

8-2013

Evaluating the Utility of Clinical Criteria for the Identification of Lynch Syndrome among Endometrial Cancer Patients

Amanda S. Bruegl

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**Evaluating the Utility of Clinical Criteria for the Identification of Lynch Syndrome among
Endometrial Cancer Patients**

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among Endometrial Cancer Patients**

A

THESIS

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences
In Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

by

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Houston, Texas

August, 2013

Evaluating the Utility of Clinical Criteria for the Identification of Lynch Syndrome among Endometrial Cancer Patients

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Background: Lynch Syndrome (LS) is a familial cancer syndrome with a high prevalence of colorectal and endometrial carcinomas among affected family members. Clinical criteria, developed from information obtained from familial colorectal cancer registries, have been generated to identify individuals at elevated risk for having LS. In 2007, the Society of Gynecologic Oncology (SGO) codified criteria to assist in identifying women presenting with gynecologic cancers at elevated risk for having LS. These criteria have not been validated in a population-based setting.

Materials and Methods: We retrospectively identified 412, unselected endometrial cancer cases. Clinical and pathologic information were obtained from the electronic medical record, and all tumors were tested for expression of the DNA mismatch repair proteins through immunohistochemistry. Tumors exhibiting loss of MSH2, MSH6 and PMS2 were designated as probable Lynch Syndrome (PLS). For tumors exhibiting immunohistochemical loss of MLH1, we used the PCR-based *MLH1* methylation assay to delineate PLS tumors from sporadic tumors. Samples lacking methylation of the *MLH1* promoter were also designated as PLS. The sensitivity and specificity for SGO criteria for detecting PLS tumors was calculated. We compared clinical and pathologic features of sporadic tumors and PLS tumors. A simplified cost-effectiveness analysis was also performed comparing the direct costs of utilizing SGO criteria vs. universal tumor testing.

Results: In our cohort, 43/408 (10.5%) of endometrial carcinomas were designated as PLS. The sensitivity and specificity of SGO criteria to identify PLS cases were 32.7 and 77%, respectively. Multivariate analysis of clinical and pathologic parameters failed to identify statistically significant differences between sporadic and PLS tumors with the exception of tumors arising from the lower uterine segment. These tumors were more likely to occur in PLS tumors. Cost-effectiveness analysis showed clinical criteria and universal testing strategies cost \$6,235.27/PLS case identified and \$5,970.38/PLS case identified, respectively.

Conclusions: SGO 5-10% criteria successfully identify PLS cases among women who are young or have significant family history of LS related tumors. However, a larger proportion of PLS cases occurring at older ages with less significant family history are not detected by this screening strategy. Compared to SGO clinical criteria, universal tumor testing is a cost effective strategy to identify women presenting with endometrial cancer who are at elevated risk for having LS.

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Abbreviations

CRC: colorectal cancer

EC: endometrial cancer

EMR: electronic medical record

EGAPP: Evaluation of genomic applications in practice and prevention

EWG: EGAPP working group

FDR: first-degree relative

IHC: immunohistochemistry

LAT: Lynch Syndrome associated tumor

LS: Lynch Syndrome

LUS: tumor arising from the lower uterine segment

MDACC: MD Anderson Cancer Center

MMR: mismatch repair

MSI: microsatellite instability

OC: ovarian cancer

Introduction

Lynch Syndrome – Definition and Clinic Based Screening Criteria

Lynch Syndrome (LS), formerly known as hereditary non-polyposis colorectal cancer (HNPCC), is a hereditary cancer syndrome characterized by an elevated prevalence of endometrial and colorectal carcinomas, and to a lesser degree other Lynch Syndrome associated tumors (LATs), among affected family members. In 1895, pathologist Aldred Warthin at University of Michigan observed a family with a significant prevalence of stomach, uterine, and small intestinal tumors. The male proband, whose index cancer was an upper gastrointestinal tumor, had 10 children and 48 relatives within a three-generation pedigree of which 17 had a diagnosis of cancer during their lifetimes (1). Through his work and subsequent study by Henry Lynch at Creighton University, the hereditary cancer syndrome now known as Lynch Syndrome was characterized (1,2).

The rarity of affected families present in any given region made this syndrome difficult for a single investigator to study epidemiology, disease natural history, and genetics. In 1990, the International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer, consisting of thirty experts representing eight different countries, met in Amsterdam to create criteria for future studies designed to investigate the underlying molecular and genetic basis of the disease (3). In 1997, the working group modified the Amsterdam criteria to include extracolonic tumors, such as endometrium, stomach, ovaries, small bowel, ureter, renal pelvis, brain, and hepatobiliary tract (4). These criteria, summarized in Table 1, ultimately led to the association of LS with germline mutations in DNA mismatch repair (MMR) genes.

In addition to the international working group establishing criteria to identify individuals with HNPCC, the National Cancer Institute (NCI) also developed criteria to address some of the weaknesses in the original Amsterdam Criteria. These weaknesses include the role of extracolonic tumors, probands with small families, and clinicopathologic characteristics associated with LS tumors (5). These criteria, summarized in Table 1, were also revised subsequently to include results of molecular diagnostic tests such as microsatellite instability testing (6).

Table 1. Summary of Amsterdam and Bethesda Criteria for the Evaluation of LS

Amsterdam I^a (All criteria must be met)	Amsterdam II^b (All criteria must be met)	Bethesda^c	Revised Bethesda^d
At least 3 relatives with CRC, one must be a first-degree relative of other 2.	At least 3 relatives with a LS-associated cancer, one should be a FDR of the other 2	Proband with 2 LS-related cancers,	CRC at age < 50
FAP has been ruled out	FAP has been ruled out	Proband with CRC and an FDR with CRC and/or LS-related cancer and/or a colorectal adenoma; one of cancers diagnosed at age <45 or adenoma diagnosed at age < 40	Synchronous, metachronous or other LS related tumors
Two affected generations	Two affected generations	CRC or EC diagnosed at age < 45	MSI-H CRC in a pt <60
At least one of CRC cases occurs at < age 50	At least one LS-associated cancer should be diagnosed < age 50	Right-sided CRC with an undifferentiated pattern on histopathology diagnosed at age < 45	CRC in ≥ 1 first degree relatives with an LS related tumor, one occurring at age <50
		Individuals with signet-ring-cell-type CRC diagnosed age < 45	CRC in ≥ 2 first- or second-degree relatives with LS-related tumors
		Individuals with adenoma diagnosed at age < 40	
		Any individual meeting Amsterdam Criteria	

a:(3)

b:(4)

c:(5)

d:(6)

1: CRC - colorectal cancer

2: FAP - familial adenomatous polyposis

3: FDR - first degree relative

4: EC - endometrial cancer

5: MSI-H – microsatellite instability high

Computer-based clinical prediction models emerged in the 2000's that attempted to provide a proband's individual risk for having a germline DNA MMR mutation. These risk calculators include PREMM_{1,2,6}, MMRPredict, and MMRPro (7-9). For each of these models, the original research population consisted of probands presenting with CRC and included the model's ability to predict germline mutations in three of the four DNA MMR genes (*MLH1*, *MSH2*, and *MSH6*). The area under the receiver-operator curves in the validation studies was greater than 0.8 in each model, indicative of a favorable predictor. These models did not address probands presenting with other LATs nor did they evaluate the model's ability to predict germline mutations in *PMS2*.

Given the favorable results from these results in predicting *MLH1*, *MSH2* and *MSH6* mutations among probands presenting with CRC, a validation study among EC probands was initiated (10). These prediction models were evaluated using a population-based cohort consisting of 563 unselected endometrial cancer cases as well as a high-risk, clinic-based cohort consisting of individuals from 129 families enrolled in the Colon Cancer Family Registry (11). These 3 prediction models had AUCs < 0.8 in both the population-based cohort and high-risk, clinic-based cohorts and were therefore deemed less useful tools in the EC population.

In 2007, the Society of Gynecologic Oncology (SGO) published a statement on risk assessment for inherited cancer syndromes among gynecologic cancer probands (12). This expert panel deemed genetic risk assessment for individuals with a 5-10% likelihood of having a germline mutation as *reasonable* and asserted that an individual with a 20-25% possibility of a germline mutation *should* undergo risk assessment. The committee published criteria corresponding to the 5-10% and 20-25% risk groups (Table 2). The

20-25% criteria resemble Amsterdam II criteria, and the 5-10% criteria resemble the revised Bethesda guidelines. Ryan et al. investigated the performance of these criteria in a cohort of 76 EC cases from familial cancer registries at Mount Sinai Hospital and in British Columbia with a known LS germline mutation. The mean age at diagnosis of EC in their cohort was 47.3 years, and 28/76 (36.8%) were diagnosed at age greater than 50. They found that SGO 20-25% criteria correctly identified 71% of individuals with a known LS germline mutation, and the 5-10% criteria correctly identified 93% of mutation carriers. The 20-25% criteria best identified *MSH2* mutations with a 78% detection rate; however, the detection rates for *MLH1* and *MSH6* were 61% and 50%, respectively. The 5-10% criteria performed equally well in the detection of *MLH1* and *MSH2* mutations and had a detection rate of 94%, but only had an 88% detection rate for identifying *MSH6* mutations. There were no known *PMS2* mutation carriers in this cohort (13). To date, these criteria have not been validated in a population-based setting.

Table 2. Society of Gynecologic Oncology (SGO) Criteria for those at 5-10% and 20-25% risk of having a germline mutation in *MLH1*, *MSH2*, *MSH6*, or *PMS2* (12).

SGO 5-10% Criteria	SGO 20-25% Criteria
EC or CRC diagnosed before age 50	Probands meeting Amsterdam II Criteria
EC or OC with a synchronous or metachronous CRC or other LAT at any age	Synchronous or metachronous EC or CRC, first cancer occurring before age 50
EC or CRC and a first degree relative with LAT diagnosed before age 50	Synchronous or metachronous OC or CRC, first cancer occurring before age 50
EC or CRC and ≥ 2 first or second degree relatives with LATs	CRC or EC with tumor testing suggestive of LS (IHC or MSI-H)
Proband with first or second degree relatives who meet these criteria	First or second degree relative with a known germline mutation

EC: Endometrial Cancer
CRC: Colorectal Cancer
OC: Ovarian Cancer

LAT: Lynch Syndrome associated tumor
MSI-H: Microsatellite Instability-High
IHC: Immunohistochemistry

Published data suggest that women with EC and mutations in *PMS2* and *MSH6* are often older with less extensive family histories of CRC or other LATs (14,15). In a study by Goodfellow et al., endometrial cancers were evaluated in a population-based fashion to characterize the role of *MSH6* mutations in endometrial cancer. In their cohort of 441 unselected endometrial cancer cases an *MSH6* mutation was identified in 1.6% (7/441). The median age at diagnosis for these carriers was 53.6 years (range: 45-71), and 5/7 cases occurred after age 50 (16).

Bonadona et al. evaluated cancer risk in a cohort of 537 LS germline mutation carriers enrolled in the French Estimation des Risques de Cancer chez les porteurs de mutation des genes MMR (ERISCAM) study. The LS cases in this cohort consisted of 248 *MLH1*, 256 *MSH2* and 33 *MSH6* mutation carriers. In their examination of the lifetime cancer risks for *MSH6* carriers, they found that the cumulative risk for 70 years of age for CRC was 12%, EC 21%, ovarian cancer 1% and 0% for stomach, small bowel, and biliary tract. These risks are substantially lower than that of *MLH1* and *MSH2* mutation carriers. Data from this study show that individuals with LS and an *MSH6* mutation have less overall risk for cancer compared to *MLH1* and *MSH2* mutation carriers (15). Much of the data we have regarding LS and EC is extrapolated from CRC registries, which tend to be dominated by *MLH1* and *MSH2* mutations. It is not clear if such data can be applied to *MSH6* mutation carriers, who have a distinct cancer risk profile. Thus, we may be underestimating and under-diagnosing the number of women with EC and LS.

Lynch Syndrome Associated Tumors (LATs)

In addition to CRC and EC, other Lynch Syndrome associated tumors (LATs) have been identified that occur in germline mutation carriers at higher rates than that of the general population. These LATs include ovary, gastric, small intestine, hepatobiliary tract, urinary tract, brain and skin (17). For women with germline mutations, the lifetime risks of EC and CRC are 39.4% and 42.7%, respectively. The risk of developing either EC or CRC is 73.4% (18). Additionally, women with germline mutations are at least equally as likely to present with a gynecologic cancer as their sentinel cancer diagnosis as they are CRC (19).

DNA Mismatch Repair (MMR)

DNA replication is a process in which errors occur at a rate of 1 of every 10^4 nucleotides. DNA polymerase, an enzyme essential to the replication process, has an innate proofreading ability which improves the fidelity of DNA replication to 1 in 10^6 (20). In addition to intra-replicative error repair, there also exist other repair systems that address errors persistent after DNA replication has been completed. The DNA MMR system identifies and repairs mismatches in nucleotide pairs following DNA replication. Figure 1 summarizes the overall process for this system. Following an error in replication, the MSH2/MSH6 heterodimer recognizes and binds to a base-pair mismatch. MLH1/PMS2 proteins are recruited, which in turn, recruit Exonuclease 1. The mismatched base-pair is excised and DNA polymerase inserts the appropriate base-pair in to the sequence (21). Germline mutations in the DNA MMR repair genes, *MLH1*, *MSH2*, *MSH6* and *PMS2*, are the genetic basis for Lynch Syndrome.

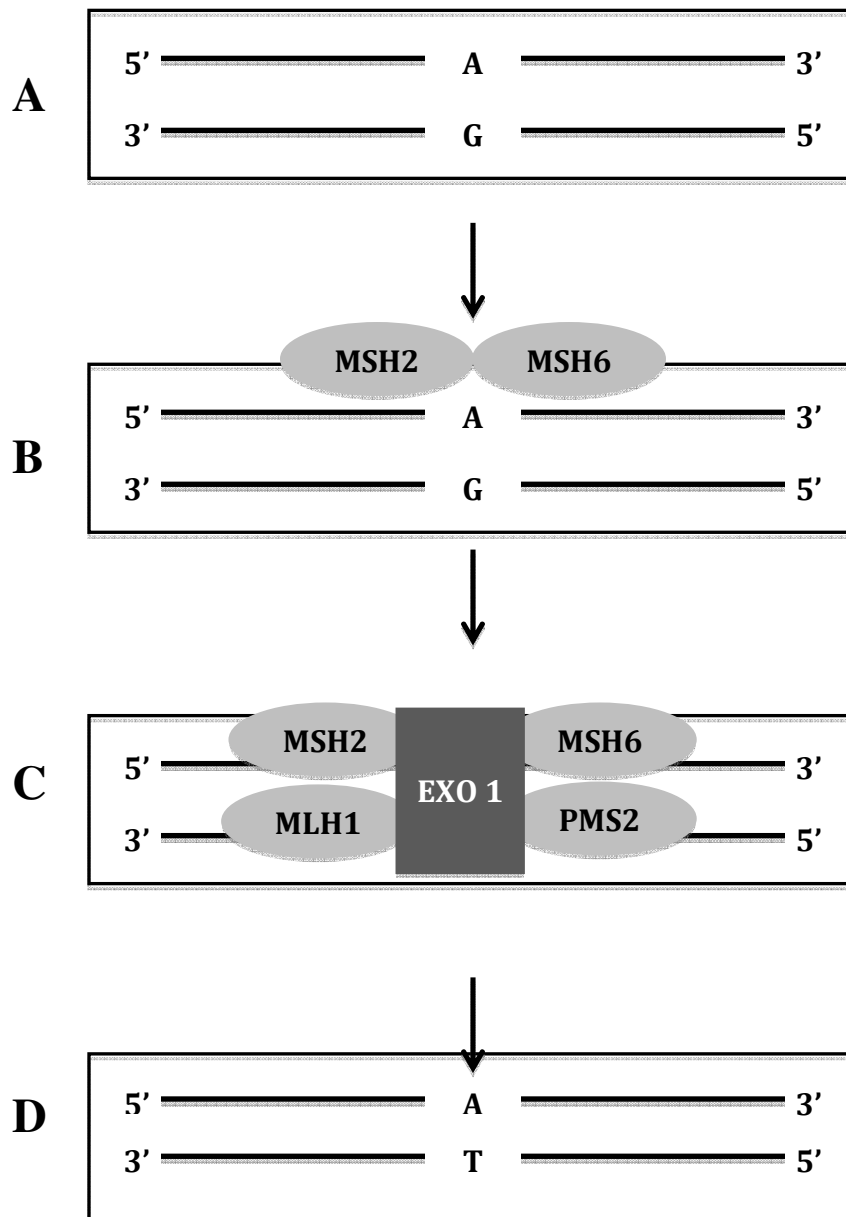


Figure 1. Schematic representation of DNA MMR system.
 A. Nucleotide mismatch occurs
 B. MSH2/MSH6 heterodimer surveys DNA, recognizes error, and initiates MMR process
 C. MLH1/PMS2 are recruited, DNA polymerase is displaced from DNA strands, exonuclease is recruited and mismatched pair is removed
 D. DNA polymerase returns and resynthesized DNA with correct strand

Molecular Diagnostic Tools In the Evaluation for Lynch Syndrome

Tissue-based Screening Methods: Microsatellite Instability, Immunohistochemistry, and MLH1 methylation

Tissue-based LS screening assays emerged in the 1990s, providing another clinical tool in identifying individuals who should proceed with germline testing. DNA microsatellites consist of multiple, tandem repeats of mono-, di-, and tri- nucleotides; such repeats are prone to errors during the DNA replication process. Errors in DNA replication resulting in a change in the number of tandem repeats is termed microsatellite instability (MSI) (22). The Bethesda Panel is a published set of microsatellite sites recommended for PCR-based MSI analysis. An earlier panel included BAT-25, BAT-26, D5S346, D2S123 and D17S250, with subsequent recommendations to add BAT40 and TGF- β R2 (6). Using the panel of 7 microsatellites, a tumor exhibiting allelic shift in 3 or more markers is designated as MSI-high (MSI-H), 1-2 markers is MSI-low (MSI-L), and no allelic shift is microsatellite stable (MSS). Lynch Syndrome associated cancers are typically MSI-H, while sporadic tumors with no defects in DNA MMR are typically MSS. MSI-L represents somewhat of a clinical conundrum (23). Nearly all MSI-L colorectal carcinomas are sporadic. However, a substantial subset of endometrial carcinomas from women known to have Lynch Syndrome mutations are MSI-L or MSS (Am J Path 2002 reference emailed to you Sat morning).

