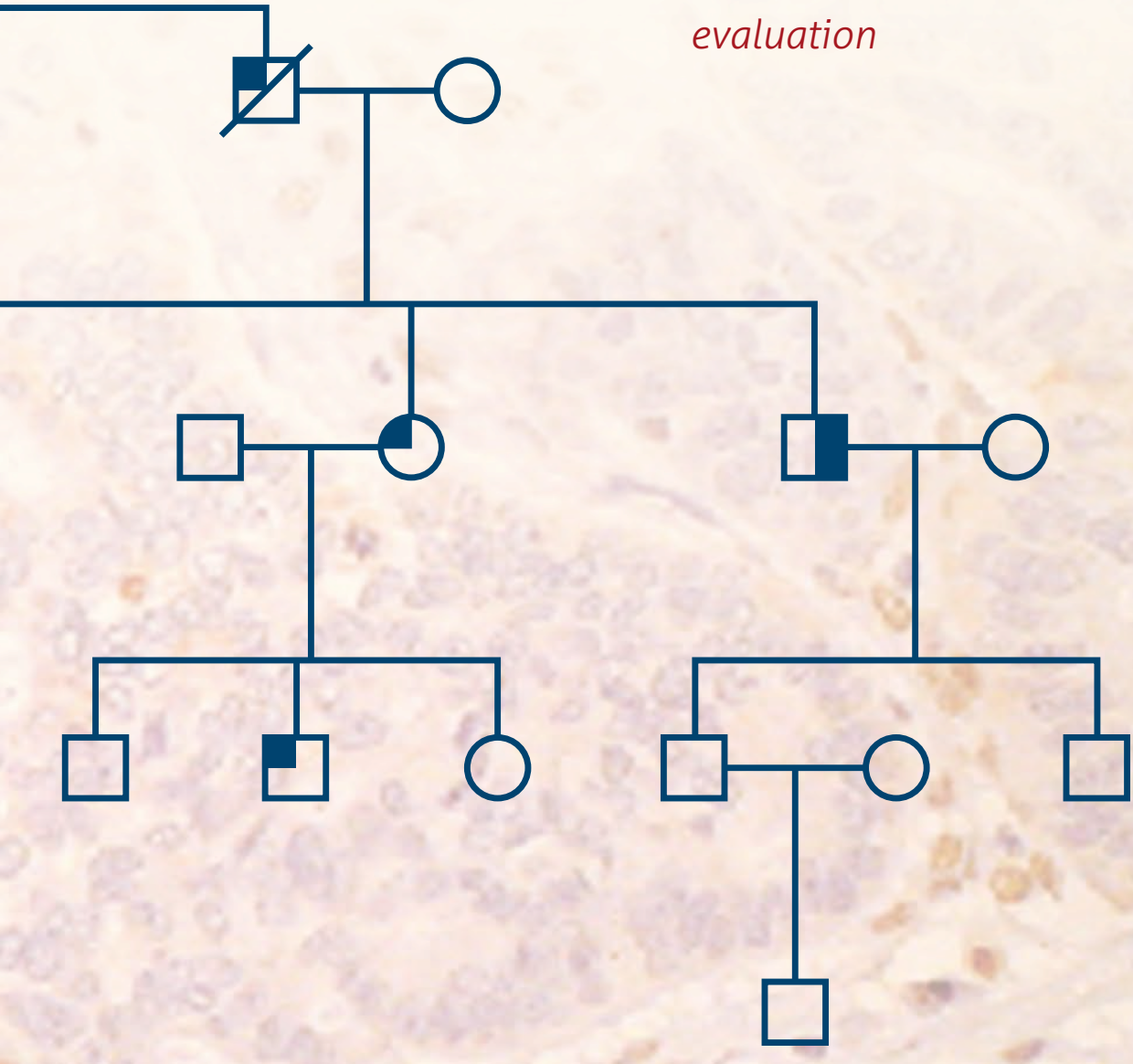
CELINE H.M. LEENEN

DIAGNOSTIC STRATEGIES FOR EARLY LYNCH SYNDROME DETECTION

from molecular testing to economic evaluation



Diagnostic Strategies for Early Lynch Syndrome Detection
From Molecular Testing to Economic Evaluation

Celine H.M. Leenen

ISBN: 978-94-6169-616-8

Layout and printing: Optima Grafische Communicatie, Rotterdam, the Netherlands
Cover: background picture of tumor tissue with absent MLH1 staining, from the Molecular Diagnostics laboratory, Department of Pathology, Erasmus University Medical Center Rotterdam.

Financial support for the printing of this thesis was kindly provided by: Nederlandse Vereniging voor Gastroenterologie; Departments of Public Health, Gastroenterology and Hepatology, Erasmus University Medical Center Rotterdam; Erasmus University Rotterdam.

Diagnostic Strategies for Early Lynch Syndrome Detection
From Molecular Testing to Economic Evaluation

Diagnostische strategieën voor vroegtijdige opsporing van het Lynch syndroom
Van moleculair onderzoek tot economische evaluatie

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
28 januari 2015 om 11.30 uur

door

Celine Helene Maria Leenen

geboren te Amsterdam

PROMOTIECOMMISSIE

Promotoren:	Prof.dr. E.J. Kuipers
	Prof. dr. E.W. Steyerberg
Overige leden:	Prof.dr. R.M.W. Hofstra
	Prof.dr. N. Hoogerbrugge
	Prof.dr. H.W. Tilanus
Copromotoren:	Dr. W.N.M. Dinjens
	Dr. M.E. van Leerdam
	Dr. A. Wagner

CONTENTS

Chapter 1

Introduction and outline of the thesis

Lynch syndrome (LS) is an autosomal dominant inherited syndrome that predisposes to multiple malignancies, in particular colorectal cancer (CRC) and endometrial cancer (EC). The lifetime risk of developing CRC for a LS mutation carrier is 25 to 70%, while women with LS carry a lifetime risk to develop EC of 13 to 65%, dependant on family history and the affected MMR gene.[1-12] In addition, LS carriers have an increased risk of up to 15% to develop other malignancies. These in particular include gastric, skin, ovarian and small bowel, as well as urinary tract cancers.[2, 13-16]

LS is caused by germline mutations in the mismatch repair (MMR) genes or deletion of the 3' region of the *TACSTD1* gene. In addition, LS can be caused rarely by germline hypermethylation of the promoter regions of the *MLH1* or *MSH2* gene.[17] Recognition of LS is of utmost importance in order to provide adequate counseling and targeted surveillance to individuals at risk. Colonoscopic surveillance has been proven to reduce CRC morbidity and mortality by 65-70%.[18-20] Furthermore, surveillance for EC by transvaginal ultrasound and tumormarker analysis may enable detection of premalignant lesions or EC at an early stage, however this requires further proof.[18, 21] Alternatively, prophylactic hysterectomy and bilateral salpingo-oophorectomy are offered to female LS mutation carriers when childbearing is complete.[22]

Since molecular and genetic testing is available for the identification of LS, dedicated treatment and surveillance can be offered to LS mutation carriers and their relatives who do carry a mutation. On the other hand, non-carriers can be relieved from the anxiety involved with the syndrome and burdensome surveillance programs.

