LYNCH SYNDROME

Improving Diagnostics and Surveillance

Anne Goverde

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Chapter 1

General introduction

Colorectal cancer (CRC) is one of the most common forms of cancer in both men and women worldwide.(1) The incidence of CRC is highest in Western countries, were the lifetime risk of developing colorectal cancer is around 5% and most patients are >60 years of age at the time of diagnosis.(1, 2) In the Netherlands, almost 15.000 new cases were diagnosed in 2017.(3) Despite the improvement of treatment, 5-year survival of CRC is still only 65%. Survival strongly depends on the stage in which colorectal cancer was found, decreasing from 90% in patients with stage I CRC to approximately 10% in patients with stage IV CRC.(4)

Colorectal cancer prevention

CRC develops from a precancerous lesion; an adenoma. Development from an adenoma into CRC (adenoma-carcinoma sequence) can take many years.(5-7) Therefore, CRC can be prevented by timely removal of adenomas.(8) Since CRC poses an important health burden, has markedly better survival when it is diagnosed at an early stage, and even has a recognizable premalignant lesion which can be removed during colonoscopy, it is an excellent candidate for population based screening.(9) In fact, in many countries population based screening for CRC has been implemented.(10) In the Netherlands, a population based screening program using fecal immunohistochemical test (FIT) for all individuals from 55-75 years of age started in 2014.(11)

Lynch syndrome

A small part of all CRCs is caused by a hereditary predisposition. In these patients, CRC often develops at a younger age. Lynch syndrome (LS) is the most common hereditary CRC predisposition, accounting for 2-3% of all CRC cases.(12-15) The first report of LS dates back to 1895, when Warthin described the family of his seamstress, in which most family members died from CRC. This hereditary predisposition to CRC was later named Lynch syndrome, after dr. Henry T. Lynch described more families with a similar phenotype. It would take years until the underlying genetic causes were found.(16)

LS is caused by autosomal dominant mutations in the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* or *PMS2*.(17-20) Additionally, although it is not a MMR gene, deletions of the 3'region of the *EPCAM* gene can also cause LS, due to hypermethylation and thereby silencing of the adjacent *MSH2* gene.(21) Similarly, although rare, germline hypermethylation of the promoter region of the *MLH1* or *MSH2* gene have been described in LS patients.(21)

The MMR genes are essential for the detection and consequent correction of mismatches that arise during DNA replication. Furthermore, the MMR genes play an important role in the induction of apoptosis in response to certain cytotoxic agents.(22,

23) In LS patients a mutant allele of one of the MMR genes is present in all cells. If a second (somatic) mutation occurs in the wild type allele, mismatches cannot be repaired, and cancer may develop.

LS patients have a lifetime risk of up to 74% of developing CRC. The cumulative risk is highly dependent on the gene involved, with lowest risk for *PMS2* and *MSH6* mutation carriers and the highest risk for *MLH1* and *MSH2* mutation carriers.(24-29) Especially in *MLH1* and *MSH2* mutation carriers, CRC often develops before the age of 50.(30-32) Furthermore, LS patients are also at risk of developing extra-colonic cancers, in particular endometrial cancer (EC) in women with a lifetime risk of 12-54%.(24-29) The risk of other types of cancer, such as ovarian, gastric, urinary tract and small intestinal cancer is also increased in LS patients.(24-29)

CRC morbidity and mortality in LS patients can be significantly reduced by intensive colonoscopy surveillance from a young age.(33-36) In these patients, colonoscopy with removal of adenomas is recommended every 2 years starting from age 25 or 2-5 years before the youngest CRC diagnose if a family member was diagnosed under 25 years of age.(25, 37, 38) In case CRC develops, (sub)total colectomy with ileorectal anastomosis should be considered instead of segmental colectomy, to reduce the risk of developing metachronous CRC. In a meta-analysis, metachronous CRC was found in 22,8% of the patients who underwent segmental colectomy despite adequate postoperative colonoscopy surveillance compared to 6% of the patients with an extended colectomy.(39) However, a decision analysis model showed that the overall gain in life expectancy for patients undergoing subtotal colectomy compared with hemicolectomy decreased with age from 2,3 years for LS patients aged 27 years to 1 year for LS patients aged 47 years and only 0,3 year for LS patients aged 67 years.(40) Therefore, in older patients segmental colectomy is probably appropriate. The benefits and increased morbidity after subtotal colectomy should be discussed with each LS patient developing CRC. After surgery, surveillance of the residual colon is still indicated.

For women with LS, gynecologic surveillance by transvaginal ultrasound, endometrial sampling and CA-125 tumor marker testing, is also recommended, although there is little evidence for the yield of this type of screening.(25, 33, 34, 37, 41-44) Women with LS can also opt for prophylactic hysterectomy and salpingo-oophorectomy after childbearing is completed to prevent the development of gynecological cancers.(25, 37, 38, 45) However, in order for LS patients to benefit from surveillance programs, they first have to be identified. Once a LS patients is identified, presymptomatic testing becomes available for family members, allowing relatives carrying the same mutation to start surveillance as well.

Identification of LS patients

As LS is an autosomal dominant predisposition, family history can be used to identify LS patients. The Amsterdam criteria were established in 1990 to select CRC patients suspect for having LS based on the age of CRC diagnosis and family history of CRC.(46) An updated version, the Amsterdam II criteria, also include extra-colonic cancers (Table 1).(47) Nevertheless, around 60% of the LS families do not fulfill these criteria.(48)

Table 1. The Amsterdam II criteria (49)

- At least 3 relatives with any LS-associated cancers
- One should be a first-degree relative of the other two
- At least two successive generations should be affected
- At least one patient should be diagnosed before age 50
- Familial adenomatous polyposis should be exclude in the CRC case(s), if any
- Tumors should be verified by pathological examination

In 1997 the Bethesda guidelines were introduced(49) followed by the revised Bethesda guidelines in 2004, which have a higher sensitivity than the Amsterdam criteria (Table 2).(50) However, the revised Bethesda guidelines still have limited sensitivity and are not well implemented in clinical practice.(51-54)

Table 2. The revised Bethesda guidelines (50)

- CRC diagnosed <50 years of age
- Synchronous or metachronous LS-associated tumors* regardless of age
- CRC with specific histology** <60 years of age
- CRC diagnosed in one or more first-degree relatives with a LS-associated tumor, with one <50 years of age
- CRC diagnosed in two or more first- or second-degree relatives with a LS-associated tumor, regardless of age

*LS-associated tumors: CRC, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, brain, sebaceous gland and small bowel cancer.

**Presence of tumor infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern.

Over the years, prediction models based on personal cancer history and family history have also been developed. Some of these models, the PREMM5, MMRpredict and MMRpro model, are available as free web-based prediction models.(55-57) Upon entering patient and family data, the probability of carrying a MMR mutation is calculated. Several studies have shown adequate performance of these prediction models in identifying LS patients among CRC patients.(13, 58-63) An advantage of the PREMM5 and MMRpro model is the fact that they can not only be used for CRC patients, but also for patients with other types of cancer or even healthy individuals. The MMRpro model however, needs extensive input including current ages of all family members,

which is less likely to be available or at least time consuming in clinical practice. Current guidelines recommend the use of prediction models as part of the strategy to identify MMR mutation carriers among patients with CRC. All methods to identify LS patients based on family history lack sensitivity especially for *MSH6* and *PMS2* mutation carriers, due to the lower penetrance. Another disadvantage of these strategies is the fact that family history is often unreliable or unavailable, limiting the yield of prediction models based on family history.(64-66)

Molecular diagnostics to identify LS patients

A method not involving family history to identify patients who are likely to have LS is based on molecular diagnostics on tumor tissue. Tumors caused by LS are characterized by MMR deficiency and show microsatellite instability (MSI) and loss of MMR protein expression.(67) Microsatellites are stretches of DNA consisting of small repetitive sequences of nucleotides, for example mononucleotide or dinucleotide repeats. In case of MMR deficiency, these sequences are prone to errors in DNA replication and therefore will become unstable resulting in microsatellites of different sizes. A pentaplex panel of five mononucleotide repeats is recommended for MSI analysis.(67) If at least two out of these five repeats show MSI, MMR deficiency in the tumor is assumed. Approximately 85% of the tumors from LS patients show MSI.(68-71) Patients with tumors displaying MSI have a better prognosis and survival than those without MMR deficiency.(69, 72) Also, for patients with tumors showing MSI, 5FU chemotherapy is not beneficial.(73-75) More recent data also suggests a role of immune checkpoint inhibitors as a therapy for MMR deficient tumors regardless of the organ involved.(76) Therefore, MSI analysis will be increasingly performed as a prognostic marker as well as for treatment options.

The second hallmark of MMR deficiency in LS associated tumors is loss of MMR protein(s) at immunohistochemistry (IHC).(67) An advantage of IHC analysis is that loss of a MMR protein not only shows MMR deficiency, but directly indicates the affected MMR protein. In tumors from *MSH6* or *PMS2* germline mutation carriers, loss of expression of the corresponding protein is seen in tumor cells. In case of a germline *MLH1* mutation, tumor cells show absent staining for MLH1 as well as PMS2 protein, since loss of MLH1 protein leads to destabilization of the PMS2 protein. Similarly, in tumors from *MSH2* mutation carriers, expression of both MSH2 and MSH6 protein is lost. Therefore, loss of a specific MMR protein or a combination of MMR proteins allows for targeted germline mutation analysis of the corresponding MMR gene. Sensitivity of IHC analysis is found to be around 83%.(71, 77) Some pathogenic mutations still allow protein formation, while the protein does not function properly. In such cases, there will be no loss of MMR

protein in tumor cells, even though the tumor is MMR deficient.(67) Such false negative results are most frequent in missense *MSH6* mutations.

While MMR deficiency in a tumor is suggestive for an underlying germline MMR mutation, it can also be seen in sporadic tumors. In sporadic tumors, MMR deficiency can be caused by epigenetic silencing of *MLH1* due to hypermethylation of the *MLH1* promoter.(67) Furthermore, MMR deficiency can be caused by two somatic MMR mutations, or one somatic MMR mutation combined with loss of heterozygosity.(78, 79)

Around 12-20% of all CRC show MMR deficiency.(12, 80-82) In cases with loss of MLH1 protein expression, *MLH1* promoter hypermethylation analysis can distinguish sporadic MMR deficient tumors from tumors likely caused by LS.(67) In cases without *MLH1* hypermethylation and in cases with loss of MSH2, MSH6 or PMS2 protein without a germline MMR mutation found, somatic mutation analysis can often clarify the cause of MMR deficiency. Somatic mutation analysis identifies two sporadic hits in >50% of the patients with a MMR deficient tumor in whom no germline MMR mutation is found.(78, 79)

The revised Bethesda guidelines were previously used to select patients in whom molecular diagnostics for LS should be performed.(50) Since these guidelines are underutilized in clinical practice leading to underdiagnosis of LS, routine molecular diagnostics for LS was proposed for CRC and EC patients.(54) In the Netherlands, the MIPA criteria were established, which entailed that pathologists could select CRC patients for MSI testing in case of 1) CRC < 50 years of age, 2) second CRC, 3) CRC and another LS-associated cancer, or 4) a colorectal adenoma with high grade dysplasia <40 years of age.(83) A multicenter study showed a high yield of routine screening for LS by MSI and IHC analysis in CRC and EC patients up to 70 years of age.(84, 85) Some even recommend universal screening of all CRC patients without an age cut-off. Off course, the more extensive the screening is, the more LS patients will be identified. However, cost-effectiveness should also be established before implementation of screening strategies.

Germline mutation analysis

A definite diagnosis of LS is made by the identification of a pathogenic germline MMR mutation. Once a pathogenic germline mutation is identified in a family, (presymptomatic) testing of relatives also allows relatives carrying the same mutation to enroll in surveillance programs. In some cases however, a variant of unknown significance (VUS) is found and the diagnosis remains uncertain. Over recent years tumor testing for LS and consecutive germline mutation analysis for LS is increasingly

performed. This will not only increase the number of LS diagnoses, but likely will also lead to more patients in whom a VUS in one of the MMR genes is found. Also, in the current era where whole exome sequencing is increasingly used for all kinds of conditions, more and more VUS are likely to be found in MMR genes. This implies the need for assays to determine pathogenicity of such VUS. Several functional assays have been developed for VUS in MMR genes.(86-89)

Surveillance programs for Lynch syndrome

After identification of LS patients, they are offered to enroll in a surveillance program, which can significantly reduce morbidity and mortality.(33-36) The goal of such intensive surveillance programs is of course the prevention or early detection of cancer. Although germline mutations in the different MMR genes result in different cancer risks, surveillance programs for LS are currently not tailored to the gene involved (Table 3).

Table 3. Recommended surveillance in Lynch syndrome patients

- Colonoscopy every 1-2 years starting from age 25 or 2-5 years before the youngest CRC diagnosis, whichever comes first.
- Gynecologic surveillance in women every year including transvaginal ultrasound and endometrial biopsy from age 40-60 years. Women can also opt for prophylactic hysterectomy and salpingo-oophorectomy after childbearing is completed.
- Testing for helicobacter pylori infection once and eradication if needed.
- Surveillance for LS-associated tumors other than CRC or EC is not recommended.

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Chapter 2

Aims and outline of the thesis

Lynch syndrome is the most common hereditary predisposition for colorectal cancer, accounting for 2-3% of all colorectal cancer cases. Furthermore, individuals with Lynch syndrome are at increased risk of developing extracolonic cancers, in particular endometrial cancer in women. The syndrome is caused by autosomal dominant mutations in the mismatch repair genes *MLH1*, *MSH2*, *MSH6* or *PMS2,* or by deletions of the 3'region of the *EPCAM* gene. The identification of individuals with Lynch syndrome is of great importance, since surveillance programs can significantly reduce their cancer morbidity and mortality.

This thesis focusses on the identification of Lynch syndrome patients, the interpretation of variants found by germline mutation analysis, and the yield of colorectal cancer surveillance for Lynch syndrome patients. The introduction in **chapter 1** includes an overview of these different aspects of Lynch syndrome.

The aim of the first part of this thesis was to determine ways in which the identification of Lynch syndrome patients can be improved. In **chapter 3**, the diagnostic yield of two prediction models for Lynch syndrome (MMRpredict and PREMM5) are reviewed in a cohort of colorectal cancer patients and an extended version of the PREMM5 model is proposed. **Chapter 4 and 5** assess the cost-effectiveness of routine screening for Lynch syndrome by molecular diagnostics in patients with colorectal cancer or endometrial cancer up to 70 years of age. Routine molecular screening for Lynch syndrome in adenomas (a precursor lesion of colorectal cancer) may have a higher benefit than screening among cancer patients, since colorectal cancer can still be prevented in these patients. Therefore, **chapter 6** evaluates the yield of screening for Lynch syndrome in adenoma patients within the national FIT-based screening program for colorectal cancer.

A definite diagnosis of Lynch syndrome can be made once a pathogenic germline mutation is identified. In some cases, a variant of unknown significance is found and the diagnosis remains uncertain. In **chapter 7 and 8** an assay for variants of unknown significance in *MLH1*, *MSH2* and *MSH6* is evaluated and several variants in these genes are analyzed.

Although the cancer risk in Lynch syndrome patients is highly dependent on the gene involved, surveillance programs are currently not tailored based on genotype. Therefore, **chapter 9** evaluates the effectiveness of colonoscopy surveillance in *MLH1*, *MSH2*, *MSH6* and *PMS2* mutation carriers.

Finally, **chapter 10** discusses the results of this thesis in perspective of the current guidelines and clinical practice.

Part I
Identification of Lynch syndrome patients

Chapter 3

Evaluation of current prediction models for Lynch syndrome: Updating the PREMM5 model to identify *PMS2* mutation carriers

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ABSTRACT

Background Until recently, no prediction models for Lynch syndrome (LS) had been validated for *PMS2* mutation carriers. We aimed to evaluate MMRpredict and PREMM5 in a clinical cohort and for *PMS2* mutation carriers specifically.

Methods In a retrospective, clinic-based cohort we calculated predictions for LS according to MMRpredict and PREMM5. The area under the operator receiving characteristic curve (AUC) was compared between MMRpredict and PREMM5 for LS patients in general and for different LS genes specifically.

Results Of 734 index patients, 83 (11%) were diagnosed with LS; 23 *MLH1*, 17 *MSH2*, 31 *MSH6* and 12 *PMS2* mutation carriers. Both prediction models performed well for *MLH1* and *MSH2* (AUC 0.80 and 0.83 for PREMM5 and 0.79 for MMRpredict) and fair for *MSH6* mutation carriers (0.69 for PREMM5 and 0.66 for MMRpredict). MMRpredict performed fair for *PMS2* mutation carriers (AUC 0.72), while PREMM5 failed to discriminate *PMS2* mutation carriers from non-mutation carriers (AUC 0.51). The only statistically significant difference between *PMS2* mutation carriers and non-mutation carriers was proximal location of colorectal cancer (77% vs. 28%, $p<0.001$). Adding location of colorectal cancer to PREMM5 considerably improved the models performance for *PMS2* mutation carriers (AUC 0.77) and overall (AUC 0.81 vs. 0.72). We validated these results in an external cohort of 376 colorectal cancer patients, including 158 LS patients.

Conclusion MMRpredict and PREMM5 cannot adequately identify *PMS2* mutation carriers. Adding location of colorectal cancer to PREMM5 may improve the performance of this model, which should be validated in larger cohorts.

INTRODUCTION

Lynch syndrome (LS) is a hereditary predisposition to colorectal cancer, endometrial cancer and other extra-colonic cancers at a young age.(1, 2) Morbidity and mortality of LS carriers can be significantly reduced by surveillance programs.(3-5) Therefore identifying LS carriers is of great importance.

LS is caused by a germline mutation in one of the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* or *PMS2*, or in the 3' end of the *EPCAM* gene and consequent hypermethylation of the *MSH2* promoter region.(6-10) As a result, tumours in LS patients are characterized by microsatellite instability (MSI) and by loss of MMR protein expression in immunohistochemistry (IHC).(11-13) Analysis of MSI and IHC, combined with *MLH1* promoter methylation analysis to exclude sporadic MMR deficient tumours, are used to identify patients with tumours likely caused by LS.(13) A definite diagnosis of LS is made when a pathogenic germline mutation is found.

The revised Bethesda guidelines were based on a set of diagnostic criteria to select patients eligible for LS screening in tumour tissue. However, due to limited sensitivity, many LS patients will likely be missed by these guidelines.(14-17) Several prediction models, such as MMRpro, MMRpredict and PREMM5 have also been developed to calculate an individual's probability of carrying a germline MMR mutation.(18-20) These models could aid in the selection of patients at high risk of having LS, for tumour analysis or direct germline mutation analysis. MMRpro is less useful in clinical practice since detailed information of all relatives is needed as input for the model.(19) However, MMRpredict and $PREMM_{1,2,6}$ (a previous version of the newly developed PREMM5 model) both performed well in previous evaluations.(21-27) An advantage of PREMM5 is that it can also be used for individuals with extracolonic malignancies and healthy individuals, as opposed to MMRpredict, which can only be used for CRC patients. Until recently, all prediction models for LS were developed with cohorts of patients carrying a *MLH1*, *MSH2*, or *MSH6* mutation. The recently published PREMM5 model is the only model that included *PMS2* mutation carriers in its development.

In this study we aimed to evaluate MMRpredict and PREMM5 in a clinical cohort and for *PMS2* mutation carriers specifically. Additionally, we aimed to identify clinical features useful for distinguishing *PMS2* mutation carriers from non-mutation carriers.

METHODS

In a retrospective, clinic-based cohort we assessed the performance of MMRpredict and PREMM5 in predicting LS mutations in general and for *MLH1*, *MSH2*, *MSH6* and *PMS2* mutations specifically. Additionally, we performed a univariate analysis to identify variables that can distinguish *PMS2* mutation carriers from patients with no MMR mutation.

Study population

We collected data for all families that were referred for genetic counselling at Erasmus MC, Rotterdam, the Netherlands, and in which colorectal cancer was analyzed for MSI and/or IHC between 2000 and 2010. Exclusion criteria were: failed or inconclusive analysis for MSI and IHC, a pathogenic mutation in *APC or MUTYH*, a variant of unknown clinical significance in one of the MMR genes or *APC*, and MSI or IHC suspect for LS while no MMR mutation was detected. To increase the number of LS families, 35 LS families outside our cohort, diagnosed after 2010, were also included in the analysis.

Analysis of MSI and IHC

MSI analysis was carried out with five markers for MSI as described previously; up to 2007 the Bethesda panel(28) was used and from 2007 onwards our center performs Promega pentaplex MSI analysis.(29) IHC for MLH1, MSH2, MSH6 and PMS2 protein *w*as performed as described previously.(13) Tumours without MSI or only a low degree of MSI and with all MMR proteins present, were considered MMR proficient. Tumours showing a high degree of MSI and/or absence of one or more MMR proteins, were considered MMR deficient. MLH1 hypermethylation analysis was performed to distinguish between sporadic MMR deficient tumours and MMR deficient tumours suspect for LS.

Germline mutation analysis

Patients with MMR deficient tumours suspect for LS underwent germline mutation analysis of the gene indicated by IHC. Germline mutation analysis of *MLH1*, *MSH2* and *MSH6* was performed by sequencing and multiplex ligation dependent probe amplification analyses. *PMS2* mutation analysis was performed as described elsewhere.(30)

Family classification

Tumour characteristics, age at diagnosis, results of molecular diagnostics and germline mutation analysis, and a detailed family history were collected from medical records. In every family the patient in whom MSI and/or IHC was analyzed, was labelled the index

patient. If more than one family member was screened for LS, the youngest CRC patient analyzed was considered the index patient. Index patients with MMR proficient tumours or sporadic MMR deficient tumours, were labelled non-mutation carriers. Families identified with a pathogenic MMR mutation were labelled LS families.

Prediction Models

For each index patient the probability of carrying a LS mutation according to MMRpredict and PREMM5 was calculated as previously described.(18, 20) For PREMM5, the equation was slightly different from the published equation, based on personal communications with F Kastrinos. See supplemental material (appendix 1) for the corrected PREMM5 equation.

Statistical analysis

Data were analyzed using SPSS statistical software version 21.0. Differences between mutation carriers and non-mutation carriers were compared using the Chi-square test or Fishers' exact test for frequencies, and by using the Mann Whitney U test for continuous data. These analysis were also performed to compare *PMS2* mutation carriers with non-mutation carriers. P-values <0.01 were considered statistically significant.

Receiver operating characteristic curves were created for MMRpredict and PREMM5 by plotting the true positive rate (sensitivity) against the false positive rate (1- specificity). Performance of MMRpredict and PREMM5 was evaluated by the area under the receiver operating characteristic curve (AUC). We compared the AUC of PREMM5 and MMRpredict for LS patients in general and for the different MMR genes specifically. Sensitivity and specificity were calculated for cut-offs previously indicated by the developers of the models (5%, 10%, 20% and 40%). These values were compared with the sensitivity and specificity of the revised Bethesda guidelines.

Model updating

Location of CRC is included in MMRpredict, but not in the PREMM5 model. To update the PREMM5 model, we used a previously proposed framework to update multinomial logistic regression models.(31) We extended the PREMM5 model using recalibration and extension. The PREMM5 model contains four linear predictors, each contributing weights to the probability of carrying a mutation in *MLH1*, *MSH2* (or *EPCAM*), *MSH6* and *PMS2*. The coefficients of the linear predictors were constrained such that the linear predictor only contributed to the calculation of the corresponding mutation. Since the original PREMM5 model was developed on a population with no *MSH6* mutation carriers with two or more CRCs, we developed two adaptations of the PREMM5 model. First we recalibrated the PREMM5 model and re-estimated the coefficient of the predictor 'Two

or more CRCs' in the linear predictor for *MSH6*. In the second adaptation we also added side of CRC as an additional predictor to the original PREMM5 model. Discriminative ability of the prediction models was quantified using the AUC. Calculations were done using R software (version 3.3.0), with estimation of the coefficients in the updated PREMM5 model using the VGAM package.

Validation of the extended PREMM5 model

For external validation of the extended PREMM5 model, we used a cohort of 376 CRC patients. Of these patients, 218 were patients with MMR proficient CRC, that where analyzed in the Erasmus Medical Center Rotterdam outside the dates of our initial cohort. LS patients (n=158) in our validation cohort were CRC patients from Leiden University Medical Center in whom an MMR mutation was found and with known location of CRC. For all patients of the validation cohort we calculated the probability of carrying an MMR mutation according to the original PREMM5 model and the extended model. The performance of both models were evaluated by comparing the AUC.

RESULTS

A total of 734 index patients were included in the study; 346 (47%) were male and mean age at time of diagnosis was 53 years (± 13 years). Overall, 569 (78%) patients fulfilled the revised Bethesda guidelines. Of the 734 index patients, 83 (11%) were diagnosed with a LS mutation; 23 *MLH1*, 17 *MSH2*, 31 *MSH6* and 12 *PMS2* mutation carriers.

Patient characteristics

Patient characteristics for mutation-positive and mutation-negative patients are shown in Table 1. Significantly more mutation carriers developed multiple CRCs (21% vs. 10%, p=0.005) and multiple LS-associated cancers in general (13% vs. 4%, p=0.002) than non-mutation-carriers. CRC patients carrying an MMR mutation had a younger age of onset (49 years vs. 53 years, p=0.002) and more often had proximal CRCs (64% vs. 28%, p<0.001) than non-mutation carriers. Among women, the frequency of EC was higher for mutation carriers than for non-mutation carriers (41% vs. 3%, p<0.001). In the mutation positive group, first and second degree relatives developed CRC at a younger age than in the mutation negative group (50 vs. 64 years, p<0.001 and 47 vs 62 years, p=0.008). First degree relatives of mutation carriers had higher rates of EC than relatives of non-mutation carriers (19% vs. 5%, p<0.001).

Table 1. Index characteristics and family history by mutation status (n=734)

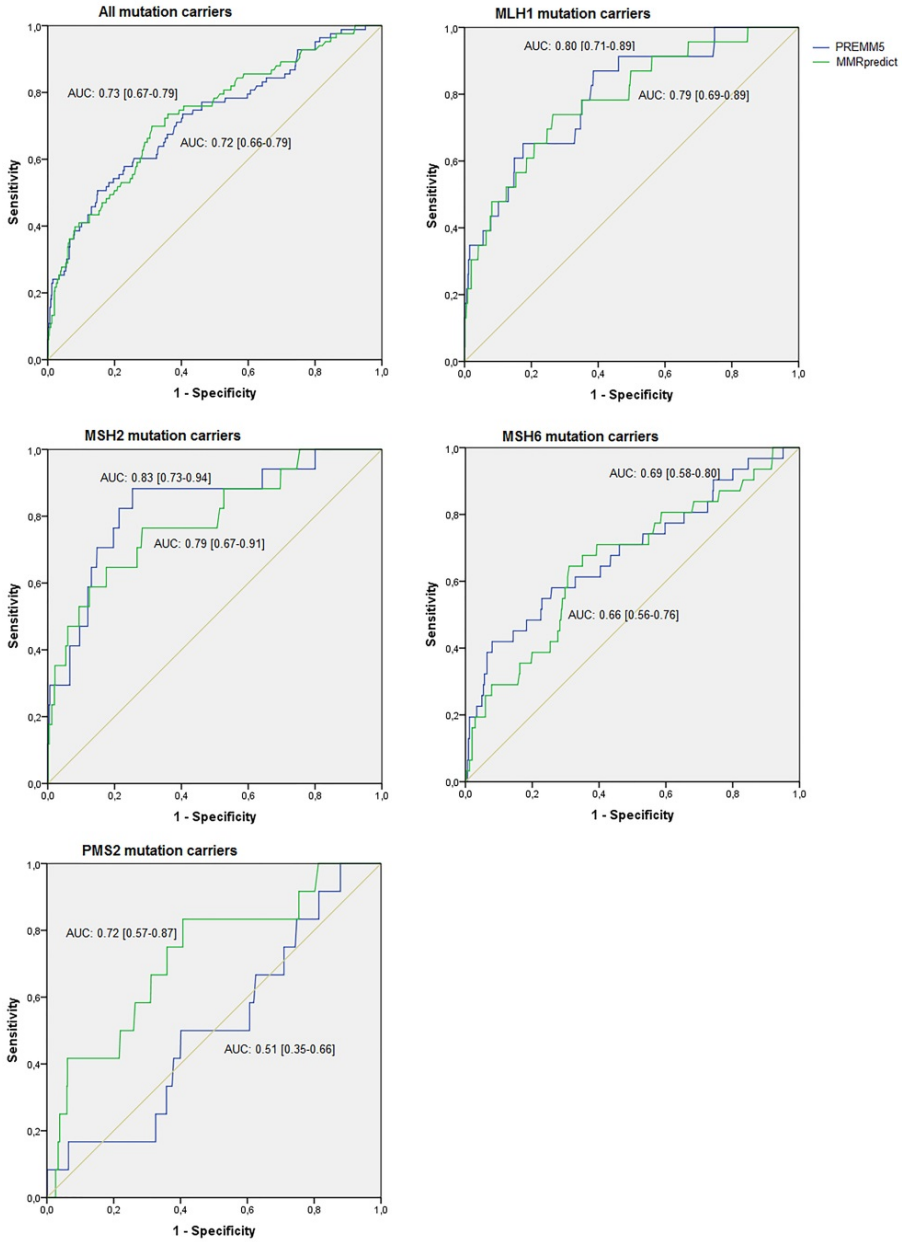
	Mutation negative, % (n)	Mutation positive, % (n)	P value
N	651	83	
Revised Bethesda guidelines	76% (494)	90% (75)	0.003
Index characteristics			
Male gender	47% (305)	49% (41)	0.66
Colorectal cancer			
Age CRC (median, IQR)	53 years [45-62]	49 years [39-59]	0.002
Proximal CRC	28% (185)	64% (53)	<0.001
≥2 CRCs	10% (66)	21% (17)	0.005
Endometrial cancer	3% (11)	41% (17)	<0.001
Age EC (median, IQR)	55 years [50-75]	54 years [49-57]	0.18
Multiple LS cancers	4% (27)	13% (11)	0.002
First degree relatives			
Colorectal cancer	55% (358)	51% (42)	0.45
≥2 FDRs with CRC	16% (107)	17% (14)	0.92
Age CRC (median, IQR)	64 years [55-71]	50 years [43-57]	<0.001
Endometrial cancer	5% (35)	19% (16)	<0.001
≥2 FDRs with EC	*0*.6% (4)	2% (2)	0.14
Age EC (median, IQR)	55 years [50-64]	50 years [45-57]	0.25
Other LS cancers	22% (142)	19% (16)	0.60
Second degree relatives			
Colorectal cancer	33% (212)	35% (29)	0.66
≥2 SDRs with CRC	12% (81)	12% (10)	0.92
Age CRC (median, IQR)	62 years [50-74]	47 years [38-64]	0.008
Endometrial cancer	3% (22)	7% (6)	0.12
≥2 SDRs with EC	0.3% (2)	2% (2)	0.07
Age EC (median, IQR)	70 years [50-76]	49 years [44-51]	0.13
Other LS cancers	16% (104)	18% (15)	0.63

Discriminative ability of prediction models

Overall, PREMM5 predicted higher probabilities of carrying a LS mutation than MMRpredict (median score 0.06 vs. 0.03, supplemental table 1). For mutation carriers, risk scores varied from 0.02 to 0.99 for PREMM5 and from 0.002 to 0.99 for MMRpredict. Both prediction models could fairly discriminate between index patients with and without an MMR mutation.(Figure 1) PREMM5 and MMRpredict had similar overall performance (AUC 0.72 [95% CI 0.66-0.79] vs. 0.73 [95% CI 0.66-0.79]). For *MLH1* and *MSH2* mutation carriers, both prediction models performed well, with AUC of 0.80 [95% CI 0.71-0.89] and 0.83 [95% CI 0.73-0.94] for PREMM5 and AUC of 0.79 [95% CI 0.69-0.89 and 0.67-0.91] for MMRpredict. Both models had a fair discriminative power for *MSH6* mutation carriers (AUC of 0.69 [95% CI 0.58-0.80] for PREMM5 and AUC of 0.66 [95% CI

0.56-0.76] for MMRpredict). MMRpredict still had fair performance for *PMS2* mutation carriers (AUC of 0.72 [95% CI 0.57-0.87]), while PREMM5 failed to discriminate *PMS2* mutation carriers from non-mutation carriers at all with an AUC of 0.51 [95% CI 0.35-0.66].

Figure 1. Performance of PREMM5 and MMRpredict in a clinical setting for all mutation carriers and for individual MMR mutations.



Sensitivity and specificity

Using a cut-off of 5% for both prediction models, PREMM5 had a higher sensitivity than MMRpredict (78% vs. 70%). This higher sensitivity came at the expense of a lower specificity (46% vs. 67%). For MMRpredict, at a 5% cut-off sensitivity for *MLH1* and *MSH2* mutation carriers were 74% and 77%, while sensitivity for *PMS2* as well as *MSH6* mutation carriers were 65% and 67%. For both models, using a cut-off of ≥20% failed to identify over 50% of the mutation carriers.

Sensitivity of the revised Bethesda guidelines decreased from 96% for *MLH1* mutation carriers to 83% for *PMS2* mutation carriers.(Supplemental table 2) Overall, the revised Bethesda guidelines had a sensitivity of 90% with a specificity of 24%. In order to reach the same sensitivity, PREMM5 and MMRpredict had a similar specificity (25%).

***PMS2* mutation carriers vs. non-mutation carriers**

Mutation carriers differed significantly from non-mutation carriers in many ways (Table 1). In contrast, there were almost no significant differences between *PMS2* mutation carriers and non-mutation carriers. Only one significant difference remained; *PMS2* mutation carriers more often had proximal CRC than patients without an MMR mutation (83% vs. 28%, p<0.001).(Table 2)

Improvement of the PREMM5 model

Since location of CRC was the only significant difference between *PMS2* mutation carriers and non-mutation carriers, we incorporated this variable in the PREMM5 model, aiming to improve the prediction model. For *PMS2* mutation carriers, the extended PREMM5 model had considerably better predictions than the original PREMM5 model (AUC 0.77 [95% CI 0.63-0.90] vs. 0.51 [95% CI 0.35-0.66])(Figure 2). At a 5% cut-off, the new PREMM5 model identified 5/6 *PMS2* mutation carriers that would have been missed by PREMM5 and 3/4 *PMS2* mutation carriers that would have been missed by MMRpredict at the same cut-off.

Adding tumour location also improved the performance of PREMM5 for identifying *MLH1* (AUC 0.92 [95% CI 0.88-0.97] vs. 0.80 [95% CI 0.71-0.89]) and *MSH6* (AUC 0.75 [95% CI 0.65-0.84] vs. 0.69 [95% CI 0.58-0.80]) mutation carriers (Figure 2). However, performance for *MSH2* mutation carriers slightly decreased (AUC 0.80 [95% CI 0.69-0.91] vs. 0.83 [95% CI 0.73-0.94]). Overall, the adjusted PREMM5 model performed better than the original PREMM5 model (AUC 0.81 [95% CI 0.76-0.86] vs. 0.72 [95% CI 0.66-0.79]) and MMRpredict (AUC 0.81 vs 0.73 [95% CI 0.66-0.79]). The adjusted prediction model can be found as supplemental material.

Table 2. Index characteristics and family history for *PMS2* mutation carriers compared with non-mutation carriers

	Mutation negative, % (n)	*PMS2* mutation positive, % (n)	P value
N	651	12	
Revised Bethesda guidelines	76% (494)	83% (10)	0.74
Index characteristics			
Male gender	47% (305)	50% (6)	0.83
Colorectal cancer			
Age CRC (median, IQR)	53 years [45-62]	46 years [40-61]	0.21
Proximal CRC	28% (185)	83% (10)	<0.001
≥2 CRCs	10% (66)	8% (1)	1.0
Endometrial cancer	3% (11)	0% (0)	1.0
Age EC (median, IQR)	55 years [50-75]		
Multiple LS cancers	4% (27)	0% (0)	1.0
First degree relatives			
Colorectal cancer	55% (358)	42% (5)	0.36
≥2 FDRs with CRC	16% (107)	8% (1)	0.70
Age CRC (median, IQR)	64 years [55-71]	62 years [45-90]	0.68
Endometrial cancer	5% (35)	17% (2)	0.14
≥2 FDRs with EC	0.6% (4)	8% (1)	0.88
Age EC (median, IQR)	55 years [50-64]	37 years [-]	0.24
Other LS cancers	22% (142)	8% (1)	0.48
Second degree relatives			
Colorectal cancer	33% (212)	17% (2)	0.35
≥2 SDRs with CRC	12% (81)	8% (1)	1.0
Age CRC (median, IQR)	62 years [50-74]	39 years [39-]	0.12
Endometrial cancer	3% (22)	8% (1)	0.35
≥2 SDRs with EC	0.3% (2)	8% (1)	0.05
Age EC (median, IQR)	70 years [50-76]	49 years [-]	0.67
Other LS cancers	16% (104)	17% (2)	1.0

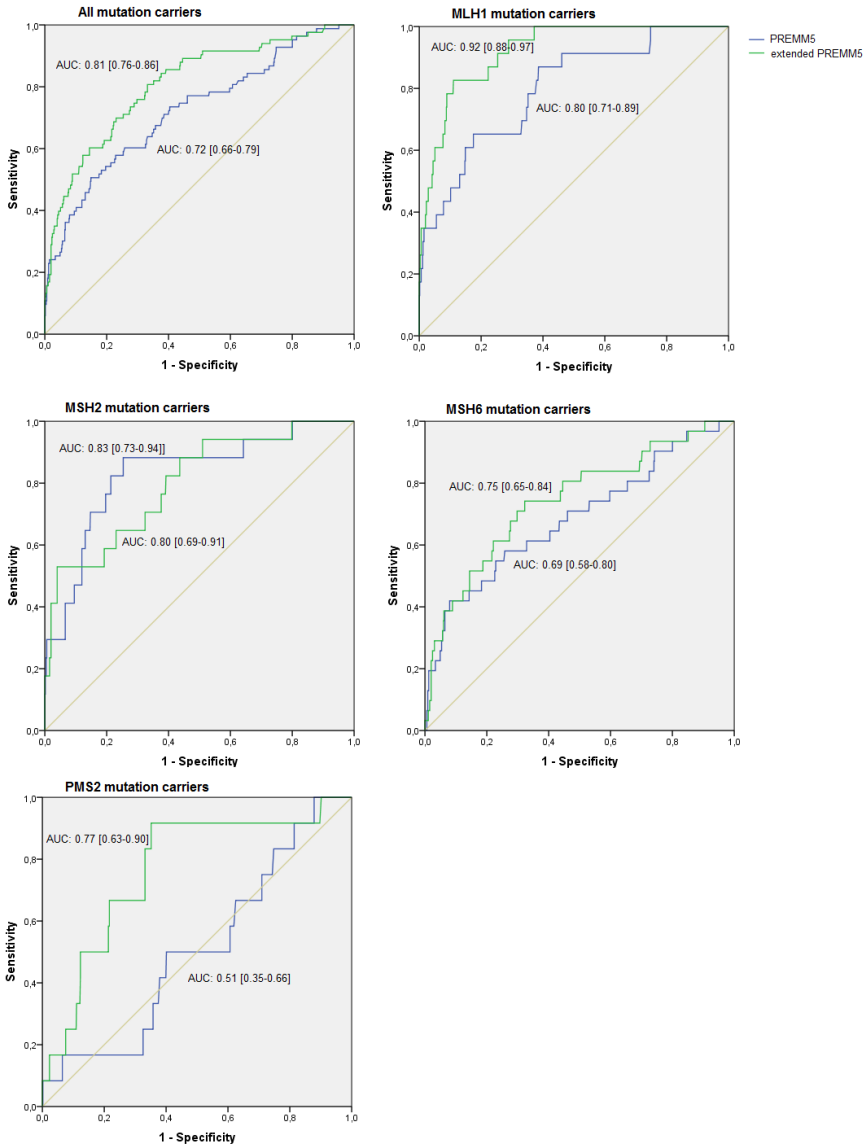
At a 5% cut-off, sensitivity of the extended PREMM5 model was higher than the sensitivity of the original PREMM5 model (92% vs 78%) with similar specificity (45% vs. 46%). Sensitivity and specificity of the extended PREMM5 model at a 5% cut off were both higher than those of the revised Bethesda guidelines (sensitivity 92% vs. 90% and specificity 45% vs. 24%)

Validation of the extended PREMM5 model

In our validation cohort, 60% of the patients were male and median age was 55 years (IQR 45-63 years). The cohort included 31 *MLH1*, 26 *MSH2*, 28 *MSH6* and 73 *PMS2* mutation carriers. Similar to the results in the initial cohort, the extended PREMM5

model had better predictions than the original PREMM5 model for *PMS2* mutation carriers (AUC 0.90 [95% CI 0.86-0.94] vs. 0.82 [95% CI 0.76-0.87]) and overall (AUC 0.92 [95% CI 0.89-0.95] vs. 0.87 [95% CI 0.84-0.91]). Performance for *MLH1, MSH2* and *MSH6* mutation carriers was also slightly better for the extended PREMM5 model than for the original PREMM5 model (AUC 0.97 [95% CI 0.94-1.00] vs. 0.95 [95% CI 0.91-0.99] for *MLH1,* 0.97 [95% CI 0.93-1.00] vs. 0.96 [95% CI 0.92-0.99] for *MSH2* and 0.86 [95% CI 0.97-0.93] vs. 0.85 [95% CI 0.77-0.93] for *MSH6* mutation carriers).

