change	NMD prediction	Tumor	Age of onset	MSI	IHC MMR	
166	M	Y	c.2764C>T	p.(Arg922*)	Predicted	CRC	71	MSI-H	MLH1 weakly+/MSH2 weakly+/MSH6−	
241	F	Y	c.651dupT	p.(Lys218*)	Predicted	CRC	48	MSI-H	MLH1 weakly+/MSH6−	
292	F	N	c.651dupT	p.(Lys218*)	Predicted	EC	50	UNK	MLH1−/MSH2−/MSH6−	
321	F	Y	c.651dupT	p.(Lys218*)	Predicted	EC	51	UNK	MLH1−/MSH2−/MSH6−	
389	M	Y	c.2982C>G	p.(Tyr994*)	Predicted	CRC	51	MSI-H	MLH1 weakly+/MSH2 weakly+/MSH6−/PMS2 weakly+	
398	F	Y	c.2117T>C	p.(Phe706Ser)	Not predicted	EC	65	MSI-H	MLH1 weakly+/MSH6−/PMS2 weakly +	
426	F	N	c.2050_2051dupCT	p.(Gly685*)	Predicted	CRC	57	MSI-H	MSH6−/PMS2 weakly+	
460	M	Y	c.2050_2051dupCT	p.(Gly685*)	Predicted	CRC	43	MSI-H	MSH2−/MSH6−/PMS2 weakly+	
462	F	Y	c.2577_2580delTTCT	p.(Ser860Leufs*7)	Predicted	CRC	38	MSI-H	MSH2 weakly+/MSH6−/PMS2 weakly+	
644	M	Y	c.1483C>T	p.(Arg495*)	Predicted	CRC	40	MSI-H	Null pattern	
						CRC	57	UNK	UNK	
679	M	Y	c.651dupT	p.(Lys218*)	Predicted	CRC	47	MSI-H	MSH2+/6− multiple times tested. MLH1 is variably +. PMS2 always +	
680	F	N	c.651dupT	p.(Lys218*)	Predicted	CRC	46	UNK	MLH1−/MSH6−/PMS2−	
						CRC	48	UNK	UNK	
						CRC	60	UNK	UNK	
706	M	N	c.3920_3927dupATCTCCCA	p.(Glu1310Ilefs*20)	Predicted	CRC	58	MSI-H	MSH6 − /MLH1−/PM2− (MLH1 hypermethylation)	Repeated IHC concluded also MSH6− /MLH1−/PM2−. Also MLH1 methylation.
						CRC	58	MSI-H	UNK	Repeated IHC concluded the same as in tumor 1.
799	M	Y	c.2805dupT	p.(Asp936*)	Predicted	CRC	39	MSI-H	MSH2−	
835	M	Y	c.2764C>T	p.(Arg922*)	Predicted	CRC	49	MSI-H	MLH1−/MSH6−	Repeated IHC was not possible.
						I	73	UNK	MSH2 weakly positive/ MSH6−	
960	M	Y	c.1634_1635delAA	p.(Lys545Argfs*17)	Predicted	CRC	50	MSI-H	MSH2−/MSH6−/PMS2−	
						CRC	69	UNK	UNK	
						CRC	72	UNK	UNK	
1333	F	Y	c.3920_3927dupATCTCCCA	p.(Glu1310Ilefs*20)	Predicted	CRC	48	MSI-H	MLH1−/MSH2 weakly +/ PMS2 not tested	
						EC	57	UNK	UNK	

CRC, colorectal carcinoma; EC, endometrial carcinoma; F, female; I, duodenum; IHC MMR, immunohistochemistry of the mismatch repair proteins (ie, MLH1, MSH2, MSH6, and PMS2); M, male; MSI, microsatellite instability; MSI-L, MSI low; MSI-H, MSI high; MSS, microsatellite stable; N, no; NGS, next-generation sequencing; NMD, nonsense-mediated decay; UNK, unknown or not tested; Y, yes

tumor with weak staining of MSH2 and loss of MSH6, VUSs in *MSH2* and *MSH3* were found. The *MSH2* VUS may explain the weak staining of MSH2.