Genetic background

LS is an autosomal dominant genetic disorder. It is caused by germline mutations in one of the human MMR genes: *MLH1*, *MSH2, MSH6* and *PMS2* or deletion of the 3' region of the *TACSTD1* gene.[17, 23-27] The protein products of the MMR genes are involved in detection and correction of mismatches and small insertion or deletion loops that arise during DNA replication, but also recognize exogenous mutations and are involved in transcription-coupled repair.[26, 28, 29] Most relevant for LS are the MutS homologue family and MutL homologue family of proteins. Two different MutS-related heterodimeric complexes are responsible for mismatch recognition: MSH2-MSH3 and MSH2-MSH6. *MSH2* is an obligatory partner in this system. MutS heterodimers signal the site of mispairing. After mismatch binding, a heterodimeric complex of MutL-related proteins, MLH1-PMS1, MLH1-PMS2 or MLH1-MLH3, is recruited and this initiates the actual mismatch repair. MLH1 is the obligate partner in this system that excises the error, followed by resynthesis. However, additional proteins are necessary to complete the repair process. Loss of DNA MMR activity greatly accelerates the rate of accumulation of mutations in genes involved in regulation of growth, regulation of apoptosis, or DNA repair.[28]

Recently, germline deletion of the 3' end of the *TACSTD1* gene, also referred to as *EPCAM* deletion, has been shown to cause LS as well.[30] The underlying mechanism is *MSH2* promoter methylation, causing silencing of *MSH2* by transcriptional read-through.[31] A recent cohort study showed that these *EPCAM* deletion carriers have a 75% cumulative risk of CRC before the age of 70 years, which does not differ significantly from that of *MSH2* mutation carriers. On the contrary, the risk of EC in *EPCAM* deletion carriers is significant lower than in *MSH2* mutation carriers.[5]

Biallelic mutations in MMR genes

On rare occasions when both parents carry an MMR gene mutation, their offspring can inherit biallelic mutations in MMR genes. This leads to a childhood cancer syndrome, referred to as constitutional mismatch repair-deficiency (CMMR-D). CMMR-D is predominantly characterized by haematological malignancies, brain tumors and gastrointestinal tumors in early childhood. Carriers of biallelic MMR gene mutations often show signs of neurofibromatosis type 1, mainly café au lait spots. More than 100 cases of children with CMMR-D have been reported in the literature.[32-37]

Microsatellite instability

The hallmark of mismatch repair deficiency is microsatellite instability (MSI). LS-MSI associated tumors are probably best recognized by the presence of this phenomenon. Microsatellites are simple repetitive DNA sequences that are found throughout the genome. In MMR deficient tumor cells, the number of nucleotide repeat units of microsatellites can deviate from the corresponding normal nucleotide repeat units. In most instances, the mutations at the microsatellite sequences result in a deletion of one or more of the mononucleotide or dinucleotide elements, resulting in a shorter sequence. Approximately 95% of all LS associated tumors exhibit MSI.[38-40] However, 15% of MSI is caused by epigenetic mechanisms. A common mechanism for loss of DNA MMR activity in CRC is epigenetic inactivation of *MLH1* gene by methylation of the promoter.[41] Determination of *MLH1* promoter hypermethylation in an MSI-high tumor with absent MLH1 staining can distinguish between sporadic MSI-high CRC and LS associated CRC.[42, 43] In the subgroup of MSI-high tumors, the acquired silencing of *MLH1* usually occurs in older patients and females.[44] Furthermore *BRAF* V600E mutation status has also been used to distinguish LS-associated from sporadic MSI-high tumors. The *BRAF* V600E mutation is associated with sporadic *MLH1* inactivation secondary to promoter hypermethylation.[45-49] Occasionally, *BRAF* mutations have been detected in LS patients.[50] Compared to determination of hypermethylation of the *MLH1* gene promoter, *BRAF* mutation analysis is less sensitive to detect sporadic MSI tumors.[49]

MSI testing

The National Cancer Institute (NCI) recommended a panel of five microsatellite sequences to assess instability including two mononucleotide (BAT25 and BAT26) and three dinucleotide (D2S123, D5S346, D17S250) repeat sequences.[39] For this panel DNA from formalin-fixed, paraffin-embedded tumor tissue and normal tissue of the same patients are compared. Preferable, tumor tissue from the youngest affected patient is used. Tumors are scored as MSI-high (MSI-H) if at least two of the five markers show instability, MSI-low if one marker shows instability and microsatellite stable (MSS) if none of the markers show instability. Biological, MSI-high tumors behave different than MSI-low and MSS tumors, and most studies categorize MSI-low as MSS. However, *MSH6* associated tumors may lack the MSI-H phenotype.[51] More recently, a different pentaplex panel comprising five mononucleotide repeats (BAT 25, BAT 26, NR 21, NR 24 and MONO 27) has been shown to be superior to the NCI panel for the detection ofMSI-H tumors, (sensitivity of 95.8% for the pentaplex panel vs 76.5% for the NCI panel and specificity of respectively 98.7% vs 97.2% for the detection of the absence of MMR protein expression).[52] A Dutch study showed that mononucleotide instability is a very early event in the development of MSI-H tumors with MMR truncating mutations compared to MMR missense mutations and non-carriers. Furthermore, in LS associated tumors, mononucleotide instability preceded dinucleotide instability.[53] These findings favor the use of the mononucleotide pentaplex panel.