Figure 2. Performance of PREMM5 and the extended PREMM5 model in a clinical setting for all mutation carriers and for individual MMR mutations.



DISCUSSION

The results of our study indicate that while the models MMRpredict and PREMM5 can adequately predict whether an individual is likely to have Lynch syndrome, they fail to identify *PMS2* mutation carriers. The performance of the PREMM5 model improved considerably by adding the location of CRC to the model. In our clinical cohort of 734 CRC patients as well as in a validation cohort of 376 CRC patients, this extended PREMM5 model not only identified *PMS2* mutation carriers more accurately, its overall performance was also better than the original PREMM5 model and the MMRpredict model.

Our results are in line with those of previous studies, where the $PREMM_{1,2,6}$ model had a slightly better overall performance than MMRpredict.(22, 32, 33) The first PREMM model, $PREMM_{1,2}$ also performed better than MMRpredict in several studies(23, 24), but had similar(25, 26) or less accurate(21) predictions in other studies. A recent meta-analysis also found pooled AUCs to be higher for the PREMM model than for MMRpredict (AUC 0.84 vs. 0.81).(27)

Although PREMM5 had better overall predictions, MMRpredict had a better performance for *PMS2* mutation carriers specifically. An explanation for this could be that the location of CRC is incorporated in the MMRpredict model but not in the $PREMM_5$ model. Proximal location of CRC is a known predictor for Lynch syndrome and in our cohort was the only significant difference between *PMS2* mutation carriers and non-mutation carriers. After adding this new variable to the existing $PREMM5_5$ model, this new model performed better than MMRpredict for *PMS2* mutation carriers. The extended $PREMM5_5$ model also performed better than the original model for *MLH1, MSH2* and *MSH6* mutation carriers and had a better overall performance.

In our validation cohort, all AUCs were much higher than in our original cohort, including those for *PMS2* mutation carriers. Selection of patients for analysis of MSI and IHC may have been less stringent at the Erasmus Medical Center Rotterdam than at the Leiden University Medical Center. Therefore, mutation carriers in our validation cohort, who were all from Leiden University Medical Center, may have had a family history more suspect for Lynch syndrome than family history of the patients in our original cohort. This could explain the higher AUCs in the validation cohort. However, in both cohorts we showed that the extended PREMM5 had better performance.

Prediction models for Lynch syndrome are not yet regularly used in current clinical practice. However, the US Multi-Society Task Force on Colorectal Cancer recommends genetic evaluation if an individual's risk of carrying an MMR gene mutation is ≥5% according to one of the prediction models MMRpro, MMRpredict or PREMM.(34) The American guideline recommends that all CRC patients undergo routine screening for LS by analysis of MSI and IHC(34), while current European guidelines recommend such routine screening in at least all CRC patients up to 70 years of age.(35) A recent study demonstrated that routine screening for LS without an age cut-off is not cost-effective.(36) A strategy using prediction models might lower the cost of screening for LS. In fact, two cost-effectiveness analyses found that strategies including prediction models were more cost-effective than those involving direct tumour testing of all CRC patients, if these prediction models were perfectly implemented.(36, 37) Additionally, prediction models could also be used in cases where no tumour tissue is available or where tumour tissue analysis failed, to assess whether an individual should be analyzed for a germline MMR mutation.

The US Multi-Society Task Force on Colorectal Cancer recommends the use of either PREMM, MMRpredict or MMRpro to assess the probability of an individual carrying an MMR mutation.(34) Since we did not include the MMRpro model in our analysis, we do not know how MMRpro would have performed in our cohort. However, MMRpro is less useful in clinical practice since extensive family data is needed as input for the model. Collection of this kind of data is very time consuming and therefore not suitable for clinical practice. PREMM5 and MMRpredict are web-based models that are easily accessible and therefore much easier to use. Also, multiple studies - including the recent meta-analysis – have shown MMRpro to have similar accuracy to $PREMM_{1,2,6}$.(21-27, 32)

Both PREMM5 and MMRpredict were far more accurate for *MLH1* and *MSH2* mutation carriers than for LS patients carrying a mutation in *MSH6* or *PMS2*. This finding is in line with a previous study that showed that carriers of mutations in *MSH6* or *PMS2* had lower risk scores than carriers of a mutation in *MLH1* or *MSH2*.(21) In our study, discrimination between non-mutation carriers and *PMS2* mutation carriers was the least accurate, in line with its more limited penetrance.

Around 15% of all Lynch syndrome cases are estimated to be caused by *PMS2* mutations.(38) In our cohort, 14% (12/83) of the Lynch syndrome patients were *PMS2* mutation carriers. To our knowledge, our study is the first to validate LS prediction models for *PMS2* mutation carriers specifically since the development of the PREMM5 model. At a 5% cut-off, our extended PREMM5 model was able to detect five out of six *PMS2* mutation carriers who would have been missed by the original PREMM5 model at

the same cut-off. Identification of Lynch syndrome carriers is highly important, since this allows not only them, but also their family members carrying the same mutation, to undergo intensive surveillance in order to prevent the development of cancer. Our new model would also identify more Lynch syndrome patients overall than the original PREMM5 model.

The performance of prediction models can differ between high-risk settings and population-based cohorts. Further validation studies should indicate whether our results can be generalized to settings with patients at low to median risk of having Lynch syndrome. Since patients in our study cohort were all referred for genetic counselling, family histories were obtained in detail and in many cases also verified by medical documents. In other settings where patients are at lower risk of having Lynch syndrome, family history is not verified and might be less reliable. Therefore, prediction models should also be validated in population-based cohorts. However, in a meta-analysis, prediction models performed better in population-based cohorts than in clinic-based cohorts.(27)

It is not known whether the current prediction models for Lynch syndrome are useful in non-Western populations. In a recent study among Korean patients, $PREMM_{1,2,6}$ was more accurate than MMRpro and MMRpredict, but still only reached an AUC of 0.71.(32) There was no association between tumour location and mutation status, so our extended PREMM5 model might not improve predictions in populations of non-Western ethnicity. However, germline analysis for *PMS2* was not performed in the Korean study, so there might have been more mutation carriers in their cohort. Another non-Western population has been studied by Khan et al, who analyzed the performance of prediction models in 15 African American patients.(22) In these patients, MMRpredict and $PREMM_{1,2,6}$ both had a high AUC of 0.89.

A main strength of our study was the large cohort, which consisted of more than 700 index patient including 83 Lynch syndrome patients. Also, our cohort included patients with *MSH6* and *PMS2* mutations. Since 12 patients were identified as a *PMS2* mutation carrier, we were able to evaluate the prediction models for each MMR mutation specifically, admittedly with considerable uncertainty.(39) Furthermore, we validated the extended PREMM5 model in a separate cohort of 376 patients including 73 *PMS2* mutation carriers.

A limitation of our study was that germline mutation analysis was not done for all index patients. Patients who had microsatellite stable tumours with normal IHC were assumed to be non-mutation carriers. However, some of these patients might still have an MMR

mutation. Also, the sample size per gene was still relatively small and it is unclear whether our results from a high-risk population apply to a population-based setting.

In conclusion, we have shown that although MMRpredict and PREMM5 can accurately predict an individual's risk of carrying a causative MMR mutation, neither model is able to identify patients with *PMS2* mutations. Adding the location of CRC to the PREMM5 model improves the performance of the model for *PMS2* mutation carriers as well as its overall performance. These findings should be validated in large cohorts from population-based settings.

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SUPPLEMENTAL MATERIAL

Equation PREMM5 model

Predicted probability of any mismatch repair gene mutation: p(any)= predicted probability of *MLH1* mutation + predicted probability of *MSH2/EPCAM* mutation + predicted probability of *MSH6* mutation+ predicted probability of *PMS2* mutation.

Predicted probability of a mutation in *MLH1*: p(*MLH1*)
Predicted probability of a mutation in *MSH2* or *EPCAM:* p(*MSH2/EPCAM*)
Predicted probability of a mutation in *MSH6:* p(*MSH6*)
Predicted probability of a mutation in *PMS2:* p(*PMS2*)
Predicted probability of no mutation: p(none) = 1- [p(*MLH1*) + p(*MSH2*) + p(*MSH6*) + p(PMS2)]

p(*MLH1*)= exp (lp(*MLH1*)) / [(1 + exp((lp(*MLH1*)) + exp(lp(*MSH2/EPCAM*)) + exp(lp(*MLH6*)) + exp(lp(PMS2)))]
p(*MSH2/EPCAM*)= exp (lp(*MSH2/EPCAM*)) / [(1 + exp((lp(*MLH1*)) + exp(lp(*MSH2/EPCAM*)) + exp(lp(*MLH6*)) + exp(lp(PMS2)))]
p(*MSH6*)= exp (lp(*MSH6*)) / [(1 + exp((lp(*MLH1*)) + exp(lp(*MSH2/EPCAM*)) + exp(lp(*MLH6*)) + exp(lp(PMS2)))]
p(*PMS2*= exp (lp(*PMS2*)) / [(1 + exp((lp(*MLH1*)) + exp(lp(*MSH2/EPCAM*)) + exp(lp(*MLH6*)) + exp(lp(PMS2)))]

lp(*MLH1*) = -5.402 + (0.901*V0) + (2.586*V1) + (3.171*V2) + (1.620*V3) + (1.275*V4) + (1.578*V5) + (0.804*V6) + (0.391*V7) + (-0.594*(V8/10)) + (0.122*(V9/10)) + (-0.458*(V10/10)).

lp(*MSH2/EPCAM*) = -4.480 + (0.933*V0) + (1.799*V1) + (2.586*V2) + (1.922*V3) + (1.582*V4) + (1.353*V5) + (0.670*V6) + (0.605*V7) + (-0.468*(V8/10)) + (0.004*(V9/10)) + (-0.470*(V10/10)).

lp(*MSH6*) = -4.672 + (0.815*V0) + (1.266*V1) + (-53.181*V2) + (1.755*V3) + (0.536*V4) + (0.549*V5) + (0.916*V6) + (0.315*V7) + (-0.099*(V8/10)) + (0.352*(V9/10)) + (-0.363*(V10/10)).

lp(PMS2) = -4.922 + (0.293*V0) + (0.990*V1) + (-0.353*V2) + (0.739*V3) + (0.394*V4) + (0.003*V5) +
(-0.425*V6) + (-0.105*V7) + (-0.089*(V8/10)) + (0.006*(V9/10)) + (-0.071*(V10/10)).

Equation based on published equation and personal communications with F Kastrinos.
All variables are equal to the original PREMM5 model: Kastrinos et al, JCO 2017.

Equation extended PREMM5 model

Predicted probability of any mismatch repair gene mutation: p(any)= predicted probability of *MLH1* mutation + predicted probability of *MSH2/EPCAM* mutation + predicted probability of *MSH6* mutation+ predicted probability of *PMS2* mutation.

Predicted probability of a mutation in *MLH1*: p(*MLH1*)
Predicted probability of a mutation in *MSH2* or *EPCAM:* p(*MSH2/EPCAM*)
Predicted probability of a mutation in *MSH6:* p(*MSH6*)
Predicted probability of a mutation in *PMS2:* p(*PMS2*)
Predicted probability of no mutation: p(none) = 1- [p(*MLH1*) + p(*MSH2*) + p(*MSH6*) + p(PMS2)]

p(*MLH1*)= exp (lp(*MLH1*)) / [(1 + exp((lp(*MLH1*)) + exp(lp(*MSH2/EPCAM*)) + exp(lp(*MLH6*)) + exp(lp(PMS2)))]
p(*MSH2/EPCAM*)= exp (lp(*MSH2/EPCAM*)) / [(1 + exp((lp(*MLH1*)) + exp(lp(*MSH2/EPCAM*)) + exp(lp(*MLH6*)) + exp(lp(PMS2)))]
p(*MSH6*)= exp (lp(*MSH6*)) / [(1 + exp((lp(*MLH1*)) + exp(lp(*MSH2/EPCAM*)) + exp(lp(*MLH6*)) + exp(lp(PMS2)))]
p(*PMS2*= exp (lp(*PMS2*)) / [(1 + exp((lp(*MLH1*)) + exp(lp(*MSH2/EPCAM*)) + exp(lp(*MLH6*)) + exp(lp(PMS2)))]

lp(*MLH1*) = -7.010 + (0.677*V0) + (1.942*V1) + (2.381*V2) + (1.216*V3) + (0.957*V4) + (1.185*V5) + (0.604*V6) +(0.294*V7) + (-0.446*(V8/10)) + (0.092*(V9/10)) + (-0.344*(V10/10)) + (3.280*V11).

lp(*MSH2/EPCAM*) = -5.269 + (0.868*V0) + (1.674*V1) + (2.407*V2) + (1.789*V3) + (1.472*V4) + (1.259*V5) + (0.624*V6) + (0.563*V7) + (-0.436*(V8/10)) + (0.004*(V9/10)) + (-0.437*(V10/10)) + (0.728*V11).

lp(*MSH6*) = -4.005 + (0.796*V0) + (1.237*V1) + (1.520*V2) + (1.714*V3) + (0.524*V4) + (0.536*V5) + (0.895*V6) + (0.308*V7) + (-0.097*(V8/10)) + (0.344*(V9/10)) + (-0.355*(V10/10)) + (0.868*V11).

lp(PMS2) = -5.511 + (0.040*V0) + (0.134*V1) + (-0.048*V2) + (0.100*V3) + (0.053*V4) + (0.000*V5) + (-0.058*V6) + (-0.014*V7) + (-0.012*(V8/10)) + (0.001*(V9/10)) + (-0.010*(V10/10)) + (2.540*V11).

V11: Side of colorectal cancer. Enter 0 for left-sided, enter 1 for right-sided.
All other variables are equal to the original PREMM5 model.

Table S1. Predicted mutation probability with PREMM5 and MMRpredict by mutation status

	Total, median (IQR)	No mutation, median (range)	*MLH1* mutation, median (range)	*MSH2* Mutation, median (range)	*MSH6* mutation, median (range)	*PMS2* mutation, median (range)	Any mutation median (range)
N	734	651	23	17	31	12	83
PREMM5	0.08 (0.04-0.15)	0.07 (0.04-0.13)	0.26 (0.14-0.64)	0.27 (0.12-0.72)	0.19 (0.07-0.39)	0.07 (0.04-0.15)	0.19 (0.08-0.42)
MMRpredict	0.03 (0.01-0.11)	0.02 (0.01-0.09)	0.21 (0.05-0.85)	0.30 (0.05-0.88)	0.07 (0.02-0.43)	0.10 (0.04-0.54)	0.12 (0.03-0.65)

Table S2. Sensitivity and specificity for clinical criteria and prediction models according to different cut-offs

Model	Cut-off	N	All mutation carriers		*MLH1*	*MSH2*	*MSH6*	*PMS2*
			Sensitivity (%)	*Specificity (%)*	*Sensitivity (%)*	*Sensitivity (%)*	*Sensitivity (%)*	*Sensitivity (%)*
Revised Bethesda guidelines		569	90	24	96	94	87	83
PREMM5	≥5%	507	87	33	96	88	87	67
	≥10%	285	69	65	83	88	61	33
	≥20%	129	49	87	56	65	48	17
MMRpredict								
	≥5%	276	70	67	74	77	65	67
	≥10%	189	53	78	65	65	39	50
	≥20%	117	43	88	52	59	39	42

Chapter 4

Cost-effectiveness of routine screening for Lynch syndrome in colorectal cancer patients up to 70 years of age

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ABSTRACT

Purpose To assess the cost-effectiveness of routine Lynch syndrome (LS) screening among colorectal cancer (CRC) patients ≤70 years of age.

Methods A population-based series of CRC patients ≤70 years was routinely screened for LS. We calculated life years gained (LYG) and incremental cost-effectiveness ratios (ICERs) for different age cut-offs and comparing age-targeted screening with the revised Bethesda guidelines.

Results Screening 1117 CRC patients identified 23 LS patients, of whom 7 were ≤50, 7 were 51-60 and 9 were 61-70 years. Additionally, 70 LS carriers were identified among relatives (14, 42 and 14 per age category). Screening amounted to 205.9 LYG or 43.6, 118.0 and 44.3 LYG per age category. ICERs were €4.226/LYG for screening CRC patients ≤60 years compared with ≤50 years and €7.051/LYG for screening CRC patients ≤70 years compared with ≤60 years. The revised Bethesda guidelines identified 70/93 (75%) LS carriers. The ICER for LS screening in CRC patients ≤70 years compared with the revised Bethesda guidelineswas €7.341/LYG. All ICERs remained <€13.000/LYG in one-way sensitivity analyses.

Conclusion Routine LS screening by analysis of microsatellite instability, immuno-histochemistry and *MLH1* hypermethylation in CRC patients ≤70 years is a cost-effective strategy with important clinical benefits for CRC patients and their relatives.

INTRODUCTION

Lynch syndrome (LS) is the most common hereditary colorectal cancer (CRC) syndrome, responsible for 2-3% of all CRC cases.(1-3) This syndrome is characterized by early onset of CRC, endometrial cancer and other extracolonic cancers.(4) Mutations in one of the four mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* or the *EPCAM* gene are the underlying defect in LS.(5) Detection of LS in CRC patients is of great importance, since affected patients and family members can benefit from LS surveillance programs, which reduce CRC incidence and mortality by 56-70%.(6, 7)

Molecular diagnostics on tumour tissue consisting of analysis for microsatellite instability (MSI) and immunohistochemical staining (IHC) for loss of MMR protein expression can identify patients at high risk of having LS.(8, 9) However, loss of MLH1 protein expression can also occur in sporadic tumours as a result of somatic *MLH1* promoter hypermethylation. Therefore, sporadic MLH1 deficient tumours can be distinguished from LS-associated tumours by *MLH1* hypermethylation analysis.(9)

The revised Bethesda guidelines have been developed to select patients eligible for MSI testing and IHC analysis based on clinical criteria.(10) These guidelines are poorly applied in clinical practice and may miss a substantial number of LS patients because of limited sensitivity.(11) Routine analysis of MSI and IHC was previously recommended in CRC patients under 50 years of age.(12) This strategy predominantly fails to identify *MSH6* and *PMS2* mutation carriers, since the mean age of CRC diagnosis in these subjects is above the age of 50 years.(13, 14) Routine screening for LS has been proposed to improve LS detection, but age cut-offs are still under debate.(15-17) Recently, the US Multi-Society Task Force on Colorectal Cancer as well as a European group of experts recommended routine LS screening by analysis of MSI or IHC and *MLH1* hypermethylation in CRC patients.(18, 19) The US guidelines support universal tumor testing for LS, while the European guidelines recommend routine LS screening in all CRC patients or in CRC patients up to 70 years of age.

We previously reported that routine analysis of MSI and IHC for MMR proteins revealed a profile compatible with LS in 4.5% of CRC patients ≤70 years of age.(20) Many of these patients were over 50 years of age.(20) The current study aimed to assess the cost-effectiveness of routine screening for LS by analysis of MSI, IHC and *MLH1* hypermethylation in CRC patients ≤70 years of age. We compared costs and health benefits for age-targeted LS screening up to 70 years of age. Also, we compared routine LS screening among CRC patients up to age 70 with LS screening based on the revised Bethesda guidelines.

METHODS

Subjects and diagnostic work-up

The present study is an extension of a prospective population-based study on the yield of routine molecular screening for LS in CRC patients up to 70 years of age.(20) Consecutive CRC patients ≤70 years of age (n=1117) from 11 Dutch hospitals were included between May 2007 and September 2009. The diagnostic approach and methods regarding tumour analyses and germline mutation analyses have been described in detail elsewhere.(20) In summary, MSI analysis and IHC for MLH1, MSH2, MSH6 and PMS2 protein expression were performed in tumour tissue of CRC patients ≤70 years of age. *MLH1* hypermethylation analysis was performed in cases with loss of MLH1 protein expression. *BRAF* mutation analysis was not included in this cost study, since previous studies have shown that *MLH1* promoter hypermethylation analysis is a superior prescreening method compared to *BRAF* mutation analysis.(21) In case tumours showed a high degree of MSI and/or absence of MMR protein without *MLH1* promoter hypermethylation, patients were suspected of having LS. These patients were offered genetic counselling and germline mutation analysis (Figure 1). In case patients suspected of having LS had deceased before they could be referred to a clinical geneticist, genetic counselling was offered to their first degree relatives. In the Netherlands, costs for genetic counselling and germline mutation analysis are covered by the mandatory basic health insurance.

If a pathogenic germline mutation was identified in one of the MMR genes or the *EPCAM* gene, patients were labelled index patients. Relatives were contacted by index patients and were offered genetic counselling and targeted mutation analysis. We collected data on the number of relatives accepting counselling and targeted mutation analysis and the number of LS carriers identified among these relatives until May 2014. This study was approved by the Institutional Review Boards of the participating hospitals.

Effectiveness

Effectiveness of LS screening was expressed in life years gained (LYG), based on the number of LS carriers detected among CRC patients and their relatives and using estimations from literature. In previous studies LS surveillance was associated with 0.09-2.5 LYG for index patients and 0.49-32.69 LYG for relatives.(12, 16, 17, 22-29) For our analysis, we directly used the reported 3% discounted LYG from previous studies. If only undiscounted LYG or LYG with a different discount rate were reported, we discounted them by 3% annually (Supplementary information, Table S1). If adherence to LS surveillance programs was not included in the reported LYG, we corrected the LYG by assuming adherence to these programs of 80% for both index patients and LS carriers among their relatives.(16) We used the median of all estimations from the literature in

our base case scenario (Table 1). If the index patient had deceased, only relatives were considered to benefit from surveillance. For CRC patients and relatives without a pathogenic mutation identified, we assumed no surveillance costs or benefits.

Costs

Direct medical costs of all analyses in the diagnostic work-up were determined following the microcosting method, which is based on comprehensive bottom-up analyses.(30) Cost data included costs of employment, material, equipment and overhead, which were obtained from the Department of Pathology and the Department of Clinical Genetics of the Erasmus MC, University Medical Center Rotterdam (Supplementary information, Table S2). Costs for *PMS2* germline mutation analysis were assumed to be similar to cost for germline mutation analysis of other MMR genes. Total costs were calculated based on the number of CRC patients and relatives analyzed. The costs for MMR gene sequencing in index patients were calculated using the total number of genes analyzed. LS surveillance costs for index patients and relatives were estimated from previous literature including costs for colonoscopy, transvaginal ultrasonography and endometrial biopsy (Table 1 and Table S2).(16, 31) Costs for gynaecological screening were only available in dollars and were converted to euros using purchasing power parity. All costs were converted to price level 2013 using the Dutch consumer price index.(32) Surveillance by colonoscopy with polypectomy every two years was assumed to start at the age of LS diagnosis or at age 25 for relatives under 25 years of age. LS surveillance was assumed to be continued until 75 years of age. For cost savings by prevention of CRC in surveillance programs, the most conservative estimate i.e. only treatment costs for the first 12 months of stage I CRC was used (Table S2). Female LS carriers were assumed to receive yearly gynaecological surveillance by transvaginal ultrasonography and endometrial biopsy starting at age 35 and continued until prophylactic surgery at 40 years of age, after childbearing is completed. Prophylactic surgery (total abdominal hysterectomy and bilateral salpo-oophorectomy) was assumed to be accepted by 19% of the index patients and 18% of their relatives.(16) LS carriers not accepting prophylactic surgery were assumed to continue yearly gynaecological surveillance up to 75 years of age. All costs were discounted by 3% annually.

Cost-effectiveness analyses

We evaluated cost-effectiveness of LS screening using a base-case cost-effectiveness model (i.e. using the most plausible parameter values), and age cut-offs of 60, and 70 years from a health care provider perspective. LS screening for CRC patients ≤50 years was the reference strategy. Incremental cost-effectiveness ratios (ICERs) per age cut-off were expressed as additional costs per LYG. In order to test the robustness of ICERs we performed one-way sensitivity analyses. Costs were assumed to range from 0.5 to 2

times as much as calculated. Ranges for all other parameters were based on literature (Table 1).

Fulfilment of the revised Bethesda guidelines

The proportion of CRC patients fulfilling the revised Bethesda guidelines was based on the literature (Table 1). In an unselected population, 26-50% of CRC patients fulfil the revised Bethesda guidelines.(1, 3, 33) We assumed only 26% of the CRC patients in our cohort fulfilled these guidelines, since this approach is unfavourable for an age-targeted screening strategy. For all index patients, a detailed family history was obtained during genetic counselling and fulfilment of the revised Bethesda guidelines was assessed by one clinical geneticist (AW).

Table 1. Parameters and values used in the cost-effectiveness analysis

Parameter	Base case	Range	Source
Median age at LS diagnosis			
Index patients	57	IQR 49-63	(20)
Relatives	41	IQR 32-56	Current study
Female index patients	61	IQR 53-66	Current study
Female relatives	38	IQR 29-56	Current study
LS surveillance			
Discounted life years gained[†]			
Female index patients	0.66	0.191-2.15	(12, 16, 23, 24, 26, 27)
Male index patients	0.66	0.092-2.15	(12, 16, 23, 24, 26, 27)
Female relatives	2.83	0.40-16.02	(12, 16, 17, 22-29)
Male relatives	2.83	0.47-16.47	(12, 16, 17, 22-29)
Interval between colonoscopies (years)	2	1-2	(18, 19)
Complication rate of colonoscopy	0.0024		(31)
Acceptance of prophylactic gynaecological surgery			
Index patients	0.19	0.10-0.30	(16)
Relatives	0.18	0.03-0.25	(16)
CRC risk and risk reduction			
Lifetime risk of developing CRC for LS carriers	0.25	0.25-0.70	(6, 7, 28, 34)
Reduction in CRC risk by LS surveillance	0.56	0.56-0.70	(6, 7)
Revised Bethesda guidelines			
Proportion of CRC patients fulfilling the revised Bethesda guidelines in an unselected CRC population	0.26	0.26-0.50	(1, 35)

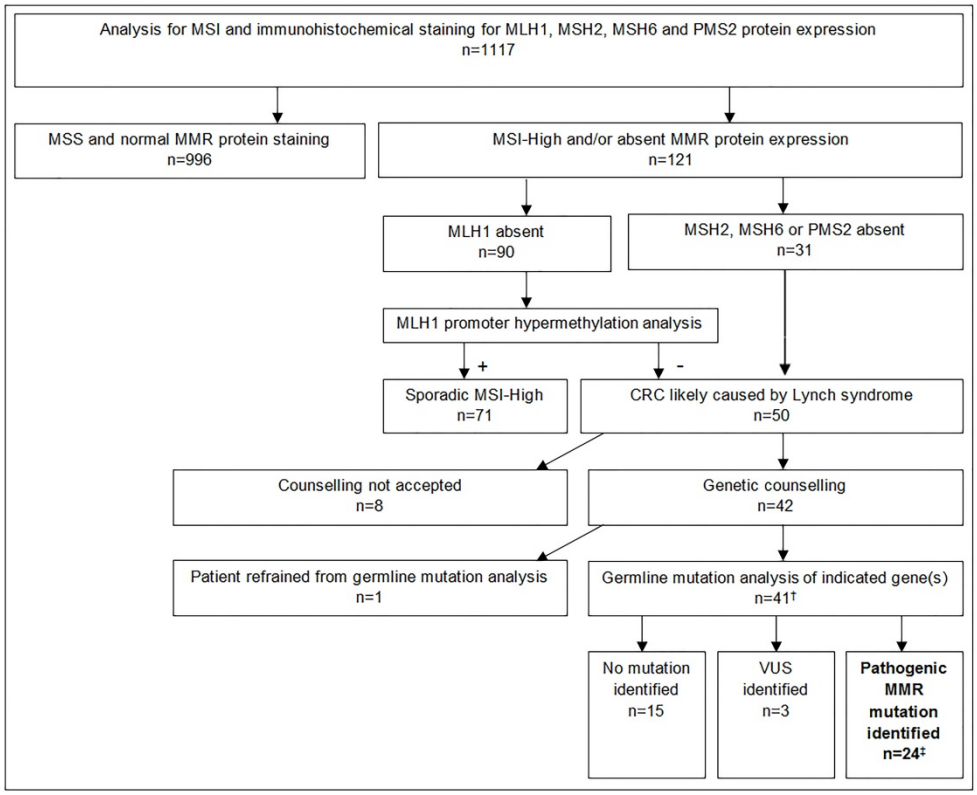
LS: Lynch syndrome; CRC: Colorectal cancer.
[†] Life years gained were discounted by 3% annually

RESULTS

In our population-based cohort 50 out of 1117 CRC patients (4.5%) were suspected of having LS by routine analysis of MSI and IHC (Figure 1). Consecutive MMR gene sequencing in 42 of these CRC patients finally identified 24 LS patients (2.1%). In one case the germline mutation was identified in stromal tissue resected along with the CRC

tissue. Since this patient nor any relatives were available for MMR gene sequencing, this patient was not considered an index patient.

Figure 1. Diagnostic work-up to detect Lynch syndrome among colorectal cancer patients ≤70 years.



MSI: microsatellite instability; MSS: microsatellite stable; MMR: mismatch repair; MSI-High, high degree of MSI; CRC; colorectal cancer; VUS: variant of unknown significance.

† In 4/41 cases germline mutation analysis was performed in a first-degree relative.

‡ In one patient a pathogenic MMR mutation was identified in stromal tissue resected during colorectal cancer surgery. This patient nor any family members were available for germline mutation analysis and this patients was excluded from the analyses.

Effectiveness of age-targeted strategies

The median age of CRC patients was 61 years (IQR 55-66); 144 CRC patients were ≤50 years, 377 CRC patients 51-60 years and 596 CRC patients 61-70 years of age. The prevalence of LS decreased from 4.9% (7/144) in the age category ≤50 years to 2.1% (8/377) in CRC patients 51-60 years of age and 1.5% (9/596) in CRC patients 61-70 years of age (Table 2).

For index patients ≤50 years of age a total of 29 first degree relatives were eligible for targeted mutation analysis, compared with 44 and 40 first degree relatives in the age categories 51-60 years and 61-70 years respectively. Genetic counselling and targeted mutation analysis was offered to these relatives and cascaded to further relatives if indicated. For each index patient a median of 3 (IQR 2-8) relatives finally accepted counselling and germline targeted mutation analysis. There was a wide range from 1-37 relatives tested for LS. In total, targeted mutation analysis was accepted by 140 relatives, identifying 70 additional LS carriers. Notably, over three times as many LS carriers were identified among relatives of CRC patients 51-60 years of age as in the other age categories (Table 2). This difference was partly attributable to one index patient in the 51-60 age category with 37 relatives tested and 16 LS carriers identified.

Based on a median estimated benefit of LS surveillance of 0.66 years per index patient and 2.83 years per relative, a total of 205.9 life years were estimated to be gained by LS screening in CRC patients ≤70 years of age. Surveillance of relatives led to the highest benefit with a total of 192.7 LYG compared with a total of 13.2 LYG for index patients.

Table 2. Number of patients screened and detection of Lynch syndrome among CRC patients and relatives

Colorectal cancer patients (n=1117)	**<50 years**	**51-60 years**	**61-70 years**	**Revised Bethesda guidelines**	**Total**
LS diagnostics in CRC patients					
Analysis for MSI and IHC	144	377	596	290	1117
MLH1 hypermethylation analysis	6	21	65	6	92
CRC patients suspected of having LS	15	15	20	27	50
Genetic counseling	12	13	17	25	42
Germline mutation analysis	11	13	17	23	41
Genes tested in CRC patients or FDR	18	22	30	30	70
LS index patients identified	7	7[†]	9	17	23[†]
Female LS index patients identified	1	3	5	8	9
LS diagnostics in relatives					
Relatives accepting genetic counseling	25	78	38	99	141
Germline mutation analysis	25	77	38	98	140
LS carriers identified among relatives	14	42	14	53	70
Female LS carriers identified among relatives	11	23	6	32	40
Life years gained					
LYG by male index patients	3.3	2.6	1.3	0.6	7.3
LYG female index patients	0.7	2.0	3.3	1.5	5.9
LYG by male relatives	8.5	51.0	22.7	10.1	82.2
LYG by female relatives	31.2	62.3	17.0	15.0	110.5
Total LYG (index and relatives)	**43.6**	**118.0**	**44.3**	**27.3**	**205.9**

Numbers of life years gained may not add up due to rounding.

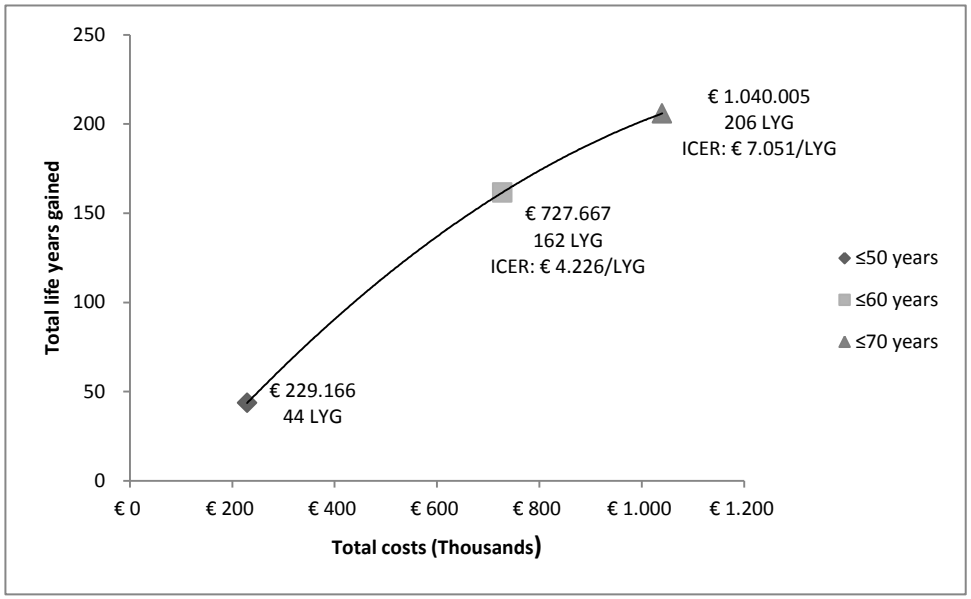
LS: Lynch syndrome; CRC: Colorectal cancer; MSI: Microsatellite instability; IHC: Immunohistochemistry; FDR: First degree relative; LYG: Life years gained.

[†] In one additional case a germline mutation was identified in stromal tissue resected along with the CRC tissue. This patient was not considered an index patient, since the patient nor any relatives were available for germline mutation analysis.

Costs and cost-effectiveness

Total costs for LS molecular screening and subsequent surveillance increased from €229.166 (€10.931 per LS carrier detected) for CRC patients ≤50 years of age to €1.040.005 (€11.183 per LS carrier detected) for CRC patients ≤70 years of age (Figure 2).

Figure 2. Total costs and life years gained (LYG) for Lynch syndrome screening in colorectal cancer patients ≤50 years of age, ≤60 years of age and ≤70 years of age. ICERs (Incremental cost-effectiveness ratios) are expressed as incremental cost per additional LYG compared with the previous strategy.



LS screening for CRC patients ≤60 years of age had an ICER of €4.226/LYG compared with screening patients ≤50 years of age. The ICER of LS screening in CRC patients ≤70 years of age compared with screening CRC patients ≤60 years of age was €7.051 per LYG (Table 3).

Table 3. Incremental costs in 2013 euro for Lynch syndrome screening in CRC patients of different age categories

Colorectal cancer patients (n=1117)	**<50 years**	**51-60 years**	**61-70 years**	**Total**
Lynch syndrome diagnostics				
CRC patients				
Molecular diagnostics	€ 32.914	€ 86.697	€ 140.220	€ 259.831
Genetic counseling	€ 3.574	€ 3.872	€ 5.064	€ 12.510
MMR gene sequencing	€ 9.680	€ 11.832	€ 16.134	€ 37.646
Relatives				
Genetic counseling	€ 4.003	€ 13.009	€ 6 .171	€ 23.183
Targeted mutation analysis	€ 7.297	€ 23.410	€ 11.249	€ 41.955
Lynch syndrome surveillance				
Colonoscopy surveillance				
Index patients	€ 27.929	€ 19.924	€ 12.358	€ 60.211
Relatives	€ 61.226	€ 174.933	€ 61.226	€ 297.385
Gynaecologic surveillance and prophylactic surgery				
Index patients	€ 5.754	€ 24.263	€ 25.634	€ 55.651
Relatives	€ 91.970	€ 183.939	€ 49.465	€ 325.374
Savings by prevention of CRC	- € 15.182	- € 43.378	- € 15.182	- € 73.743
Total costs (minus savings)	€ 229.166	€ 498.501	€ 312.338	€ 1.040.005
Total life years gained	43.6	118.0	44.3	205.9
Costs per life year gained	reference	€ 4.226	€ 7.051	-

DISCUSSION

Our economic evaluation indicates that routine screening for LS in CRC patients ≤70 years of age by analysis of MSI, IHC and *MLH1* hypermethylation is cost-effective according to currently accepted standards. In a one-way sensitiviy analysis, expanding routine screening for LS from CRC patients ≤50 years of age to CRC patients ≤60 years of age never exceeded €10.000/LYG. Costs for LS screening among CRC patients 61-70 years of age were €7.051/LYG in our base case analysis and remained <€13.000/LYG in one-way sensitivity analysis. The cost-effectiveness threshold of any diagnostic strategy depends on a healthcare system's willingness to pay for each LYG. In the Dutch healthcare system, willingness to pay depends on severity of the disease and most interventions will be considered cost-effective if costs remain under €40.000/LYG.(36) In the UK and US a threshold of $50.000/LYG (approximately €40.000/LYG) is commonly used in cost-effectiveness analyses for cancer screening. However, thresholds over $50.000/LYG can also be justified.(37)

Our sensitivity analysis confirmed the finding of other studies that the assumed benefit (LYG) from LS surveillance has a tremendous effect on ICERs, especially LYG assumed for relatives.(12, 16, 17, 23, 24, 27) The benefit of LS surveillance programs for relatives that we estimated from literature ranged from 0.40 LYG to 16.74 LYG per relative (Table 1 and Table S1). These extreme differences reflect the impact of assumptions made on uncertain parameters such as CRC risk for LS carriers, the method and risk reduction of

LS surveillance and assumed adherence to LS surveillance programs. In our base case analysis we used the median of all estimations from literature to attain plausible estimates. In our one-way sensitivity analyses we considered the full range of estimates from the literature, which resulted in ICERs well within currently accepted thresholds for cost-effectiveness. Specifically, all ICERs remained under €13.000/LYG.