The atypical IHC results in 5 cases could be explained by a somatic event. Somatic events, including somatic pathogenic variants or LOH, may explain deficient staining of one of the MMR proteins other than MSH6.[62] However, the atypical staining patterns in the investigated tumors were not solely explained by somatic variants, except for case 706. There, NGS showed a second hit in *MSH6* in both tumors (LOH and in the other tumor somatic duplication, namely, c.3254dupC, p.(Phe1088Leufs*5) causing absence of staining of MSH6, and MLH1 hypermethylation causing absence of staining of MLH1 and PMS2. LOH analysis demonstrated a loss of the allele carrying the germline *MSH6* variant in the other unsolved tumors, indicating that these tumors were sporadic in nature and, therefore, not associated with LS.

A limitation of this study was the retrospective, highly selective nature of this cohort. Family pedigrees and patient data were gathered through clinical genetics departments, and clinical data concerning MSI and IHC results were verified by the genetic counselor when relevant for patient counseling. Therefore, MSI and IHC results were not known in all cases, and other discordant results were potentially missed. Additional testing for heritability in this cohort was mainly initiated in Amsterdam or (repeated) Bethesda-positive families, as UTS became increasingly common in The Netherlands after 2014.[63] Furthermore, pathogenic variants resulting in the retention of antigenicity are less likely to be identified by UTS screening and may, therefore, be underrepresented in our cohort.

Finally, many of the MSI and IHC results dated from the period of 1995 to 2000 and were, therefore, less reliable, as previously explained. Thus, they were more likely to include discordant outcomes compared with current techniques.

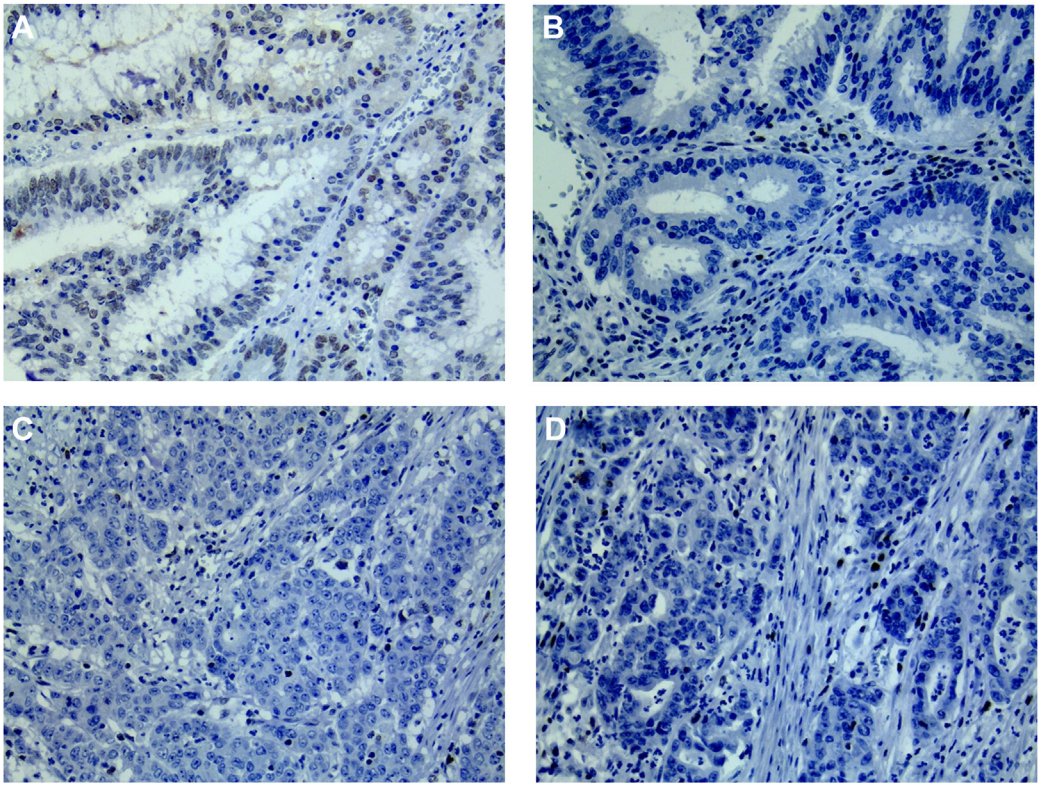

Figure 2.
Focal positive staining results of (A) MSH2 and (B) absence of staining results of MSH6 of ID no. 645 (colorectal carcinoma at 53 year) and absence of staining of (C) MSH2 and (D) MSH6 of ID no. 645 (colorectal carcinoma at 61 year).