Immunohistochemistry testing

Immunohistochemistry (IHC) on the tumor tissue can be performed to determine the affected MMR protein. Specific antibodies are used to check for staining of the MLH1, MSH2, MSH6 and PMS2 proteins. This will give an indication which MMR gene is most eligible for DNA analyses. In case of an *MLH1* germline mutation, there is no expression of MLH1 protein in tumor cell nuclei, illustrating the pathological nature of the mutation. MLH1 is the stabilizing protein partner of PMS2. The inactivation of MLH1 usually also leads to destabilization of the PMS2 protein and thus additional absent staining of PMS2. The same phenomenon is seen in case of a *MSH2* germline mutation, leading to absent protein staining of both MSH2 and MSH6 in tumor cell nuclei. In case of a *PMS2* or *MSH6* germline mutation, isolated loss of PMS2 or MSH6 protein is generally seen by IHC staining.[28, 54] In case there is no MLH1 expression in tumor cells, the methylation status of the *MLH1* promoter should be determined.[55]

IHC is especially indicative for MMR mutations that result in truncation of the protein, such as nonsense, frameshift, splicesite mutations, and large genomic rearrangements. In case of missense mutations, IHC is not always diagnostic. The missense can lead to loss of the enzymatic activity, but the expression of the protein can be preserved, leading to a false negative result.[28, 54] IHC has a high sensitivity in predicting mutations in *MSH2*

(92%), *MSH6* (90%) and *MLH1* (71%; when including both MLH1 and PMS2 staining).[54] Discordant results do occur in MSI and IHC analysis.[26, 56]

The clinical use of MSI status as predictive marker in CRC patients

Because MSI is the hallmark of LS associated tumors, MSI testing is used for the identification of patients at high risk for LS. MSI tumors tend to have a better prognosis.[39] In a systematic review, including 7642 CRC cases (1277 MSI-H) overall survival was significantly better in the MSI group (HR 0.65 :95% CI 0.59-0.71).[57] However, others showed that only *TP53* mutation or DNA ploidy status and not MSI status retained statistical significant associations with disease free survival in the multivariate analyses.[58, 59] A study from Norway showed that neither MSI status nor DNA ploidy predicted distant metastasis, disease free survival and disease-specific survival.[60] More prospective data are necessary to determine the exact prognostic value of MSI status. With regard to treatment, several studies have shown that MSI-H tumors do not benefit from 5-fluorouracil (5-FU) adjuvant chemotherapy.[61-63] Recently, Sargent *et al* evaluated MMR status as predictor of adjuvant chemotherapy benefit in 507 patients with stage II and III colon cancer from five RCTs. No benefit in disease free survival from 5-FU was observed in patients with MSI (HR: 1.39; 95 % CI 0.46–4.15; p = 0.56), compared to patients with MSS tumors (HR: 0.67; 95 % CI 0.48–0.93; p = 0.02).

Germline mutation analysis

Germline mutation analyses should be performed on the selected group of patients with MSI-H tumors and/ or loss of protein expression by IHC without MMR gene promoter hypermethylation. It is obvious that analysis should start first with the gene that is indicated by the loss of protein expression. Mutation analyses techniques for *MLH1*, *MSH2* and *MSH6* are commercially available. The genetic diagnosis of a *PMS2* germline mutation is difficult, because of the large number of pseudogenes which confound diagnostic DNA sequencing.[64, 65] The technical details of germline mutation analyses will not be discussed in this introduction. Hundreds of distinct genomic variants have been identified throughout the four MMR genes. A large number of these alterations are missense variants, intronic variants and synonymous changes. The determination of the pathological significance of these variants is difficult.[54] An international database is available with the known MMR gene variants (www.med.mun.ca/MMRvariants/). When a family is tested negative for any of the known germline mutations, it is possible that a present germline mutation escaped detection by the used method. From previous cohort studies it is known that by routine molecular testing for LS, a substantial part of suspected LS (sLS) patients test negative for germline MMR gene mutations.[66-69] In these patients a germline mutation could have been missed or somatic mutations in MMR genes occur.[68, 69]

Identification of Lynch syndrome

Clinical criteria

Apart from several clinical and pathological features, LS lacks clear phenotypic characteristics. This makes identification of LS challenging. Several clinical features can help to identify LS. LS presents with an earlier mean age of onset of CRC (mean approximately 45 years in *MLH1* and *MSH2* carriers and approximately 55 years in *MSH6* carriers) compared with an average age of onset of CRC in the general population between 60-70 years.[13, 70, 71] There is a predominance of right-sided colon cancers, approximately 70% of all cancers are proximal to the splenic flexure.[40] Synchronous and metachronous CRC do occur more frequent.[72] Besides CRC there is an excessive risk, mainly for EC, but also other extracolonic cancers do occur within the pedigree including ovary, stomach, small bowel, hepatobiliary tract, pancreas, upper uro-epithelial tract, brain and skin tumors.[2, 13, 14, 73] Specific pathology features are associated with LS related CRC. These features include the presence of tumor infiltrating lymphocytes, a Crohn's-like lymphocytic

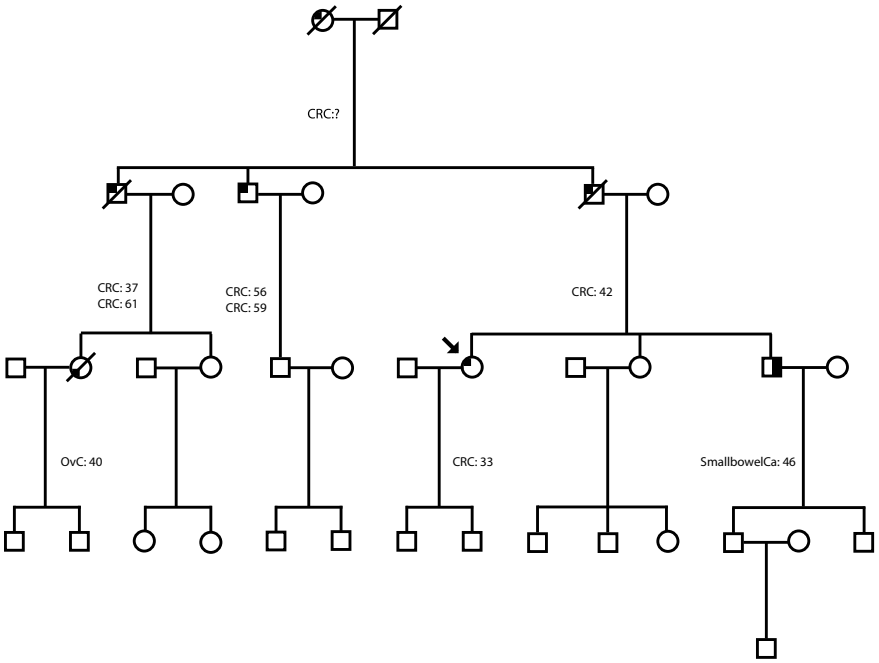


Figure 1. Example of a Lynch syndrome family fulfilling the Amsterdam criteria II and the revised Bethesda guidelines. ⟶ Arrow indicating initial counselee; □ male; ○ female; ◪ CRC, colorectal cancer; ◨ small bowel cancer; ⊘ OvC, ovarian cancer; **/** deceased

reaction, mucinous or signet ring differentiation, and a medullary or undifferentiated and solid growth pattern.[74-77] LS patients develop adenomas at the same rate as subjects without the mutation, but the onset of adenoma is earlier and there seems to be an accelerated transition to carcinoma.[75] For the identification of LS families based on clinical criteria, a thorough family history is essential. A family history should include all cancers and age of onset in first and second-degree relatives (an example pedigree is shown in Figure 1).