LS screening in CRC patients ≤70 years of age identified over three times as many LS index patients as only screening CRC patients ≤50 years of age. Also, LS carriers among family members of these index patients were identified. We found a median of 3 relatives that were tested for each index patient. However, there was a very wide range from 1-37 relatives that were tested per index patient for a total of 140 relatives. Interestingly, in our study over three times as many LS carriers were identified among relatives of CRC patients 51-60 years of age compared with the other age categories. This difference was partly caused by a very large familiy with 37 relatives tested and 16 LS carriers identified, which we may consider as a statistical outlier. Furthermore, the 51-60 years age group contained 1.5 times as many first degree relatives eligible for genetic testing compared with index patients ≤50 years of age and had a higher prevalence of LS among tested relatives compared with the 61-70 years age category. An older age of siblings from 61-70 year old CRC patients compared with siblings from younger CRC patients might explain this difference in LS prevalence, due to the reduced life expectancy of LS carriers. Our study may still underestimate the number of LS patients ultimately detected among relatives. Relatives currently refraining from targeted mutation analysis as well as minors not yet eligible for genetic testing could request genetic testing at a later time. Also, CRC patients suspected of LS who currently refrained from genetic testing might opt for MMR gene sequencing in the future, thereby further increasing the identification of LS carriers among CRC patients and their relatives. Further studies are necessary on these issues.

Our results are in line with previous studies using decision-analytic models, in which LS screening by only IHC testing or analysis for MSI for CRC patients >50 years of age was found to be cost-effective.(16, 17, 26, 27) In one study LS screening of CRC patients ≤60 years of age led to an ICER of $33.800/LYG (€25.000/LYG) compared with screening patients ≤50 years of age. Expanding the age limit for LS screening to CRC patients ≤70 years of age resulted in an ICER of $44.200/LYG (€33.000/LYG).(16) In contrast, a recent Dutch study found an ICER of only €2.703 for LS screening in CRC patients ≤70 years of age compared with LS screening of CRC patients ≤50 years of age.(26) However, this study did not include costs for gynaecological surveillance. Furthermore, LYG for relatives in their study was 6.9 to 7.22 years which is higher than assumed in other studies on cost-effectveness of LS screening. Interestingly, the assumed incidence of CRC in LS

carriers was higher than in other studies, which accounts for their high estimate of LYG per relative tested compared to other recent studies on cost-effectiveness of LS screening. Since we used the median of all estimates for LS benefit from the current literature, our ICERs are in between those found by Sie et al. and those found by recent studies assuming benefit for LS carriers among relatives under 1 LYG.

Sensitivity of the revised Bethesda guidelines was 74% in our cohort. In previous literature, the sensitivity of these guidelines was 72-88%.(19) To assess cost-effectiveness of age-targeted LS screening compared with the revised Bethesda guidelines, we assumed only 26% of CRC patients in our cohort fulfilled the revised Bethesda guidelines. We assumed 100% adherence to the revised Bethesda guidelines, while in clinical practice molecular diagnostics for LS may be performed in only 11-14% of the patients fullfilling these guidelines.(11, 38) In a previous study, low rates of failure to apply the revised Bethesda guidelines made LS screening by molecular diagnostics the preferred strategy.(16) In our study, the ICER for LS screening among CRC patients ≤70 years of age compared with testing according to the revised Bethesda guidelines remained <€13.000/LYG. Age-targeted LS screening may be much easier and therefore even more cost-effective to implement in clinical practice than clinical criteria based on family history.

LS screening without any age cut-off is presumed to further increase benefit for LS carriers. US guidelines recommended LS screening of all CRC patients by IHC or MSI analysis as a possible screening strategy.(18) However, it is unclear whether the benefit of universal LS screening will come at acceptable costs. In our population-based cohort, the prevalence of LS decreased with increasing age of CRC diagnosis. Recently, universal tumor testing for LS was not found to be cost-effective by a model constructed by Barzi et al.(22) Interestingly, the combination with predictive models was found to be cost-effective, however only in case of available family history which is known to be an important clinical challenge. In line with these findings, a German research group also concluded that the most cost-effective strategy involved family-history assessment.(25) A recent international validation study confirmed the validity and potential clinical usefulness of prediction models to direct testing.(39)

Strengths of this study are the use of real life data of index patients and their relatives, inclusion of hypermethylation analysis in the diagnostic work-up, our detailed analysis of diagnostic costs, and inclusion of gynaecological surveillance. To our knowledge this study is the first cost analysis for LS screening using cost data and family data directly derived from a prospective population-based cohort of CRC patients. In contrast to studies that fully rely on assumptions in cost-effectiveness models, we aimed to stay

close to prospectively collected data. Furthermore, minimal cost savings by CRC prevention were used in the calculations. In practice, cost savings from LS screening are likely to be much higher.

This study also has several limitations. First, we did not correct LYG for quality of life. As posed by some, being identified as LS carrier might not have an impact on quality of life and it has been suggested that it is not necessary to include quality of life in cost-effectiveness analyses of life saving strategies.(40, 41) However, two previous cost-effectiveness analyses of LS screening did find an impact on the ICER by including quality of life.(17, 42) Secondly, costs and benefit from surveillance for extracolonic cancers other than gynaecological cancers were not included in our analyses, since these are not generally recommended and the actual benefit of such surveillance is unclear. We also did not include costs for prophylactic colectomy or aspirin chemoprevention. The use of chemoprevention by aspirin in LS carriers is not yet implemented as results of the CAPP3 study are pending.(43) Third, we did not perform a full probabilistic sensitivity analysis. Furthermore, in this study we did not evaluate cost-effectiveness of MSI analysis and IHC alone. In previous studies, LS screening by IHC alone was found to be more cost-effective than LS screening by MSI analysis alone or MSI analysis and IHC combined.(16, 17) Finally, in this study we did not include the use of prediction models for LS detection, since detailed family history was not available from all patients. MMRpro, MMRpredict and $PREMM_{1,2,6}$ have been proposed as prescreening tools for LS.(44-46) It has been suggested that a combined strategy using IHC and prediction models among CRC patients <70 years of age improves the cost-effectiveness of LS detection.(22, 47) Prediction models may exclude CRC patients with a minimal risk of having LS from molecular diagnostics. Further research should therefore focus on validation of prediction models in population-based cohorts and evaluate the combination with molecular testing for LS.

In conclusion, routine screening for LS in CRC patients up to 70 years of age is a cost-effective strategy according to currently accepted standards with important clinical benefits for LS carriers among CRC patients and their relatives. Our findings support the recent recommendation for LS screening by analysis of MSI or IHC and *MLH1* hypermethylation in all CRC patients ≤70 years of age.(18, 19)

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SUPPLEMENTAL MATERIAL

Table S1. Data on Life years gained (3% discounted) by index patients and relatives

	Females	Males	Source
Index patients	0,191	0,092	Ladabaum et al 2011
	0,14	0,14	Calculated from Sie et al 2014
	0,53	0,53	Calculated from Snowsill et al 2014
	0,79	0,79	Calculated from Ramsey et al 2003
	0,79	0,79	Calculated from Ramsey et al 2001
	2,15	2,15	Calculated from Kievit et al 2005
Median	**0,66**	**0,66**	
Relatives	0,40	0,47	Calculated from Severin et al 2015
	0,49	0,51	Ladabaum et al 2011
	0,63	0,63	Barzi et al 2015
	0,64	0,64	Calculated from Snowsill et al 2014
	1,07	1,07	Mvundura et al 2010
	2,83	2,83	Calculated from Kievit et al 2005
	3,58	3,58	Calculated from Vasen et al 2010
	6,91	6,91	Calculated from Syngal et al 1998
	8,58	8,58	Calculated from Sie et al 2014
	16,02	16,02	Calculated from Ramsey et al 2001
	16,74	16,74	Calculated from Ramsey et al 2003
Median	**2,83**	**2,83**	

If adherence to LS surveillance programs was not included in the reported LYG, we corrected LYG by assuming adherence of 80% for both index patients and LS carriers among their relatives.

Table S2. Cost data in 2013 euros for Lynch syndrome diagnostics and surveillance

	Cost	Source
Lynch syndrome diagnostics[†]		
Analysis for microsatellite instability	€ 89	Dept. of Pathology, Erasmus MC
IHC testing for MMR protein expression	€ 135	Dept. of Pathology, Erasmus MC
MLH1 hypermethylation analysis	€ 99	Dept. of Pathology, Erasmus MC
Genetic counseling for index patients	€ 298	Dept. of Clinical Genetics, Erasmus MC
Genetic counseling for relatives	€167	Dept. of Clinical Genetics, Erasmus MC
MMR gene sequencing (per gene)	€ 538	Dept. of Clinical Genetics, Erasmus MC
Targeted mutation analysis for relatives	€ 304	Dept. of Clinical Genetics, Erasmus MC
Lynch syndrome surveillance		
Surveillance colonoscopy including polypectomy	€ 393	Goede et al 2013
Treatment of complications after colonoscopy	€ 1.250	Goede et al 2013
Treatment cost of stage I CRC (first 12 months)	€ 12.100	Goede et al 2013
Transvaginal ultrasound	€ 100	Ladabaum et al 2011
Endometrial biopsy	€ 204	Ladabaum et al 2011
Prophylactic hysterectomy and salpo-oophorectomy	€ 14.920	Ladabaum et al 2011

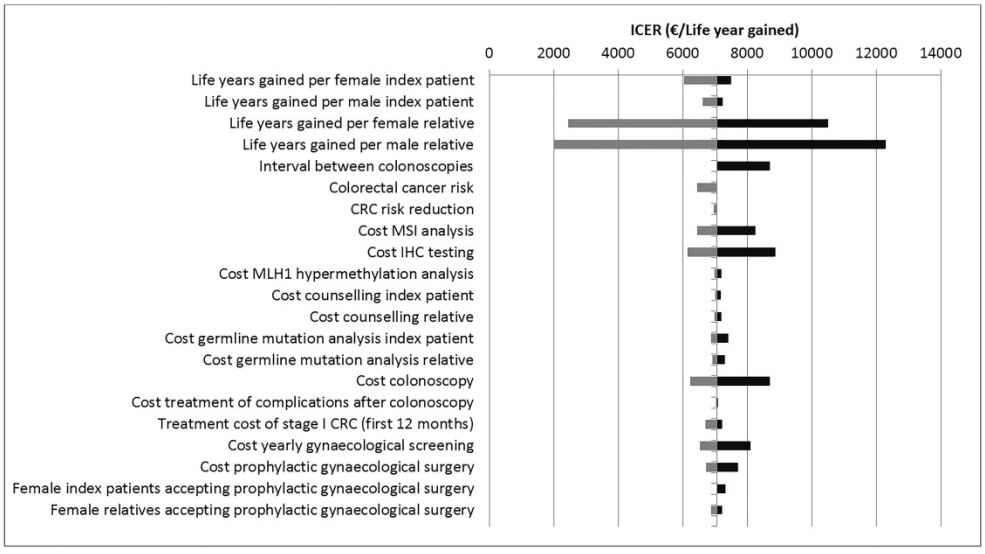
IHC: Immunohistochemistry; MMR: Mismatch repair; CRC: Colorectal cancer.

[†] Costs were determined following the microcosting method and included costs of employment, material, equipment and overhead

Table S3. Incremental costs in 2013 euro for Lynch syndrome screening in CRC patients ≤70 years of age and the revised Bethesda guidelines

Colorectal cancer patients (n=1117)	**Revised Bethesda guidelines**	**Total ≤70 years**
Lynch syndrome diagnostics		
CRC patients		
Molecular diagnostics	€ 65.681	€ 259.831
Genetic counseling	€ 7.447	€ 12.510
MMR gene sequencing	€ 16.134	€ 37.646
Relatives		
Genetic counseling	€ 16.345	€ 23.183
Targeted mutation analysis	€ 29.490	€ 41.955
Lynch syndrome surveillance		
Colonoscopy surveillance		
Index patients	€ 46.184	€ 60.211
Relatives	€ 223.039	€ 297.385
Gynaecologic surveillance and prophylactic surgery		
Index patients	€ 51.121	€ 55.651
Relatives	€ 261.989	€ 325.374
Savings by prevention of CRC	- € 55.307	- € 73.743
Total costs (minus savings)	€ 662.123	€ 1 040.005
Total life years gained	154.4	205.9
Costs per life year gained	Reference	€ 7.341

Figure S1. One-way sensitivity analysis for the ICER of routine screening for Lynch syndrome in colorectal cancer patients ≤70 years compared with Lynch syndrome screening in colorectal cancer patients ≤60 years of age.



Chapter 5

Cost-effectiveness of routine screening for Lynch syndrome in endometrial cancer patients up to 70 years of age

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ABSTRACT

Purpose To assess cost-effectiveness of routine screening for Lynch syndrome (LS) in endometrial cancer (EC) patients ≤70 years of age.

Methods Consecutive EC patients ≤70 years of age were screened for LS by analysis of microsatellite instability, immunohistochemistry and *MLH1* hypermethylation. Costs and health benefit in life years gained (LYG) included surveillance for LS carriers among EC patients and relatives. We calculated incremental cost-effectiveness ratios (ICERs) comparing LS screening among EC patients ≤70 years with ≤50 years and the revised Bethesda guidelines.

Results Screening for LS in 179 EC patients identified 7 LS carriers; 1 was ≤50 and 6 were 51-70 years. Per age category 18 and 9 relatives were identified as LS carrier. Screening resulted in 74,7 LYG (45,4 and 29,3 LYG per age category). The ICER for LS screening in EC patients ≤70 compared with ≤50 years was €5.252/LYG. The revised Bethesda guidelines missed 4/7 (57%) LS carriers among EC patients. The ICER for LS screening in EC patients ≤70 years of age compared with the revised Bethesda guidelines was €6.668/LYG. Both ICERs remained <€16.000/LYG in sensitivity analyses.

Conclusion Routine LS screening in EC patients ≤70 years is a cost-effective strategy, allowing colorectal cancer prevention in EC patients and their relatives.

INTRODUCTION

Lynch syndrome (LS) is a hereditary cancer syndrome characterized by early onset of colorectal cancer (CRC), endometrial cancer (EC) and other extracolonic cancers.(1, 2) Over half of the women who develop multiple LS associated cancers will present with a gynaecological malignancy, in most cases EC.(3) LS carriers among EC patients are at high risk of developing CRC. It is important to identify LS in EC patients, since colonoscopy surveillance prevents development of CRC in these patients and their affected family members.(4-6)

Tumors from LS patients display certain characteristics: microsatellite instability (MSI) and loss of mismatch repair (MMR) protein expression in immunohistochemistry (IHC).(7) In cases with loss of MLH1 protein expression, *MLH1* hypermethylation analysis can be done to distinguish between sporadic MLH1 deficient tumors and tumors likely caused by LS.(7) Mean age at CRC diagnosis in LS carriers is 44-61 years, compared with 69 years in sporadic CRC cases.(8) Recent guidelines recommend routine LS screening by analysis of MSI and/or IHC among CRC patients up to 70 years of age.(8, 9) Guidelines for LS screening among EC patients, however, are ambiguous. Mean age at EC diagnosis among LS carriers is between 48-62 years, compared with 65 years for sporadic cases of EC.(8) A European group of experts recommends routine LS screening among EC patients up to 70 years of age(9), while an American task force advises an age-cut of 50 years.(8) Although several age cut-offs as well as universal LS screening among EC patients have been suggested, data on the cost-effectiveness of such strategies are scarce.(10-12) Furthermore, only one of these cost-effectiveness analyses calculated effectiveness in life years gained and none of these studies included costs and health benefit for relatives of LS patients in their analyses.

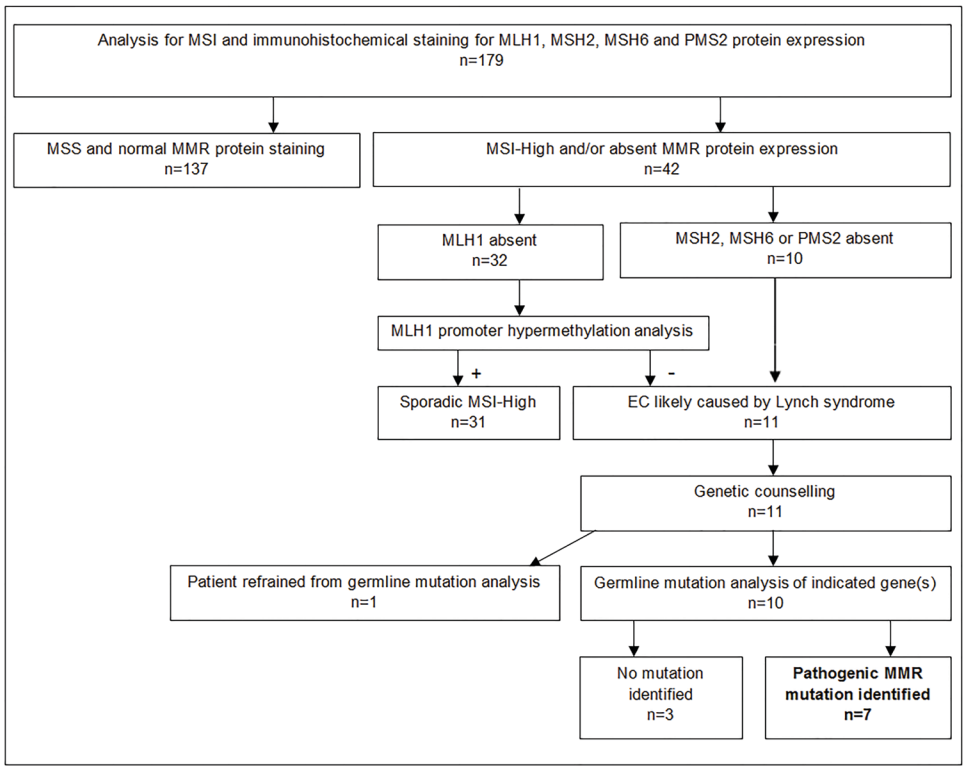
In 2012 we reported on a population-based cohort of EC patients ≤70 years of age routinely screened for LS by analysis of MSI, IHC and *MLH1* hypermethylation. A profile compatible with LS was detected in 6% of these EC patients and all but one of these patients (10/11) were over 50 years of age.(13) In the current study, we used data from this population-based cohort including costs and benefit for relatives to assess whether routine LS screening among EC patients up to 70 years of age is cost-effective.

METHODS

Subjects and diagnostic work-up

We used data from a prospective population-based study on the yield of routine molecular screening for LS in EC patients up to 70 years of age, described in detail previously.(13) In summary, consecutive EC patients ≤70 years of age (n=179) from 8 Dutch hospitals were routinely screened for LS by analysis of MSI, IHC for MLH1, MSH2, MSH6 and PMS2 protein expression. *MLH1* hypermethylation analysis was performed in cases with loss of MLH1 protein expression. Patients with tumor characteristics suspect for LS, i.e. a high degree of MSI and/or absence of MMR protein without *MLH1* promoter hypermethylation, were offered genetic counselling and germline mutation analysis (Figure 1).

Figure 1. Diagnostic work-up to detect Lynch syndrome among endometrial cancer patients ≤70 years.
MSI: microsatellite instability; MSS: microsatellite stable; MMR: mismatch repair; MSI-High: high degree of MSI; EC: endometrial cancer.



Patients identified with a pathogenic germline mutation in one of the MMR genes *MLH1*, *MSH2*, *MSH6*, or *PMS2*, or in the *EPCAM* gene, were labelled index patients. Relatives of index patients were offered genetic counselling and targeted mutation analysis via the index patients. We registered the number of relatives accepting genetic counselling and targeted mutation analysis, the number of LS carriers identified among relatives and their age at time of LS diagnosis up to February 2015. This study was approved by the Institutional Review Boards of the participating hospitals.

Effectiveness

Health benefit from LS screening in life years gained (LYG) was calculated, based on the number of LS patients detected among EC patients and their relatives. In previous studies LS surveillance resulted in 0.15-2.50 LYG for female index patients with CRC and 0.49 to 32.69 LYG for relatives (Table 1).(14-25) No data is available on LYG by LS surveillance for EC index patients. For these patients we therefore used the median of all LYG from literature for female index patients with CRC. Similarly, we used the median of all reported LYG for relatives for LS carriers that were identified among family members of index patients (Table 1). Most studies only reported 3% discounted LYG and if undiscounted LYG were reported, we first discounted them by 3% annually (Supplementary information, Table S1). For studies that did not include the adherence to LS surveillance programs in the reported LYG, we assumed an adherence of 80% for index patients as well as LS carriers among their relatives.(16) We assumed that EC patients and relatives without a pathogenic mutation identified would not undergo LS surveillance. Also, no health benefit or surveillance costs were calculated for deceased cases.

Costs

Detailed direct medical costs for LS diagnostics (i.e. MSI analysis, IHC, *MLH1* hypermethylation analysis, genetic counseling and germline mutation analysis) in EC patients and their relatives were calculated using the microcosting method.(26) Cost data for *PMS2* germline mutation analysis were not available and were assumed to be similar to costs for germline mutation analysis of other MMR genes. Total costs for routine LS screening, including costs for LS diagnostics, LS surveillance for LS carriers, and savings by prevention of CRC development, were determined based on the number of EC patients and relatives analyzed. Index patients and relatives were assumed to undergo LS surveillance, consisting of colonoscopy with polypectomy every two years, starting at the age patients were diagnosed with LS (or at age 25 for younger relatives) up to 75 years of age. Additionally, female LS carriers among relatives were assumed to undergo gynaecologic surveillance consisting of yearly transvaginal ultrasonography and endometrial biopsy from age 35 until prophylactic surgery at 40 years of age. We

assumed that 18% of these relatives accepted prophylactic surgery (total abdominal hysterectomy and bilateral salpo-oophorectomy).(16) LS carriers not accepting prophylactic surgery were assumed to continue yearly gynaecological surveillance up to 75 years of age. We did not include costs for prophylactic colectomy or aspirin chemoprevention, since both are not recommended as standard clinical care by current guidelines. All costs for LS surveillance were derived from literature. For cost savings by prevention of CRC in surveillance programs, the most conservative estimate (only treatment costs for the first 12 months of stage I CRC) was used. Costs for gynaecological screening were only available in dollars and were converted to euros using purchasing power parity. All costs were converted to price level 2013 using the Dutch consumer price index(27) and were discounted by 3% annually. All cost data are summarized in Supplemental Table 2.

Table 1. Parameters and values used in the cost-effectiveness analysis.

Parameter	Base case value	Range	Source
Median age at LS diagnosis			
Index patients	**59**	**IQR 53-62**	**(13)**
Relatives	**46**	**IQR 36-54**	**Current study**
Female Relatives	**46**	**IQR 36-57**	**Current study**
LS surveillance[†]			
Discounted life years gained			
Index patients[‡]	**0.68**	**0.13-2.14**	**(15, 16, 18, 19, 21, 22)**
Female relatives	**2.80**	**0.40-17.82**	**(14-25)**
Male relatives	**2.80**	**0.47-17.82**	**(14-25)**
Interval between colonoscopies (years)	**2**	**1-2**	**(8, 9)**
Complication rate of colonoscopy	**0.0024**		**(28)**
Acceptance of prophylactic gynaecological surgery	**0.18**	**0.03-0.25**	**(16)**
CRC risk and risk reduction			
Lifetime risk of developing CRC for LS carriers	**0.25**	**0.25-0.70**	**(5, 6, 23, 29)**
Reduction in CRC risk by LS surveillance	**0.56**	**0.56-0.70**	**(5, 6, 30)**

LS: Lynch syndrome; CRC: Colorectal cancer

[†] Life years gained were discounted by 3% annually

[‡] Health benefit for CRC index patients was used for index patients, since no data are available on LYG for index patients with endometrial carcinoma

Revised Bethesda guidelines

For EC patients who received genetic counselling, a detailed family history was obtained, and one clinical geneticist (AW) assessed if these patients fulfilled the revised Bethesda guidelines. Family history for patients who did not receive genetic counselling was not available. We assumed that these patients would not fulfil the revised Bethesda guidelines, biasing our analysis against age-targeted LS screening.

Cost-effectiveness analyses

Incremental costs and health benefit were calculated for LS screening among EC patients using an age cut-off of 70 years compared with an age cut-off of 50 years. The incremental cost-effectiveness ratio (ICER) indicates the additional costs per LYG. We also calculated the ICER of routine LS screening in EC patients ≤70 years of age compared with a screening strategy according to the revised Bethesda guidelines. We performed one-way sensitivity analyses in order to test the robustness of the ICERs. Costs were assumed to range from 0.5 to 2 times as much as calculated. Ranges for all other parameters were based on literature (Table 1).

RESULTS

Routine LS screening among 179 EC patients identified 11 patients (6.1%) suspected of having LS (Figure 1). All patients suspected of having LS received genetic counselling. Consecutive germline mutation analysis was accepted by 10/11 patients and finally identified 7 LS patients (3.9%).

Effectiveness of screening

The median age of EC patients was 61 years (IQR 57-66); 15 EC patients were ≤50 years and 164 patients were 51-70 years of age. The prevalence of LS decreased was 6.7% (1/15) in EC patients ≤50 years of age and 3.7% (6/164) in EC patients 51-70 years of age.(Table 2). Per index patient a median of 3 (IQR 2-8) relatives accepted genetic counselling and germline mutation analysis. In total, germline mutation analysis was accepted by 73 relatives, resulting in identification of 27 additional LS carriers. Surveillance of LS carriers among EC patients ≤70 years of age and their relatives amounted in a total of 74.7 LYG. Only a small portion of the health benefit was attributed to LS surveillance among index patients (4.8 LYG vs. 69.9 LYG for relatives). Although LS screening among EC patients ≤50 years of age only identified a single LS carrier, a higher benefit was found for this group than for screening among the older age category (44.8 LYG vs. 25.2 LYG). The high amount of LYG found in the younger age group was caused by a large number of 50 relatives tested, identifying 18 LS carriers.

Table 2. Number of patients screened and detection of Lynch syndrome among EC patients and relatives

Endometrial cancer patients (n=179)	**<50 years (n=14)**	**51-70 years (n=165)**	**Total**
LS diagnostics in EC patients			
Analysis for MSI and IHC	14	165	179
MLH1 hypermethylation analysis	0	32	32
EC patients suspected of having LS	1	10	11
Genetic counseling	1	10	11
Germline mutation analysis	1	9	10
Genes tested in EC patients or FDR	1	11	12
LS index patients identified	1	6	7
LS diagnostics in relatives			
Relatives accepting genetic counseling	50	23	73
Germline mutation analysis	18	9	27
LS carriers identified among relatives	11	7	18
Life years gained			
LYG by index patients			
LYG by male relatives	0.7	4.1	4.8
LYG by female relatives	19.6	5.6	25.2
Total LYG (index and relatives)	25.2	19.6	44.8

Numbers may not add up due to rounding

LS; Lynch syndrome, EC: Endometrial cancer; MSI: Microsatellite instability; HC: Immunohistochemistry; FDR: First degree relative

Costs and cost-effectiveness

Total costs for LS screening increased from €150.817 (€7.938 per LS carrier detected) for EC patients ≤50 years of age to €304.442 (€8.954 per LS carrier detected) for EC patients ≤70 years of age. LS screening for EC patients ≤70 years of age had an ICER of €5.252/LYG compared with screening patients up to 50 years of age (Table 3). In one-way sensitivity analysis the assumed LYG for LS carriers among relatives impacted the ICER the most. The ICER for screening EC patients ≤70 years of age compared with screening patients with EC diagnosed ≤50 years of age never exceeded €13.000/LYG (Figure 2).

Revised Bethesda guidelines

LS screening according to the revised Bethesda guidelines would have identified 3/7 (43%) index patients and 22/27 (81%) relatives. Using favorable assumptions, total cost for this strategy were €193.066 (€7.723 per LS carrier detected), with a total health benefit of 58.0 LYG. The ICER for routine LS screening in CRC patients ≤70 years compared with the revised Bethesda guidelines was €6.668/LYG. In sensitivity analysis this ICER never exceeded €16.000/LYG.

Figure 2. One-way sensitivity analysis for the ICER of routine screening for Lynch syndrome in endometrial cancer patients using an age cut-off of 70 years compared with an age cut-off of 50 years.

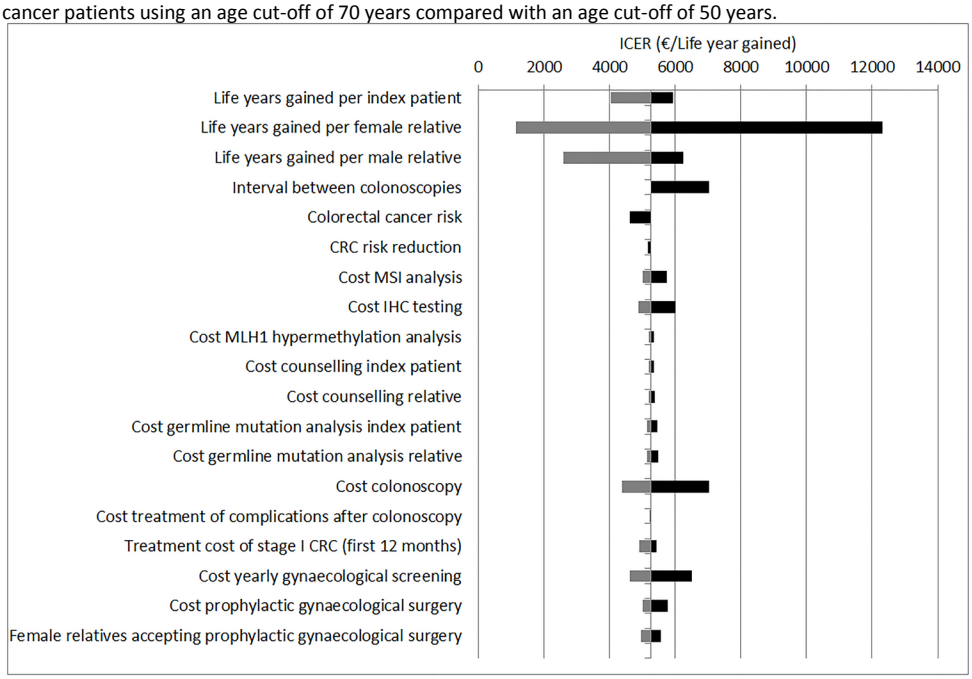


Table 3. Incremental costs in 2013 euro for Lynch syndrome screening in EC patients of different age categories

	<50 years	**61-70 years**	**Total**
Lynch syndrome diagnostics			
Endometrial cancer patients			
Molecular diagnostics	€ 3.142	€ 40.212	€ 43.354
Genetic counseling	€ 298	€ 2.979	€ 3.276
Germline mutation analysis	€ 538	€ 5.916	€ 6.454
Relatives			
Genetic counseling	€ 8.172	€ 3.836	€ 12.008
Targeted mutation analysis	€ 15.201	€ 6.993	€ 22.194
Lynch syndrome surveillance			
Colonoscopy surveillance			
Index patients	€ 3.883	€ 15.597	€ 19.481
Relatives	€ 64.902	€ 36.507	€ 101.410
Savings by prevention of colorectal cancer	- € 18.240	- € 10.260	- € 28.499
Gynaecologic surveillance and prophylactic surgery in relatives	€ 72.920	€ 51.845	€ 124.765
Total costs (minus savings)	€ 150.817	€ 153.625	€ 304.442
Total life years gained	45.4	29.3	74.7
Costs per life year gained	reference	€ 5.252	-

Numbers may not add up due to rounding

DISCUSSION

Based on this population-based cohort of EC patients routine screening for LS in EC patients ≤70 years of age by analysis of MSI, IHC and *MLH1* hypermethylation is a cost-effective strategy. In sensitivity analysis, the ICER for routine LS screening among EC patients up to 70 years of age compared to EC ≤50 years of age remained <€13.000/LYG (<$15.000/LYG). For life-saving interventions a threshold of €40.000/LYG (or $50.000/LYG) is commonly accepted, and some authors advocate even higher thresholds.(31, 32)

In this cost-effectiveness analysis, we used the median of all estimates from literature for the health benefit that results from LS screening. Previous studies, as well as our own sensitivity analysis, showed that assumptions for health benefit in relatives have a tremendous effect on the ICER.(15-19) Since there are no estimates available for health benefit of EC index patients, we used the median of all estimates for CRC index patients. However, we expect that index patients with EC will benefit more from LS surveillance than index patients with CRC, since mortality rate is higher in LS patients diagnosed with CRC than in LS patients diagnosed with EC.(33)

Most of the relatives tested for LS in our study were related to the one index patient ≤50 years of age. In this family 50 relatives were tested compared to a total of 23 relatives for all index patients >50 years of age together. However, since the index patient ≤50 years and all her relatives were our reference group, the large number of relatives tested in this family did not impact the ICER. The focus of our study lies on the incremental costs and benefit for LS screening among EC patients 51-70 years of age and their relatives. For these EC patients, between 1 and 11 relatives accepted germline mutation analysis. Previous studies on cost-effectiveness of LS screening among CRC patients have assumed that a total of 2-8 relatives would be tested per index patient.(16, 17, 21) Among the EC patients in our cohort aged 51-70 years this would result in a total of 12-48 relatives tested for LS. The total of 23 relatives accepting germline mutation analysis we found in this age group is at the lower half of these estimates. In time, far more LS carriers might be identified among relatives of the index patients in our study. Relatives refraining from germline mutation analysis and minors not yet eligible for genetic testing, could still be tested at a later time and enroll in LS surveillance programs. This would increase the costs of our analyses, however the increased benefit in LYG would likely lead to lower ICERs.

Only a few studies have been published on cost-effectiveness of LS screening among EC patients. All of these studies concluded that some form of LS screening among EC

patients was cost-effective.(10-12) However, none of these studies included health benefit for relatives; one study calculated costs/LYG for index patients, another calculated costs/index patient detected, and the third study calculated costs/possible index patient detected. Two studies found that LS screening by IHC among all EC patients was cost-effective compared with clinical criteria, the Amsterdam criteria and the SGO criteria respectively.(10, 12) In contrast, Kwon et al found that LS screening by IHC analysis of all EC patients who had one or more first degree relatives with a LS associated cancer, was more cost-effective than routine IHC analysis among EC patients using an age cut-off of 50 or 60 years.(11) In our study, we used a higher age cut-off of and almost half of the LS carriers we identified among EC patients, were over 60 years of age. Using favourable assumptions for the revised Bethesda guidelines, the ICER for LS screening among EC patients up to 70 years of age compared with these guidelines remained <€16.000/LYG. We believe that an age cut-off will be much easier to use in clinical practice, since previous research among CRC patients showed that clinical criteria for LS screening based on family history are poorly applied in clinical practice.(34, 35)

In our study analysis for MSI as well as IHC for loss of mismatch repair protein was performed for all patients. In studies for LS screening among CRC patients, IHC analysis alone was more cost-effective than LS screening by MSI analysis or analysis of MSI and IHC combined.(16, 17) Omitting MSI analysis from our diagnostic work-up would most likely also result in a lower ICER. In CRC patients, combining IHC analysis and prediction models for LS may further increase the cost-effectiveness of LS screening.(36) However, such prediction models are not well applicable on EC patients.(37)

LS screening among EC patients up to 70 years of age identified 7 times more index patients than only screening EC patients up to 50 years of age. Screening all EC patients for LS regardless of age is proposed by some authors and will undoubtedly detect even more LS carriers.(38) Little is known, however, about the ultimate costs and benefit of universal LS screening among EC patients. One study reported an ICER of $648.494 (>€500.000) for screening all EC patients for LS by IHC analysis compared with IHC analysis for EC patients with at least one first degree relative with a LS associated cancer.(11) However, only LYG for index patients were included in this analysis, and not the health benefit for relatives, which has a far greater impact on ICERs.

Current guidelines recommend routine LS screening for CRC patients up to 70 years of age.(8, 9) For EC patients, however, LS screening is ambiguously recommended with age cut-offs of 50 years or 70 years. In clinical practice EC patients are usually not screened for LS, even if they fulfill the revised Bethesda guidelines.(39, 40) Therefore, in clinical practice, many LS carriers among EC patients will remain undetected. Identifying these LS

carriers can greatly improve their survival and in an even larger extend improve survival of their relatives.

In our study benefit of LS screening was based on the decrease in mortality by LS surveillance. We only used conservative estimates for the cost saved by a decrease in CRC morbidity. We did not account for the gained quality of life by the reduction of morbidity. Furthermore, the decrease in mortality and morbidity by LS surveillance will impact cost-effectiveness by allowing LS patients to continue their daily activities including working. Additional strategies such as informing patients on signs and symptoms of cancer could further contribute to early diagnosis of cancer, thereby contributing to the cost-effectiveness of LS screening. Also, encouraging LS carriers to make lifestyle choices such as not smoking could increase the benefit of LS surveillance programs. Finally, aspirin treatment to prevent development of cancer would add to the costs of LS screening, but may further increase its benefit. We did not include these strategies in our analysis, since there are no exact data on their benefit.

To our knowledge, this study is the first cost-effectiveness analysis for LS screening among EC patients that includes costs and health benefit for relatives. Other strengths of this study are the detailed analysis of costs, the use of data from a population-based cohort prospectively screened for LS, the inclusion of *MLH1* hypermethylation analysis in LS diagnostics, and the use of conservative estimates if assumptions were needed.

Our study also has several limitations. First, the data set was small with only 7 LS index patients and all patients from a Dutch population, which may not be representative for other populations. However, in a recent study among American EC patients the prevalence of LS in patients with endometroid type EC without an age cut-off was at least 3,89%, which corresponds well with the LS prevalence in our cohort (7/179, 3,9%).(41) Secondly, we did not include LS surveillance for extracolonic cancers other than gynaecological cancers in the cost-effectiveness analysis, since health benefit from such surveillance is still unclear. For LS carriers, lifetime risk of developing other LS-associated cancers such as small-bowel cancer and gastric cancer are below 20%, while lifetime risks for CRC and EC are up to 70%.(9) Furthermore, we only performed one-way sensitivity analyses, as opposed to full probabilistic sensitivity analyses. Also, it is unclear whether all studies from which we used the number of LYG corrected for other causes of death. We use the median of all LYG from literature for our base case scenario. Recently, the assumptions from Mvundura et al. were updated by accounting for death from other causes amongst other recalculations. Grosse et al found that accounting for other causes of death specifically resulted in a 5% lower number for LYG.(25) However, if we assumed 5% lower LYG in our study, all ICERs would still remain <€17.000/LYG and therefore well

below the currently accepted standards for cost-effectiveness. Finally, LYG were not corrected for quality of life, as this has been reported to be unnecessary for benefit of LS detection.(42, 43)

In conclusion, routine screening for LS by analysis of MSI, IHC and *MLH1* hypermethylation in EC patients up to 70 years of age is a cost-effective strategy according to currently accepted standards, allowing prevention of CRC in EC patients and their relatives. Implementation in clinical practice should be considered. In the Netherlands, routine screening for LS using IHC is now being implemented. Further research should focus on the cost-effectiveness of LS screening in larger cohorts of EC patients including routine LS screening without an age cut-off.

