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Universal Immunohistochemistry for Lynch Syndrome: A Systematic Review and Meta-analysis of 58,580 Colorectal Carcinomas

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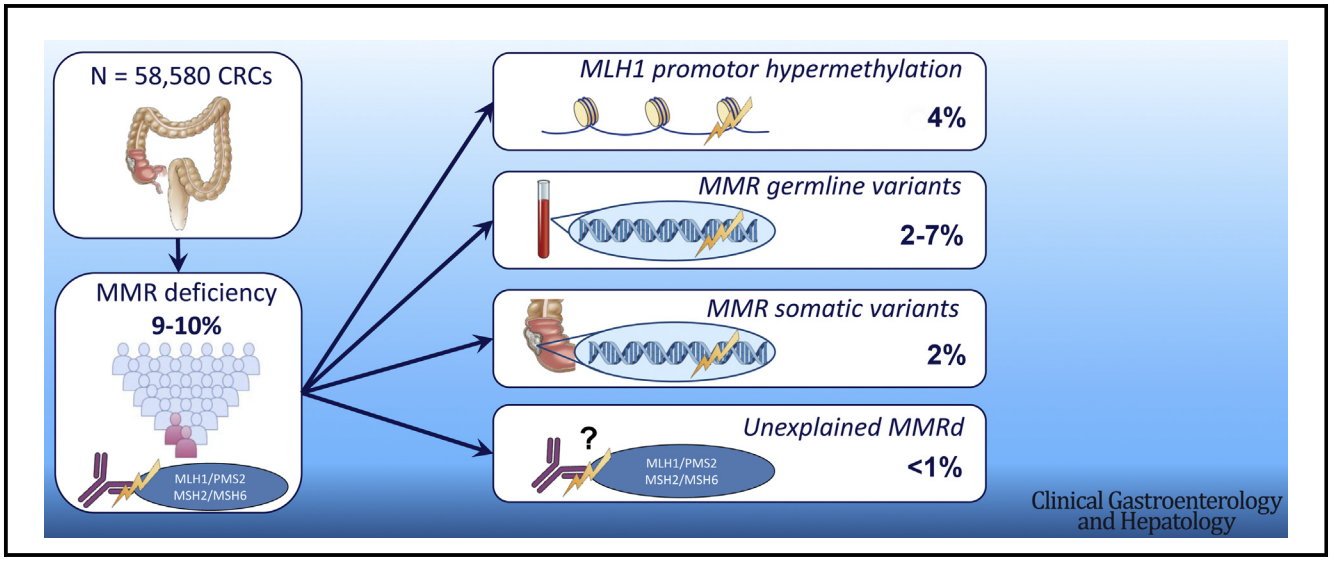

BACKGROUND & AIMS: Lynch syndrome is a form of hereditary colorectal cancer (CRC) caused by pathogenic germline variants (PV) in DNA mismatch repair (MMR) genes. Currently, many Western countries perform universal immunohistochemistry testing on CRC to increase the identification of Lynch syndrome patients and their relatives. For a clear understanding of health benefits and costs, data on its outcomes are required: proportions of Lynch syndrome, sporadic MMR-deficient (MMRd) cases, and unexplained MMRd cases.

METHODS: Ovid Medline, Embase, and Cochrane CENTRAL were searched for studies reporting on universal MMR immunohistochemistry, followed by MMR germline analysis, until March 20, 2020. Proportions were calculated, subgroup analyses were performed based on age and diagnostics used, and random effects meta-analyses were conducted. Quality was assessed using the Joanna Briggs Critical Appraisal Tool for Prevalence Studies.

RESULTS: Of 2723 identified articles, 56 studies covering 58,580 CRCs were included. In 6.22% (95% CI, 5.08%–7.61%; $I^2 = 96\%$) MMRd was identified. MMR germline PV was present in 2.00% (95%

[a]Authors share co-first authorship. [b]Authors share co-senior authorship.

Abbreviations used in this paper: CRC, colorectal cancer; MMR, mismatch repair; IHC, immunohistochemistry; LLS, Lynch-like syndrome; LOH, loss of heterozygosity; LS, Lynch syndrome; MMRd, mismatch repair deficient; MSI, microsatellite instability.

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CI, 1.59%–2.50%; I^2 = 92%), ranging from 1.80% to 7.27% based on completeness of diagnostics and age restriction. Immunohistochemistry outcomes were missing in 11.81%, and germline testing was performed in 76.30% of eligible patients. In 7 studies, including 6848 CRCs completing all diagnostic stages, germline PV and biallelic somatic MMR inactivation were found in 3.01% and 1.75%, respectively; 0.61% remained unexplained MMRd.

CONCLUSIONS: **Age, completeness, and type of diagnostics affect the percentage of MMR PV and unexplained MMRd percentages. Complete diagnostics explain almost all MMRd CRCs, reducing the amount of subsequent multigene panel testing. This contributes to optimizing testing and surveillance in MMRd CRC patients and relatives.**

Keywords: Lynch Syndrome; Universal Tumor Screening; Mismatch Repair Deficiency; Immunohistochemistry; Colorectal Cancer.

See editorial on page e354.

Individuals with Lynch syndrome (LS) have an increased risk of developing multiple tumors, particularly colorectal cancer (CRC) and endometrial cancer. This increased cancer risk is caused by pathogenic germline variants in the DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, *PMS2*, or a deletion of the 3' end of the *EpCAM* (TACSTD1) gene, resulting in hypermethylation of the *MSH2* promoter.[1–5] When a second hit arises in the corresponding MMR allele, mismatch repair is hampered, leading to MMR deficiency (MMRd). MMRd causes accumulation of insertions and deletions in short repetitive DNA sequences, a phenomenon called microsatellite instability (MSI).