Preferentially, all cancers should be proven either by pathology report or medical record. However, the use of family history of cancer as a predictive marker is insensitive and can be difficult to assess accurately in a clinical setting.[78, 79] In 1990 a set of selection criteria for families with LS (previously referred to as HNPCC) was established in order to provide a basis for uniformity in collaborative studies, known as the Amsterdam Criteria I.[80] Because the Amsterdam Criteria I did not take into account the extracolonic cancers that are part of the syndrome, the Amsterdam Criteria II were published in 1999.[81] These criteria are only based on clinical data. Furthermore, the criteria aimed to provide a common nomenclature for the selection of families for studies and comparison of study results.[81] The Amsterdam criteria II do have a limited sensitivity of about 40% and specificity around 90% when also *MSH6* mutation carriers are taken into account.[82, 83] In order to optimize the identification of LS patients, the Bethesda guidelines have been developed in 1997 to select a group of cancer patients in whom MSI analyses should be performed. The revised Bethesda guidelines, published in 2004 are currently international being used to select mainly CRC patients for further MSI analyses (Table 1[84]).
When *MLH1*, *MSH2* and *MSH6* mutation carriers are taken into account, the sensitivity of the revised Bethesda guidelines is around 77-95% with a specificity around 30-60%.[76, 82, 83, 85] The revised Bethesda guidelines are not optimal and have been criticized for being too complex in clinical practice. Furthermore, there is a marked underutilization by adapting the Bethesda guidelines, leading to underdiagnosis of LS in CRC patients.[55]

Table 1. The revised Bethesda Guidelines for testing colorectal cancer for MSI.[84]

One of the following criteria need to be met to fulfill the guidelines:
1. Diagnosed with colorectal cancer before the age of 50 years;
2. Synchronous or metachronous colorectal or other LS-related tumors (which include stomach, bladder, ureter, renal pelvis, biliary tract, brain (glioblastoma), sebaceous gland adenomas, keratoacanthomas and carcinoma of the small bowel), regardless of age;
3. Colorectal cancer with a high-microsatellite instability morphology* that was diagnosed before the age of 60 years;
4. Colorectal cancer with one or more first-degree relatives with colorectal cancer or other LS-related tumors. One of the cancers must have been diagnosed before the age of 50 years;
5. Colorectal cancer with two or more relatives with colorectal cancer or other LS-related tumors, regardless of age.

* Presence of tumor-infiltrating lymphocytes, Crohn's like lymphocytic reaction, mucinous or signet-ring differentiation, or medullary growth pattern.
LS, Lynch syndrome; MSI, microsatellite instability

Prediction models

In addition, several prediction models have been developed to predict the likelihood of carrying a germline mutation.[27, 82, 85, 86] Mutation prediction models predict the probability of a mutation using logistic regression or Bayesian methods. These models use information based on personal and family history as input to predict the probability of mutation carriership. A major advantage of prediction models is that these models give a quantitative estimation of the likelihood of mutation carrier ship instead of a bivariate (yes/no) assessment as provided by the clinical diagnostic criteria. Furthermore, these models can be used for individuals for whom tumor samples are not available and for individuals in whom germline testing finds no mutation. Mutation prediction models are thus potentially useful in clinical practice to optimize the identification of LS. External validation of the prediction model MMRpro on clinic-based families showed a good performance. This model was more sensitive and specific than the existing clinical guidelines (Amsterdam criteria and revised Bethesda guidelines) and the Leiden model for identifying individuals who may benefit from MMR germline testing.[85] Another study, validating the $PREMM_{1,2}$ model in a population based cohort identified all *MLH1* and *MSH2* mutation carriers, however the number of identified mutation carriers (N=8) was very low, limiting the generalizability of this study.[87] A Dutch external validation study demonstrated that the $PREMM_{1,2}$ model and Edinburgh model had the best performance in clinic-based families compared to the Leiden, UK-Ams and UK-alt models, showing the highest discriminative ability and the best calibration.[88] More recently, Green *et al* evaluated the Leiden, MMRpro, $PREMM_{1,2,6}$ and the MMRpredict model in CRC patients younger than 75 years of age, and demonstrated the best performance for selecting patients requiring additional workup for determining LS using the MMRpredict model.[89] To enable easy calculation of the predicted probabilities in clinical practice, the MMRpro (http://bcb.dfci.harvard.edu/bayesmendel/mmrpro.php), MMRpredict (http://hnpccpredict.hgu.mrc.ac.uk/) and $PREMM_{1,2,6}$ (http://www.dfci.org/premm) models are available as a free web-based model, as shown in Table 2.[90] Despite the good performance of the prediction models, these models are not yet widely used in clinical setting.

Routine molecular screening

In view of the above described disadvantages of current clinical guidelines, routine molecular screening by MSI testing and IHC in CRC and EC patients under the age of 50 years, with a central role of the pathologist, was introduced in the Netherlands (MIPA: MSI-testing-Indicated-by-a-Pathologist; Table 3[78]). The MIPA criteria recommend MSI analysis in patients newly diagnosed with CRC before age 50, or before age 70 in patients diagnosed with two LS-associated cancers. A central role for the pathologist by direct selection of patients by tumor tissue simplifies the diagnostic procedure compared to the revised Bethesda guidelines. However, this strategy predominantly fails to

Table 2. Overview of prediction models for the identification of mismatch repair gene mutation carriers (adapted from Kastrinos *et al*[90]).

