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Evidence Summary MOAC-3 REQUIRES UPDATING

A Quality Initiative of the Program in Evidence-Based Care (PEBC), Cancer Care Ontario (CCO)

Screening for Lynch Syndrome by Immunohistochemistry, *BRAF* Mutations Analysis, and *MLH1* Promoter Methylation Analysis for Patients in Ontario with Colorectal or Endometrial Cancers

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Evidence Summary: MOAC-3

Screening for Lynch Syndrome by Immunohistochemistry, *BRAF* Mutations Analysis, and *MLH1* Promoter Methylation Analysis for Patients in Ontario with Colorectal or Endometrial Cancers

THE PROGRAM IN EVIDENCE-BASED CARE

The Program in Evidence-Based Care (PEBC) is an initiative of the Ontario provincial cancer system, Cancer Care Ontario (CCO). The PEBC mandate is to improve the lives of Ontarians affected by cancer through the development, dissemination, and evaluation of evidence-based products designed to facilitate clinical, planning, and policy decisions about cancer control.

The PEBC is a provincial initiative of CCO supported by the Ontario Ministry of Health and Long-Term Care (OMHLTC). All work produced by the PEBC is editorially independent from the OMHLTC.

INTRODUCTION

Lynch syndrome (LS), previously known as hereditary nonpolyposis colorectal carcinoma (HNPCC), is a variably penetrant autosomal dominant genetic condition that increases susceptibility to cancer. It is caused by defects in the mismatch repair (MMR) genes and results in deficient MMR. LS carries a high risk of colorectal cancer (CRC) and endometrial cancer (EC), with lifetime risk estimates ranging from 12% to 48% for CRC and 15% to 54% for EC, depending on the gene and mutation carried (i.e., *MLH1*, *MSH2*, *MSH6,* or *PMS2*) (1-5). Identification of LS tumours, compared with sporadic tumours (no germline mutation that confers increased susceptibility to cancer), in patients presenting with CRC and EC will assist with the early identification of subsequent malignancies and help identify at-risk family members, allowing for implementation of effective surveillance and screening. By screening for LS, the reduction in risk of CRC to relatives has been found to be up to 62% (6).

In Ontario, an estimated 8814 new cases of CRC were diagnosed in 2013. Of those, 4226 were diagnosed in patients younger than 70 years of age (7). The prevalence of LS among CRC patients has been found to be 2% to 4% (5). In Ontario, an estimated 1929 cases of uterine cancer were diagnosed in 2013 with 1386 of those diagnosed in women younger than 70 years (7, 8). Of those 1386, 85% to 93% are diagnosed as EC (9,10). The prevalence of LS among EC patients has been found to be 2.5% (9-11).

Germline mutation testing is the "gold standard" for the diagnosis of LS. However, it requires patient consent and, thus, MMR-deficient tumours are typically identified through microsatellite instability (MSI) testing to identify tumours with high-frequency MSI (MSI-H) and, more recently, immunohistochemistry (IHC) testing to detect loss of protein expression for one or more MMR proteins (i.e., MLH1, MSH2, MSH6, PMS2).

There has been some debate about the most effective and appropriate screening mechanism and strategy for detecting LS. Historically, guidelines such as the revised Amsterdam criteria (1999) and revised Bethesda criteria (2002) have been used by health care programs to determine who should be tested and in what manner (12, 13). Assessment of MSI is widely accepted as a primary method for identifying individuals who may have HNPCC. The original (1997) Bethesda guidelines proposed a panel of five microsatellite markers for the uniform analysis of MSI in HNPCC (14). The 2002 revision recommended testing a secondary panel of mononucleotide markers (13). Other criteria commonly examined to diagnose LS

include tumour site (colorectal, endometrial, etc.), number of affected relatives, generations affected, and age.

The lack of ability of MSI to specify a gene for mutation analysis is a strong disadvantage, and if germline mutation testing is restricted to MSI-H cases, it may also be somewhat less sensitive than IHC testing in the detection of LS due to the MSH6 mutation (15); IHC can identify *MSH6* cases that may not show high MSI and, thus, can be missed by MSI testing (16).

IHC testing for LS is emerging as a common standard of care in many countries around the world, including Canada and the United States. It is readily available in diagnostic pathology laboratories and antibodies to the four proteins associated with LS (MLH1, MSH2, MSH6, PMS2) are commercially available. In Ontario, experts at the 2011 symposium on hereditary gastrointestinal cancer, held at the Zane Cohen Centre at Mount Sinai Hospital in Toronto, focused on optimal approaches to screening for LS and reached unanimous agreement that MMR reflex IHC testing (MMR-IHC) is a viable screening option to detect LS (17). They concluded that testing by IHC for MLH1, MSH2, MSH6, and PMS2, should be performed on tumours from all patients with CRC or EC cancer who are younger than 70 years of age.

As a next step in the testing algorithm, if IHC testing shows abnormal *MLH1* expression, *BRAF* testing is recommended. *BRAF V600E* mutations are extremely rare in patients with LS and can be used as a screening tool to identify tumours with sporadic methylation of the *MLH1* gene promoter. However, rare cases of *BRAF* mutations can occur in patients with LS; thus, the committee recommended that all patients with an *MLH1*-deficient tumour should be referred for genetic counselling, regardless of *BRAF* results, if they are younger than 50 years or have a family history meeting the Ministry of Health and Long-term Care (MOHLTC) clinical testing criteria (see Appendix I).

Should no *BRAF* V600E mutation be found (i.e., wild type), then the specimen should undergo *MLH1* promoter methylation testing. If the promoter is not hyper-methylated it is recommended that the patient be referred for genetic counselling. If the promoter is hyper-methylated and the patient is younger than 50 years or has a family history meeting the MOHLTC clinical testing criteria, they should be referred for genetic counselling. In all other cases, no further action is required (see Appendix II Figure 1a).

The testing algorithm for endometrial tumour specimens is similar to the above with the exception of the *BRAF* testing element. Because *BRAF* mutations have not been associated with endometrial cancer, methylation testing should be performed on cases where IHC testing detects a deficiency in MLH1 protein levels, while *BRAF* testing may be skipped (see Appendix II Figure 1b).

This systematic review reports on the most recent literature assessing the effectiveness of IHC, compared with MSI, as a first-line screening tool for identifying LS, and the utility of B*RAF V600E* mutation and *MLH1* promoter methylation status tumours as predictive markers of germline MMR mutation status of CRC and EC patients.

RESEARCH QUESTIONS

This Working Group developed the following research question for this systematic review in consultation with the Molecular Oncology Advisory Committee (MOAC).

1. For patients with CRC or EC, what is the evidence for using IHC testing to identify a) tumours that are MSI-H and b) tumours that are LS?

2. How does IHC testing compare with MSI testing for identifying the MMR deficiencies characterizing LS in CRC tumours and endometrial tumours?

3. For tumours that show abnormal MLH1 protein expression by IHC
    a. How effective is *BRAF V600E* testing at differentiating between sporadic versus LS-associated CRC?
    b. How effective is *MLH1* promoter methylation testing at differentiating between sporadic versus LS-associated CRC and EC?
    c. How effective is a combination of *BRAF V600E* testing and *MLH1* promoter methylation testing at differentiating between sporadic versus LS-associated CRC and EC?

METHODS

This systematic review was developed using a planned two-stage method, summarized here and described in more detail below.

(1) Search of existing systematic reviews: If one or more existing systematic reviews (defined as describing search databases, search time period, search terms, and study selection criteria; and having at least one eligible article that met our study selection criteria for original studies) were identified that addressed the research questions and were of reasonable quality, then those systematic reviews would form the core of the evidentiary base.

(2) Review of the primary literature: This review would be conducted if no existing systematic reviews were located and/or accepted.

The Program in Evidence-Based Care (PEBC) is supported by the Ontario MOHLTC. All work produced by the PEBC is editorially independent from the Ministry.

Post hoc refinements to the original project plan

After an initial literature search and document scoping, the Working Group members approved some modifications to the research. This was done to avoid duplication and enhance the quality of the research output, while expediting the completion of this evidence summary.

Questions 1 and 2

Regarding the diagnostic utility of IHC and MSI testing, a search in MEDLINE and EMBASE resulted in over 200 articles being identified as potentially addressing the inclusion criteria as originally stated. An overview of some of these studies identified the following main issues to be considered as the research progressed:

- Some studies report IHC data in MSI-H cases only; thus, limiting the ability to examine the comparability of the two tests.
- The majority of studies do not perform mutation testing in cases that that do not exhibit MSI and/or with normal IHC staining, limiting the ability to calculate sensitivity and specificity for these two tests in predicting LS.
- Study populations are very diverse, ranging from the inclusion of all newly diagnosed cancer patients to those suspected of having LS because of young age at diagnosis and/or presence or absence of a family history of cancer
- Studies include diverse combinations and inclusions of the four IHC proteins (MLH1, MSH2, MSH6, and PMS2) and a variety of MSI markers, making comparisons among studies difficult.
- Some sample sizes are very small.

An overview by Shia et al. 2008 (16) among pre-2008 pertinent literature data in CRC, found that IHC with *MLH1*, *MSH2*, *MSH6,* and *PMS2* resulted in a predictive value that was comparable to that of MSI testing. Thus, given the abundance of literature in this area, especially in recent years, literature from mid-2007 to the present was searched using our own search strategy to address the issues in the literature as stated above. These involved:

- Limiting sample size to a minimum of 30 tumours.
- Incorporating studies that include tumours that have been tested for both IHC and MSI.
- Including studies that test at least the core panel of two MSI markers (BAT25, BAT26).

Because very few of the studies perform germline mutation testing on all subjects, it was not possible to exclude studies based on partial population testing of mutations.