Nevertheless, LS is the underlying cause in only part of the MMR-deficient tumors. Sporadic events such as hypermethylation of the *MLH1* promoter[6] or biallelic somatic inactivation of a MMR gene also can cause MMRd. Distinguishing between pathogenic MMR germline variants (LS) and sporadic MMRd is relevant for accurate assessment of cancer risks in these individuals and the need for cascade screening in their relatives.[7] These individuals and their relatives then can be offered tailored surveillance and other preventive measures.[8,9]

Indications for the presence of potential pathogenic variants causative for LS can be found by performing MSI analyses using DNA isolated from tumors. However, a cheaper and less labor intensive method is immunohistochemistry (IHC) for MMR proteins[10] because absence of at least 1 protein is indicative for underlying MMRd. Both methods, which are usually performed by the Pathology Department, are deemed acceptable to indicate an underlying DNA MMR defect.

Until recently, LS diagnostics were performed only in cases with a clinical suspicion for LS, for example, when the revised Bethesda criteria were met.[11] However, adhering to this approach results in half of the LS cases among CRC patients remaining undetected.[12] This problem stimulated the implementation of universal, unselected IHC testing (also called *reflex testing*) for CRC in many Western countries over the past decade. After an MMR-deficient protein staining result, patients usually are offered germline genetic testing of the corresponding gene or genes.

The proportions of germline variants and sporadic variants in MMR testing varies greatly between studies.[13–15] The variation in studies likely is owing to differences in testing strategies, such as performance of *BRAF* pathogenic variant analysis without analysis of *MLH1* promoter hypermethylation, preselection of CRC cases (based on age or other criteria) to be tested for MMRd, or a lack of complete diagnostics being performed. More accurate proportions of LS and unexplained MMRd cases are warranted: not only to determine the benefits and costs of universal MMR testing, but also in relation to the quest for more suitable recommendations in clinical guidelines.

Therefore, we performed a systematic review with random-effects meta-analyses and meta-regressions in unselected CRC patients tested with universal MMR IHC. Our aim was to evaluate the outcomes of MMR IHC in terms of proportions of LS, sporadic MMRd, and unexplained MMRd.

Materials and Methods

The protocol for this systematic review and meta-analysis was preregistered at the PROSPERO database (registration ID: CRD42019130651, https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42019130651).

This manuscript was drafted according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines.[16]

Literature Search, Data Extraction, and Quality Assessment

Ovid Medline, Embase, and Cochrane CENTRAL were searched systematically up to May 20, 2019, for studies that assessed universal IHC testing for MMR proteins in CRCs, followed by at least MMR germline testing. On March 20, 2020, the search was repeated. Studies were eligible for inclusion if universal IHC for at least 1 MMR protein was performed in a series of CRCs that were described in that study as population-

based or consecutive, regardless of study design. We manually searched the reference lists of retrieved reports for additional studies meeting our inclusion criteria. Studies were assessed using Rayyan Intelligent Systematic Review (Cambridge, MA),[17] and duplicates subsequently were removed as described by Bramer et al.[18] Studies were screened independently by 2 authors (E.L.E. and A.S.W.) by evaluating the title and abstract, followed by full-text evaluation. In case of disagreement, a third author made the final decision (M.N.).

Data from included studies were extracted independently by 2 authors (E.L.E. and A.S.W.). A standardized format was used according to which data were extracted. In case of missing data, the corresponding authors were contacted for remaining results by e-mail. In case of other missing data, we contacted corresponding authors for remaining results by e-mail. When a cohort was described multiple times, the article providing us with the most information regarding diagnostic LS assays was used and shown in analyses (supplemented by other data from the matching cohort). In addition, authors were contacted for individual data. When articles included multiple cohorts, of which at least 1 was an unselected population, we approached corresponding authors for separate data on this unselected cohort. Subsequently, only these unselected cohorts were included in further analyses.

Potential bias in studies was assessed according to the Joanna Briggs Institute critical appraisal tool for prevalence studies.[19] Studies were scored based on 9 questions: sample frame, whether the study participants were sampled in the appropriate way, adequate sample size, description of study subjects, data analysis, methods for the identification of the condition, measurement of this condition, and adequate response rate. Items were scored individually by 2 authors (E.L.E. and A.S.W.).

For a full description of the literature search, data extraction, missing data, and quality assessment see Supplementary Table 1 through 3.

Outcomes: Main Analysis

The outcome consisted of the proportion of patients with LS (including chances of carrying a germline variant during the genetic workflow), sporadic MMRd, and unexplained MMRd after (at least) universal MMR IHC and MMR germline analysis had been performed. Causes of deficient MMR protein staining were assessed in included studies and divided in MMR germline events, sporadic *MLH1* events (pathogenic *BRAF* variant and/or *MLH1* promoter hypermethylation), and somatic variants (biallelic somatic pathogenic variant or 1 somatic variant with loss of heterozygosity [LOH] of the other allele). In addition, meta-analyses were performed for the probability of carrying a germline variant in different circumstances.

What You Need to Know

Background

Lynch syndrome (LS) patients have an increased risk of developing colorectal cancer (CRC) in particular owing to germline pathogenic variants in DNA mismatch repair (MMR) genes. To identify LS, universal CRC MMR immunohistochemistry is performed routinely in Western countries, although its outcomes (in age categories, with subsequent diagnostic tests) have not been analyzed thoroughly.

Findings

In our systematic review and meta-analysis, the proportion of LS patients varied depending on age and diagnostic tests performed, but was highest (7.3%) in patients younger than age 50. In 58,580 CRCs, 4.3% remained unexplained. In our subgroup analysis with all diagnostic assays performed, this was 0.6%, representing 11.1% of MMR-deficient (MMRd) CRCs.