In addition to MSI analysis, the development of monoclonal antibodies against DNA MMR proteins has allowed IHC to become another technique available in evaluating for LS. Tumors with positive nuclear expression of these proteins typically have an intact MMR system. Loss of protein expression in tumor cell nuclei with

preserved stromal cell staining is suggestive of LS. Individuals with mutations in *MLH1* will typically have IHC loss of MLH1 and PMS2, while patients with *MSH2* mutations will have IHC loss of MSH2 and MSH6, due to the dominant role of MLH1 and MSH2 in heterodimer formation of the MMR complex. Individuals with germline mutations in *MSH6* or *PMS2* typically show only IHC loss of the corresponding MMR protein (24,25).

Approximately 15-20% of all endometrial and colorectal adenocarcinomas have epigenetic silencing of the *MLH1* promoter secondary to methylation (26-28). Between 65-96.9% of endometrial tumors exhibiting MLH1 loss have methylation at the *MLH1* promoter region (29-32). By performing this PCR-based assay as part of the LS evaluation, unnecessary and costly genetic testing can be avoided. Despite the availability of this assay, it is not universally utilized in published clinical studies (33-36). One reason is that PCR-based technology may not be as readily available in all pathology laboratories, whereas performance of IHC is fairly straightforward, less expensive, and more universally available.

Performance of MSI and IHC

The utilization of MSI or IHC to aid in the evaluation for LS varies across different institutions based on resources and departmental standards. A recent study by Bartley *et. al* examined the concordance/discordance between MSI and IHC in patients undergoing tumor testing in the evaluation for LS (37). The majority of tumors evaluated were colorectal adenocarcinomas (88%), with a smaller fraction of endometrial adenocarcinomas (7%). They found an overall concordance of MSI and IHC results in 97.8% of tumors.

The overall sensitivity of MSI and IHC analyses for identifying LS germline mutations in CRCs is similar, with rates of 83% and 94%, respectively (38). The slightly decreased sensitivity of MSI is attributed to *MSH6* and *PMS2* mutations, which more often tend to be associated with MSS and MSI-L tumors (39,40).

BRAF and Endometrial Cancer

In colorectal cancers, 5-10% of sporadic, MSI-H tumors will be associated with a mutation in the *BRAF* gene, a component of the MAPK pathway (41). The prevalence of this mutation in CRCs has made *BRAF* analysis a component of standard tumor testing in evaluation CRCs for LS. In particular, *BRAF* mutation analysis may be useful in patients with CRCs with IHC loss of MLH1, when an *MLH1* methylation assay is not available.

BRAF mutations are exceedingly rare in EC (30,34,42), so this test has not been incorporated into the clinicopathologic algorithms for LS evaluation in EC patients.

Pathologic Features Associated with Lynch Syndrome

There are certain pathologic features in EC that have been shown to be associated with LS tumors. These features include tumors arising from the lower uterine segment (LUS), tumors with peritumoral lymphocytes, and presence of tumor infiltrating lymphocytes (43,44). LUS tumors are a relatively rare phenomenon, occurring in only 3.5% of all endometrial adenocarcinomas; however, the prevalence of LS in this subset of patients is 29% (44). The presence of peritumoral lymphocytes, lymphocytes surrounding a tumor at scanning power microscopically, has an odds ratio of 2.8 in predicting PLS tumors (43). Similarly, tumor infiltrating lymphocytes (TILs) are aggregates of lymphocytes located within the tumor and have an odds ratio of 3.1 in predicting PLS tumors (43).

A study investigating the value of young age and the pathologic features of peritumoral lymphocytes, TILs and heterogeneous tumors (tumors consisting of 2 histologies with each contributing at least 10% to tumor volume) in identifying DNA MMR defects was performed by Garg et al. Individuals who were either younger than 50 years of age, had any of the aforementioned pathologic findings, or whose primary physician requested tumor screening were included in their analysis. Their study found that utilizing age and these features improved detection of tumors that correlated with DNA MMR defects (45). It should be mentioned here that endometrial carcinomas with these unique features (young age of diagnosis, heterogeneous histology, LUS anatomic location, and TILs) can indeed be associated with Lynch Syndrome. But, because population-based studies are lacking, it is less certain whether the majority of Lynch Syndrome-associated endometrial carcinomas have these unique features.

National Recommendations of Ideal Screening Strategy for LS

In 2009, the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working group (EWP) published their recommendations regarding LS screening among CRC patients. They placed less emphasis on using family history as an initial triage tool, recommending that all patients with CRCs undergo evaluation for LS in the form of MSI and/or IHC testing and (46). The reasoning behind the removal of family history resulted from several factors. First, Amsterdam II and Revised Bethesda criteria, both heavily dependent on utility of family history, have far from optimal sensitivity or specificity. Next, obtaining an accurate family history often requires a skilled professional such as a genetic counselor. This requires both time and clinical resources in addition to patient compliance. Finally, these criteria work less well for

patients from small families or in scenarios in which the family history of cancer is not known. Although the working group removed family history as a requirement, it did not deter the health care team from utilizing this information as part of the full evaluation for LS.

A recent publication from the Cleveland Clinic evaluated three universal tumor testing regimens and found the approach that led to the best detection rate and follow up was universal testing in which the surgeon and genetic counselor both played lead roles in patient notification and follow-up (47). A national consensus on the ideal method of screening for LS among EC patients is not available. While the publication of SGO criteria helped to define the likelihood of identifying a Lynch Syndrome mutation in women with EC, this group did not provide recommendations on methods of patient screening.

Germline Testing

The traditional “gold standard” for diagnosing LS is to perform sequencing to detect a known germline mutation in one of the DNA MMR genes. This involves sequencing of the coding region of the gene and, in the case of *PMS2*, the performance of multiplex ligation-dependent probe amplification (MLPA) to detect mutations in the *PMS2* pseudogene. Mutations in MMR genes are not in hot spots, as is the case for *KRAS* or *BRAF* mutations. Sequencing has excellent sensitivity for detecting point mutations and minor insertions and deletions, but accurate detection of large deletions, insertions or gene rearrangements is a limitation of conventional sequencing technology (48). It is unclear whether individuals with molecular diagnostic testing results (IHC loss of MMR protein, MSI-H) suggestive of LS with negative germline testing are truly negative for

Lynch Syndrome or if their mutations have genetic features that make identification by conventional sequencing methods difficult. This is a controversial topic in the field of Lynch Syndrome research.

Cancer Prevention for Individuals with Lynch Syndrome

After the time of sentinel cancer diagnosis, an individual has a cumulative 1.5-3% yearly risk of developing a second LAT (49). The identification of LS at the time of sentinel cancer diagnosis allows for heightened cancer screening in the individual as well as testing and appropriate screening of first degree relatives (FDRs). Heightened CRC screening, via colonoscopy every 1-2 years, decreases the incidence of CRC among individuals with LS by 62% (50). The ideal methods of screening for endometrial and ovarian cancers are not known, but many experts agree that yearly evaluation of the endometrial cavity with endometrial biopsy and/or transvaginal ultrasound is a reasonable approach.

Women with known germline mutations may elect to undergo prophylactic hysterectomy and/or bilateral salpingo-oophorectomy to reduce their risk of developing endometrial or ovarian cancer. A study by Schmeler et. al compared gynecologic cancer outcomes among germline mutation carriers between those who did and did not undergo prophylactic surgery. Findings showed a 33% incidence of EC among those who were managed expectantly compared to 0% in the prophylactic surgery group. Incident cases of ovarian cancer were too small to determine statistical significance (51).

Costs Associated with Screening Strategies

There are ample data supporting effective identification of patients with LS and prevention of subsequent cancers in both affected individuals and primary tumors in first

degree relatives (FDRs). The concept of cost analysis to ascertain the benefit of various health strategies emerged in the 1990s as a tool to assist in determining the best treatment and prevention strategies both in terms of clinical effectiveness as well as cost effectiveness.

Four different subtypes of cost analysis exist: cost minimization analysis, cost effectiveness analysis, cost utility analysis, and cost-benefit analysis. Cost minimization analysis is perhaps the simplest evaluation, comparing strategies of equal effectiveness to determine which is the least expensive. Cost effectiveness analysis compares alternative strategies for a specific condition or disease and evaluates both cost and outcome. Cost utility analysis incorporates mortality and morbidity into alternative strategies being compared, utilizing quality adjusted life year (QALY) as its metric of measurement. Lastly, cost-benefit analysis compares alternative strategies with different effectiveness and different costs (52). These strategies are often employed utilizing computer-based algorithms based on large cohorts of hypothetical patients. Assumptions are then placed into the model based on published literature and individual analysis inclusion criteria.

Regardless of the type of analysis used for a study, there are three key elements at the foundation of any economic healthcare study. First, define the approaches being compared, such as standard of care vs. a new strategy for a disease treatment. Second, the perspective of which we are basing costs relays whether this is from the viewpoint of the patient, hospital/care provider, or society. Lastly, the outcome(s) being measured should be clearly defined. This can be either very broad or quite focused in nature depending on the goals of the study (52).

In response to the recommendations by the EWG in 2009 that all CRCs should be offered laboratory evaluation of their tumors, Mvundura et al. investigated the cost effectiveness of such an initiative and compared it to age-targeted testing (testing all cases occurring before age 50). The strategies being compared were: 1) IHC testing for all DNA MMR proteins with genetic sequencing based on IHC results for those with loss of MSH2, MSH6 or PMS2 and those with loss of MLH1 with negative *BRAF* testing; 2) IHC testing for all DNA MMR proteins and IHC directed genetic sequencing for those with loss of any protein type; 3) MSI testing of all tumors with gene sequencing for all MSI-H tumors; and, 4) genetic sequencing of all tumors. They utilized a decision model involving 150,000 hypothetical patients. The costs accounted for in this study were those associated with identifying LS for each strategy as well as the costs associated with genetic counseling, genetic testing, surveillance for CRC, and complications of colonoscopy for both the individual and his/her first-degree relatives. The outcome measure was both discounted life-years (LY) saved and quality-adjusted life years (QALY). The authors concluded that the most effective economic strategy was Strategy 1 (53).

Kwon and colleagues initiated a similar study in EC to evaluate the cost-effectiveness of several different screening strategies. They used a Markov Monte Carlo simulation model to perform a cost analysis comparing six different models for detecting LS in EC. The strategies compared were: 1) those meeting Amsterdam II criteria; 2) women aged younger than 50 at diagnosis with at least one first degree relative (FDR) with an LAT; 3) IHC of all women aged younger than 50 at diagnosis with targeted germline testing based on IHC results; 4) IHC of all women aged younger than 60 at

diagnosis with targeted germline testing; 5) IHC for any woman with at least one FDR had an LAT; 6) IHC of all EC cases. The incremental cost effectiveness ratio (ICER) was the metric used to compare these groups; it is “the additional cost of a specific strategy divided by its health benefit compared with an alternate strategy”(33). In general, an ICER of \$50,000 per QALY or less has been arbitrarily accepted as a cost-effective strategy (54). They found that IHC triage of women with EC who had 1 FDR with an LAT was the most favorable screening strategy with an ICER of \$9,126 per year of life gained (33).

Hypothesis and Specific Aims

The absence of population-based evaluations of SGO 5-10% clinical criteria in the endometrial carcinoma literature has prompted us to investigate its performance in an unselected cohort of EC patients. These criteria parallel the Bethesda Guidelines, which were derived from data from colorectal cancer registries. Given the fact that these guidelines are extrapolated from CRC patient data, we hypothesize that SGO 5-10% criteria (which will be referred to as SGO Criteria from this point on) will fail to identify a majority of LS patients in the EC population.

Specific Aim 1. Evaluate the performance of SGO Criteria in detecting individuals at elevated risk for LS in endometrial cancer.

- A1) Calculate the sensitivity and specificity of SGO Criteria in identifying probable Lynch Syndrome (PLS) EC cases in a convenience sample in which molecular testing has already been performed.
- A2) Determine the number of individuals who meet MDACC institutional screening criteria who actually receive referrals for genetic counseling.
- B) Calculate the sensitivity and specificity of SGO Criteria in an *unselected, sequential cohort* of EC cases in identifying PLS cases.

Specific Aim 2. Determine if clinicopathologic features distinguish sporadic EC from PLS EC.

- A) Determine if historical risk factors of LS such as low BMI, young age at diagnosis and strong family history are significantly different between sporadic EC and PLS EC cases.
- B) Compare clinicopathologic characteristics between sporadic and PLS EC cases to determine if alternative clinical screening criteria exist.
- C) Determine the utility of *MLH1* methylation analysis in the evaluation of endometrial carcinomas with IHC loss of MLH1.

Specific Aim 3. Perform a simplified cost-effectiveness analysis comparing direct costs of utilizing a clinical history-based model, SGO Criteria, to a universal tumor testing model (immunohistochemistry and *MLH1* methylation analysis when indicated).

Methods

Institutional Approval

This study received Institutional Review Board Approval by the University of Texas, MD Anderson Cancer Center IRB (PA12-0131).

Patient Population

Convenience Cohort

350 EC patients were identified who had previously undergone IHC analysis for DNA MMR protein expression at MD Anderson Cancer Center, with complete clinicopathologic information available for 337. The majority in this cohort, 149 (44.2%), were from a protocol in which EC cases were selected based solely on matched frozen tumor tissue availability (55). Fifty-five EC cases came from a study evaluating the prevalence of LS among women diagnosed with EC prior to age 50, a risk factor known to increase an individual's risk for having LS (56). An additional 52 cases were derived from a study that evaluated women with synchronous endometrial and ovarian cancers, an indicator in Revised Bethesda guidelines for tissue testing for LS (57). Fifty-one cases were obtained from a study evaluating the concordance between MSI testing and IHC in LATs, with most of these patients recruited based on young age and family history of LATs (37). An additional 17 patients were obtained from a study investigating the association of LS and LUS tumors (44). The remaining 13 EC patients were known to have LS germline mutations.

Unselected, Sequential Cohort

Using a Department of Pathology database, we identified patients diagnosed with endometrial cancer between 2004-2011 who had undergone surgery at the University of Texas, MD Anderson Cancer Center. EC cases were included if the woman was 18 years of age or greater, surgery was performed at MD Anderson Cancer Center, and sufficient tissue was available for molecular analysis. All histotypes of endometrial carcinoma were included. Selection was not based on historical risk factors for LS such as young age at diagnosis or family history.

Genetic Counseling Referral Cohort

To assess the genetic counseling referral process, we retrospectively reviewed clinical and pathologic data for endometrial cancer cases at MD Anderson Cancer Center between 2007-2010. Patient clinical history was obtained from the electronic medical record. At the time of this analysis, institutional referral criteria were as follows: 1) any patient with a history of CRC; 2) any patient with an FDR with CRC or EC; 3) any patient with a relative of any degree with EC or CRC diagnosed before age 50; or 4) any patient with relatives who have a known LS germline mutation. Patients were classified as either “meets criteria for genetic counseling referral” or “genetic counseling referral not warranted.” Then, genetic counseling referrals were charted for each patient in the “meets criteria” category.

Data Collection

Clinical data were extracted from the electronic medical record (EMR). Data recorded included race/ethnicity, age at EC diagnosis, age of menarche and menopause, gravidity/parity, history of cancer, history of any hormone replacement therapy, BMI (kg/m^2), past medical history of diabetes/hypertension/thyroid disease, family history of cancer, and total number of first-degree relatives (FDRs).

Tumor characteristics were derived from pathology reports generated by 6 gynecologic pathologists. Recorded pathologic data included: tumor histology, tumor location (corpus/LUS), tumor grade, surgical stage, largest tumor dimension (cm), depth of invasion, and presence of lymph-vascular space invasion (LVSI).

Immunohistochemistry

Immunohistochemical analyses for the nuclear protein expression of MLH1, MSH2, MSH6, and PMS2 were performed on sections from formalin-fixed, paraffin-embedded endometrial carcinomas. IHC was performed using standard techniques for MLH1 (G168-15, 1:25; BD Biosciences Pharmingen), MSH2 (FE11, 1:100; Calbiochem), MSH6 (44, 1:300; BD Biosciences Pharmingen), and PMS2 (Alb-4, 1:125; BD Biosciences Pharmingen (37,57)). A tumor exhibiting nuclear loss of protein expression by light microscopic examination was designated as negative for that MMR protein. The presence of nuclear staining in surrounding stromal and normal tissues served as internal positive controls (Figure 2).