In conclusion, this study identified a variety of causes for discordant MSI and IHC results in *MSH6* variant carriers. The pathologist and also the clinician should keep in mind that germline *MSH6* variant carriers can be missed by UTS, which may be due to either retention of staining or to discordant MSI and/or atypical staining patterns. The pathologist should be aware that MSI and IHC MMR, whether or not as reflex testing, is the first step in the genetic diagnosis of LS. The gastroenterologist's referral of the patient to a genetic counselor is mainly based on these results and conclusions reported by the pathologist. Therefore, in the case of normal MSI and/or IHC results, it should be mentioned that further diagnostics for inheritable colon cancer, including LS, should still be considered in cases with a strong family history or young age of cancer diagnosis. Specifically, atypical staining patterns deserve further investigation using germline or tumor analyses, preferably as part of a larger gene panel, especially in families with a high suspicion of LS.

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Author Contributions

A.-S.vd.W.-'t L. performed conceptualization and methodology, formal analysis, investigation, writing – original draft and review and editing, and visualization. D.T. conducted formal analysis and investigation and contributed to writing – review and editing. C.M.T. performed formal analysis and writing – review and editing. M.S.v.K. contributed to methodology and writing – review and editing. L.P.v.H., H.J.P.G., F.A.M.D., A.W., E.L.E., T.G.W.L., M.M.d.J., S.W.B.-t.B., F.E.B., E.B.G.G., and N.d.W. contributed to writing – review and editing. J.T.v.W. conducted conceptualization, methodology, writing – review and editing. H.M. conducted methodology, writing – review and editing. M.S. conducted conceptualization, writing – original draft, methodology, investigation, writing – review and editing. M.N. contributed toward conceptualization, methodology, formal analysis, investigation, writing – original draft and review and editing, visualization, supervision, project administration, funding acquisition.

Data Availability

The data set including the patients with a discordant MSI and or IHC result is included in the Supplementary data. Data of the overall cohort used during the current study are available from the corresponding author upon reasonable request.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Ethical Approval and Consent to Participate

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 Code of Conduct for responsible use of human tissue in the context of health research (Federation of Dutch Medical Scientific Societies).