Model	Ascertainment	Outcome	Method	Molecular testing included	Predictors	Link
Dutch model[27]	Clinic-based	Point mutations in *MLH1*, *MSH2*	Logistic regression analysis	No	Young age at CRC diagnosis in the family; fulfillment of Amsterdam criteria; presence of endometrial cancer in the family	http://www5.utsouthwestern.edu
Amsterdam – plus[86]	Derivation and validation: clinic-based	Point mutations in *MLH1*, *MSH2*, *MSH6*	Logistic regression analysis	No	Fulfillment of Amsterdam criteria; number of relatives with CRC and endometrial cancer; number of relatives with multiple CRC or endometrial cancer; mean age at diagnosis of CRC and endometrial cancer; number of relatives with > 5 colonic adenomas	N/A
MMRpredict[82]	Derivation: population-based CRC patients < 55 years Validation: population-based CRC patients < 45 years	Overall estimate *MLH1*, *MSH2*, *MSH6*	2 stage: Multivariate logistic regression analysis. MSI/ IHC data to refine prediction	Yes	Proband: age, gender, tumor's location, multiple tumors Family: CRC age (dichotomized at 50 years), endometrial cancer in 1st degree relative)	http://hnpccpredict.hgu.mrc.ac.uk/
MMRpro[85]	Derivation: population and clinic-based Validation: clinic-based	Gene-specific estimate *MLH1*, *MSH2*, *MSH6*	Mendelian and Bayesian analysis	Yes	Proband and family: CRC and endometrial cancer status, age at diagnosis, relation to the proband, current age or at last follow- up, MSI/IHC, germline testing result	http://bcb.dfci.harvard.edu/bayesmendel/mmrpro.php
$PREMM_{1,2,6}$[152]	Derivation: clinic-based Validation: population and clinic-based	Gene-specific estimate *MLH1*, *MSH2*, *MSH6*	Logistic regression analysis	No	Proband: age, gender, number of CRC, other Lynch-associated tumors Family: age and number of 1st and 2nd degree relatives with CRC and other Lynch-associated tumors	http://www.dfci.org/premm

CRC, colorectal cancer; IHC, immunohistochemistry; MSI, microsatellite instability; N/A, not available.

Table 3. Dutch guidelines for MSI-testing (www.oncoline.nl).

Adapted from: MIPA[78], January 2008
The pathologist is advised to request MSI-testing (and immunohistochemistry of the MMR proteins in the following patients:
1. CRC or EC before the age of 50 years
2. A second CRC befor the age of 70 years
3. CRC before the age of 70 years AND another synchronous or previous LS-associated tumor*

*colorectal, endometrial ovarian, gastric, small-bowel, pancreas, hepatobilliary tract, renal pelvis or ureter cancer and brain tumors, sebaceaous gland adenomas and keratoacanthomas.
CRC, colorectal cancer; EC, endometrial cancer; LS, Lynch syndrome

identify *MSH6* and *PMS2* mutation carriers, since the mean age of CRC diagnosis in these subjects is above the age of 50 years.[9, 70] Several large international cohort studies have been conducted to evaluate the yield of molecular screening for LS among unselected CRC and EC patients.[66,91] Hampel *et al* included a total of 1566 unselected CRC and EC patients. In a total of 44 probands (2.8%) and 109 relatives an MMR mutation was detected as part of the study.[14, 66, 92] When applying the revised Bethesda guidelines in the study cohort, it would lead to only 72% LS detection. In a large Spanish prospective cohort (EPICOLON) by Pérez-Carbonell *et al* 2093 CRC patients were studied.[91] In tumor tissue of the included unselected CRC patients MSI and IHC testing was done.[91] In tumor tissue of 180 CRC patients (8.6%) MSI and/ or loss of expression of the MMR proteins was found. In fourteen CRC patients an MMR germline mutation was confirmed (0.7%). Of the detected LS carriers, twelve CRC patients fulfilled the revised Bethesda guidelines (86%) in concordance with Hampel *et al*.[66] These data suggest that if the revised Bethesda guidelines are used in clinical practice, still an important percentage of LS carriers would be missed.

Furthermore, cost-effectiveness for LS screening was evaluated by several studies using Markov models. Recently, Sie *et al* evaluated expansion of the age limit for LS screening to CRC patients ≤ 70 years of age and this resulted in an ICER of $44,000/ LYG (€32,381/LYG).[93] This finding was in concordance with other cost evaluations.[78, 94-96] Ladabaum *et al* included also the use of prediction models in a large cost-effectiveness study using a Markov model.[93] The combination of IHC with *BRAF* testing was found to be the preferred strategy according to their model. Number of tested relatives of mutation carriers appeared to be an important determinant in analyses. Overall, the model based cost-effectiveness studies suggest that screening for LS could be cost-effective. However, cost-effectiveness analysis based on real life prospective data is lacking.

Family communication

Despite the potential benefits of genetic testing for LS as previously described, a Dutch study by Ramsoekh *et al* on the interest in genetic testing for hereditary colorectal can-

cer syndromes showed that almost half of the subjects in this cohort of family members at risk did not opt for genetic testing for LS at a median follow-up time after identification of the family specific mutation of 82 months, ranging 10-140 months.[97] Known reasons for refraining from genetic testing include problems with health insurance and mortgage.[98, 99]

In most European countries the communication regarding presence of an MMR gene mutation within a family occurs by means of the family-mediated approach. When a pathogenic mutation is detected, the LS mutation carrier or counselee is asked to inform all at risk relatives. During the counseling process, communication strategies to inform relatives are discussed with the counselee, including a letter to inform relatives. This approach implies that family members are responsible to inform their relatives on the diagnosis of LS and the possibility of genetic testing. Currently, literature on experiences with the family mediated approach is scarce.[99] A previous qualitative study in the Netherlands among 30 individuals from LS families showed that motivation to disclose seemed to increase if there were more cancer cases in the family. Disrupted family relations were found to be an important reason for non-disclosure. The way family members communicate about LS may also influence whether or not at-risk family members decide to opt for genetic testing.[100, 101] Knowledge on the challenges with regards to informing family members may help to improve counseling procedures and uptake of genetic testing.

AIMS AND OUTLINE OF THE THESIS

In this thesis various aspects of diagnostic strategies for LS were evaluated. The aim of this thesis was to evaluate whether routine molecular testing for LS in CRC and EC patients older than 50 years of age can contribute to early LS detection. In addition, cost-effectiveness and prediction models were evaluated. Furthermore, we aimed to investigate barriers to genetic testing and family communication.

The first part of this thesis focuses on challenges and pitfalls in tumor tissue analyses for LS and the more rare CMMR-D. The second and main part of this thesis consists of population and clinical based studies on LS diagnostic strategies. The papers in this thesis are mainly derived from a prospective multicenter population based study named LIMO (Lynch syndroom Immunohistochemisch en MSI Onderzoek). Clinical data are derived from the prospective cohort of LS families at the department of Clinical Genetics, Erasmus MC.