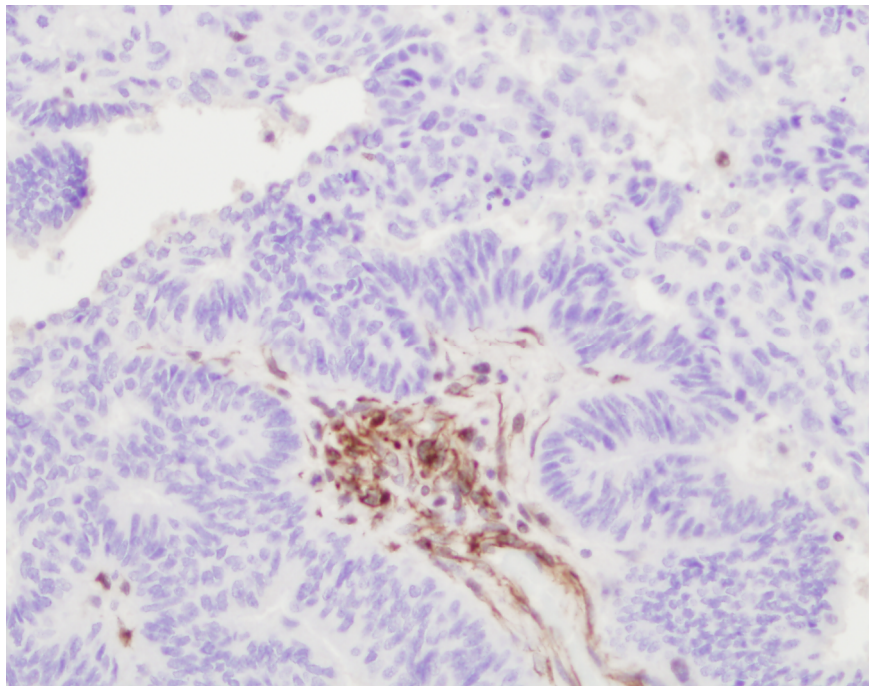
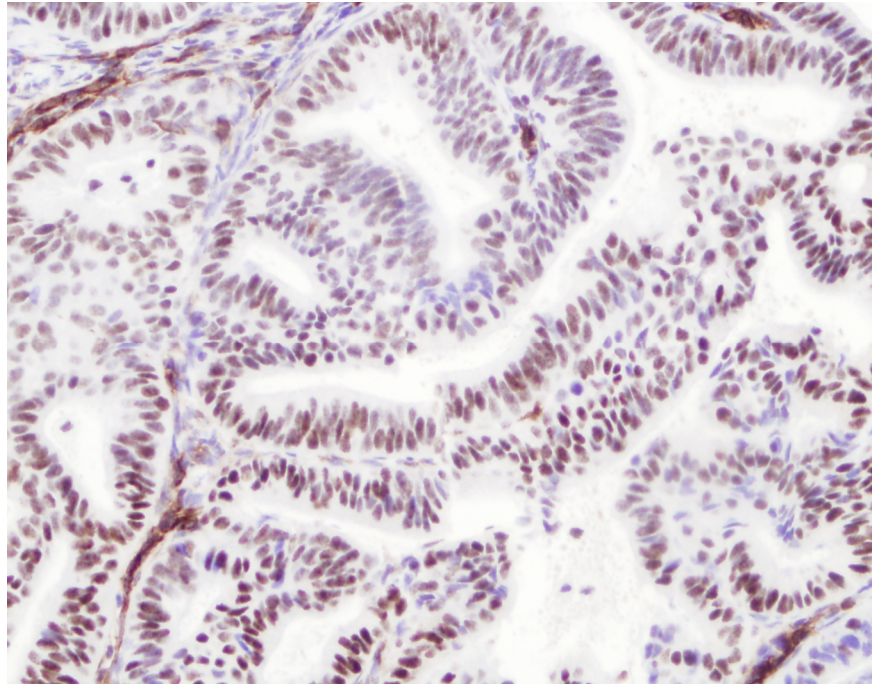


Figure 2A and 2B. Example of IHC retention (A) and loss (B) of MMR protein expression. Non-nuclear staining of both tumor cell nuclei and stromal cell nuclei in (A) and retention of stromal cell nuclear staining in (B) without presence of tumor staining.

MLH1 Methylation

For cases in which there was IHC loss of MLH1 protein expression, PCR-based *MLH1* promoter methylation analysis was performed. DNA was isolated from formalin-fixed, paraffin-embedded tissue sections that were microdissected with a scalpel blade to provide relatively pure tumor samples for analysis. Isolated DNA was treated with bisulfite to convert methylated cytosine to uracil. The treated DNA was then amplified using fluorescently labeled PCR primers that were specific for methylated (M) or the unmethylated (U) versions of *MLH1* (*MLH1*-M forward, 5_-gatagcgatttttaacgc-3_ and *MLH1*-M reverse, 5_-tctataaataactaaatctcttcg-3_; *MLH1*-U forward, 5_-agagtggatagtgatttttaagt-3_ and *MLH1*-U reverse, 5_-actctataaattactaaatctcttca-3_). Amplified PCR products were then detected using capillary electrophoresis and GeneScan software. Chromatograms for tumor were compared to those generated for the RKO colon carcinoma cell line (positive control known to have loss of MLH1 protein due to *MLH1* promoter methylation) and the leukemia cell line K562 (negative control with no *MLH1* methylation) (Figure 3) (57).

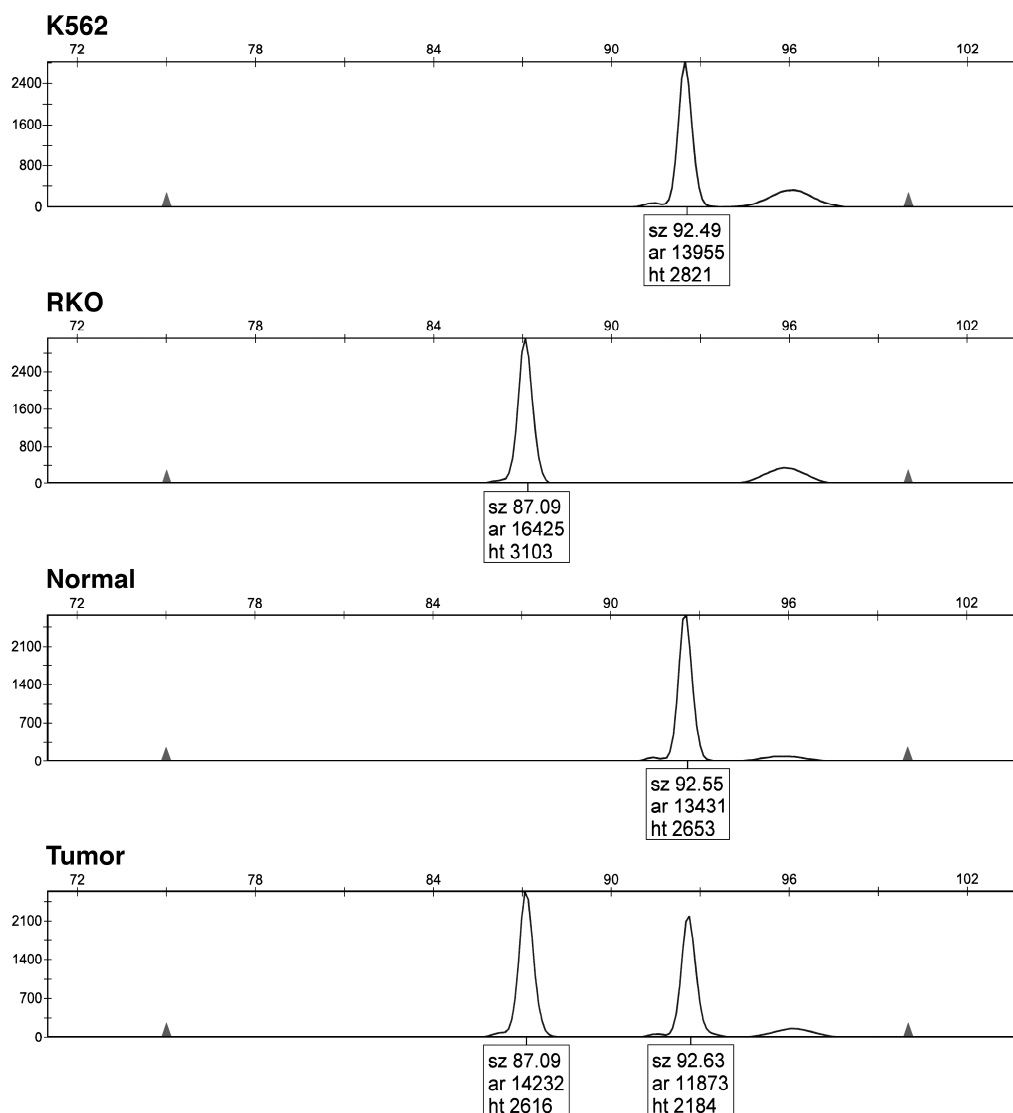


Figure 3. For cases with IHC loss of MLH1, PCR-based *MLH1* promoter methylation analysis was performed. Rows 1 and 2 are negative and positive controls, respectively. Row 3 and 4 show normal and tumor tissue from the same patient, respectively. The absence of a second peak in the normal tissue and presence of a second peak in the tumor indicates methylation of the *MLH1* promoter in the tumor.

Definition of Probable Lynch Syndrome (PLS) and Sporadic EC Tumors

Tumors with intact IHC nuclear staining for all 4 DNA MMR proteins and those with MLH1 loss and *MLH1* promoter methylation were designated sporadic tumors. Tumors with absent nuclear staining for MSH2, MSH6 or PMS2 were designated as PLS. Tumors exhibiting MLH1 loss with absence of *MLH1* promoter methylation were also designated as PLS.

Statistical Analysis

Statistical analyses were conducted using SAS software, version 9.2 (SAS, Inc. Cary, NC). Clinical and pathological criteria were compared across a variety of groups. Fisher's, chi-squared, Mann-Whitney, or t-test were conducted to test association across groups depending on the distribution of the data. CART analysis was performed to attempt to select a set of variables that would predict Lynch Syndrome, but none of the models were good fits. Sensitivity and specificity were also calculated for SGO Criteria in its ability to predict PLS EC tumors in both the convenience sample cohort and unselected, sequential cohort.

Cost-effectiveness Analysis

The cohort of 412 unselected, sequential EC cases was used to perform a simplified cost-effectiveness analysis. The direct costs of utilizing SGO Criteria were compared to universal tumor testing (IHC and *MLH1* methylation analysis when indicated) of all EC tumors. Effectiveness was expressed in two ways: 1.) the number of women with EC in which PLS was identified, and 2.) the total number of women with EC in which PLS was identified as well as identification of LS in their FDRs. Both technical and professional costs were collected for genetic counseling visits, IHC for the 4 DNA

MMR proteins, *MLH1* promoter methylation assay for tumors with IHC loss of MLH1, and single gene germline testing. The cost of single site genetic testing was used for identification of mutations in FDRs.

Cost analyses were performed using both institutional costs and Medicare reimbursement fees. MDACC institutional cost data were provided by the Department of Clinical Revenue and Reimbursement for specific procedure codes (CPT codes) derived from a review of billing records and CPT codes. Table 3 shows a list of items and costs included in these analyses. Medicare reimbursement figures were obtained from the Physician Fee Schedule and Laboratory Fee Schedule (<http://www.cms.gov/Medicare/Medicare-Fee-for-Service-Payment/PhysicianFeeSched/index.html>). All cost amounts are shown in 2012 US dollars.

In the SGO Criteria model, the original 412 EC cases were stratified according to whether or not they fulfilled SGO 5-10% criteria and only those tumors meeting criteria underwent further work-up with IHC for DNA MMR proteins, *MLH1* methylation when indicated, and genetic counseling. In the universal tumor testing model, all 412 EC cases underwent IHC following hysterectomy, and PLS patients underwent genetic counseling and germline testing.

Table 3. Unit costs included in screening strategies (expressed in 2012 US dollars)¹

	MDACC institutional costs	Medicare reimbursement amounts
Initial genetic counseling consultation (1 hr) ^a	\$264	\$210
Follow-up genetic counseling visits (30 min) ^b	\$132	\$104
IHC for MLH1, MLH2, MSH6, and PMS2 ^c	\$349	\$422
MLH1 promoter methylation assay for tumor with IHC loss of MHL1 ^d	\$316	\$125
Single gene germ-line testing ²	\$1300	\$1300
Single site testing ^{3,4}	\$475	\$475

¹ Includes technical and professional components

² Cost of germline testing was obtained from Myriad ABN worksheet

³ Cost of single site testing was obtained from Myriad ABN worksheet

⁴ Single site testing intended for first degree relatives of pts with endometrial cancer who were identified as having Lynch Syndrome based upon positive germline test results

^a MDACC costs derived from CPT code 96040 in 2010 billing statements and converted to 2012 US dollars. CPT 99215 and CPT 99213 were used to determine Medicare reimbursement amounts from the 2012 Fee Schedule.

^b Costs adapted from CPT code 99214.

^c CPT code 88342 used for each individual IHC DNA MMR protein

^d CPT codes 83900, 83909, 83912 from 2012.

The following assumptions were made for the cost effectiveness analysis: 1) women with PLS would all be willing to undergo genetic counseling and recommended germline testing; 2) women identified as PLS by SGO Criteria had an average of 5.3 first degree relatives and women identified as PLS by universal tumor testing had an average of 5.5 first degree relatives (these numbers were extracted from the electronic medical record); 3) all FDRs recommended to undergo screening and testing would be compliant.

Finally, to evaluate the impact on the incremental cost per additional case of Lynch Syndrome identified, we varied our assumptions regarding the proportion of FDRs who would have positive germline tests (e.g. single-site test based upon initial germ line mutation found in women with PLS) from 25% to 75%. These estimates of 25%, 50%, and 75% are based on the variable rate at which immunohistochemistry is found to predict germline mutations (48,58). The incremental cost-effectiveness ratio (ICER) is an estimate of the cost per unit of effectiveness of different screening strategies. We varied the percentages of FDRs we assumed would test positive, between 25%-75%, to evaluate how cost-effectiveness ratios would differ based on these assumptions within a given strategy. The ICER is calculated as the difference in costs between alternative screening strategies divided by the difference in effectiveness between screening strategies (Figure 4).

$$\text{Incremental Cost Effectiveness Ratio (ICER)}_{\text{SGO}} = \frac{\text{SGO Criteria Costs} - \text{Baseline Costs}}{\text{SGO Effectiveness} - \text{Baseline Effectiveness}}$$

$$\text{Incremental Cost Effectiveness Ratio (ICER)}_{\text{UTT}} = \frac{\text{UTT Costs} - \text{SGO Criteria Costs}}{\text{UTT Effectiveness} - \text{SGO Effectiveness}}$$

Figure 4. Equations used to derive the ICER for SGO (comparing SGO to no intervention) and ICER for Universal tumor testing (UTT) (comparing UTT to SGO criteria costs). Effectiveness is the number of PLS cases identified.

Results

Aim 1A1. Performance of SGO Criteria in a Convenience Sample of EC Cases

Three-hundred-and-fifty cases of endometrial cancer were identified that had previously undergone immunohistochemical analysis for DNA MMR protein expression at MD Anderson Cancer Center, and complete clinicopathologic information was available for 337 (Figure 5). There was an average of 5.3 FDR per PLS case identified.

In this cohort, 56/337 (16.6%) were PLS EC tumors. IHC loss of MLH1/PMS2 with unmethylated *MLH1* occurred in 14/56 (25%), loss of MSH2/MSH6 in 24/56 (42.8%), loss of MSH6 only in 11/56 (19.6%) and loss of PMS2 only in 7/56 (12.5%). The median BMI of all PLS cases was 32.0, and median age at diagnosis was 49.