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SUPPLEMENTAL MATERIAL

Table S1. Data on Life years gained (3% discounted) by index patients and relatives

	Females	Males	Source
Index patients	0,191		Ladabaum et al 2011
	0,13		Calculated from Sie et al 2014
	0,54		Calculated from Snowsill et al 2014
	0,82		Calculated from Ramsey et al 2003
	0,82		Calculated from Ramsey et al 2001
	2,14		Calculated from Kievit et al 2005
Median	0,68		
Relatives	0,40	0,47	Calculated from Severin et al 2015
	0,49	0,51	Ladabaum et al 2011
	0,63	0,63	Barzi et al 2015
	0,68	0,68	Calculated from Snowsill et al 2014
	0,80	0,80	Mvundura et al 2010
	2,80	2,80	Calculated from Kievit et al 2005
	3,82	3,82	Calculated from Vasen et al 2010
	7,36	7,36	Calculated from Syngal et al 1998
	8,09	8,09	Calculated from Sie et al 2014
	17,05	17,05	Calculated from Ramsey et al 2001
	17,82	17,82	Calculated from Ramsey et al 2003
Median	2,80	2,80	

Table S2. Cost data in 2013 euros for Lynch syndrome diagnostics and surveillance

	Cost	Source
Lynch syndrome diagnostics[†]		
Analysis for microsatellite instability	€ 89	Dept. of Pathology, Erasmus MC
IHC testing for MMR protein expression	€ 135	Dept. of Pathology, Erasmus MC
MLH1 hypermethylation analysis	€ 99	Dept. of Pathology, Erasmus MC
Genetic counseling for index patients	€ 389	Dept. of Clinical Genetics, Erasmus MC
Genetic counseling for relatives	€ 256	Dept. of Clinical Genetics, Erasmus MC
MMR gene sequencing (per gene)	€ 538	Dept. of Clinical Genetics, Erasmus MC
Targeted mutation analysis for relatives	€ 304	Dept. of Clinical Genetics, Erasmus MC
Lynch syndrome surveillance		
Surveillance colonoscopy including polypectomy	€ 393	Goede et al 2013
Treatment of complications after colonoscopy	€ 1.250	Goede et al 2013
Treatment cost of stage I CRC (first 12 months)	€ 12.100	Goede et al 2013
Transvaginal ultrasound	€ 100	Ladabaum et al 2011
Endometrial biopsy	€ 204	Ladabaum et al 2011
Prophylactic hysterectomy and salpo-oophorectomy	€ 14.920	Ladabaum et al 2011

IHC: Immunohistochemistry; MMR: Mismatch repair; CRC: Colorectal cancer.

[†] Costs were determined following the microcosting method and included costs of employment, material, equipment and overhead

Chapter 6

Routine molecular analysis for Lynch syndrome among adenomas or colorectal cancer within a national CRC screening program

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ABSTRACT

Background & aims It is important to identify individuals with Lynch syndrome because surveillance programs can reduce their morbidity and mortality from colorectal cancer (CRC). We assessed the diagnostic yield of immunohistochemistry to detect Lynch syndrome in patients with advanced and multiple adenomas within our national CRC screening program.

Methods We performed a prospective study of all participants (n=1101; 55% male; median age, 66 years; interquartile range, 61–70 years) referred to the Erasmus MC in The Netherlands after a positive result from a fecal immunohistochemical test, from December 2013 to December 2016. Colon tissues were collected from patients with advanced adenomas, ≥4 non-advanced adenomas, or CRC, and analyzed by immunohistochemistry to identify patients with loss of mismatch repair (MMR) proteins (MLH1, MSH2, MSH6, or PMS2)—a marker of Lynch syndrome. Specimens from patients with loss of MLH1 were analyzed for *MLH1* promoter hypermethylation. Patients with a MMR-deficient tumor or adenoma without *MLH1* promoter hypermethylation were referred for genetic analysis.

Results At colonoscopy, 456 patients (41%) (65% male; mean age, 67 years; interquartile range, 63–71 years) were found to have CRC and/or an adenoma eligible for analysis by immunohistochemistry. Of 56 CRCs, 7 (13%) had lost an MMR protein and 5 had hypermethylation of the *MLH1* promoter. Analyses of tumor DNA revealed that 2 patients without *MLH1* promoter hypermethylation had developed sporadic tumors. In total, 400 patients with adenomas were analyzed. Of the examined adenomas, 208 (52%) had a villous component and/or high-grade dysplasia: 186 (47%) had a villous component and 41 (10%) had high-grade dysplasia. Only 1 adenoma had lost an MMR protein. This adenoma was found to have 2 somatic mutations in *MSH6*.

Conclusion In a CRC screening program in The Netherlands for individuals aged 55-75 years, routine screening for Lynch syndrome by immunohistochemistry analysis of colon tissues from patients with advanced and multiple adenomas identified no individuals with this genetic disorder.

INTRODUCTION

Lynch syndrome (LS) is the most common hereditary predisposition for colorectal cancer (CRC) and is caused by germline mutations in one of the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6*, or *PMS2* or in the *TACSTD1* gene.(1-5) LS patients are at increased risk of developing CRC as well as extracolonic cancers.(6, 7) Periodic colonoscopy leads to a considerable (65%) reduction of morbidity and mortality(8, 9), therefore identifying individuals with LS is highly important.

Microsatellite instability (MSI) and immunohistochemistry (IHC) for loss of MMR protein expression on tumour tissue can identify patients with tumours likely caused by LS.(10) Current guidelines recommend routine screening for LS by IHC and/or MSI analysis among CRC patients up to the age of 70 years or even universally.(6, 7) A definite diagnosis of LS is made when a pathogenic germline mutation is found.

In current practice, patients with premalignant disease are not routinely screened for LS. However, LS screening in adenoma patients could yield more clinical benefit, since in adenoma patients with proven LS a tailored surveillance strategy can prevent future development of CRC. In a previous study, 125 patients with advanced adenoma up to the age of 45 years were routinely screened for LS by IHC and MSI analysis. LS was diagnosed in 3 (2,4%) of these patients.(11)

In the Netherlands, the population aged 55-75 is offered screening for CRC by faecal immunohistochemical test for haemoglobin (FIT) and colonoscopy in case of a positive FIT.(12) While patients with a strong family history of CRC may already be diagnosed with LS under the age of 55, we expect that LS patients without a clear family history of CRC, especially *MSH6* and *PMS2* mutation carriers, are participating in the population screening program. Therefore, in this study we aimed to evaluate the yield of routine IHC analysis in consecutive patients with CRC or advanced or multiple adenomas within the national screening program for CRC.

METHODS

In this prospective population-based study we performed IHC staining for MLH1, MSH2, MSH6 and PMS2 protein in patients with CRC or advanced or multiple adenomas within the Dutch national CRC screening program.

Study population

In the Dutch CRC screening program, men and women from 55-75 years of age are offered a faecal immunohistochemical test (FIT) every two years. In the invitation letter for the CRC screening program, exclusion criteria when not to participate the FIT based CRC screening are enclosed, namely: individuals who have a previous history of CRC, a history of IBD, current bowel complaints, underwent a colonoscopy in the previous five years or have a short life expectancy. These people are encouraged not to participate and in doubt advised to discuss this with their gastroenterologist or family doctor.

Patients with a positive FIT are referred to a nearby certified colonoscopy center, based on the colonoscopy capacity of these centers. In general, the participation rate of the population based CRC screening program is 70% with a FIT positivity rate of 5-7%(12) We collected data for all patients that were referred to our center after having a positive FIT within the Dutch national CRC screening program. Patients were included between December 2013 and December 2016. For all patients, we collected data on age, personal and family cancer history and colonoscopy findings including the number of adenomas and histology of CRC and adenomas.

IHC staining for MMR proteins was performed for all patients with CRC or advanced adenomas found at colonoscopy. For all patients, the most advanced lesion was analyzed. Adenomas were considered advanced if they showed high-grade dysplasia, consisted of ≥25% villous component or were ≥10mm in size according to the endoscopy report. Also, if patients had at least four synchronous non-advanced adenomas, IHC staining was performed on the largest of these adenomas.

This study was approved by the Medical Ethics Committee (MEC-2013-496) of Erasmus MC Rotterdam, the Netherlands. All patients were informed about the study at the intake visit and received an information leaflet of the study. Patients could indicate if they wanted to be informed on the results of routine molecular analyses of their tissue.

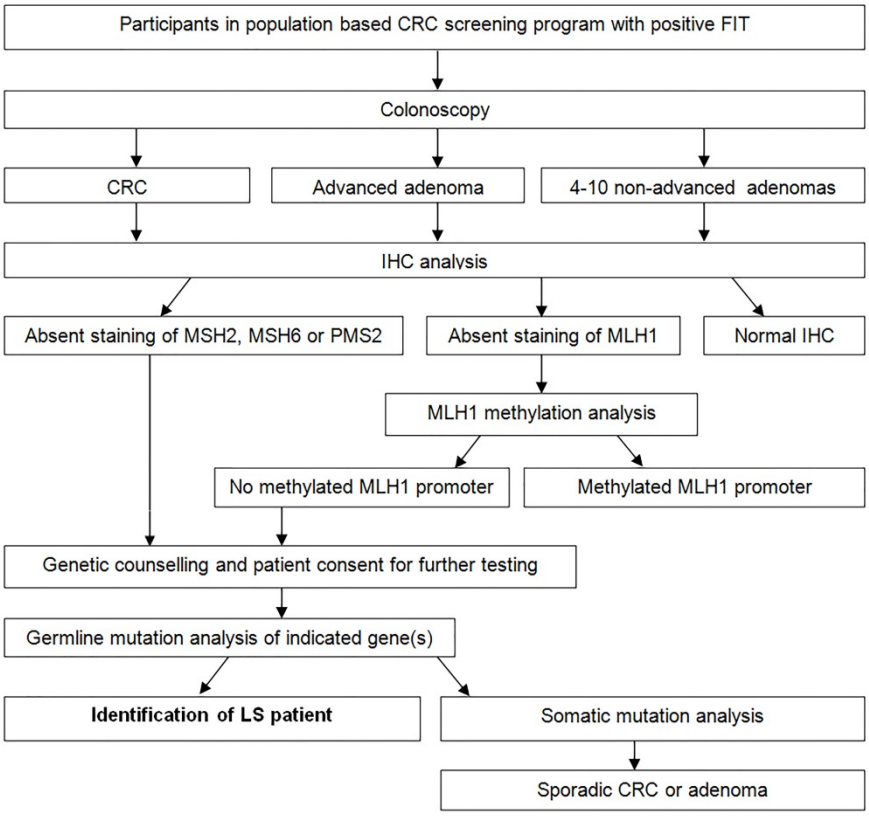
IHC analysis

IHC for MLH1, MSH2, MSH6 and PMS2 protein *w*as performed on CRC or adenoma tissue as described previously.(10) Tumours or adenomas in which one or more MMR proteins were absent, were considered MMR deficient. In cases with loss of MLH1 protein expression, we performed *MLH1* promoter hypermethylation analysis to distinguish sporadic MMR deficient tumours from MMR deficient tumours suspect for LS.

Patients suspected of having LS

Patients with a MMR deficient tumour or adenoma suspect for LS were referred for genetic counselling and were offered germline mutation analysis of the gene indicated by IHC. For patients with IHC results suspect for LS, in whom no germline pathogenic MMR mutation was found, we performed somatic mutation analysis as described previously.(13) Figure 1 shows a flowchart of the study.

Figure 1. Flowchart of the study



RESULTS

A total of 1101 patients with a positive FIT were included in the study; 610 (55%) were male and median age at time of intake visit was 66 [62-70] years. Patient characteristics are shown in Table 1.

Personal and family cancer history

Two (0,2%) patients had a previous history of CRC. Seven (1,4%) women had a personal history of endometrial cancer. Two of these women had CRC at colonoscopy, both without aberrant IHC. Other LS-associated tumours were present in 19 (1,7%) patients.

Most patients did not have a family history of CRC. In total, 172 (16%) patients had at least one first degree relative with CRC. Median age of CRC in first degree relatives was 68 [60-75] years. Median PREMM5 scores were 0,3% [IQR 0,2-0,5]. Scores for PREMM5 were >5% in 4 patients. The highest PREMM5 score was 9%. In this patient, a *MSH2* mutation was found in family members, but he was not a carrier of the familial mutation.

Table 1. Patients characteristics

	Total	**Patients included for IHC**
N	1101	456
Male gender	618 (56%)	296 (65%)
Age (years, IQR)	66 [62-70]	67 [63-71]
Personal cancer history		
CRC	2 (0,2%)	1 (0,2%)
EC	7 (1,4%)	2 (1,3%)
Other LS associated cancer	19 (1,7%)	9 (2,0%)
First degree relatives		
CRC	172 (16%)	82 (18%)
≥2 with CRC	18 (1,6%)	8 (1,8%)
EC	13 (1,2%)	7 (1,5%)
PREMM5 >5%	4 (0,4%)	3 (0,7%)

IHC

At colonoscopy, 456 (42%) patients (65% male; median age 67 years [63-71 years] had a CRC or adenoma eligible for IHC analysis.

Adenoma patients

In total, 400 adenoma patients were analyzed; 370 patients with advanced adenoma and 30 patients with at least four non-advanced adenomas. Of the examined adenomas, 208 (52%) had a villous component and/or high grade dysplasia (186 (47%) with villous component and 41 (10%) with high grade dysplasia). A total of 162/370 (44%) adenomas were judged advanced based on size ≥10 mm alone. The adenoma patients had a median age of 66 [63-71] years and 65% were male.

Only one adenoma patient had aberrant IHC. This was a 67 years old woman with a large (35mm) tubulovilleus adenoma. IHC showed loss of MSH6 protein in the adenoma cells.

There was no personal or family history of CRC or other LS-associated cancers. No germline *MSH6* mutation was found whereas somatic mutation analysis in adenoma cells showed two somatic *MSH6* mutations (c.892C>T; p.R298* and c.1872_1873insA; p.S625Ifs*15).

Colorectal cancer patients

A total of 56 CRC were eligible for IHC in this study. These patients had a median age of 69 [63-72] years and 64% were male. Seven (13%) showed aberrant IHC with loss of MLH1 and PMS2 protein expression. Five of these cases had *MLH1* promoter hypermethylation. In both cases without *MLH1* promoter hypermethylation no germline *MLH1* mutation was found. Somatic mutation analysis showed two somatic hits in their tumour (*MLH1* mutation c.1459C>T; p.R487* and LOH of the wildtype allele in the first patient and *MLH1* mutations c.307-1G>A; p.? and c.1549G>T; p.G517* in the second patient).

Table 2. Results of molecular diagnostics

	N	Age (years, IQR)	Male gender	MMR deficiency	MHL1 promoter methylation	Germline MMR mutation	Somatic MMR mutations
Patients included for IHC	456	67 [63-71]	296 (65%)	8	5	0	3
Colorectal cancer	56	69 [63-72]	36 (64%)	7	5	0	2
Advanced adenoma	370	66 [62-71]	237 (64%)				
Villous component	186	65 [61-69]	124 (67%)	1	0	0	1
High grade dysplasia	42	67 [63-74]	30 (73%)	0	0	0	0
4-10 non-advanced adenomas	30	67 [63-74]	23 (77%)	0	0	0	0

DISCUSSION

Previous studies showed that loss of MMR expression can be found in 50-84% of adenomas from LS patients(14-17). There is no data on the percentage of MMR deficiency among sporadic adenomas. In this study we were able to examine whether MMR deficiency is common in adenomas and whether screening for LS in adenoma patients within a colorectal cancer screening program is efficient. Screening 400 adenoma patients showed that MMR deficiency is not common. In our study, only 1/400 adenomas showed loss of MMR protein due to somatic MMR mutations. We detected 7 CRC cases with MMR deficiency and all proved to be sporadic. No LS patients were identified in our cohort of 456 adenoma and colorectal cancer patients.

Our data suggest that screening all adenomas for LS is likely not an effective strategy. However, screening among a subset of adenoma patients might still be effective. Our

cohort consisted of an older age group (median age 67 years, IQR 63-71 years). The yield of LS screening may be higher in younger adenoma patients. In one study there was a significant association between MMR deficiency in adenomas and age <50 years.(18) Our results are slightly different from our previous prospective study in a young age group, in which we found MMR deficiency in 4/125 (2,4%) advanced adenoma patients <45 years of age. In all 3 cases with an MMR deficient adenoma without *MLH1* hypermethylation from that study a pathogenic MMR mutation was found.(11) In contrast, in a recent retrospective study only 1 out of 208 advanced adenoma patients under 50 years of age had aberrant IHC.(19) However, high risk patients may have been excluded from this retrospective study. The one patient with a MMR deficient adenoma is likely to have LS, since she previously had endometrial cancer. In another study, 10/187 (5,4%) adenomas with high grade dysplasia showed loss of MMR protein expression.(18) In both studies no germline mutation analysis was performed. Table 3 shows the results of our study in comparison with previous studies on LS screening by IHC analysis in adenomas.

Table 3. Studies on Lynch syndrome screening by IHC analysis in adenomas

	Current study	**Van Lier et al 2012**	**Basterra et al 2016**	**Mendelsohn et al 2017**
	Prospective	Prospective	Retrospective	Retrospective
Population	FIT positive in population based CRC screening program	Advanced adenoma <45 years of age	Adenoma showing high grade dysplasia	Adenoma <50 years of age
Adenomas	400	125	187	208
Ages	55-75 years	<45 years	All ages included	<50 years
HGD	41	30	187	3
Advanced adenomas	370	125	187	208
Aberrant IHC	1	4	10	1
MLH1 hypermethylation	-	1	Not performed	Not performed
Somatic MMR mutations	1	-	Not performed	Not performed
Pathogenic germline mutation	0	3	Not performed	Not performed (LS likely based on cancer history)

In routine diagnostics, 35 new LS families were identified in our center during the study period. The index patient is usually identified after development of (colorectal) cancer and/or based on family history, since IHC analysis on adenomas is not current practice. However, two adenoma patients were diagnosed with LS after aberrant IHC; a man with a large tubulovillous adenoma with high grade dysplasia at 27 years of age had a *MSH6* mutation and a woman aged 34 with a tubulovillous adenoma (and a sister with CRC at age 22) had a *MSH2* mutation.

Compared to the general population, LS patients more often develop adenomas with a villous component, high grade dysplasia and probably an increased size.(20-22) Furthermore, adenomas with a villous component or high grade dysplasia are most likely to show MSI and loss of MMR protein expression in LS patients.(14-17) In our study, a

total of 162/370 (44%) adenomas were judged advanced based on size ≥10 mm alone. In LS patients, these adenomas are less likely to show loss of MMR protein expression. Therefore, screening only adenomas with villous component or high grade dysplasia is expected to have a higher diagnostic yield. In fact, Basterra et al. found a high percentage (5,4%) of MMR deficiency among adenomas with high grade dysplasia. Nevertheless, MMR deficiency in these patients could still be due to *MLH1* hypermethylation or sporadic MMR mutations. No MMR deficiency was found in adenomas with high grade dysplasia in our study. However, only 41 cases with high grade dysplasia were included. Mendelsohn et al. found MMR deficiency in only 1/208 advanced adenoma patients under 50 years of age, but included only 3 adenomas with high grade dysplasia.

Selecting adenoma patients for LS screening based on family history or prediction models for LS might also be an effective strategy. Two cost-effectiveness analyses found that selecting CRC patients for LS screening using prediction models was more cost-effective than universal testing, if these prediction models were perfectly implemented.(23, 24) In our cohort, 16% of the patients had at least one first degree relative with CRC. Most patients in our cohort had PREMM5 values <1% and only four patients had PREMM5 scores >5%.

In LS patients, three different molecular pathways in colorectal cancer development were recently proposed.(25) Some adenomas from LS patients are MMR proficient, while most adenomas from LS patients seem to arise from MMR deficient crypt foci. Also, MMR deficient crypts may directly advance to CRC without formation of an adenoma. In the general population, it is not known whether the same pathways may apply in different proportions, or whether MMR deficiency, if it develops, always arises at a later stage in carcinogenesis. Around 12-20% of CRC are MMR deficient and most of the sporadic MMR deficient CRC show *MLH1* promoter hypermethylation.(26-29) In our study, 12,5% of CRC were MMR deficient and 8,9% of all CRC had *MLH1* promoter hypermethylation. If MMR deficiency is an early event in the adenoma-carcinoma sequence in the general population, we expected to find aberrant IHC in a similar proportion of the adenoma patients. The results of our study, with aberrant IHC in only 1/370 advanced adenomas (due to somatic *MSH6* mutations in a large tubulovillous adenoma) and no adenomas with loss of *MLH1* protein expression, support the hypothesis that MMR deficiency is a relatively rare and late event in the sporadic adenoma-carcinoma sequence.

To our knowledge, this is the first study on the yield of LS screening among patients within a population-based CRC screening program. A main strength of our study was its

setting, a population-based cohort, and the prospective collection of data. Also, germline and somatic mutation analysis was performed for all patients suspected of having LS by IHC. Our cohort was relatively large with 400 adenoma patients included for IHC analysis.

Our study has several limitations. Since individuals with a history of colorectal disease or a clear family history of CRC may not undergo FIT, there may be a selection of individuals unlikely to have LS. However, family history may not always be helpful to identify LS patients. Especially *MSH6* or *PMS2* mutation carriers may not have been identified before they reach the age of enrolment in a population based CRC screening program. Secondly, patients without a positive FIT were not included in our study. Nonetheless, we expect that patients without a positive FIT are not more likely to have LS than patients with a positive FIT. The results of our study may not be generalizable to all CRC screening programs. Population based screening programs with other screening methods (i.e. colonoscopy based instead of FIT based),including younger patients, or with lower uptake, may result in a different selection of the population. An optimal study would include colonoscopies in all individuals of different age groups regardless of FIT and IHC analysis of all adenomas. However, this does not seem feasible.

In conclusion, our results indicate that routine screening for LS by IHC among adenoma patients within a population based screening program for CRC is not effective. Further research should focus on IHC analysis in younger populations.

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Part II
Variants of unknown significance

Chapter 7

Diagnosing Lynch syndrome: Identification of pathogenic *MLH1* and *MSH2* variants in clinical practice

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ABSTRACT

Lynch syndrome (LS) is the most common hereditary predisposition to colorectal cancer. It is caused by inactivating germline mutations in DNA mismatch repair (MMR) genes, particularly *MLH1* and *MSH2*. To establish a definitive LS diagnosis, a pathogenic MMR gene mutation must be identified. Proper diagnosis of LS is important to ensure patients and their family members benefit from appropriate LS surveillance programs, as well as to relieve non-carriers from the physical and psychological burden of such surveillance programs. LS diagnosis is straightforward when patients carry nonsense and frameshift mutations that unambiguously abrogate MMR. Many patients however harbor missense variants whose functional implications are unclear. Such variants of uncertain significance (VUS) pose a clinical problem. To help physicians diagnose LS, we recently developed a genetic screen for the identification of pathogenic *MLH1* and *MSH2* variants. In the present study we used the screen to investigate the phenotype of 10 *MLH1* and 8 *MSH2* VUS found in suspected-LS patients from two medical centers in the Netherlands. Twelve of the VUS were found to abrogate MMR and thus cause LS. This study demonstrates the genetic screen is a useful tool for diagnosing LS in clinical practice.

INTRODUCTION

The hereditary colorectal cancer (CRC) predisposition Lynch syndrome (LS) accounts for approximately 5% of all CRC cases.(1) LS patients are also at increased risk of developing extracolonic cancer such as endometrial, ovarian and gastric cancer.(2, 3)

The underlying cause of LS is an inactivating germline mutation in one of the DNA mismatch repair (MMR) genes *MLH1*, *MSH2, MSH6* or *PMS2*, or a deletion in the 3' region of the *EPCAM* gene that affects *MSH2* expression.(4–8) The DNA MMR genes are essential for the maintenance of genome fidelity during DNA replication. Together they detect and correct base-base mismatches as well as insertion-deletion loops that may arise during erroneous DNA replication and induce cell death if adducts caused by certain DNA damaging agents are not removed.(9–12) LS patients usually inherit a mutant and a wild type copy of one of the DNA MMR genes. The single wild type allele can provide enough functional protein for the MMR system to work; however, upon somatic loss of the wild type allele, cells become MMR deficient. Consequently, mismatches that arise during DNA replication cannot be corrected and a mutator phenotype will develop that predisposes to cancer.(1, 13, 14)

LS tumors are characterized by the absence of immunohistochemical staining (IHC) for one of the MMR proteins and microsatellite instability (MSI). MSI reflects the hypermutable condition that develops in MMR-deficient cells; in these cells the errors made by DNA polymerase during the replication of repetitive DNA sequences like $(CA)_n$ or $(A)_n$ are not detected and corrected by DNA MMR, leading to length alterations that can easily be detected.(15–17) IHC and MSI analyses on tumor tissue are often used to identify patients who may suffer from LS. For a definitive LS diagnosis, however, germline mutation analysis must reveal a defect in one of the DNA MMR genes.(18) Germline nonsense and frameshift mutations in the MMR genes or 3' deletion of the *EPCAM* gene can unambiguously be assigned to abrogate MMR and hence to cause LS. Missense mutations, however, pose a clinical problem. These mutations only alter a single amino acid, often with unclear functional consequences, leaving their clinical significance uncertain.(19)

An unambiguous LS diagnosis enables the identification of patients and family members who will benefit from LS surveillance programs.(13) LS surveillance can reduce morbidity and mortality of LS carriers by 62%.(20) If a variant of uncertain significance (VUS) is identified, it is unclear whether a patient will benefit from this intensive and burdensome surveillance. Moreover, targeted germline analysis in relatives to determine which family members can benefit from LS surveillance is also not possible in these cases. Therefore, determining the pathogenicity of VUS in MMR genes is of great clinical importance.

We recently presented a genetic screen for the identification of pathogenic *MLH1* and *MSH2* DNA MMR gene variants associated with LS.(21, 22) In the current study we used

the genetic screen to determine the pathogenic phenotype of variants in the *MLH1* and *MSH2* genes found in clinical practice.

RESULTS

Genetic screen for the identification of pathogenic *MLH1* and *MSH2* variants

Here we demonstrate the clinical application of a genetic screen that was previously developed for the identification of pathogenic *MLH1* and *MSH2* variants.(21, 22) The screen allows many variants to be studied in parallel in three simple steps: 1) the *MLH1* or *MSH2* mutation of interest is introduced into mouse embryonic stem cells hemizygous for *Mlh1* or *Msh2*; 2) cells are subsequently selected for loss of MMR capacity; 3) the presence of the intended mutation is confirmed in the MMR-deficient cells.

In hemizygous $Mlh1^{+PUR/\Delta}$ and $Msh2^{+PUR/\Delta}$ mouse embryonic stem cells (mESCs) one of the endogenous *Mlh1* or *Msh2* alleles was deleted (*Δ*), respectively, and a *puromycin* resistance gene was inserted adjacent to the remaining *Mlh1* or *Msh2* allele that retained wild type activity (*+PUR*). Introduction of the desired mutation into the single *Mlh1* or *Msh2* allele leads to expression of solely the variant protein, allowing immediate investigation of its phenotype. Cells were site-specifically mutated by oligonucleotide-directed gene modification (oligo targeting).(23) Oligo targeting has an efficiency around 10^{-3}. To determine whether the 1 in 1000 oligo targeted mESCs that incorporated the mutation consequently became MMR-deficient, cells were exposed to the DNA damaging agent 6-thioguanine (6TG). 6TG is highly toxic to MMR-proficient but only mildly toxic to MMR-deficient cells.(9) Therefore the appearance of 6TG-resistant colonies suggests MMR-deficient mESCs were generated. MMR deficiency may arise due to the introduced mutation but also due to loss of heterozygosity events that delete the one functional *Mlh1* or *Msh2* allele. To select against loss of heterozygosity, Puromycin selection was performed simultaneously. 6TG/Puromycin-resistant colonies were sequenced to confirm the presence of the intended mutation in the selected MMR-deficient cells (Figure 1).

Screening clinically relevant variants

The genetic screen was used to investigate the pathogenicity of 18 VUS that were found in the *MLH1* and *MSH2* genes in 21 suspected-LS families from the Erasmus Medical Center Rotterdam and the Netherlands Cancer Institute Amsterdam (Table S1 presents clinical data). Of the 10 *MLH1* VUS, 7 were identified in 6TG-resistant colonies and hence suggested to abrogate MMR: *Mlh1 M1T*, *M35N*, *E37K*, *L586F*, *P652L*, *A685V* and *R691W* (Table 1A, Figure S1A). Five of the 8 screened *MSH2* VUS led to 6TG-resistance: *MSH2 C333Y*, *L421P*, *I685R*, *Q690E* and *IVS15-3C>G* (Table 1B, Figure S1B). *Mlh1* variants *A31C*, *V213M*, *T549A* and *MSH2* variants *N127S*, *R534C*, *N596S* were not found in 6TG-resistant colonies and therefore do not appear to attenuate MMR capacity (Table 1).

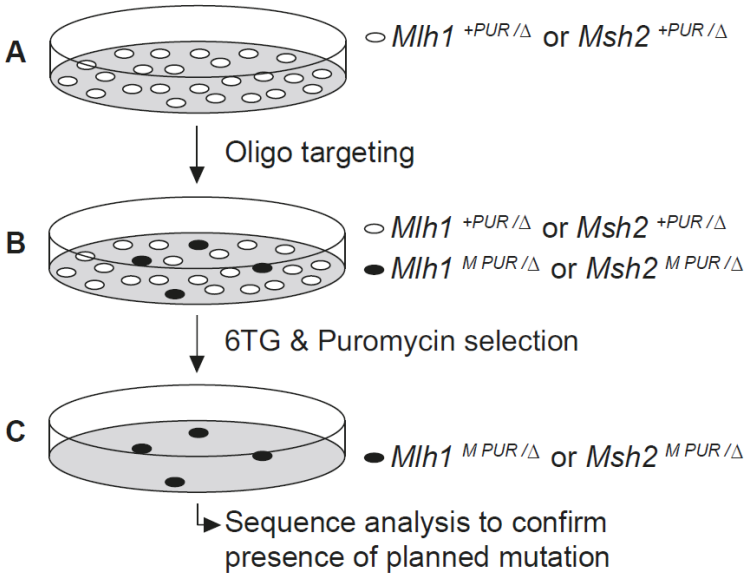

Figure 1. Genetic screen for the identification of pathogenic *MLH1* and *MSH2* variants.
(A) The mutation of interest was introduced into *Mlh1*+PUR/Δ or *Msh2*+PUR/Δ mESCs by oligo targeting.
(B) Oligo targeting has a site directed mutagenesis efficiency of ±10-3. To determine if the 1 in 1000 mESCs that incorporated the base change (annotated as *Mlh1* M PUR/Δ or *Msh2* M PUR/Δ) following oligo targeting had lost MMR capacity, the mESCs were exposed to 6TG. MMR-deficient cells are 6TG resistant while MMR-proficient cells die in response to 6TG exposure. To exclude cells that became MMR-deficient due to loss of heterozygosity events from further analyses, Puromycin selection was performed simultaneously.
(C) 6TG/Puromycin-resistant cells were sequenced to confirm the presence of the planned mutation.

Phenotypic assessment of detected MMR-attenuating variants

The 12 VUS that were found to attenuate MMR in the genetic screen were detected in patients with hallmarks of LS, *i.e.*, their tumors showed MSI-H and lacked MMR proteins. To confirm the missense mutations are causative for the observed phenotype, Western blot analyses as well as functional assays examining MSI and methylation-damage-induced mutagenesis were performed with the 6TG-resistant variants.

Protein levels in the variant cell lines were determined by Western blot analysis and quantified with respect to the amount of MLH1 and MSH2 observed in MMR-proficient *Mlh1*$^{+PUR/\Delta}$ and *Msh2*$^{+PUR/\Delta}$ mESCs, respectively (Figure 2).

A

Variant in man	Variant in mice	Nucleotide change	Colonies encoding mutation / number sequenced colonies
M1T	M1T	A>G	10 / 12
A31C	A31C	GC>CA	0 / 12
		GC>TG	0 / 7
M35N	M35N	CA>GT	1 / 12
E37K	E37K	C>T	9 / 11
V213M	V213M	C>T	0 / 9
		G>A	0 / 7
T545A	T549A	T>C	0 / 12
		A>G	0 / 8
L582F	L586F	G>A	10 / 12
P648L	P652L	G>A	11 / 12
A681V	A685V	G>A	5 / 12
R687W	R691W	G>A	9 / 12

B

Variant in man	Variant in mice	Nucleotide change	Colonies encoding mutation / number sequenced colonies
N127S	N127S	T>C	0 / 1
		A>G	0 / 2
C333Y	C333Y	C>T	2 / 7
L421P	L421P	A>G	12 / 12
R534C	R534C	G>A	0 / 10
		C>T	0 / 5
N596S	N596S	T>C	0 / 9
		A>G	0 / 3
I685R	I685R	AT>CG	2/ 5
Q690E	Q690E	G>C	8 / 8
IVS15-3C>G	IVS15-3C>G	C>G	9 / 12

Table 1. Identification of pathogenic *MLH1* and *MSH2* VUS using the genetic screen.
The genetic screen was used to investigate the MMR-abrogating effect of (A) 10 *MLH1* variants and (B) 8 *MSH2* variants. Variants are annotated according to their amino acid number and change in men and mice as well as the base alteration. Each variant was initially introduced with the antisense oligonucleotide. If the screening protocol with the antisense oligonucleotide did not give rise to 6TG/Puromcyin-resistant colonies encoding the mutation of interest, the screen was repeated with the sense oligonucleotide (lower row where two rows are present in the 'Nucleotide change' column). Antisense and sense oligonucleotides can have differing genome integration frequencies depending on their target site; by repeating the screen we reduce the chance that a variant was missed in 6TG/Puromycin-resistant colonies due to low mutation introduction efficiencies. The final column describes how many of the sequenced 6TG-resistant colonies encoded the desired mutation. We always aimed to sequence 12 colonies unless fewer survived the selection protocol.

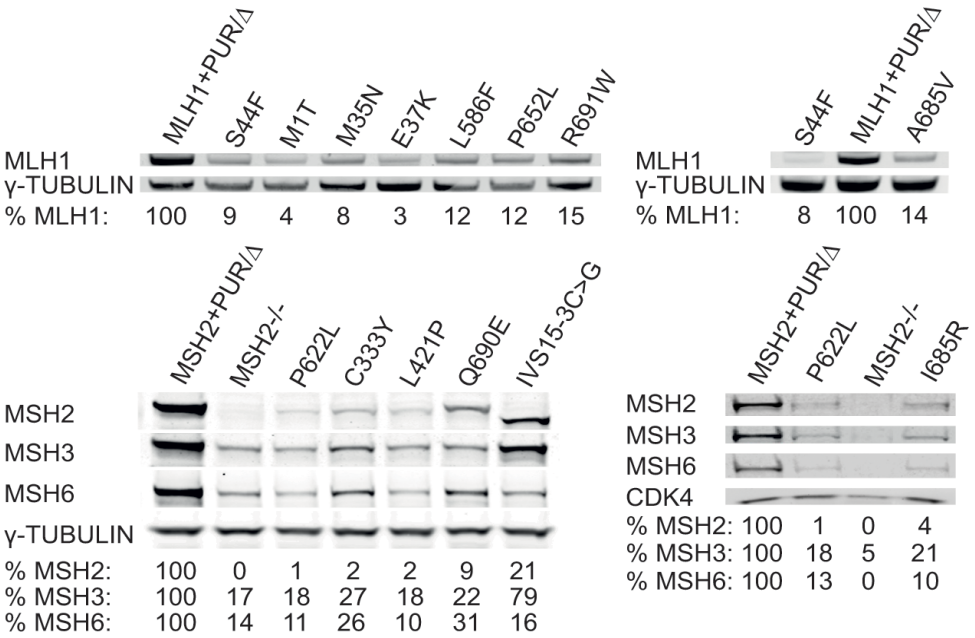

Figure 2. Western blot analysis of detected pathogenic *Mlh1* and *Msh2* mutations
MLH1 and γ-TUBULIN levels were analyzed in whole cell lysates of the *Mlh1* variant cell lines. Relative MLH1 levels compared to *Mlh1*+PUR/Δ mESCs are displayed in percentages. MSH2, MSH3, MSH6 and γ-TUBULIN or CDK4 levels were assessed in the *Msh2* variant cell lines. Relative MSH2, MSH3 and MSH6 levels were quantified with respect to the amount of protein in *Msh2*+PUR/Δ mESCs. γ-TUBULIN and CDK4 functioned as loading controls. *Mlh1* S44F/Δ, *Msh2* P622L/Δ, and *Msh2* -/- mESCs were used as pathogenic controls.

Mlh1$^{+PUR/\Delta}$ and *Msh2*$^{+PUR/\Delta}$ mESCs maintain an active MMR system with 60-80% of the protein quantity observed in wild-type mESCs.(21, 22) MLH1 levels in the *Mlh1* mutant cell lines fell to 3-15%. Similarly, MSH2 quantities in the *Msh2* mutant cell lines dropped to 2-21%. The reduction in MSH2 levels was accompanied by a decrease in the amount of MSH3 and MSH6. MSH3 and MSH6 form a heterodimer with MSH2 to detect insertion-deletion loops and base-base mismatches, respectively. Without MSH2, both MSH3 and MSH6 are less stable. Only the *MSH2 IVS 15-3C>G* mESCs maintained relatively high MSH3 levels. This variant, which is located in the splice acceptor site of exon 15 led to protein truncation, which primarily appeared to affect complex formation with MSH6 rather than with MSH3.

To assess MSI in the 6TG-resistant variant cell lines, we used a $(G)_{10}$-*neo* slippage reporter. This reporter is composed of a *neomycin* resistance gene (*neo*) that is rendered out of frame by a preceding $(G)_{10}$ repeat. The *neo* gene only becomes in-frame when DNA polymerase slippage errors on the $(G)_{10}$ repeat, such as the deletion of one G or insertion of two Gs, are not noticed by the DNA MMR system. Hence, the number of Geneticin-resistant colonies that arise is indicative of the frequency of *neo*-restoring slippage events and provides a quantitative readout of the MMR capacity of the

cells.(24) The slippage rates, *i.e.*, the chance of a slippage event occurring during one cell division, of the *Mlh1* and *Msh2* variant cell lines ranged from $2.6x10^{-4}$ to $7.6x10^{-4}$; comparable to the rates observed for the *Mlh1 S44F* and *Msh2 P622L* pathogenic controls and on average ±500 times higher than the slippage rates of $Mlh1^{+PUR/\Delta}$ ($1.2x10^{6}$) and $Msh2^{+PUR/\Delta}$ ($7.3x10^{-7}$) mESCs (Figure 3).

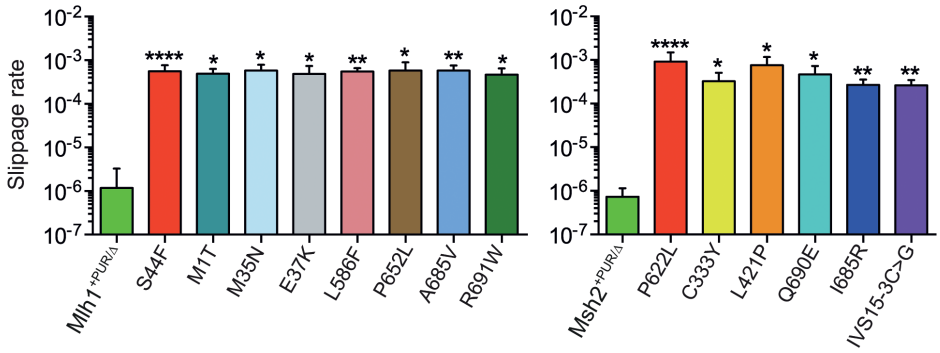

Figure 3. MSI analysis in *Mlh1* and *Msh2* variant cell lines
MSI in the variant cell lines was investigated using a (G)10-neo slippage reporter. This reporter is composed of a neo gene that is rendered out of frame by a disrupting (G)10 repeat. In MMR-proficient cells, slippage events on the (G)10 repeat that bring the neo in frame will be detected and corrected. In MMR-deficient cells, such slippage events go unnoticed and lead to the generation of Geneticin-resistant cells. The number of Geneticin-resistant cells gives an indication of the slippage rates in the variant cell lines. The slippage rate is defined as the emergence of a Geneticin-resistant cell per cell division. The slippage rates of the detected pathogenic variant cell lines was compared to the MMR-proficient *Mlh1*+PUR/Δ and *Msh2*+PUR/Δ and the MMR-deficient *Mlh1* S44F and *Msh2* P622L controls. Statistical differences were calculated using a two-tailed, unpaired t-test with Welch's correction. Asterisks indicate values significantly higher than those of the MMR-proficient controls: *P<0.05; **P<0.01; ****P<0.0001.