Implications for patient care

The extent to which MMRd CRCs can be explained depends highly on the completeness of the diagnostic assays in LS diagnostics, particularly *MLH1* promotor hypermethylation and somatic mutation analysis. Explaining MMRd CRCs is relevant in clinical practice for offering accurate surveillance to patients as well as their relatives.

Outcomes: Subgroup Analyses

Not all included studies performed additional assays besides MMR IHC and MMR germline analyses to determine potential explanations of the deficient MMR protein staining, as shown in Supplementary Figure 1. As a consequence, we expected to encounter missing data in our main analysis as a result of, among others, diagnostic tests that were not (completely) performed or outcomes that were not (completely) mentioned. When studies performed other assays than IHC and germline analysis, these studies were included for subgroup analyses in an effort to assess diagnostic outcomes more precisely. Therefore, a series of subgroup analyses were conducted of studies that performed IHC for all 4 MMR proteins (complete IHC) vs studies with incomplete MMR IHC (subgroup analysis 1); studies that performed *MLH1* promoter hypermethylation with or without *BRAF* analysis vs studies with only BRAF(V600E) analysis vs studies without methylation or BRAF(V600E) analysis (subgroup analysis 2); studies including only participants younger than age 50 years vs studies including participants younger than age 70 years vs studies including participants without age restrictions (subgroup analysis 3); and studies that performed somatic DNA

analysis for all 4 DNA MMR genes vs studies with incomplete or no somatic mutation analysis (subgroup analysis 4). For a full description of the subgroup analyses see Supplementary Table 1.

Statistical Analyses

Data in each category were extracted from the included full-text studies (number of CRCs tested, outcomes of IHC, germline analyses, and somatic analyses) and summed. To compare raw proportions resulting from subgroup analyses, chi-squared tests were performed. Afterward, pooled proportion meta-analyses were performed using a random-effects model (generalized linear mixed model) to account for study heterogeneity. Meta-analyses were performed to assess the following proportions: the number of CRCs that required subsequent germline analysis (meta-analysis 1), the probability of carrying a germline variant in case of a CRC (meta-analysis 2), the probability of carrying a germline variant in case of MMR-deficient protein staining (meta-analysis 3), and the probability of carrying a germline variant when germline analyses were performed (meta-analysis 4). For these meta-analyses, only studies reporting on the corresponding outcomes were included. Because of the potential to explain heterogeneity between studies, various factors including country, end of study period, and applied inclusion and exclusion criteria of individual included studies (such as exclusion of patients with inflammatory bowel disease, polyposis syndromes, or a history of CRC) were included as covariates in the meta-regression. Meta-analyses were performed only when 5 studies or more were available.

The Cochrane Q statistic and I^2 with corresponding 95% CI were used to assess heterogeneity between studies.[20] In case of an I^2 greater than 75%, heterogeneity between studies was considered high and a random-effects meta-regression was performed. Because of the potential to explain heterogeneity between studies, various factors including country, end of study period, and inclusion and exclusion criteria were included as covariates in the meta-regression. In addition, the presence of publication bias was investigated by visual inspection of funnel plots and formal testing using the Egger test ($P < .05$ was considered statistically significant). Statistical analyses were performed using SPSS (IBM Statistics, NY) version 25 and R (R: A Language and Environment for Statistical Computing. Vienna, Austria) package meta.

Results

Literature Search

The electronic database search identified 2723 articles; 5 articles were identified subsequently during a manual search.[13,21–24] Of these, 116 articles were deemed eligible for full-text review, which resulted in inclusion of 54 articles. The remaining 62 articles were excluded for various reasons (Figure 1). The included cohorts overlapped (partially) in 11 studies. After repeating the search, eventually 56 articles (corresponding to 58 data sets) were included. An overview of included cohorts and their associated characteristics can be found in Supplementary Table 4.

Universal Immunohistochemistry Outcomes: Main Cohort and Subgroup Analysis 1

Pooled data identified 58,580 CRCs, where universal IHC was performed for at least 1 MMR protein (See Table 1, Figure 2, and Supplementary Figures 2–7 for an overview of the performed analyses). Because of missing data or failed IHC testing, IHC results were unknown for 11.81% of all CRCs (n = 6920). MMR-proficient protein staining was identified in 78.14% (n = 45,776 CRCs) of all CRCs, whereas 10.04% (n = 5884) showed an MMR-deficient protein staining (Table 1). When studies that performed IHC for all 4 MMR proteins (with complete IHC, subgroup analysis 1, Supplementary Figure S2) were compared with studies with incomplete IHC, the former group contained significantly more abnormal IHCs ($P < .001$) (Table 1). In the main cohort of 58,580 CRCS, MLH1/PMS2, MSH2/MSH6, MSH6, and PMS2 were absent in 69.26% (n = 4075), 16.69% (n = 982), 5.73% (n = 337), and 4.71% (n = 277) of abnormal IHCs, respectively. Other staining patterns were seen in 2.84% (n = 167) of CRCs with MMR-deficient protein staining. In the remaining 0.78% (n = 46) MMR-deficient CRCs, specific IHC outcomes were not mentioned (Figure 3). These percentages were comparable with those identified in subgroup analysis 1 regarding studies with IHC performed for all 4 MMR proteins (Table 1, Supplementary Figure 2). Upon exclusion of CRCs with MMR deficiency owing to *MLH1* promoter hypermethylation (n = 2099; 51.51% of MLH1-deficient CRCs), the proportion of MMR-deficient CRCs in our main analysis was found to represent 6.22% (95% CI, 5.08%–7.61%; $I^2 = 96\%$) of all CRCs. Therefore, germline analyses were deemed necessary in 6.22% of the total study population (Supplementary Figure 8). The extent to which complete IHC was performed did not significantly affect the amount of identified pathogenic MMR germline variants later in the diagnostic process ($P = .913$) (Table 1).