In **Chapter 2** of the thesis we focus on the molecular diagnostics in a CMMR-D family with biallelic *PMS2* mutations from our clinical research cohort. In **Chapter 3** we further investigate somatic MMR gene aberrations in microsatellite-unstable CRC and EC of suspected LS patients negative for germline MMR gene mutations. It is important to evaluate whether somatic mutations may be the cause of the MSI-high tumor, since this may relieve family members from the intensive surveillance program that is currently advised to suspected LS carriers.

Early detection of LS remains challenging and the optimal LS diagnostic strategy is still under debate. We evaluated routine molecular testing in tumor tissue of CRC and EC patients up to the age of 70 years and in advanced adenomas of patients until 45 years of age in the large population based LIMO study (**Chapter 4 and 5**). Cost–effectiveness of routine molecular testing for LS in CRC patients up to 70 years of age is evaluated in **Chapter 6**. Another promising strategy to detect LS families based on familial risk are LS prediction models. Since validation of LS prediction models was lacking, an international validation study of prediction models for LS was conducted, including MMRpro, MMRpredict, and $PREMM_{1,2,6}$ (**Chapter 7**).

In **Chapter 8** we performed a cross-sectional survey among individuals with a personal or family history of LS to explore communication about a familial mutation and barriers to genetic testing.

Finally, **Chapter 9** presents an overview and general discussion of this thesis.

Part I:

Challenges and pitfalls in molecular analyses

Chapter 2

Pitfalls in molecular analysis for mismatch repair deficiency in a family with biallelic *PMS2* germline mutations

Celine H.M. Leenen, Willemina R.R. Geurts-Giele, Hendrikus J. Dubbink, Roel E. Reddingius, Ans M. van den Ouweland, Carli M.J. Tops, Heleen M. van de Klift, Ernst J. Kuipers, Monique E. van Leerdam, Winand N.M. Dinjens, Anja Wagner

Clinical Genetics 2011;80(6):558-65

ABSTRACT

Heterozygous germline mutations in the mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* cause Lynch syndrome. Biallelic mutations in the MMR genes are associated with a childhood cancer syndrome referred to as constitutional mismath repair-deficiency (CMMR-D). This is predominantly characterized by hematological malignancies and tumors of bowel and brain, often associated with signs of neurofibromatosis type 1 (NF1). Diagnostic strategies for selection of patients for MMR gene analysis include analysis of microsatellite instability (MSI) and immunohistochemical analysis (IHC) of MMR proteins in tumor tissue.

We report the clinical characterization and molecular analyses of tumor specimens from a family with biallelic *PMS2* germline mutations. This illustrates pitfalls of present molecular screening strategies. Tumor tissues of five family members were analyzed for MSI and IHC. MSI was observed in only one of the analyzed tissues. However, IHC of brain tumor tissue of the index patient and his sister showed absence of PMS2 expression, and germline mutation analyses showed biallelic mutations in *PMS2*: p.Ser46Ile and p.Pro246fs. The same heterozygous mutations were confirmed in the father and mother, respectively.

These data support the conclusion that in case of a clinical phenotype of CMMR-D, it is advisable to routinely combine MSI analysis with IHC for expression of MMR proteins. With inconclusive or conflicting results, germline mutation analysis of the MMR genes should be considered after thorough counseling of the patients and/or their relatives.

INTRODUCTION

Heterozygous germline mutations in mismatch repair (MMR) genes *MLH1*, *MSH2*, *MSH6* and *PMS2* cause Lynch syndrome. [23-25, 102, 103] Carriers of heterozygous MMR gene mutations are at high-risk for developing colorectal carcinomas (CRC) and extracolonic neoplasias such as endometrial, small bowel, ureter, renal pelvis, stomach, ovarian, and brain tumors. In Lynch syndrome carriers, these malignancies usually develop during the 4th and 5th decade of life. Biallelic mutations in MMR genes lead to a childhood cancer syndrome. This is predominantly characterized by haematological malignancies, brain tumors and gastrointestinal tumors in early childhood. Carriers of biallelic MMR gene mutations often show signs of neurofibromatosis type 1 (NF1), mainly café au lait (CAL) spots. This childhood cancer syndrome is often referred to as constitutional mismatch repair-deficiency (CMMR-D). To our knowledge, a total of 107 cases of children with CMMR-D have been reported in the literature.[32-37]

Diagnostic strategies for fast selection of patients with an MMR gene defect suspected for Lynch syndrome include analysis of microsatellite instability (MSI) and immunohistochemical (IHC) analysis of tumor tissue for expression of MMR proteins.[39, 104, 105] However, the sensitivity of molecular tests in tumor tissue of patients with CMMR-D is unclear. MSI and absent MMR protein staining have been described in gastrointestinal tumors of patients with CMMR-D.[35] In contrast, tumor tissue of most reported CMMR-D patients with brain tumors did not show MSI.[34, 106] Here, we report a family with childhood brain tumors and early onset CRC with biallelic germline mutations in the *PMS2* gene that underscores pitfalls of the present molecular screening strategy.

CASE REPORT

Family data

At age 7 the index patient was diagnosed with an anaplastic glial brain tumor (Figure 1: pedigree, individual IV.2). His older sister (individual IV.1) had died from a primitive neuroectodermal brain tumor (PNET) at 4 years of age. Both children had multiple large CAL spots (Figure 2) and the index patient showed freckling. The younger sister of the index patient (individual IV.3) showed one CAL spot. Both non-consanguineous parents were from Dutch origin and showed no signs of NFI. At the time of counseling the family history of both parents was not suggestive for Lynch syndrome. One maternal uncle had been diagnosed with CRC (individual II.5) at age 62. The parents of the index patient declined endoscopic screening. However, within two years after the diagnosis of the brain tumor of our index patient, father was diagnosed with CRC at age 43 (pT4N2M1, Dukes stage D). The paternal mother (individual II.2) was diagnosed with CRC during

the same period of time at age 84. The mother of our index patient then underwent surveillance colonoscopy and one adenoma with low grade dysplasia was removed.