In addition to elevated spontaneous mutagenesis events, MMR-deficient cells also experience increased methylation-damage-induced mutagenesis. To assess if the *Mlh1* and *Msh2* variants increased methylation-damage-induced mutagenesis, mESCs were exposed to the methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and the number of cells that consequently acquired mutations was quantified.(25) MNNG creates lesions in the genome that give rise to mismatches when replicated. In MMR-proficient cells, these mismatches elicit futile repair attempts that ultimately lead to cell death. In MMR-deficient cells however, these mismatches are not recognized, which allows cell survival albeit at the expense of methylation-damage-induced mutagenesis. To quantify the frequency of mutation accumulation, MNNG-treated cells were exposed to a high dose of 6TG for an extended period of time. MMR-proficient or -deficient mESCs do not survive this stringent 6TG selection scheme unless they have acquired an inactivating mutation in *Hprt* because HPRT is required for 6TG to act as a DNA damaging agent. All detected *Mlh1* and *Msh2* variant cell lines had elevated MNNG-induced mutator phenotypes compared to the MMR-proficient control cell lines (Figure 4).

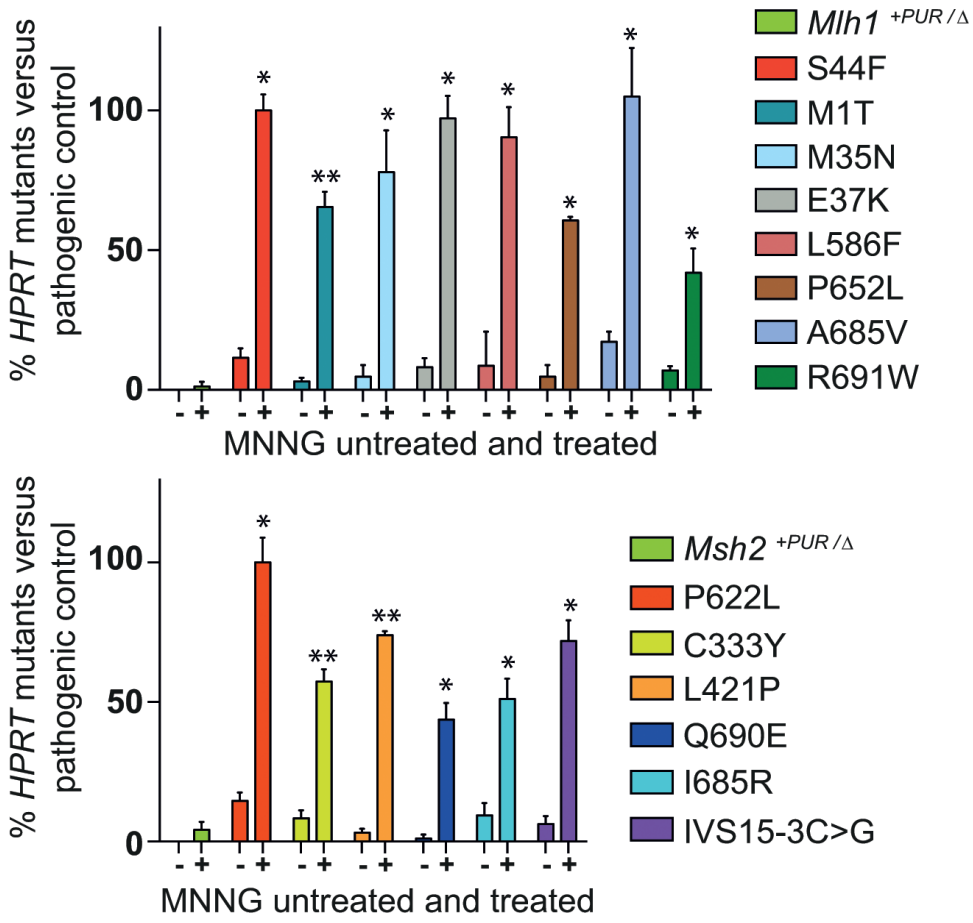

Figure 4. Methylation-damage-induced mutagenesis in *Mlh1* and *Msh2* variant cell lines
To assess the level of methylation-damage-induced mutagenesis in the variant cell lines, mESCs were exposed to 0 or 4 µM MNNG and the number of cells that consequently acquired mutations in Hprt was quantified by exposing mESCs to a high dose of 6TG for an extended period of time; only HPRT-deficient cells are resistant to this treatment. The spontaneous (-) and MNNG induced (+) mutation frequencies of the variant cell lines were compared to the MMR-proficient *Mlh1*+PUR/Δ and *Msh2*+PUR/Δ and the MMR-deficient *Mlh1* S44F and *Msh2* P622L control mESCs where the numbers of Hprt mutant cells observed for the 4 µM MNNG-treated pathogenic controls were set at 100%. Statistical differences between the MNNG-treated MMR-proficient controls and MNNG-treated variant cell lines were calculated using a one-tailed, unpaired t-test with Welch's correction. Asterisks indicate values significantly higher than those of the MNNG-treated MMR-proficient *Mlh1*+PUR/Δ and *Msh2*+PUR/Δ controls: *P<0.05; **P<0.01.

Phenotypic assessment of a non-detected *MLH1* variant

According to the clinical data, *MLH1 A31C* was suspicious of being pathogenic: the patient developed a MSI-H tumor for which IHC was not conclusive but no *MLH1* promoter methylation was observed. However, our genetic screen did not identify this variant as MMR abrogating. To exclude *MLH1 A31C* somehow escaped detection by our

screen, we generated *Mlh1*$^{A31C/\Delta}$ mESCs using CRISPR/Cas9 technology (Figure S1C, Figure S2) and analyzed their MMR capacity using the MSI and MNNG-induced mutagenesis assays as well as Western blot analysis (Figure 5). *Mlh1*$^{A31C/\Delta}$ mESCs had normal MLH1 levels, and did not show increased MSI or methylation-damage-induced mutagenesis. Thus, *Mlh1 A31C* did not attenuate MMR, consistent with the screening result.

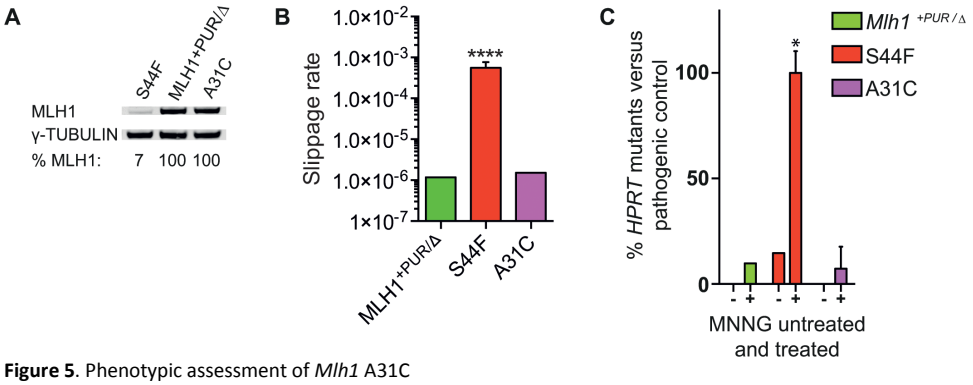

Figure 5. Phenotypic assessment of *Mlh1* A31C

(A) Western blot analysis: MLH1 and γ-TUBULIN levels were analyzed in *Mlh1* A31C/Δ mESCs. γ-TUBULIN functioned as the loading control. The relative MLH1 level in the variant cell line compared to *Mlh1*+PUR/Δ mESCs is indicated.

(B) MSI was investigated using the (G)10-neo slippage reporter. The slippage rate (the emergence of a Geneticin-resistant cell per cell division) of the *Mlh1* A31C/Δ cells was compared to the rate in MMR-proficient *Mlh1*+PUR/Δ and MMR-deficient *Mlh1* S44F control cell lines. Statistical differences were calculated using a two-tailed, unpaired t-test with Welch's correction. ****, indicates the slippage rates of *Mlh1* S44F/Δ mESCs were significantly higher than Mlh1+PUR/Δ mESCs: P<0.0001.

(C) Methylation-damage-induced mutagenesis assay: the accumulation of Hprt mutations in *Mlh1* A31C/Δ mESCs was analyzed following exposure to 0 or 4 μM MNNG. The spontaneous (-) and MNNG induced (+) mutation frequency in the *Mlh1* A31C/Δ mESCs was compared to the MMR-proficient *Mlh1*+PUR/Δ and the MMR-deficient *Mlh1* S44F controls. Statistical differences between the MNNG-treated MMR-proficient control and MNNG-treated variant cell lines were calculated using a one-tailed, unpaired t-test with Welch's correction. Asterisks indicate the value was significantly higher than that of the MNNG-treated MMR-proficient Mlh1+PUR/Δ control: *P<0.05.

DISCUSSION

DNA MMR gene VUS pose an important clinical problem. Until the pathogenic phenotype of a VUS is established, a patient cannot be diagnosed with LS.(18) This uncertainty may inadvertently exclude non-mutation carriers from highly beneficial surveillance programs or unnecessarily expose mutation carriers to the physical and psychological burden of regular screening.(13)

To help physicians diagnose LS we recently developed a genetic screen for the identification of pathogenic *MLH1* and *MSH2* variants.(21, 22) The genetic screen

combines oligo targeting in *Mlh1*$^{+PUR/\Delta}$ and *Msh2*$^{+PUR/\Delta}$ mESCs with 6TG selection and sequence analysis to distinguish pathogenic DNA MMR gene variants from polymorphisms. The relative simplicity of this approach allows many VUS to be studied in parallel and makes it a promising tool for clinical genetics laboratories confronted with DNA MMR VUS. In the present study we demonstrate the application of our genetic screen in clinical practice.

We investigated the phenotype of 18 VUS found in the *MLH1* and *MSH2* genes of suspected-LS patients seen at the Erasmus Medical Center Rotterdam and the Netherlands Cancer Institute Amsterdam. Variants *MLH1 M1T*, *M35N*, *E37K*, *L582F*, *P648L*, *A681V*, *R687W* as well as *MSH2 C333Y*, *L421P*, *I685R*, *Q690E*, *IVS15-3C>G* were found to abrogate MMR thus confirming the diagnosis LS, while variants *MLH1 A31C*, *V213M*, *T545A* and *MSH2 N127S*, *R534C*, *N596S* were not found to be pathogenic.

The screening results for the detected pathogenic mutations are in line with the clinical data.(Table S1) All 7 *MLH1* mutations were identified in patients with MSI-H tumors of which 6 had lost MLH1 and PMS2 expression (in the remaining case IHC was inconclusive). Similarly, the 5 *MSH2* mutations were identified in cases highly suspicious of LS, *i.e.*, patients had developed MSI-H CRC that did not stain for MSH2 or MSH6. Except for one *MLH1* case, all *MSH2* and *MLH1* mutation carriers belonged to families with a clear history of gastrointestinal and/or genitourinary cancer.

Western blot analyses and MSI assays demonstrated the detected pathogenic mutations led to a drop in protein levels as well as a strongly increased MSI. As expected, reduced MSH2 levels were accompanied by reduced MSH6 and MSH3 levels as the stability of these proteins relies on their interaction with MSH2. *Msh2 IVS15-3C>G* mESCs maintained the highest MSH2 level of 21%. Interestingly, this is also the only detected pathogenic *Msh2* variant cell line that maintained relatively large quantities of MSH3. The Western blot data suggest *Msh2 IVS15-3C>G* led to truncation of MSH2, which moderately destabilized the protein and interfered with MSH2/MSH6 but not MSH2/MSH3 dimerization. Whether MSH2 IVS15-3C>G is able to detect insertion-deletion loops and prevent slippage events at microsatellites with larger repeats is unclear as the MSI assay in this study used a $(G)_{10}$ repeat that specifically monitors MSH2/MSH6 activity rather than MSH2/MSH3 activity. The pathogenicity of *MSH2* variant Q690E is consistent with a previous assay in which this variant was found to display high levels of mono-nucleotide instability despite intermediate tolerance to 6TG.(26)

The genetic screen identified *MLH1* variants *A31C*, *V213M* and *T545A* as not pathogenic. *MLH1 V213M* was found in a MSS tumor with normal IHC. According to the International Society for Gastrointestinal Hereditary Tumours (InSiGHT) colon cancer variant database (27), which categorizes DNA MMR gene variants based on their likelihood of being pathogenic considering the available data, *MLH1* variant *V213M* is not pathogenic. This was based on studies demonstrating *MLH1 V213M* did not influence protein expression, stability, subcellular localization, protein-protein interactions, or MMR capacity.(28–30)

MLH1 variant *T545A* was found in an MSI-H tumor that did not stain for the presence of MLH1 or PMS2. This phenotype may however be explained by the *c.677+1delG* mutation that was identified in the other *MLH1* allele. *MLH1 c.677+1delG* is a classified pathogenic mutation; based on available data its likelihood of being pathogenic is >0.99.(19) *MLH1 A31C* was identified in a MSI-H tumor for which IHC was inconclusive but no *MLH1* promoter methylation was observed. Consistent with our screening result, we demonstrated that independently generated $Mlh1^{A31C/\Delta}$ mESCs (using CRISPR/Cas9-assisted base-pair substitution) maintained a fully functional DNA MMR system. This is in line with the *in vitro* data obtained by Drost et al. (31) that also indicated *MLH1 A31C* is MMR proficient.

According to the genetic screen *MSH2* variants *N127S, R534C* and *N596S* did not abrogate MMR. All 3 variants were found in patients with MSS tumors that showed normal IHC, suggesting they are indeed unlikely to affect MMR activity. The InSiGHT database classified *MSH2 N127S* as not pathogenic. *In vitro* studies have demonstrated this variant is capable of interacting with MSH6 as well as recognizing and repairing mismatches.(32, 33) The presence of two not-pathogenic variants, *MLH1 V213M* and *MSH2 N127S*, in a single CRC patient is remarkable and underscores the necessity for careful phenotypic assessment of MMR gene mutations. Although VUS *R534C* was identified in a tumor that did not show the hallmarks of LS, the patient did have a family history of cancer and met the revised Bethesda guidelines. *In silico* algorithms suggest *MSH2 R534C* may be pathogenic.(34, 35) The consulted literature and the InSiGHT database did not give any further description of *MSH2 R534C*; to confirm the accuracy of our screening result, the MMR capacity of $Mlh1^{R534C/\Delta}$ mESCs should be assessed independently. VUS *N596S* belongs to the uncertain class in the InSiGHT database. *In silico* algorithms suggest the variant does not interfere with MMR activity.(34, 35) Consistently, clinical studies indicate *N596S* is a rare polymorphism because it did not co-segregate with disease and N596 is not conserved: in MMR-proficient yeast a serine is found at the analogous position.(36–38)

The present study further confirms the strength of the genetic screen we developed for the characterization of *MSH2* and *MLH1* DNA MMR gene variants. All detected variants were shown to abrogate MMR activity and one of the non-detected VUS was confirmed to be MMR-proficient. With the advances in whole genome sequencing, it will become increasingly common to determine an individual's entire DNA sequence and the variations it harbors. To use this data for the diagnosis and treatment of patients, techniques need to be in place in clinical genetics laboratories that interrogate the functional implications of rare sequence variants. The presented genetic screen is one approach that allows pathogenic DNA MMR gene variants to be distinguished from polymorphisms and hence can contribute to the identification of LS patients.

METHODS

MLH1 and *MSH2* VUS in clinical cohort

We studied 18 VUS found in the *MLH1* and *MSH2* genes of suspected-LS patients seen at the department of Clinical Genetics at the Erasmus Medical Center Rotterdam or the Family Cancer Clinic at the Netherlands Cancer Institute Amsterdam. Tumor characteristics, age at diagnosis, results of molecular diagnostics and germline mutation analyses, as well as a detailed family history of these cases were collected from medical records (Table S1). Analysis for MSI was performed with the Bethesda panel for cases before 2007 (39) or with the Promega pentaplex MSI analysis from 2007 onwards.(40) IHC for MLH1, MSH2, MSH6 and PMS2 protein *w*as performed as described previously.(41) Germline mutation analysis of *MLH1* and *MSH2* was performed by sequencing and multiplex ligation dependent probe amplification. The *in silico* prediction models MAPP-MMR (34) and PolyPhen (35) were used to estimate the chance of a variant being deleterious. MAPP-MMR scores were calculated on http://mappmmr.blueankh.com/ and PolyPhen scores on http://genetics.bwh.harvard.edu/pph2/.

Genetic screen for the identification of pathogenic *MLH1* and *MSH2* VUS

The genetic screen for the characterization of *MLH1* and *MSH2* variants was performed in $Mlh1^{+PUR/\Delta}$ and $Msh2^{+PUR/\Delta}$ mESCs (21, 22), respectively. $7x10^5$ $Mlh1^{+PUR/\Delta}$ or $Msh2^{+PUR/\Delta}$ mESCs were seeded on 6 wells and 24 h later exposed to 7.5 µl *Trans*IT-siQuest® transfection agent (Mirus) plus 3 µg single-stranded locked-nucleic-acid-modified DNA oligonucleotides (LMOs) encoding the desired mutation in 250 µl serum-free medium.(23) After 24h, the medium was refreshed and after 2 days $1.5x10^6$ LMO-exposed cells were transferred to 10 cm plates for 6TG (250 nM) (Sigma-Aldrich®) and Puromycin (1.8 µg/ml) (Sigma-Aldrich®) selection. The 12 largest 6TG/Puromycin-resistant mESCs were picked, expanded and sequenced to confirm the presence of the planned mutation.

Generation of $Mlh1^{A31C/\Delta}$ mESCs

$Mlh1^{A31C/\Delta}$ mESCs were created by CRISPR/Cas9-assisted gene modification.(42) Complementary primers CACCGATCATCTCTTTGATAGCAT and AAACATGCTATCAAAGA GATGATC (Sigma-Aldrich®) were phosphorylated, annealed and ligated into the *Bbs*I site (enabled by the underlined bases in the primers) of a px330 vector (43) that also contains a *puromycin* resistance gene (PGK1-Puro-PGK1pA). The vector was subsequently transfected into $Mlh1^{+PUR/\Delta}$ mESCs: $7x10^5$ $Mlh1^{+PUR/\Delta}$ mESCs were seeded on a gelatin-coated 6 well in BRL-conditioned medium and exposed to 0.25 µg vector plus 2.25 µg homology directed repair (HDR) oligonucleotide template (CTCAGTCGGG GAACTCGGCTCCGCTCCCTCCGTACCAGTTTTCTATCATCTCTTTGATACAATTTGCCGGCCGCTG AATGACTTCCCCCGCCGCTATGCGGTTCACTACCGTCTCGTCCA) and 6.25 µl Trans-IT LTI transfection agent (Mirus) in 250 µl optiMem (Gibco) after 24 h. The HDR oligonucleotide template was complementary to the *Mlh1* target site except for the two central bases (underlined) that encode the desired *A31C* alteration as well as a silent mutation that inactivates the PAM site (underlined). The PAM site was inactivated to prevent continued Cas9 cutting after successful gene modification. The following day, one-third

of the transfected mESCs was transferred to a gelatin-coated 6 well and cultured in BRL-conditioned medium containing 1.8 µg/ml Puromycin for 2 days. Six days post transfection, $1.5x10^6$ mESCs were transferred to a gelatin-coated 10 cm plate in BRL-conditioned medium. After 10 days, colonies were picked, expanded and sequenced to identify cells carrying the *Mlh1 A31C* mutation.

Phenotypic assessment of detected *Mlh1* and *Msh2* variants as well as *Mlh1*$^{A31C/\Delta}$ mESCs

The phenotypes of the detected 6TG-resistant variants as well as *Mlh1*$^{A31C/\Delta}$ mESCs were further characterized using MSI assays (24), methylation-damage-induced mutagenesis assays (25) and Western blot analyses (21, 22), as described previously.

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SUPPLEMENTAL MATERIAL

Figure S1. Sequences of detected pathogenic *Mlh1* and *Msh2* variants as well as *Mlh1* A31C/Δ mESCs. (A) Sequences of 6TG-resistant *Mlh1* and (B) *Msh2* variants as well as (C) *Mlh1* A31C/Δ mESCs. One-letter amino acid codes are illustrated below the nucleotide sequences. WT stands for the wild-type *Mlh1* or *Msh2* sequences.

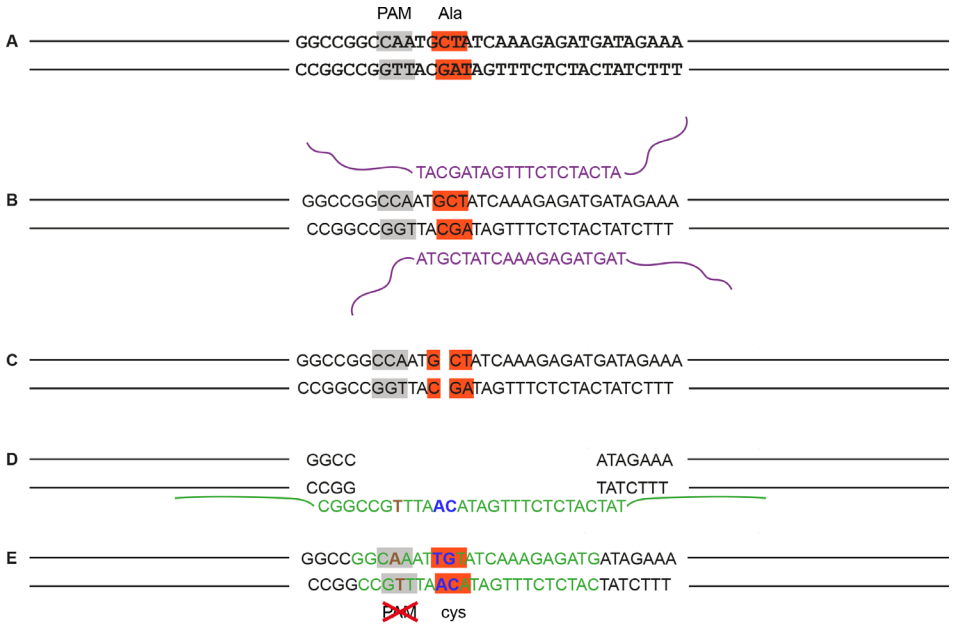

Figure S2. Generation of *Mlh1* A31C/Δ mESCs using CRISPR/Cas9 technology.
Mlh1 A31C/Δ mESCs were created by CRISPR/Cas9-assisted gene modification. A) The nucleotide sequence at the target site is presented: the alanine at residue 31 is highlighted in red and the PAM site in grey. The gRNA sequence is underlined in purple and the DSB site indicated by Δ. B) The break was repaired by homology directed repair (HDR) using an oligonucleotide template (green). The HDR oligonucleotide template is complementary to the target site except at residue 31 and the PAM site: at residue 31 a cysteine is encoded (blue) and at the PAM site, a neutral nucleotide change is introduced (brown) to eliminate the PAM and prevent re-introduction of the break by Cas9. C) HDR of the Cas9 introduced break using the oligonucleotide template lead to introduction of the cysteine at residue 31 and a silent mutation abolishing the PAM site.

Table S1. Clinical data collected for 18 VUS found in the MLH1 and MSH2 genes in 21 suspected-LS families in the Netherlands.

Variant	Index patient	MSI	IHC	Other tumor analysis	Other variants	Relatives	Revised Bethesda Guidelines	InSiGHT class	Prediction models	Screen results
MLH1 M1T	CRC 53 yrs Female	MSI-H	MLH1-PMS2-	No methylation		FDR: Testicular cancer	No	NA	PolyPhen: 0.973	Pathogenic
MLH1 A31C	CRC 52 yrs Male	MSI-H	NA	No methylation		FDR: - Tubulovillous adenoma and 2 hyperplastic polyps		3	MAPP-MMR: 3.26 PolyPhen: 1	Not pathogenic
MLH1 M35N	CRC 52 yrs Male	MSI-H	MLH1-PMS2-			FDR: - CRC 67 - CRC 50 and EsoC/GaC 78 - PaC - OvC 69 (MSS, IHC normal) - CRC 78 (carrier of VUS) SDR: - CRC 46 (MSS, VUS in tumor)	Yes	5	MAPP-MMR: 20.02 PolyPhen: 0.968	Pathogenic
MLH1 E37K	OvC and UtC 45 yrs Female	MSI-H	MLH1 failed PMS2 weak			FDR: - CRC 46 - UtC 43 SDR: - CRC 59 - CRC 55 and GaC 65 - CRC 61 - CRC 48	Yes	3	MAPP-MMR: 18.32 PolyPhen: 1	Pathogenic
MLH1 V213M	CRC 28 yrs Female	MSS	IHC failed		*MSH2 N127S*	SDR: - CRC 52	Yes	1	MAPP MMR: 1.99 PolyPhen: 0.607	Not pathogenic
MLH1 T545A	CRC 30 yrs Male	MSI-H	MLH1-PMS2-		Pathogenic mutation *c.677+1delG* on other *MLH1* allele		Yes	3	MAPP-MMR: 4.65 PolyPhen: 0.971	Not pathogenic
MLH1 L582F	CRC 68 yrs Male	MSI-H	MLH1-PMS2-	No methylation		FDR: - CRC <60 - CRC 66 (MSS, normal IHC) - OvC <55 TDR: - BrainC 11	Yes	3	MAPP-MMR: 2.24 PolyPhen: 0.997	Pathogenic
MLH1 L582F	CRC (3x) 26 yrs Male	No tissue	No tissue	Homozygous for VUS		FDR: - CRC 31 and glioblastoma 30 (also homozygous for VUS) *Fathers family:* SDR: - CRC 59 TDR: - CRC 40 - CRC 58 *Mothers family:* TDR: - 2 relatives CRC	Yes	3	MAPP-MMR: 2.24 PolyPhen: 0.997	Pathogenic

MLH1 L582F	CRC 60 yrs Male	MSI-H	MLH1-PMS2-			FDR: - CRC 62 - Cancer 67	No	3	MAPP-MMR: 2.24 PolyPhen: 0.997	Pathogenic
MLH1 L582F	CRC 60 yrs Male	MSI-H	MLH1-PMS2-			TDR: - CRC 37 (carrier VUS) - CRC 62 (carrier VUS)	Yes	3	MAPP-MMR: 2.24 PolyPhen 0.997	Pathogenic
MLH1 P648L	CRC 32 yrs Female	MSI-H	MLH1-PMS2-	No methylation		FDR: - CRC 45 - CRC 41 SDR: - CRC 46 (carrier VUS) - CRC 46 - CRC 74	Yes	5	MAPP-MMR: 6.87 PolyPhen: 1	Pathogenic
MLH1 A681V	EC 51 yrs Female	MSI-H	MLH1-PMS2-	No methylation				3	MAPP-MMR: 5.5 PolyPhen: 0.993	Pathogenic
MLH1 R687W	CRC 44 yrs Male	MSI-H	MLH1-PMS2-	No methylation Retention VUS, LOH of *MLH1*		FDR: - BoneC and melanoma 74 - OvC 53 SDR: - Two relatives CRC TDR: - CRC 63 (carrier VUS, MSI-H, MLH1-, retention VUS and LOH of *MLH1)*	Yes	5	MAPP-MMR: 3.87 Polyphen: 1	Pathogenic
MSH2 N127S	CRC 28 yrs Female	MSS	IHC normal		*MLH1 V213M*	SDR: - CRC 52	Yes	1	MAPP MMR: 3.26 PolyPhen: 0.912	Not pathogenic
MSH2 C333Y	CRC 58 yrs Female	MSI-H	MSH2-MSH6-			FDR: - CRC (carrier VUS, MSI-H, MSH2-/MSH6-, no LOH or methylation of *MSH2*) - CRC - OvC 53 - Meningeoma 40 SDR: - Two relatives CRC - UtC TDR: - Three relatives CRC 44-67 - UtC	Yes	3	MAPP-MMR: 12.95 PolyPhen: 1	Pathogenic
MSH2 L421P	CRC 44 yrs Female	MSI-H	MSH2-MSH6-			FDR: - Renal cell carcinoma <48 SDR: - CRC 34 - BlC 59 - GaC <42 - UtC <53 TDR: - CRC 22 (carrier VUS)	Yes	3	MAPP-MMR: 16.26 PolyPhen: 0.998	Pathogenic
MSH2 R534C	CRC 45 yrs BlC 63 yrs Male	BlC MSS CRC no tissue	BlC IHC normal CRC no tissue			FDR: - GaC 67 - BrainC 47 - Cervical cancer 27	Yes	NA	MAPP-MMR: 17.11 PolyPhen: 1	Not pathogenic

MSH2 N596S	CRC 51 yrs Female	MSS	IHC normal			FDR: - CRC 59 - Testicular cancer 37 - Carcinoid carcinoma appendix 31 and seminoma in situ 41 SDR: - CRC 42 - CRC 70 TDR: - CRC 71	Yes	3	MAPP-MMR: 1.84 PolyPhen: 0.012	Not pathogenic
MSH2 I685R	CRC 32/ 34 yrs Male	MSI-H	MSH2-MSH6-			SDR: - CRC 35, - CRC		NA	MAPP-MMR: 24.76 PolyPhen: 0.15	Pathogenic
MSH2 Q690E	CRC 41 yrs Female	MSI-H	MSH2-MSH6-			FDR: - CRC 50 (MSI-H, MSH2-MSH6-) SDR: - 2x CRC 79 (MSI-H, MSH2-/MSH6-) - CRC 80 and 81 and BlC 82 (carrier VUS, BlC MSS, IHC normal) TDR: - CRC 48 (MSS, IHC normal)	Yes	3	MAPP-MMR: 10.23 PolyPhen: 0.998	Pathogenic
MSH2 IVS15-3C>G	CRC (2x) 60 yrs Male	MSI-H	MSH2-MSH6-			FDR: - CRC 32 - Leukemia 37 SDR: - CRC 58, 71 and 76 - Three other relatives CRC - GaC	Yes	NA		Pathogenic

Clinical data was collected by the Erasmus Medical Center Rotterdam and the Netherlands Cancer Institute Amsterdam. The table annotates the sex and age of the patients as well as the types of tumors they and their family members developed: CRC, colorectal cancer; UtC, uterine cancer; OvC, ovarian cancer; BlC, bladder cancer; EsoC, Esophageal cancer; GaC, gastric cancer; PaC, pancreatic cancer; BoneC, bone cancer; BrainC, brain cancer. Tumor pathology (MSI, IHC and other tumor analysis) data is indicated: MSS, microsatellite stable; MSI-H, high microsatellite instablility; IHC, immunohistochemistry: -, protein is absent. The 'Other variants' column indicates any other MMR gene variant that was detected in the patients. The index patient's cancer family history is described for FDR, first degree relatives; SDR, second degree relatives; and TDR, third degree relatives. The table also indicates whether index patients met the Revised Bethesda guidelines. The InSiGHT classification is shown for each MMR gene variant: 5, pathogenic; 3, uncertain; 1, not pathogenic; NA, not available. In silico prediction models MAPP-MMR and PolyPhen were used to forecast the effect of variants on MMR. MAPP-MMR scores >4.55 are expected to affect MMR function. Polyphen scores >0.2 are considered probably damaging. The final column presents the results from the genetic screen.

Chapter 8

Suspected Lynch syndrome associated *MSH6* variants: A functional assay to determine their pathogenicity

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ABSTRACT

Lynch syndrome (LS) is a hereditary cancer predisposition caused by inactivating mutations in DNA mismatch repair (MMR) genes. Mutations in the *MSH6* DNA MMR gene account for approximately 18% of LS cases. Many LS-associated sequence variants are nonsense and frameshift mutations that clearly abrogate MMR activity. However missense mutations whose functional implications are unclear are also frequently seen in suspected-LS patients. To conclusively diagnose LS and enroll patients in appropriate surveillance programs to reduce morbidity as well as mortality, the consequences of these variants of uncertain clinical significance (VUS) must be defined. We present an oligonucleotide-directed mutagenesis screen for the identification of pathogenic *MSH6* VUS. In the screen, the *MSH6* variant of interest is introduced into mouse embryonic stem cells by site-directed mutagenesis. Subsequent selection for MMR-deficient cells using the DNA damaging agent 6-thioguanine (6TG) allows the identification of MMR abrogating VUS because solely MMR-deficient cells survive 6TG exposure. We demonstrate the efficacy of the genetic screen, investigate the phenotype of 26 *MSH6* VUS and compare our screening results to clinical data from suspected-LS patients carrying these variant alleles.

INTRODUCTION

Lynch syndrome (LS) is an autosomal dominant predisposition to a variety of malignancies at a young age, mainly colorectal cancer (CRC) and endometrial cancer (EC).(1) It is caused by inactivating germline mutations in the DNA mismatch repair (MMR) genes *MLH1*, *MSH2, MSH6* or *PMS2*, or a deletion in the 3' region of the *EPCAM* gene that affects *MSH2* expression.(2-6)

The DNA MMR system is essential for the fidelity of DNA replication. Its primary function is the correction of base-base mismatches and insertion-deletion loops that may arise during DNA replication. Base-base mismatches are recognized by the MSH2-MSH6 heterodimer while MSH2-MSH3 detects loops of unpaired bases. Following mismatch binding, the MSH heterodimers recruit another heterodimer, MLH1-PMS2, to coordinate removal and resynthesis of the error-containing strand.(7-9) A second function of the DNA MMR system is to mediate the toxicity of certain DNA damaging agents such as methylating agents and thiopurines. These DNA damaging agents create adducts in the genome that give rise to mismatches when replicated. The DNA MMR system recognizes the mismatches but will remove the incorporated nucleotide rather than the lesion itself, creating a repetitive cycle of nucleotide incorporation and deletion that ultimately leads to DNA breakage and cell death.(10, 11) In the absence of MMR, cells tolerate methylation damage, but consequently show high levels of DNA damage-induced mutagenesis on top of a strongly elevated level of spontaneous mutagenesis.(12)

LS patients inherit a functional and a mutant copy of one of the DNA MMR genes. For cells to become MMR-deficient and develop a mutator phenotype that accelerates carcinogenesis, somatic loss of the wild-type allele is required.(13) Microsatellite instability (MSI), *i.e.*, length alterations of repetitive sequences like $(CA)_n$ or $(A)_n$, and loss of immunohistochemical staining (IHC) for MMR proteins are considered hallmarks of LS tumors. Analysis of MSI and IHC on tumor tissue can identify patients who may suffer from LS. For a definitive LS diagnosis, however, sequence analyses must reveal a pathogenic germline mutation in one of the DNA MMR genes or the 3' region of *EPCAM.*(14, 15) Many LS-associated sequence variants are nonsense and frameshift mutations that clearly truncate the protein and unambiguously abrogate MMR activity. Missense mutations that only alter a single amino acid are also frequently identified in suspected-LS patients. The functional implications of these variants are less clear. Consequently, the diagnosis of suspected-LS patients carrying missense variants is difficult in the absence of clear segregation and functional data. As long as the phenotype of these variants of uncertain significance (VUS) is unclear, non-carriers cannot safely be discharged from burdensome surveillance programs.(16) Surveillance programs have proven to significantly reduce morbidity and mortality in LS patients (1, 17, 18), but pose unnecessary psychological and physical stress on carriers of innocent VUS as well as pressure on preventive healthcare. Therefore, techniques that characterize MMR gene VUS and enable the identification of individuals at risk are urgently needed.

While in the past primarily *MSH2* and *MLH1* were sequenced to identify LS-causing mutations, in recent years *MSH6* has been gained fame for causing LS due to the advancement of DNA sequencing. However, *MSH6* mutation carriers can be difficult to diagnose because they may not entirely fulfill the criteria for LS diagnosis: their age at cancer onset is generally later than for *MLH1* and *MSH2* mutation carriers, and their tumors occasionally stain for MSH6 and have no or low MSI.(19-21) We therefore extended the applicability of the oligonucleotide-directed mutagenesis screen we recently described for the identification of pathogenic *MSH2* variants to *MSH6* variants.(22) The genetic screen uses oligonucleotide-directed gene modification (oligo targeting) (23) to introduce variant codons into the endogenous *Msh2* gene of mouse embryonic stem cells (mESCs) and subsequently identifies pathogenic variants by selecting for cells that are resistant to the thiopurine 6-thioguanine (6TG). Here we present the applicability of this screen for the characterization of *MSH6* VUS.

RESULTS

Genetic screen for the identification of pathogenic *MSH6* mutations

The oligonucleotide-directed mutagenesis screen takes a four step approach to the identification of pathogenic *MSH6* mutations (Figure 1): 1) site-directed mutagenesis to introduce the variant of interest into a subset of *Msh6*$^{+/-}$ mESCs, 2) selection for cells that consequently lost MMR capacity, 3) PCR analysis to exclude cells that lost MMR capacity due to loss of the *Msh6*$^{+}$ allele (loss of heterozygosity events), 4) sequence analysis to confirm the presence of the planned mutation in the MMR-deficient cells.

mESCs provide a good study model because the human and mouse MSH6 amino acid sequences share over 86% identity (Figure S1) and mouse models can be made from these cells if VUS need to be studied *in vivo*. *Msh6*$^{+/-}$ mESCs only contain one wild type *Msh6* allele (*Msh6*$^{+}$); the other allele was disrupted by a *puromycin*-resistance gene and therefore inactivated (*Msh6*$^{-}$).(24) Hence introduction of a specific mutation into the one active *Msh6* allele will lead to expression of solely the variant protein and allow immediate investigation of its phenotype. To achieve this, *Msh6* was site-specifically mutated by oligo targeting, a gene modification technique that uses short single-stranded locked-nucleic-acid-modified DNA oligonucleotides (LMOs) (with either sense or antisense orientation) to substitute a single base pair at a desired location. LMO-directed base-pair substitution can be achieved at an efficiency of 10^{-3}; thus, about 1 in every 1000 LMO-exposed *Msh6*$^{+/-}$ mESCs will contain the desired mutation.(23) To determine whether the substitution abrogated *Msh6* activity and this subset of cells consequently lost MMR activity, LMO-exposed mESCs were treated with 6TG. The thiopurine DNA damaging agent 6TG is highly toxic to MMR-proficient but only moderately toxic to MMR-deficient cells.(11) Therefore, the appearance of colonies that survived mild 6TG selection is indicative for loss of MMR capacity. Loss of MMR capacity may arise due to the introduced mutation or due to loss of heterozygosity events that

caused loss of the functional *Msh6* allele. To exclude the latter from further investigation, a PCR that detected the presence of both the disrupted and non-disrupted *Msh6* alleles was performed.(24) 6TG-resistant colonies that maintained both *Msh6* alleles were sequenced to confirm the presence of the planned mutation.

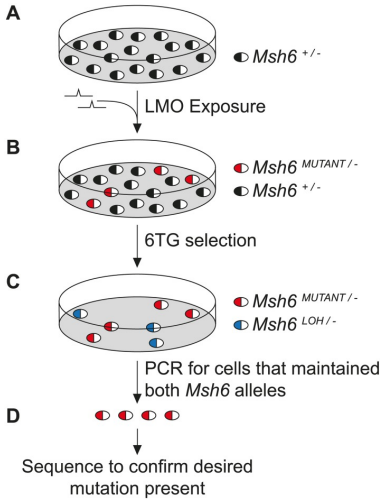

Figure 1. Oligonucleotide-directed mutagenesis screen for the detection of pathogenic *MSH6* variants.
(A) *Msh6*$^{+/-}$ mESCs were exposed to LMOs encoding the mutations of interest. LMO-exposure introduced the mutation into the endogenous *Msh6* gene in ±1 per 1000 *Msh6*$^{+/-}$ mESCs. (B) To determine if the subset of cells carrying the mutation in the *Msh6*$^{+}$ allele had lost MMR activity, the mESCs were treated with 6TG. MMR-proficient cells die in response to 6TG exposure while MMR-deficient cells are 6TG resistant. (C) Cells may also lose MMR capacity due to loss of heterozygosity (LOH) events deleting the *Msh6*$^{+}$ allele. To exclude these cells from further investigation, a PCR was performed that detected the presence of both *Msh6* alleles. (D) 6TG-resistant LMO-exposed mESCs that maintained the *Msh6*$^{+}$ allele were sequenced to confirm the presence of the planned mutation.

