Validity of a two-antibody testing algorithm for mismatch repair deficiency testing in cancer; a systematic literature review and meta-analysis

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INTRODUCTION
Mismatch repair deficiency (MMRd) occurs in ~10–15% of colorectal carcinomas (CRC) and ~25–30% endometrial carcinomas (EOG). MMRd can be caused by somatic MLH1 promoter hypermethylation, somatic mutations combined with loss of heterozygosity or bi-allelic mutations. Furthermore, MMRd can also result from germline mutations (Lynch syndrome) in mismatch repair (MMR) genes in combination with a second hit on the wildtype allele. Lynch syndrome accounts for ~20% of MMR-deficient CRC and ~10% of EOG. Many professional societies have recommended reflex MMR testing for CRC and EC by initial MLH1, PMS2, MSH2 and MSH6 IHC to triage for subsequent germline testing. Subsequent MLH1 promoter hypermethylation or BRAF testing is performed in cases with isolated MLH1 or MSH2 loss or combined MLH1/MSH2 loss alone. In the six articles with the main aim of investigating the two-antibody testing algorithm all MMRd cases were detected with the two-antibody testing algorithm, there were no cases with isolated MLH1 or MSH2 loss or combined MLH1/MSH2 loss alone. This suggests that high detection rate of MMRd of the two-antibody testing algorithm supports its use in clinical practice by specialized pathologists. Staining of all four antibodies should remain the standard in cases with equivocal results of the two-antibody testing algorithm. Finally, educational sessions in which staining pattern pitfalls are discussed will continue to be important.

MMRd testing is no longer simply a matter of screening for individuals who have undiagnosed Lynch syndrome. Patients with cancer types with high mutational burden, specifically MMRd colorectal, endometrial, gastric, bladder, breast, ovarian, bile duct/gall bladder, pancreatic, small cell lung and thyroid carcinomas have been shown to benefit from checkpoint inhibition. As a result, MMRd has been approved by the FDA as a pan-cancer predictive biomarker for checkpoint inhibition. Therefore, correctly identifying cancers with MMRd enables targeted and effective treatment with checkpoint inhibitors in addition to Lynch syndrome screening.

MMRd, microsatellite instability (MSI), tumor mutational burden (TMB) and programmed death-ligand 1 (PD-L1) IHC have all been recognized as predictive biomarkers of the efficacy of checkpoint inhibition. Next generation sequencing (NGS) can be used to index MSI or TMB. MSI can also be objectified by specific polymerase chain reaction (PCR) testing with a panel of microsatellite markers. Both NGS and MSI testing require DNA isolation from the tumor, are costly and take at least a few days to generate a result. As MMR IHC is a sensitive, relatively fast and cheap method to index MSI, it is an appealing alternative to NGS and MSI testing for targeted treatment with checkpoint inhibitors.
identify MMRd, it is frequently put forward as the preferred test. In contrast to MSI testing, MMR IHC provides information with regard to the involved gene. Furthermore, it may identify cases with subclonal loss of expression (loss of expression in a specific area of the tumor), which may be missed by localized DNA extractions. Other benefits include the long-term experience pathologists have with evaluating MMR proteins (specifically in the context of screening for Lynch syndrome) and the very little material needed for adequate evaluation. The rise in checkpoint inhibitor therapy has led to an increase of the number of MMR IHC tests performed in routine diagnostic pathology.

The mismatch repair proteins function as two heterodimer complexes: PMS2 forms a stable heterodimer with MLH1, while MSH6 dimerizes with MSH2. If either PSM2 or MSH6 loses protein function MLH1 and MSH2 can form a heterodimer with another protein, e.g., PMS1, MLH3 or MSH3, resulting in retained or slightly diminished immunohistochemical expression of MLH1 and MSH2. However, PMS2 and MSH6 are not able to form alternative heterodimers resulting in loss of function of the entire heterodimer complex. Subsequently MMR protein expression as objectified with IHC is expected to be negative in both MMR proteins of the affected heterodimer. This knowledge forms the biological rationale for a two-antibody MMR IHC screening which is limited to immunostaining for PMS2 and MSH6, as proposed by Shia et al.