BRAF *(V600E) and* MLH1 *Promotor Hypermethylation: Subgroup Analysis 2*

In addition, outcomes of studies that performed *MLH1* promoter hypermethylation assays with or without *BRAF* (V600E) testing were compared with those in studies that only performed *BRAF* analysis and

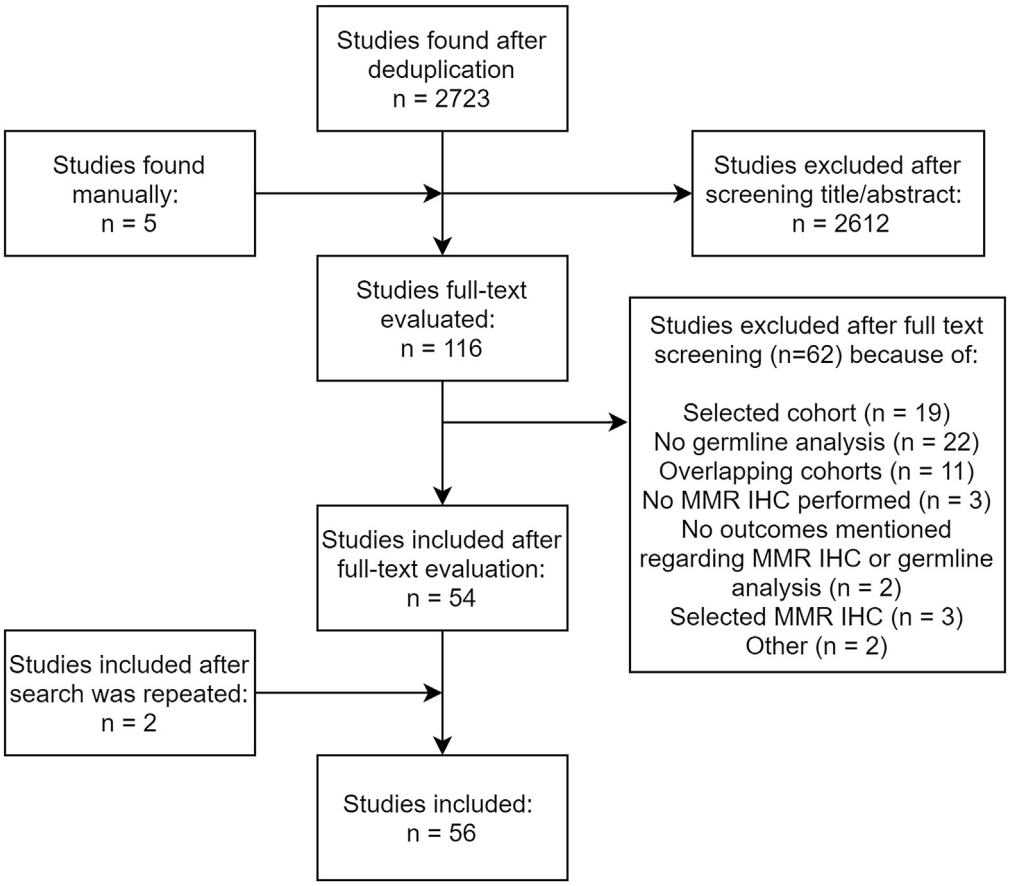

Figure 1. Flowchart of study inclusion. IHC, immunohistochemistry; MMR, mismatch repair.

studies that performed neither of these analyses (subgroup analysis 2). Thirteen studies did not perform or did not mention outcomes of *MLH1* promoter hypermethylation assays or *BRAF* (V600E) testing. In studies that performed *MLH1* promoter hypermethylation assays with or without *BRAF* (V600E) testing, 60.55% (n = 1742) of MLH1-deficient protein staining was found to be explained by *MLH1* promoter hypermethylation or a *BRAF* variant, compared with 41.56 % (n = 357) in studies performing *BRAF* analysis alone (Table 1, Supplementary Figures 3 and 4). In studies that performed *MLH1* promoter hypermethylation assays with or without *BRAF* mutation analysis, absence of MLH1 was explained significantly more often than studies that only performed *BRAF* (P <.001) (Table 1). However, performance of these analyses did not yield significantly more pathogenic MMR germline variants (P < .001) (Table 1, Supplementary Figures 3 and 4).

Germline Analysis

All included studies performed germline analysis for at least 1 MMR gene. However, in only 42 studies, germline analysis was performed for all 4 MMR genes; that is, for the gene or genes corresponding to the protein or proteins that were absent at the MMR-deficient protein staining. All included studies together, irrespective of the genes for which they had performed germline analysis, reported 3785 CRCs with any MMR-deficient protein staining, without *MLH1* hypermethylation. Of these CRCs with MMR-deficient protein staining, without *MLH1* hypermethylation, 31.65% (n = 1198) were found to carry a germline variant in 1 of the MMR genes. This corresponds with an overall pooled proportion of 33.50% (95% CI, 26.06%–41.86%; I^2 = 93%) of MMR-deficient CRCs caused by a pathogenic MMR germline variant (Supplementary Figure 9). When compared with the total of 58,580 CRCs, the proportion of identified pathogenic germline variants represents 2.00% (95% CI, 1.59%–2.50%; I^2 = 92%; n = 1198) of these CRCs (Supplementary Figure 10).

In non-*MLH1* hypermethylated MMR-deficient protein staining, pathogenic germline variants were found in the genes *MLH1*, *MSH2*, *EpCAM*, *MSH6*, and *PMS2* in 10.62%, 10.67%, 0.92%, 5.18%, and 3.30%, respectively (Supplementary Table 5). During an assessment per protein individually, the following percentages of germline variants were found: in nonmethylated MLH1-negative cases, 20.34% was explained by germline *MLH1* variants; in MSH2 this was 44.70% (including *EpCAM*), in MSH6 this was 58.16%, and in PMS2 this was 45.13% (Supplementary Table 6).