Proof of principle

To demonstrate the ability of the oligonucleotide-directed mutagenesis screen to distinguish pathogenic *MSH6* mutations from polymorphisms, a proof of principle study was performed with *MSH6* variants G1139S and L1087R that were previously proven to be pathogenic and not pathogenic, respectively (25), as well as all classified pathogenic and not pathogenic missense variants described in the International Society for Gastrointestinal Hereditary Tumours (InSiGHT) colon cancer variant database (http://insight-group.org/). This database uses available clinical, *in vitro* and *in silico* data to categorize DNA MMR gene sequence variants according to a five-tiered classification scheme as: class 5, Pathogenic; 4, Likely pathogenic; 3, Uncertain; 2, Likely not pathogenic; and 1, Not pathogenic.(26) *Msh6*$^{+/-}$ mESCs were first exposed to antisense oriented LMOs encoding the desired base-pair substitution. If subsequent 6TG selection did not reveal resistant colonies encoding the planned mutation, the screen was repeated with sense oriented LMOs.

LMO-mediated introduction of both pathogenic and not pathogenic variants led to the appearance of 6TG-resistant colonies. The vast majority of 6TG-resistant colonies obtained with LMOs encoding *polymorphisms* had lost the wild-type *Msh6* allele by loss of heterozygosity events, as inferred from allele-specific PCR analysis. Sequencing of the few 6TG-resistant colonies that had retained both *Msh6* alleles (±6%), did not detect any mutation (Figure 2A). These background colonies apparently arose from cells that for unknown reasons survived 6TG exposure. Of the 6TG-resistant colonies that emerged following LMO-mediated introduction of *pathogenic mutations*, ±40% still contained both *Msh6* alleles. Sequence analysis detected pathogenic mutations in all but one of these 6TG-resistant colonies (Figure 2B; Figure S2A). Thus, the oligonucleotide-directed mutagenesis screen detected all 4 pathogenic mutations and not one of the 5 non-pathogenic variants, indicating it is capable of distinguishing pathogenic *MSH6* mutations from polymorphisms.

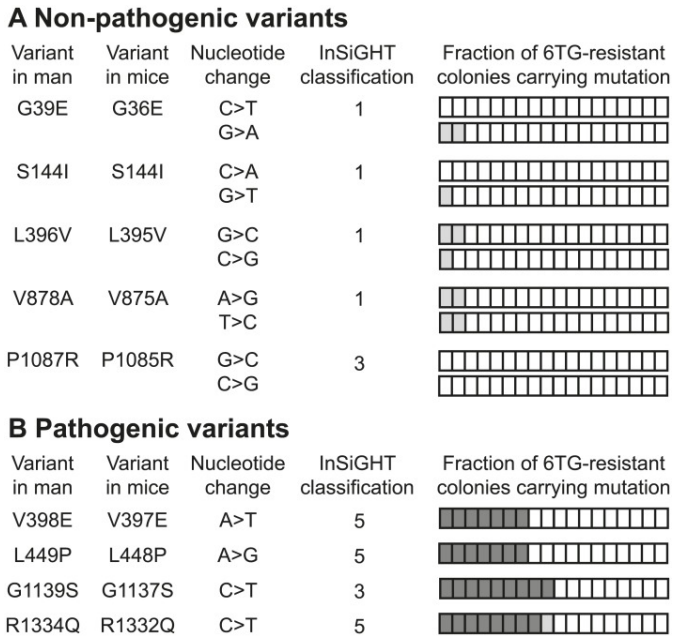

Figure 2. Distinguishing pathogenic *MSH6* variants from polymorphisms.
(A) Five known non-pathogenic variants and (B) four pathogenic mutations tested in the proof of principle study. Variants are annotated according to their amino acid change and location in men and mice. The nucleotide change was first introduced by antisense-oriented LMOs. If no 6TG-resistant colonies encoding the mutation appeared, the screening protocol was repeated with sense-oriented LMOs (lower row where two rows are present). The fourth column presents the InSiGHT classification of each variant where 5 is pathogenic, 3 is uncertain and 1 is not pathogenic. At variance with the InSiGHT classification, a previous study demonstrated variant G1139S is pathogenic and L1087R is not pathogenic (25). The bars in the 'Fraction of 6TG-resistant colonies carrying mutation' column represent the 18 6TG-resistant colonies that were investigated further. The white portions represent colonies in which the *Msh6*$^{+}$ allele was lost by LOH; the light grey portions illustrate the fraction of background colonies that apparently survived 6TG selection but maintained the *Msh6*$^{+}$ allele without the planned mutation; the dark grey portions represent the fractions of colonies that maintained the *Msh6*$^{+}$ allele and encoded the mutation of interest.

Screening variants of uncertain significance

We used the oligonucleotide-directed mutagenesis screen to investigate the phenotype of 18 *MSH6* VUS described in literature and the InSiGHT database as well as 8 *MSH6* VUS detected in suspected-LS patients from the Erasmus Medical Center Rotterdam and the Radboud University Medical Center Nijmegen (see Tables S1 and S2 for clinical data (27-38); see Figure S3 for location of variants in *MSH6* (39, 40)). Of the 26 variants, 18 were not present in 6TG-resistant colonies and hence do not appear to affect MMR activity. Mutations R510G, A586P, G683D, F703S, L1060R, E1191K, T1217D and T1217I were identified in 6TG-resistant colonies by sequence analysis (Figure 3A and B; Figure S2B). The MMR abrogating effect of all *Msh6* variants conferring 6TG-resistance was further characterized by Western blot analyses, MSI assays and methylation-damage-induced mutagenesis assays.

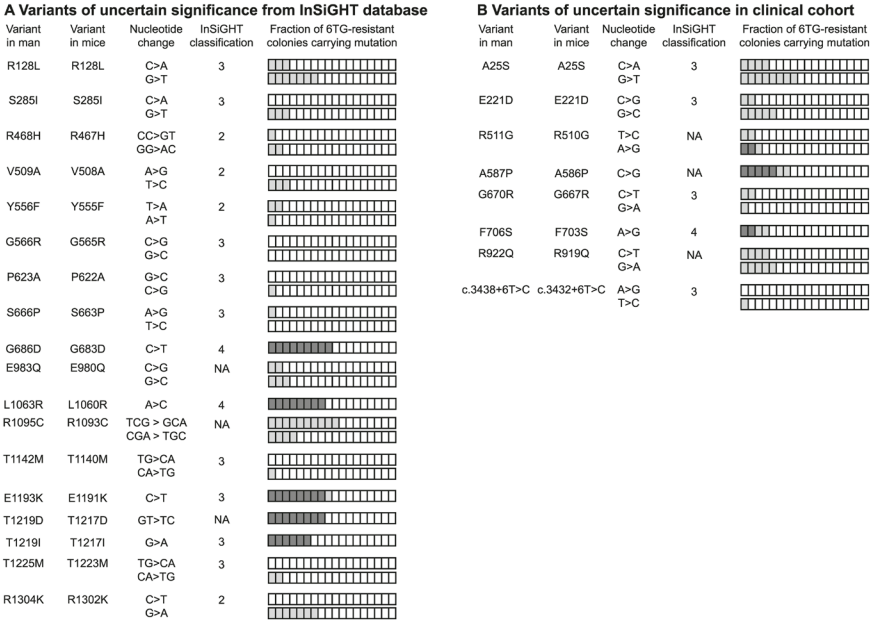

Figure 3. Identification of pathogenic *MSH6* VUS.

The genetic screen was used to analyze (A) 18 VUS selected from literature and the InSiGHT database as well as (B) 8 VUS identified in patients from two medical centers in the Netherlands. Variants are displayed according to their amino acid number and change in men and mice. The 'Nucleotide change' column presents the one or two base alteration introduced by the LMOs. If antisense-oriented LMOs did not give rise to 6TG-resistant colonies encoding the mutation of interest, the screen was repeated with sense-oriented LMOs (lower row where two rows are present for the variant). The InSiGHT classification of each variant is indicated: 4, likely pathogenic; 3, uncertain; 2, likely not pathogenic; NA, not available. The bars in the 'Fraction of 6TG-resistant colonies carrying mutation' column represent the 18 6TG-resistant colonies that were analyzed for the presence of the planned mutation: the white segments represent LOH events; the light grey segments represent background colonies that maintained the *Msh6*$^{+}$ allele but did not encode the planned mutation; the dark grey segments display the fractions of colonies that maintained the *Msh6*$^{+}$ allele and encoded the mutations of interest.

Phenotypic assessment of identified MMR abrogating *Msh6* variants

The effect of the identified MMR abrogating mutations on MSH6 and MSH2 protein levels was evaluated by Western blot analyses (Figure 4). MSH6 and MSH2 form a heterodimer; consequently, a drop in MSH6 levels is often associated with a slight decrease in MSH2 protein stability. Protein levels were quantified with respect to *Msh6*$^{+/-}$ mESCs, which maintain a functional MMR system with about two-third of the MSH6 level observed in *Msh6*$^{+/+}$ mESCs.(25) Known pathogenic mutations V397E, L448P, G1137S and R1332Q reduced MSH6 levels to 7-33% of that seen in *Msh6*$^{+/-}$ mESCs. The R1332Q mutation is located in the splice donor site of exon 9 which may explain the appearance of a shorter protein. The drop in MSH6 levels seen for the known pathogenic mutations was mirrored by variants A586P, G683D, F703S and L1060R that reduced protein levels to 7-24%. Variants R510G, E1191K, T1217D and T1217I maintained relatively high MSH6 levels of 59-79%.

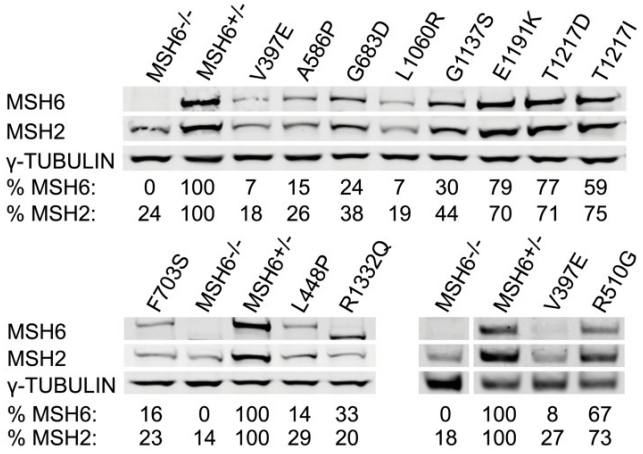

Figure 4. Western blot analysis of mESCs expressing *Msh6* variants.
MSH6, MSH2 and γ-TUBULIN levels were analyzed in whole cell lysates. MSH6 and MSH2 levels in the variant cells lines were quantified with respect to the protein levels seen in *Msh6*$^{+/-}$ mESCs.

MSI in *MSH6* mutation carriers is largely restricted to mononucleotide markers.(41) To investigate the effect of the detected *Msh6* variants on MSI we used a $(G)_{10}$-*neo* slippage reporter. The neomycin resistance gene (*neo*) in this reporter is rendered out of frame by a preceding $(G)_{10}$ repeat. When DNA polymerase slippage errors at the $(G)_{10}$ repeat such as the deletion of one G or insertion of two Gs remain unnoticed, the *neo* becomes in frame and generates Geneticin-resistant cells. Hence the number of Geneticin-resistant colonies is indicative of the frequency of *neo*-restoring slippage events and the MMR capacity of the cells.(42) The slippage rates, *i.e.*, the chance of a slippage event occurring during one cell division, in 6TG-resisant *Msh6* VUS expressing mESCs ranged from $5.3x10^{-5}$ to $5.1x10^{-4}$; which is around the average rate of $1.9x10^{-4}$ observed for the known pathogenic mutations and 140 to 1340-fold higher than the slippage rate of $3.8x10^{-7}$ seen for *Msh6*$^{+/-}$ MMR-proficient mESCs (Figure 5).

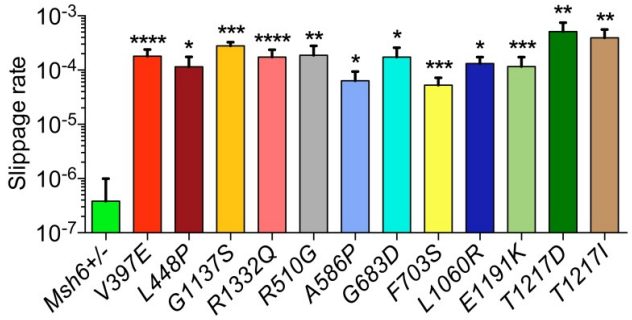

Figure 5. MSI analysis of mESCs expressing *Msh6* variants.
To quantify the level of MSI, a $(G)_{10}$-*neo* slippage reporter was introduced into variant mESCs. Spontaneous DNA polymerase slippage events on the $(G)_{10}$ repeat that are not corrected can bring the *neo* in frame, rendering cells Geneticin-resistant. Slippage rates (the emergence of a Geneticin-resistant cell per cell division) of VUS expressing cells are compared to the MMR-proficient *Msh6*$^{+/-}$ cell line and MMR-deficient *Msh6*$^{V397E/-}$, *Msh6*$^{L448P/-}$, *Msh6*$^{G1137S/-}$, and *Msh6*$^{R1332Q/-}$ pathogenic controls. Statistical differences were calculated using one-tailed, unpaired t-test with Welch's correction. Asterisks indicate values significantly higher than those of the MMR-proficient *Msh6*$^{+/-}$ control: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

In addition to increased spontaneous mutagenesis events, MMR-deficient cells also experience increased methylation-damage-induced mutagenesis .(43) To study the influence of the detected MMR attenuating *Msh6* variants on methylation-damage-induced mutagenesis, mESCs were exposed to the methylating DNA damaging agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and the number of cells that consequently attained mutations was quantified. In MMR-proficient cells, DNA replication across MNNG-induced O^6-methylguanine lesions is impaired by futile cycles of MMR, ultimately leading to cell death and suppression of methylation-damage-induced mutagenesis. Under MMR-deficient conditions, however, the MNNG-induced mismatches are not recognized and remain in the genome leading to the accumulation of mutations. To provide a quick read out for the frequency of mutation accumulation, we measured the number of MNNG-exposed cells that became resistant to a high dose of 6TG for an extended period. Solely cells that carry an inactivating mutation in *Hprt* survive stringent 6TG treatment because HPRT is required for 6TG to behave as a DNA damaging agent. All detected *Msh6* variant cell lines showed an elevated MNNG-induced mutator phenotype when compared to the MMR-proficient *Msh6*$^{+/-}$ mESCs (Figure 6).

Phenotypic assessment of a non-detected *Msh6* variant

According to literature *MSH6-G566R* may be pathogenic (33, 44), yet our screen did not identify this variant in 6TG-resistant colonies. Hence we investigated whether the MMR abrogating effect of *Msh6-G565R* could have been missed by the screen due to technical difficulties. Rather than applying 6TG selection after oligonucleotide-directed mutagenesis, we purified *Msh6*$^{G565R/-}$ mESCs using a Q-PCR-based protocol (Figure S2C) (25) and subsequently examined their MMR capacity. Exposure of *Msh6*$^{G565R/-}$ cells to increasing doses of 6TG revealed that they were equally sensitive to 6TG as *Msh6*$^{+/-}$ cells (Figure 7A). In the MSI assay, *Msh6*$^{G565R/-}$ mESCs did not experience significantly more

slippage events than the MMR-proficient control (Figure 7B). Thus, *Msh6-G565R* did not attenuate MMR consistent with the oligonucleotide-directed mutagenesis screening result.

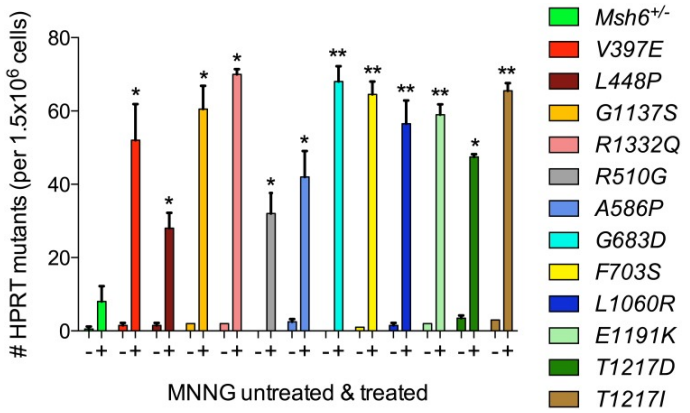

Figure 6. MNNG-induced mutagenesis in mESCs expressing *Msh6* variants.
Variant MSH6 expressing mESCs were exposed to MNNG and the number of cells that consequently acquired mutations in *Hprt* quantified (29). *Hprt*-defective mESCs were identified by long-term exposure to a high dose of 6TG (10 µg/ml). The spontaneous (-) and MNNG induced (+) mutation frequency was compared to MMR-proficient *Msh6*$^{+/-}$ mESCs and MMR-deficient *Msh6*$^{V397E/-}$, *Msh6*$^{L448P/-}$, *Msh6*$^{G1137S/-}$, and *Msh6*$^{R1332Q/-}$ pathogenic controls. The statistical differences between MNNG-treated *Msh6*$^{+/-}$ mESCs and MNNG-treated variant cell lines was calculated using a one-tailed, unpaired t-test with Welch's correction. Asterisks indicate values significantly higher than those of the MNNG-treated MMR-proficient *Msh6*$^{+/-}$ control: **P*<0.05; ***P*<0.01.

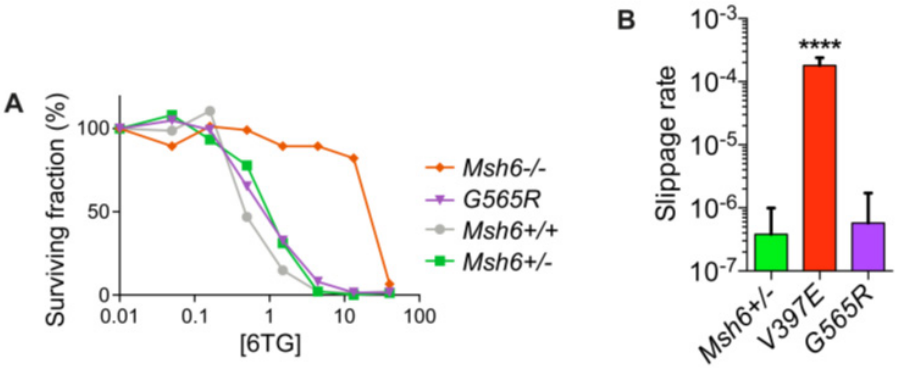

Figure 7. MMR capacity of *Msh6*$^{G565R/-}$ mESCs.
The MMR activity of *Msh6*$^{G565R/-}$ mESCs was investigated using two assays. (A) 6TG survival assay. The colony-forming capacity of *Msh6*$^{G565R/-}$ mESCs as well as MMR-deficient *Msh6*$^{-/-}$ and MMR-proficient *Msh6*$^{+/-}$ and *Msh6*$^{+/+}$ cells was determined in response to increasing doses of 6TG. (B) MSI in the *Msh6*$^{G565R/-}$ mESCs was investigated using the $(G)_{10}$-*neo* slippage reporter. The slippage rate (the emergence of a Geneticin-resistant cell per cell division) in *Msh6*$^{G565R/-}$ cells was compared to the rate in MMR-proficient *Msh6*$^{+/-}$ and MMR-deficient *Msh6*$^{V397E/-}$ control cell lines. Statistical differences were calculated using one tailed, unpaired t-test with Welch's correction. **** indicates significantly higher than the mismatch repair proficient *Msh6*$^{+/-}$ control: *P*<0.0001.

DISCUSSION

The results of our study demonstrate the oligonucleotide-directed mutagenesis screen we previously described for the characterization of *MSH2* VUS (22) can be extended to *MSH6* VUS. Combining oligo targeting in *Msh6*$^{+/-}$ mESCs with 6TG selection and sequence analysis allows pathogenic *MSH6* variants to be distinguished from polymorphisms. The efficacy of the genetic screen was established in a proof of principle study with 4 known pathogenic *MSH6* mutations and 5 polymorphisms. This number was low because of the paucity of *MSH6* variants that were classified with 100% certainty. Not one of the 5 non-pathogenic variants was identified as MMR abrogating. Also, among the 26 *MSH6* VUS we subsequently analyzed, not one of the 4 variants classified as likely not pathogenic was identified as pathogenic by our screen. Finally, functional assays established that one of the VUS that was not detected as pathogenic by the screen indeed did not influence MMR activity (G565R). Hence the false positive rate of the screen, *i.e.*, the chance the screen identified a VUS as MMR abrogating while it was *a priori* or *a posteriori* identified as (likely) non-pathogenic was <1/10, giving a specificity >90.0%. The sensitivity of the genetic screen is a measure of the false negative rate; it is the likelihood that a pathogenic mutation is not detected. All 6 InSiGHT classified pathogenic and likely pathogenic variants as well as the previously proven pathogenic G1139S mutation were recognized as MMR abrogating by the screen, translating to a sensitivity of >85.7%.

We used the oligonucleotide-directed mutagenesis screen to investigate the MMR capacity of 26 *MSH6* VUS. Eight of these were found in suspected-LS patients from two medical centers in the Netherlands. From this clinical cohort, the mouse equivalent of mutations R511G, A587P and F706S were detected by our screen and shown to abrogate MMR. However, R510G and F703S were detected in only 2/5 and 2/4 6TG-resistant colonies, respectively, that had retained two *Msh6* alleles, while the other pathogenic variants were present in virtually all colonies diploid for *MSH6* (Figures 2B, 3A and 3B). The poorer recovery of R510G and F7103S mutants may have been due to a lower success rate of LMO-mediated base-pair substitution. The pathogenic phenotype observed for these three variants is in line with clinical data: all three variants were detected in patients with MSI-H LS-related tumors and with a family history of LS-related tumors. In the case of VUS A587P and F706S, relatives with LS-related tumors carried the same mutation. IHC also demonstrated MSH6 was absent in the patients encoding MSH6-A587P and MSH6-F706S; the IHC data for MSH6-R511G were inconclusive.

The other 5 variants in the clinical cohort, A25S, E221D, G670R, R922Q and c.3438+6T>C, were not identified as MMR abrogating. VUS E221D, G670R and R922Q were found in patients who also harbored a second, known pathogenic mutation in one of the DNA MMR genes that was likely causative for the LS phenotype. E221D was also detected in a second patient who was 83 years old and did not have a family history suspicious for LS. MSH6-A25S was found in a typical LS tumor, *i.e.*, a colon tumor showing MSI, loss of heterozygosity of *MSH6*, and loss of MSH6 protein expression. The patient however only had one relative with a colorectal tumor and this tumor was not MSI-high and stained

positive for all MMR proteins. A previous *in vitro* study also suggested MSH6-A25S is not pathogenic (45); it could be that the tumor arose due to a missed somatic mutation. VUS c.3438+6T>C was found in a patient with a family history suspicious of LS. We however do not know if the relatives with LS-associated cancers also carried this specific *MSH6* sequence variant. IHC failed in the index patient carrying the c.3438+6T>C variant, therefore we cannot exclude that a somatic mutation or *MLH1* hypermethylation caused the MSI in the tumor. Tumor tissue of one family member was tested and showed no MSI and normal IHC. It is also possible that the genetic screen was unable to identify c.3438+6T>C as pathogenic due to differences between the human and mouse *MSH6* sequences. While the *MSH6* coding sequence is highly conserved, intron sequences are more variable between species (Figure S4 shows human and mouse sequence around c.3438+6). Hence there is a chance that variant c.3438+6T>C affects splicing in man but not in mice. According to several splice site prediction programs (NNSPLICE, GeneSplicer, Human Splicing Finder), however, c.3438+6T>C does not affect splicing.

The other 18 *MSH6* VUS we studied were attained from literature and the InSiGHT database. The genetic screen found 5 of these variants abrogate MMR: G686D, L1063R, E1193K, T1219D and T1219I. The detection of G686D and L1063R is in line with their InSiGHT classification, which describes the mutations as likely pathogenic. Variant E1193K has previously been suggested to cause LS in studies that identified the mutation in patients with ECs that were MSI and did not stain for MSH6.(27, 31) Not much clinical data is available for VUS T1219D but $Msh6^{T1217D}$ mice were demonstrated to have increased cancer susceptibility.(46) VUS T1219I has been described in a CRC patient who had a family history of CRC and a MSI tumor that stained positive for MSH6, the latter being consistent with the high levels of this variant protein we observed in mESCs. Both clinical and *in vitro* data indicate MSH6-T1219I abrogates MMR activity.(29, 45)

MSH6 VUS R128L, R468H, V509A, Y556F, P623A, S666P, E983Q, R1095C, T1255M and R1304K were not identified as pathogenic in our screen. These sequence variants were classified as likely not pathogenic by InSiGHT, identified in patients with *MLH1* promoter methylation or with MSS and MSH6 positive tumors, or observed in patients for whom little clinical data was available. VUS S285I, G565R and T1142M were also not detected as MMR attenuating by our screen, yet they seem suspicious for pathogenicity based on available data. MSH6-T1142M was previously suggested to be probably pathogenic based on clinical data describing the variant in a 27 year old patient with polyps who met the Bethesda guidelines, had a 61 year old mother with polyps, and did not carry pathogenic mutations in any other MMR gene nor showed *MLH1* promoter methylation in the tumor.(35) VUS S285I and G566R were detected in CRC patients with MSI (low and high, respectively) tumors that had loss of heterozygosity of *MSH6*.(33) Cyr and Heinen (44) investigated the effect of these two mutations on mismatch binding and processing: variant S285I was not found to have a specific MMR attenuating effect but variant G566R was suggested to abrogate MMR by interfering with the ATP-dependent conformational change that must take place to activate downstream repair pathways upon mismatch binding. We therefore purified $Msh6^{G565R/-}$ mESCs and assessed their MMR capacity. The $Msh6^{G565R/-}$ cells behaved like MMR-proficient $Msh6^{+/-}$ mESCs, confirming the result of

the oligonucleotide-directed mutagenesis screen. Despite the good performance of our screen and the high amino acid conservation of MSH6, we cannot exclude *Msh6-G565R* was not identified as pathogenic due to differences between mice and men. To fully dissuade this argument we will need to develop the oligonucleotide-directed mutagenesis screen in human cells.

The oligonucleotide-directed mutagenesis screen presented here is a relatively simple tool that can be used to investigate the pathogenic phenotype of many *MSH6* VUS in parallel. While the evolutionary conservation of MMR justifies the use of mouse cells for the majority of VUS, testing of splice-site and intronic mutations necessitates adaptation to human cells. Also, as long as uncertainty exists about its specificity and sensitivity, functional testing needs to be combined with clinical data and *in silico* estimations to arrive at a reliable classification of VUS. Conforming the updated American College of Medical Genetics and Genomics (ACMG) standards and guidelines for sequence variant interpretation, we are currently transferring our functional tests to certified Clinical Genetics laboratories and creating an infrastructure where test results are compared and interpreted taking into account all available data. In this way, LS mutation carriers can be identified with the highest certainty and enrolled in tailored surveillance programs while relatives without the mutation can be excluded from surveillance.

METHODS

Oligonucleotide-directed mutagenesis screen to identify pathogenic *MSH6* variants

The genetic screen was developed in *Msh6*$^{+/-}$ mESCs, which contain one active *Msh6* allele (*Msh6*$^{+}$) and one *Msh6* allele that was disrupted by the insertion of a *puromycin* resistance marker (*Msh6*$^{-}$).(24) The *MSH6* variants under investigation were introduced into the *Msh6*$^{+/-}$ mESCs by oligo targeting using LMOs.(23) 7x10^{5} *Msh6*$^{+/-}$ mESCs were seeded in BRL-conditioned medium on gelatin-coated 6 wells and exposed to a mixture of 7.5 µl *Trans*IT-siQuest® transfection agent (Mirus), 3 µg LMOs and 250 µl serum-free medium the following day. After 3 days, 1.5x10^{6} LMO-exposed cells were transferred to gelatin-coated 10 cm plates and subjected to 6TG (250 nM) (Sigma-Aldrich®) selection. After 10 days the 18 largest 6TG-resistant colonies were picked. Cells that became 6TG-resistant due to loss of heterozygosity events were excluded from further analyses using a PCR specialized to detect the presence of both the disrupted and non-disrupted *Msh6* alleles.(24) 6TG-resistant mESCs that maintained both *Msh6* alleles were sequenced to confirm the presence of the planned mutation.

Western blot analysis

Western blot analyses were performed as described in Wielders et al.(25) Rabbit polyclonal antibodies against mMSH2 (1:500) (47) and mMSH6 (1:500) (24) as well as mouse polyclonal antibody against γ-Tubulin (1:1000; GTU-88 Sigma-Aldrich®) were used as primary antibodies. Protein bands were visualized using IRDye® 800CW goat anti-rabbit IgG and IRDye 800CW® goat anti-mouse IgG secondary antibodies (Li-cor) and the

Odyssey scan. The infrared fluorescent signals measured by the Odyssey scan are directly proportional to the amount of antigen on the Western blots, allowing quantification of the protein bands.

Microsatellite instability assay

mESCs were electroporated with the $(G)_{10}$-*neo Rosa26* targeting vector as described in Dekker et al.(48) The $(G)_{10}$-*neo Rosa26* targeting vector is composed of a promoterless *histidinol* resistance gene as well as a *neomycin* resistance gene (*neo*) that is rendered out of frame by a preceding $(G)_{10}$-repeat.(42) Once electroporated, 10^6 cells were seeded on gelatin-coated 10 cm plates in BRL-conditioned medium and exposed to Histidinol (3mM) (Sigma-Aldrich®). Successful integration of the vector into the *Rosa26* locus of the Histidinol-resistant colonies routinely occurs at a frequency of ±95% and was confirmed by Southern blot analyses. The individual successfully targeted colonies were subsequently expanded to 10^7 cells and transferred to gelatin-coated 10 cm plates at a density of 10^5 cells per plate for Geneticin selection (600 µg/ml) (Life Technologies). After 10 days, the number of Geneticin-resistant colonies was counted and the slippage rate of the variant mESCs calculated using the formula: $0.6 \times \text{Geneticin}^{\text{total}} = N \times p \times \log(N \times p)$, where $\text{Geneticin}^{\text{total}}$ is the number of Geneticin-resistant colonies, *N* the number of cells to which the culture was expanded, and *p* the number of mutations per cell division. Experiments were performed in quadruplicate and statistical differences calculated using a one-tailed, unpaired t-test with Welch's correction.

MNNG-induced mutagenesis assay

The MNNG-induced mutagenesis assay was performed as described in Claij and te Riele.(43) 2.5×10^6 variant mESCs were seeded on an irradiated mouse embryonic fibroblasts feeder layer in 10 cm plates and exposed to 0 or 4µM MNNG (Sigma-Aldrich®) for 1h the following day. 40 µM O^6-benzylguanine was present in the medium from 1h prior to the MNNG treatment until 6 days after, at which point 1.5×10^6 cells were transferred to gelatin-coated 160 cm^2 plates for 6TG selection (10 µg/ml). After two weeks of 6TG selection, the number of resistant colonies and hence the frequency of MNNG-induced *Hprt* mutants could be determined. Experiments were performed in duplo and the statistical difference between MNNG-treated $Msh6^{+/-}$ mESCs and MNNG-treated variant cell lines calculated using a one-tailed, unpaired t-test with Welch's correction.

Generation of $Msh6^{G565R/-}$ mESCs

$Msh6^{G565R/-}$ mESCs were made as described by Wielders et al.(25) Variant G565R was introduced into $Msh6^{+/-}$ mESCs by oligo targeting and a pure $Msh6^{G565R/-}$ mESC clone was obtained by consecutive rounds of seeding and mutation specific PCR: oligonucleotide-exposed cells were expanded and subsequently seeded on a 96-well plate at a density of 5000 cells per well. A mutation-specific quantitative PCR was used to identify wells that contained $Msh6^{G565R/-}$ mESCs. Positive wells were reseeded at lower density and positive wells again identified by Q-PCR. A pure clone was finally obtained by seeding single cells per well. Sequence analysis confirmed the creation of $Msh6^{G565R/-}$ mESCs.

6TG DNA damage response assay

The 6TG sensitivity of *Msh6*$^{G565R/-}$ mESCs was investigated by exposing the variant cell line to increasing doses of 6TG, as described in Wielders et al.(49) MMR-deficient *Msh6*$^{-/-}$ and MMR-proficient *Msh6*$^{+/-}$ and *Msh6*$^{+/+}$ mESCs were taken along for comparison.

Clinical data

We investigated the pathogenic phenotype of *MSH6* VUS that were found in suspected-LS patients at the Clinical Genetics departments of the Erasmus Medical Center Rotterdam and Radboud University Medical Center Nijmegen. We collected tumor characteristics, age at diagnosis, results of molecular diagnostics and germline mutation analysis, and family history from medical records. MSI analysis was performed with the Bethesda panel (50) or with the Promega pentaplex MSI analysis.(51) IHC for MLH1, MSH2, MSH6 and PMS2 protein was performed as described previously.(52) Germline mutation analysis of *MSH6* was performed by sequencing and multiplex ligation dependent probe amplification. The *in silico* prediction model PolyPhen (53) was used to estimate the chance of a variant being deleterious.

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SUPPLEMENTAL MATERIAL

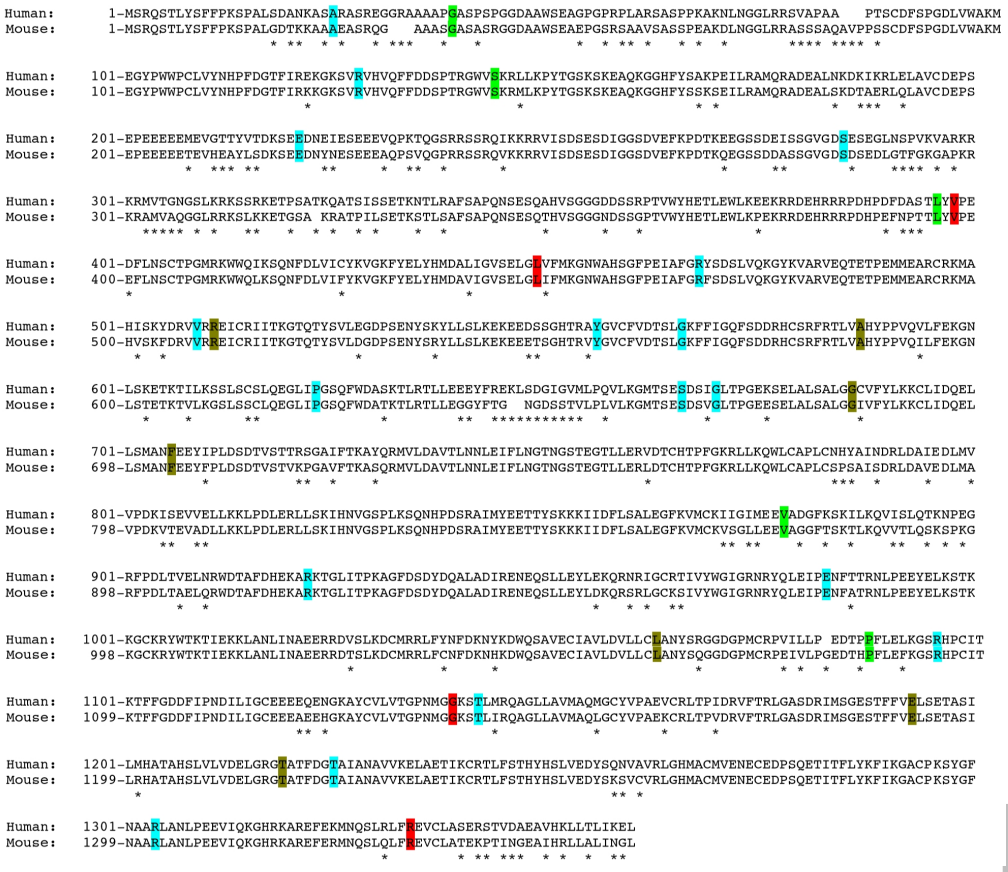

Figure S1. Alignment of human and mouse MSH6 amino acid sequences demonstrating conservation of studied variants.
Asterisks mark amino acids that are not conserved between the human (upper row) and mouse (lower row) MSH6 proteins. The positions of the studied MSH6 variants are highlighted: known pathogenic variants in red, known not-pathogenic variants in green, detected 6TG-resistant variants in mustard, non-detected variants in blue.

Figure S2. Sequences of *Msh6* variants detected by genetic screen and *Msh6*$^{G565R/-}$ mESCs. *Msh6* sequences in mESCs expressing (A) pathogenic variants in proof of principle study, (B) VUS detected in 6TG-resistant colonies, and (C) variant *Msh6-G565R*. Note that in most cases the sequences are a superposition of the variant allele and the normal sequence of the *Msh6*$^{-}$ allele. One-letter amino acid codes are annotated below the nucleotide sequences. *Msh6* WT is the wild-type *Msh6* sequence.

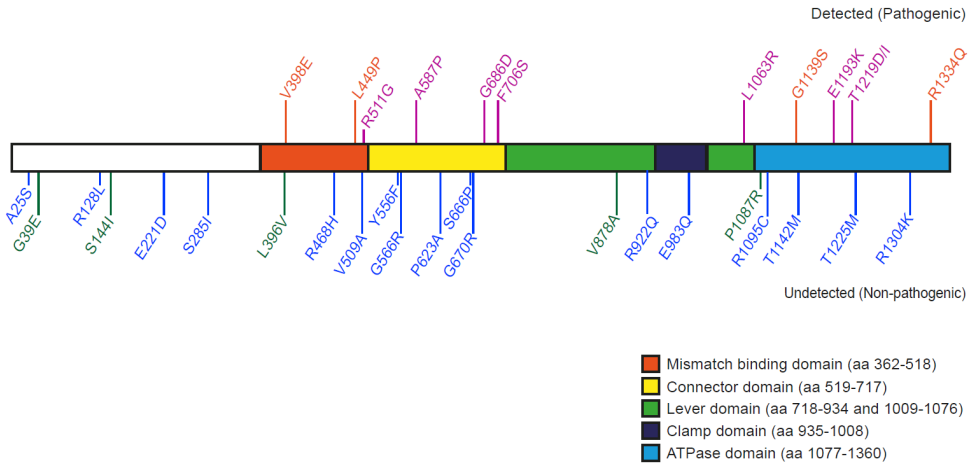

Figure S3. Location of the studied mutations in the MSH6 protein.
The MSH6 domains are displayed in different colors (1, 2). The studied mutations are annotated according to their amino acid number and change. The detected variants are depicted above the MSH6 domains: in orange are the 4 mutations in the proof of principle study, in purple are the 6TG-resistant VUS. Undetected variants are displayed below the MSH6 domains: in green are the non-pathogenic variants in the proof of principle study, in blue are the VUS that did not give rise to 6TG-resistance.
1. Warren JJ, et al. (2007) Structure of the human MutSα DNA lesion recognition complex. *Cell* 26:579-92.
2. Terui H, Akagi K, Kawame H, Yura, K (2013) CoDP: predicting the impact of unclassified genetic variants in MSH6 by the combination of different properties of the protein. *J Biomed Sci* 20:25.