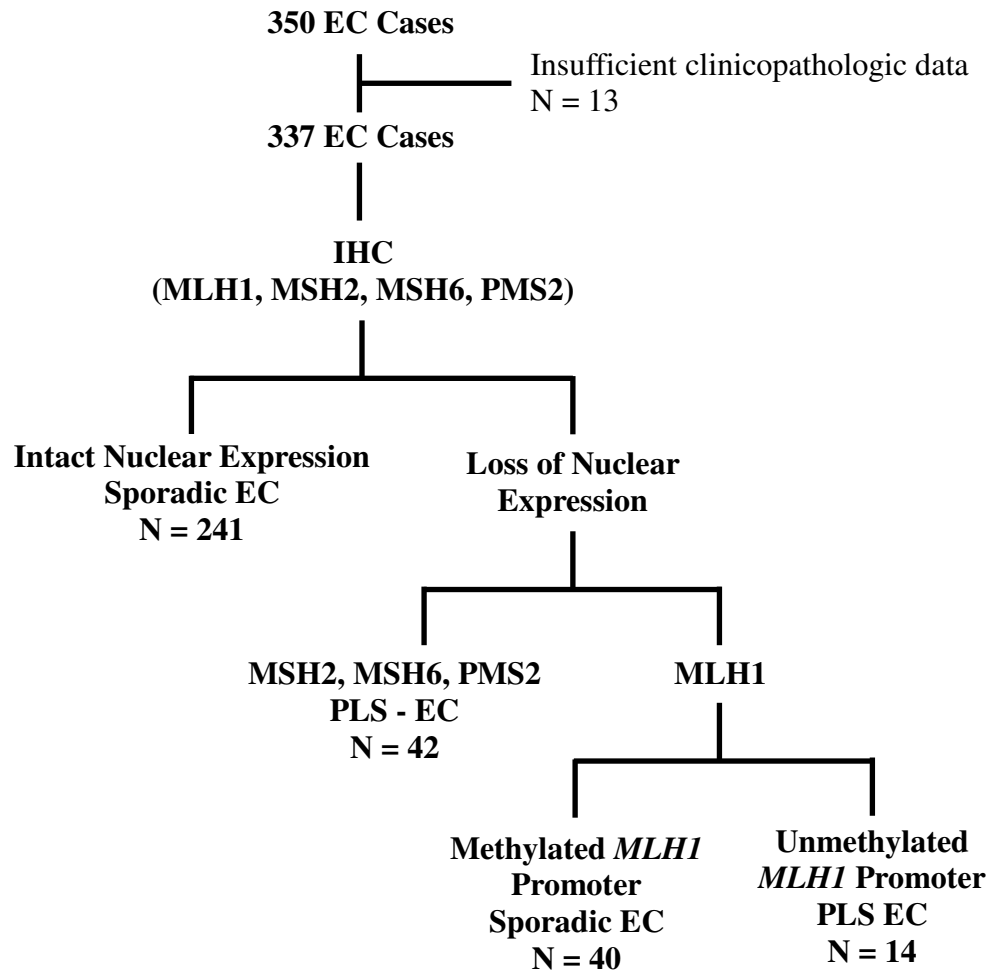


Figure 5. Immunohistochemical and *MLH1* methylation results for a convenience sample of 350 Endometrial Cancer Cases.

EC: endometrial cancer
 IHC: immunohistochemistry
 PLS: probable Lynch Syndrome

Sensitivity and specificity of SGO Criteria were 76.8% and 44.4%, respectively. For EC cases meeting SGO Criteria, mean age at diagnosis was 48 years, mean BMI was 33.6, 17.7% had a family history of EC and 46.9% had a family history of CRC. Tumor location in the lower uterine segment (LUS) was in 25.6% of these cases. In the 43/56 PLS cases meeting SGO Criteria, IHC loss of MLH1/PMS2 occurred in 11, MSH2/MSH6 in 21, MSH6 only in 7, and PMS2 only in 4.

Of the 13 (23.2%) patients not captured by clinical criteria, mean age was 68 years, mean BMI 34.4, no cases had a family history of CRC and one patient had a first-degree relative with a gynecologic cancer of unknown origin. EC cases missed when employing SGO screening criteria were associated with IHC loss of MLH1/PMS2 in 3, MSH2/MSH6 in 3, MSH6 only in 4, and PMS2 only in 3 instances. Tumor arising from the LUS occurred in 33.3% of these cases. If LUS tumors were made a component of SGO Criteria, sensitivity and specificity in this cohort become 85.7% and 40.2%, respectively.

AIM 1A2. Number of individuals meeting MDACC Criteria Referred for Genetic Counseling

Between 2007-2010, 1064 endometrial cancer patients new to MDACC were seen in the gynecology oncology clinic. Of these, 156 (14.7%) met institutional guidelines for referral to a genetic counselor to assess risk for LS. Forty-nine of these patients (30.2%) received a recommendation to see a genetic counselor (Figure 5). Of the 107 EC patients meeting criteria but not referred to genetic counseling, a large number had compelling personal or family histories of cancer. One EC patient had a prior history of CRC, 57 had a FDR with CRC (5 diagnosed at less than age 50), and 29 had a FDR with EC (3 diagnosed at less than age 50). Six EC patients had a FDR history significant for both EC and CRC. Additionally, only 12/30 EC patients who had a second degree relative with an LAT diagnosed before age 50 received a referral.

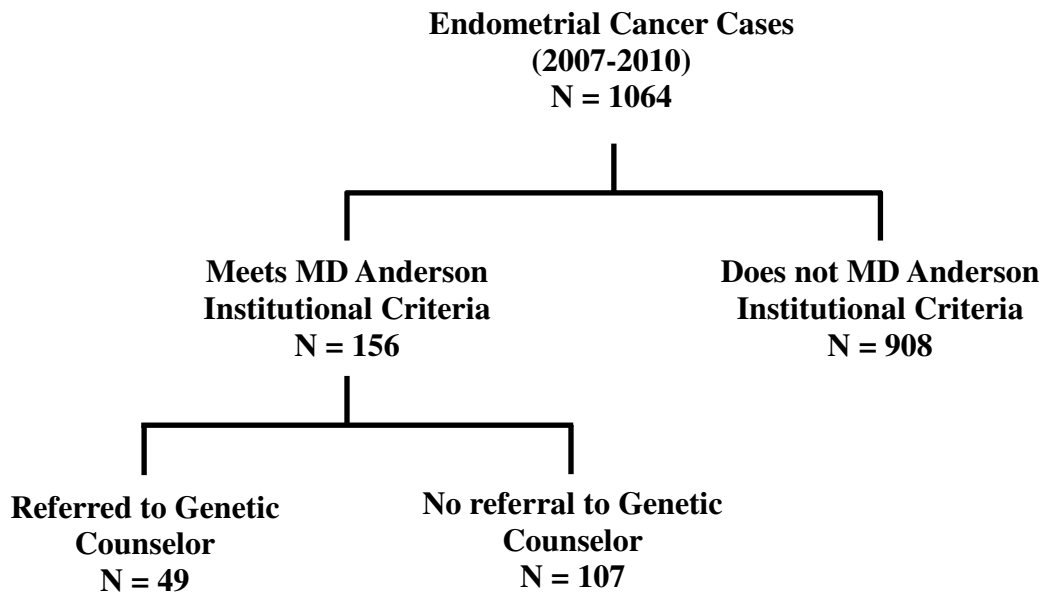


Figure 6. Referral experience of endometrial cancer patients who meet MDACC Gynecologic Oncology Center Genetic Counseling Criteria for Lynch Syndrome evaluation from 2007-2010.

Aim 1B. Performance of SGO Criteria in an unselected cohort of EC cases

From the results of the convenience sample study in Aim 1A1, it was noted that a substantial subset of PLS patients were older than age 50 and/or did not have compelling family histories of CRC or EC. Therefore, we hypothesized that LS screening in a population-based fashion would identify patients who would otherwise not be identified when screening is based on historical risk factors. Four hundred twelve consecutive, unselected EC cases met inclusion criteria and underwent molecular testing (Figure 7). There was one case in which immunohistochemistry and three cases in which *MLH1* methylation were unsuccessful. Of the 411 cases with complete IHC results, 118 had loss of at least one MMR protein (90 MLH1/PMS2, 12 MSH2/MSH6, 9 MSH6 and 7 PMS2). Of those with loss of MLH1/PMS2, 72/90 (80%) had methylation of the *MLH1* promoter (Figure 7). The total number of PLS EC cases in our series was 43 (10.5%). There was an average of 5.5 FDRs per PLS EC case identified in this cohort.

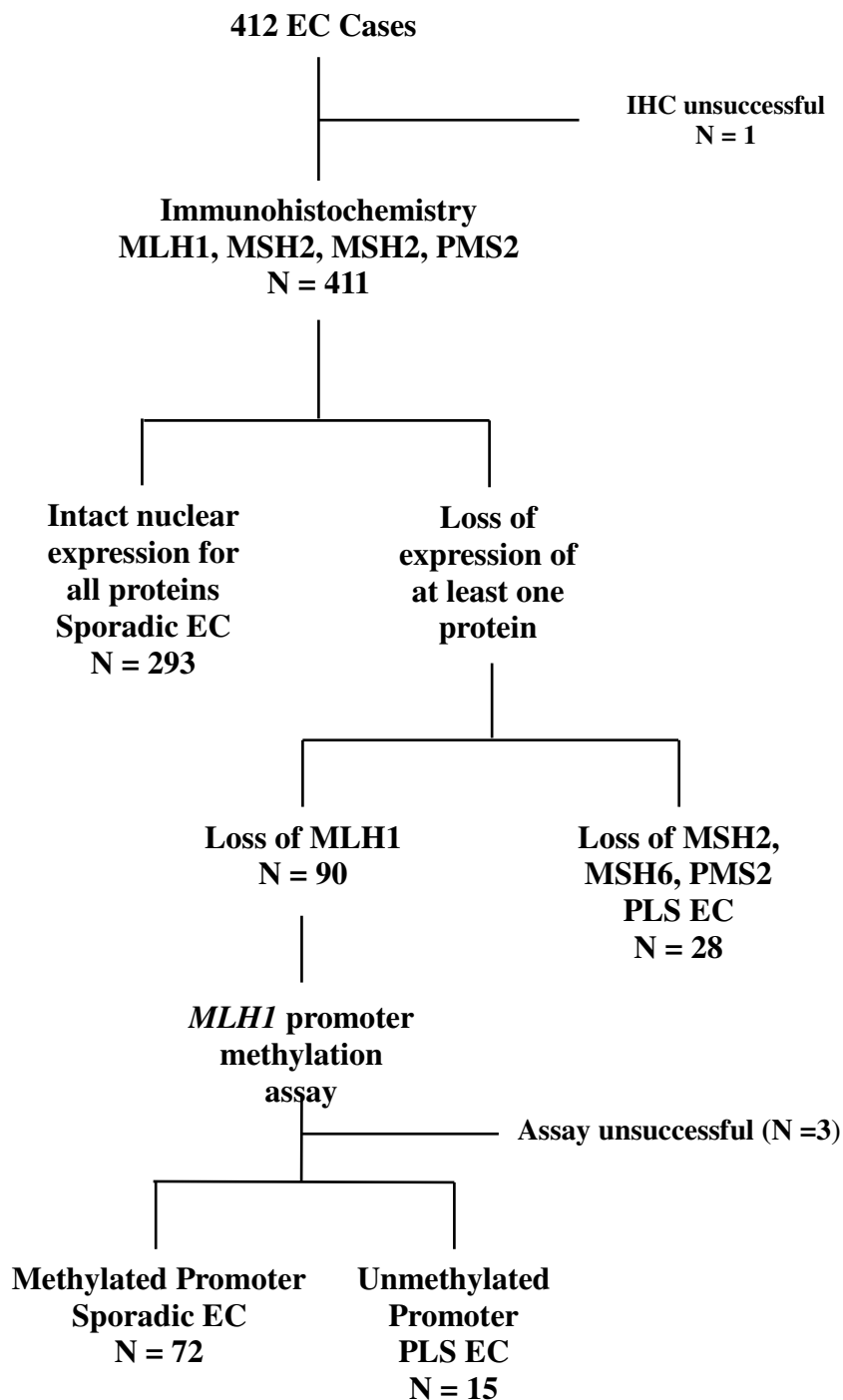


Figure 7. IHC and *MLH1* methylation results of the unselected, sequential cohort of EC cases.

Demographic and pathologic information for this cohort is summarized in Table 4. The median age at diagnosis was 60.5 with a range of 18-92. Most patients were of white ethnicity, older than age 50 years, obese ($\text{BMI} \geq 30 \text{ kg/m}^2$), and had unremarkable family histories for EC or CRC. The median tumor size was 4.3 cm. Most tumors were endometrioid histology, low grade, early stage, and located in the corpus.

Table 4. Demographic and pathologic data for the cohort of unselected, sequential EC cases.

Characteristic	Number (%)
Race	
White	270 (66.2)
Non-White	138 (33.8)
Age (years)	
< 50	62 (15.2)
≥ 50	346 (84.8)
History of Any Cancer	
Yes	53 (13.0)
No	355 (87.0)
Synchronous Tumor (any type)	
Yes	27 (6.6)
No	381 (93.4)
BMI (kg/m ²)	
< 30	138 (33.9)
≥ 30	269 (66.1)
History of Diabetes	
Yes	98 (24.0)
No	310 (76.0)
Family History of EC	
Yes	39 (9.8)
No	360 (90.2)
Family History of CRC	
Yes	66 (16.5)
No	335 (83.5)
Histology	
Endometrioid	336 (82.4)
Non-endometrioid	72 (17.6)
Grade	
1&2	299 (73.3)
3	109 (26.7)
Stage	
I & II	326 (79.9)
III & IV	82 (20.1)
Tumor Location	
Corpus	395 (96.8)
Lower Uterine Segment	13 (3.2)

The sensitivity and specificity of SGO Criteria and its associated 95% confidence intervals are 34.1% (20.5, 49.9) and 77.6 % (72.9,81.8), respectively. The sensitivity of SGO Criteria by type of IHC loss is presented in Table 5. SGO Criteria perform best in patients with tumors exhibiting IHC loss of MLH1/PMS2 or MSH2/MSH6 and perform poorly in tumors with IHC loss of only MSH6 or PMS2.

Table 5. Sensitivity and specificity of SGO Criteria in identifying PLS EC cases.

	Sensitivity (95% CI)	Specificity (95% CI)
Probable Lynch Syndrome (N = 43)	32.6 (19.1 – 48.5)	77.2 (72.7 – 81.6)
MLH1/PMS2 (N = 15)	40.0 (16.3 – 67.7)	---
MSH2/MSH6 (N = 12)	42.7 (15.2 – 72.3)	---
MSH6 (N = 9)	22.2 (3.0 – 60.0)	---
PMS2 (N = 7)	14.3 (0.3 – 57.9)	---

Ninety-seven of 408 (23.8%) of all EC cases fulfilled SGO Criteria. Of these, 14/97 (14.4%) were PLS EC tumors based on tissue testing and 83/97 (85.6%) that would be characterized as sporadic based on tissue testing. This also results in 29/43 (67.4%) PLS tumors that did not meet criteria that would go undetected in a clinical screening criteria based referral system.

For the 43 patients identified by tissue testing as PLS, the patterns of IHC loss between those that do and do not meet SGO Criteria were examined (Figure 7). Tumors with IHC loss of MLH1 and MSH2 were the predominant types of PLS tumors among those that fulfill SGO Criteria (11/14). Among those failing to meet criteria (n=29), there is a fairly equal distribution of types of IHC protein loss. IHC loss of MLH1/PMS2 occurred in 9, MSH2/MSH6 in 7, MSH6 only in 7, and PMS2 only in 6.

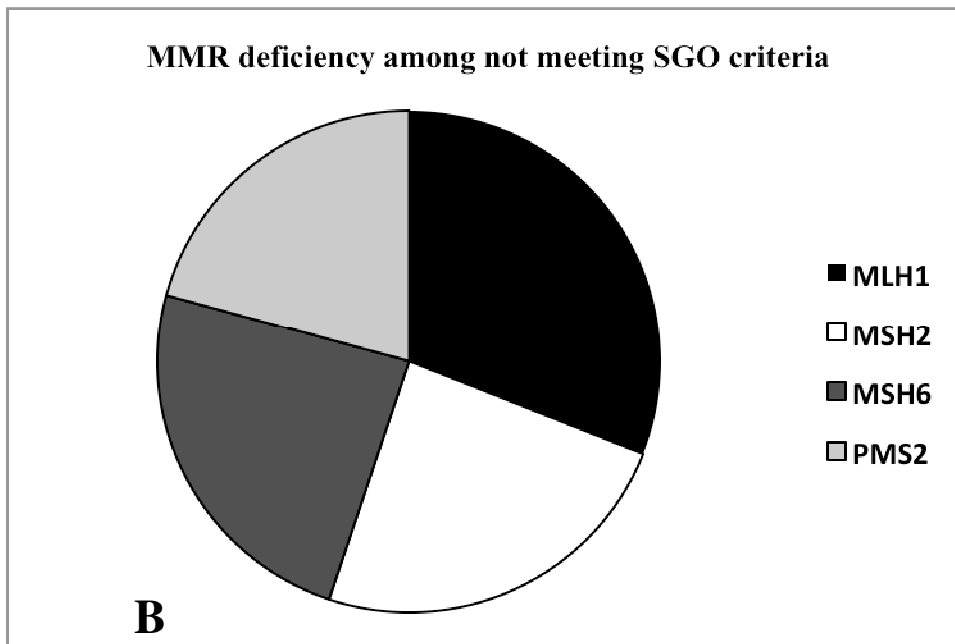
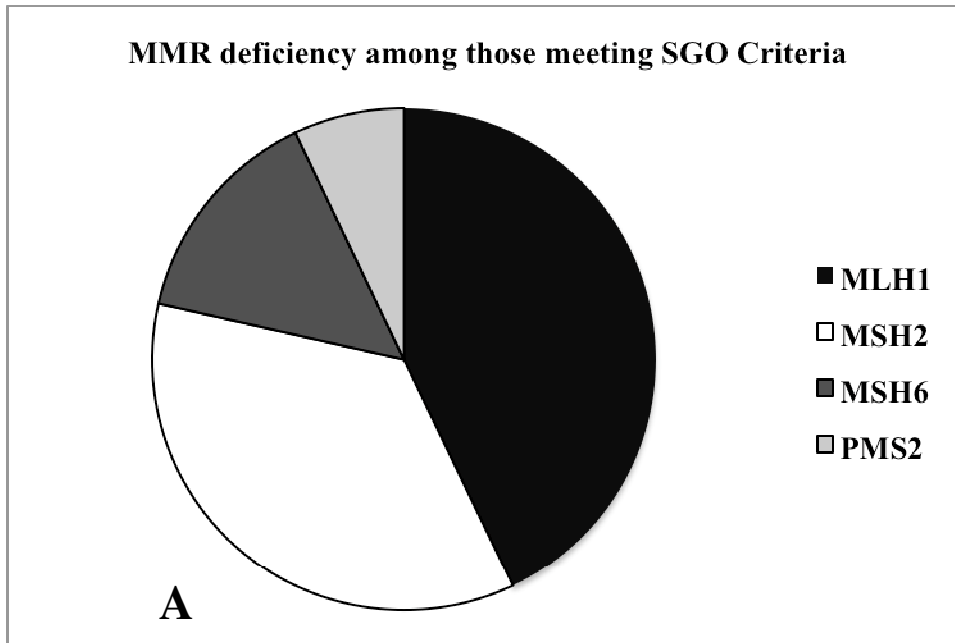


Figure 8. Type of IHC protein loss for EC cases do (A) and do not (B) meet SGO Criteria.