Table 1. Overview Subgroup Analyses and Outcomes

	(Subgroup) analysis[a]	Cohorts, n	Sample size, n	Retained IHC, %	Abnormal IHC, %	Unknown IHC results, %	*BRAF*(V600E) and/or hypermethylation of total cohort (and of absent *MLH1* staining),[b] %	Germline tested of abnormal IHC without *MLH1* hypermethylation,[b] %	Germline variants in patients germline tested,[c] %	Germline variants, %	Explained by double somatic variants (or LOH), %	Unexplained MMRd[d] (of which truly unexplained), %
Main analysis		58	58,580	78.14	10.04	11.81	3.58 (51.51)	76.30	36.65	1.98	0.24	4.24 (N/A)
1	Complete IHC	39	47,850	85.45	10.59	3.96	3.96 (53.35)	77.50	36.85	1.97	0.25	4.37 (N/A)
	Incomplete IHC	19	10,730	63.47	7.62	28.91	1.91 (39.05)	64.17	35.46	2.05	0.07	4.17 (N/A)
2	Methylation with or without *BRAF*(V600E)	33	39,351	85.30	10.41	4.29	4.43 (60.55)	82.60	37.14	1.83	0.32	3.77 (N/A)
	Only *BRAF*(V600E)	12	13,886	82.56	9.41	8.03	2.57 (41.56)	63.73	41.56	1.80	0.12	5.11 (N/A)
	No methylation or *BRAF*(V600E) tested	13	5343	49.95	9.00	41.05	N/A	N/A	28.13	3.61	N/A	5.84 (N/A)
3	Age inclusion <50 y	5	784	71.94	14.54	13.52	[e]	[e]	41.53	7.27	[e]	[e]
	Age inclusion <70 y	3	1998	66.87	8.06	25.07	[e]	[e]	[e]	5.01	[e]	[e]
	All ages of onset included	50	55,798	82.08	10.05	7.87	3.63 (51.52)	76.77	35.64	1.80	0.24	4.42 (N/A)
4	Complete somatic mutation analysis	7	6848	90.74	9.21	0.04	3.69 (64.71)	94.33	56.82	3.01	1.75	0.77 (0.61)
	Incomplete or no somatic mutation analysis	51	51,732	80.19	10.15	9.66	3.55 (49.87)	74.58	35.16	1.85	0.02	4.85 (N/A)

IHC, immunohistochemistry; LOH, loss of heterozygosity; MMR, mismatch repair; MMRd, mismatch repair deficiency; N/A, not applicable, not tested.

[a]Percentages shown are for the total cohort unless otherwise mentioned. Flowcharts of subgroup analysis: Complete IHC, Methylation with or without BRAF(V600E), Only BRAF(V600E), Age inclusion <50 years, Age inclusion <70 years, and Complete somatic mutation analysis can be found in Supplementary Figures 2 through 7. Flowcharts of the remaining subgroups are available upon request.

[b]For studies performing germline testing in all patients, regardless of IHC outcomes, the amount of patients tested was adjusted to the amount of MMR-deficient protein staining without *BRAF* V600E mutation or *MLH1* promotor hypermethylation.

[c]In studies mentioning these proportions.

[d]Total group of unexplained MMRd including unexplained MMRd despite of somatic testing, not tested, failed or unknown test results. Between brackets unexplained proportion after complete somatic testing.

[e]Because of an abundance of unknown data, the proportions of this subgroup analysis are based on the results of 1 study. Therefore, these proportions are not shown.

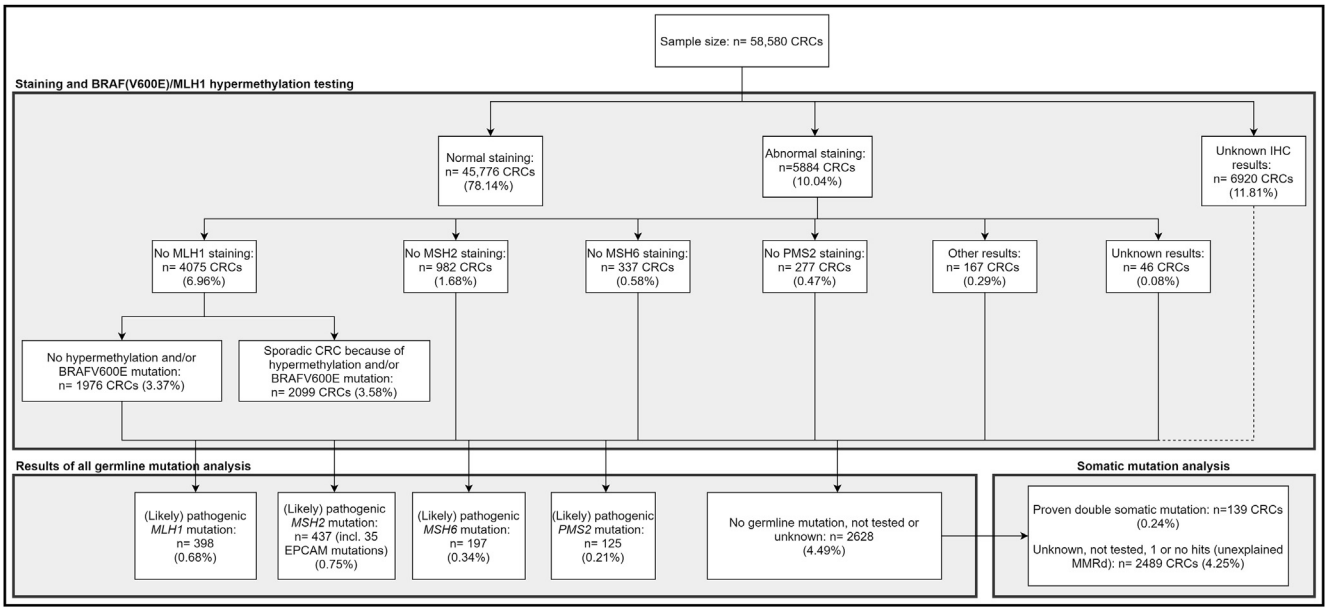

Figure 2. Complete overview of all articles. CRC, colorectal cancer; IHC, immunohistochemistry; MMRd, mismatch repair deficiency.