The two-antibody testing algorithm may be an attractive alternative to the two-antibody testing algorithm. The effectiveness of the two-antibody testing algorithm for the determination of MMR status assignment was assessed by calculation of a weighed percentage of MMRd cases that would not have been identified by this approach (cases with either isolated loss of MLH1/MSH2 or double loss of MLH1 and MSH2), in order to provide a scientific basis for guideline recommendations.

**MATERIALS AND METHODS**

This systematic review was conducted and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines.

**Search strategy**

A computerized literature search of PubMed, Embase and Cochrane library was conducted for all peer-reviewed studies that reported on MMR IHC in humans. The search strategy was devised in collaboration with an information specialist and consisted of a combination of Medical Subject Headings (MESH terms) and free text words with the following combined keywords: ‘immunohistochemistry’ and ‘mismatch repair’ including all relevant keyword variations. The original search strategy can be found in Supplementary Information 1 (S1). The search was performed in April 2020. Articles were limited to English language and publication dates after June 2007 due to the introduction of automated staining technology and more representative reporting patterns. The references of primary selected studies were scrutinized for additional records that were not identified through database search.

**Eligibility criteria**

Two reviewers, senior pathology residents (KA & TTD), independently selected and identified the appropriate studies based on prespecified inclusion and exclusion criteria using the online tool Rayyan. Where consensus could not be reached, the senior pathologist (TB) made the final decision. Articles were eligible for inclusion when a detailed description of all four MMR IHC staining patterns for each individual lesion was reported and the combination of MMR IHC results were unequivocal to the reviewers. Exclusion criteria were: reviews and meta-analyses containing no original data, case reports, nonhuman studies and studies with insufficient data for analysis (reporting immunohistochemistry results on <20 cases).

**Risk of bias assessment**

The quality of the selected studies was rated based on the quality of the immunohistochemical staining process and interpretation. Adequateness of IHC staining and interpretation was determined for each article based on the following quality parameters: use of whole slides, reporting a clear definition of MMR IHC loss, use of NORDIQC approved immunohistochemical stains, evaluation by ≥2 evaluators and evaluation by at least one pathologist. A quality score between 0 and 5 was given according to how the IHC staining and interpretation was determined for each article based. Articles were rated between 0 and 5. Articles meeting 4 or 5 of the quality parameters were considered to be of high quality.

The percentage of MMRd was expected to vary among the articles, due to biological differences in MMRd prevalence in cancer types and different inclusion criteria used (e.g., some studies only included MSI-high specimens).
To avoid selection (sampling) bias the analysis was performed only on the MMRd cases, MMRp samples were not included in the analysis.

**Data extraction**

The following information was extracted: authors, lesion type, specimen type, hospital(s) and country/countries of specimen inclusion, dates between which patients were included, use of whole slides or tissue microarray (TMA), definition of loss of MMR IHC staining used, antibody clones used for MMR IHC, type of IHC evaluator (pathologists yes/no), number of evaluators, if the aim of the study was to test the validity of the two-antibody testing algorithm compared to the traditional four antibody approach (yes/no), number of specimens with IHC reported for all 4 MMR proteins and number of MMRd specimens. The number of MMRd specimens was further specified as follows: number of specimens with isolated loss of PMS2, MLH1, MSH2 or MSH6, number of specimens with dual loss of PMS2/MLH1, MSH2/MSH6, PMS2/MSH6, PMS2/MSH2, MSH6/MLH1 or MLH1/MSH2, the number of cases with loss of 3 or 4 MMR proteins and their combination. In the event MMR status interpretation was adjusted after addition molecular tests, only the original stain scores (raw IHC data) were collected.

Cases in which the terms “subclonal”/“heterogeneous”/“focal”/“patchy” were used to describe the MMR IHC results were considered to have loss of MMR expression if the description was further clarified as an abrupt loss of nuclear MMR protein expression in clearly demarcated tumor areas with positive internal control. Cases that were reported to show “weak” or “equivocal” MMR staining were excluded, as this pattern is most likely due to artefactual loss (e.g., fixation artefact). Owing to many variables between country of inclusion of specimen cohort and specimen type, country of inclusion of specimen cohort was categorized in 5 groups (USA/Canada, Europe/Scandinavia, Asia/Middle East, Australia/New Zealand and other) and specimen type was categorized in 4 organ system groups (Gastro-intestinal (GI), Gynecological (GYN), Dermatological (DERM) and Other).