Select clinical and pathologic characteristics between those that do and do not meet clinical criteria were further investigated (Table 6). Those patients meeting SGO Criteria had a younger age at diagnosis, stronger family history of EC and CRC, and a higher frequency of tumors arising from the lower segment than those who do not meet criteria. Regardless of whether or not criteria are met, the majority of PLS EC cases do not have a family history of EC or CRC.

Table 6. Features of EC patients who do and do *not* meet SGO Criteria.

Total PLS N = 43	Meets SGO Criteria N = 14	Does <i>Not</i> Meet SGO Criteria N = 29
Median age at diagnosis	48.5	63.0
Average BMI (kg/m ²)	32.0	33.0
Family History of EC ¹	2/14 (14.3%)	2/29 (6.9%)
Family History of CRC ²	4/14 (28.6%)	4/29 (13.8%)
LUS Tumor ³	3/14 (21.4%)	2/29 (6.9%)

¹EC, endometrial cancer

²CRC, colorectal cancer

³LUS, tumor arising from lower uterine segment

Specific Aim 2A. Evaluation of Historical LS risk factors

Young age at cancer diagnosis is one of two fundamental characteristics present in published clinical screening criteria for LS. Our data show that 62/408 (15.2%) of all EC occurred in patients younger than 50 years of age. Of these 62 cases, 10/62 (16.1%) were PLS tumors, and 10/43 (23.2%) of all PLS tumors occurred in this age category. Thus, 75% of PLS cases in this cohort occur at older ages. Figure 9 shows the distribution of EC cases by age. The median age at diagnosis is 61 years for both sporadic EC and PLS EC groups. The age range for patients with sporadic tumors is wider (18-92) than for PLS (42-87). PLS cases did not occur at less than age 40, whereas sporadic EC occurred as young as 18 years. After age 40, the proportion of individuals with PLS or sporadic EC at any age is similar.

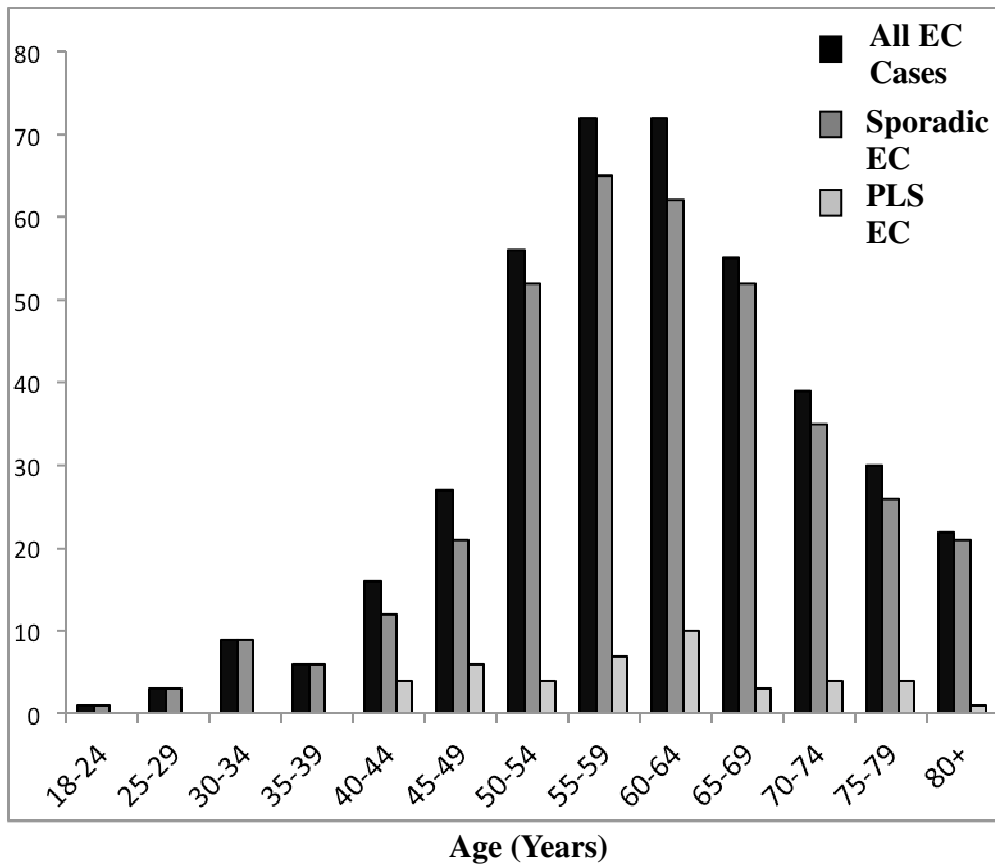


Figure 9. Distribution by age of EC cases for all EC tumors, sporadic tumors, and PLS tumors.

The other fundamental characteristic of published screening criteria is a strong family history of EC or CRC. Less than 30% of EC patients in this cohort had a family history of either EC or CRC. Table 7 shows the family histories of EC, CRC and either EC or CRC for the sporadic and PLS EC groups. Family history of these cancers does not distinguish sporadic from PLS.

Table 7. Family history of EC, CRC, and EC or CRC in sporadic and PLS EC cases in the unselected, sequential cohort.

Family History	Sporadic EC N (%)	PLS EC N (%)	p-value
EC	35 (9.8)	4 (9.8)	> 0.99
CRC	58 (16.2)	8 (19.0)	0.63
EC or CRC	87 (24.2)	12 (28.6)	0.54

Specific Aim 2B. Comparison of clinicopathologic features in PLS and Sporadic EC to determine if alternative screening criteria exist

Clinicopathologic characteristics were compared between the sporadic and PLS patients in our sequential, unselected cohort (Table 8). With the exception of tumors arising from the lower uterine segment (LUS), there was no statistically significant difference identified between these two groups. Although not statistically significant, there was a trend toward younger age at diagnosis (23.3%), lower BMI (39.5%), personal history of hypertension (58.1%) and smaller tumor (3.6 cm) among the PLS EC cases.

From the complete list of all clinicopathologic data collected, a Classification and Regression Tree (CART) analysis was performed to determine if more ideal screening criteria could be generated that could effectively delineate sporadic from PLS EC tumors. This analysis yielded no superior criteria.

Table 8. Comparison of clinical and pathologic features between sporadic and PLS EC cases.

Clinical Features	Sporadic EC N (%)	PLS EC N (%)	p -value
Age (years)			
< 50	52 (14.2)	10 (23.3)	0.12
≥ 50	313 (85.8)	33 (76.7)	
BMI (kg/m ²)			
< 30	121 (33.2)	17 (39.5)	0.41
≥ 30	243 (66.8)	26 (60.5)	
History of Diabetes			
Yes	89 (24.4)	9 (20.9)	0.62
No	276 (75.6)	34 (79.1)	
History of Hypertension			
Yes	201 (55.1)	18 (41.9)	0.10
No	164 (44.9)	25 (58.1)	
Pathologic Features	Sporadic EC N (%)	PLS EC N (%)	p -value
Histology			
Endometrioid	299 (81.9)	37 (86.0)	0.67
Non-endometrioid	66 (18.1)	6 (14.0)	
Stage			
I & II	289 (79.2)	37 (86.0)	0.42
III & IV	76 (20.8)	6 (14.0)	
Grade			
1 & 2	267 (73.2)	32 (74.4)	0.86
3	98 (26.8)	11 (25.6)	
Depth of myometrial invasion			
< 50%	257 (70.4)	32 (74.4)	0.85
≥ 50%	108 (29.6)	11 (25.6)	
Tumor Location			
Corpus	357 (97.8)	38 (88.4)	0.007
Lower uterine segment	8 (2.2)	5 (11.6)	
Largest tumor dimension (cm)			
Mean	4.3	3.6	0.13
Median	4	3.5	

Next, a receiver-operator curve (ROC) was generated in which age was varied within the SGO Criteria by 1-year intervals (Figure 10). Ideally, the area under the curve is 0.8 or greater. Varying age does not improve the sensitivity and specificity profiles of SGO Criteria. Increases in sensitivity come at the cost of decreases in specificity.

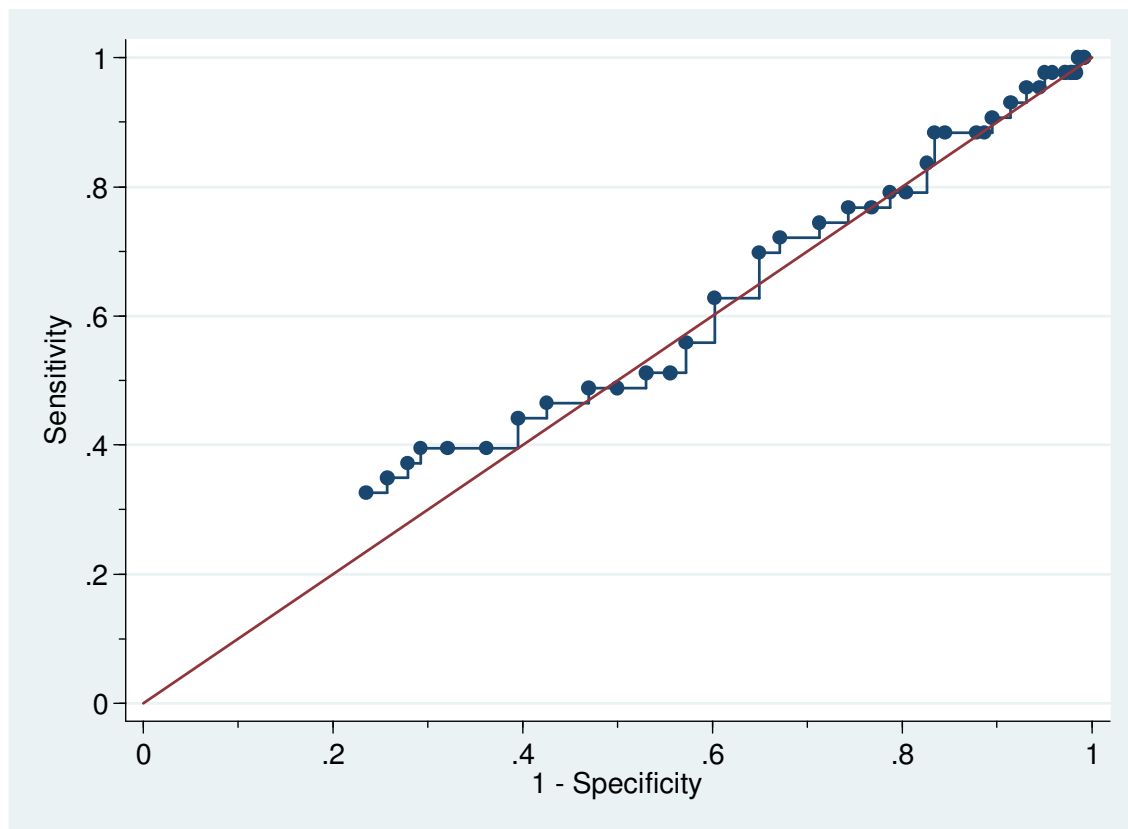


Figure 10. Receiver-operator curve (ROC) depicting SGO Criteria with age criteria being varied in 1-year intervals.

Finally, clinicopathologic variables for the different types of PLS were compared (Table 9). There is a trend toward younger median age at diagnosis between those exhibiting MSH2/MSH6 loss and PMS2 loss only; however, the age range is fairly similar across all types of MMR deficiency types. There were more individuals with a BMI (kg/m^2) < 30 and no family history of EC in these two groups as well. However, overall, there were no clinicopathologic variables that distinguished any of these groups.

Table 9. Comparison of clinicopathologic characteristics by MMR deficiency type.

	MLH1/PMS2 No methylation N = 15	MSH2/MSH6 N = 12	MSH6 N = 9	PMS2 N = 7	p-value
Median age at diagnosis	62	56	62	56	0.50
(Range)	(43-79)	(42-71)	(50-76)	(45-87)	---
BMI < 30	8 (53%)	10 (83.3%)	8 (88.9%)	2 (28.6%)	0.30
FH EC¹	3 (21.4%)	0	1 (12.5%)	0	0.31
FH CRC²	3 (20%)	3 (25%)	1 (12.5%)	1 (14.3%)	0.95
LUS³ Tumor	1 (6.7%)	1 (8.3%)	0	3 (42.9%)	0.05
Endometrioid Histology	13 (86.7%)	9 (75%)	8 (88.9%)	7 (100%)	0.62
Stage I & II	13 (86.7%)	8 (66.7%)	9 (100%)	7 (100%)	0.14
Meets SGO Criteria	6 (40.0%)	5 (41.7%)	2 (22.2%)	1 (14.3%)	0.62

¹EC, endometrial cancer²CRC, colorectal cancer³LUS, tumor arising from lower uterine segment

Specific Aim 2C. Utility of MLH1 Methylation Analysis among EC with MLH1 IHC loss

Most EC with MLH1 IHC loss are sporadic secondary to *MLH1* methylation rather than familial due to *MLH1* mutation. We investigated whether any clinical or pathological features can reliably distinguish the MLH1 IHC negative (sporadic) and MLH1 IHC negative (PLS) endometrial cancer patients. Utilizing the convenience sample of 337 EC cases, IHC loss of MLH1 nuclear expression was detected in 54/337 (16%) of endometrial tumors. Of these, 40/54 (74.1%) endometrial tumors demonstrated *MLH1* promoter methylation and were designated as sporadic endometrial carcinomas. The remaining 14/54 (25.9%) lacked MLH1 methylation and were designated as PLS. The proportion of *MLH1* methylated tumors is comparable to that seen in several other studies consisting of unselected endometrial cancer patients with a range of 65-96.9% (Figure 11) (29-32).

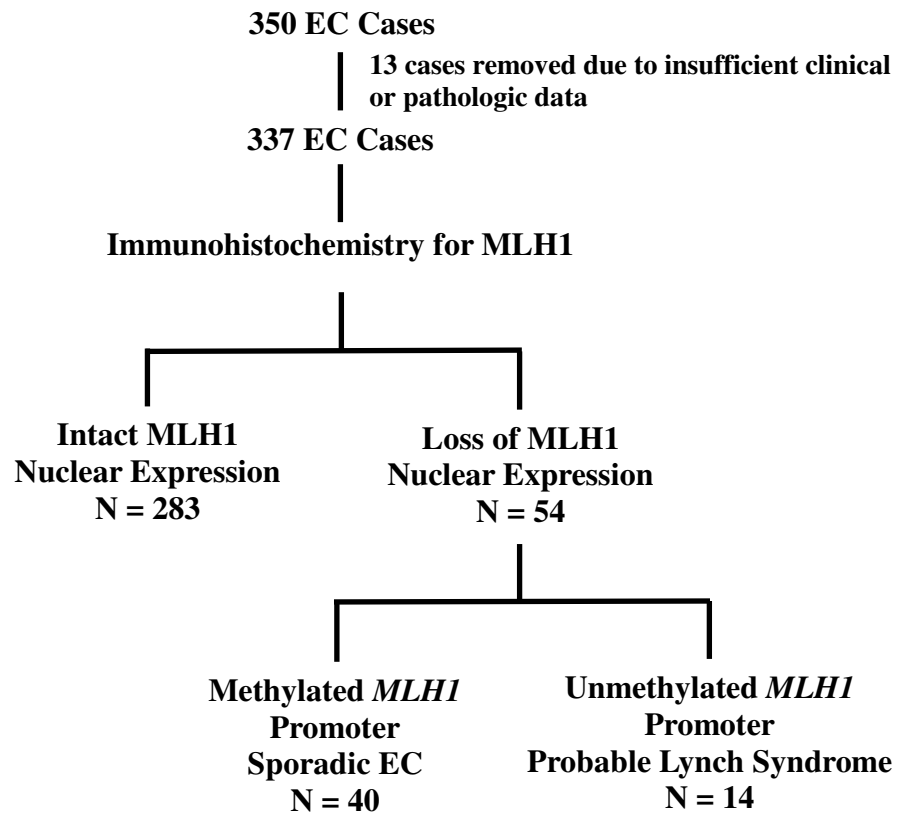


Figure 11. Results of IHC for MLH1 and *MLH1* methylation in convenience sample cohort of EC cases.