Supplementary Material

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References

1. Dominguez-Valentin M, Sampson JR, Seppälä TT, et al. Cancer risks by gene, age, and gender in 6350 carriers of pathogenic mismatch repair variants: findings from the Prospective Lynch syndrome Database. *Genet Med*. 2020;22(1):15–25. https://doi.org/10.1038/s41436-019-0596-9
2. Lindor NM, Burgart LJ, Leontovich O, et al. Immunohistochemistry versus microsatellite instability testing in phenotyping colorectal tumors. *J Clin Oncol*. 2002;20(4):1043–1048. https://doi.org/10.1200/JCO.2002.20.4.1043
3. Seppälä TT, Latchford A, Negoi I, et al. European guidelines from the EHTG and ESCP for Lynch syndrome: an updated third edition of the Mallorca guidelines based on gene and gender. *Br J Surg*. 2021;108(5):484–498. https://doi.org/10.1002/bjs.11902
4. Leclerc J, Vermaut C, Buisine MP. Diagnosis of Lynch syndrome and strategies to distinguish Lynch-related tumors from sporadic MSI/dMMR tumors. *Cancers (Basel)*. 2021;13(3):467. https://doi.org/10.3390/cancers13030467
5. Adar T, Rodgers LH, Shannon KM, et al. Universal screening of both endometrial and colon cancers increases the detection of Lynch syndrome. *Cancer*. 2018;124(15):3145–3153. https://doi.org/10.1002/cncr.31534
6. Chika N, Eguchi H, Kumamoto K, et al. Prevalence of Lynch syndrome and Lynch-like syndrome among patients with colorectal cancer in a Japanese hospital-based population. *Jpn J Clin Oncol*. 2017;47(2):108–117. https://doi.org/10.1093/jjco/hyw178
7. Dong L, Jin X, Wang W, et al. Distinct clinical phenotype and genetic testing strategy for Lynch syndrome in China based on a large colorectal cancer cohort. *Int J Cancer*. 2020;146(11):3077–3086. https://doi.org/10.1002/ijc.32914
8. Haraldsdottir S, Rafnar T, Frankel WL, et al. Comprehensive population-wide analysis of Lynch syndrome in Iceland reveals founder mutations in MSH6 and PMS2. *Nat Commun*. 2017;8:14755. https://doi.org/10.1038/ncomms14755
9. Jiang W, Cai MY, Li SY, et al. Universal screening for Lynch syndrome in a large consecutive cohort of Chinese colorectal cancer patients: high prevalence and unique molecular features. *Int J Cancer*. 2019;144(9):2161–2168. https://doi.org/10.1002/ijc.32044
10. Kang SY, Park CK, Chang DK, et al. Lynch-like syndrome: characterization and comparison with EPCAM deletion carriers. *Int J Cancer*. 2015;136(7):1568–1578. https://doi.org/10.1002/ijc.29133
11. Pearlman R, Haraldsdottir S, de la Chapelle A, et al. Clinical characteristics of patients with colorectal cancer with double somatic mismatch repair mutations compared with Lynch syndrome. *J Med Genet*. 2019;56(7):462–470. https://doi.org/10.1136/jmedgenet-2018-105698
12. Guyot D'Asnières De Salins A, Tachon G, Cohen R, et al. Discordance between immunochemistry of mismatch repair proteins and molecular testing of microsatellite instability in colorectal cancer. *ESMO Open*. 2021;6(3):100120. https://doi.org/10.1016/j.esmoop.2021.100120
13. Sugawara T, Sato N, Shimizu D, et al. Efficient screening strategy for Lynch syndrome in Japanese endometrial cancer. *Tohoku J Exp Med*. 2015;235(2):117–125. https://doi.org/10.1620/tjem.235.117
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. *Gynecol Oncol*. 2015;137(2):306–310. https://doi.org/10.1016/j.ygyno.2015.01.541
15. Mas-Moya J, Dudley B, Brand RE, et al. Clinicopathological comparison of colorectal and endometrial carcinomas in patients with Lynch-like syndrome versus patients with Lynch syndrome. *Hum Pathol*. 2015;46(11):1616–1625. https://doi.org/10.1016/j.humpath.2015.06.022
16. Mills AM, Sloan EA, Thomas M, et al. Clinicopathologic comparison of Lynch syndrome-associated and "Lynch-like" endometrial carcinomas identified on universal screening using mismatch repair protein immunohistochemistry. *Am J Surg Pathol*. 2016;40(2):155–165. https://doi.org/10.1097/PAS.0000000000000544