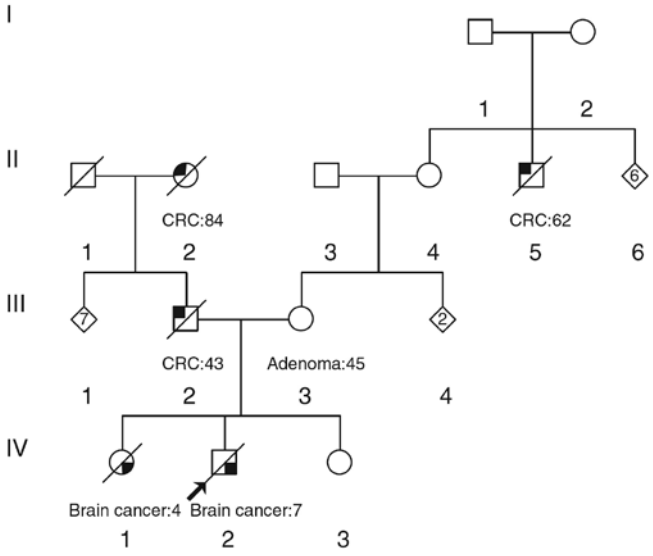


Figure 1. Pedigree of the reported family. Patient details on malignancy, adenomas and age of diagnosis in years are given. ⟶ index patient; □ male; ○ female; ◇ males and females; ◧ CRC, colorectal cancer; ◪ brain cancer; **/** deceased.

MSI and IHC analyses

Tissues of five family members including CRC tissues from the father and his mother, the colonic adenoma from the mother and brain tumor specimens from the index case and his sister were analyzed for MSI and IHC aberrations (Table 1). MSI analysis was performed on DNA retrieved from paraffin-embedded tumor tissues, using five mononucleotide repeat MSI markers (Promega pentaplex) as previously described.[55, 106] As controles, normal leukocyte DNA from the index patient, DNA from paraffin-embedded normal tissue from the father (III.2), DNA from paraffin-embedded normal tissue from the grandmother (II.2), and unrelated normal DNA were used. The MSI marker profiles of all these normal DNA samples (3 family members, one unrelated normal DNA) were identical, demonstrating the absence of MSI in normal tissues. IHC analysis was performed for four mismatch repair proteins: MLH1, MSH2, MSH6 and PMS2, according to the standard procedure.[55] The brain tumor of the index patient showed an MSI pattern with additional fragments of increased size of markers NR-21 and BAT-26. Surprisingly, microsatellite stability (MSS), was observed in the brain tumor of the sister of the index patient (Figure 3.a). IHC analysis of brain tumor tissues from both children showed

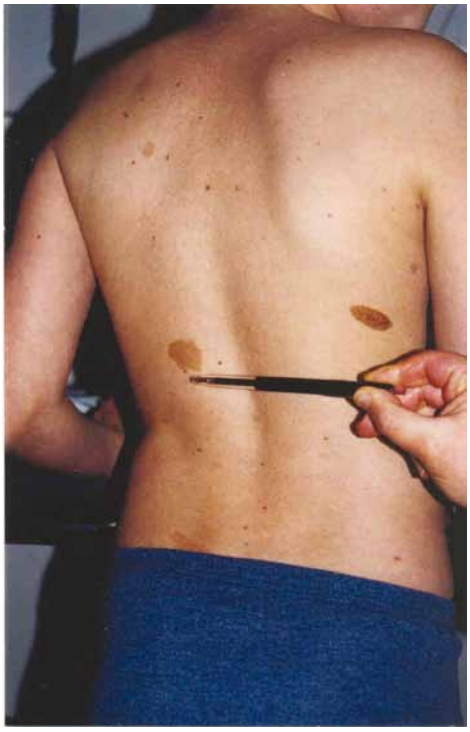


Figure 2. Café-au-lait spots of the index patient.

Table 1. Summary of results of the molecular and immunohistochemical analyses of tissues from the studied family.

Case	Malignancy	Age at diagnosis (years)	Skin lesions	*NF1* gene mutation	Analysis of MSI	IHC	LOH analysis *PMS2*	*PMS2* gene mutation
II.2	Adenocarcinoma of the rectum	84	ND	ND	MSS	Normal	ND	None
III.2	Adenocarcinoma of the transverse colon	43	None	ND	MSS	Normal	no LOH	Heterozygous p.Ser46Ile
III.3	One adenoma (low grade dysplasia)	45	None	ND	MSS	Normal	ND	Heterozygous p.Pro246fs
IV.1	PNET	4	CAL spots > 6, haemangioma leg	ND	MSS	PMS2 absent	ND	Compound heterozygous Pro246fs, p.Ser46Ile
IV.2	Anaplastic glial brain tumor	7	CAL spots > 6, axillary melanotic freckling	None	MSI-H	PMS2 absent	ND	Compound heterozygous Pro246fs, p.Ser46Ile

CAL, café au lait; IHC, immunohistochemistry of MLH1, MSH2, MSH6 and PMS2; LOH, loss of heterozygosity analysis by sequencing; ND, not determined; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSS, microsatellite stable; PNET, primitive neuroectodermal brain tumor.

absence of PMS2 expression in the tumor and normal cells. Tumor specimens from all other family members were MSS and showed normal expression of the MMR proteins in the tumor and normal tissue.

