Tumor Mismatch Repair Immunohistochemistry and DNA *MLH1* Methylation Testing of Patients With Endometrial Cancer Diagnosed at Age Younger Than 60 Years Optimizes Triage for Population-Level Germline Mismatch Repair Gene Mutation Testing

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Terms in blue are defined in the glossary, found at the end of this article and online at www.jco.org.

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ABSTRACT

Purpose

Clinicopathologic data from a population-based endometrial cancer cohort, unselected for age or family history, were analyzed to determine the optimal scheme for identification of patients with germline mismatch repair (MMR) gene mutations.

Patients and Methods

Endometrial cancers from 702 patients recruited into the Australian National Endometrial Cancer Study (ANECs) were tested for MMR protein expression using immunohistochemistry (IHC) and for *MLH1* gene promoter methylation in *MLH1*-deficient cases. MMR mutation testing was performed on germline DNA of patients with MMR-protein deficient tumors. Prediction of germline mutation status was compared for combinations of tumor characteristics, age at diagnosis, and various clinical criteria (Amsterdam, Bethesda, Society of Gynecologic Oncology, ANECs).

Results

Tumor MMR-protein deficiency was detected in 170 (24%) of 702 cases. Germline testing of 158 MMR-deficient cases identified 22 truncating mutations (3% of all cases) and four unclassified variants. Tumor *MLH1* methylation was detected in 99 (89%) of 111 cases demonstrating *MLH1*/PMS2 IHC loss; all were germline *MLH1* mutation negative. A combination of MMR IHC plus *MLH1* methylation testing in women younger than 60 years of age at diagnosis provided the highest positive predictive value for the identification of mutation carriers at 46% versus $\leq 41\%$ for any other criteria considered.

Conclusion

Population-level identification of patients with MMR mutation-positive endometrial cancer is optimized by stepwise testing for tumor MMR IHC loss in patients younger than 60 years, tumor *MLH1* methylation in individuals with *MLH1* IHC loss, and germline mutations in patients exhibiting loss of MSH6, MSH2, or PMS2 or loss of *MLH1*/PMS2 with absence of *MLH1* methylation.

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INTRODUCTION

Lynch syndrome is an autosomal dominantly inherited syndrome caused by mutations in the DNA mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, or *PMS2*. Cancer risks are greatly increased for mutation carriers,¹⁻³ the most common malignancy being colorectal cancer, with a reported cumulative risk of 25% to 70%.^{4,5} Female carriers have an additional lifetime risk of endometrial cancer of

16% to 71%,^{1,5,6} and a 40-fold risk of endometrial cancer following colorectal cancer diagnosis.⁷ Efficient, sensitive, and cost-effective approaches to identify mutation carriers will facilitate optimal clinical management of carriers, in addition to their at-risk family members. Cancer risk and mortality is proven to be reduced by colonoscopy or risk-reducing surgery,⁸⁻¹⁰ whereas exogenous progestins are indicated for chemoprevention of endometrial cancer.¹¹

The revised Amsterdam criteria,¹² revised Bethesda criteria,¹³ and Society of Gynecologic Oncology (SGO) guidelines¹⁴ were developed to aid identification of possible MMR gene mutation carriers based on their personal and family cancer history (including age at onset and multiple cancers) and tumor characteristics (Table 1). Similarly, the Australian National Endometrial Cancer Study (ANECs) criteria were developed to identify endometrial cancer patients with increased likelihood of cancer because of a high-risk mutation in any hereditary cancer gene.¹⁵ However, several reports indicate family history is not consistently recorded,¹⁵⁻¹⁷ and family history–based criteria seem to have low sensitivity, even when family history is recorded.¹⁸⁻²¹ Consequently, there is increasing literature advocating identification of possible MMR mutation carriers through universal

testing of colorectal and endometrial cancers for the distinctive molecular features associated with mismatch repair defects caused by a germline MMR gene mutation.^{18,22-28} Tumor MMR deficiency may be detected as microsatellite instability (MSI) or loss of MMR protein expression by immunohistochemistry (IHC). IHC testing is generally preferred because of lower cost, faster turnaround time, wider availability in routine diagnostic laboratories, reported increased sensitivity,²⁹ and ability to direct germline mutation testing.^{22-27,30}

For colorectal cancer, most MLH1/PMS2-deficient tumors are due to somatic inactivation caused by *MLH1* gene promoter methylation, with *MLH1* methylation accounting for 40% of mutation-negative microsatellite-unstable colorectal cancers.³¹ Furthermore, somatic *MLH1* methylation is tightly linked to *BRAF* V600E somatic

Table 1. Amsterdam II, Revised Bethesda, Society of Gynecologic Oncology, and ANECs Criteria

The Amsterdam II criteria

1. At least three relatives with a Lynch-associated cancer (colorectal, endometrial, small bowel, ureter, or renal pelvis cancer)
2. One should be a first-degree relative of the other two
3. At least two successive generations should be affected
4. At least one should be diagnosed at < 50 years of age
5. Familial adenomatous polyposis (FAP) should be excluded in the colorectal cancer case(s) if any*
6. Tumors should be verified by pathologic examination*

Revised Bethesda guidelines

1. Colorectal cancer diagnosed at < 50 years of age
2. Presence of synchronous or metachronous colorectal, or other Lynch-associated cancers (endometrial, ovarian, stomach, pancreas, small bowel, biliary tract, ureter and renal pelvis, glioblastoma as seen in Turcot syndrome, sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome)
3. Colorectal cancer with microsatellite instability-high tumor histology diagnosed at < 60 years of age†
4. Colorectal cancer diagnosed in one or more first-degree relatives with a Lynch-associated cancer, with one cancer diagnosed at < 50 years of age
5. Colorectal cancer diagnosed in two or more first- or second-degree relatives with Lynch-associated cancers, regardless of age

The SGO criteria‡

SGO25: Patients with ≥ 20% to 25% chance of having an inherited predisposition to endometrial, colorectal, and related cancers and for whom genetic risk assessment is recommended

1. Patients with endometrial or colorectal cancer who meet the revised Amsterdam criteria (as above)
2. Patients with synchronous or metachronous endometrial and colorectal cancer with the first cancer diagnosed before age 50 years
3. Patients with synchronous or metachronous ovarian and colorectal cancer with the first cancer diagnosed before age 50 years
4. Patients with endometrial or colorectal cancer with evidence of a mismatch repair defect (ie, microsatellite instability or immunohistochemical loss of expression of *MLH1*, *MSH2*, *MSH6* or *PMS2*)§
5. Patients with a first- or second-degree relative with a known mismatch repair gene mutation||

SGO10: Patients with ≥ 5% to 10% chance of having an inherited predisposition to endometrial, colorectal, and related cancers and for whom genetic risk assessment may be helpful

1. Patients with endometrial or colorectal cancer diagnosed before age 50 years
2. Patient with endometrial or ovarian cancer with a synchronous or metachronous colon or other Lynch-associated tumors (colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, glioblastoma as seen in Turcot syndrome, sebaceous gland adenomas and keratoacanthomas in Muir-Torre syndrome) at any age
3. Patients with endometrial or colorectal cancer and a first-degree relative with a Lynch-associated tumor diagnosed before age 50 years
4. Patients with endometrial or colorectal cancer diagnosed at any age with two or more first- or second-degree relatives (ie, parents, siblings, aunts, uncles, nieces, nephews, grandparents, and grandchildren) with Lynch-associated tumors, regardless of age
5. Patients with a first- or second-degree relative that meets the above criteria

ANECs criteria

1. Endometrial cancer diagnosed at < 50 years of age in the patient
2. Presence of multiple Lynch-associated cancers¶ (a second primary or synchronous) in the patient
3. Any prior cancer in the patient
4. At least 1 first-degree relative with endometrial cancer diagnosed at < 50 years of age
5. At least 3 first-degree relatives with Lynch-associated cancers, regardless of age
6. At least 2 first- or second-degree relatives with Lynch-associated cancers, regardless of age
7. At least 1 case of breast, thyroid, or Lynch-associated cancer diagnosed in the family at < 50 years of age

Abbreviations: ANECs, Australian National Endometrial Cancer Study; SGO, Society of Gynecologic Oncology.