Outcomes for Patients Younger Than Age 50 Years and Younger Than Age 70 Years: Subgroup Analysis 3

Five studies included only patients younger than age 50 years, 3 studies included only patients younger than age 70 years, and the remaining 50 studies did not apply an age cut-off (subgroup analysis 3). Studies that included only patients younger than age 50 years comprised a total of 1998 CRCs, of which IHC outcomes were known for 1497 patients (Supplementary Figure 5). Studies that included only patients younger than age 70 years comprised 784 CRCs, of which IHC outcomes were known for 678 patients (Supplementary Figure 6). When compared with the total amount of CRCs, a pathogenic MMR germline variant was found in 7.27% (n = 57) of all 784 CRCs in patients younger than age 50 years and in 5.06% (n = 101) of all 1998 CRCs in patients younger than age 70 years (Table 1). Consequently, age restrictions for IHC testing in patients younger than age 50 and age 70 years eventually will yield significantly more pathogenic MMR germline variants ($P < .001$) (Table 1).

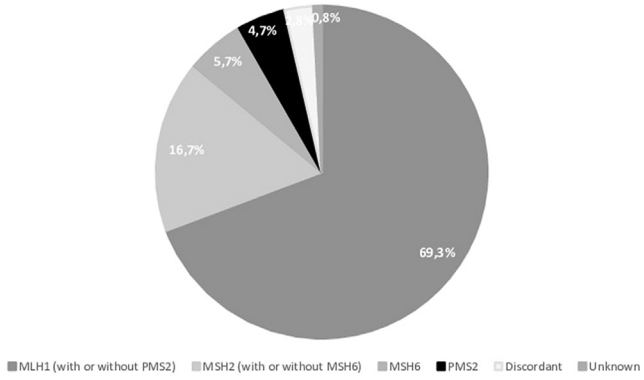

Figure 3. Distribution of mismatch repair (MMR)-deficient protein staining patterns in the total cohort of 5884 MMR-deficient colorectal cancers (CRCs). Here, CRCs with unknown immunohistochemistry outcomes were known to have an MMR-deficient protein staining, but their pattern was not specified.

Pursuit of Germline Testing

In 30 of 56 included studies, the number of patients pursuing germline DNA diagnostics could be determined. Of all CRCs with MMRd without *MLH1* hypermethylation, germline testing was performed in 76.30% (Table 1). The amount of germline variants identified with DNA diagnostics was described in 41 of 56 included studies. Of all patients who actually pursued germline testing (76.30% of patients with abnormal IHC, without *MLH1* promoter hypermethylation or *BRAF* mutation, in whom germline testing was deemed necessary), a pathogenic variant was found in 37.90% of patients, corresponding to an overall pooled proportion of 37.60% (95% CI, 30.51%–45.26%; $I^2 = 89\%$) (Supplementary Figure 11, Table 1).

Somatic DNA Analysis: Main Cohort

In 10 of the 56 included articles, somatic DNA analysis was performed for at least 1 MMR gene during the study period. In most of these articles, only outcomes were mentioned and, therefore, the total amount of cases with somatic analysis performed could not be determined. Somatic DNA analysis resulted in identification of 140 biallelic somatic variants or a somatic variant with LOH (95 in *MLH1*, 35 in *MSH2*, 7 in *MSH6*, and 3 in

PMS2), representing 0.24% of the total CRC cohort. After *MLH1* promoter hypermethylation analysis, germline analysis, and somatic analysis, this led to 2489 CRCs remaining unexplained MMR deficient (Figure 2, Table 1). When compared with the total amount of 58,580 CRCs, this corresponds with a percentage of 4.24%. Because only 10 studies performed complete or partial somatic DNA analysis, this percentage contains many MMR-deficient CRCs in which no somatic testing was performed. The proportion of unexplained MMRd therefore was assessed further in studies with complete somatic analysis for the gene (*MLH1*, *MSH2*, *MSH6*, or *PMS2*), of which the corresponding protein was not present at the MMR-deficient protein staining.