Question 3

Two very recent (2012 and 2014) systematic reviews assessed the utility of *BRAF V600E* and *MLH1* methylation status of EC (18) and CRC (19) tumours as predictive markers of germline MMR mutation status. Parsons et al. (19) found *BRAF V600E* mutation and *MLH1* promoter methylation to be strong predictors of negative MMR mutation status. Metcalf et al. (18) showed that BRAF mutations occur infrequently and, thus, should be discounted as a suitable marker for predicting negative MMR status in EC patients. The authors go on to state that "known MMR mutation status, and further studies of EC cohorts with known MMR mutation status are necessary to quantify the utility of tumour MLH1 promoter methylations as a marker of negative germline MMR mutation status in EC patient."(18) For this review, more recent literature on *BRAF V600E* and *MLH1* methylation status, outside these previous studies' timeframes (2011 for CRC to the present/2013 for EC to the present), was searched using our own search strategy.

Existing Systematic Reviews

A systematic search was conducted in OVID MEDLINE (mid-2007 or 2011/2013 through August 2014), EMBASE (mid-2007 or 2011/2013 to 2014 week 25), the Cochrane library (Mid-2007 or 2011/2013 to August 2014), and ASCO conference proceedings (2007 or 2011/2013 to August 2014). The keywords included 'lynch syndrome', 'MSI', 'microsatellite instability', 'IHC', 'immunohistochemistry', 'HNPCC BRAF', 'HNPCC *BRAF V600E'*, 'HNPCC *MLH1* methylation', 'HNPCC *MLH1* promoter methylation', 'Lynch BRAF', 'Lynch *BRAF V600E'*, 'Lynch *MLH1* methylation', and 'Lynch *MLH1* promoter methylation', ' endometri*', 'colorectal'. Systematic reviews were included if:

1. They evaluated randomized or non-randomized controlled trials of patients with CRC or EC that have been evaluated with IHC and MSI, or *BRAF V600,* or *MLH1* promoter methylation.
2. The literature search strategy for the existing systematic review is reproducible, reported and appropriate.
3. The existing systematic review reported the sources searched as well as the dates that were searched.

Primary Literature Systematic Review

Assuming that no existing systematic review was identified, or that identified reviews were incomplete in some fashion, a systematic review of the primary literature was also planned. This review would be reduced in scope, such as a reduction in subject areas covered, time frames covered, etc., based on the scope of incorporated existing reviews. The criteria described below are written assuming no existing reviews would be incorporated.

Literature Search Strategy

Details of the literature search strategy are included in Appendix III.

Study Selection Criteria and Protocol

Articles were selected for inclusion in this systematic review of evidence if they were fully published reports or published abstracts of randomised or non-randomised control trials. Inclusion Criteria:

For Question 1 and 2 (as noted above):

- Limiting sample size to a minimum of 30 tumours.
- Incorporating studies that include tumours that have been tested by both IHC and MSI.
- Including studies that test at least the core panel of two MSI markers (BAT25, BAT26).

For Question 3:

- Studies assessing a cohort of patients with tumours
- Studies screening for the *BRAF V600E* mutation in tumours to differentiate between sporadic and LS-associated cancers
- Studies screening for *MLH1* promoter methylation in tumours to differentiate between sporadic and LS-associated cancers

Exclusion Criteria:

- Case studies, letters, comments, or editorials.
- Non-English publications.

A review of the titles and abstracts that resulted from the search was performed independently by one of the reviewers (JB). For those items that warranted full-text review, JB reviewed each item independently. However, in cases where there was uncertainty in including certain articles, a second reviewer (AP) was asked to review.

Data Extraction and Assessment of Study Quality and Potential for Bias

Data from the included studies were independently extracted by JB. If more than one publication addressed the same study, only the most updated or recent versions of the data were reported in the result. All extracted data and information were audited by an independent auditor.

The QUADAS-2 (Quality Assessment of Diagnostic Accuracy Studies-2) (20) was used to assess study quality in four key domains: patient selection, index test(s), reference standard, and flow and timing. The signaling questions in each domain are rated in terms of risk of bias (low, high, unclear) and concerns regarding applicability (low, high, unclear), with associated signaling questions to help with bias and applicability judgments.

The potential for bias in the domain of **patient selection** was assessed on the basis of the enrollment of the study sample (consecutive, random, or convenience), the avoidance of a case-control design, and the avoidance of inappropriate patient exclusions.

The potential for bias in the domain of the **index tests** (IHC/MSI) were assessed according to whether results were interpreted without knowledge of the results of germline testing and whether a pre-specified threshold was used for the index tests.

The potential for bias in the domain of the **reference standard** (i.e., the gold standard used to confirm a diagnosis of LS – germline mutation testing) was judged on the basis of whether the germline mutation testing was likely to correctly classify the target

condition (LS) and whether the results were interpreted with knowledge of the MSI/IHC results.

The potential for bias in the domain of **flow and timing** was assessed on the basis of inappropriate intervals between MSI/IHC and germline testing, standardized administration of testing among patients, all patients receiving germline testing, and equal inclusion of patients in the analysis. Specific questions addressed for each domain are in Appendix IV.

Each signaling question required a 'yes', 'no', or 'unclear' response. We developed decision rules to consolidate 'yes', 'no', or 'unclear' responses to the questions into a single 'yes', 'no', or 'unclear' response for each risk of bias domain. The decision rules are shown in Appendix IV.

Synthesizing the Evidence

Specificity and sensitivity across the studies could not be calculated due to the fact that sequencing, genotyping for MSI and MMR was not done on every sample in the study; in other words, in most cases, germline mutation analysis was conducted only on individuals that exhibited high MSI levels and/or with abnormal (deficient) expression for the MLH1, MSH2, MSH6, or PMS2 proteins. Thus, to assess the ability of IHC, and the compared ability of IHC and MSI, to detect tumours that are LS, we present positive predictive value (PPV - the probability that subjects with a positive screening test truly have the disease) and the proportion of tumours correctly determining LS detected by IHC/MSI divided by the number of cases of LS determined by germline mutation testing.

To assess the ability of IHC to detect tumours that are MSI-H, Kappa was used to measure the agreement between the two diagnostic tests of interest, with 0 indicating no more agreement that can be expected on the basis of chance and the value 1 indicating perfect agreement. For this review, a Kappa value lower than 0.4 represent poor agreement, values between 0.4 and 0.75 fair to good agreement, and values higher than 0.75 excellent agreement.

For ease of view, studies are presented according to whether the authors choose to look at all four IHC proteins or a selected group (e.g., MLH1, MSH2 only). Finally, we assessed studies by selected (population selected based on characteristics typically associated with LS [e.g., age, family history, etc]) and unselected populations (population not selected based on characteristics typically associated with LS) to deal with the diverse populations presented in the studies and the influence of prevalence on PPV.

Studies assessing the effectiveness of *BRAF* and *MLH1* promoter methylation to differentiate between LS and sporadic tumours (questions 3a, 3b, 3c) are presented for both MMR mutation-negative and -positive tumours. Not enough data were presented by studies to assess *MLH1* promoter methylations by promoter regions tested (A, B, C, and D).

When clinically homogenous results from two or more trials were available, a meta-analysis would be conducted using the Review Manager software (RevMan 5.1) available from the Cochrane Collaboration (21).

RESULTS

As previously mentioned, an article by Shia et al. 2008 (16) examined pre-2008 pertinent literature data in CRC and found that IHC assessment of MLH1, MSH2, MSH6, and PMS2 protein levels resulted in a predictive value that was comparable to that of MSI testing. Thus, a literature search was performed on literature from mid-2007 to the present, for both EC and CRC.

As previously mentioned, two very recent (2012 and 2014) systematic reviews assessed the utility of *BRAF V600E* and *MLH1* methylation status of EC (18) and CRC (19) tumours as

predictive markers of germline MMR mutation status of cancer patients. We have searched more recent literature.

Primary Literature Systematic Review

The primary literature review yielded a total of 1976 articles from all identified databases, after duplicates were removed. Of these, 1844 were excluded after reviewing the titles and abstracts. After reviewing the remaining 132 articles, 113 were excluded for not meeting eligibility criteria. A total of 19 articles were included in this review. The articles selection process and reasons for exclusions are summarized in the PRISMA flow diagram in Figure 1.

Literature Search Results

Sixteen studies were eligible for inclusion in the primary literature systematic review for questions 1 and 2. Nine full-text articles examined MSI and IHC testing in CRC patients and seven examined EC tumours.

Four articles examined the effectiveness of *BRAF V600E* and/or *MLH1* promoter methylation to distinguish sporadic tumours from LS tumours and were eligible for inclusion. Three examined CRC tumours and one examined EC tumours (all full text).

Study Design and Quality

Among the 19 studies examining both CRC and EC, 14 were prospective cohort studies (22-35) and the remaining five were retrospective (36-40).

Table 1a shows the risk of bias and applicability for studies examining IHC and MSI for CRC using the QUADAS-2 tool. Five of the nine studies were assessed as having high risk of bias for patient selection, mainly due to the selection of patients who were more likely to have LS based on age and family history. All studies were rated as low risk on the domain of index testing, mainly due to the fact that our inclusion criteria specified studies testing for both MSI and IHC. Only one test was scored as having a low risk of bias for reference standard (germline testing) and all studies scored as high risk on the domain of flow and timing, due to the fact that they did not provide germline testing to the entire population. All studies scored low for concern about applicability for the domains of patient selection and index testing. All studies were rating as high concern on applicability for the reference standard, again, mainly due to the fact that germline mutation analysis was conducted only on individuals that exhibit high MSI levels and/or with abnormal (deficient) expression for the MLH1, MSH2, MSH6, or PMS2 proteins.

Table 1b shows the risk of bias and applicability for studies examining IHC and MSI for EC. Three of the seven studies were assessed as having high risk of bias for patient selection and all were rated as low risk on the domain of index testing. For all studies, it was unclear as to the risk of bias for the reference standard (germline testing) and, with the exception of one, studies scored as high risk on the domain of flow and timing. All studies scored low for concern about applicability for the domains of patient selection and index testing. All studies, except for one, were rating as high concern on applicability for the reference standard.