*This Amsterdam II criterion was not assessable for relatives of probands in this study.

†This Bethesda guideline criterion was not assessable for this study.

‡All patients assessed for SGO criteria were identified via an endometrial cancer diagnosis.

§Immunohistochemical testing results were used as evidence of a mismatch repair defect.

||This SGO criterion was not assessable for this study.

¶Lynch-associated cancers include endometrial, colorectal, ovarian, stomach, pancreas, small bowel, biliary tract, bladder, ureter and renal pelvis, and brain tumors.

mutation in colorectal tumors. Thus, tests for tumor *MLH1* methylation or *BRAF* V600E mutation are used as a negative predictive marker of *MLH1* germline mutation status in the triage of patients with colorectal cancer for MMR gene mutation screening.^{29,31}

With regard to endometrial cancer, 10% to 20% of patients show loss of tumor *MLH1*/PMS2 expression, but only a minority of these cases are mutation carriers.^{32,33} Endometrial tumor *MLH1* methylation status has recently been proposed for inclusion in universal screening protocols for Lynch Syndrome in endometrial cancer patients.^{28,34} However, no previous study has validated *MLH1* methylation as a negative predictive marker of *MLH1* mutation status in endometrial tumors by assessing *MLH1* methylation status in MMR gene mutation carriers, noncarriers, and MMR immuno-normal patients. This is essential to address the effectiveness of *MLH1* methylation screening, with other predictors of mutation status, and define the optimal triage for population-level MMR mutation testing of patients with endometrial cancer.

The aim of this study was to compare a combination of strategies, including MMR IHC testing, *MLH1* methylation testing, age at diagnosis, and family history of cancer, to determine the most efficient and clinically feasible scheme for identifying MMR mutation carriers among patients with endometrial cancer in the population. For this purpose, we used a well-characterized, population-based cohort of women diagnosed with endometrial cancer unselected for age or family history from the ANECS.

PATIENTS AND METHODS

The study was approved by the QIMR Berghofer Medical Research Institute Human Research Ethics Committee and the participating hospitals and cancer registries. All participants provided informed written consent.

Patients With Endometrial Cancer

In Australia, it is a legal requirement for cancer diagnoses to be reported to state-based registries. Eligible cases were women 18 to 79 years of age living in Australia with histologically confirmed epithelial endometrial cancer (International Classification of Diseases, 10th revision, C54) newly diagnosed between July 2005 (May 2005 in Queensland) and December 2007. Cases were recruited by nurses who liaised with treatment clinics, physicians, and state-based cancer registries across Australia. A total of 1,459 eligible participants consented to participate in ANECS (55% of identified, 67% of invited; Fig 1 details reasons for nonparticipation). Participants completed a detailed questionnaire providing epidemiologic information and reported cancers in first- and second-degree relatives. They were asked to provide a blood sample and consent to retrieve relevant medical, oncology, pathology, and genetics records. Information on tumor pathology characteristics was abstracted in standardized format from clinical pathology reports. Formalin-fixed paraffin-embedded tumor material was collected when possible for molecular testing (702 participants).

Molecular and Genetic Testing

IHC analysis of *MLH1*, *MSH2*, *MSH6*, and *PMS2* was performed on formalin-fixed, paraffin-embedded tissue sections, as described previously.³⁶ Based on the pattern of MMR protein loss in tumor tissues, DNA from peripheral-blood samples was tested for germline MMR gene mutations, including multiplex ligation-dependent probe amplification using SALSA kits P003, P003-B2, P008, P072-B2, and P248-A2 (MRC-Holland, Amsterdam, the Netherlands) to detect large genomic insertion/deletion rearrangements of the MMR genes and deletions in the 3' end of *EPCAM* and exon sequencing.³⁷ *PMS2* mutation testing was performed using a long-range polymerase chain reaction method.³⁸ Variants predicted to encode a truncated protein were

considered pathogenic, and variants of uncertain clinical significance were excluded from analysis.

DNA methylation (*MLH1*) analysis was performed as previously described³⁶ for all tumors from patients exhibiting *MLH1*/PMS2 IHC loss or other patterns of MMR deficiency when there was sufficient DNA available for bisulfite conversion and methylation analysis and also for a random subset of 77 MMR immuno-normal individuals. *MLH1* methylation was quantitatively reported based on the percentage of methylated reference (PMR) calculations,³⁹ using $\geq 4\%$ PMR as cutoff for positive methylation status.

Statistical Analysis

Data were analyzed using SPSS 20.0 statistical software for Windows (SPSS, Chicago, IL). Qualitative variables were compared using the χ^2 test, and normally distributed continuous variables were compared using the *t* test. A *P* value less than .05 was considered statistically significant. Performance characteristics (ie, sensitivity, specificity, negative and positive predictive values) of selected clinical criteria and tumor tests were calculated with respect to the presence of a germline MMR gene mutation.

RESULTS

Demographic, clinical, and tumor-related characteristics of the patients, as well as detailed family history and fulfillment of clinical criteria, are summarized in Table 2. There was no significant difference in these characteristics between the 702 MMR IHC-tested and the 757 ANECS cases without tumor material, except for fulfillment of SGO25 criteria—reflecting inclusion of MMR IHC results in these criteria.

Considering cases with MMR IHC results (Fig 1), 170 (24%) of 702 demonstrated loss of expression of at least one MMR protein (MMR deficiency). MMR-deficient cases showed mostly loss of *MLH1*/PMS2 expression (127 of 170; 75%), followed by *MSH2*/*MSH6* loss (22 of 170; 13%), solitary *MSH6* loss (20 of 170; 12%), and solitary *PMS2* loss (one of 170; 1%). Germline MMR gene mutation analysis was performed on the 158 MMR-deficient cases with DNA available for testing. A truncating gene mutation was identified in 22 of 158 MMR-deficient cases, equating to an overall carrier frequency of 3%. Mutations were most frequently identified in *MSH6* (10 of 22), followed by *MSH2* (eight of 22), *MLH1* (three of 22), and *PMS2* (one of 22). In addition, four exonic variants of uncertain significance (predicted missense or in-frame deletions) were identified in individuals exhibiting *MSH6* tumor loss. Bioinformatic analysis indicates that three of these *MSH6* variants have a very high prior probability of pathogenicity ($\geq 89\%$; see Fig 1 footnote), and if confirmed to be disease-causing mutations, the carrier frequency would increase to 4%.

Characteristics of the 26 carriers of pathogenic mutations or unclassified variants are detailed in Appendix Table A1 (online only). Mean age at diagnosis for pathogenic mutation carriers was 52 years, with an average body mass index (BMI) of 27.1. Of the pathogenic mutation carriers, two were adopted, four fulfilled the Amsterdam II criteria, another 13 fulfilled the revised Bethesda guidelines, and another two met both the SGO10 and ANECS criteria.