Studies With Complete Somatic Analysis: Subgroup Analysis 4

In 7 of the 56 included studies, complete somatic DNA analysis was performed. Pooled data identified 6848 CRCs in these studies (subgroup analysis 4) (Table 1 and Supplementary Figure 7).[12,25–29] Of these, 9.17% (n = 628) of all CRCs had an MMR-deficient protein staining, corresponding with 5.48% (n = 375) of all CRCs after exclusion of sporadic MLH1-deficient protein staining. Of germline tested patients, 56.82% were found to carry a germline variant in 1 of the MMR genes. The amount of pathogenic MMR germline variants corresponds with 3.01% of the total cohort of 6848 CRCs, which is significantly more when compared with studies with incomplete or no somatic DNA analysis ($P < .001$) (Table 1). In non-*MLH1* hypermethylated MMR-deficient protein staining, pathogenic germline variants were found in genes *MLH1*, *MSH2*, *MSH6*, and *PMS2* in 13.35%, 17.20%, 12.96%, and 8.73%, respectively (Supplementary Table 5). When assessing this per protein individually, the following percentages of germline variants were found: in nonmethylated MLH1-negative cases, 37.68% was explained by germline *MLH1* variants, in MSH2 this was 55.84% (including *EpCAM*), in MSH6 this was 79.03%, and in PMS2 this was 78.57% (Supplementary Table 6). Of the remaining 173 CRCs in which no pathogenic germline variant could be identified, somatic analysis was performed in the majority of CRCs. Somatic mutation analysis identified biallelic mutations or 1 somatic mutation with LOH in 68.79% of this group (n = 119) of remaining CRCs, corresponding to 1.75% of all 6848 CRCs. In 21 cases no germline or somatic testing was performed, and in 3 cases no sufficient testing result could be obtained. In 18 cases only 1 or no somatic hits were found. Conclusively, MMRd remained unexplained because of failed testing, no testing, or 1 or no somatic hits found in 24.28% of the remaining CRCs (n = 42), corresponding to 11.11% of the nonmethylated MMRd CRCs and 0.61% of this total cohort. Conclusively, in studies with complete somatic analysis, significantly fewer CRCs remained unexplained than in studies with incomplete or no somatic mutation analysis ($P < .001$) (Table 1).

Meta-Regressions, Publication Bias, and Quality Assessment

To explore potential causes of heterogeneity between studies, random-effects meta-regressions were performed, which reduced I^2 only marginally (Table 2). In addition, no evidence for publication bias was found in data concerning IHC or germline outcomes. Upon quality assessment, most cohorts were estimated to have a low or unclear risk of bias. A potential risk of bias was found mostly in questions regarding adequate sample size (25 cohorts, 43.10%) and sampling (17 cohorts, 29.31%) (Supplementary Tables 2 and 3).

Discussion

In this systematic review and meta-analysis, we investigated the proportion of LS, sporadic MMRd, and unexplained MMRd cases in 58,580 unselected CRCs, subjected to universal MMR IHC. Approximately 1 of 10 CRCs had an MMR-deficient protein staining, of which *MLH1* promotor hypermethylation explained the absence in approximately half of the MLH1-deficient protein staining. This proportion roughly varied between 4 of 10 CRCs (solely *BRAF* mutation analysis performed in MLH1-deficient staining) and 6 of 10 CRCs (additional *MLH1* promoter hypermethylation assay performed). After IHC and *MLH1* promoter hypermethylation analyses had been performed, 6% of all 58,580 CRCs were deemed candidates for further germline and somatic analyses. Of all patients pursuing germline genetic testing, a pathogenic MMR germline variant was identified in 38%. This resulted in the identification of a pathogenic MMR germline variant in 2% of all CRCs (corresponding to 33% of all MMRd CRCs). This percentage increased to 7.27% and 5.01% of all CRCs in patients younger than age 50 and 70, respectively. Although significantly more pathogenic MMR germline variants were found in these groups than in studies without age restrictions for IHC testing, older LS patients will be missed.[12] After application of germline and somatic mutation analyses, 4.24% of the total cohort (corresponding with 68.48% of MMRd CRCs without *MLH1* hypermethylation or *BRAF* variants) in our main analysis remained unexplained. In studies with complete somatic mutation analysis, this percentage decreased to 0.61% of the total cohort (corresponding with 11.11% of MMRd CRCs). This finding therefore stresses the value of complete diagnostic analyses in LS diagnostics.

The results of our main analysis are comparable with previous reports of universal testing, in which an estimated 2.2% to 3.0% of all CRCs were related to Lynch syndrome.[30,31] As reported previously, most of the MMR-deficient staining lacked MLH1 or MSH2 and, therefore,

Table 2. Overview of the Meta-Regression With I^2 After Random-Effect Meta-Analyses (Crude) and After Meta-Regression, per Meta-Analysis Performed

Meta-analysis	Proportion (95% CI)	I^2, crude	I^2, meta-regression	Studies, n
Deficient MMR protein staining (without *BRAF* variants and *MLH1* promoter hypermethylation) in total CRCs (meta-analysis 1)	6.22% (5.08%–7.61%)	96.30%	93.64%	37
MMR germline variants in total CRCs (meta-analysis 2)	2.00% (1.59%–2.50%)	92.40%	88.79%	52
MMR germline variants in CRCs with deficient MMR protein staining (without *BRAF* variants and *MLH1* promoter hypermethylation) (meta-analysis 3)	33.50% (26.06%–41.86%)	92.80%	87.99%	37
MMR germline mutations in patients in whom germline mutation analysis was performed (meta-analysis 4)	37.60% (31.51%–45.26%)	89.30%	82.33%	41

NOTE. Meta-regression was performed for country, inclusion and exclusion criteria, and year of study.
CI, confidence interval; CRC, colorectal cancer; MMR, mismatch repair.

most pathogenic germline variants were found in these genes.[32] Conversely, in our subgroup analysis with complete somatic mutation analysis, the overall proportion of MMRd CRCs caused by a pathogenic germline variant in *MSH6* was found to be higher. Because 4 of these studies were performed in Europe, this phenomenon potentially could be explained by a higher carrier frequency of germline *MSH6* variants in this region.[33,34] In addition, our results indicate that the found percentage of biallelic somatic mutations or germline variants depend on the specific MMR protein lacking at staining. For *MSH6* and *PMS2*, germline variants are more common than for *MLH1 a*nd *MSH2*. In case MMR-deficient protein staining lacks MLH1 or MSH2, chances are higher of detecting biallelic somatic variants in corresponding genes as a cause for colorectal carcinogenesis. Future cost-effectiveness analyses therefore should assess the optimal order of tumor and germline analyses in patients with MMRd CRCs. In addition, when discussing the consequences of germline testing with patients in clinical practice, it should be realized that although the total number of germline variants identified is the highest in *MLH1* and *MSH2*, the actual chance of finding a germline variant in individual cases is the highest for *MSH6* and *PMS2*.