Table 1c shows the risk of bias and applicability for studies examining *BRAF* and/or *MLH1* promoter methylation for EC. All three studies were assessed as having high risk of bias for patient selection and all were rated as low risk on the domain of index testing. For all studies, it was unclear as to the risk of bias for the reference standard (germline testing) and, all studies scored as high risk on the domain of flow and timing. All studies scored low for concern about applicability for the domains of patient selection and index testing. All studies were rating as high concern on applicability for the reference standard.

Table 1d shows the risk of bias and applicability for studies examining *BRAF* and/or *MLH1* promoter methylation for EC. The single study showed low risk of bias for the domains of patient selection and index testing. It was unclear as to the risk of bias for the reference standard (germline testing) and flow and timing was considered as being at high risk of bias. The study scored low for concern about applicability for the domains of patient selection and index testing and rated as high concern on applicability for the reference standard.

Figure 1: Flow Diagram of Search Results

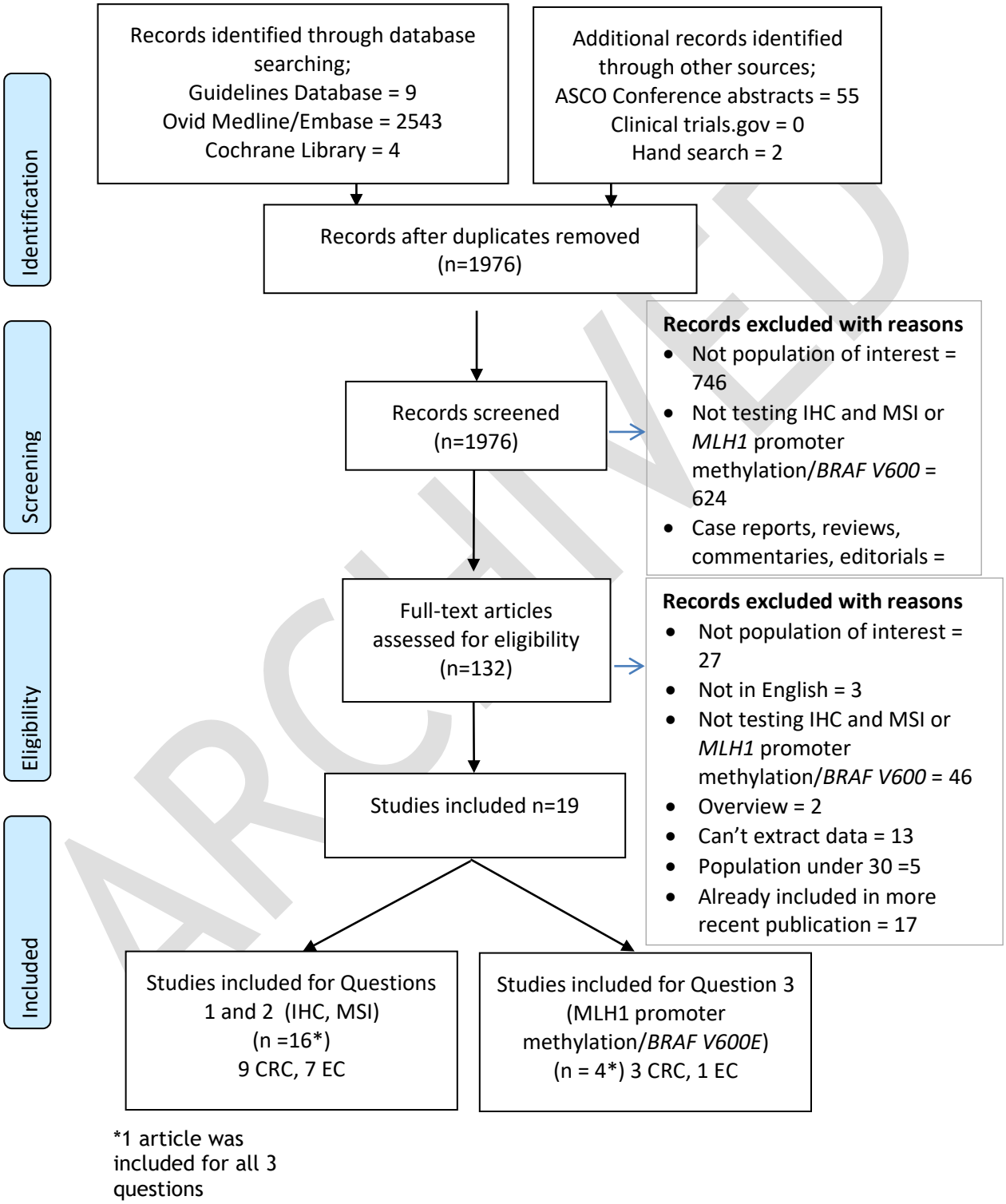

Table 1a. Risk of bias and applicability for studies examining IHC and MSI for colorectal cancer - Results of QUADAS 2 quality assessment

	Risk of bias				Applicability Concerns		
Author	**Patient Selection**	**Index Test**	**Reference Standard**	**Flow & Timing**	**Patient Selection**	**Index Test**	**Reference Standard**
Bonnet et al, 2012 (22)	X	√	X	X	√	√	X
Canard et al, 2012 (24)	√	√	?	X	√	√	X
Hampel et al, 2008 (27)	√	√	√	X	√	√	X
Mueller et al, 2009 (30)	X	√	?	X	√	√	X
Perez-Carbonell et al, 2012 (31)	√	√	?	X	√	√	X
Stigliano et al, 2014 (33)	√	√	?	X	√	√	X
van Lier et al, 2011 (34)	X	√	?	X	√	√	X
Yan et al, 2008 (36)	X	√	?	X	√	√	X
Yoon et al, 2011 (35)	X	√	?	X	√	√	X

√ = low risk; X = high risk; ? = unclear

Table 1b. Risk of bias and applicability for studies examining IHC and MSI for endometrial cancer - Results of QUADAS 2 quality assessment

	Risk of bias				Applicability Concerns		
Author	**Patient Selection**	**Index Test**	**Reference Standard**	**Flow & Timing**	**Patient Selection**	**Index Test**	**Reference Standard**
Egoavil et al, 2013 (25)	X	√	?	X	√	√	X
Ferguson et al, 2014 (26)	√	√	?	?	√	√	?
Leenen et al, 2012 (28)	√	√	?	X	√	√	X
Lu et al, 2007 (29)	X	√	?	X	√	√	X
Modica et al, 2007 (37)	√	√	?	X	√	√	X
Peterson et al, 2012 (32)	√	√	?	X	√	√	X
Walsh et al, 2008 (38)	X	√	?	X	√	√	X

√ = low risk; X = high risk; ? = unclear

Table 1c. Risk of bias and applicability for studies examining *BRAF V600E* and/or *MLH1* promoter methylation for colorectal cancer - Results of QUADAS 2 quality assessment.

	Risk of bias				Applicability Concerns		
Author	**Patient Selection**	**Index Test**	**Reference Standard**	**Flow & Timing**	**Patient Selection**	**Index Test**	**Reference Standard**
Bonnet et al, 2012 (22)	X	√	X	X	√	√	X
Capper et al, 2013 (39)	X	√	?	X	√	√	X
Thiel et al, 2013 (40)	X	√	?	X	√	√	X

√ = low risk; X = high risk; ? = unclear

Table 1d. Risk of bias and applicability for studies examining *BRAF V600E* and/or *MLH1* promoter methylation for endometrial cancer - Results of QUADAS 2 quality assessment.

	Risk of bias				Applicability Concerns		
Author	**Patient Selection**	**Index Test**	**Reference Standard**	**Flow& Timing**	**Patient Selection**	**Index Test**	**Reference Standard**
Buchanan et al, 2014 (23)	√	√	?	X	√	√	X

√ = low risk; X = high risk; ? = unclear

Abbreviations: IHC = immunohistochemistry; MSI = microsatellite instability

Outcomes

Meta-analyses of the study results were not feasible because patient characteristics, the number of MMR system proteins detected by IHC and MSI markers analyzed, along with the testing assays, were very different among the eligible studies.

Studies Assessing MSI and IHC (Q1 and Q2)

Table 2 shows the characteristics of the nine studies examining MSI and IHC for CRC tumours for Questions 1 and/or 2. Eight were prospective cohort studies (22, 24, 27, 30, 31, 33-35) and one was retrospective (36). Sample sizes ranged from 71 (30) to 2093 (31). Four studies used selected populations (22, 30, 33, 34), with patients selected based on their age, personal history of CRC or EC, family history, or a combination of the three. The remaining studies used unselected populations (24, 27, 31, 35, 36). The mean age at diagnosis ranged from 42 (36) to 77 years (24). Five studies examined protein levels of all four MMR system components with IHC (27, 30, 31, 33, 34), three examined three (MLH1, MSH2, MSH6) (22, 24, 36) and one examined only two (MLH1, MSH2) (35). Most studies defined MSI-H if at least one to three markers showed instability.

Table 3 shows the characteristics of the seven studies examining MSI and IHC for EC tumours for Questions 1 and or 2. Five were prospective cohort studies (25, 26, 28, 29, 32) and the remaining two were retrospective (37, 38). Sample sizes ranged from 90 (37) to 179 (28). Two studies used selected populations (29, 38), with patients selected based on their age, personal history of CRC or EC, family history, or a combination of the three. The remaining studies used unselected populations (25, 26, 28, 32, 37). The mean age at diagnosis ranged from 44 (29) to 68 years (25). Six studies examined all four MMR system proteins detected by IHC (25, 26, 28, 32, 37, 38), and one examined three (MLH1, MSH2, MSH6) (29). Most studies defined MSI-H if at least one to three markers showed instability.

Q1a: For patients with CRC or EC, what is the evidence for using IHC testing to identify tumours that are MSI-H?