**Article selection**

Articles reporting on the same specimen cohort were identified by using a combination of hospital(s) and country/countries of specimen inclusion, dates between which patients were included, lesion type, specimen type and authors. For articles with overlapping cohorts the article that met most quality parameters was included. If all articles met the same amount of quality parameters, the article with the most MMRd cases was selected.

**Data analysis and statistics**

Our primary outcome was defined as the proportion of cases that would not have been identified as MMRd by the two-antibody testing algorithm, which uses PMS2 and MSH6 staining only, but showed either individual loss of MLH1/MSH2 or combined loss of MLH1 and MSH2 when using the four antibody approach. Henceforward these cases will be called non-dimeric loss cases, as the staining pattern cannot be explained by the heterodimeric function of the MMR proteins. Our secondary outcomes were the proportions of cases with non-dimeric loss in specific organ systems. The proportions of interest were calculated from the relevant numerator and denominator and were derived using a random effects model (arcsine regression). Proportions were presented along with 95% confidence intervals. To account for the differences in sample sizes between studies, a weighted mean of the estimates from each study was used. The following independent study variables were included in the meta-analysis: number of specimens with MMR IHC identified MMRd and number of cases with MMR IHC loss that would not have been identified by the two-antibody testing algorithm (single isolated loss of MLH1 or MSH2 and dual loss of MLH1 and MSH2). The sensitivity of the two antibody approach to identify MMRd compared to the gold standard of the four antibody approach could not be calculated as MMRp cases were excluded. A chi-square test was used to identify differences in the distribution of variables among categorized subgroups (e.g., specimen type, high/low quality articles).

Statistical analyses were performed in R statistical software, using the metafor package (version 4.0.3) and IBM SPSS Statistics (version 25). Forest plots were produced in R, showing the individual study results and weighted estimates together with 95% CI. The degree of heterogeneity across articles was examined using visual inspection of data and the I² statistic. The R data script can be found in Supplementary Information 2 (S2).

**RESULTS**

**Article selection**

The literature search yielded a total of 1704 original articles (PubMed 1644, Embase 1161, Cochrane library 38, manual 1). After screening titles and abstracts, 995 articles were excluded. Seven hundred and nine articles were eligible for full-text evaluation, 559 were excluded for various reasons, and 131 articles were included in the meta-analysis (Fig. 2).
Data distribution
Of the 131 included articles, 78 focused on gastro-intestinal cancers, 39 on cancer of the female genital tract, 6 on dermatological lesions, 4 on mixed cohorts and 4 on other specimen types (breast-, head and neck-, oral- or urological specimens) (Table 1). In total, there were 47,745 cancers of which 9014 were MMRd, varying from 20 to 661 MMRd cases per article. Non-dimeric MMR IHC patterns were reported in 298 cases. These 298 included 172 cases of isolated MLH1 loss (130 in GI, 39 in GYN and 2 in other specimen types), 114 cases of isolated MSH2 loss (88 in GI, 12 in GYN and 14 in other specimen types) and 12 cases of combined MLH1 and MSH2 loss (9 in GE and 3 in GYN) (Table 2). Cases with non-dimeric loss were reported across 40 studies. Thirteen cases, which were reported among 3 articles, were excluded due to the description of MMR IHC as “weak” or “equivocal”.

Risk of bias assessment
The risk of bias assessment for each article is shown in Supplementary Information 3 (S3). Twenty articles met four or five quality parameters and were considered to be of high quality. Of all the articles 51% used whole slides (n = 67), 63% reported a clear definition of IHC loss (n = 83), 19% used only NORDIQC approved immunohistochemical stains (n = 25), 31% were evaluated by ≥2 evaluators (n = 40) and 31% were evaluated by one or more pathologist (n = 41). The other articles either failed to meet the quality parameter or did not report on the given parameter. The articles with the highest percentage of unexpected MMR IHC results (isolated MLH1 or MSH2 loss or combined MLH1/MSH2 loss) are described in Supplementary Information 4 (S4).