17. 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. *J Clin Oncol*. 2015;33(36):4301–4308. https://doi.org/10.1200/JCO.2015.63.9518
18. Frolova AI, Babb SA, Zantow E, et al. Impact of an immunohistochemistry-based universal screening protocol for Lynch syndrome in endometrial cancer on genetic counseling and testing. *Gynecol Oncol*. 2015;137(1):7–13. https://doi.org/10.1016/j.ygyno.2015.01.535
19. Watkins JC, Nucci MR, Ritterhouse LL, Howitt BE, Sholl LM. Unusual mismatch repair immunohistochemical patterns in endometrial carcinoma. *Am J Surg Pathol*. 2016;40(7):909–916. https://doi.org/10.1097/PAS.0000000000000663
20. Rubio I, Ibáñez-Feijoo E, Andrés L, et al. Analysis of Lynch syndrome mismatch repair genes in women with endometrial cancer. *Oncology*. 2016;91(3):171–176. https://doi.org/10.1159/000447972
21. Najdawi F, Crook A, Maidens J, et al. Lessons learnt from implementation of a Lynch syndrome screening program for patients with gynaecological malignancy. *Pathology*. 2017;49(5):457–464. https://doi.org/10.1016/j.pathol.2017.05.004
22. Dillon JL, Gonzalez JL, DeMars L, Bloch KJ, Tafe LJ. Universal screening for Lynch syndrome in endometrial cancers: frequency of germline mutations and identification of patients with Lynch-like syndrome. *Hum Pathol*. 2017;70:121–128. https://doi.org/10.1016/j.humpath.2017.10.022
23. Suerink M, Kilinç G, Terlouw D, et al. Prevalence of mismatch repair deficiency and Lynch syndrome in a cohort of unselected small bowel adenocarcinomas. *J Clin Pathol*. 2021;74(11):724–729. https://doi.org/10.1136/jclinpath-2020-207040
24. Inácio A, Silva AL, Pinto J, et al. Nonsense mutations in close proximity to the initiation codon fail to trigger full nonsense-mediated mRNA decay. *J Biol Chem*. 2004;279(31):32170–32180. https://doi.org/10.1074/jbc.M405024200
25. Shyu AB, Wilkinson MF, van Hoof A. Messenger RNA regulation: to translate or to degrade. *EMBO J*. 2008;27(3):471–481. https://doi.org/10.1038/sj.emboj.7601977
26. Li D, Hoodfar E, Jiang SF, et al. Comparison of universal versus age-restricted screening of colorectal tumors for Lynch syndrome using mismatch repair immunohistochemistry: a cohort study. *Ann Intern Med*. 2019;171(1):19–26. https://doi.org/10.7326/M18-3316
27. Brennan B, Hemmings CT, Clark I, Yip D, Fadia M, Taupin DR. Universal molecular screening does not effectively detect Lynch syndrome in clinical practice. *Ther Adv Gastroenterol*. 2017;10(4):361–371. https://doi.org/10.1177/1756283X17690990
28. 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. *Am J Surg Pathol*. 2015;39(10):1370–1376. https://doi.org/10.1097/PAS.0000000000000459
29. Wu Y, Berends MJ, Mensink RG, et al. Association of hereditary nonpolyposis colorectal cancer-related tumors displaying low microsatellite instability with MSH6 germline mutations. *Am J Hum Genet*. 1999;65(5):1291–1298. https://doi.org/10.1086/302612
30. Pan S, Cox H, Willmott J, et al. Discordance between germline genetic findings and abnormal tumor immunohistochemistry staining of mismatch repair proteins in individuals with suspected Lynch syndrome. *Front Oncol*. 2023;13, 1069467. https://doi.org/10.3389/fonc.2023.1069467
31. Okkels H, Lindorff-Larsen K, Thorlasius-Ussing O, et al. MSH6 mutations are frequent in hereditary nonpolyposis colorectal cancer families with normal pMSH6 expression as detected by immunohistochemistry. *Appl Immunohistochem Mol Morphol*. 2012;20(5):470–477. https://doi.org/10.1097/PAI.0b013e318249739b
32. Overbeek LI, Ligtenberg MJ, Willems RW, et al. Interpretation of immunohistochemistry for mismatch repair proteins is only reliable in a specialized setting. *Am J Surg Pathol*. 2008;32(8):1246–1251. https://doi.org/10.1097/pas.0b013e31816401bb