MLH1 methylation testing was performed on 230 tumors (Fig 1). This included all 153 MMR-deficient tumors with sufficient material available (encompassing 16 mutation carriers) and a randomly selected subset of 77 tumors with immuno-normal (MMR-proficient) results as reference. *MLH1* methylation was not detected in the tumors from the two *MLH1* mutation carriers tested, but was detected in 99

MMR Mutation Testing Triage of Patients With Endometrial Cancer

Fig 1. Selection, recruitment, mismatch repair (MMR) tumor, and MMR gene germline testing of Australian National Endometrial Cancer Study (ANECs) population-based endometrial cancer cases. Details of nonpolymorphic MMR gene variants identified are shown in Appendix Table A1 (online only). The prior probabilities of pathogenicity for *MSH6* variants of uncertain clinical significance from bioinformatics analysis as per Thompson et al³⁵ are as follows: *MSH6* c.3694_3696del p.(Val1232del), 0.97; *MSH6* c.2314C>T p.(Arg772Trp), 0.89; *MSH6* c.3725_3727del p.(Arg1242_Thr1243delinsPro), 0.99; *MSH6* c.3469G>A p.(Gly1157Ser), 0.41. (For deletion variants, the prior probability of pathogenicity is that for the highest prior possible for a missense substitution at that residue.) No unclassified variants in the *MLH1* or *MSH2* genes were detected in this study. Methylation analysis was performed for all MMR-deficient patients with sufficient tumor DNA available for bisulfite conversion and methylation analysis and for a subset of 77 randomly selected patients with immuno-normal MMR protein tumor expression. The two immuno-normal cases (2.6%; 95% CI, 0.7% to 9.0%) demonstrating methylation showed 5.2% and 9.3% methylation compared with reference. In cases with loss of *MLH1*/*PMS2* expression only, *MLH1* methylation was detected in 99 of 111 (89.2%; 95% CI, 82.1% to 93.7%) mutation-negative cases, and in eight of 10 (80.0%; 95% CI, 49.0% to 94.3%) mutation-unknown cases. The individual with *MSH6* loss and *MLH1* methylation (reference value 11.9%) also demonstrated immunohistochemistry (IHC) loss of *MLH1* and *PMS2*. Overall, a truncating gene mutation was identified in 22 of 158 MMR-deficient cases with germline DNA available for testing (13.9%, 95% CI, 9.4% to 20.2%), equating to an overall carrier frequency of 3.1% (95% CI, 2.1% to 4.7%). NEG, negative for MMR gene deleterious mutation or unclassified variant of uncertain clinical significance; POS, positive for MMR gene deleterious mutation; UNK, mutation status unknown (no germline DNA available for testing); UV, positive for MMR gene unclassified variant.

(89%) of 111 mutation-negative cases with loss of *MLH1*/*PMS2* expression only and in one tumor from a *MSH6* gene mutation carrier in whom the tumor demonstrated loss of *MSH6*, *MLH1*, and *PMS2*. No other MMR-deficient tumors demonstrated *MLH1* methylation. In the reference group of MMR-proficient tumors, only 2/77 (3%) were *MLH1* methylation-positive (PMR values 5.2% and 9.3%).

The distribution of MMR IHC, *MLH1* methylation, and mutation status according to age at endometrial cancer diagnosis is shown in Table 3. The proportion of mutation carriers by age group was 10% for patients younger than 50 years, 5% for patients 50 to 59 years of age, and 1% for patients older than 60 years. Most mutation carriers were between 50 and 59 years of age (11 of 21; 52%), followed by

younger than 50 years (seven of 21; 33%), and then older than 60 years (three of 21; 14%).

Performance characteristics of selected screening strategies are shown in Table 4. Screening strategies based on family history/clinical criteria alone all performed poorly (positive predictive value [PPV] < 22%). As expected from the triage approach used to select patients for sequencing, universal MMR IHC tumor testing demonstrated 100% sensitivity in identifying patients with endometrial cancer with a MMR germline mutation, but poor PPV (13%). PPV ranged from 17% to 33% when considering MMR IHC testing in combination with age younger than 60 years, BMI less than 30, or family history of Lynch cancers.

Table 2. Characteristics of Patients With Endometrial Cancer Included in This Study Versus Those Excluded

Characteristic	IHC Not Tested (n = 757)			IHC Tested (n = 702)			P
	No.	%	95% CI	No.	%	95% CI	
Age at diagnosis, years							
Mean		61.0			61.8		
Range		26.4-80.0			27.1-79.8		
< 50	91	12.0	9.9 to 14.5	75	10.7	8.6 to 13.2	.340
50-59	247	32.6	29.4 to 36.1	212	30.2	26.9 to 33.7	
≥ 60	419	55.4	51.8 to 58.9	415	59.1	55.4 to 62.7	
BMI							
Mean		31.2			31.3		
Range		16.9-67.8			18.7-75.5		
Normal (18.50-24.99)	190	25.1	22.1 to 28.3	149	21.2	18.4 to 24.4	.294
Overweight (25.00-29.99)	178	23.5	20.6 to 26.7	173	24.6	21.6 to 28.0	
Obese (≥ 30.00)	338	44.7	41.1 to 48.2	319	45.4	41.8 to 49.1	
Unknown	51	6.7	5.2 to 8.8	61	8.7	6.8 to 11.0	
Multiple primary malignancies	59	7.8	6.1 to 9.9	65	9.3	7.3 to 11.6	.316
Histology and FIGO grade							.176
Endometrioid							
Grade 1	417	55.1	51.5 to 58.6	327	46.6	42.9 to 50.3	
Grade 2	167	22.1	19.3 to 25.2	189	26.9	23.8 to 30.3	
Grade 3	63	8.3	6.6 to 10.5	56	8.0	6.2 to 10.2	
Grade unknown	1	0.1	0 to 0.7	0	0		
Serous	37	4.9	3.6 to 6.7	54	7.7	5.9 to 9.9	
Clear cell	19	2.5	1.6 to 3.9	17	2.4	1.5 to 3.8	
Carcinosarcoma (MMMT)	24	3.2	2.1 to 4.7	28	4.0	2.8 to 5.7	
Other*	29	3.8	2.7 to 5.5	31	4.4	3.1 to 6.2	
FIGO stage (2009)							.182
I-II	667	88.1	85.6 to 90.2	617	87.9	85.3 to 90.1	
III-IV	61	8.1	6.3 to 10.2	72	10.3	8.2 to 12.7	
Stage unknown	29	3.8	2.7 to 5.5	13†	1.9	1.1 to 3.1	
First-degree relative(s) with colorectal cancer	91	12.0	9.9 to 14.5	98	14.0	11.6 to 16.7	.270
First-degree relative(s) with endometrial cancer	34	4.5	3.2 to 6.2	36	5.1	3.7 to 7.0	.570
Fulfills Amsterdam II criteria	26	3.4	2.4 to 5.0	26	3.7	2.5 to 5.4	.782
Fulfills revised Bethesda guidelines	257	34.0	30.7 to 37.4	254	36.2	32.7 to 39.8	.372
Fulfills Society of Gynecological Oncology (SGO) guidelines:							
SGO25‡ (including IHC test results)	27	3.6	2.5 to 5.1	188	26.8	23.6 to 30.2	< .001
SGO25‡ (excluding IHC test results)	27	3.6	2.5 to 5.1	29	4.1	2.9 to 5.9	.575
SGO10	404	53.4	49.8 to 56.9	372	53.0	49.3 to 56.7	.885
Fulfills ANECS criteria	431	56.9	53.4 to 60.4	401	57.1	53.4 to 60.7	.942

Abbreviations: ANECS, Australian National Endometrial Cancer Study; FIGO, International Federation of Gynecology and Obstetrics; IHC, immunohistochemistry; MMMT, malignant mixed Müllerian tumor; SGO, Society of Gynecologic Oncology.

*Includes adenocarcinoma, undifferentiated, mucinous subtypes, mixed subtypes including a serous and/or clear cell (minor) component, and other epithelial.