Clinical and pathologic characteristics for the endometrial cancer patients with and without *MLH1* promoter methylation are shown in Table 10. There was no statistical difference between median age of diagnosis, median body mass index (BMI), or family history of CRC or EC between the *MLH1* methylated and unmethylated promoter groups. A personal history of diabetes was statistically more common in the unmethylated group.

Investigation of tumor-specific characteristics revealed no statistically significant differences between the two groups with respect to histology, FIGO stage, endometrioid grade, lymphatic/vascular space invasion, tumor location, or tumor size (Table 10). Depth of myometrial invasion was the only pathologic characteristic that was statistically different between the two groups. Deep myometrial invasion was seen in 37.5% of *MLH1* methylated tumors, whereas 71.4% of the *MLH1* unmethylated tumors had myometrial invasion greater than or equal to 50% myometrial thickness. In this cohort, 25/54 of the patients had endometrial carcinomas with depth of myometrial invasion greater than or equal to 50% total myometrial thickness making this a criterion of low specificity.

Table 10. Patient and tumor characteristics for EC in the convenience sample cohort with IHC loss of MLH1, stratified by presence or absence of *MLH1* promoter methylation.

	Methylated <i>MLH1</i> n (%)	Unmethylated <i>MLH1</i> n (%)	P - value
Patient Characteristics			
Age			
Median age at diagnosis	57	52	0.4295
Age range	31-92	42-79	
Median Body Mass Index	32.9	30.9	> 0.999
<30	13 (33.3)	5 (35.7)	
≥ 30	26 (66.7)	9(64.3)	
Family History of EC¹	4 (10.5)	3 (21.4)	0.370
Family History of CRC²	7 (18.4)	3 (21.4)	0.999
Diabetes	4 (10)	6 (42.9)	0.013
Hypertension	23 (57.5)	6 (42.9)	0.371
Tumor Characteristics			
Histology			
Endometrioid	35 (87.5)	11(78.6)	0.413
Non-Endometrioid	5 (12.5)	3(21.4)	
FIGO Stage³			
I & II	27 (67.5)	11 (78.6)	0.515
III & IV	13 (32.5)	3 (21.4)	
Endometrioid Tumor Grade			
1 or 2	26 (74.3)	9 (81.8)	> 0.999
3	9 (25.7)	2 (18.1)	
Median depth of myometrial invasion (mm)⁴	9.0	9.5	0.487
< 50% myometrial invasion	25 (62.5)	4 (28.5)	0.035
≥ 50% myometrial invasion	15 (37.5)	10 (71.4)	
Lymphatic/vascular space invasion	24 (60.0)	8 (57.1)	> 0.999
Tumor location			
Corpus	37 (92.5)	11 (78.6)	0.173
Lower uterine segment	3 (7.5)	3 (21.4)	
Tumor Size			
< 4 cm	21 (52.5)	8 (57.1)	> 0.999
≥ 4 cm	19 (47.5)	6 (42.9)	

¹EC, endometrial cancer

²CRC, colorectal cancer

³FIGO stage I and II denote endometrial carcinomas confined to the uterus. FIGO stages III and IV represent extra-uterine spread of tumor.

⁴Depth of myometrial invasion ≥ 50% total myometrial thickness is associated with increased risk of lymph node metastasis.

The sensitivity and specificity of various clinical screening criteria and selected patient characteristics are presented in Table 11. Young age of EC diagnosis was included here, as this is a common feature included in many different clinical screening criteria for LS. BMI less than 30 was also included, because it has been previously reported that endometrial cancer patients with LS have a lower BMI than patients with sporadic endometrial cancer (56). Single factors such as young age, BMI less than 30, family history of colorectal cancer, and family history of EC showed poor overall sensitivity and specificity in ability to predict *MLH1* methylation status accurately. The SGO criteria had a moderate sensitivity (71.4%) and specificity (69.2%). Amsterdam II criteria had a high specificity, 94.9%, at the expense of sensitivity, only 14.3%. When the statistically significant factors from Table 10, deep myometrial invasion and patient history of diabetes, were added to SGO 5-10% criteria, sensitivity increased to 100%, but specificity was low at 35.9%. Overall, SGO 5-10% criteria had the best sensitivity and specificity profile of the screening criteria evaluated in this cohort of EC cases.

Table 11. Sensitivity and specificity of selected clinical characteristics and screening criteria in predicting presence or absence of *MLH1* methylation in endometrial carcinomas with MLH1 loss by immunohistochemistry

	Sensitivity	Specificity
Age < 50	50.0	77.5
Body mass index < 30	33.3	35.7
History of diabetes	42.8	90.0
Myometrial invasion > 50%	71.4	62.5
Family history colorectal cancer	21.4	81.6
Family history endometrial cancer	21.4	89.5
Amsterdam II Criteria	14.3	94.9
SGO Criteria	71.4	69.2
SGO Criteria or $\geq 50\%$ myometrial invasion or diabetes	100	35.9

Since SGO Criteria performed the best in this cohort, we sought to further examine clinicopathologic features of the 4 EC cases not captured by the criteria. Table 12 presents patient clinical and pathologic characteristics of the 4 EC cases lacking *MLH1* promoter methylation (PLS) that were not captured by SGO criteria (in other words, 4 patients designated as sporadic endometrial cancer rather than PLS endometrial cancer). In each case, patients are older than age 50 years, have a body mass index greater than 30, and there is no family history of CRC. One patient has an LUS tumor, and all but one of the patients had deep myometrial invasion.

Case	Age at EC Diagnosis ¹	BMI	DM ²	Family History of EC	Family History of CRC ³	Meets Amsterdam II Criteria	Tumor Location (Corpus or LUS ⁴)	FIGO Stage	Tumor Grade	Tumor Size (cm)	Histology ⁵	Depth of Uterine Wall Invasion
1	61	51.8	No	Cousin age > 50	No	No	Corpus	II	1	13	E	> 50%
2	64	30.9	No	No	No	No	LUS	II	1	3	C	> 50%
3	71	38	Yes	Mother unknown gyn cancer age > 50	No	No	Corpus	IA	2	5.5	M	< 50%
4	79	31	Yes	No	No	No	Corpus	IIIC2	2	1.9	E	> 50%

Table 12. Characteristics of EC cases with immunohistochemical loss of MLH1 and absence of *MLH1* methylation (PLS) that were incorrectly designated as sporadic by SGO criteria

¹EC, endometrial cancer

²DM, diabetes mellitus

³CRC, colorectal cancer

⁴LUS, tumor arising from lower uterine segment

⁵E, endometrioid carcinoma; C, clear cell carcinoma; M, mixed endometrioid and sarcomatoid carcinoma

Given these findings in the convenience sample cohort, these characteristics were also evaluated in the population-based cohort (Table 13). As in the convenience sample cohort, there was no statistically significant difference between the clinical characteristics of median age of diagnosis and family history of EC or CRC. The significant association of diabetes between unmethylated and methylated *MLH1* found in the convenience cohort was not present in the population-based cohort. This suggests that the statistically significant difference found in the convenience sample may be due to Type I error. Investigation of tumor-specific characteristics revealed no statistically significant differences between the two groups with respect to histology, FIGO stage, endometrioid grade, lymphatic/vascular space invasion, tumor location, median tumor size or depth of myometrial invasion. The statistically significant difference of deep myometrial invasion found among unmethylated tumors in the convenience sample cohort may be a result of Type I error or the inherent biases of the endometrial cancer cases used to generate the convenience sample.

Table 13. Patient and tumor characteristics for EC in the consecutive, unselected cohort with IHC loss of MLH1, stratified by presence or absence of *MLH1* promoter methylation.

	Methylated <i>MLH1</i> n (%)	Unmethylated <i>MLH1</i> n (%)	P - value
Patient Characteristics			
Age			
Median age at diagnosis	63	62	0.235
Age range	49-92	42-79	
Age			
< 50	1 (1.4)	4 (26.7)	0.003
≥ 50	71 (98.6)	11 (73.3)	
Family History of EC¹	5 (7.1)	3 (21.4)	0.123
Family History of CRC²	5 (7.0)	3 (20.0)	0.140
History of Diabetes	16 (22.2)	6 (40.0)	0.192
Meets SGO Criteria	6 (8.5)	6 (40.0)	0.005
Tumor Characteristics			
Histology			
Endometrioid	70 (97.2)	13 (86.7)	0.136
Non-Endometrioid	2 (2.8)	2 (13.3)	
FIGO Stage³			
I & II	57 (79.2)	13 (86.7)	0.725
III & IV	15 (20.8)	2 (13.3)	
Endometrioid Tumor Grade			
1 or 2	59 (81.9)	12 (80.0)	> 0.999
3	13 (18.1)	3 (20.0)	
Median depth of myometrial invasion (mm)⁴			
0 mm	10 (13.9)	3 (20.0)	0.736
< 50% myometrial invasion	41 (56.9)	7 (46.7)	
≥ 50% myometrial invasion	21 (29.2)	5 (33.3)	
Lymphatic/vascular space invasion	39 (54.9)	8 (53.3)	> 0.999
Tumor location			
Corpus	70 (97.2)	14 (93.3)	0.437
Lower uterine segment	2 (2.8)	1 (6.7)	
Largest Median Tumor Dimension (cm)	4	3.5	0.547

¹EC, endometrial cancer

²CRC, colorectal cancer

³FIGO stage I and II denote endometrial carcinomas confined to the uterus. FIGO stages III and IV represent extra-uterine spread of tumor.

⁴Depth of myometrial invasion ≥ 50% total myometrial thickness is associated with increased risk of lymph node metastasis

Specific Aim 3. Simplified Cost Analysis

Based on the data presented in Tables 5-9, it was determined that universal tumor testing identified the greatest number of PLS EC cases. However, tissue testing of all patients could be a cost-prohibitive process. To investigate this further, a simplified cost-effectiveness analysis was performed using the cohort of 412 endometrial cancer patients.

SGO Criteria were applied to the unselected cohort of 412 EC cases, identifying 97 women meeting criteria for further evaluation through tissue testing and genetic counseling, resulting in a total cost of \$93,529.08 (Figure 12, Table 14). Of these 97 identified by the SGO model, 15 are PLS as defined by tissue testing. The total cost per PLS case detected in the SGO model was \$6,143.21. It is estimated that 4-11 of these 15 PLS cases would have a germline mutation detected based on germline mutation detection rates among those with positive tumor testing results of 25-75%.

The average number of FDRs for those meeting SGO criteria was 5.3. Based on this and the range of estimated germline mutation rates among PLS EC cases, 21-48 FDRs would be eligible for single site gene mutation analysis and enhanced LS screening. The estimated costs for screening both PLS cases and their FDRs in this strategy is \$3,055.46-\$6,423.55 per case based on germline detection rates of 25-75% (Table 14).

Applying the universal tumor testing model identified 43 EC patients warranting further work-up through genetic counseling and germline testing (Figure 13). The total cost of this screening strategy was \$256,726.36, with cost per PLS case identified of \$5,970.38. It is estimated that 11-32 of these 43 patients would have a positive germline mutation (Table 14).

The average number of FDRs for those included in the universal tumor-testing model was 5.5. Based on this and the range of estimated germline mutation rates among PLS EC cases, 60-176 FDRs would be eligible for single site gene mutation analysis and enhanced LS screening. The estimated costs for screening both PLS cases and their FDRs in this strategy is \$3,003.35-\$6,526.52 per case based on germline mutation detection rates of 25-75% (Table 14).

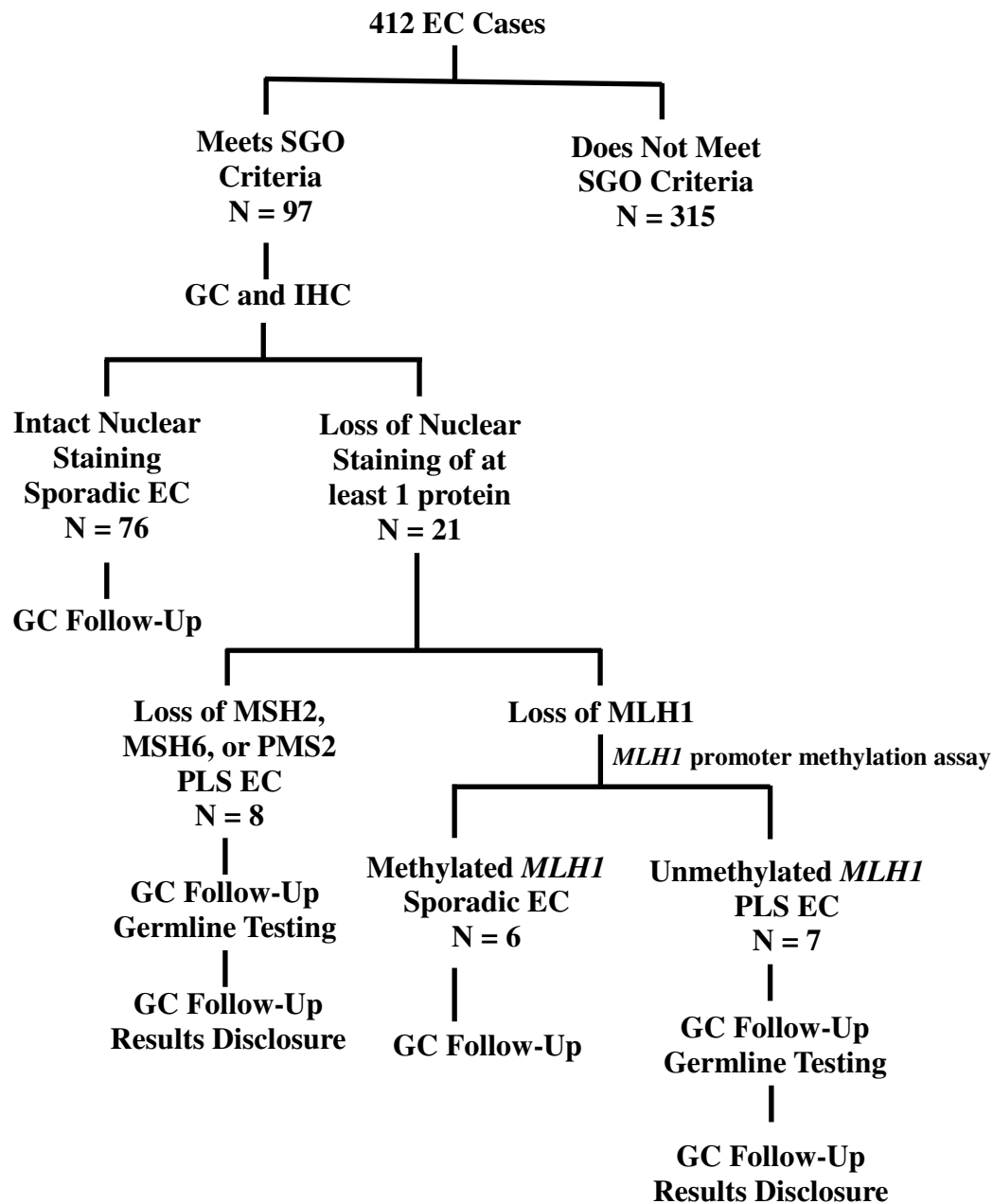


Figure 12. Cost analysis schema utilizing SGO 5-10% Clinical Criteria as a screening model.

1. GC, Genetic Counseling
2. PLS, Probable Lynch Syndrome
3. There was one case in which *MLH1* promoter methylation did not work and this was included in the cases that would go on to receive genetic counseling and germline testing.