33. Mangold E, Pagenstecher C, Friedl W, et al. Tumours from MSH2 mutation carriers show loss of MSH2 expression but many tumours from MLH1 mutation carriers exhibit weak positive MLH1 staining. *J Pathol*. 2005;207(4):385–395. https://doi.org/10.1002/path.1858
34. Watson N, Grieu F, Morris M, et al. Heterogeneous staining for mismatch repair proteins during population-based prescreening for hereditary nonpolyposis colorectal cancer. *J Mol Diagn*. 2007;9(4):472–478. https://doi.org/10.2353/jmoldx.2007.060162
35. Sarode VR, Robinson L. Screening for Lynch syndrome by immunohistochemistry of mismatch repair proteins: significance of indeterminate result and correlation with mutational studies. *Arch Pathol Lab Med*. 2019;143(10):1225–1233. https://doi.org/10.5858/arpa.2018-0201-OA
36. Shia J, Ellis NA, Paty PB, et al. Value of histopathology in predicting microsatellite instability in hereditary nonpolyposis colorectal cancer and sporadic colorectal cancer. *Am J Surg Pathol*. 2003;27(11):1407–1417. https://doi.org/10.1097/00000478-200311000-00002
37. 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. *J Mol Diagn*. 2008;10(4):293–300. https://doi.org/10.2353/jmoldx.2008.080031
38. Hechtman JF, Rana S, Middha S, et al. Retained mismatch repair protein expression occurs in approximately 6% of microsatellite instability-high cancers and is associated with missense mutations in mismatch repair genes. *Mod Pathol*. 2020;33(5):871–879. https://doi.org/10.1038/s41379-019-0414-6
39. Engel C, Forberg J, Holinski-Feder E, et al. Novel strategy for optimal sequential application of clinical criteria, immunohistochemistry and microsatellite analysis in the diagnosis of hereditary nonpolyposis colorectal cancer. *Int J Cancer*. 2006;118(1):115–122. https://doi.org/10.1002/ijc.21313
40. Ryan E, Sheahan K, Creavin B, Mohan HM, Winter DC. The current value of determining the mismatch repair status of colorectal cancer: a rationale for routine testing. *Crit Rev Oncol Hematol*. 2017;116:38–57. https://doi.org/10.1016/j.critrevonc.2017.05.006
41. Pearlman R, Markow M, Knight D, et al. Two-stain immunohistochemical screening for Lynch syndrome in colorectal cancer may fail to detect mismatch repair deficiency. *Mod Pathol*. 2018;31(12):1891–1900. https://doi.org/10.1038/s41379-018-0058-y
42. McCarthy AJ, Capo-Chichi JM, Spence T, et al. Heterogenous loss of mismatch repair (MMR) protein expression: a challenge for immunohistochemical interpretation and microsatellite instability (MSI) evaluation. *J Pathol Clin Res*. 2019;5(2):115–129. https://doi.org/10.1002/cjp2.120
43. Chen W, Pearlman R, Hampel H, et al. MSH6 immunohistochemical heterogeneity in colorectal cancer: comparative sequencing from different tumor areas. *Hum Pathol*. 2020;96:104–111. https://doi.org/10.1016/j.humpath.2019.11.003
44. Scheiderer A, Riedinger C, Kimball K, Kilgore L, Orucevic A. Reporting subclonal immunohistochemical staining of mismatch repair proteins in endometrial carcinoma in the times of ever-changing guidelines. *Arch Pathol Lab Med*. 2022;146(9):1114–1121. https://doi.org/10.5858/arpa.2021-0201-OA
45. Abdullah M, Sudoyo AW, Utomo AR, Fauzi A, Rani AA. Molecular profile of colorectal cancer in Indonesia: is there another pathway? *Gastroenterol Hepatol Bed Bench*. 2012;5(2):71–78. PMID 24834203.
46. Kuismanen SA, Moisio AL, Schweizer P, et al. Endometrial and colorectal tumors from patients with hereditary nonpolyposis colon cancer display different patterns of microsatellite instability. *Am J Pathol*. 2002;160(6):1953–1958. https://doi.org/10.1016/s0002-9440(10)61144-3
47. Wong YF, Cheung TH, Lo KW, et al. Detection of microsatellite instability in endometrial cancer: advantages of a panel of five mononucleotide repeats over the National Cancer Institute panel of markers. *Carcinogenesis*. 2006;27(5):951–955. https://doi.org/10.1093/carcin/bgi333