†Stage at diagnosis according to the 1988 FIGO system for these 13 individuals was Stage 2 tumor (n = 2), Stage 3 tumor (n = 10), and unknown (n = 1).

‡SGO25 includes IHC testing as one of several selection criteria (Table 1). Analysis excluding tumor mismatch repair IHC test results was performed for comparison.

Including *MLH1* methylation testing in MMR IHC-based strategies improved PPVs: 36% overall, 46% for women younger than 60 years. Similar PPVs were seen for a stratified approach testing MSH2/MSH6 IHC for all diagnoses and *MLH1*/PMS2 IHC only for women younger than 60 years with *MLH1* unmethylated (40%) or for women with family history meeting Bethesda guidelines (35%). The area under the curve was ≥ 91% for these four strategies only.

Based on the performance analysis (Table 4) and considering estimated costs for selected IHC screening strategies (Appendix Table A3, online only), testing women younger than 60 years at diagnosis for MMR IHC and *MLH1* methylation was optimal in terms of number of carriers detected for the lowest number of diagnostic tests and was the

most cost-effective approach. Further cost savings would be possible by modulating this strategy to a two-stain (MSH6/PMS2) IHC screening approach, as recently proposed.²⁸

The three mutation carriers diagnosed at age more than 60 years presented with family history meeting Amsterdam II criteria (one *MSH2* carrier) or Bethesda/SGO10/ANECS clinical criteria (two *MSH6* carriers). There were also 18 patients in this study with normal tumor MMR protein expression who fulfilled the Amsterdam II criteria, with some potentially carrying MMR gene mutations encoding immunostable MMR-deficient proteins. These observations were used to inform development of the proposed testing scheme outlined in Figure 2, which incorporate family history and/or tumor MSI analysis as secondary mechanisms for referral.

Table 3. Age at Diagnosis of Probands by MMR IHC, *MLH1* Tumor Methylation, and Mutation Status

MMR IHC and MMR Gene Mutation Status	< 50 Years	50-59 Years	≥ 60 Years	Total
Immuno-normal	57	157	318	532
MLH1/PMS2 loss (n = 117)				
<i>MLH1</i> mutation negative, <i>MLH1</i> methylated	2	27	70	99
<i>MLH1</i> mutation negative, <i>MLH1</i> unmethylated	0	5	7	12
<i>MLH1</i> mutation positive, <i>MLH1</i> unmethylated	1	1	0	2
MSH2/MSH6 loss (n = 20)				
<i>MSH2</i> mutation negative	2	4	6	12
<i>MSH2</i> mutation positive	3	4	1	8
MSH6 loss (n = 20)				
<i>MSH6</i> mutation negative	2	1	3	6
<i>MSH6</i> mutation positive	3	5	2	10
<i>MSH6</i> unclassified variant positive	1	2	1	4
PMS2 loss (n = 1)				
<i>PMS2</i> mutation positive	0	1	0	1
No. pathogenic mutation carriers identified/ No. tested for MMR IHC and <i>MLH1</i> methylation	7/71	11/207	3/408	21/686
%	9.9	5.3	0.7	3.1
95% CI	4.9 to 19.0	3.0 to 9.3	0.3 to 2.2	2.0 to 4.6
No. pathogenic mutation carriers identified/ Total no. of pathogenic mutation carriers in cohort	7/21	11/21	3/21	21/21
%	33.3	52.4	14.3	100
95% CI	17.2 to 54.6	32.4 to 71.7	5.0 to 34.6	84.5 to 100

NOTE. This table presents characteristics for the 686 probands who had tumor MMR IHC results, germline DNA available for testing if demonstrating tumor loss of MMR expression (n = 158), and tumor DNA available for *MLH1* methylation tests if demonstrating tumor loss of *MLH1* expression (n = 113). Germline DNA was not available for 10 individuals with *MLH1*/PMS2 loss (eight of whom demonstrated *MLH1* tumor methylation), and two individuals with *MSH2*/MSH6 loss. Four individuals with *MLH1*/PMS2 loss were untested for *MLH1* methylation (including one *MLH1* mutation carrier, aged 29 years at diagnosis, and three individuals with no identified *MLH1* mutation).

Abbreviations: MMR, mismatch repair; IHC, immunohistochemistry.

DISCUSSION

In this study, we report results from the largest series to date of unselected endometrial cancer cases characterized for MMR protein expression by IHC, *MLH1* gene promoter methylation, and germline MMR gene mutation testing. We have demonstrated the utility of *MLH1* methylation testing as a negative predictive marker for triaging patients with endometrial cancer for genetic testing and established the most efficient and clinically feasible strategy to identify MMR gene mutation carriers in newly diagnosed patients with endometrial cancer at the population level. This strategy involves tumor MMR IHC testing in patients diagnosed at age ≤ 60 years and subsequent testing for *MLH1* methylation in *MLH1*/PMS2-deficient cases (Fig 2).

Mutation testing was performed only on cases demonstrating tumor MMR protein loss, and thus sensitivity was recorded as 100% for universal MMR IHC testing to identify MMR gene mutation carriers. MMR IHC in combination with methylation testing showed the highest combined values for sensitivity (100%) and specificity (95%). Applying an age restriction of 60 years at diagnosis to MMR IHC testing produced the next highest combined values for sensitivity (84%) and specificity (97%), while also attaining the highest PPV (46%) and retaining a high negative predictive value (99%).

Clinical criteria were less effective for identifying mutation carriers, with high specificity but low sensitivity (Amsterdam II), modest sensitivity and specificity (revised Bethesda), or high sensitivity but poor specificity (SGO10, ANECS). Our results indicate that a considerable proportion of mutation carriers would not be identified using the Amsterdam II criteria (~86%), the revised Bethesda guidelines (~24%), SGO10 (~14%), or ANECS criteria

(~14%). Family history information alone is thus inadequate to prioritize patients with endometrial cancer at high risk for Lynch syndrome for germline testing.

Recent studies have shown that MMR IHC or MSI testing of patients with endometrial cancer is feasible for identification of MMR mutation carriers.^{28,33,40,41} Suggestions to improve specificity and decrease testing costs have included screening for patients younger than 50 years,^{36,40,42} IHC in combination with first-degree family history of Lynch syndrome cancers,⁴³ and/or two-stain *MSH6*/PMS2 IHC testing.²⁸ Our results indicate that 67% of mutation carriers would be missed if IHC testing was limited to patients 50 years of age, and family history criteria combined with universal IHC similarly reduces sensitivity. Although BMI less than 30 has been proposed as a simple clinical predictor of mutation status,^{40,44} 27% of carriers in our unselected series were obese and would have been missed by applying this criterion.