In our overall analysis, in 4.42% of the total cohort no cause could be identified for the MMRd. This percentage likely contains many sporadic MMR-deficient CRCs because in most studies no somatic testing was performed.[7,35] In the subgroup analysis with complete somatic mutation analysis only 11.11% of MMRd CRCs remained truly unexplained, corresponding with 0.61% of this subgroup's total sample size. This estimate of unexplained MMRd is comparable with a recent study with complete germline and somatic analyses performed in a cohort of 3602 CRCs of patients younger than age 70.[36] Because MMRd is caused mainly by a variant in 1 of the MMR genes, part of the cases with unexplained MMRd could be explained by previously missed explanatory variants, either in the tumor or in the germline. We therefore recommend complete assessment of the MMR genes before performance of multigene panel testing. In the future, tumor whole sequencing also likely will contribute to finding previously missed germline or somatic variants.[37,38] Furthermore, other explanations also must be sought for the unexplained MMRd when no germline or somatic variants in the MMR genes are found, for example, by assessment of *POLE* and *POLD1*.[39] Notably, somatic MMR variants also can occur secondary to germline variants in *POLE*, *POLD1*, and *MUTYH*. Assessment of these genes could yield an explanatory germline variant owing to double-somatic MMR variants, especially in patients who developed CRC at a young age.[39,40]

A better understanding of unexplained MMRd is of considerable clinical importance. Patients are referred to as having unexplained MMRd or having Lynch-like syndrome (LLS). Varied definitions of unexplained MMRd that are common in the literature hamper further research on this topic. For example, the term LLS is sometimes used to refer to either unexplained MMRd or to double-somatic MMR variants.[41] We suggest, as Katz et al[42] have commented previously, that use of the term LLS should be avoided and henceforth unexplained MMRd should be defined as follows: high MSI tumors that, upon MMR-deficient protein staining for at least 1 MMR protein, could not be explained by a pathogenic MMR somatic or germline variant. The former includes both *MLH1* promoter hypermethylation, double-somatic MMR variants, or LOH.

Our study had several limitations. First, substantial heterogeneity between studies was observed, despite exclusion of studies that performed nonuniversal IHC and even after performing random-effects meta-regression analyses. Recent studies have shown that the percentage of MMR deficiency depends on the tumor type

and the continent where the study was performed.[43–45] Therefore, this was taken into account in our meta-regression, possibly explaining part of the heterogeneity. Another explanation might be the differences in the diagnostic tract: for example, in approximately one third of the studies, not all 4 MMR genes were analyzed. In addition, *MLH1* promoter hypermethylation was not performed in all studies. The order of analyses also differed between studies. In addition, the use of different techniques could have affected outcomes because of false-negative or false-positive tests, or missed somatic mosaicism.[41] To compare studies, we assumed that microsatellite stable CRCs would have normal IHC in studies that first performed MSI analyses and only tested high MSI tumors for pathogenic MMR germline variants. However, in light of tumors with a germline variant but MMR proficient protein staining, some MMR germline variants might be missed, even when applying universal tumor testing. In particular, the previous literature has suggested that patients with a germline *MSH6* variant may be under-represented,[27,46,47] although some articles maintain that this percentage is actually very low.[48]

Second, on many occasions, we were hindered by missing data regarding IHC and germline testing results. Besides not having analyzed all MMR genes, some studies also did not mention specific outcomes of tests performed. In addition, only a minority of included studies mentioned specific methods to assess variants in the 3' end of *PMS2*, while germline analyses in this region are known to be difficult because of the presence of pseudogenes.[49,50] Because missing data hampered accurate calculations of proportions in our main analysis, we chose to perform multiple subgroup analyses to calculate the desired proportions more accurately.

Third, results of this study probably underestimate the number of actual pathogenic germline variant carriers in CRC cases because not all patients who are eligible for germline DNA testing were actually tested. The reasons for this may be 2-fold: on the one hand, patients may not have had the opportunity to proceed to germline testing because of nonreferral[51]; on the other hand, personal reasons may have played a role, exemplified by some of our included studies.[12,52,53] Although IHC testing is known to be an efficient and cost-effective way to detect LS in patients diagnosed with CRC at younger than age 70 years,[54,55] the efficiency and cost effectiveness of this approach also depends on the uptake of germline testing.[55,56] Barriers for germline DNA testing therefore should be identified and decreased as much as possible.

A key strength of our study was the very large number of included patients, and this systematic review and meta-analysis estimated the proportion of explained MMRd in a large group of patients. In addition, because estimates of unexplained MMRd in earlier literature generally were higher than in our subgroup analysis, but usually were assessed in studies lacking complete LS diagnostics, we are confident that the present study more accurately estimates the proportion of truly unexplained MMRd CRCs.

In conclusion, the percentage of germline MMR-deficient cases and unexplained cases is highly dependent on the completeness and type of diagnostics used. In studies that completed all diagnostic stages, germline variants were found in 3.01% of CRCs. In addition, complete diagnostics led to a percentage of unexplained MMRd of only 0.61%. The relevance of complete diagnostics therefore should be stressed in current guidelines. This can help navigate gastroenterologists, surgeons, and clinical geneticists through the genetic workflow in patients with CRC and is relevant for future cost-benefit studies. However, more research should be performed to accurately characterize the small and potentially heterogeneous group of unexplained MMRd.

Supplementary Material

Note: To access the supplementary material accompanying this article, please click here.

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Conflicts of interest
The authors disclose no conflicts.