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Human amino acid sequence:   M  G  G  K  S  T  L  M  R  Q  INTRON
Human DNA sequence:          ATGGGGGGCAAGTCTACGCTTATGAGACAGGTAACTGATTCTTAA
Mouse DNA sequence:          ATGGGGGGCAAGTCTACACTCATAAGACAGGTAATTTGTTCTTCA
Mouse amino acid sequence:   M  G  G  K  S  T  L  I  R  Q  INTRON
```

Figure S4. Alignment of human and mouse sequences around human *MSH6 c.3438+6T*.
Depicted are the exon and intron sequences around position *c.3438+6* in human *MSH6* (upper) as well as the corresponding mouse sequence (lower). The amino acid codons are marked in blue and green and the corresponding amino acids are indicated above and below the sequences. *hMSH6 c.3438+6T* and *mMSH6 c.3432+6T* are highlighted in red.

Table S1. Clinical data available for 18 *MSH6* VUS that were selected for screening from literature and the InSiGHT database

Variant	Tumor	MSI	IHC	Other tumor analysis	Revised Bethesda guidelines	Relatives with cancer	References	InSiGHT class	PolyPhen prediction	Screen result
R128L	EC 68 yrs	MSI-H	MSH2+ MSH6+ MLH1-	*MLH1* promoter methylation	No	None	(27, 31)	3	Possibly damaging	Not Pathogenic
S285I	CRC 69 yrs	MSI-L		Loss of *MSH6* heterozygosity in tumor		- Sister breast cancer 44 yrs and ovarian cancer 49 yrs - Father lung cancer 69 yrs - Mother CRC 74 yrs	(33)	3	Possibly damaging	Not Pathogenic
R468H	CRC <55 yrs	MSS	MSH2+ MSH6+ MLH1+		No		(28)	2	Probably damaging	Not Pathogenic
R468H	CRC 50 yrs				Yes		(38)	2	Probably damaging	Not Pathogenic
V509A	CRC 45 yrs					- Father CRC - Mother CRC	(30)	2	Probably damaging	Not Pathogenic
V509A	LS related						(36)	2	Probably damaging	Not Pathogenic
V509A	LS related						(32)	2	Probably damaging	Not Pathogenic
Y556F	EC 50 yrs				Yes	- Relative with same variant developed CRC 50 yrs - Three other relatives not carrying the variant developed CRC 43 yrs, CRC 42 yrs, Stomach cancer 29 yrs	(37)	2	Possibly damaging	Not Pathogenic

G566R	CRC 62 yrs	MSI-H		Loss of *MSH6* heterozygosity in tumor	No	- Sister CRC 73 yrs	(33)	3	Probably damaging	Not Pathogenic
P623A								3	Benign	Not Pathogenic
S666P	CRC 39 yrs				Yes		(38)	3	Possibly damaging	Not Pathogenic
G686D								4	Probably damaging	Pathogenic
E983Q								NA	Probably damaging	Not Pathogenic
L1063R	CRC 27 yrs					- Sibling CRC - 3 uncles/aunts CRC	InSIGHT database	4	Probably damaging	Pathogenic
R1095C								NA	Probably damaging	Not Pathogenic
T1142M	Polyps 27 yrs			No *MLH1* promoter methylation	Yes	- Mother polyps 61 yrs	(35)	3	Probably damaging	Not Pathogenic
E1193K	EC 60 yrs	MSI-H	MSH2- MSH6- MLH1+	No *MLH1* promoter methylation	No	- Brother skin cancer 60 yrs	(27, 31)	3	Probably damaging	Pathogenic
E1193K	EC 59 yrs	MSI-H	MSH2+ MSH6- MLH1+	No *MLH1* promoter methylation	No	- None	(27, 31)	3	Probably damaging	Pathogenic
T1219D								NA	Probably damaging	Pathogenic
T1219I	CRC 37 yrs	MSI-H	MSH2+ MSH6+ MLH1+		Yes	- Parent CRC 36 yrs - 2nd degree relative CRC - 2 3rd degree relatives had cancer of unknown origins	(29)	3	Probably damaging	Pathogenic
T1225M	EC 61 yrs	MSI-H			No		(31)	3	Probably damaging	Not Pathogenic

T1225M	CRC 49 yrs	MSS	MSH6+		No	- Parent CRC 49 yrs	(34)	3	Probably damaging	Not Pathogenic
T1225M	CRC 49 yrs				Yes	- Relative CRC 53 yrs - Relative CRC 59 yrs	(37)	3	Probably damaging	Not Pathogenic
R1304K								2	Possibly damaging	Not Pathogenic

For each of the 18 VUS we aimed to collect clinical data describing the type of tumors found in patients encoding these mutations. Where no data is presented, we did not find this information about the specific *MSH6* variant in the consulted literature. Cancer type and age of onset are noted: CRC, colorectal cancer; EC, endometrium cancer; LS related, Lynch syndrome related tumor. We annotated the MSI status of each tumor: MSS, microsatellite stable; MSI-L, microsatellite instable low; MSI-H, microsatellite instable high. Tumor IHC is also presented: +, protein is present; -, protein is absent in tumor. Also indicated is whether the index patients met the Bethesda, Amsterdam I, Amsterdam II guidelines or not any of the guidelines, as well as the patients' family cancer history. The reference column presents the literature from which the clinical data was retrieved. The InSiGHT classification is shown for each tumor: 1, not pathogenic; 2, likely not pathogenic; 3, uncertain; 4, likely pathogenic; 5, pathogenic; NA, not available. PolyPhen scores were calculated on http://genetics.bwh.harvard.edu/pph2/. In the final column the results from our screen are presented.

Table S2. Clinical data of 8 *MSH6* variants collected from medical centers in the Netherlands

Variant	Tumor	MSI	IHC	Other tumor analysis	Other variants	Revised Bethesda guidelines	Relatives with cancer	InSiGHT class	PolyPhen prediction	Screen result
A25S	CRC 43 yrs female	MSI-H	MSH6-	LOH of *MSH6* in tumor		Yes	- Father CRC 66 yrs (MSS, IHC normal)	3	Benign	Not Pathogenic
E221D	CRC or GaC 83 yrs female	No tissue	No tissue	No tissue		No	- Daughter UtC 33 yrs - Father abdominal tumour 59 yrs	3	Benign	Not Pathogenic
E221D	CRC 44 yrs male	MSI-H	MSH2- MSH6-	NA	*MSH2* VUS L421P, which is pathogenic in our screen. (Cousin CRC 22 yrs only VUS L421P)	Yes	- Mother renal cell carcinoma <48 yrs - Uncle CRC 34 yrs - Uncle bladder cancer 59 yrs - Cousin CRC 22 yrs (MSI-H, IHC MSH2-MSH6-) - Grandmother UtC <53 yrs	3	Benign	Not Pathogenic
R511G	UtC 62 yrs female	MSI-H	Suspect MSH2+ MSH6+	NA		Yes	- Brother CRC 45 yrs (IHC normal) - Sister UtC 55 yrs	NA	Probably damaging	Pathogenic

A587P	CRC 59 yrs male	MSI-H	MSH6-	NA		No	- Sister UtC 56 yrs (MSI-H, IHC MSH6-, retention VUS) - Grandfather CRC	NA	Probably damaging	Pathogenic
G670R	UtC 58 yrs CRC 71 yrs female	MSI-H	MSH6-	NA	Pathogenic mutation (del exon 1+2) on same allele.	Yes		3	Probably damaging	Not Pathogenic
F706S	UtC 48 yrs female	MSI-H	Partly MSH6-	NA		Yes	- Sister CRC 36 yrs, ovarian cancer 38 yrs, breast cancer 50 yrs - Father CRC 54 yrs (retention of VUS) - Paternal aunt CRC 62 yrs (1 marker MSI, 2 markers MSS, IHC partly MSH2-MSH6-, retention VUS)	4	Probably damaging	Pathogenic
R922Q	CRC 39 yrs male	No tissue	No tissue	No tissue	Pathogenic mutation *MSH6* (Asp936X) on other allele		- No other relatives with LS associated tumors	NA	Possibly damaging	Not Pathogenic
c.3438+6 T>C	CRC 60 yrs female	MSI-H	Failed	NA			- Brother CRC 50 yrs - Brother Pancreas carcinoma 72 yrs - Sister CRC 38yrs - Sister CRC 81 yrs - Nephew CRC 57 yrs (MSS, IHC normal)	3		Not Pathogenic

Clinical data was collected by the Erasmus Medical Center Rotterdam and Radboud University Medical Center Nijmegen for the 8 variants in the clinical cohort. The table annotates the sex and age of the patients as well as the types of tumors they developed: CRC, colorectal cancer; GaC, gastric cancer; UtC, uterine cancer. Tumor pathology (MSI, IHC and other tumor analysis) data is indicated: MSS, microsatellite stable; MSI-L, microsatellite instable low; MSI-H, microsatellite instable high; IHC+, protein is present in the tumor; IHC-, protein is absent; LOH, loss of heterozygosity of *MSH6*. The 'Other variants' column describes any other MMR gene variant that was detected in the patients. Whether the index patients met the Revised Bethesda guidelines is displayed as well as the patients' family cancer history. The InSiGHT classification is shown for each tumor: 3, uncertain; 4, likely pathogenic; NA, not available. PolyPhen scores were calculated on http://genetics.bwh.harvard.edu/pph2/. In the final column the results from our screen are presented.

Part III
Surveillance for Lynch syndrome

Chapter 9

Yield of Lynch syndrome surveillance for individual mismatch repair genes

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Submitted for publication

ABSTRACT

Objective To assess the yield of Lynch syndrome (LS) surveillance for *MLH1*, *MSH2*, *MSH6* and *PMS2* mutation carriers.

Design Data on colonoscopy surveillance including histopathology was collected for all patients diagnosed with LS in our center. We compared the development of (advanced) adenomas and colorectal cancer (CRC) between patients with *MLH1*, *MSH2*, *MSH6* and *PMS2* mutations.

Results A total of 1836 follow-up years was collected for 264 patients; 55 *MLH1*, 44 *MSH2*, 143 *MSH6* and 22 *PMS2* mutation carriers (median follow-up of 6 years per patient). At first colonoscopy CRC was found in eight patients. During 916 follow-up colonoscopies, CRC was found in nine patients. No CRC was found in *MSH6* or *PMS2* mutation carriers. There were no significant differences in the number of colonoscopies with adenomas or advanced adenomas between the different gene mutation carrier groups. Median time of adenoma development was 3 years (IQR 2-6 years). There were no significant differences in time to development of an adenoma. However, for *MSH6* mutation carriers there was a trend of longer follow-up time to development of advanced adenoma and a significant longer time to development of advanced neoplasia (advanced adenoma or CRC) than in the other mutation carrier groups. Six patients died during follow-up; three of these patients died from pancreatic cancer.

Conclusion Since no CRC was found during follow-up in *MSH6* mutation carriers and advanced neoplasia was found in shorter follow-up time in *MLH1* and *MSH2* mutation carriers, the colonoscopy interval in *MSH6* mutation carriers might be increased to three years instead of the regular two year interval.

INTRODUCTION

Lynch syndrome (LS) is the most common hereditary colorectal cancer (CRC) syndrome and is responsible for approximately 2-3% of all CRC cases(1-4). This syndrome is characterized by early onset of CRC, endometrial cancer and other extracolonic cancers.(1) The lifetime risk of CRC in these patients is up to 70%.

Lynch syndrome is caused by mutations in one of the four mismatch repair (MMR) genes *MLH1, MSH2, MSH6* and *PMS2* or in the 3' end of the *EPCAM* gene.(5-9) The identification of a pathogenic mutation in a cancer patient allows presymptomatic genetic testing in family members who have not yet developed cancer. Intensive surveillance programs with colonoscopy every 1-2 years from age 25 and removal of adenomas can reduce CRC incidence and mortality in these individuals by 56-70%.(10-12)

Lifetime risk of developing CRC is highest for *MLH1* and *MSH2* mutation carriers, and CRC develops less frequently and at a later age in individuals with a mutation in *MSH6* or *PMS2*.(13-18) However, although mutations in the different MMR genes result in different CRC risks, all LS carriers are currently offered the same surveillance interval regardless of the gene involved.

Most studies on surveillance programs for LS have only included patients with a mutation in *MLH1* or *MSH2*. Therefore, *MSH6* and *PMS2* mutation carriers may be enrolled in too stringent surveillance programs. In this study we aimed to evaluate the yield of Lynch syndrome surveillance for the individual MMR gene carrier groups. Additionally, we aimed to develop recommendations for tailored surveillance programs based on the causal MMR gene.

METHODS

In this retrospective cohort study we compared the development of colorectal adenomas and CRC between patients with *MLH1*, *MSH2*, *MSH6* and *PMS2* mutations that participate in LS surveillance programs.

Study population

After genetic counselling and germline mutation analysis, all LS patients in our center are offered intensive surveillance for CRC consisting of colonoscopy every 2 years, starting at the age of 25 years. We collected data on all patients diagnosed with LS in our center by

germline mutation analysis and participating in a LS surveillance program. Patients who had developed CRC before the identification of the MMR mutation were excluded from the study. Patients were contacted for informed consent to collect medical records on their LS surveillance. For patients with written informed consent data on age, gender, MMR gene involved, number of colonoscopies performed, interval between colonoscopies, and findings at each examination including histopathology were collected up to September 2017. Patients who declined colonoscopy surveillance were excluded from the study. Colonoscopies performed prior to the results of germline mutation analysis were also included in the study. Adenomas were considered advanced in case of a villous component, high-grade dysplasia and/or ≥10mm in size. We compared the development of (advanced) adenomas and CRC between patients with *MLH1*, *MSH2*, *MSH6* and *PMS2* mutations.

Statistical analysis

Data were analyzed using SPSS statistical software version 21.0. Differences between the mutation carrier groups were tested using the Chi square test for categorical variables and the one-way ANOVA for quantitative variables. Cumulative incidence of adenomas and CRC were calculated using the Kaplan-Meier time-to-event analysis and Cox regression analysis. P-values <0.05 were considered statistically significant.

RESULTS

A total of 314 LS carriers were included in this study; 116 (37%) were male and median age at LS diagnosis was 44 years (age ranged from 18-82 years).

Informed consent and colonoscopy data were available for 264 (84%) patients from 113 different families. Median age at time of the first colonoscopy was 44 years [IQR 35-56 years, total range 20-80 years]. Of these patients, 55 had a *MLH1*, 44 a *MSH2*, 143 a *MSH6* and 22 a *PMS2* mutation. Patient characteristics are shown in Table 1.

Index colonoscopy

At first colonoscopy, adenomas were found in 70 (27%) and advanced adenomas (villous component, high grade dysplasia and/or ≥10mm in size) were found in 33 (13%) patients. There was no significant difference in the number of colonoscopies in which adenomas or advanced adenomas were detected for the different gene mutation groups. CRC was found in 8 (3%) patients (age range 42-69 years, 3 *MLH1*, 1 *MSH2* and 4 *MSH6* mutation carriers).

Table 1. Patient characteristics

	Total	MLH1	MSH2	MSH6	PMS2	P
N	264	55	44	143	22	
Male	99 (38%)	23 (42%)	16 (36%)	54 (38%)	6 (28%)	0,69
Age at first colonoscopy	44 years (IQR 35-56, range 20-80)	38 years (IQR 31-53, range 20-75)	39 years (IQR 28-52, range 22-66)	49 years (IQR 38-57, range 25-77)	50 years (IQR 35-57, range 26-80)	0,001
First colonoscopy						
Adenomas	70 (27%)	15 (27%)	10 (23%)	37 (26%)	8 (36%)	0,69
1	36 (14%)	11 (20%)	4 (9%)	18 (13%)	3 (14%)	
2	23 (9%)	3 (6%)	5 (11%)	11 (8%)	4 (18%)	
>2	11 (4%)	1 (2%)	1 (2%)	8 (5%)	1 (5%)	
Advanced adenoma	33 (13%)	8 (15%)	4 (9%)	17 (12%)	4 (18%)	0,71
CRC	8 (3%)	3 (6%)	1 (2%)	4 (3%)	0	0,01
Observation years	1836 years	416 years	424 years	899 years	97 years	
Median number of observation years	6 years (IQR 2-10, range 0-32)	6 years (IQR 2-10, range 0-31)	8 years (IQR 2-15, range 0-32)	6 years (IQR 2-10, range 0-27)	4 years (IQR 2-6, range 0-11)	0,01
Median number of colonoscopies	4 years (IQR 2-6, range 1-19)	4 years (IQR 2-7, range 1-15)	5 years (IQR 2-8, range 1-19)	4 years (IQR 2-6, range 1-14)	3 years (IQR 2-4, range 1-9)	0,04

Colorectal cancer during surveillance

A total of 916 follow-up colonoscopies were performed in 220 patients. CRC was found in nine patients, all within 43 months after a colonoscopy; four in *MLH1* mutation carriers and five in *MSH2* mutation carriers (age range 43-72 years).(Supplemental table 1). In 3/9 CRC patients, colonoscopy was performed after a delayed interval (≥33 months). In 5/9 CRC patients, at least one adenoma was detected (and removed) at the previous colonoscopy. During follow-up no CRC was found in *MSH6* or *PMS2* mutation carriers.

Adenoma detection during surveillance

Adenomas were found in 191 (21%) and advanced adenomas in 31 (3%) colonoscopies (Table 2 and Figure 1). During surveillance, there were no significant differences in the number of colonoscopies with adenomas or advanced adenomas between the different gene mutation carrier groups. In total, 264 adenomas were found in 101 different patients (46% of the patients). Mean number of adenomas per procedure (MAP) was 0,28. Mean number of adenomas per positive procedure (MAP+) was 1,38. There were no significant differences in MAP or MAP+ between the different gene mutation carrier groups.

Table 2. Colonoscopies with adenomas

	Total	MLH1	MSH2	MSH6	PMS2	P
N	191	36	42	97	16	
Male	79 (41%)	15 (42%)	18 (43%)	39 (40%)	7 (44%)	0,99
Age at diagnosis	57 years (IQR 47-66, range 27-79)	49 years (IQR 41-60, range 27-78)	56 years (IQR 46-67, range 29-74)	61 years (IQR 53-68, range 31-79)	56 years (IQR 38-59, range 34-76)	<0,001
Colonoscopy interval	3 years (IQR 2-6, range 0-24)	4 years (IQR 2-8, range 0-24)	3 years (IQR 1-6, range 0-17)	3 years (IQR 2-6, range 0-14)	1 year (IQR 1-4, range 0-6)	0,09
Mean number of adenomas (MAP+)	1,38	1,19	1,40	1,40	1,63	0,49
Advanced adenoma	31 (16%)	8 (22%)	9 (21%)	10 (10%)	4 (25%)	0,16
High-grade dysplasia	9 (5%)	3 (8%)	2 (5%)	3 (3%)	1 (6%)	0,66
Villous component	15 (8%)	3 (8%)	4 (10%)	5 (5%)	3 (19%)	0,36
Size ≥10mm	17 (9%)	4 (11%)	3 (7%)	6 (6%)	4 (25%)	0,18

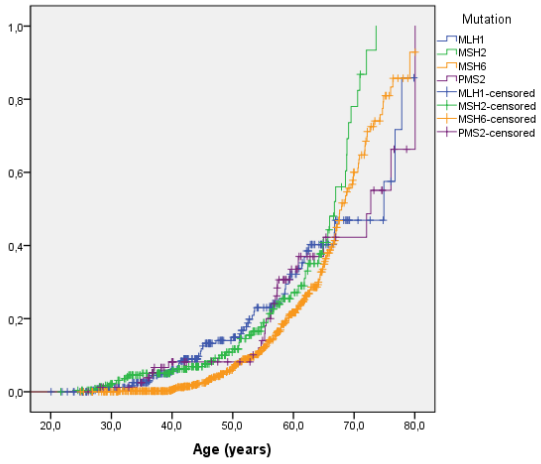

Figure 1. Cumulative incidence of adenomas during follow-up.

Median time of adenoma development (calculated as the time since the last adenoma identified or since the first colonoscopy in case no previous adenomas were found) was 3 years (IQR 2-6 years). There were no significant differences in time to development of an adenoma or advanced adenoma between the gene mutation carrier groups adjusted for age and gender (p=0,24 and p=0,13).(Figure 2A and 2B)

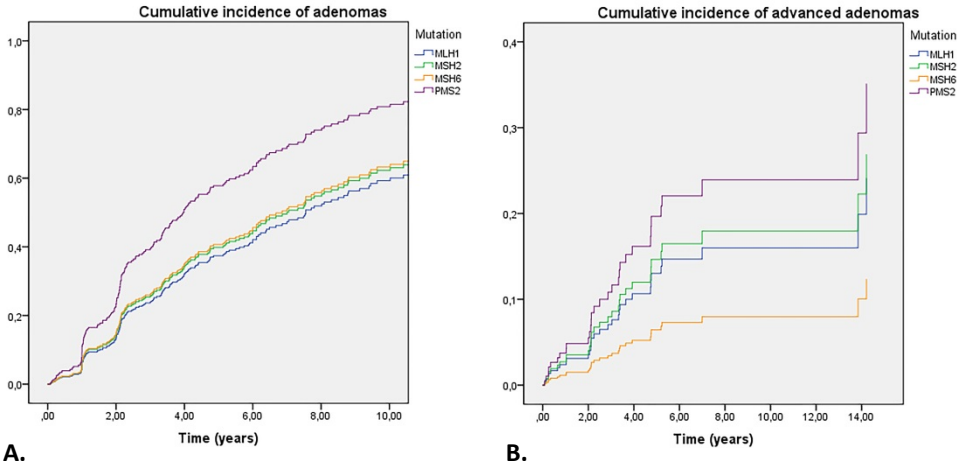

Figure 2. Cumulative incidence of adenomas (A) and advanced adenomas (B) during follow-up.

However, in *MSH6* mutation carriers advanced neoplasia (advanced adenoma or colorectal carcinoma) was found after a significant longer follow-up time than in other mutation carriers (p=0,01, Figure 3).

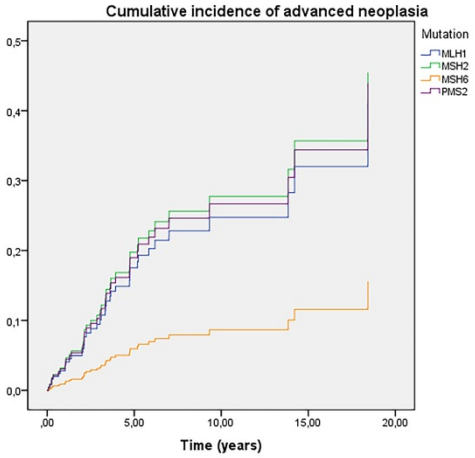

Figure 3. Cumulative incidence of advanced neoplasia.

Survival

Six patients died (3 *MSH2* and 3 *MSH6* mutation carriers). Only one patient died from CRC (*MSH6* mutation carrier aged 53), which was diagnosed at the first colonoscopy. In total 3/6 patients died from pancreatic cancer (1 *MSH6* and 2 *MSH2* mutation carriers). Patient characteristics are shown in Supplemental table 2.

DISCUSSION

The results of our study indicate a significant difference in advanced neoplasia development between *MSH6* mutation carriers and *MLH1/MSH2* mutation carriers. As no CRC was found during follow-up in *MSH6* mutation carriers and advanced neoplasia was found after longer follow-up time in these patients, we propose that the colonoscopy interval in *MSH6* mutation carriers might be increased to three years instead of every two years.

Adenomas

In line with a recent study, in our study no adenomas were detected during most colonoscopies (79% and 82% respectively). During follow-up, at least one adenoma was found in 46% of the patients, which is also comparable to the recent Portugese study in which adenomas were detected in 54% of the LS patients under surveillance.(19) However, the adenoma detection rate up to age 60 was lower than in previous studies.(19, 20) The lower detection rate in our cohort may be explained by differences in risk factors for adenoma development (for example, male sex or lifestyle factors). Also, other studies included results from the first colonoscopy in their results, which we excluded from the surveillance analysis. At the first colonoscopy, a higher percentage of advanced lesions were found than in follow-up colonoscopies in our cohort, likely due to a longer time period in which adenomas could progress.

As opposed to other CRC predisposition syndromes (Familial adenomatous polyposis, serrated polyposis syndrome), Lynch syndrome is not characterized by a high number of adenomas. During follow-up colonoscopies, we did not find many adenomas per procedure (MAP 0,28 and MAP+ 1,38). There were no significant differences in the number of colonoscopies in which adenomas or advanced adenomas were found between the different gene mutation carrier groups, similar to a previous study.(19) For *PMS2* mutation carriers, a relatively high number of adenomas and also advanced adenomas was found during a short follow-up time. At least in part, this is probably caused by the older age of inclusion of these patients. Also, the quality of colonoscopies has advanced over the years and *PMS2* mutation carriers predominantly underwent colonoscopies in more recent years than *MLH1* or *MSH2* mutation carriers who underwent colonoscopies going back 20 years. However, due to the small sample size, no clear conclusions could be drawn for *PMS2* mutation carriers.

Median time of adenoma development was 3 years (IQR 2-6 years) and did not differ significantly between the MMR mutation carrier groups adjusted for age and gender. Considering a median time of adenoma development of 3 years, the colonoscopy interval

of 2 years seems adequate. In the general population, removal of adenomas can prevent the development of CRC.(21) In LS patients, several different pathways for carcinogenesis are proposed.(22, 23) Adenomas can arise similar to those in the general population with an accelerated progression to CRC.(24) However, some crypts can become MMR deficient and then develop into an MMR deficient adenoma or directly progress to a CRC without an adenoma being formed first.(22, 23) We hypothesize that adenomas may be more prone to develop a second hit in an MMR gene than normal crypts, leading to faster progression to CRC in adenomas than in normal crypts. Contrary to adenomas, crypt foci with MMR deficiency cannot be identified at colonoscopy, and therefore colonoscopy surveillance will not be able to prevent CRC developing through this pathway. This is likely the cause of at least part of the interval CRC in LS patients under surveillance. Off course, surveillance colonoscopies are expected to find these cancers in an earlier stage, thereby enabling better treatment options and survival.

Colorectal cancer

In *MLH1* and *MSH2* mutation carriers advanced neoplasia (advanced adenoma or colorectal carcinoma) was found in shorter follow-up time than in MSH6 mutation carriers. Progression to CRC may be faster in *MLH1* and *MSH2* mutation carriers than in *MSH6* and *PMS2* mutation carriers, since MLH1 and MSH2 proteins play a central role in the MMR complexes. Further research should focus on in vitro studies comparing the effect of loss of different MMR proteins on oncogenesis.

None of the *MSH6* or *PMS2* mutation carriers in our study developed CRC during follow-up, in line with their lower penetrance. Although no CRC was found in *PMS2* mutation carriers, there was no evidence for a slower progression to advanced neoplasia in *PMS2* mutation in our cohort. This rather unexpected finding might be due to the small number of *PMS2* mutation carriers included. If development of advanced neoplasia is indeed slower in *MSH6* mutation carriers compared to *MLH1* and *MSH2* mutation carriers, this would justify longer intervals between colonoscopies in these patients. Expanding the intervals in these patients will lower their colonoscopy burden and might increase the adherence to surveillance programs.

During follow-up, CRC was found in 9/220 (4%) patients. The small percentage of CRC compared to the cancer incidence in patients not undergoing surveillance points toward the value of CRC surveillance in LS patients. In a recent study including 1942 LS carriers, despite colonoscopy surveillance, the most frequent type of cancer was CRC. Cumulative incidence of CRC ate age 70 years in that study ranged from 10% in *PMS2* mutation carriers to 46% in *MLH1* mutation carriers.(17) In our study, the lower percentage of CRC

is probably due to the smaller sample size. Also, especially younger patients enrolled in our study could still develop CRC in time.

Half of the CRCs in our cohort were diagnosed at the first screening colonoscopy. In three out of nine patients in whom CRC was found during follow-up, there was a clear delay between surveillance colonoscopies (≥33 months). This stresses the importance of early identification of LS patients and their adherence to surveillance programs.

Differences in the age of onset of adenomas or CRC could not be determined in our study, since not many patients were enrolled from a young age. CRC risk in *MSH6* and *PMS2* mutation carriers is known to be lower than in *MLH1* and *MSH2* mutation carriers. Also, CRC more often occurs at a later age in these patients, indicating that the starting age of CRC surveillance could be later in *MSH6* and *PMS2* mutation carriers. However, in spite of the reduced penetrance Goodenberger et al described that 8% of *PMS2* mutation carriers who developed CRC, were diagnosed under 30 years of age.(25)

Genotype-phenotype correlation in LS patients may not just be caused by the MMR gene involved, but could also be influenced by the type of mutation. Patients with truncating *PMS2* mutations may have a more severe phenotype than those with non-truncating mutations.(26) This should be evaluated further in larger cohorts, since it could potentially lead to more personalized surveillance programs.

Survival

Strikingly, half of the patients who died in our cohort, died from pancreatic cancer. Screening for pancreatic cancer is not recommended in LS patients, unless there is a family history of pancreatic cancer.(27) In a recent study, a high risk of pancreatic cancer was not found.(28) Combined risk of gastric, duodenal, bile duct or pancreatic cancer up to 75 years of age was 7-21% depending on the gene involved.(28) However, pancreatic cancer has a high mortality rate. As surveillance programs may cause less LS patients to die from CRC or EC, other cancer types such as pancreatic cancer may become more important for their survival. Only one patient died from CRC. In this patient, CRC was found at the first colonoscopy and therefore could not be prevented by the surveillance program.

Strengths and limitations

Strengths of our study were the comparison of adenoma development between the different mutation carrier groups and the relatively large cohort of *MSH6* mutation carriers included. However, only a small number of *PMS2* mutation carriers and young patients could be included. Also, there was no control group. A randomized controlled

trial with a part of the LS patients undergoing no or limited LS surveillance would enable clear conclusions on the yield of LS surveillance programs. However, considering the cancer risk in these patients such a study is unethical. Our study included patients with a long follow-up time, especially in *MLH1* and *MSH2* mutation carriers colonoscopy data went years back. For patients undergoing colonoscopies in the 80's or 90's, the quality of colonoscopy was likely lower than of those performed in more recent years. Finally, there could be information bias, since we were not able to include data from all the patients diagnosed with LS.

In conclusion, the results of our study imply that a longer interval (three years instead of two years) between colonoscopies for *MSH6* mutation carriers might be justified. Follow-up data on larger cohorts of MMR mutation carriers from a young age are needed.

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SUPPLEMENTAL MATERIAL

Table S1. Colorectal cancer patients

Sex	Age	Gene	Colonoscopy interval (months)	Previous findings	Side	Stage
M	59	MLH1	25	No adenomas	L	?
M	43	MLH1	15	No adenomas	L	T3N0M0
F	56	MSH2	33	No adenomas	R	T3N0M0
M	57	MSH2	15	1 tubular adenoma (low grade dysplasia)	R	T3N0M0
F	72	MSH2	13	2 tubular adenomas (low grade dysplasia)	R	
F	53	MSH2	17	2 tubular adenomas (low grade dysplasia)	R	T?N0M0
M	56	MLH1	43	1 tubular adenoma (low grade dysplasia)	?	?
F	47	MSH2	19	No adenomas	R	T2N0M0
M	50	MLH1	36	1 tubular adenoma (low grade dysplasia)	L	T3N0M0
F	69	MLH1	First colonoscopy	-	R	T1N0M0
F	50	MSH6	First colonoscopy	-	R	T3N0M0
F	49	MSH6	First colonoscopy	-	L	T3N0M0
M	42	MLH1	First colonoscopy	-	R	TisN0M0
M	58	MSH6	First colonoscopy	-	R	T3N0M0
M	57	MSH2	First colonoscopy	-	R	?
M	55	MLH1	First colonoscopy	-	R	T2N0M0
F	52	MSH6	First colonoscopy	-	L	T3N0M0

Table S2. Patients who died during follow-up

Sex	Age	Gene	Cause	Previous cancers
F	74	MSH2	Pancreatic cancer	Endometrial, breast, colorectal and small bowel cancer
F	56	MSH6	Lung cancer	-
F	69	MSH6	Pancreatic cancer	Endometrial cancer
F	53	MSH6	Colorectal cancer	-
F	79	MSH2	Not cancer related	-
M	44	MSH2	Pancreatic cancer	-

Part IV
General discussion and appendix

Chapter 10

General discussion and conclusions

Lynch syndrome (LS) is the most common hereditary colorectal cancer (CRC) predisposition and accounts for 2-3% of all CRC cases. Individuals with LS are also at increased risk of developing extracolonic cancers, especially endometrial cancer in women. LS is caused by autosomal dominant mutations in the mismatch repair genes *MLH1*, *MSH2*, *MSH6* or *PMS2,* or by deletions of the 3'region of the *EPCAM* gene. Since surveillance programs can significantly reduce cancer morbidity and mortality in LS patients, the identification of these individuals is of great importance.

IDENTIFICATION OF LYNCH SYNDROME PATIENTS

Prediction models

Several prediction models that can calculate the probability that an individual has LS are available. The prediction models MMRpro, MMRpredict and PREMM5 are available online free of charge.(1-3) Despite the easy web-based access, MMRpro is less useful in clinical practice since extensive information on all family members is needed, such as current age of every healthy relative. All three models have been shown to perform well for the identification of LS patients.(4-10) However, in many studies germline mutation analysis was not performed for all patients and therefore LS patients may have been missed. Especially in *MSH6* and *PMS2* mutation carriers, family history is less informative due to the lower cancer risk compared to *MLH1* and *MSH2* mutation carriers.

In fact, **chapter 3** shows that while the prediction models MMRpredict and PREMM5 can adequately predict whether an individual is likely to have Lynch syndrome caused by mutations in *MLH1*, *MSH2* and *MSH6*, they fail to identify *PMS2* mutation carriers. To improve the performance of the PREMM5 model, we added the location of the CRC to the model, as it is known that CRC in LS patients more often develops proximal from the splenic flexure than CRC in the general population. This addition to the PREMM5 model improved the overall performance as well as the identification of *PMS2* mutation carriers. These results were also confirmed in a validation cohort. The MMRpredict model already includes the side of CRC as a variable in the model and therefore could not be improved further. Interestingly, MMRpredict can only be used for CRC patients, whereas PREMM5 can also calculate the probability of a healthy individual to have LS and could therefore be used in different clinical settings.

The US Multi-Society Task Force on Colorectal Cancer recommends germline mutation analysis in individuals with a risk of carrying a MMR gene mutation ≥5% according to one of the prediction models MMRpro, MMRpredict or PREMM.(11) The authors who developed the PREMM5 model suggest a cut-off of 2,5% in order to identify *PMS2*

mutation carriers.(3) Strategies including prediction models to identify LS patients might lower the cost of screening for LS. Cost-effectiveness analyses found that strategies including prediction models and/or family-history assessment were more cost-effective than those involving direct tumor testing of all CRC patients, if these prediction models were perfectly implemented.(12, 13) However, family data is not always reliable or available and in practice, even the more compact revised Bethesda guidelines were shown to be underutilized.(14, 15) Routine screening of CRC patients by molecular diagnostics seems much more likely to be well implemented than the revised Bethesda guidelines or prediction models in clinical practice. In fact, in a cost-effectiveness study underutilization of the revised Bethesda guidelines made routine LS screening by molecular diagnostics the preferred strategy.(13) Another use for prediction models could be to select patients from population based screening programs in whom screening for LS could be performed. In a meta-analysis, prediction models indeed performed better in population-based cohorts than in clinic-based cohorts.(10) Also, in cases where no tumor tissue is available or where tumor tissue analysis failed, prediction models could help assess whether an individual should be analyzed for a germline MMR mutation. In high risk populations however, such as patients referred to a clinical geneticist, prediction models will likely be less useful. In such a selected patient group, molecular diagnostics for LS as a first step has relatively low costs, a priori chance of finding a MMR mutation is assumed to be high depending on the guidelines for referral and off course specificity of prediction models is not 100%. Furthermore a clinical geneticist will always draw an extensive pedigree and not only weighs the number of family members who developed cancer and the ages of onset of different types of cancer, but also takes into account if there are many relatives who have not developed cancer or if the family is very small with a relatively high frequency of cancer. Since family size has decreased over the last decades prediction models may increasingly lead to lower predictions in LS patients. In settings with a very low capacity of molecular testing and/or germline mutation analysis, prediction models may play a larger role in order to optimize the use of the available resources.

Routine molecular screening for Lynch syndrome

Colorectal cancer patients

Analysis of microsatellite instability (MSI) and immunohistochemistry (IHC) on tumor tissue can be used as a screening tool to identify patients who are likely to have LS.(16) For a long time, routine screening for LS was performed for CRC patients <50 years of age, thereby missing mostly *MSH6* and *PMS2* mutation carriers. **Chapter 4** shows routine screening for LS in CRC patients ≤70 years of age by analysis of MSI, IHC and MLH1 hypermethylation is cost-effective according to currently accepted standards. Expanding the age limit for routine screening for LS among CRC patients from 50 to 60 years of age

remained <€10.000/LYG in sensitivity analysis. The incremental cost effectiveness ratio (ICER) for further expansion of the age limit from 60 to 70 years of age never exceeded €13.000/LYG. These ICERs are well within the currently accepted thresholds for cost-effectiveness of €40.000/LYG in the Dutch healthcare system.(17) Several studies indicate that a screening strategy using only IHC instead of MSI analysis and IHC together may be even more cost-effective.(13, 18)

In our study, the ICER for LS screening among CRC patients ≤70 years of age compared with testing according to the revised Bethesda guidelines remained <€13.000/LYG and therefore cost-effective in the Dutch setting. Furthermore, age-targeted LS screening will be much easier to use and by better implementation may be even more cost-effective in clinical practice than criteria based on family history. In most Western countries cost-effectiveness threshold similar to the Dutch setting are used, and some authors advocate even higher thresholds.(17, 19) However, in low-income countries lower cost-effectiveness thresholds might cause screening for LS not to be cost-effective.(20)

International guidelines for screening for LS have indeed been extended in recent years, recommending screening for LS by analysis of MSI and/or IHC in CRC patients up to 70 years.(11, 21) The American guidelines even recommend universal LS screening for CRC patients, without any age cut-off.(11) Of course, this will probably increase the number of LS patients identified. However, it is unclear whether this is also cost-effective, as the likelihood of underlying LS decreases with the increase of the age of CRC diagnosis. In **chapter 4**, LS was diagnosed in 4,9% of the CRC patients ≤50 years of age, 2,1% of the patients aged 51-60 years of age and 1,5% of the patients 61-70 years of age. Also, as most CRC patients are >70 years of age, there would be an enormous increase of patients screened for LS, with a low a priori risk of having LS. In one cost-effectiveness study universal LS screening in CRC patients was indeed not cost-effective.(12) However, as MSI analysis may also be increasingly performed in order to determine whether 5FU chemotherapy is beneficial, universal screening for LS may become part of routine diagnostics. It is important to secure the routing of the results of MSI and/or IHC analysis in clinical practice, in order to offer adequate counseling and germline mutation analysis in patients with aberrant results. Also, patients with CRC at a very young age or with a strong family history of CRC should still be referred to a clinical geneticist, even if the results of molecular diagnostics are not suspect for LS. For example, in these families, there might be a different CRC predisposition.

Endometrial cancer patients

Similar to LS screening in CRC patients, routine molecular screening is also recommended for endometrial cancer (EC) patients.(11, 21) While not many studies on the cost-

effectiveness of LS screening among EC patients have been performed, all concluded that some form of LS screening among these patients was cost-effective.(22-24) In **chapter 5**, the ICER for routine screening for LS among EC patients up to 70 years of age compared to 50 years of age remained <€13.000/LYG. EC patients will likely benefit more from LS surveillance than CRC patients, since development of CRC can still be prevented in these patients and the mortality rate for LS patients diagnosed with CRC is higher than in LS patients diagnosed with EC.(25) Therefore, the cost-effectiveness of routine LS screening among EC patients may be even higher than for CRC patients. More research is needed in larger cohorts of EC patients to determine the cost-effectiveness of screening for LS in these patients with an age cut-off over 70 years, or even universal screening. In cohort studies of EC patients without an age cut-off, a prevalence of LS of at least 2,1-3,9% was found.(26-28)

The cost-effectiveness of screening for LS among CRC and EC patients is mainly based on the identification of healthy relatives carrying the same MMR mutation, since these relatives will benefit the most from surveillance programs for LS. This stresses the importance of informing family members about the diagnosis and offering them genetic counseling and germline testing. In both our cost-effectiveness studies, a median of 3 relatives were tested for each index patient. However, there was a very wide range from 1-50 relatives that were tested per index patient. In the Netherlands, as in most countries, the index patient will inform relatives about the LS diagnosis. Most patients are satisfied with the current approach, where the diagnosis will be communicated by a family member.(29) During genetic counseling of an index patient, known risk factors for failure to communicate the diagnosis to relatives (such as disrupted family relations) should be evaluated in order to provide adequate assistance to communicate the diagnosis to family members. An important reason for relatives to refrain from germline mutation analysis in cancer predisposition syndromes is the possible effect on life insurance policies.(30, 31) It is important to inform relatives at risk of having LS about the possibility to enroll in LS surveillance programs if they refrain from germline mutation analysis. Also, education on symptoms of CRC and EC could be beneficial in these patients even if they opt not to participate in a surveillance program.

Adenoma patients

Molecular diagnostics for LS can also be performed in tissue other than CRC or EC. Little is known about the yield of LS screening for other types of cancer, such as bladder cancer or ovarian cancer. In two studies, a high percentage of 52% and 66% of the sebaceous neoplasms showed MMR deficiency.(32, 33) Unfortunately, germline mutation analysis was performed in only a small number of patients. Currently, no routine screening is recommended for other types of LS-associated cancer.