Eight studies assessed the ability of IHC to detect tumours that were MSI-H for CRC. Kappas ranged from 0.73 (33) to 0.83 (36) for studies examining selected populations. For studies examining unselected populations, with the exception of one study showing a 0.58 result (35), Kappas suggested excellent agreement between the two tests, with scores ranging from 0.81 (31) to 0.95 (24) (see Table 4 and Appendix V).

Seven studies assessed the ability of IHC to detect tumours that are MSI-H for EC. Kappas ranged from 0.74 (29) to 0.75 (38) for studies examining selected populations and from 0.74 (37) to 1.00 (28) in unselected population, suggesting good to excellent agreement between the two tests for both groups (see Table 5 and Appendix VI).

Q1b: For patients with CRC or EC, what is the evidence for using IHC testing to identify tumours that are LS?

Table 6 shows studies assessing IHC and MSI in detecting LS among CRC patients. Overall, the ability of IHC to identify tumours that were LS ranged from 74% (30) to 100% (34) in selected populations and from 79% (31) to 94% (27) in unselected populations. As expected, the PPVs were higher in selected populations, ranging from 48% (22, 33) to 59% (30); in unselected populations they ranged from 7% (31) to 30% (36) (Table 6).

Table 7 shows studies assessing IHC and MSI for detecting LS for EC. Overall, the ability of IHC to identify tumours that were LS was 100% (29) in selected populations and 88% (25) to 100% (26, 28) in unselected populations. The PPV for the one study, with a selected population, was 38% (29). For unselected populations, the PPVs ranged from 12% (25) to 28% (26) (Table 7).

Q2. How does IHC testing compare with MSI testing for identifying the MMR deficiencies characterizing LS in CRC tumours and EC tumours?

Overall, IHC was comparable to MSI in its ability to identify tumours that were LS for CRC patients, ranging from 74% (30) to 100% (34) for IHC versus 91% (30) to 100% (33, 34) for MSI in selected populations, and 79% (31) to 94% (27) for IHC versus 84% (24) to 100% (27, 31) for MSI in unselected populations. For the most part, PPVs were comparable for IHC and MSI in both selected and unselected populations, with the exception of two studies (30, 33) having MSI PPVs much higher than those of IHC (MSI 75% versus IHC 59% (30); MSI 79% versus IHC 48% (33)) (Table 6).

Overall, IHC was comparable to MSI in its ability to identify tumours that were LS for EC, 100% versus 100% (29) of the time in selected populations, and 88% (25) to 100% (26, 28) for IHC versus 75% (25) to 100% (26, 28) for MSI in unselected populations. PPVs were comparable for IHC and MSI, with 38% (29) for IHC and 32% (29) for MSI in selected populations; in unselected populations, PPVs ranged from 12% (25) to 28% (26) for IHC and 13% (25) to 29% (26) for MSI (Table 7).

Table 2. Characteristics of studies examining MSI and IHC for colorectal cancer tumours

Author, year; study design	Patient selection	Age at diagnosis (range); Sex	IHC proteins analyzed	MSI markers	MSI-H (unstable) defined
Bonnet et al, 2012 (22); prospective cohort	307 CRC patients that met at least one of three clinical criteria were included: (1) CRC before 50 years, (2) personal history of colorectal or endometrial cancer, (3) first-degree relative history of colorectal or endometrial cancer (selected)	Mean ± SD age 53±15 yrs); 48% male	MLH1, MSH2, MSH6	BAT26, BAT25, D5S346, D2S123, D17S250	5 markers high level of MSI (MSI-H) 1 or more
Canard et al, 2012 (24); prospective cohort	1040 CRC patients (unselected)	Sporadic: 77 yrs (21–98); 33% male Possible LS: 55 yrs (16–86); 66% male	MLH1, MSH2, MSH6	NR21, NR22, NR24, BAT25, BAT26	Tumours were scored as MSI-H if at least 3 of the 5 markers showed instability
Hampel et al, 2008 (27) (see Hempel 2005); prospective cohort	483 tumours from unselected patients with CRC	NR; 52% male	MLH1, MSH2, MSH6, PMS2	5 or 6 polymorphic markers (BAT25, BAT26, D2S123, D5S346, and D18S69 or D17S250 or both)	MSI-H defined as instability shown by two or more markers,
Mueller et al, 2009 (30) (see Syngal 1999, 2000); prospective cohort	71 suspected HNPCC cases, 48 with tumours (selected)	NR	MLH1, MSH2, MSH6, PMS2	NR	Tumours screened for MSI using 5 and 10 MSI marker panels recommended by the National Cancer Institute consensus groups
Perez-Carbonell et al, 2012 (31); prospective cohort	2093 patients with CRC from the EPICOLON I and II cohorts (unselected)	Median age 70.5 yrs (26-101); 60% male	MLH1, MSH2, MSH6, PMS2	BAT26 and NR24	Tumours classified as MSI when either of the two markers was unstable.
Stigliano et al, 2014 see also: Sanchez et al, 2012 (33) (abstract); prospective cohort	117 CRC patients aged ≤ 50 yrs (selected) (no family history n=70; with Am.II criteria n=40; family history w/o Am.II criteria n=7 (selected)	Mean age at diagnosis 42 yrs (20–50); 37% male	MLH1, MSH2, MSH6, PMS2	NR	NR

Table 2. Characteristics of studies examining MSI and IHC for colorectal cancer tumours

Author, year; study design	Patient selection	Age at diagnosis (range); Sex	IHC proteins analyzed	MSI markers	MSI-H (unstable) defined
van Lier et al, 2011 (34) (LIMO study group); prospective cohort (not Q1b only – can't determine how many IHC predict MSI)	1117 CRC patients ≤70 yrs and patients with advanced colorectal adenomas ≤45 yrs (selected)	Median age 61 yrs; 57% male	MLH1, MSH2, MSH6, PMS2	NR	Tumours with more than one unstable marker categorized as having a high degree of microsatellite instability (MSI-H).
Yan et al, 2008(36); retrospective cohort	227 CRC tumours receiving both IHC and MSI testing. (unselected)	Mean age 41.5 (21–68); 54% male	MLH1, MSH2, MSH6	Two mono-(BAT25, BAT26), and three di-(D2S123, D5S346, D17S250) repeat markers	MSI-H when 2–5 markers identified as unstable.
Yoon et al, 2011(35) (see also Yoon 2010) (Q1a only – no GMA); prospective cohort	2028 sporadic CRC samples (unselected)	Mean age 59.9 (SD±11); 60% male	MLH1, MSH2	BAT25, BAT26, D5S346, D2S123, and D17S250	MSI-H, two or more unstable markers

Selected population = population selected based on characteristics typically associated with Lynch syndrome (e.g., age, family history, etc); Unselected population = a population not selected based on characteristics typically associated with Lynch syndrome

Abbreviations: Am.II criteria = Amsterdam criteria (revised); CRC = Colorectal cancer; GMA = Germline mutation analysis; HNPCC = Hereditary nonpolyposis colorectal cancer; IHC = Immunohistochemistry; MSI = Microsatellite instability; NR = Not reported; w/o = Without; yrs = Years

Table 3. Characteristics of studies examining MSI and IHC for endometrial cancer tumours

Author, year	Patient selection	Age at diagnosis (range)	IHC proteins analyzed	MSI markers	MSI-H (unstable) defined
Egoavil et al, 2013 (25); prospective cohort	173 consecutive patients with newly diagnosed EC (unselected)	Mean 63 yrs (29-90)	MLH1, MSH2, MSH6, PMS2	BAT26, BAT25, NR21, R24, NR27	Diagnosis of MSI was considered positive when two or more markers showed an altered pattern
Ferguson et al, 2014 (26); prospective cohort	118 consecutive patients with EC (unselected)	Median age 61 yrs (26-91)	MLH1, MSH2, MSH6, PMS2	BAT25, BAT26, D17S250, D5S346, D2S123	Positive for MSI if ≥1 markers unstable
Leenen et al, 2012(28); prospective cohort	179 consecutive patients ≤70 newly diagnosed with EC (unselected)	Median age 61 yrs (IQR 57–66)	MLH1, MSH2, MSH6, PMS2	(BAT-25, BAT-26, NR-21, NR-24 and MONO-27)	Tumour with >1 unstable marker categorized as MSI-H

Table 3. Characteristics of studies examining MSI and IHC for endometrial cancer tumours

Author, year	Patient selection	Age at diagnosis (range)	IHC proteins analyzed	MSI markers	MSI-H (unstable) defined
Lu et al, 2007 (29); prospective cohort	100 women younger than 50 years of age at the time of diagnosis (selected)	Median age 44.0 yrs (24-49)	MLH1, MSH2, MSH6	BAT25, BAT26, BAT40, D2S123, D5S346, D173250	Tumours showing allelic shift at two or more markers were classified as MSI-H
Modica et al 2007 (37); (Q1a only – no germline mutation testing); retrospective cohort	90 patients selected from a gynecologic database that contained 473 patients whose tumours had been tested for MSI (unselected)	Mean age 63.8 yrs (37 to 86).	MLH1, MSH2, MSH6, PMS2	BAT25, BAT26, D2S123, D5S346, D17S250	Tumours classified as MSI-H if at least 2 of the 5 markers displayed band shifting in tumour DNA when compared with normal tissue DNA
Peterson et al, 2012 (32) (Q1a only – no GMA); prospective cohort	96 cases of EC (unselected)	Mean age 66 (42-92)	MLH1, MSH2, MSH6, PMS2	4 mononucleotide and 6 dinucleotide repeat markers, including BAT26, D17S250, D5S346, ACTC, D18S55, BAT40, D10S197, BAT34c4, MYCL, and BAT25	Tumours showing instability in 30% of markers were considered to be MSI-H
Walsh et al, 2008 (38) (Q1a only – no GMA); retrospective cohort	146 EC patients ≤50 yrs of age (selected)	Mean age 45.1 yrs (28-50)	MLH1, MSH2, MSH6, PMS2	10 MSI markers included BAT25, BAT26, BAT34C4, and BAT40; dinucleotide markers D5S346, D17S250, ACTC, D18S55, D10S197	Tumours classified as MSI-H if ≥30% of the markers showed instability