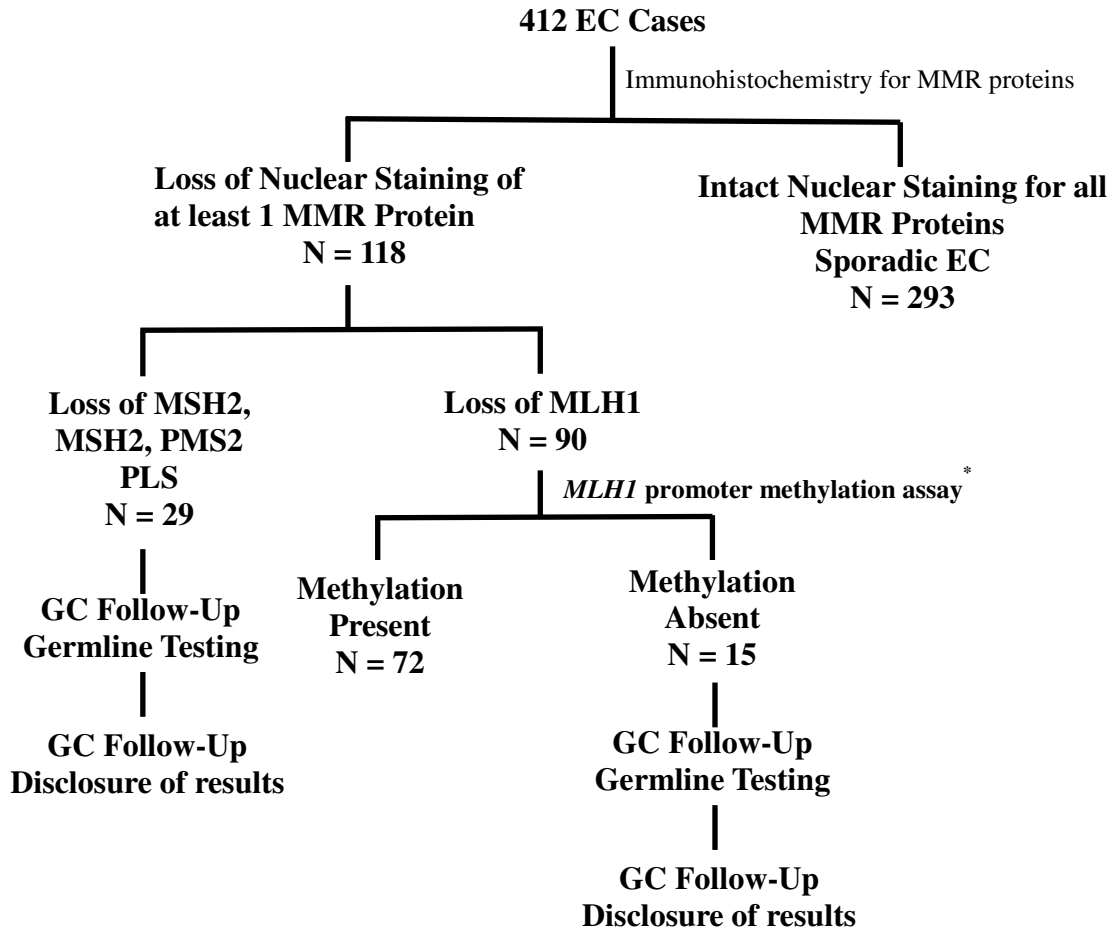


Figure 13. Cost analysis schema utilizing universal tumor testing via immunohistochemistry and *MLH1* methylation analysis when indicated

1. GC, Genetic Counseling
2. PLS, Probable Lynch Syndrome

* There were three cases in which *MLH1* methylation was unsuccessful. One case met clinical referral criteria and was included in genetic counseling decision tree costs; the other two cases had no risk factors for LS and were not included in the genetic counseling costs with this model.

Table 14. Comparison of direct Medicare costs associated with SGO Criteria and universal tumor testing models.

	SGO	Universal
Endometrial Cancer Cases (N = 412)		
# pts who undergo IHC testing	97	412
# pts who have loss of expression of IHC	21	118
# pts who undergo MLH methylation testing	13	90
# pts seen by genetic counselor	97	43
# PLS identified by strategy	15	43
# PLS estimated to have positive germline test (Detection rates of 25%, 50%, and 75% germline detection)	4, 8, 11	11, 22, 32
Estimated Costs for Screening Strategies		
Cost to Screen 412 EC Cases	\$93,529.08	\$256,726.36
Average cost per PLS case detected	\$6,235.27	\$5,970.38
First degree relatives (FDRs)		
# FDRs eligible for germline testing if 25%, 50%, or 75% of PLS cases have an identifiable germline mutation	21, 42, 58	60, 121, 176
Assuming 50% of PLS cases have an identifiable germline mutation:		
# of FDRs who will be germline positive for LS if 25%, 50% or 75% inherit the same mutation	11, 21, 32	30, 61, 91
Estimated Costs For Screening Including both PLS and FDRs (Assuming 50% of PLS have germline mutation)		
Cost per LS case identified if 25% of FDRs have positive germline mutation:	\$6,432.55	\$6,526.52
Cost per LS case identified if 50% of FDRs have a positive germline mutation:	\$4214.43	\$4,088.90
Cost per LS case identified if 75% of FDRs have a positive germline mutation:	\$3,055.46	\$3,003.35

Next, we calculated the incremental cost effectiveness ratio (ICER) using both MD Anderson Cancer Center Costs and Medicare costs (Table 15). The calculations assumed that 50% of PLS patients tested would have a germline mutation detected and varied the number of FDRs having an identifiable germline mutation between 25-75%. Comparing SGO Criteria to no screening at all, the ICER using Medicare costs vary between \$3,055.46/LS case (assuming 75% of FDRs are germline positive) and \$6,432.55/LS case (assuming 25% of FDRs will be germline positive). Using MD Anderson costs, the ICERs vary between \$3,202.67/LS case and \$6,742.46/LS case. The SGO Criteria model costs an additional \$3,000-\$7,000 more per LS case identified than doing no screening at all.

Comparing the universal testing model to SGO Criteria, the ICER using Medicare costs vary between \$2,974.89-\$6,580.62/LS case, and the MD Anderson costs vary between \$2,859.95-\$6,326.56 /LS case. This means that universal testing costs an additional \$2,800-\$6,600 more per LS case than using SGO Criteria.

There is not a universally accepted ICER value that is interpreted as a favorable or unfavorable value. The ICER is one component of many factors that a health care administrator or provider can use to determine which intervention strategy would work best for his/her practice. In this cohort, one could choose to utilize the SGO criteria model and spend \$2,859.95-\$6,326.56 per LS case to identify 4-11 germline Lynch Syndrome mutations in a population of 412 EC cases and potentially impact 21-58 FDRs. For an additional \$2,800-\$6,600 investment per LS case identified in the universal tumor testing model, 11-32 germline Lynch Syndrome mutations would be identified and 60-

176 FDRs could be impacted. This type of information can help to determine which strategy best benefits the specific patient population being examined.

Table 15. Incremental cost effectiveness ratios (ICER) using direct MDACC institutional costs and direct Medicare costs.

	# LS cases identified (pts + FDRs)	MDACC Costs (\$)	MDACC ICER \$/case	Medicare Costs (\$)	Medicare ICER, \$/case
If 75% of FDRs have positive germline tests					
No screening	0	\$0	\$0/case	\$0	\$0/case
SGO screening	40	\$122	\$3,202.67	\$122,218.44	\$3,055.46
Universal screening	113	\$336,883.14	\$2,859.95	\$339,379.04	\$2, 974.80
If 50% of FDRs have positive germline tests					
No screening	0	\$0	\$0/case	\$0	\$0/case
SGO screening	29	\$128,106.68	\$4,417.47	\$122,218.44	\$4,214.43
Universal screening	83	\$336,883.14	\$3,866.23	\$339,379.04	\$4,021.49
If 25% of FDRs have positive germline tests					
No screening	0	\$0	\$0/case	\$0	\$0/case
SGO screening	19	\$128,106.68	\$6,742.46	\$122,218.44	\$6,432.55
Universal screening	52	\$336,883.14	\$6,326.56	\$339,379.04	\$6,580.62

Discussion

Endometrial Cancer Patient Population

The EC population used for this study is derived from a large NCI designated cancer center so there exists a potential for referral bias. However, published data from a large, national epidemiologic analysis of 161,513 EC cases show that the MDACC EC population is comparable in terms of baseline clinicopathologic characteristics (59). Table 16 shows selected characteristics including the number diagnosed at age < 50, endometrioid histology, and early stage disease between our population and the overall U.S. population. The greatest difference between the two groups is that there is a higher proportion of women with grade 2 and 3 tumors in the MDACC population. Despite these differences, there are no published data stating that Lynch Syndrome associated endometrial tumors occur preferentially within a certain grade of tumor, so it is reasonable to believe that the MDACC data can be generalized to other endometrial cancer patient populations.

Table 16. Clinicopathologic data of EC Cases for MD Anderson Cancer Center and U.S. Population.

	MDACC % of EC population	U.S. Population¹ % of EC population
Diagnosed at age < 50	15.2	13.9
Histology		
Endometrioid	82.4	85.3
Non-endometrioid	17.6	14.7
Grade		
1	12.3	48.7
2	61.0	35.8
3	26.7	16.1
Stage²		
I	72.8	74.7
II - IV	27.2	25.3

¹ Derived from reference (59)

²Data from reference (57) was derived from the SEER database, in which staging is recorded using the local/regional/distant categories. MDACC surgical staging data is according to the FIGO staging system. FIGO Stage I and SEER “Local” category both refer to disease limited to the uterus. Direct comparisons cannot be made for other FIGO stages.

In addition to having comparable overall EC demographics, the results of the immunohistochemical and *MLH1* analyses are comparable to both national and international published data. A study by Backes et al. involved performing immunohistochemical analysis for expression of MLH1, MSH2, MSH6, and PMS2 on all tumors in a series of 140 unselected, endometrial cancers. Tumors with intact nuclear staining were considered sporadic, and tumors with absent MSH2 and/or MSH6 were referred for genetic counseling. *MLH1* promoter methylation analysis was not performed. For patients with tumors exhibiting loss of MLH1 and/or PMS2, those diagnosed at age > 60 years and no FDRs with endometrial or colorectal cancer received no further follow up, and those younger than age 60 years or having FDRs with EC or CRC received referral for genetic counseling. Their study showed that 21.4% of all EC tumors exhibited IHC nuclear loss of at least one MMR protein (60). A similar, population-based study was undertaken by Leenen et al. in which 183 sequential, unselected EC tumors of women diagnosed at ≤ 70 years underwent both MSI and IHC testing. Individuals with MSI-H tumors and absent IHC expression of MSH2, MSH6, or PMS2 were referred for genetic counseling. MSI-H tumors with IHC loss of MLH1 underwent *MLH1* methylation analysis, and those with absent methylation were referred to genetic counseling. Their study found a rate of 23.5% of IHC loss among all EC tumors, and 96.9% of tumors with IHC loss of MLH1 were methylated (34). A summary of these published findings, including data from our study, are summarized in Table 17. These findings support the idea that the results of this thesis research are potentially generalizable to other endometrial cancer patient populations.

Table 17. Comparison of MD Anderson results to similarly designed, population-based endometrial cancer national and international studies.

	MDACC¹ N = 408	Ohio State² N = 140	Netherlands³ N = 179
% IHC Loss	28.9	21.4	23.5
% MLH1 Loss	22.0	17.1	17.9
% methylated MLH1 promoter	82.7	Not performed	96.9
% with PLS EC	10.5	Estimate (7.7) ⁴	6 (3-11)

1: MDACC: MD Anderson Cancer Center

2: (60)

3: (34)

4: Ohio State calculation of % PLS is based on an approximate 80% methylation rate of tumors with IHC loss of MLH1.

SGO Clinical Criteria

Society of Gynecologic Oncology clinical screening criteria for Lynch Syndrome among endometrial cancer patients was codified in 2007. As can be seen from Tables 1 and 2, these criteria strongly resemble Amsterdam II and revised Bethesda Guidelines. Thus, many of the clinical recommendations for identifying endometrial cancer patients at elevated risk for having Lynch Syndrome have been extrapolated from colorectal cancer registries.

Ryan et al. evaluated Amsterdam II, revised Bethesda, and SGO criteria in a cohort of 76 endometrial cancer cases with known Lynch Syndrome germline mutations identified through databases from the British Columbia Familial Cancer Registry and Mount Sinai Hospital Familial Gastrointestinal Cancer Registry. They found that SGO 5-10% criteria identified 93% of EC cases with known germline mutations. The detection rates of *MLH1*, *MSH2*, and *MSH6* using SGO 5-10% criteria were 94%, 94%, and 88%, respectively. There were no *PMS2* mutations in this patient population. They concluded that SGO 5-10% Criteria performed best in identifying germline Lynch Syndrome mutations in endometrial cancer cases (13). These criteria have not been validated in either a clinic-based referral population or a population-based setting.

The predominance of *MLH1* and *MSH2* mutation carriers within Lynch Syndrome registries, including the three in which mutation carriers from the Ryan et al. study were derived, results in validation of existing criteria for these types of mutations. The relative paucity of *MSH6* and *PMS2* mutation carriers in Lynch Syndrome registries suggests that these mutations are rare or that they are missed by current screening strategies. Work by Senter and colleagues investigated 99 individuals with immunohistochemical loss of

PMS2 in 99 Lynch Syndrome associated tumors (91 CRC, 5 EC, 1 gastric, 1 small bowel, and 1 transition cell of renal pelvis) and performed PCR-based mutation analysis. Cases were obtained both through clinic-based screening criteria as well as in the population-based setting; 8/99 (8.1%) were endometrial cancers. They found that 62% of cases with IHC loss of PMS2 had a detectable *PMS2* germline mutation. Among germline carriers, 9.1% met Amsterdam II guidelines, 65.5% met revised Bethesda Guidelines, and 25.5% met no published clinical criteria (58). The fact that only a quarter of germline *PMS2* mutation carriers meet published clinical guidelines supports the possibility that its prevalence in Lynch Syndrome associated cancers may be under-estimated.

Published data on cumulative cancer risk to age 70 among individuals with known germline *MSH6* mutations have been shown to be less than that for *MLH1* and *MSH2* mutations carriers (61). Hendriks et. al examined the lifetime cancer risks among 146 individuals with germline mutations in *MSH6* who met Amsterdam II criteria, compared to a cohort of *MLH1* and *MSH2* germline mutation carriers. For male *MSH6* mutation carriers, the cumulative colorectal cancer risk is 69%; for female *MSH6* mutation carriers, the cumulative colorectal cancer risk is 30% and the cumulative risk for endometrial cancer is 70%. Additional clinical screening criteria were not evaluated in their study, but *MSH6* mutations carriers have been found to not meet standard clinical criteria in several previously published studies (62,63).

The performance of SGO Criteria in our population of identifying individuals at elevated risk (IHC loss of *MLH1*, *MSH2*, *MSH6* or *PMS2* with absence of *MLH1* promoter methylation) is consistent with these other published results. Clinical criteria perform best at detecting those with IHC loss of *MLH1* or *MSH2*, but perform quite

poorly at identifying those with loss of *MSH6* or *PMS2*. Based on the findings of our study and other published literature, clinical criteria preferentially identify only a subset of patients with endometrial cancer at risk for having Lynch Syndrome.

The underlying cause for an older median age at diagnosis for *MSH6* or *PMS2* mutations has not been elucidated. Work by Chen and colleagues examined single nucleotide polymorphisms (SNPs) of genes playing key roles in the cell cycle in a population of individuals with identifiable germline Lynch Syndrome mutations identified through a CRC registry. They utilized CART analysis and found that certain SNPs were associated with either earlier median onset of CRC diagnosis or a later age of onset. One SNP association they found associated with older age at diagnosis was individuals with wildtype *E2F2* and *AURKA* variant had a median age of diagnosis of 70 (64). It is possible that genetic variants may also play a role in endometrial cancers in individuals harboring *MSH6* and *PMS2* mutations that might explain the older median age at diagnosis in this subgroup.

In the colorectal cancer literature, there have been modifier genes identified that may account for some of the variability seen among germline Lynch Syndrome mutation carriers. Wijnen and colleagues explored the role of established SNPs associated with CRC (8q24.21, 18q21.1, 15q13.3, 8q23.2, 10p14, and 11q23.1) in a population of 675 patients with germline Lynch Syndrome mutations. They found that an individual with either the SNP rs3802842 (11q23.1) or rs16892766 (8q23.3) as well as a germline Lynch Syndrome mutation had a greater risk for developing CRC than individuals without these SNPs (65). This work was validated by Talseth-Palmer et al. in a cohort of 684 individuals with confirmed germline mutations in Lynch Syndrome genes. They found

the association between the SNPs on 11q23.2 and 8q23.2 was valid for only those with *MLH1* mutations (66). Talseth-Palmer et al. subsequently performed a combined analysis of data derived from the Wijnen et al. and Talseth-Palmer et al. studies to further characterize the role of these SNPs among *MLH1* germline mutation carriers. Among individuals with Lynch Syndrome, they found the SNP at 11q23.1 was associated with an increased CRC risk compared to individuals without Lynch Syndrome. Additionally, Lynch Syndrome mutation carriers with the SNP at 8q23.2 were diagnosed at earlier ages (67). These studies call attention to modifier genes and their impact on the variability seen within colorectal cancer among Lynch Syndrome patients. There are currently no published studies examining the role of modifier genes among the endometrial cancer cases in patients with Lynch Syndrome.

SGO Clinical Criteria among Endometrial Cancer Patients with IHC loss of MLH1

Approximately 15-20% of all endometrial and colorectal tumors will exhibit IHC loss of MLH1; however, between 60-90% of these tumors are considered sporadic rather than hereditary because they have epigenetic silencing of the *MLH1* promoter through a methylation event (26-28,30). Despite the body of research that supports performing the PCR-based *MLH1* methylation assay when evaluating tumors exhibiting IHC loss of MLH1, it is not routinely performed in the published literature describing clinical screening algorithms for LS (33-36). Though the reasons for this are not entirely clear, one possible explanation could be that some clinical laboratories do not have access to PCR-based testing or only have access to simpler PCR analyses such as hot-spot sequencing for mutational analyses.

This sub-analysis was designed to determine if any clinical, pathologic, or clinical screening tool could effectively replace using the PCR-based *MLH1* methylation analysis in the evaluation of endometrial cancer patients for possible Lynch Syndrome. Therefore, our evaluation was limited to ECs with loss of MLH1 in our convenience sample cohort. From the data presented in Tables 10-13, we conclude that no combination of clinical, pathologic, or clinical criteria is superior to *MLH1* methylation in evaluating tumors with IHC loss of MLH1.