Combining *MSH2*/MSH6 IHC for all cases with *MLH1*/PMS2 IHC for only Bethesda cases exhibits good sensitivity and specificity. However, assuming it is possible to overcome limitations owing to poor recognition of family history,¹⁵⁻¹⁷ this scheme is not the most cost-effective (Appendix Table A3). Using the suggested scheme in Figure 2, systematic MMR IHC testing for cases diagnosed at age ≤ 60 years would result in nearly 60% fewer MMR IHC tests compared with universal MMR IHC testing, but miss only 1% of carriers. The proposed scheme also allows for IHC testing of later-onset cases presenting with a strong family history indicative of Lynch syndrome to identify some missed cases. We have shown previously that deficiencies in recording of family history of endometrial cancer cases at diagnosis is least marked for cases with strong family history (50%

Table 4. Performance Characteristics of Selected Clinical Criteria and Tumor Tests for the Identification of Patients With Endometrial Cancer Having a Germline MMR Gene Mutation

Criteria Fulfilled by Endometrial Cancer Probands	Probands Fulfilling Criteria (N = 698)			MMR Mutation Carriers Fulfilling Criteria (N = 21)				Sensitivity		Specificity		Positive Predictive Value		Negative Predictive Value		AUC*
	No.	%	95% CI	N	%	95% CI	%	%	95% CI	%	95% CI	%	95% CI	%	95% CI	
Amsterdam II	25	3.6	2.4 to 5.2	3	14.3	5.0 to 34.6	14.3	3.8 to 37.4	96.8	95.0 to 97.9	12.0	3.2 to 32.3	97.3	95.7 to 98.4	0.56	
Revised Bethesda	253	36.3	32.8 to 39.9	16	76.2	54.9 to 89.4	76.2	52.5 to 90.9	65.0	61.2 to 68.6	6.3	3.8 to 10.3	98.9	97.2 to 99.6	0.71	
SGO10	370	53.0	49.3 to 56.7	18	85.7	65.4 to 95.0	85.7	62.6 to 96.2	48.0	44.2 to 51.8	4.9	3.0 to 7.7	99.1	97.1 to 99.8	0.67	
SGO25†																
Including IHC test results	184	26.4	23.2 to 29.8	21	100	84.5 to 100	100	75.9 to 100	75.9	72.5 to 79.1	11.4	7.4 to 17.1	100	99.1 to 100	0.88	
Excluding IHC test results	28	4.0	2.8 to 5.7	6	28.6	13.8 to 50.0	28.6	12.2 to 52.3	96.8	95.0 to 97.9	21.4	9.0 to 41.5	97.8	96.2 to 98.7	0.63	
ANECs	399	57.2	53.5 to 60.8	18	85.7	65.4 to 95.0	85.7	62.6 to 96.2	43.7	40.0 to 47.6	4.5	2.8 to 7.2	99.0	96.8 to 99.7	0.65	
Age at diagnosis < 60 years	285	40.8	37.2 to 44.6	18	85.7	65.4 to 95.0	85.7	62.6 to 96.2	60.6	56.8 to 64.2	6.3	3.9 to 10.0	99.3	97.7 to 99.8	0.73	
Age at diagnosis < 60 years + BMI < 30	119	17.1	14.4 to 20.0	13	61.9	40.9 to 79.3	61.9	38.7 to 81.0	84.3	81.3 to 87.0	10.9	6.2 to 18.3	98.6	97.2 to 99.4	0.73	
MMR IHC loss for all ages	166	23.8	20.8 to 27.1	21	100	84.5 to 100	100	80.8 to 100	78.6	75.3 to 81.6	12.7	8.2 to 18.9	100	99.1 to 100	0.89	
MMR IHC loss < 60 years	71	10.2	8.1 to 12.6	18	85.7	65.4 to 95.0	85.7	62.6 to 96.2	92.2	89.8 to 94.0	25.4	16.1 to 37.3	99.5	98.5 to 99.9	0.89	
MMR IHC loss for all ages + BMI < 30	86	12.3	10.1 to 15.0	16	76.2	54.9 to 89.4	76.2	52.5 to 90.9	89.7	87.1 to 91.8	18.6	11.3 to 28.8	99.2	98.0 to 99.7	0.83	
MMR IHC loss < 60 years + BMI < 30	40	5.7	4.2 to 7.7	13	61.9	40.9 to 79.3	61.9	38.7 to 81.0	96.0	94.2 to 97.3	32.5	19.1 to 49.2	98.8	97.5 to 99.4	0.79	
MMR IHC loss for all ages + 1 FSDR	89	12.8	10.5 to 15.4	15	71.4	50.1 to 86.2	71.4	47.7 to 87.8	89.1	86.4 to 91.3	16.9	10.1 to 26.6	99.0	97.8 to 99.6	0.80	
MMR IHC loss for < 60 years + 1 FSDR	45	6.5	4.9 to 8.5	12	57.1	36.5 to 75.5	57.1	34.4 to 77.4	95.1	93.1 to 96.6	26.7	15.1 to 42.2	98.6	97.3 to 99.3	0.76	
MMR IHC loss for all ages + <i>MLH1</i> unmethylated	58	8.3	6.5 to 10.6	21	100	84.5 to 100	100	80.8 to 100	94.5	92.5 to 96.1	36.2	24.3 to 49.9	100	99.3 to 100	0.95	
MMR IHC loss for < 60 years + <i>MLH1</i> unmethylated	39	5.6	4.1 to 7.6	18	85.7	65.4 to 95.0	85.7	62.6 to 96.2	96.9	95.2 to 98.0	46.2	30.4 to 62.6	99.5	98.6 to 99.9	0.91	
IHC for MSH2/MSH6 for all ages + (IHC <i>MLH1</i> /PMS2 only for < 60 years + <i>MLH1</i> unmethylated)	52	7.5	5.7 to 9.6	21	100	84.5 to 100	100	80.8 to 100	95.4	93.5 to 96.8	40.4	27.3 to 54.9	100	99.3 to 100	0.98	
IHC for MSH2/MSH6 for all ages + (IHC <i>MLH1</i> /PMS2 only for < 60 years and those who meet revised Bethesda guidelines)	58	8.4	6.6 to 10.7	20	95.2	77.3 to 99.2	95.2	74.1 to 99.8	94.4	92.3 to 95.9	34.5	22.8 to 48.2	99.8	99.0 to 100	0.95	

NOTE. This analysis was limited to 696 probands who had tumor MMR IHC results, germline DNA available for testing if demonstrating tumor loss of MMR expression (n = 158), and tumor DNA available for *MLH1* methylation tests if demonstrating tumor loss of *MLH1* expression (n = 113). *MLH1* methylation status was considered for sensitivity and specificity analyses only for individuals exhibiting MMR IHC loss of *MLH1* and PMS2 alone. See Table 1 for details of the Amsterdam II, revised Bethesda, Society of Gynecology, and the ANECs criteria assessed.

Abbreviations: AUC, area under the curve; BMI, body mass index; FSDR, first- and second-degree relative(s); IHC, immunohistochemistry; MMR, mismatch repair.

*Analysis for area under the receiver operating characteristic curve was conducted. The ideal AUC is 0.80 or greater.

†SGO25 includes IHC testing as one of several selection criteria (see Table 1). Analysis excluding tumor MMR IHC test results was performed for comparison.

Fig 2. Proposed scheme for identification of germline mismatch repair (MMR) mutation carriers at diagnosis of endometrial cancer. (*) Class 5 from qualitative or quantitative analysis according to criteria accepted by InSiGHT (<http://www.insight-group.org/classifications>; Thomson et al^{39a}). (†) Consider microsatellite instability (MSI) testing for patients with high clinical suspicion. (‡) Unclassified variants can be submitted for further analyses at www.insight-group.org. IHC, immunohistochemistry; MSI-H, microsatellite-high instability; MSI-L, microsatellite-low instability; MSS, microsatellite stable.

recorded for Amsterdam *v* 25% for Bethesda) and that strong family history can prompt self-referral.¹⁵