Selected population = population selected based on characteristics typically associated with Lynch syndrome (e.g., age, family history, etc); Unselected population = a population not selected based on characteristics typically associated with Lynch syndrome
Abbreviations: EC = Endometrial cancer; GMA = Germline mutation analysis; IHC = Immunohistochemistry; IQR Interquartile range; MSI = Microsatellite instability; Yrs = Years

Table 4. Studies assessing the ability of IHC to detect tumours that are MSI-H for *colorectal cancer* (Q1a)*

			Level of Agreement		
Author, year	**MMR proteins analyzed**	**Sample Size**	**Kappa**	**SE of Kappa**	**95% CI**
Selected Population					
Yan et al, 2008	MLH1, MSH2, MSH6	227	0.83	0.038	0.75-0.90
Bonnet et al, 2012	MLH1, MSH2, MSH6	268	0.95	0.026	0.91-1.00
Mueller et al, 2009	MLH1, MSH2, MSH6, PMS2	48	0.87	0.072	0.73-1.00
Stigliano et al, 2013	MLH1, MSH2, MSH6, PMS2	117	0.73	0.076	0.58-0.88
Unselected Population					
Yoon et al, 2011	MLH1, MSH2	2028	0.58	0.03	0.52-0.64
Canard et al, 2011	MLH1, MSH2, MSH6	1040	0.95	0.017	0.91-0.98
Hampel et al, 2008	MLH1, MSH2, MSH6, PMS2	483	0.82	0.039	0.74-0.89
Perez-Carbonell, 2012	MLH1, MSH2, MSH6, PMS2	2093	0.81	0.025	0.77-0.86

Selected population = population selected based on characteristics typically associated with Lynch syndrome (e.g., age, family history, etc); Unselected population = a population not selected based on characteristics typically associated with Lynch syndrome
Abbreviations: IHC = Immunohistochemistry; MMR = Mismatch repair; MSI-H = High-frequency microsatellite instability
*See Appendix V for more detailed calculations

Table 5. Studies assessing the ability of IHC to detect tumours that are MSI-H for *endometrial cancer* (Q1a)*

			Level of Agreement		
Author, year	**MMR proteins**	**Sample Size**	**Kappa**	**SE of Kappa**	**95% CI**
Selected Population					
Lu et al, 2007	MLH1, MSH2, MSH6	100	0.74	0.076	0.59-0.89
Walsh et al, 2008	MLH1, MSH2, MSH6, PMS2	146	0.75	0.065	0.62-0.88
Unselected Population					
Egoavil et al, 2013	MLH1, MSH2, MSH6, PMS2	173	0.77	0.053	0.67-0.87
Ferguson et al, 2014	MLH1, MSH2, MSH6, PMS2	114	0.93	0.040	0.85-1.00
Leenen et al, 2012	MLH1, MSH2, MSH6, PMS2	179	1	0	1.0-1.0
Modica et al, 2007	MLH1, MSH2, MSH6, PMS2	90	0.74	0.073	0.60-0.88
Peterson et al, 2012	MLH1, MSH2, MSH6, PMS2	96	0.84	0.063	0.72-0.96

Selected population = population selected based on characteristics typically associated with lynch syndrome (e.g. age, family history, etc); Unselected population = a population not selected based on characteristics typically associated with lynch syndrome
Abbreviations: IHC = Immunohistochemistry; MMR = Mismatch repair; MSI-H = High-frequency microsatellite instability
*See Appendix VI for more detailed calculations

Table 6. Studies assessing IHC and MSI for detecting Lynch syndrome for *colorectal cancer* tumours (Q1b, Q2)

Author, year	MMR proteins analyzed	PPV [a] (%)		# LS pred./#LS[b](%)	
		IHC	MSI	IHC	MSI
Selected Population					
Bonnet al, 2012	MLH1,MSH2,MSH6	19/40 **(48%)**	19/40 **(48%)**	19/20 **(95%)**	19/20 **(95%)**
Mueller et al, 2009	MLH1,MSH2,MSH6,PMS2	17/29 **(59%)**	21/28 **(75%)**	17/23 **(74%)**	21/23 **(91%)**
Stigliano et al, 2014	MLH1,MSH2,MSH6, PMS2	14/29 **(48%)**	19/24 **(79%)**	14/18 [c] **(78%)**	19/19 **(100%)**
van Lier et al, 2011	MLH1,MSH2,MSH6,PMS2	CD	CD	26/26 **(100%)**	26/26 **(100%)**
Unselected Population					
Canard et al, 2012	MLH1,MSH2,MSH6	23/79 **(29%)**	21/77 **(28%)**	23/25 **(92%)**	21/25**(84%)**
Yan et al, 2008	MLH1,MSH2,MSH6	24/79 **(30%)**	27/97 **(28%)**	24/28 **(86%)**	27/28 **(96%)**
Hampel et al, 2008	MLH1,MSH2,MSH6,PMS2	17/71 **(24%)**	18/64 **(28%)**	17/18 **(94%)**	18/18 **(100%)**
Perez-Carbonell, 2012	MLH1,MSH2,MSH6,PMS2	11/153 **(7%)**	14/152 **(9%)**	11/14 **(79%)**	14/14 **(100%)**

[a] PPV = T he probability that subjects with a positive screening test truly have the disease
[b] # LS correctly determined by test (IHC or MSI) /# confirmed LS
[c] One inconclusive results for MSI
Selected population = population selected based on characteristics typically associated with LS (e.g., age, family history, etc); Unselected population = a population not selected based on characteristics typically associated with LS
Abbreviations: CD = Cannot be determined from information given; IHC = Immunohistochemistry; LS = Lynch syndrome; MMR = Mismatch repair; MSI = Microsatellite instability; PPV = Positive predictive value

Table 7. Studies assessing IHC and MSI for detecting Lynch syndrome for *endometrial cancer* tumours (Q1b, Q2)

Author, year	MMR proteins analyzed	PPV [a] (%)		# LS pred./#LS[b](%)	
		IHC	MSI	IHC	MSI
Selected Population					
Lu et al, 2007	MLH1,MSH2,MSH6	9/24 **(38%)**	8/25 **(32%)**	9/9 **(100%)**	8/8 **(100%)**
Unselected Population					
Egoavil et al,2013	MLH1,MSH2,MSH6,PMS2	7/58 **(12%)**	6/47 **(13%)**	7/8 **(88%)**	6/8 **(75%)**
Ferguson et al, 2014	MLH1,MSH2,MSH6,PMS2	7/25 **(28%)**	6/21 **(29%)**	7/7 **(100%)**	6/6 **(100%)**
Leenan et al, 2012	MLH1,MSH2,MSH6,PMS2	7/41 **(17%)**	7/42 **(17%)**	7/7 **(100%)**	7/7**(100%)**

[a] PPV = The probability that subjects with a positive screening test truly have the disease
[b] # LS correctly determined by test (IHC or MSI) /# confirmed LS
Selected population = population selected based on characteristics typically associated with LS (e.g., age, family history, etc); Unselected population = a population not selected based on characteristics typically associated with LS
Abbreviations: IHC = Immunohistochemistry; LS = Lynch syndrome; MMR = Mismatch repair; MSI = Microsatellite instability; PPV = Positive predictive value

Studies Assessing BRAF V600E and/or MLH1 Promoter Methylation (Q3)

Table 8 shows the characteristics of the three studies examining *BRAF V600E* and/or *MLH1* methylation for CRC tumours for Question 3. One was a prospective cohort study (22) and two were retrospective (39, 40). Sample sizes ranged from 91 (39) to 307 (22). All three studies used selected populations and the mean age at diagnosis ranged from 50 years (39, 40) to 53 years (22). All three assessed the ability of *BRAF V600E* to differentiate between sporadic and LS tumours; none assessed *MLH1* methylation testing. One tested *MLH1*-deficient tumours (22) and the other two tested MSI-H tumours (39, 40).

Table 9 shows the characteristics of one study examining *MLH1* methylation for EC tumours for Question 3 (23). This was a prospective cohort studies with a sample size of 702. An unselected population was used and the mean age at diagnosis was approximately 68 years. *MLH1*-deficient tumours were tested (23).

Q3a: For tumours that show abnormal MLH1 expression by IHC, how effective is BRAF V600E testing at differentiating between sporadic versus LS-associated CRC?

Three studies assessed the effectiveness of the test to determine *BRAF V600E* for CRC patients. Bonnet el al (22) tested 27 of 33 *MLH1* tumours for the *BRAF* mutation and found five carriers. Germline testing was performed on three of the five tumours and all three were negative for LS. Conversely, eight LS tumours tested for *BRAF* were found to be negative. In a study of 91 MSI-H tumours, 11 of 11 tumours classified as *BRAF V600E* mutation positive were positive by IHC, and 79 of 80 tumours classified as *BRAF* wild type showed negative staining (39). Theil et al (40) collected a control sample of 17 cases to test *BRAF V600E* mutation status in LS versus non-mutated MSI-H CRC. Among the 11 confirmed LS cases, none were found to have a *BRAF V600E* mutation. Among the six non-LS cases, three had a *BRAF* mutation (40). No studies assessed the *BRAF V600E* test for EC.

Q3b: For tumours that show abnormal MLH1 expression by IHC, how effective is MLH1 promoter methylation testing at differentiating between sporadic versus LS-associated CRC and EC?

One study assessed the effectiveness of *MLH1* promotor methylation testing in differentiating between LS and sporadic EC. Buchanan et al (23) tested 153 MMR-deficient tumours and a randomly selected subset of 77 tumours with MMR-proficient results as reference. *MLH1* methylation was not detected in the tumours from the two *MLH1* mutation carriers tested, but was detected in 99 of 111 mutation-negative cases with loss of *MLH1*/*PMS2* expression. In the reference group of MMR-proficient tumours, only two of 77 were *MLH1* methylation-positive. No studies assessed *MLH1* methylation testing for CRC.