48. Libera L, Sahnane N, Carnevali IW, et al. Microsatellite analysis of sporadic and hereditary gynaecological cancer in routine diagnostics. *J Clin Pathol*. 2017;70(9):792–797. https://doi.org/10.1136/jclinpath-2017-204348
49. Wang Y, Shi C, Eisenberg R, Vnencak-Jones CL. Differences in microsatellite instability profiles between endometrioid and colorectal cancers: a potential cause for false-negative results? *J Mol Diagn*. 2017;19(1):57–64. https://doi.org/10.1016/j.jmoldx.2016.07.008
50. Palomaki GE, McClain MR, Melillo S, Hampel HL, Thibodeau SN. EGAPP supplementary evidence review: DNA testing strategies aimed at reducing morbidity and mortality from Lynch syndrome. *Genet Med*. 2009;11(1):42–65. https://doi.org/10.1097/GIM.0b013e31818fa2db
51. Hendriks YM, Wagner A, Morreau H, et al. Cancer risk in hereditary nonpolyposis colorectal cancer due to MSH6 mutations: impact on counseling and surveillance. *Gastroenterology*. 2004;127(1):17–25. https://doi.org/10.1053/j.gastro.2004.03.068
52. Goel A, Nagasaka T, Hamelin R, Boland CR. An optimized pentaplex PCR for detecting DNA mismatch repair-deficient colorectal cancers. *PloS One*. 2010;5(2):e9393. https://doi.org/10.1371/journal.pone.0009393
53. You JF, Buhard O, Ligtenberg MJ, et al. Tumours with loss of MSH6 expression are MSI-H when screened with a pentaplex of five mononucleotide repeats. *Br J Cancer*. 2010;103(12):1840–1845. https://doi.org/10.1038/sj.bjc.6605988
54. Pagin A, Zerimech F, Leclerc J, et al. Evaluation of a new panel of six mononucleotide repeat markers for the detection of DNA mismatch repair-deficient tumours. *Br J Cancer*. 2013;108(10):2079–2087. https://doi.org/10.1038/bjc.2013.213
55. Verma L, Kane MF, Brassett C, et al. Mononucleotide microsatellite instability and germline MSH6 mutation analysis in early onset colorectal cancer. *J Med Genet*. 1999;36(9):678–682.
56. Xicola RM, Llor X, Pons E, et al. Performance of different microsatellite marker panels for detection of mismatch repair-deficient colorectal tumors. *J Natl Cancer Inst*. 2007;99(3):244–252. https://doi.org/10.1093/jnci/djk033
57. Zhang L. Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. Part II. The utility of microsatellite instability testing. *J Mol Diagn*. 2008;10(4):301–307. https://doi.org/10.2353/jmoldx.2008.080062
58. Wahlberg SS, Schmeits J, Thomas G, et al. Evaluation of microsatellite instability and immunohistochemistry for the prediction of germ-line MSH2 and MLH1 mutations in hereditary nonpolyposis colon cancer families. *Cancer Res*. 2002;62(12):3485–3492. PMID 12067992.
59. Chen W, Hampel H, Pearlman R, et al. Unexpected expression of mismatch repair protein is more commonly seen with pathogenic missense than with other mutations in Lynch syndrome. *Hum Pathol*. 2020;103:34–41. https://doi.org/10.1016/j.humpath.2020.07.001
60. van Riel E, Ausems MG, Hogervorst FB, et al. A novel pathogenic MLH1 missense mutation, c.112A > C, p.Asn38His, in six families with Lynch syndrome. *Hered Cancer Clin Pract*. 2010;8(1):7. https://doi.org/10.1186/1897-4287-8-7
61. Morak M, Käsbauer S, Kerscher M, et al. Loss of MSH2 and MSH6 due to heterozygous germline defects in MSH3 and MSH6. *Fam Cancer*. 2017;16(4):491–500. https://doi.org/10.1007/s10689-017-9975-z
62. Haraldsdottir S, Hampel H, Tomsic J, et al. Colon and endometrial cancers with mismatch repair deficiency can arise from somatic, rather than germline, mutations. *Gastroenterology*. 2014;147(6):1308–1316.e1. https://doi.org/10.1053/j.gastro.2014.08.041
63. van Lier MG, Leenen CH, Wagner A, et al. Yield of routine molecular analyses in colorectal cancer patients ≤70 years to detect underlying Lynch syndrome. *J Pathol*. 2012;226(5):764–774. https://doi.org/10.1002/path.3963