Although it has been assumed that *MLH1* methylation testing is a necessary inclusion in triage of patients with endometrial cancer for MMR gene testing,^{28,34} our study provides essential data to prove the efficacy of *MLH1* methylation as a negative predictive marker of *MLH1* mutation status for population-level testing of patients with endometrial cancer. *MLH1* methylation occurred in a very high proportion (90%) of endometrial cancer cases demonstrating loss of *MLH1*/*PMS2* expression and no identifiable germline mutation, with prevalence increasing with age at diagnosis. Only 3% of MMR-proficient endometrial tumors were methylated (and at low levels), and none of the tumors from *MLH1* mutation carriers were methylated. Although testing for *MLH1* methylation was once considered to be technically complex and costly,^{45,46} it is now a clinically feasible, robust, and cost-effective prescreening method for selecting patients exhibiting tumor *MLH1* protein loss for germline analysis.^{28,29,47} The MethyLight assay (Qiagen, Valencia, California; used in this study and previous colorectal cancer studies⁴⁸) and methylation specific-multiplex probe amplification³⁰ have both proven to be robust methods. *MLH1* methylation testing is reported to be more cost-effective compared with *BRAF* V600E mutation testing^{47,49} in the diagnostic

algorithm for *MLH1*-deficient colorectal cancer, and *BRAF* V600E mutations occur too infrequently in endometrial cancer to be considered a useful alternative predictor for negative germline MMR mutation status.⁵⁰

Results from our unselected series supports previous studies demonstrating that *MSH6* mutations are the most prevalent of all MMR gene mutations in patients with endometrial cancer.⁵¹ *MSH6* mutations were identified in 1.4% of patients, comparable to estimates of 1% to 3.8% reported previously.^{33,40,51,52} It is also worthy to note that most MMR mutation carriers (17 of 22) presented with tumors of endometrioid subtype, indicating that the triage proposed in this study will not be enhanced by considering histologic subtype as previously suggested.⁵³⁻⁵⁵

Similar to findings from another population-based study³³ and a hospital-based series using various age and pathology selection criteria,²⁸ our results also demonstrated that DNA testing fails to identify mutations in many cases with tumor *MSH2* deficiency or *MSH6* deficiency (60% and 30%, respectively). Because such cases are generally deemed to be of high clinical suspicion of Lynch syndrome, further study is required to determine the underlying cause of MMR deficiency in such instances, including assessment of somatic methylation of the *MSH2* gene promoter⁵⁶ or *MSH6* promoter. Similarly,

10% of mutation-negative cases in this series showed *MLH1* deficiency and no evidence of *MLH1* methylation, indicating the need to explore alternative reasons for MMR deficiency, including intronic or other “cryptic” mutations,^{57,58} somatic mosaicism, or double somatic hits.^{59,60}

We are aware of some limitations of the study. First, analysis did not include all endometrial cancer probands diagnosed in the study period. Over-representation of MMR carriers could possibly result from increased research participation for individuals with a family history of cancer or modest survival bias of MSI-H patients. However, such bias reported for MSI-H colorectal tumors⁶¹⁻⁶³ is not established for MSI-H endometrial tumors,^{64,65} and comparison to national Cancer Registry data suggests that age, histology and grade distribution of ANECS participants does not differ greatly from that for all women diagnosed in Australia. Second, family history of cancer was self-reported by participants, but previous studies indicate that reporting accuracy is better for relatives free of cancer than those with cancer,⁶⁶ so such possible bias would result in even poorer performance of family history–based criteria. Although material was not available for all study participants, participant characteristics did not differ according to availability of tumor material for testing. Furthermore, mutation testing directed by tumor IHC analysis would have missed MMR protein-stable mutations.⁶⁷⁻⁶⁹ For this reason, the testing scheme in Figure 2 includes tumor MSI testing in combination with family history as secondary trigger for mutation testing. The strengths of this study were investigation of an unselected endometrial cancer cohort to inform population-level screening programs, comparison of the performance of molecular criteria with clinical criteria based on detailed family history, and confirmation of *MLH1* methylation as a negative predictor of mutation status essential for efficient triage of incident endometrial cancer cases for mutation testing.

In conclusion, using a large population-based study of endometrial cancer, we have demonstrated that the combined molecular testing of tumor MMR protein expression and tumor DNA *MLH1* methylation for patients with endometrial cancer diagnosed at age less than 60 years optimizes detection of mutation carriers.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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GLOSSARY TERMS

DNA methylation: Methylation of bases contained in the DNA double helix, resulting in a loss of gene function. Generally occurring on cytosine residues in the DNA, methylation is important in regulating cell growth and differentiation and has resulted in the testing of DNA methyltransferase inhibitors as anticancer agents and differentiation agents.

immunohistochemistry: The application of antigen-antibody interactions to histochemical techniques. Typically, a tissue section is mounted on a slide and incubated with antibodies (polyclonal or monoclonal) Specific to the antigen (primary reaction). The antigen-antibody signal is then amplified using a second antibody conjugated to a complex of peroxidase-antiperoxidase, avidin-biotin-peroxidase, or avidin-biotin alkaline phosphatase. In the presence of substrate and chromogen, the enzyme forms a colored deposit at the sites of antibody-antigen binding. Immunofluorescence is an alternate approach to visualize antigens. In this technique, the primary antigen-antibody signal is amplified using a second antibody conjugated to a fluorochrome. On ultraviolet light absorption, the fluorochrome emits its own light at a longer wavelength (fluorescence), thus allowing localization of antibody-antigen complexes.

Lynch syndrome: Hereditary nonpolyposis colorectal cancer (HNPCC). A cancer syndrome characterized by Henry T. Lynch in 1966, this genetic condition has a high risk of colon cancer as well as other cancers including endometrial, ovary, stomach, small intestine, hepatobiliary tract, upper urinary tract, brain, and skin.

MMR (mismatch repair genes): Genes that recognize and correct errors in DNA replication leading to single base-pair mismatches or insertions/deletions in small repetitive tracts of DNA known as microsatellites.

population-based study: Study in which the patients are drawn from a defined population in a manner that is representative of the source population studied. Such a design can avoid bias arising from the selective factors that guide affected individuals to a particular medical facility, allowing for greater generalizability of the findings.

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Appendix

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Table A1. Nonpolymorphic MMR Gene Variants Identified in MMR IHC-Loss Patients With Endometrial Cancer