Q3c: For tumours that show abnormal MLH1 expression by IHC, how effective is a combination of BRAF V600E testing and MLH1 promoter methylation testing at differentiating between sporadic versus LS-associated CRC?

There were no studies that compared *MLH1* promoter methylation and *BRAF V600E* together with respect to their combined abilities to differentiating between sporadic versus LS-associated CRC.

Table 8. Study characteristics for studies *BRAF V600* for *colorectal cancer* tumours.

Author, year; study design	Total Population	Age at diagnosis; Sex	Tests used	Tumours tested for BRAF	BRAF Mutation Testing Methods
Bonnet et al, 2012 (22); prospective cohort	307 CRC patients that met at least one of three clinical criteria: (1) CRC before 50 years, (2) personal history of colorectal or endometrial cancer, (3) first-degree relative history of colorectal or endometrial cancer (selected)	Mean age 53 yrs (±SD 15); 48% male	BRAF	*MLH1* deficient tumours	Allelic discrimination using Taqman probes
Capper et al, 2013 (39); retrospective cohort	91 MSI-H CRC specimens (selected)	Mean age 50 yrs (26-92); 53% male	BRAF	All MSI-H cases	IHC stained *BRAF V600E* mutation-specific antibody VE1
Thiel et al, 2013 (40); retrospective cohort	137 consecutive CRC patients, control group of 17 "likely LS" patients and an additional 181 consecutive tumours (selected)	Mean age 50 yrs (range 23–77); 46% males	BRAF	MSI-H	IHC stained BRAF V600 antibody, qPCR

Selected population = Population selected based on characteristics typically associated with Lynch syndrome (e.g., age, family history, etc); Unselected population = A population not selected based on characteristics typically associated with Lynch syndrome
Abbreviations: BRAF = *BRAF V600E* mutation; CRC = Colorectal cancer; IHC = Immunohistochemistry; MSI-H = High-frequency microsatellite instability; qPCR = quantitative polymerase chain reaction; yrs = Years

Table 9. Study characteristics for studies *MLH1* methylation for *endometrial cancer* tumours.

Author, year; Study design	Total Population	Age at diagnosis	Tests used	Tumours tested for meth	Methylation Assay (region tested)
Buchanan et al, 2014 (23); prospective cohort	702 newly diagnosed EC patients (unselected)	Mean 61.8 yrs (27.1-79.8);	Meth	Cases with loss of *MLH1* expression	MS-MLPA

Selected population = Population selected based on characteristics typically associated with Lynch syndrome (e.g., age, family history, etc); Unselected population = A population not selected based on characteristics typically associated with Lynch syndrome
Abbreviations: EC = Endometrial cancer; Meth = *MLH1* promoter hypermethylation; MS-MLPA = Methylation-specific multiplex ligation-dependent probe-amplification technique; yrs = Years

DISCUSSION

The high Kappa scores in this review indicate that IHC testing is an effective testing method for identifying tumours that are MSI-H. As well, evidence in this report indicates that IHC testing is useful in identifying tumours that are LS, with overall percentages of tumours being correctly identified ranging from 73% to 100%. Likewise, IHC was relatively comparable to MSI in its ability to identify tumours that were LS, with percentages of identifying tumours ranging from 74% to 100% for IHC versus 75% to 100% for MSI. Four studies examined *MLH1* promoter methylation and/or *BRAF V600E* mutation status and, for the most part, obtained negative test results for LS tumours, indicating that, for CRC and EC patients, these tests are good indicators for triaging *MLH1*-negative patients to germline MMR mutation testing.

In contrast to the good to excellent Kappas presented in Tables 3 and 5, the Kappa of 0.58 calculated from data presented in Yoon et al (35) indicated only fair agreement between the two tests. Yoon et al (35) conceded that "the sensitivity of IHC in detecting MSI-H was lower than that reported in previous studies." They hypothesize that a false interpretation of IHC might be a result of technical limitations or the occurrence of focal or cytoplasmic staining" (35). They also suggest that "the accuracy of IHC might also have been limited by the fact that only two IHC antibodies were used (MLH1, MSH2)" (35). None the less, Kappa values from the remaining studies assessing the ability of IHC to detect tumours that are MSI-H showed good to excellent agreement between the two tests. Although we did not extract this type of data, this potentially indicates a need for technical quality assessment or validation of IHC testing.

For the most part, PPVs were comparable for IHC and MSI in selected populations for CRC, with the exception of two studies having MSI PPVs much higher than those of IHC (MSI 75% versus IHC 59% (30); MSI 79% versus IHC 48% (33) (Table 5). According to Stigliano et al (33) in their study, "IHC was misleading, as it showed a lack of expression of MMR genes in three MSI-H patients in which the germline mutation analysis did not reveal any deleterious mutation." They state that "the main factors potentially affecting IHC staining are tissue processing, antigen retrieval procedures, the type of fixative and duration, condition of tissue fixation." For the most part, PPVs were comparable for IHC and MSI in unselected populations for CRC. However, the large population-based cohort used in Perez-Carbonell et al had a much lower PPV (7%) compared with the other three studies (31), mainly based on its low prevalence of LS. The authors speculate that this may be due to the lower rates of MSI in Mediterranean populations due to dietary, toxic or other environmental factors (31). Again, this addressed the need for quality assessment or validation for IHC testing.

For EC studies, IHC and MSI were comparable in their ability to identify tumours that were LS for both selected and unselected populations. However, the unselected population used in Ferguson et al had a much higher PPV (28% for IHC versus 29% for MSI) compared with the other two studies, mainly based on its higher prevalence of LS in this EC population (6%). The authors speculate that "younger patients with a strong family history of cancer may have been more motivated to participate in this trial, thereby introducing bias in the study population and possibly accounting for the observed mutation rate." However, the authors do not provide study characteristics (specifically age) for individuals who refused study participation (26).

This review demonstrates one of the major gaps in the literature. That is, most studies do not perform mutation testing in cases that that do not exhibit MSI and/or with normal IHC staining, limiting the ability to examine false negatives and true negatives for these two tests in predicting germline mutations. For this very reason, most studies in this review were rated as having a high risk of bias on the domains of the "reference standard" and "flow and timing" in our quality assessment. However, one recent study included in this review did offer

genetic testing to all study participants and found that both IHC and MSI testing had high sensitivity (100%) and specificity (>78%) for EC patients (26). The study also found improved performance of IHC and MSI testing among women younger than 60 years of age, with a significant impact on the PPV from 28% to >58% (26). The authors conclude that "although MSI testing maybe an effective alternative, IHC is less expensive and directs gene-specific germline mutation testing, thereby offering an overall cost savings." The authors state that "further studies are needed to ensure adequate implementation of these universal screening programs to maximize the identification of women at risk of LS, and to determine the most cost-effective strategies to prevent cancers in these LS families."

While both methylation of the *MLH1* promoter and *BRAF V600E* mutation testing have been used in several studies, most studies used the tests to rule out further testing for germline MMR status and, thus, negative tumours were not tested. We found only four studies that actually conducted germline mutation analysis on a subsample of *MLH1* promoter and/or *BRAF V600E*-negative and -positive tumours. Again, all of these studies mostly obtained negative results for LS on *MLH1* promoter and/or *BRAF V600E*-positive tumours. However, until more studies include germline testing on both negative and positive *MLH1* promoter and/or *BRAF V600E* tumours we cannot, with confidence, determine their ability to triage potential LS patients for germline testing.

CONCLUSIONS

This study found IHC to be a very efficient test to identify tumours that are MSI-H and tumours that are associated with LS. As well, methylation of the *MLH1* promoter and *BRAF V600E* testing has been shown to be effective in distinguishing between tumours that are sporadic versus LS tumours. However, more research is needed on complete populations in order to accurately calculate sensitivities and specificities to truly assess the efficacies of these tests.

CONFLICT OF INTEREST

The authors, members, and reviewers reported that they had no conflicts of interest. In accordance with the PEBC Conflict of Interest Policy, the evidence summary authors, and the Molecular Oncology Advisory Group members were asked to disclose potential conflicts of interest.

INTERNAL REVIEW

The evidence summary was reviewed by the Director of the PEBC. The Working Group was responsible for ensuring the necessary changes were made.

Approval by Molecular Oncology Advisory Committee

After internal review, the report was presented to the MOAC. The MOAC reviewed the document and formally approved it on September 18, 2015.

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Appendix I – Risk categories for individuals eligible for screening for a genetic susceptibility to colon cancer – Ministry of Health and Long-Term Care (July 2009).

Testing for Hereditary Non-Polyposis Colon Cancer (HNPCC)
If a tumour sample is unavailable, germline testing may proceed on the youngest, living, affected individual from families meeting criteria 1 & 2 ONLY.

1. Affected and unaffected individuals from families with a known HNPCC causing mutation.

2. Affected individuals whose families meet the Amsterdam criteria. The family must meet all of the following criteria:
   - Three affected relatives with any combination of colorectal, endometrial, small bowel, ureter, transitional cell kidney cancer (urothelial), sebaceous adenoma/carcinoma and/or keratoacanthoma.
   - One should be a first-degree relative of the other two.
   - At least two successive generations should be affected.
   - At least one diagnosis must be before age 50 years.
   - Tumour type should be confirmed by review of pathology or other medical records.

3. Affected individuals from families with:
   Three affected individuals, one with colorectal cancer, and the other two with any combination of: colorectal, endometrial, small bowel, ureter, sebaceous adenoma/carcinoma, ovarian, pancreatic, kidney (transitional cell cancer only), gastric, primary brain or primary hepatobiliary cancer.
   - Two of the three family members must be in a first-degree relationship.
   - At least one diagnosis younger than 50 years of age.
   - Familial adenomatous polyposis should be excluded.
   - Tumours should be verified by pathological examination.