Percentage of isolated or combined MLH1/MSH2 IHC loss
Meta-analysis of 131 studies yielded a weighted percentage of 1.1% (95% CI 0.53–18.87, S5) for MMRd cases that were reported to show either isolated MLH1 or MSH2 loss or combined loss of MLH1 and MSH2 alone (with retained PMS2 and MSH6). Considerable heterogeneity was present (I² = 87%), representing differences in results between studies. Therefore, subgroup analysis was performed.

Subgroup analysis
In six articles with the primary aim of investigating the two vs four antibody approach, no cases with non-dimeric loss were described in 505 MMRd cases (0%, CI 0.00–0.00, heterogeneity I² 0%, S6). Organ system subgroup analysis showed a similarly low weighted percentage in gynaecology oriented articles and dermatology oriented articles, respectively 0.29% (95% CI 0.02–0.85, heterogeneity I² 75%, Fig. 3) and 0.39% (95%CI 0.00–2.19, heterogeneity I² 64%, S7). A percentage of non-dimeric loss cases of 1.54% (95%CI 0.61–2.39, heterogeneity I² 90%, Fig. 4) was observed in the articles describing lesions of the gastrointestinal tract. Heterogeneity was substantial (I² > 50%) in all subgroups regarding quality score, individual quality parameters and the countries of inclusion of the specimen cohort.

Distribution of MMRd
Finally, we divided the articles into those without non-dimeric loss cases, articles with <5% non-dimeric loss cases and articles with >5% non-dimeric loss cases and compared these in terms of our predefined quality parameters. The group of articles without non-dimeric loss cases had a significantly higher percentage of high-quality articles (p < 0.05, chi-square test), compared to the ones that did report cases of non-dimeric loss. A significant difference was found for definition of nuclear loss (p < 0.05, chi-square test). No significant differences were found for the other individual quality parameters (pathologist scored, whole slide usage, ≥2 evaluators), publication date, cancer type, country of inclusion of specimen cohort and university/non university hospital.

Molecular findings
Nine of the 40 articles that report non-dimeric loss cases provided some additional molecular data for these specific cases. Giraldez et al.48 described one case with isolated loss of MLH1 protein expression. This case was microsatellite unstable with no MLH1 promoter hypermethylation and a pathogenic variant in MLH1 was not identified. Timmerman et al.49 reported on one case with isolated MLH1 loss with confirmed hypermethylation. Microsatellite instability was reported in 16 out of 18 cases with isolated MLH1 or MSH2 loss by Siraj et al., but subsequent testing for germline pathogenic variants or somatic MLH1 hypermethylation was not performed. The MSH6 and PMS2 antibodies used in this study were not NORDIQC approved, and this was the only study in our meta-analysis that used the PMS2 clone C-20 by Santa Cruz Biotechnology, Dallas, Texas, USA.60 Three cases (2x CRC and 1x
skin lesion, respectively) with isolated MSH2 loss were reported to have a matching germline MSH2 pathogenic variant. MMR IHC was re-evaluated in one of these cases and showed focal retained staining of MSH6, initially interpreted as positive\textsuperscript{61–63}. Cavazza et al.\textsuperscript{64} reported on one case with isolated MLH1 loss and one with isolated MSH2 loss, with identification of germline MSH2 pathogenic variants in both cases. In this study, the methodology of IHC staining and scoring was poorly described and no specifics were provided about the identified variants\textsuperscript{64}. None of the articles provided images of the MMR immunohistochemical staining patterns of the non-dimeric loss cases described.

**DISCUSSION**

Reflex testing with four MMR protein immunohistochemistry for defining MMR status to triage patients at higher risk for Lynch syndrome and identify patients that may benefit from checkpoint inhibition is increasingly recommended in clinical guidelines throughout the world. The implementation of these guidelines resulted in a significant increase in MMR testing in pathology laboratories. Based on biological rationale, it has been suggested that a testing algorithm that uses just two MMR antibodies (against MSH6 and PMS2) is adequate for assessing MMR status. This is the first systematic review and meta-analysis on the performance of this two-antibody testing algorithm.