Patient	Age at Diagnosis (years)	BMI	Tumor Grade	FIGO Stage	Histotype	Tumor MMR IHC Deficiency	Germline MMR Mutation	MMR Classification
1	54	24.8	3	1	Serous	<i>MLH1/PMS2</i>	MLH1: c.1464_1468del p.(Lys488Asnfs*13)	Pathogenic
2	29	35.0	2	3	Endometrioid	<i>MLH1/PMS2</i>	MLH1: c.208-?_306+?del p.(Lys70_Glu102del)	Pathogenic
3	49	23.4	2	1	Endometrioid	<i>MLH1/PMS2</i>	MLH1: c.1489dupC p.(Arg497Profs*6)	Pathogenic
4	48	25.7	3	1	Adenocarcinoma	<i>MSH2/MSH6</i>	MSH2: c.(?-68)_(*272_?)del p.?	Pathogenic
5	52	30.4	3	2	Endometrioid	<i>MSH2/MSH6</i>	MSH2: c.163del p.(Arg55Glyfs*9)	Pathogenic
6	45	26.4	3	1	Endometrioid	<i>MSH2/MSH6</i>	MSH2: c.(?-68)_1386+?del p.?	Pathogenic
7	51	22.3	2	1	Endometrioid	<i>MSH2/MSH6</i>	MSH2: c.942 + 3A>T p.(Val265_Gln314del)	Pathogenic
8	54	21.5	2	3	Endometrioid	<i>MSH2/MSH6</i>	MSH2: c.1286delA p.(Gln429Argfs*9)	Pathogenic
9	57	30.9	1	2	Endometrioid	<i>MSH2/MSH6</i>	MSH2: c.892C>T p.(Gln298*)	Pathogenic
10	66	26.6	1	1	Endometrioid	<i>MSH2/MSH6</i>	MSH2: c.793-?_942+?del p.(Val265_Gln314del)	Pathogenic
11	36	19.8	1	1	Endometrioid	<i>MSH2/MSH6</i>	MSH2: c.2635-?_(*272_?)del p.?	Pathogenic
12	59	24.5	2	1	Endometrioid	<i>PMS2</i>	PMS2: c.736_741delinsTGTGTGTGAAG p.(Pro246Cysfs*3)	Pathogenic
13	51	20.5	1	3	Serous	<i>MSH6</i>	MSH6: c.3513_3514del p.(Asp1171Glufs*5)	Pathogenic
14	57	33.7	2	1	Endometrioid	<i>MSH6</i>	MSH6: c.3573dupT p.(Val1192Cysfs*2)	Pathogenic
15	53	22.3	3	1	Endometrioid	<i>MSH6</i>	MSH6: c.(?-152)_(*93_?)del p.?	Pathogenic
16	54	27.9	2	1	Endometrioid	<i>MSH6</i>	MSH6: c.2731C>T p.(Arg911*)	Pathogenic
17	61	29.4	1	1	Endometrioid	<i>MSH6</i>	MSH6: c.3996_4000dup p.(Arg1334Hisfs*14)	Pathogenic
18	58	35.3	3	1	Endometrioid	<i>MSH6</i>	MSH6: c.458-?_627+?del p.(Ser154Argfs*8)	Pathogenic
19	49	23.2	2	1	Serous	<i>MSH6</i>	MSH6: c.458-?_627+?del p.(Ser154Argfs*8)	Pathogenic
20*	66	28.4	1	1	Endometrioid	<i>MSH6</i> and <i>MLH1/PMS2</i>	MSH6: c.1870_1871dup p.(Ser625Alafs*11)	Pathogenic
21	35	37.9	2	3	Mucinous	<i>MSH6</i>	MSH6: c.3142C>T p.(Gln1048*)	Pathogenic
22	47	27.1	2	3	Endometrioid	<i>MSH6</i>	MSH6: c.3202C>T p.(Arg1068*)	Pathogenic
23	45	25.4	3	1	Endometrioid and clear cell	<i>MSH6</i>	MSH6: c.3694_3696del p.(Val1232del)	Unclassified variant†
24	58	28.7	2	1	Endometrioid	<i>MSH6</i>	MSH6: c.2314C>T p.(Arg772Trp)	Unclassified variant‡
25	64	29.1	1	1	Endometrioid	<i>MSH6</i>	MSH6: c.3725_3727del p.(Arg1242_Thr1243delinsPro)	Unclassified variant§
26	59	26.6	3	1	Endometrioid	<i>MSH6</i>	MSH6: c.3469G>A p.(Gly1157Ser)	Unclassified variant

NOTE. Of the pathogenic mutation carriers, three (13.6%; 95% CI, 4.8% to 33.3%) had no family history of any cancer, 15 (68.2%; 95% CI, 47.3% to 83.6%) had at least one first- or second-degree relative with endometrial and/or colorectal cancer, and three (13.6%; 95% CI, 4.8% to 33.3%) had prior colorectal cancer.

Abbreviations: AMSII, Amsterdam II criteria; ANECS criteria, Australian National Endometrial Cancer Study criteria; CRC, colorectal cancer; EC, endometrial cancer; FDR, first-degree relatives; MMR, mismatch repair; N/D, not done—no material available for *MLH1* methylation analysis; SDR, second-degree relatives; SGO10, Society of Gynecologic Oncology criteria (5% to 10% risk); SGO25, Society of Gynecologic Oncology criteria (20% to 25% risk); SGO25noIHC, Society of Gynecologic Oncology criteria (20% to 25% risk) excluding immunohistochemistry testing as one of its criterion.

*This individual has a *MLH1* methylated reference value of 11.9%.

†The prior probability of pathogenicity for this variant is 0.97.

‡The prior probability of pathogenicity for this variant is 0.89.

§The prior probability of pathogenicity for this variant is 0.99.

||The prior probability of pathogenicity for this variant is 0.41.

MMR Mutation Testing Triage of Patients With Endometrial Cancer

Table A1. Nonpolymorphic MMR Gene Variants Identified in MMR IHC-Loss Patients With Endometrial Cancer (continued)

Patient	<i>MLH1</i> Methylation	Prior Cancer	Family History of Lynch Syndrome Cancer	Family History of Other Cancer	Fulfilled Amsterdam Criteria	Fulfilled Revised Bethesda Guidelines	Fulfilled Other Clinical Criteria
1	No	CRC	SDR CRC	FDR breast	No	Yes	SGO10, ANECS, SGO25noIHC
2	N/D	Thyroid	SDR CRC, FDR EC	—	Yes	Yes	SGO10, ANECS, SGO25noIHC
3	No	—	FDR CRC, FDR stomach cancer	SDR breast	No	Yes	SGO10, ANECS
4	No	—	SDR CRC, FDR EC	—	Yes	Yes	SGO10, ANECS, SGO25noIHC
5	N/D	—	FDR CRC, SDR CRC	—	No	Yes	SGO10, ANECS
6	No	—	SDR CRC, FDR EC, SDR EC	—	Yes	Yes	SGO10, ANECS, SGO25noIHC
7	N/D	—	FDR CRC, SDR CRC, SDR EC	—	No	Yes	SGO10, ANECS
8	No	CRC	—	—	No	Yes	SGO10, ANECS, SGO25noIHC
9	N/D	Breast	Adopted–family history unknown	Adopted–family history unknown	No	Yes	RBG, SGO10, ANECS
10	N/D	—	FDR CRC, SDR CRC, FDR EC	SDR breast cancer	Yes	Yes	SGO10, ANECS, SGO25noIHC
11	No	—	FDR CRC, SDR CRC	—	No	Yes	SGO10, ANECS
12	n/d	—	—	SDR breast cancer	No	No	No
13	No	—	—	SDR breast cancer	No	No	No
14	No	—	SDR CRC , FDR EC	—	No	Yes	SGO10, ANECS
15	No	—	FDR CRC	SDR breast cancer	No	Yes	SGO10, ANECS
16	No	—	—	—	No	No	No
17	No	Breast	FDR CRC, FDR stomach cancer	FDR breast cancer	No	Yes	SGO10, ANECS
18	No	CRC	SDR CRC	FDR breast cancer	No	Yes	SGO10, ANECS, SGO25noIHC
19	No	Melanoma	—	SDR breast cancer	No	Yes	SGO10, ANECS
20*	Yes	—	FDR CRC	—	No	Yes	SGO10, ANECS
21	N/D	—	—	—	No	No	SGO10, ANECS
22	No	—	FDR EC	—	No	No	SGO10, ANECS
23	No	—	FDR CRC	SDR breast cancer	No	Yes	SGO10, ANECS
24	No	Breast	FDR EC	FDR breast cancer, SDR breast cancer, SDR thyroid cancer	No	Yes	SGO10, ANECS
25	N/D	—	Adopted–family history unknown	Adopted–family history unknown	No	No	No
26	No	—	—	—	No	No	No

Table A2. Characteristics of Patients With Endometrial Cancer Having Normal Tumor Mismatch Repair Immunohistochemical Results: Comparison of Patients With Versus Without Methylation Testing Results