4. Individual affected with colorectal cancer (CRC) and a second primary HNPCC-associated cancer (as listed in #3). This includes synchronous and metachronous CRCs. At least one primary cancer must be diagnosed before 55 years of age. Families are eligible with or without family history of HNPCC-associated cancer, and tumours should be verified by pathological examination.

5. Individual diagnosed with CRC under the age of 35. Families are eligible with or without family history of HNPCC-associated cancer, and tumours should be verified by pathological examination.

6. One case of CRC before 50 years of age, with a first- or second-degree relative with one of the following HNPCC-related cancers diagnosed before 50 years of age: colorectal, endometrial, small bowel, ureter, urothelial, sebaceous adenoma/carcinoma or keratoacanthoma.

7. Individuals with immunodeficient tumours (regardless of family history) as follows

- *MSH2*-deficient tumour ± *MSH6* deficiency (sequence and multiplex ligation-dependent probe-amplification technique [MLPA] of *MSH2* gene only)
- *MSH6* (only)-deficient tumour (sequence and MLPA of *MSH6* gene only)
- *MLH1*-deficient tumour in individual younger than 60 years of age (sequence and MLPA of *MLH1* gene only)

Appendix II

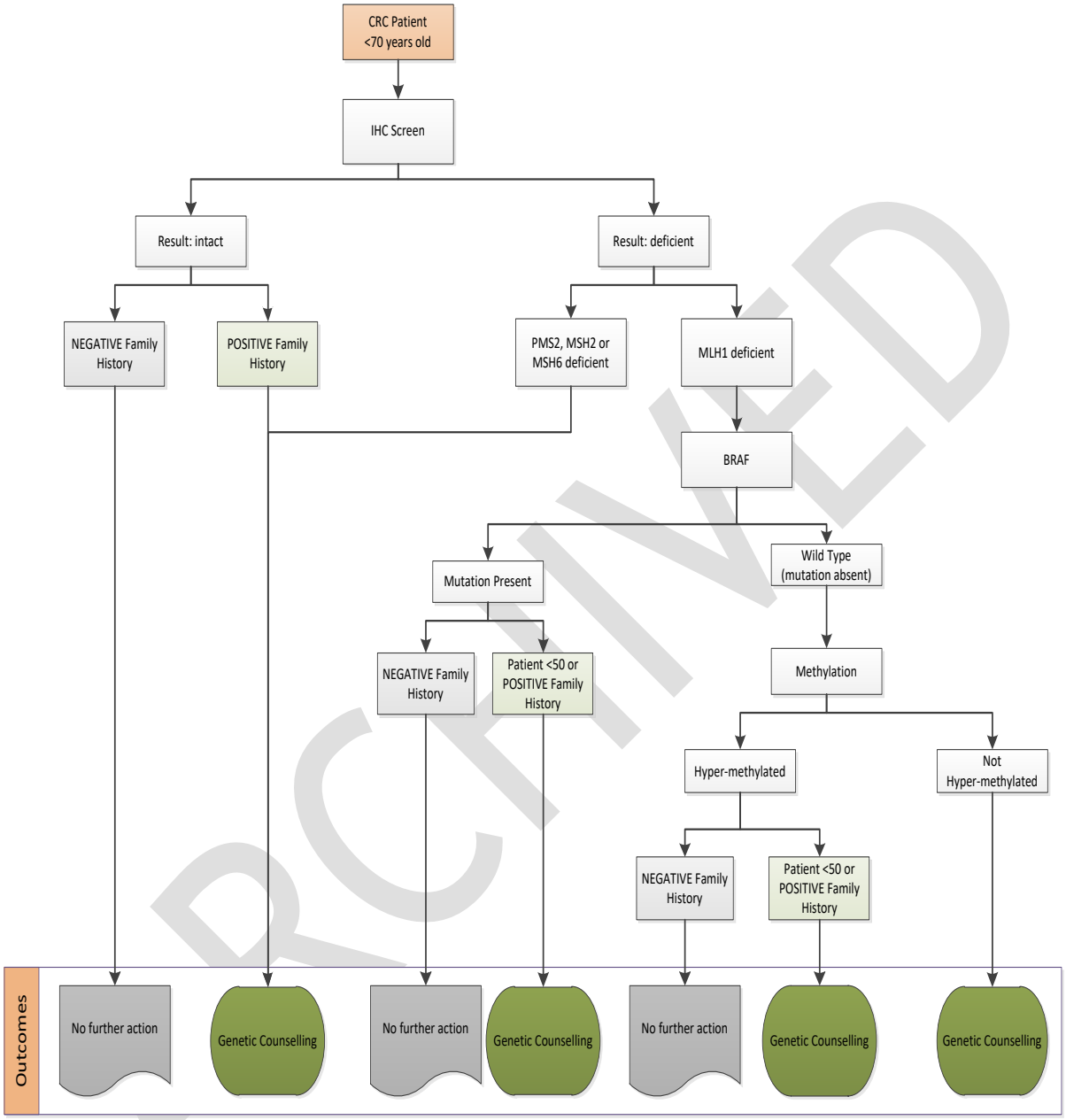

Figure 1a: Testing Algorithm Colorectal Cancer

Abbreviations: CRC = Colorectal cancer; IHC = Immunohistochemistry

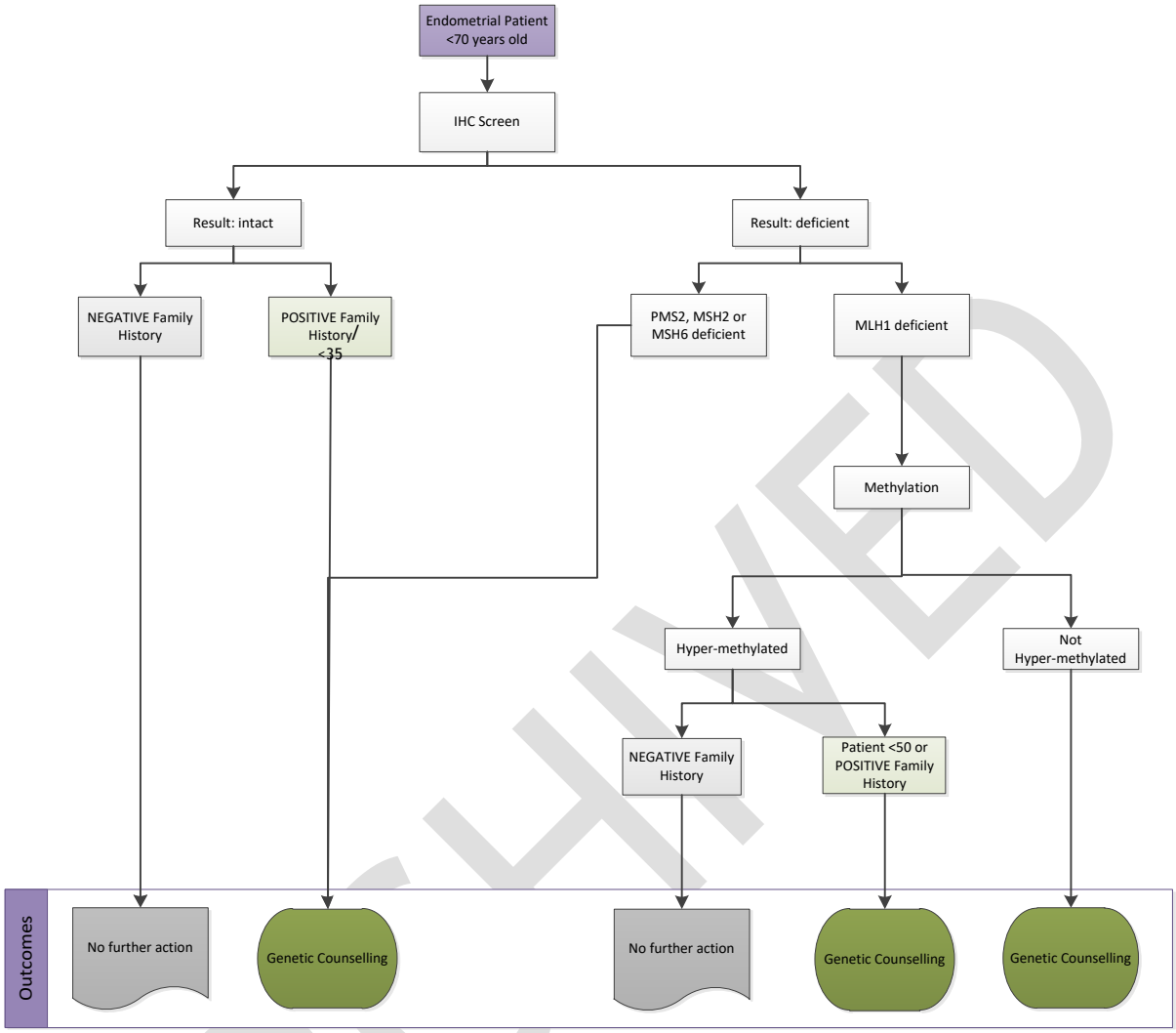

Figure 1b: Testing Algorithm Endometrial Cancer

Abbreviation: IHC = Immunohistochemistry

Appendix III: MOAC-3 - literature search strategy Lynch Syndrome (MEDLINE)