Loss of MMR expression is found in 50-84% of the adenomas from LS patients.(34-37) Screening for LS among patients with these precancerous lesions could provide an opportunity to identify LS index patients in whom development of cancer can be prevented. **Chapter 6** shows that screening for LS in advanced adenomas found in a the national FIT-based CRC screening program is probably not effective. Screening among a subset of adenoma patients such as younger adenoma patients, however, might still be effective. In a previous study 3/125 (2,4%) advanced adenoma patients under 45 years of age were diagnosed with LS.(38) In LS patients, adenomas with a villous component or high grade dysplasia are most likely to show MSI and loss of MMR protein expression.(34-37) Therefore, screening only adenomas with a villous component or high grade dysplasia might be effective. In one study 5,4% of the adenomas (10/187) with high grade dysplasia showed aberrant IHC. However, *MLH1* hypermethylation analysis, germline mutation analysis and somatic mutation analysis were not performed, so it is unclear how many of these patients actually could be diagnosed with LS.(39) Further research should focus on screening for LS by IHC analysis in adenomas from younger populations and/or with a villous component and high grade dysplasia.

In clinical practice, molecular diagnostics for LS in adenoma tissue will be performed in selected cases, for example in families where there is no tumor available for testing. In recent years, at least two families have been diagnosed with LS in our center after IHC analysis on adenoma tissue. The first index patient was a man with a large tubulovilleus adenoma with high grade dysplasia at age 27 in whom a germline *MSH6* mutation was found. In the second family a tubulovilleus adenoma was analyzed from a 34-year old woman, since CRC tissue from her sister (with CRC at age 22) was not available. A germline *MSH2* mutation was identified in this patient.

MMR deficiency is found in 12-20% of all CRC.(40-43) The MMR deficiency can be caused by an underlying germline MMR mutation, but can also be seen in some sporadic CRC. In these CRC, MMR deficiency is caused by hypermethylation of the MLH1 promoter or two somatic hits.(16, 44, 45) There is no data on the percentage of MMR deficiency among sporadic adenomas. Based on the results of **chapter 6** with none of the adenomas showing MLH1 hypermethylation and only one adenoma in which two somatic mutations were found, MMR deficiency seems uncommon and not an early event in the adenoma-carcinoma sequence in the general population.

VARIANTS OF UNKNOWN SIGNIFICANCE

In individuals suspected of having LS based on family history or the results of molecular diagnostics, germline mutation analysis is performed to confirm the diagnosis. In some patients, a variant of unknown significance (VUS) is found. The inability to determine the pathogenicity of such a DNA variant causes uncertainty for the index patients as well as their relatives. If a pathogenic variant is classified as a VUS, presymptomatic testing is not offered to family members. Moreover, relatives will be less likely to comply with surveillance recommendations until the diagnosis of LS is confirmed despite their risk of developing cancer.(46) In contrast, family members not at an increased risk of developing CRC may also undergo invasive surveillance procedures that they do not need.

Co-segregation analysis of the VUS with MMR deficient tumors in family members and in silico prediction models are used for the classification of VUS. However, co-segregation is not always possible, especially in smaller families. With the decrease in family size co-segregation will be less informative. Functional assays have been developed to determine the pathogenicity of VUS in the MMR genes.(47-50)

With the expanding screening for LS , germline mutation analysis of the MMR genes will increasingly be performed. This will not only increase the number of LS patients being diagnosed, but will probably lead to more patients in whom a VUS in one of the MMR genes is found. Also, with the widespread use of whole exome sequencing, a germline VUS in one of the MMR genes can be an incidental finding. Therefore, the need for functional assays to distinguish pathogenic MMR gene variants from polymorphisms will increase. Once a VUS has been classified as either pathogenic or non-pathogenic, this classification can also be used for other patients carrying the same variant.

The MMR genes are responsible for the repair of mismatches arising during DNA replication and can induce cell death in response to cytotoxic agents.(51, 52) In 2016, a functional assay for MMR gene variants was developed which starts with oligo targeting in embryonic stem cells from mice to insert a variant of interest in the *MLH1* or *MSH2* gene.(49) These cells are subsequently treated with 6TG, which has a cytotoxic effect by inducing double-strand DNA breaks. If the MMR system in the cells functions properly, apoptosis will be induced as a result of the cytotoxicity of 6TG. In case of a pathogenic MMR mutation, however, cells can become resistant to DNA-methylating agents and cell death will not be induced. Hence, survival of cultures after 6TG treatment implies pathogenicity of the inserted variant. After identification of cultures resistant for 6TG, DNA sequencing is performed to confirm the presence of the VUS of interest in the cells

and to exclude MMR deficiency due to a somatic second hit in the cells. **Chapter 7** concerns the evaluation of 18 VUS in *MLH1* and *MSH2* that were identified in 21 different families, using this functional assay. Seven *MLH1* VUS and five *MSH2* VUS were pathogenic according to the 6TG resistance in the cells. Western blot analysis showed that these cells had significantly lower protein levels for the corresponding MMR gene. All six other VUS were likely not pathogenic, which in four cases was in line with the (lack of) family history of LS-associated tumors in these patients and results of MSI and IHC analysis. In one patient, loss of MLH1 and PMS2 protein expression in the tumor is likely caused by the known pathogenic *MLH1* mutation c.677+1delG that was found simultaneously with the VUS. The *MLH1* VUS A31C was found in a patient with a tumor showing MSI without *MLH1* hypermethylation. An additional evaluation of this VUS using CRISPR/Cas9 technology and MNNG-induced mutagenesis assays also showed MMR proficiency, which is in line with a previous report on the variant.(47) Off course, a variant could have a different effect on human cells than mice. Alternatively, a different mutation in *MLH1* missed by DNA sequencing might be causing the phenotype in this patient, for example an intronic mutation.

Non-pathogenicity of the *MLH1* variants A31C, V213M and T549A and *MSH2* variants N127S and N596S is in line with previous reports on these VUS.(47, 53-55) In silico predictions indicate that the *MSH2* VUS R534C may be pathogenic.(56, 57) Although this VUS was identified in a patient with a MMR proficient tumor, he did develop two LS-associated tumors and had a family history of cancer. Still, environmental factors and/or genetic factors other than the MMR genes could play a role in the development of cancer in this family. Other functional assays could be used to confirm the results of our assay.

Chapter 8 shows that the genetic screen for MMR genes based on oligo targeting and selection for 6TG resistance, can also be used to determine pathogenicity of VUS in *MSH6*. Four known pathogenic variants were indeed resistant for 6TG treatment and five variants previously classified as non-pathogenic were also classified as non-pathogenic by the assay. After this proof of principle analysis, 26 VUS from clinical practice and from the International Society for Gastrointestinal Hereditary Tumours (INSIGHT) database were evaluated. Three out of the eight VUS found in clinical practice were indicated to be pathogenic, which was in line with the family history of LS-associated tumors in these patients and the presence of MMR deficiency in their tumors. Three other variants classified as non-pathogenic were found in patients in whom a simultaneously identified pathogenic MMR mutation was identified, explaining their phenotype. The last two VUS that were indicated to be non-pathogenic by the assay, were found in cases in whom no other cause of their MMR deficient tumors was identified. For one of these two VUS,

A25S, the classification as non-pathogenic was in line with a previous functional assay also indicating the variant not to be pathogenic.(48) The CRC in the patient carrying this VUS showed MSI, loss of MSH6 protein expression and loss of heterozygosity of *MSH6.* However, family history was not highly suspect for LS and a second somatic hit may have been missed in the tumor. In contrast, the second VUS that was classified as non-pathogenic, c.3438+6T>C, was identified in a patient with a family history of LS-associated tumors. However, IHC on tumor tissue failed and tumor analysis in a relative showed MMR proficiency. Unfortunately, segregation analysis could not be performed in this family. In this case, there could still be another explanation for the MMR deficient tumor in the index patient, such as somatic MMR mutations or MLH1 hypermethylation. However, especially since intron sequences are more variable between species, pathogenicity of the c.3438+6T>C variant cannot be excluded with absolute certainty, due to differences in the mouse and human DNA sequences. There could be a splicing effect from this variant in humans, which is not present in mice, although several splice site prediction programs do not predict aberrant splicing by c.3438+6T>C.

The correct classification of a VUS in the MMR genes is highly important, because of the implications it has for eligibility for cancer surveillance. In order to obtain the most reliable classification, results of functional assays should be used in combination with other methods for the classification of VUS. These methods include clinical data such as cancer history, molecular diagnostics on tumor tissue of the index patient as well as relatives, and co-segregation analysis, and also the use of in silico prediction programs. Also, different functional assays could be used complimentary in cases with uncertain results, to achieve a high level of evidence for the classification of VUS and proper enrollment of individuals at high risk of developing cancer in tailored surveillance programs.

SURVEILLANCE IN LYNCH SYNDROME PATIENTS

The goal of identifying LS patients is of course to inform them on their increased cancer risk and to decrease cancer morbidity and mortality in these patients by surveillance programs. Guidelines recommend CRC surveillance by colonoscopy every 1-2 years starting from age 25. Since colonoscopies are invasive and burdensome, patients should not undergo unnecessary procedures. Despite the differences in cancer risk between the different MMR gene mutation carriers, surveillance is the same for all LS patients regardless of which MMR gene is involved. The yield of LS colonoscopy surveillance for carriers of a mutation in *MLH1*, *MSH2*, *MSH6* and *PMS2* is assessed in **chapter 9**. In most sessions no adenomas or CRC were found and there were no significant differences in

the number of adenomas that were found between the groups. However, the risk of finding advanced neoplasia increased at a slower pace in *MSH6* mutation carriers than in the other MMR gene mutation carriers groups. None of the 121 *MSH6* or 20 *PMS2* mutation carriers in our study developed CRC during follow-up, in line with their lower penetrance. Considering this slower pace of progression to advanced neoplasia, the fact that no CRC was found in these patients during follow-up and considering the size of the groups included, the colonoscopy interval in *MSH6* mutation carriers may be less stringent than for *MLH1* and *MSH2* mutation carriers. Expanding the colonoscopy interval in these patients could lower the colonoscopy burden and might thereby even increase the adherence to the surveillance program. The fact that 3/9 CRCs that were found during follow-up were diagnosed after a delay between colonoscopies, emphasizes the importance of adherence to these programs. However, three patients with a *MLH1* or *MSH2* mutation developed CRC despite timely colonoscopies. These interval cancers are likely due to an alternative pathway for CRC development in LS, with MMR deficiency arising in a colonic crypt and direct progression to CRC without the development of an adenoma.(58, 59) Since no precursor lesion can be identified for CRC developing through this pathway, prevention by colonoscopic surveillance may be difficult. Nevertheless, early detecting of these CRC would improve survival in LS patients.

Due to the small number of *PMS2* mutation carriers included, no clear conclusions could be drawn for this group. However, based on the lower cancer risk in *PMS2* mutation carriers, less frequent surveillance will probably be sufficient in these patients. Moreover, in a recent study no survival differences were found between LS patients undergoing colonoscopy surveillance yearly, every two years or every three years. At least for *MSH6* and *PMS2* mutation carriers, a colonoscopy interval of three years could therefore be justified. The median age of CRC is also higher in *MSH6* and *PMS2* mutation carriers than in *MLH1* and *MSH2* mutation carriers. In our study only a small number of young patients were included and we were unable to determine whether surveillance in *MSH6* and *PMS2* mutation carriers could start at a later age. However, in a study among CRC patients with a *PMS2* mutation, 8% were diagnosed <30 years of age.(60) Larger cohorts of young MMR mutation carriers should be analyzed prospectively in order to determine the proper age to commence surveillance for all MMR genes. Similar studies on the yield of LS surveillance for gynecological cancer are needed. Also, patients with *EPCAM* gene mutations should be included in evaluations on LS surveillance. While their risk of developing CRC is comparable to the risk in *MSH2* mutation carriers, the risk of developing EC is lower, implying less intensive gynecologic surveillance in these patients.(61)

The MMR gene involved is probably not the only factor that determines genotype-phenotype correlation in LS patients. For example, truncating *PMS2* mutations were shown to cause a more severe phenotype than non-truncating mutations.(62) Also, studies identified possible modifiers in *MLH1* mutation carriers.(63, 64) Increasing knowledge on all these factors that can influence the phenotype of a patient can in time lead to truly personalized surveillance programs.

In three out of five patients who died during follow-up in our cohort, the cause of death was pancreatic cancer, which is a cancer type with a very high mortality rate. In literature an increased risk of developing pancreatic cancer in LS is described by some but not others.(65-67) As CRC can be prevented or detected early in LS patients, other tumor types may become of increasing importance for survival of LS patients.

FUTURE DIRECTIVES

The guidelines for the identification of LS patients have been improved over the years. Considering the low cost of IHC analysis and high benefit of diagnosing LS (in index patients but most of all in healthy relatives by cascade testing), further expansion of screening for LS may still be cost-effective. Future research could focus on the identification of LS patients in tissue types that are currently not screened for LS, such as routine screening in ovarian and bladder cancers, or sebaceous adenomas. Although screening for LS in older adenoma patients was not effective, screening for LS in young adenoma patients should still be evaluated given the potentially high benefit of such a screening strategy with the ability to prevent the development of cancer in index patients. Also, the implementation of the current guidelines should be monitored, since the revised Bethesda guidelines were not well-used.

As VUS found in the MMR genes pose an important problem for index patients as well as their relatives, effort needs to be undertaken to properly classify these variants. Clinical data, results for molecular diagnostics on tumor tissue, co-segregation analysis, in silico prediction programs and functional assays should be part of the routing to classify these VUS.

In order to achieve surveillance programs tailored to the gene involved or even more personalized surveillance, large datasets of LS patients undergoing surveillance are needed. International collaborations are key in order to reach the numbers needed for a proper evaluation of surveillance programs, which should be stimulated by funding programs.

A promising technique which may change the surveillance of LS patients altogether, is the liquid biopsy. Liquid biopsies, such as the analysis of circulating tumor cells or cell free DNA (cfDNA) and micro-RNA in blood show great potential for monitoring disease progression and response to treatment in cancer patients.(68-71) If early stage cancers or even adenomas can be reliably detected through a blood test, this would reduce the number of invasive colonoscopy procedures needed in LS patients. Also, extracolonic tumors for which currently no screening is possible, might be identified by surveillance using liquid biopsies.

In conclusion, the identification of LS has improved with the expanded screening for LS among cancer patients, functional assays are available to test VUS in the MMR genes and evidence for tailored surveillance programs for LS is emerging. Future research should focus on further improvement of the identification of LS patients (also among patients who have not yet developed cancer), the classification of VUS in the MMR genes, development of tailored surveillance programs for LS based on genotype, and the potential use of liquid biopsies in these surveillance programs.

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Appendix

SUMMARY

This thesis focusses on Lynch syndrome, the most common hereditary predisposition for colorectal cancer. Lynch syndrome accounts for 2-3% of all colorectal cancer cases. The syndrome is caused by autosomal dominant mutations in the mismatch repair genes *MLH1*, *MSH2*, *MSH6* or *PMS2,* or by deletions of the 3'region of the *EPCAM* gene. Individuals with Lynch syndrome not only have an increased risk of developing colorectal cancer, but also of developing extracolonic cancers, especially endometrial cancer in women.

Chapter 1 consists of a general introduction on the subject, followed by the aims and outline of this thesis in **chapter 2**.

Identification of Lynch syndrome patients
Surveillance programs can significantly reduce morbidity and mortality in individuals with Lynch syndrome. However, in order to be offered surveillance, these individuals first have to be identified. Therefore, identification of individuals with Lynch syndrome is of great importance.

Prediction models
Several prediction models that can calculate the probability that an individual has Lynch syndrome are available. In **chapter 3**, two of these prediction models, MMRpredict and PREMM5, were evaluated in a cohort of 734 colorectal cancer patients. Both models could fairly predict whether an individual is likely to have Lynch syndrome, but PREMM5 failed to identify Lynch syndrome patients with a *PMS2* mutation, with an AUC of 0.52. We extended the PREMM5 model with the location of colorectal cancer as a new variable, which improved the identification of *PMS2* mutation carriers (AUC 0.77) as well as its overall performance (0.81 vs. 0.72). These results were also confirmed in a validation cohort of 376 colorectal cancer patients. The extended PREMM5 model could for example be used to identify individuals eligible for Lynch syndrome diagnostics in populations with a low a priori risk of having Lynch syndrome, such as a population-based screening program for colorectal cancer.

Routine molecular screening for Lynch syndrome
Tumors from Lynch syndrome patients show microsatellite instability and loss of mismatch repair protein expression at immunohistochemistry. Therefore, analysis of microsatellite instability and immunohistochemistry on tumor tissue can be used as a screening tool to identify patients likely to have Lynch syndrome.

Previous guidelines recommended molecular screening for Lynch syndrome in all colorectal cancer patients up to 50 years of age. In **chapter 4 and 5** we assessed the cost-effectiveness of routine molecular screening for Lynch syndrome in colorectal cancer patients and endometrial cancer patients up to 70 years of age. The incremental cost effectiveness ratio for expanding the age limit for routine Lynch syndrome screening among these patients from 50 to 70 years of age remained <€13.000 per life year gained in sensitivity analysis. This is well within the thresholds for cost-effectiveness (<€40.000 per life year gained). Current international guidelines now recommend routine screening for Lynch syndrome by analysis of microsatellite instability and/or immuno-histochemistry in patients with colorectal cancer or endometrial cancer up to at least 70 years of age.

The individuals benefiting the most from Lynch syndrome screening among colorectal and endometrial cancer patients, are healthy relatives of the index patients carrying the same mutation, since cancer can still be prevented in these individuals. Identification of Lynch syndrome patients among patients with adenomas (a precursor lesion of colorectal cancer) would ensure a similarly high benefit for the index patients. However, in **chapter 6**, no Lynch syndrome patients were identified by screening for Lynch syndrome among all advanced adenomas in the population based CRC screening program. Therefore, routine screening of all adenomas is probably not effective. Screening for Lynch syndrome among a subset of adenoma patients such as younger adenoma patients, however, might still be effective.

Variants of unknown significance

A definite diagnosis of Lynch syndrome is made when a pathogenic mutation in one of the mismatch repair genes *MLH1*, *MSH2*, *MSH6* or *PMS2,* or a deletion of the 3'region of the *EPCAM* gene is found. In some cases, a variant of unknown significance is found and the diagnosis remains uncertain. The correct classification of a variant in the mismatch repair genes is highly important, because of the implications it has for eligibility for cancer surveillance.

In **chapter 7 and 8** a functional assay to classify variants of unknown significance in the mismatch repair genes is evaluated and 26 variants in *MLH1*, *MSH2* and *MSH6* are analyzed. Seven variants in *MLH1*, five variants in *MSH2* and three variants in *MSH6* were indicated to be pathogenic according to this functional assay. In most cases the classification was in line with clinical data, prediction programs and results of other functional assays.

Surveillance in Lynch syndrome patients

The ultimate goal of identifying Lynch syndrome patients is to inform them on their increased cancer risk and to decrease their cancer morbidity and mortality by surveillance programs. Guidelines recommend colorectal cancer surveillance by colonoscopy every 1-2 years starting from age 25. Although the cancer risk in Lynch syndrome patients is highly dependent on the gene involved, all patients are currently offered the same surveillance. Therefore, **chapter 9** evaluates the yield of colonoscopy surveillance in *MLH1*, *MSH2*, *MSH6* and *PMS2* mutation carriers.

At most colonoscopies no adenomas or colorectal cancer were found and there were no significant differences in the number of adenomas that were found between the groups. However, the risk of finding advanced neoplasia (advanced adenoma or colorectal cancer) increased at a slower pace in *MSH6* mutation carriers than in the other MMR gene mutation carriers groups. Considering this slower pace of progression to advanced neoplasia and the fact that none of the 121 *MSH6* mutation carriers developed colorectal cancer during follow-up, the colonoscopy interval in *MSH6* mutation carriers may be less stringent than for *MLH1* and *MSH2* mutation carriers. The interval between colonoscopies might be increased to three years for MSH6 mutation carriers. Due to the small number of *PMS2* mutation carriers included, no clear conclusions could be drawn for this group. However, based on the lower cancer risk in *PMS2* mutation carriers, less frequent surveillance will probably also be sufficient in these patients.

Finally, **chapter 10** discusses the results of this thesis in perspective of the current guidelines and clinical practice.

SAMENVATTING

Dit proefschrift richt zich op Lynch syndroom, de meest voorkomende erfelijke aanleg voor darmkanker. Lynch syndroom is de oorzaak van 2-3% van alle darmkankers. Het syndroom wordt veroorzaakt door (autosomaal dominante) fouten in het DNA (mutaties) in de mismatch herstelgenen *MLH1*, *MSH2*, *MSH6* of *PMS2,* of door het missen van een stuk DNA aan de achterkant van het *EPCAM*-gen. Mensen met Lynch syndroom hebben niet alleen een verhoogd risico op darmkanker, maar ook op het ontwikkelen van andere tumoren, met name baarmoederkanker bij vrouwen.

Hoofdstuk 1 omvat een algemene introductie over het onderwerp, gevolgd door de doelstelling en opbouw van het proefschrift in **hoofdstuk 2**.

Identificatie van Lynch syndroom patiënten

Periodieke controles kunnen het ontstaan van darmkanker en de sterfte aan kanker bij Lynch syndroom patiënten fors verlagen. Echter, om deze controles aan te kunnen bieden, is het opsporen van mensen met Lynch syndroom van groot belang.

Predictiemodellen

Verschillende predictiemodellen die de kans kunnen berekenen dat een persoon Lynch syndroom heeft zijn beschikbaar. In **hoofdstuk 3** werden twee van deze predictiemodellen, MMRpredict en PREMM5, geëvalueerd in een groep van 734 patiënten met darmkanker. Beide modellen konden redelijk goed voorspellen of iemand Lynch syndroom heeft, maar PREMM5 kon Lynch syndroom patiënten met een *PMS2* mutatie niet goed identificeren. De voorspelling was slechts in 52% van de gevallen juist.
We hebben het PREMM5 model uitgebreid met de locatie van de darmkanker als nieuwe variabele (rechts- of linkszijdig). Hiermee verbeterde zowel de identificatie van *PMS2* mutatiedragers (77% goed voorspellend) als de algehele prestatie van het model (van 71% goed voorspellend naar 81% goed voorspellend). Deze resultaten werden bevestigd in een tweede (validatie) groep van 376 darmkanker patiënten. Het uitgebreide PREMM5 model kan bijvoorbeeld worden gebruikt op mensen te identificeren die in aanmerking komen voor diagnostiek naar Lynch syndroom in populaties met een lage algemene kans op Lynch syndroom, zoals binnen het landelijke bevolkingsonderzoek naar darmkanker.

Routinematige moleculaire screening voor Lynch syndroom

Microsatellieten zijn repeterende stukjes in het DNA. Wanneer de mismatch herstelgenen niet goed functioneren, treden gemakkelijk fouten op in deze herhalingen, waardoor microsatellieten van verschillende lengtes ontstaan. Dit wordt microsatelliet instabiliteit genoemd. Tumoren van Lynch syndroom patiënten worden gekenmerkt door

microsatelliet instabiliteit en het verlies van eiwit van de mismatch herstelgenen bij kleuring van de tumor (immunohistochemie). Daarom kan analyse van microsatelliet instabiliteit en immunohistochemie worden ingezet als screening om patiënten te identificeren die mogelijk Lynch syndroom hebben.

Eerdere richtlijnen adviseerden moleculaire screening voor Lynch syndroom bij alle darmkanker patiënten tot 50 jaar. In **hoofdstuk 4 en 5** berekenden we de kosten-effectiviteit van routinematige screening op Lynch syndroom bij alle patiënten met darmkanker of baarmoederkanker tot 70 jaar. De extra kosten voor het verleggen van de leeftijdsgrens van 50 naar 70 jaar bij deze patiënten bleef <€13.000 per gewonnen levensjaar. Dit valt ruim binnen de grenzen voor kosteneffectiviteit (<€40.000 per gewonnen levensjaar). De huidige internationale richtlijnen adviseren routinematige screening op Lynch syndroom middels analyse van microsatelliet instabiliteit en/of immunohistochemie bij alle patiënten met darmkanker of baarmoederkanker tot 70 jaar.

Wanneer bij een patiënt met darmkanker of baarmoederkanker Lynch syndroom wordt vastgesteld, hebben met name gezonde familieleden van deze patiënt die dezelfde mutatie dragen, hier baat van, omdat het ontstaan van kanker bij hen nog kan worden voorkomen. Het identificeren van Lynch syndroom bij patiënten met adenomen (een voorstadium van darmkanker) zou dezelfde hoge winst voor patiënten zelf betekenen. Echter in **hoofdstuk 6** werd bij routinematige screening op Lynch syndroom bij alle patiënten met gevorderde adenomen in het bevolkingsonderzoek naar darmkanker geen enkele patiënt met Lynch syndroom geïdentificeerd. Routinematige screening van alle adenomen lijkt daarom niet zinvol. Screening van een deel van deze patiënten, zoals jongere patiënten, zou wel effectief kunnen zijn.

Varianten van onbekende klinische betekenis

De definitieve diagnose Lynch syndroom wordt gesteld wanneer een ziekteverwekkende mutatie in een van de mismatch herstelgenen *MLH1*, *MSH2*, *MSH6* or *PMS2,* of verlies van een stuk DNA aan de achterkant van het *EPCAM*-gen wordt aangetoond. In sommige gevallen wordt een DNA-variant gevonden waarvan we niet zeker weten of het tot Lynch syndroom kan leiden, waardoor de diagnose onzeker blijft. De juiste classificering van varianten in de mismatch herstelgenen is zeer belangrijk, vanwege de implicaties die dit heeft voor het controleadvies.

Hoofstuk 7 en 8 omvat de evaluatie van een test om varianten, waarvan we niet weten of ze ziekteverwekkend zijn, in de mismatch herstelgenen te classificeren. We hebben de analyse gedaan op 26 varianten in *MLH1*, *MSH2* en *MSH6.* Zeven varianten in *MLH1*, vijf varianten in *MSH2* en drie varianten in *MSH6* waren ziekteverwekkend volgens de

functionele test. In de meeste gevallen kwam dit goed overeen met klinische data, predictie programma's en de resultaten van andere functionele testen.

Surveillance van Lynch syndroom patiënten

Het doel van de identificatie van Lynch syndroom patiënten is om hen te informeren over het verhoogde risico op kanker en middels periodieke controles het ontstaan van kanker en de sterfte verlagen. Periodieke controles middels colonoscopie elke 1-2 jaar wordt geadviseerd vanaf 25-jarige leeftijd. Hoewel het kankerrisico bij Lynch syndroom patiënten sterk afhankelijk is van het betrokken gen, wordt alle patiënten momenteel dezelfde controles aangeboden. Daarom wordt in **hoofdstuk 9** de opbrengst van deze colonoscopie controles geëvalueerd voor *MLH1*, *MSH2*, *MSH6* en *PMS2* mutatiedragers.

Bij de meeste colonoscopieën werden geen adenomen (voorstadium van darmkanker) of darmkanker gevonden en er waren geen significante verschillen in het aantal adenomen dat werd gevonden tussen de verschillende patiëntengroepen. Echter het risico op gevorderde neoplasie (een gevorderd adenoom of darmkanker) liep voor *MSH6* mutatiedragers langzamer op dan voor de andere groepen. Op basis van deze langzamere progressie en het feit dat geen van de 121 *MSH6* mutatiedragers darmkanker ontwikkelden gedurende de studieperiode, behoeven *MSH6* mutatiedragers waarschijnlijk minder frequente controles dan mensen met een mutatie in *MLH1* of *MSH2*. Het interval tussen colonoscopieën zou daarom mogelijk verlengd kunnen worden tot drie jaar voor *MSH6* mutatiedragers. Vanwege het kleine aantal *PMS2* mutatiedragers konden voor deze groep geen sterke conclusies worden getrokken, maar gebaseerd op het lagere kankerrisico bij *PMS2* mutaties is minder frequente controle ook bij deze patiënten waarschijnlijk voldoende.

Ten slotte worden in **hoofdstuk 10** de resultaten van dit proefschrift besproken in relatie tot de huidige richtlijnen en de klinische praktijk.

PUBLICATIONS

A Goverde*, H Houlleberghs*, M Dekker, H Lantermans, MJ Bruno, FBL Hogervorst, ME van Leerdam, M Ruijs, MCW Spaander, A Wagner, RMW Hofstra, H te Riele (*equal contribution), Diagnosing Lynch syndrome: Identification of pathogenic *MLH1* and *MSH2* variants in clinical practice. *Manuscript in preparation*.

A Goverde, A Wagner, E Viskil, MJ Bruno, RMW Hofstra, MCW Spaander, Yield of Lynch syndrome surveillance for individual MMR genes. *Submitted for publication.*

A Goverde, A Wagner, MJ Bruno, RMW Hofstra, M Doukas, MM van der Weiden, HJ Dubbink, WNM Dinjens, MCW Spaander, Routine molecular analysis for Lynch syndrome among adenomas and colorectal cancer within a national screening program. *Accepted for publication in Gastroenterology.*

A Goverde, MCW Spaander, D Nieboer, AMW van den Ouweland, WNM Dinjens, HJ Dubbink, CJ Tops, SW ten Broeke, MJ Bruno, RMW Hofstra, EW Steyerberg, A Wagner, Evaluation of prediction models for Lynch syndrome: Updating the PREMM5 model to identify *PMS2* mutation carriers. *Fam Cancer. 2018 Jul;17(3):361-370*.

H Houlleberghs, **A Goverde***, J Lusseveld*, M Dekker, MJ Bruno, F Menko, AR Mensenkamp, MCW Spaander, A Wagner, RMW Hofstra, H te Riele (*equal contribution), Suspected Lynch syndrome associated *MSH6* variants: a functional assay to determine their pathogenicity. *PLoS Genet. 2017 May 22;13(5):e1006765*.

A Goverde, MCW Spaander, HC van Doorn, HJ Dubbink, AMW van den Ouweland, CM Tops, SG Kooi, J de Waard, RF Hoedemaeker, MJ Bruno, RMW Hofstra, EW de Bekker-Grob, WNM Dinjens, EW Steyerberg, A Wagner, Cost-effectiveness of screening for Lynch syndrome in endometrial cancer patients ≤70 years. *Gynecol Oncol. 2016 Dec;143(3): 453-459*.

A Goverde*, CHM Leenen*, EW de Bekker-Grob, A Wagner, MGF van Lier, MCW Spaander, MJ Bruno, CM Tops, AMW van den Ouweland, HJ Dubbink, EJ Kuipers, WNM Dinjens, ME van Leerdam, EW Steyerberg (*equal contribution), Cost-effectiveness of routine screening for Lynch syndrome in colorectal cancer patients up to 70 years of age. *Genet Med. 2016 Oct;18(10):966-73.*

Publications not in this thesis:

A Goverde, SE Korsse, A Wagner, ME van Leerdam, N Krak, J Stoker, HR van Buuren, RMW Hofstra, MJ Bruno, P Dewint, E Dekker, MCW Spaander, Small-bowel surveillance in patients with Peutz-Jeghers syndrome: Comparing Magnetic Resonance Enteroclysis and Double Balloon Enteroscopy. *J Clin Gastroenterol. 2017 Apr;51(4):e27-e33*.

WR Geurts-Giele, CHM Leenen, HJ Dubbink, IC Meijssen, E Post, HF Sleddens, EJ Kuipers, **A Goverde**, AMW van den Ouweland, MG van Lier, EW Steyerberg, ME van Leerdam, A Wagner, WNM Dinjens, Somatic aberrations of mismatch repair genes as a cause of microsatellite-unstable cancers. *J Pathol. 2014 Dec;234(4):548-59*.

PHD PORTFOLIO

Courses

2017	Update BROK (Basiscursus Regelgeving Klinisch Onderzoek)
2015	Genetics course
2015	Biomedical English writing and communication
2014	Introductory course on Statistics and survival analysis
2014	Research Integrity
2014	Biomedical English writing
2014	Research Management
2014	Course on Patient Oriented Research (CPO)
2013	Open Clinica
2013	BROK (Basiscursus Regelgeving Klinisch Onderzoek)
2013	Medical Statistics
2013	Molecular diagnostics
2013	Course on SPSS
2013	Courses by Medical Library

Seminars and workshops

2018	PhD day Erasmus MC
2013-2018	Research meetings department of Clinical Genetics
2013-2016	Journal club department of Gastroenterology and Hepatology
2014	PhD day department of Gastroenterology and Hepatology
2014	PhD day Erasmus University Rotterdam
2013	PhD day Erasmus MC

Oral presentations at (inter)national conferences

2018	Yield of Lynch syndrome surveillance for individual MMR genes. Digestive Disease Week, Washington DC, USA
2017	Routine molecular analysis for Lynch syndrome in patients with advanced adenoma or colorectal cancer within a national CRC screening program. InSiGHT meeting, Florence, Italy
2017	Routine molecular analysis for Lynch syndrome in patients with advanced adenoma or colorectal cancer within a national CRC screening program. Digestive Disease Week, Chicago, USA

2016 Evaluation of prediction models for Lynch syndrome: Updating the $PREMM_{1,2,6}$ model to identify PMS2 mutation carriers.
Assistenten Landelijk overleg Genetische counseling, Vereniging Klinische Genetica Nederland, Utrecht, the Netherlands

2016 Cost-effectiveness of routine screening for Lynch syndrome in endometrial cancer patients up to 70 years of age.
International Congress of Human Genetics, Kyoto, Japan

2016 Evaluation of prediction models for Lynch syndrome: Updating the $PREMM_{1,2,6}$ model to identify PMS2 mutation carriers.
International Congress of Human Genetics, Kyoto, Japan

2016 Chair concurrent oral session ICHG
International Congress of Human Genetics, Kyoto, Japan

2015 Cost-effectiveness of routine screening for Lynch syndrome in colorectal cancer patients up to 70 years of age. (poster pitch)
United European Gastroenterology Week, Barcelona, Spain

2015 Cost-effectiveness of routine screening for Lynch syndrome in colorectal cancer patients up to 70 years of age.
Conference Nederlandse Vereniging voor Gastro-Enterologie, Veldhoven, the Netherlands

2015 Small-bowel surveillance in patients with Peutz-Jeghers syndrome: Comparing Magnetic Resonance Enteroclysis and Double Balloon Enteroscopy.
Conference Nederlandse Vereniging voor Gastro-Enterologie, Veldhoven, the Netherlands

Poster presentations at (inter)national conferences

2018 Yield of Lynch syndrome surveillance for individual MMR genes.
Joint meeting UK and Dutch Clinical Genetics societies, Utrecht, the Netherlands

2017 Routine molecular analysis for Lynch syndrome in patients with advanced adenoma or colorectal cancer within a national CRC screening program.
United European Gastroenterology Week, Barcelona, Spain

2017 Routine molecular analysis for Lynch syndrome in patients with advanced adenoma or colorectal cancer within a national CRC screening program
European Society of Human Genetics, Copenhagen, Denmark

2016	Cost-effectiveness of routine screening for Lynch syndrome in endometrial cancer patients up to 70 years of age. Nederlandse Vereniging voor Humane Genetica, Amsterdam, the Netherlands
2016	Small-bowel surveillance in patients with Peutz-Jeghers syndrome: Comparing Magnetic Resonance Enteroclysis and Double Balloon Enteroscopy. Digestive Disease Week, San Diego, USA
2016	Evaluation of prediction models for Lynch syndrome: Updating the $PREMM_{1,2,6}$ model to identify PMS2 mutation carriers. Digestive Disease Week, San Diego, USA
2015	Cost-effectiveness of routine screening for Lynch syndrome in colorectal cancer patients up to 70 years of age. United European Gastroenterology Week, Barcelona, Spain
2015	Small-bowel surveillance in patients with Peutz-Jeghers syndrome: Comparing Magnetic Resonance Enteroclysis and Double Balloon Enteroscopy United European Gastroenterology Week, Barcelona, Spain
2015	Cost-effectiveness of routine screening for Lynch syndrome in endometrial cancer patients up to 70 years of age. American Society of Human Genetics, Baltimore, USA
2015	Cost-effectiveness of routine screening for Lynch syndrome in colorectal cancer patients up to 70 years of age. Digestive Disease Week, Washington, Verenigde Staten

Teaching

2017	Training Genetic Counseling, second year medical students
2017	Lecture Hereditary diseases in a nutshell, minor Clinical Genetics
2016	Tutor Introduction to clinical practice (KBP), first year medical students
2016	Training Dysmorfology examination, medical students
2015	Tutor first year medical students
2015	Training Genetic Counseling, second year medical students
2015	Assisting practicum Basic Introduction Course on SPSS, PhD students
2015	Lecture Lynch syndrome, second year medical students
2015	Assisting practicum Basic Introduction Course on SPSS, PhD students

Awards

2018	Certificate of Recognition Digestive Disease Week, Washington DC, USA
2017	Certificate of Recognition Digestive Disease Week, Chicago, USA
2015	Poster champ award and poster of excellence United European Gastroenterology Week, Barcelona, Spain

DANKWOORD

Een groot aantal mensen heeft bijgedragen aan de totstandkoming van dit proefschrift. Een aantal personen wil ik hier specifiek noemen.

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A

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Anne Goverde werd op 15 januari 1988 in Rotterdam geboren. Na het behalen van haar gymnasium diploma aan het Emmauscollege in Rotterdam, begon zij in 2005 met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam. Na het doorlopen van de co-schappen behaalde zij haar artsexamen in januari 2013. Aansluitend startte zij in 2013 als arts-onderzoeker bij de afdeling Klinische Genetica en de afdeling Maag-, Darm- en Leverziekten van het Erasmus MC Rotterdam, onder begeleiding van promotoren prof. dr. R.M.W. Hofstra en prof. dr. M.J. Bruno en copromotoren dr. A. Wagner en dr. M.C.W. Spaander. Dit proefschrift focust op de diagnostiek naar Lynch syndroom en de surveillance van deze patiënten. Vanaf 2016 werkt Anne als arts-assistent bij de afdeling Klinische Genetica van het Erasmus MC Rotterdam. Per 1 januari 2018 is zij hier in opleiding tot Klinisch Geneticus.