Characteristic	Methylation Not Tested (n = 455)			Methylation Tested (n = 77)			P
	No.	%	95% CI	No.	%	95% CI	
Age at diagnosis, years							
Mean		61.4			63.1		
Range		27.1-79.8			44.8-76.6		
< 50	53	11.7	9.0 to 14.9	3	3.9	1.3 to 10.9	.196
50-60	136	29.9	25.9 to 34.3	21	27.3	18.6 to 38.1	
≥ 60	265	58.2	53.7 to 62.7	53	68.8	57.8 to 78.1	
BMI							
Mean		31.6			31.7		
Range		18.7-75.5			19.6-58.6		
Normal (18.50-24.99)	99	21.8	18.2 to 25.8	13	16.9	10.1 to 26.8	.793
Overweight (25.00-29.99)	104	22.9	19.2 to 26.9	20	26.0	17.5 to 36.7	
Obese (≥ 30.00)	213	46.8	42.3 to 51.4	37	48.1	37.3 to 59.0	
Unknown	1	0.2	0 to 1.2	0	0		
Multiple primary malignancies	39	8.6	6.3 to 11.5	5	6.5	2.8 to 14.3	.540
Histology and FIGO grade							.895
Endometrioid	367	80.7	76.8 to 84.0	64	83.1	73.2 to 89.9	
1	242	53.2	48.6 to 57.7	27	35.1	25.3 to 46.2	
2	94	20.7	17.2 to 24.6	31	40.3	30.0 to 51.4	
3	28	6.2	4.3 to 8.8	6	7.8	3.6 to 16.0	
Unknown	88	19.3	16.0 to 23.2	13	16.9	10.1 to 26.8	
Serous	41	9.0	6.7 to 12.0	6	7.8	3.6 to 16.0	
Clear cell	11	2.4	1.4 to 4.3	1	1.3	0.2 to 7.0	
Carcinosarcoma (MMMT)	18	4.0	2.5 to 6.2	4	5.2	2.0 to 12.6	
Other*	18	4.0	2.5 to 6.2	2	2.6	0.7 to 9.0	
FIGO stage (2009)							.529
I-II	402	88.4	85.1 to 91.0	69	89.6	80.8 to 94.6	
III-IV	46	10.1	7.7 to 13.2	6	7.8	3.6 to 16.0	
Stage unknown†	7	1.5	0.8 to 3.1	2	2.6	0.7 to 9.0	
First-degree relative(s) with colorectal cancer	63	13.9	11.0 to 17.3	7	9.1	4.5 to 17.6	.254
First-degree relative(s) with endometrial cancer	22	4.8	3.2 to 7.2	2	2.6	0.7 to 9.0	.556
Fulfills Amsterdam II criteria	16	3.5	2.2 to 5.6	2	2.6	0.7 to 9.0	.999
Fulfills revised Bethesda guidelines	157	34.5	30.3 to 39.0	26	33.8	24.2 to 44.9	.900
Fulfills Society of Gynecologic Oncology (SGO) guidelines‡							
SGO25 (including IHC test results)	16	3.5	2.2 to 5.6	2	2.6	0.7 to 9.0	.999
SGO25 (excluding IHC test results)	16	3.5	2.2 to 5.6	2	2.6	0.7 to 9.0	.999
SGO10	240	52.8	48.2 to 57.3	37	48.1	37.3 to 59.0	.446
Fulfills ANECS criteria	255	56.0	51.5 to 60.5	42	54.6	43.5 to 65.2	.807

Abbreviations: ANECS criteria, Australian National Endometrial Cancer Study criteria; BMI, body mass index; FIGO, International Federation of Gynecology and Obstetrics; MMMT, malignant mixed Müllerian tumor; SGO, Society of Gynecologic Oncology.

*Includes adenocarcinoma, undifferentiated, mucinous subtypes, mixed subtypes including a serous and/or clear cell (minor) component, and other epithelial.

†Stage at diagnosis according to the 1988 FIGO system for these nine individuals was stage 2 tumor (n = 1), stage 3 tumor (n = 2), and unknown (n = 1).

‡SGO25 includes IHC testing as one of several selection criteria (Table 1). Analysis excluding tumor MMR IHC test results was performed for comparison.

Table A3. Total Cost Per Carrier for Selected Screening Strategies (expressed in 2013 AUD dollars)

Screening Strategy	IHC Testing for MSH2/MSH6*			IHC Testing for MLH1/PMS2*			MMR Gene Promoter Methylation for MLH1†			Genetic Counseling for Cases Prioritized for Genetic Testing‡			Single MMR Gene Germ-Line Testing (sequencing + MLPA)\$			Mutation Carrier Identified	Total Cost Per Carrier	Cost Saving Per Carrier Compared With Universal IHC Screening
	No. Tested	No. of Stains	Total Cost	No. Tested	No. of Stains	Total Cost	No. Tested	No. NA	Total Cost	No. for GC	Total Cost	No. Tested	No. Total Cost					
MMR IHC loss for all ages (universal IHC testing)	698	2	\$30,712	698	2	\$30,712	NA	NA	166	\$66,400	166	\$182,600	21	\$14,782.10	—			
MMR IHC loss < 60 years	285	2	\$12,540	285	2	\$12,540	NA	NA	71	\$28,400	71	\$78,100	18	\$7,310.00	\$7,472.10			
MMR IHC loss for all ages + MLH1 unmethylated	698	2	\$30,712	698	2	\$30,712	124	\$37,200	58	\$23,200	58	\$63,800	21	\$8,839.24	\$5,942.86			
MMR IHC loss for < 60 years + MLH1 unmethylated	285	2	\$12,540	285	2	\$12,540	42	\$12,600	39	\$15,600	39	\$42,900	18	\$5,343.33	\$9,438.76			
IHC for MSH2/MSH6 only + IHC MLH1/PMS2 for those who meet revised Bethesda guideline + age < 60 years	698	2	\$30,712	88	2	\$3,872	NA	NA	130	\$52,000	130	\$143,000	20	\$11,479.20	\$3,302.90			
IHC for MSH2/MSH6 only + IHC MLH1/PMS2 for those age < 60 years + MLH1 unmethylated	698	2	\$30,712	256	2	\$11,264	42	\$12,600	52	\$20,800	52	\$57,200	21	\$6,313.14	\$8,468.95			
2 stain MSH6/PMS2 IHC loss for all ages	698 + 42	1	\$16,280	698 + 124	1	\$18,084	NA	NA	166	\$66,400	166	\$182,600	21	\$13,493.52	\$1,288.57			
2 stain MSH6/PMS2 IHC loss < 60 years	285 + 29	1	\$6,908	285 + 42	1	\$7,194	NA	NA	71	\$28,400	71	\$78,100	18	\$6,700.11	\$8,081.98			
2 stain MSH6/PMS2 IHC loss for all ages + MLH1 unmethylated	698 + 42	1	\$16,280	698 + 124	1	\$18,084	123	\$36,900	58	\$23,200	58	\$63,800	21	\$7,536.38	\$7,245.71			
2 stain MSH6/PMS2 IHC loss for < 60 years + MLH1 unmethylated	285 + 29	1	\$6,908	285 + 42	1	\$7,194	42	\$12,600	39	\$15,600	39	\$42,900	18	\$4,733.44	\$10,048.65			

NOTE. The two best cost-effective strategies are bolded.

Abbreviations: GC, genetic counseling; IHC, immunohistochemistry; MLPA, multiplex ligation-dependent probe amplification; MMR, mismatch repair; NA, not applicable. *\$22 per IHC test. MBS code 72,847. Unit cost derived from the Australian Medicare Benefits Schedule (MBS; July 2013) (www.mbsonline.gov.au).

†\$300 per methylation test. Cost estimates derived from two independent clinical genetic testing services in Australia.

‡\$400 for two genetic consultations. MBS code 132 and code 133 (www.mbsonline.gov.au).

\$1,100 per germline test, includes sequencing and MLPA. Cost estimates derived from two independent clinical genetic testing services in Australia.

||Costing for two-stain approach assumes initial IHC for MSH6 and PMS2 only, followed by MSH2 IHC only for individuals exhibiting tumor MSH6 IHC loss, and MLH1 IHC only for individuals exhibiting tumor PMS2 IHC loss.