Our analysis showed a weighted percentage of 1.1% of reported cases with non-dimeric loss that would not have been identified using a two antibody approach. Considering the overall low percentage of cases with non-dimeric loss, especially in tumors of the GYN and DERM subgroups (<0.5%), implementation of the two-antibody testing algorithm seems adequate for both screening to identify patients at higher risk of having Lynch syndrome as well as identifying patients that may benefit from checkpoint inhibition therapy. The slightly higher percentage of 1.54% of non-
dimeric staining patterns in the GI group is accompanied by high interstudy heterogeneity, unexplained by any of the variables (e.g., publication year, use of NORDIQC approved antibodies). Four out of 6 articles with the aim of investigating the two vs four antibody approach were GI focused and found no cases with non-dimeric loss of staining. This absence (0%) of non-dimeric staining patterns in all articles with the primary aim of investigating the two vs four antibody approach demonstrates that unexpected staining

Fig. 4 Meta-analysis forest plot showing data from all Gastrointestinal oriented articles. The analysis included 78 articles.
patterns will not lead to misclassification as MMRd when evaluators have their primary focus on scoring MMR IHC47–50.57. However, these studies were conducted by expert pathologists, and thus a four antibody approach is advisable to assure correct MMR status assignment in less experienced hands.

Issues with correct interpretation of MMR IHC staining might be a possible explanation for the reporting of non-dimeric loss of staining. Sometimes MMR protein expression is reduced in intensity, heterogeneous, focal or patchy. This is most frequently the result of inadequate fixation of tissue. In these instances the four antibody approach seems justified as the combination of stains will support the pathologist’s interpretation. The use of dated archived material can also result in lesser quality of the immunohistochemical stains. MMR staining was observed to be more intense and homogeneous in biopsies compared to resection specimens and thus easier to interpret in the former, most likely due to more uniform and complete fixation48. The use of biopsy material for MMR IHC is therefore preferred over the use of resection specimens. Secondary downregulation of the MMR genes should also be considered as a possible cause for unexpected staining patterns. Environmental factors such as tissue hypoxia and oxidative stress have been shown to significantly reduce the expression of MMR genes at RNA level and result in suppression of DNA mismatch repair. These factors may be caused by prolonged ischemia or delayed fixation49.

Another possible explanation for noncanonical staining patterns is the practice of neo-adjuvant treatment prior to MMR-assessment; this may also influence expression patterns, especially of MSH650,51. As neo-adjuvant therapy in endometrial cancer is rare but common in gastro-intestinal cancers, this might partly explain the higher percentage of non-dimeric loss found in gastro-intestinal cancers. The interpretation of the MMR proteins in the context of the two-antibody testing algorithm should therefore always be evaluated with care and when in doubt about heterogeneity, focal or inequivalent staining patterns subsequent MSH2 and MLH1 should be performed. When possible, it is preferable to perform MMR IHC on (biopsy) specimens taken before neo-adjuvant therapy. Another challenge is the clinical interpretation of subclonal or regional loss of MMR expression. Subclonal loss is believed to an acquired MMR defect arises during tumorgenesis. Whether subclonal MMR loss can be observed in a germline context requires further study52. The predictive value of subclonal loss for checkpoint inhibition therapy is being investigated53.

In conclusion, the results of our study support the use of the two-antibody testing algorithm, starting with PMS2 and MSH6, in clinical practice. Using this approach at least 98.9% of MMRd cases will be detected. However, our study suggests that the number of misclassified cases may be as low as zero when MMR IHC is interpreted by specialist pathologists. Educational sessions in which staining pattern pitfalls are discussed will remain important. Staining of all four antibodies should be performed when there is MMRd or any doubt about the interpretation of the staining patterns observed with the two-antibody testing algorithm.

DATA AVAILABILITY

All data generated and/or analyzed during the current study are included in this published article and its supplementary information files.

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