Several published reports support that the prevalence of *MLH1* methylation increases with age (31,32). An investigation by Whelan et al. examined 40 endometrial carcinomas with immunohistochemical loss of MLH1 and compared their clinicopathologic information to 40 endometrial carcinomas with intact MLH1. It is unclear if their endometrial cases were recruited from a population-based setting or from a clinic-based referral population. They found a significant difference between the age of endometrial cancer diagnosis in *MLH1* methylated (mean age 56.1 years) versus unmethylated (mean age of 65.4 years) cases (31). Work by Zaubert and colleagues also investigated *MLH1* methylation in the endometrial cancers, examining differences between women diagnosed at less than age 50 compared to women diagnosed at greater than or equal to age 50. They found that 61.9% of MSI-H tumors were unmethylated in the younger group with a median age of diagnosis of 42.6, and 17.1% of MSI-H tumor were unmethylated in the older group with a median age of diagnosis of 64.6. As can be seen in tables 10 and 13, we found no statistically significant difference in either our convenience sample cohort or population-based cohort in terms of median age of diagnosis between methylated and unmethylated *MLH1* EC tumors. Our data agree with

other published data that *MLH1* methylation of EC tumors increases with increasing age, but age does not accurately predict methylation status.

Previous investigators have demonstrated that there are macroscopic and microscopic pathologic features of endometrial carcinomas that correlate with a diagnosis of Lynch Syndrome. Westin et al. examined endometrial carcinomas arising from the lower uterine segment (LUS) and found that 29% (10/35) were Lynch Syndrome-associated (44). Among these 10 cases, 9/10 had loss of MSH2 by IHC and 1 had loss of MLH1 without *MLH1* methylation. In both our convenience sample and population-based cohorts, LUS tumor location did not distinguish between the methylated and unmethylated *MLH1* tumors with IHC loss of MLH1.

When immunohistochemistry for DNA mismatch repair proteins is used as part of the evaluation for Lynch Syndrome in endometrial or colorectal cancers, absence of MLH1 immunohistochemical protein expression is a poor predictor for a germline mutation (38), as most of these tumors will also have somatic methylation of the *MLH1* gene (sporadic carcinoma), rather than germline mutation (Lynch Syndrome). Further, our sub-analysis supports that clinical and pathologic screening criteria poorly predict which endometrial cancers with IHC loss of MLH1 are likely to have presence or absence of *MLH1* methylation. In our convenience sample cohort of 54 endometrial cancer cases with immunohistochemical loss of MLH1, 14/54 cases would be candidates for germline *MLH1* testing. SGO criteria correctly identifies 10/14 unmethylated tumors. If SGO criteria were solely used without the *MLH1* methylation assay, 22/54 patients would undergo germline testing for *MLH1*, thereby subjecting 12 women to unnecessary and expensive germline testing. We conclude that *MLH1* promoter methylation testing is a

valuable component of clinical laboratory tumor testing for Lynch Syndrome among patients with endometrial cancers exhibiting immunohistochemical loss of MLH1.

Historical Risk Factors for Lynch Syndrome and Generation of New Criteria

From the data presented in this thesis, historically accepted risk factors such as young age at cancer diagnosis, strong family history, and non-obese BMI ($< 30 \text{ kg/m}^2$) are not as useful at delineating sporadic endometrial cancer from Lynch Syndrome associated cases. Several studies have shown that the prevalence of Lynch Syndrome due to defects in all DNA MMR genes is increased in women diagnosed with endometrial cancer at age younger than 50 years. In the study by Lu et al., 11% (11/100) of women presenting with endometrial cancer at age younger than 50 years had tumor testing results suggestive of Lynch Syndrome and 9% had an identifiable germline mutation. Of the 9 women with identifiable germline mutations, 7 had an *MSH2* mutation, 1 had an *MLH1* mutation, and 1 had an *MSH6* mutation (56). In a similar study performed by Walsh et al., 18% (26/146) of women with endometrial cancer diagnosed at less than age 50 years had molecular diagnostics testing (IHC, MSI, and *MLH1* methylation) consistent with Lynch Syndrome. In their study, there were 6 *MLH1*, 13 *MSH2*, and 7 *MSH6* PLS tumors. In both of these studies, those with presumed *MSH2* mutations were the most likely to present with Lynch Syndrome associated endometrial cancer at a younger age (68).

Obesity is an important determinant of endometrial cancer risk in the sporadic patient population (69). The relationship between BMI and Lynch Syndrome associated endometrial cancer has been investigated previously in patients younger than age 50 as well as in cohorts consisting of all ages. Lu et al. found that a median BMI of 27.6

among Lynch Syndrome endometrial cancer cases was significantly lower than the median BMI of 37.5 among sporadic cases in a set of EC patients diagnosed at age less than 50. The sensitivity and specificity of $\text{BMI} \leq 30$ for predicting Lynch Syndrome was 56% and 65%, respectively (56). Another study by Schmeler et al. found that 56% of 188 EC patients under the age of 50 were obese ($\text{BMI} \geq 30.0$), and all six patients with Lynch Syndrome were either normal weight or overweight ($\text{BMI} 25.0\text{-}29.9$) (70). In one additional study, McCourt et al. evaluated microsatellite instability in a series of 473 sequential endometrial carcinomas and found that patients with MSI-high tumors had a significantly lower BMI (30.3) than those with microsatellite-stable tumors (32.7) (29). While these studies support that BMI may play a role in differentiating between sporadic and Lynch Syndrome associated endometrial tumors, the results from our study reveal no statistically significant difference between $\text{BMI} < 30$ and a diagnosis of PLS EC. This could be due in part to the increases in obesity in the U.S. population, which may obscure a previously significant differentiator between sporadic and Lynch Syndrome associated tumors. Also, the median age of EC diagnosis in our unselected patient cohort was well above 50 (age 61). It is possible that BMI is a distinguishing feature only in younger EC patients.

A strong family history of certain cancers played a pivotal role in the initial identification and characterization of Lynch Syndrome and continues to be a principal component in widely accepted clinical screening algorithms such as Amsterdam II, revised Bethesda guidelines, MMRPro, PREMM, MMRPredict, and SGO Criteria. Unfortunately, family history is not perceived to be as helpful as it once was. In 2009, the EGAPP working group de-emphasized the role of family history when evaluating

individuals for risk of Lynch Syndrome, recommending a universal tumor testing approach. This recommendation was due in part to the poor overall sensitivity and specificity profiles of clinic-based screening criteria such as Amsterdam II or Revised Bethesda guidelines as well as suboptimal recording patient history by clinicians (46). We found no statistically significant differences in family history of EC or CRC between our PLS EC cases and sporadic EC cases. Further, the majority of all patients in our cohort did not have a family history of either tumor. Additionally, average family size in the 2000s is not the same as it was when Lynch Syndrome was first characterized in the early 1900s. In Dr. Warthin's original Family G, there was a male proband with 10 children. In our current cohort, the average number of siblings among our endometrial cancer cases was 3.4, and the average number of children was 2.1. As family sizes decrease, the probability of detecting a high proportion of cancers also decreases. The utility of family history in identifying patient's at risk for hereditary cancer syndromes may be decreasing in the current generations.

Further analyses to modify SGO Criteria or generate new clinical criteria to better identify Lynch Syndrome among endometrial cancer patients could not be generated from our cohort of 408 cases. In order to capture the maximal number of women with Lynch Syndrome who present with endometrial cancer, it may be necessary to adopt a universal tumor testing approach, as is increasingly being recommended for colorectal cancer patients.

Cost Analysis

There are few published cost analyses that evaluate screening methodologies for Lynch syndrome in women presenting with endometrial cancer. Kwon et. al evaluated several different screening strategies using a Monte Markov simulation model to evaluate the costs (IHC for DNA MMR genes, genetic counseling, germline testing, colonoscopy for detected LS cases, and average lifetime cost of CRC treatment) relative to the benefits (life expectancy) and generated an ICER for each screening strategy. They found that triaging all women with endometrial cancer who had a first-degree relative having a Lynch Syndrome-associated cancer at any age with immunohistochemistry was the most cost effective method (33). If we were to apply this screening strategy to our existing population-based cohort of 412 EC cases, 60 (14.6%) individuals would undergo immunohistochemical analysis, with 9 of these having tumor testing suggestive of Lynch Syndrome. This leaves 34 individuals with tumor testing consistent with Lynch Syndrome that would go undiagnosed using the Kwon et. al screening strategy. Though determined cost effective by Kwon's analysis, one must determine if the cost savings is worth the potential health-related implications of missing the diagnosis in 34 individuals and the impact this might have on both them and their first-degree relatives.

Work by Dinh and colleagues thoroughly evaluated screening strategies for colorectal and endometrial cancer using a Monte Carlo simulation of 100,000 hypothetical individuals modeled after the U.S. population starting at age 20. They compared direct medical costs for risk-assessment based screening (PREMM_{1,2,6}) starting at different age at diagnosis and compared this to universal 4-gene mutation sequencing of all individuals. Unique to their study, they evaluated an individual's risk *prior* to the

development of EC or CRC and the detection/prevention of these cancers was also incorporated in to costs/benefits. They found that risk-assessment of all individuals between the ages of 25-35 with PREMM_{1,2,6} followed by genetic testing for those with a risk score of $\geq 5\%$ was the most cost-effective strategy (71). At the time of this publication, the study by Mercado et al evaluating the PREMM_{1,2,6}, MMRPro, and MMRPredict algorithms in endometrial cancer had not yet been published. Dinh's study utilized PREMM_{1,2,6} as its sole risk-assessment strategy and found it to have an area under the curve (AUC) of 0.77; an AUC greater than 0.80 is deemed favorable. None of these screening strategies have been validated in the *PMS2* germline mutation population.

In our analysis, the cost per PLS case identified using a universal tumor testing strategy comprised of immunohistochemistry and *MLH1* methylation analysis when indicated is \$5,970.38. While this could be considered a favorable cost, creating an ideal cost analysis strategy is difficult. As can be seen by both our work and the work from others described above, there can be immense shift in costs as different assumptions and costs are added or removed from the models. The variation in perspective from different physician groups, different cancer patient populations, and different societies can also influence how such cost analyses are perceived.

Compliance with Published Screening Criteria Guidelines

As we strive to identify the best model for identifying Lynch Syndrome among endometrial cancer patients, it is important to be aware of the compliance of physicians with meeting referral guidelines. In our analysis, only 30% of endometrial cancer patients meeting institutional referral criteria actually received a recommendation to see a genetic counselor. This low rate is comparable to other published studies examining

compliance with screening recommendations. In the BRCA population, Meyer et al. examined the medical records of 3,765 ovarian cancer patients, of which 23.8% were determined to be at substantial risk for having a BRCA mutations. Only 12% of these patients were referred to genetic counseling at the beginning of their study, and this number increased to 48% following a clinic-based intervention strategy. Despite a dramatic improvement in referral habits over the course of the study, 50% of women at substantial risk were not offered genetic counseling at the end of the study period (72). Grover et al. evaluated the genetic counseling referral rate among colorectal cancer patients who met revised Bethesda Guidelines. In their cohort of 387 patients, 75 (19%) met referral criteria, but only 13 (17%) of these patients received a referral for genetic counseling and further risk assessment (73).

These data, coupled with results from our analysis, show a very poor compliance with published hereditary cancer syndrome screening guidelines. If published guidelines have excellent sensitivity and specificity for detecting disease, it would seem reasonable to create systems within the clinic-based setting to improve history-taking and referral rates. In the case of SGO Criteria, it may not be as beneficial to invest resources in improved referral when only a 32% of patients at elevated risk are identified. There has been one study in the CRC literature examining referral rates after implementation of a universal tumor testing regimen comprised of microsatellite instability testing and immunohistochemistry in all CRC cases. Heald and colleagues evaluated three different referral programs: 1) test results were sent only to the surgeon; 2) test results went to both the surgeon and genetic counselor, and the genetic counselor would send a follow up e-mail to the surgeon; and, 3) test results went to both the surgeon and genetic counselor,

and the genetic counselor would contact the patient and arrange a follow-up appointment. They found that the multidisciplinary approach (Options 2 and 3) resulted in 10-fold quicker follow-up for patients when compared to a single individual approach (Option 1). When the genetic counselor was the primary point of patient follow-up (Option 3), 100% of patients meeting referral criteria received a referral, whereas only 55% of those in Option 1 and 82% of those in Option 2 received a referral (47).

Study Limitations

This study has several limitations. First, it is a retrospective study and all clinicopathologic data was abstracted from the electronic medical record. This particularly affects acquisition of family history. Ideally, a full 3-generation family history is obtained from a genetic counseling professional and patient's fulfillment of clinical screening criteria is based on that assessment. Many patients have recall bias or vague ideas of the original location of a family member's tumor. For example, they know that a family member had a gynecologic cancer but unsure of whether it was cervix, uterine, or ovary. Our study delineated whether or not a patient met criteria based on self-reported history and genetic counseling data when available.

Additionally, we have based our assessment of SGO Criteria on its ability to detect elevated-risk EC tumors defined by those with IHC loss of a DNR MMR protein (absence of *MLH1* methylation when indicated) and not on its ability to detect germline mutations. At this point in time, patients have been notified of their tumor testing results and are in various stages of genetic counseling follow-up. Many previously published studies have used germline testing results as the "gold" standard. DNA sequencing is known for high sensitivity in detecting point mutations and small insertions, but the large

deletions, insertions, or gene arrangements are not as easily detected (48). Thus, when a patient has tumor molecular diagnostic testing results suggestive of LS but negative germline testing, the question remains whether or not that patient truly has Lynch Syndrome.

Of the myriad of studies using molecular diagnostics testing to evaluate for Lynch Syndrome, there is only one publication that addresses tumor test positive/germline negative cases. Rodriguez-Soler et al. evaluated 1,705 consecutive colorectal patients for Lynch Syndrome by performing MSI and IHC testing on all tumors (74). They examined clinicopathologic data of patients as well as age-adjusted incidence of cancer in family members between sporadic, tumor test positive/germline negative, and germline positive individuals. They found that germline positive patients were more likely to fulfill revised Bethesda guidelines than the tumor test positive/germline negative. Additionally, they found that the familial incidence of colorectal cancer was greatest in germline mutation carriers, next highest in tumor test positive/germline negative, and least in families with an individual with a sporadic colorectal cancer. Risk of endometrial cancer and other Lynch Syndrome associated tumors were not included in this study. More investigation needs to be done to determine the optimal approach to managing tumor test positive/germline negative patients, particularly since published guidelines support universal tumor testing of all colorectal cancers.

Lastly, the assumptions built in to our simplified cost analysis help to generate an estimate of direct costs, but it does not encompass many other factors that can be part of costs/benefits of any healthcare decision. To begin with, our model assumes a 100% genetic counseling referral rate for endometrial cancer patients meeting SGO Criteria,

when published rates for various criteria in clinical practice vary from 17-48%. Thus, costs associated with the SGO model are a higher estimate than what would likely occur in reality (72,73). Our model also assumes that all patients either meeting SGO Criteria or with tumor testing suggestive of Lynch Syndrome will accept referral for genetic counseling and/or germline testing. Compliance of endometrial cancer patients with genetic counseling referrals to evaluate for Lynch Syndrome may not be 100%. In a single institution study, Backes and colleagues surveyed the 47/384 EC patients who met institutional criteria for genetic counseling referral through a mailed questionnaire and follow-up phone call. Immunohistochemistry was performed on all endometrial cancers and referral for genetic counseling was recommended for: 1) individuals with loss of MLH1/PMS2 diagnosed at less than age 60; 2) individuals with loss of MLH1/PMS2 with a family history of a Lynch Syndrome associated tumor in a first-degree relative; 3) any individual with loss of MSH2/MSH6, MSH6 only, or PMS2 only. A total of 26/47 (55.3%) responded to the questionnaire and 20/26 (77%) stated that they had been referred to see a genetic counselor. Despite referral, only 9/20 (45%) saw a genetic counselor and 8/9 underwent germline testing. They found the two most common reasons for not seeing a genetic counselor were lack of insurance/cost for seeing a genetic counselor and anxiety related to the results (75).

There are many more costs that can be incorporated into a cost analysis, such as the cost of more frequent colonoscopy in LS cases, cost of colonoscopy complications, the cost of failing to prevent a colorectal cancer in an undetected LS case, the cost of a preventable EC or CRC in first-degree family members, and the emotional “cost” associated with the anxiety of testing. There are also different measures of effectiveness

that could be used such as number of cancers prevented or cancers diagnosed at an early stage. Referring to Figure 4, it is easy to see how subtle changes in the assumptions and inputs into the model can cause more dramatic changes in ICER, our metric for cost effectiveness comparison. Lastly, costs for this analysis were calculated using 2012 U.S. dollars and 2012 CPT codes. Inflation, CPT codes, and Medicare reimbursements can all change year to year. Therefore, rather than focusing on absolute costs, for our cost analysis it is more useful to consider the relative cost between the SGO screening method and the universal tumor testing method.

Conclusions

This is the first large, single-institution study examining endometrial cancer and Lynch Syndrome in a population-based fashion with a universal testing model starting with immunohistochemical analysis of all endometrial tumors. Our evaluation of the Society of Gynecologic Oncology 5-10% Clinical Criteria's ability to detect probable Lynch Syndrome endometrial cancer cases has shown that SGO Criteria identify only a small subset of PLS EC cases in the population-based setting. Universal tumor testing of EC (IHC and *MLH1* methylation when indicated) is a cost-effective alternative that detects more individuals at elevated risk, providing more opportunity for cancer prevention among women and their families.

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Vita

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