Section A: Disease and/or population	1. exp colorectal neoplasms/su
	2. exp colorectal neoplasms/
	3. (colorectal or colon$ or rectal or rectum or recto$).tw.
	4. or/1-3
	5. exp UTERUS/
	6. uterus.mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
	7. exp UTERINE NEOPLASMS/ or exp UTERINE DISEASES/
	8. uterine.tw.
	9. exp ENDOMETRIAL NEOPLASMS/
	10. or/5-9
Section B: Intervention or diagnostic test	11. lynch syndrome.mp. or exp lynch syndrome, Hereditary Nonpolyposis/
	12. Germ-Line Mutation/ or Hereditary Nonpolyposis/ or hereditary nonpolyposis.mp. or Microsatellite Repeats/
	13. HNPCC.tw.
	14. (*MLH1* or *MSH2* or *MSH6* or h*MSH2* or h*MLH1* or hPMS or h*PMS2* or h*MSH6* or hMLH3).tw.
	15. Or/11-14
	16. (immunohistochemistry or IHC).mp. or exp Immunohistochemistry/ [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier]
	17. (microsatellite instability or MSI-H or MSI).mp. or Colorectal Neoplasms/ or Microsatellite Instability/ or Mutation/ or DNA, Neoplasm/ or Carcinoma/
	18. 16 or 17
	19. 15 or 18
Section C: Study design (only for capturing meta-analysis and systematic reviews)	20. practice guidelines.mp. or exp Practice Guideline/
	21. meta-Analysis as topic/
	22. meta analysis.pt.
	23. (meta analy$ or metaanaly$).tw.
	24. (systematic review$ or pooled analy$ or statistical pooling or mathematical pooling or statistical summar$ or mathematical summar$ or quantitative synthes?s or quantitative overview).tw.
	25. (systematic adj (review$ or overview?)).tw.
	26. (exp Review Literature as topic/ or review.pt. or exp review/) and systematic.tw.
	27. Or/20-25
	28. (cochrane or embase or psychlit or psyclit or psychinfo or psycinfo or cinahl or cinhal or science citation index or scisearch or bids or sigle or cancerlit).ab.
	29. (reference list$ or bibliograph$ or hand-search$ or relevant journals or manual search$).ab.
	30. (selection criteria or data extraction or quality assessment or jadad scale or methodological quality).ab.
	31. (study adj selection).ab.

	32. 28 or 29
	33. review.pt.
	34. 30 and 31
	35. 26 or 27 or 28 or 29 or 33
Section D: Exclusion strategy	36. (comment or letter or editorial or note or erratum or short survey or news or newspaper article or patient education handout or case report or historical article).pt.
Combining Sections A, B, C, and D	37. (4 and 10 and 19 and 35) not 36
Limiting the final search by date and language	38. limit 37 to (English language and humans and yr="1999 - Current")

MOAC-3 - literature search strategy – (Embase)
Lynch Syndrome

Section A: Disease and/or population	1. exp colorectal carcinoma/ or exp hereditary colorectal cancer/ or exp hereditary nonpolyposis colorectal cancer/ or colorectal.mp. or exp colorectal cancer/
	2. endometrial cancer.mp. or exp endometrium cancer/
	3. uterine.mp. or exp uterus/
	4. or/1-3
Section B: Intervention or diagnostic test	5. lynch syndrome.mp. or exp hereditary nonpolyposis colorectal cancer/
	6. lynch syndrome.mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword]
	7. (immunohistochemistry or IHC).ti.
	8. (microsatellite instability or MSI-H or MSI).ti,ab.
	9. or/5-8
Section C: Study design (for MA only)	10. guidelines.mp. or exp practice guideline/
	11. (meta analy$ or metaanaly$).tw.
	12. (systematic review$ or pooled analy$ or statistical pooling or mathematical pooling or statistical summar$ or mathematical summar$ or quantitative synthes?s or quantitative overview).tw
	13. (systematic adj (review$ or overview?)).tw.
	14. (systematic or selection criteria or data extraction or quality assessment or jadad scale or methodological quality).ab.
	15. (study adj selection).ab.
	16. or/10-15
Section D: Exclusion strategy	17. (case report$ or editorial$ OR comment$ OR letter$).pt.
	18. (editorial OR note OR letter erratum OR short survey OR abstract).pt. OR abstract report/ OR letter/ OR case study/
	19. Animal/ not Human/
	20. or/17-19
Combining Sections A, B, C, and D	21. (4 and 9 and 16) not 20
Limiting the final search by date and language	22. limit 21 to (English language and humans and yr="1999 - Current")

Appendix IV

The following table summarizes QUADAS-2 and lists all signalling, risk of bias and applicability rating questions.

DOMAIN	PATIENT SELECTION	INDEX TEST	REFERENCE STANDARD	FLOW AND TIMING
Description	Describe methods of patient selection: Describe included patients (prior testing, presentation, intended use of index test and setting):	Describe the index test and how it was conducted and interpreted:	Describe the reference standard and how it was conducted and interpreted:	Describe any patients who did not receive the index test(s) and/or reference standard or who were excluded from the 2×2 table (refer to flow diagram): Describe the time interval and any interventions between index test(s) and reference standard:
Signaling questions(yes/no/unclear)	Was a consecutive or random sample of patients enrolled?	Were the index test results interpreted without knowledge of the results of the reference standard?	Is the reference standard likely to correctly classify the target condition?	Was there an appropriate interval between index test(s) and reference standard?
	Was a case-control design avoided?	If a threshold was used, was it pre-	Were the reference standard results interpreted without	Did all patients receive a reference standard?

	Did the study avoid inappropriate exclusions?	specified?	knowledge of the results of the index test?	Did all patients receive the same reference standard? Were all patients included in the analysis?
Risk of bias: High/low/unclear	Could the selection of patients have introduced bias?	Could the conduct or interpretation of the index test have introduced bias?	Could the reference standard, its conduct, or its interpretation have introduced bias?	Could the patient flow have introduced bias?
Concerns regarding applicability: High/low/unclear	Are there concerns that the included patients do not match the review question?	Are there concerns that the index test, its conduct, or interpretation differ from the review question?	Are there concerns that the target condition as defined by the reference standard does not match the review question?	

Appendix IV (con't): Decision rules to consolidate responses to QUADAS-2 signalling questions into responses to QUADAS-2 risk of bias questions Domains

Domain 1: Patient Selection (3 questions each domain)
All yes's* = low risk of bias;
All no's* = high risk of bias; or
All unclear* = unclear.
Mixed categories default to the lowest category:
2 yes, 1 no* = high risk of bias;
2 yes, 1 unclear* = unclear;
2 no, 1 yes* = high risk of bias;
2 no, 1 unclear* = high risk of bias;
2 unclear, 1 no* = high risk of bias;
2 unclear, 1 yes* = unclear; or
1 yes, 1 no, 1 unclear* = unclear.

Domains 2 and 3 : Index Test and Reference Standard (2 questions each domain)
Both yes's* = low risk of bias;
Both no's* = high risk of bias;
Both unclear* = unclear;
1 yes, 1 no* = high risk of bias;
1 yes, 1 unclear* = unclear; or
1 no, 1 unclear* = high risk of bias.

Domain 4: Flow and Timing (4 questions each domain)
All yes's* = low risk of bias;
All no's* = high risk of bias; or
All unclear* = unclear.
Mixed categories default to the lowest category:

Appendix V: Q1a - Studies assessing the ability of IHC to detect tumours that are MSI-H for colorectal cancer

	MMR proteins	Sample Size	yes/yes	yes/no	no/yes	no/no	Level of Agreement Kappa	SE of Kappa	95% CI
Bonnet et al, 2012	*MLH1*, *MSH2*, *MSH6*	268	38	1	2	227	0.95	0.026	0.905-1.000
Canard et al, 2011	*MLH1*, *MSH2*, *MSH6*	1040	95	3	7	935	0.945	0.017	0.911-0.979
Hampel et al, 2008	*MLH1*, *MSH2*, *MSH6*, *PMS2*	483	56	6	15	406	0.817	0.039	0.741-0.893
Mueller et al, 2009	*MLH1*, *MSH2*, *MSH6*, *PMS2*	48	27	1	2	18	0.871	0.072	0.729-1.000
Perez-Carbonell et al, 2012	*MLH1*, *MSH2*, *MSH6*, *PMS2*	2093	127	25	28	1913	0.814	0.025	0.765-0.863
Stigliano et al, 2013	*MLH1*, *MSH2*, *MSH6*, PMS3	117	21	3	8	85	0.732	0.076	0.584-0.880
Yan et al, 2008	*MLH1*, *MSH2*, *MSH6*	227	79	19	0	129	0.825	0.038	0.751-0.899
Yoon et al, 2011	*MLH1*, *MSH2*	2028	128	75	79	1746	0.582	0.03	0.523-0.642

	IHC-absent	IHC-present
MSI-H	yes/yes	yes/no
MSS	no/yes	no/no

Abbreviations: IHC = Immunohistochemistry; MSI-H = High-frequency microsatellite instability; MSS = Microsatellite stable

Appendix VI: Q1a- Studies assessing the ability of IHC to detect tumours that are MSI-H for endometrial cancer

	MMR proteins	Sample Size	yes/yes	yes/no	no/yes	no/no	Kappa	Level of Agreement SE of kappa	95% CI
Egoavil et al, 2013	*MLH1*, *MSH2*, *MSH6*, *PMS2*	173	44	14	3	112	0.769	0.053	0.666-0.872
Ferguson et al, 2014	*MLH1*, *MSH2*, *MSH6*, *PMS2*	114	27	0	3	84	0.930	0.040	0.852-1.000
Leenen et al, 2012	*MLH1*, *MSH2*, *MSH6*	179	42	0	0	137	1	0	1.0-1.0
Lu et al, 2007	*MLH1*, *MSH2*, *MSH6*, *PMS2*	95	22	3	7	63	0.742	0.076	0.592-0.892
Modica et al, 2007	*MLH1*, *MSH2*, *MSH6*, *PMS2*	85	41	4	7	33	0.739	0.073	0.596-0.883
Peterson et al, 2012	*MLH1*, *MSH2*, *MSH6*, *PMS2*	93	23	5	1	64	0.84	0.063	0.717-0.963
Walsh et al, 2008	*MLH1*, *MSH2*	146	27	2	11	106	0.75	0.065	0.622-0.877

	IHC-absent	IHC-present
MSI-H	yes/yes	yes/no
MSS	no/yes	no/no

Abbreviations: IHC = Immunohistochemistry; MSI-H = High-frequency microsatellite instability; MSS = Microsatellite stable