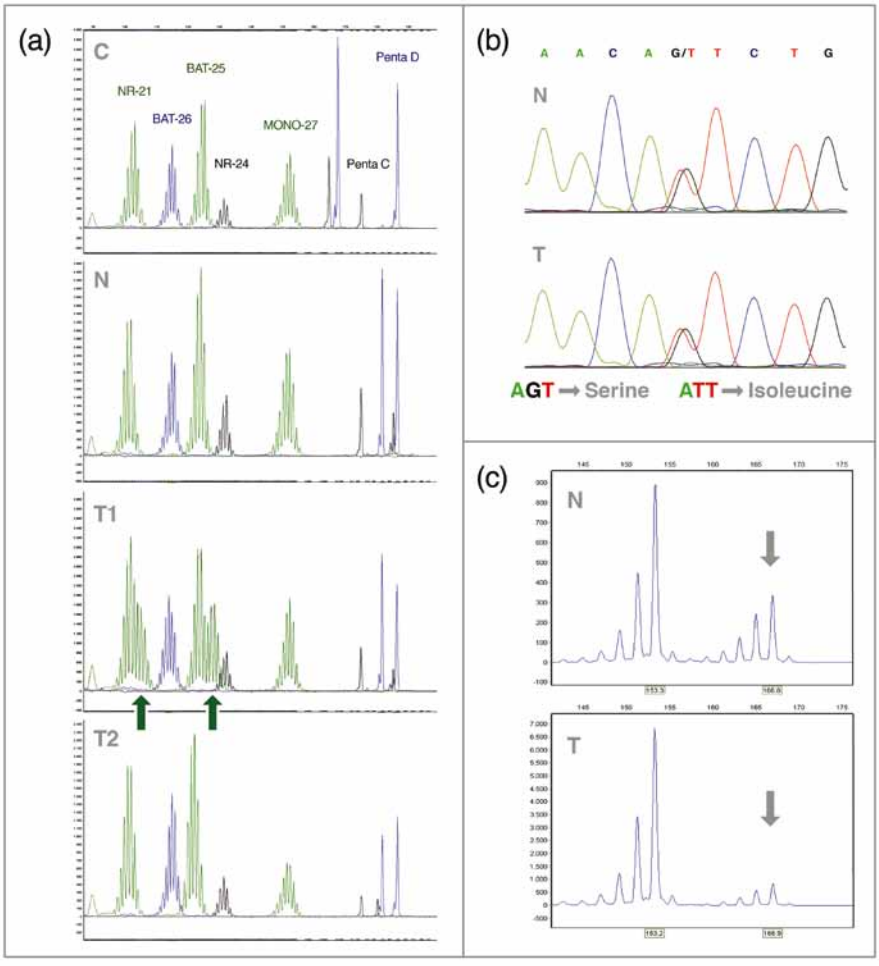


Figure 3. MSI analysis of the brain tumors of the index patient and his sister (individual IV.2 and IV.1) and LOH analysis of the father of the index patient (individual III.2).
a) Promega pentaplex MSI results of unrelated normal control DNA (C), index patients' normal leukocyte DNA (N), index patient's brain tumor DNA (T1) and his sister's brain tumor DNA (T2). MSI (markers NR-21 and BAT-25, instability indicated by arrows) is only observed in the tumor of the index patient and not in his sisters'tumor.
b) Sequencing results of exon 2 of the *PMS2* gene, showing the heterozygous p.Ser46Ile (c.137G>T) mutation in normal tissue (N) and CRC (T) of father. No LOH is detected in tumor tissue (T).
c) Result of LOH analysis with a dinucleotide polymorphic microsatellite at the P53 locus in normal tissue (N) and CRC (T) of father. Arrows indicate the relative loss of the larger allele in the CRC compared to the normal DNA.

Germline mutation analysis

Mutation analysis of the *NF1* gene was performed in the index patient but a mutation could not be identified. Mutation analysis in a blood sample of the index patient identified the compound heterozygous mutations, p.Pro246fs and p.Ser46Ile. Both mutations were also found in DNA derived from brain tissue from his sister. No mutation analysis was performed in the younger sister of the index patient. The heterozygous mutations p.Ser46Ile and p.Pro246fs were confirmed in the father and mother, respectively, indicating the compound heterozygous pattern in the index patient and his sister. The paternal grandmother appeared not to carry the p.Ser46Ile mutation as present in the father of the index patient. In both the index patient and his father no germline mutation was detected in the *MLH1*, *MSH2* and *MSH6* gene.

Additional molecular analysis: loss of heterozygosity

To asses whether the tumor from the father of the index patient was caused by the *PMS2* germline mutation, loss of heterozygosity (LOH) analysis was performed (Figure 3.b). For that purpose, DNA extracted from normal and tumor tissue of the father was sequenced, according to a previously described method.[106] No LOH of the *PMS2* locus was found, while the tumor percentage was high enough to detect LOH, which was indicated by the presence of LOH at the *P53* and *APC* loci (Figure 3.c).

DISCUSSION

The above-mentioned family displays a CMMR-D phenotype in the presence of compound heterozygous *PMS2* mutations (p.Ser46Ile and p.Pro246fs). MSI was only found in the brain tumor of the *PMS2* compound heterozygous index patient. The brain tumor of his compound heterozygous sister as well as the CRCs of the father and his mother and the colorectal adenoma of the mother were MSS. Immunohistochemical analysis showed absence of PMS2 staining in both the brain tumor and normal tissue of the index patient and his sister, but not in the analysed CRCs of their father and grandmother.

PMS2 is considered a tumor suppressor gene.[24] In tumors of carriers of a heterozygous *PMS2* mutation, MSI and absence of immunohistochemical staining of PMS2 can be expected due to loss of the wild-type allele. In case of a biallelic germline mutation MSI and especially absence of PMS2 expression can be expected already in normal tissue, as well as in tumor tissue.

Both parents of the index patients were found to carry a heterozygous *PMS2* mutation. The p.Pro246fs mutation of the mother (individual III.3) is a previously described pathogenic frameshift mutation.[107] The p.Ser46Ile missense germline mutation of father (individual III.2) has been found in seven cases in a cohort of 400 selected Dutch patients

suspected for an MMR gene defect. In contrast, this mutation was not detected in 927 controls (unpublished data of the Department of Human and Clinical Genetics, LUMC). Also, the amino acid involved in this mutation is positioned in a highly conserved small helix domain (codon 35-48) and in addition serine and isoleucine have very different physical and chemical properties. In the literature there is a clear overrepresentation of p.Ser46Ile in patients with PMS2 negative tumors.[9, 65, 108-110] These findings support the pathogenicity of this mutation.

Surprisingly no MSI and IHC aberrations were found in the CRC of the father. Eight additional CRCs in heterozygous carriers of the p.Ser46Ile mutation have been reported (Table 2). Unfortunately, only data on the MSI status of the tumor tissues of three of these eight patients were available, all displaying MSI. Absent PMS2 expression was found in all described tumors in contrast to our observations in the CRC of father. Because additionally no loss of heterozygosity of the *PMS2* locus was detected in the tumor of father, a role of *PMS2* in the development of the early onset CRC of the father can not be demonstrated at the moment. It is possible that other CRC susceptibility genes are involved. Since the tumor tissue of father's CRC was found to be MSS, it is unlikely, however, that this concerns the other MMR genes. Also, germline mutation analyses of

Table 2. Genetic and clinical summary of nine reported cases (including our case) of patients with CRC and the heterozygous *PMS2* mutation: c.[137G>T], p.Ser46Ile.

Patient	Case	Malignancy	Age at diagnosis of malignancy (years)	Analysis of MSI	IHC	Reference
1	1	CRC: cecum	32	NA	PMS2 absent	Senter *et al*, 2008[9]
2	2	CRC: cecum	47	NA	PMS2 absent	Senter *et al*, 2008[9]
3	3	CRC: sigmoid	44	NA	PMS2 absent	Senter *et al*, 2008[9]
4	5	CRC: transverse	43	NA	PMS2 absent	Senter *et al*, 2008[9]
5	6	CRC: sigmoid	62	NA	PMS2 absent	Senter *et al*, 2008[9]
6	Patient 1	CRC	31	MSI-H	PMS2 absent	Nakagawa *et al*, 2004[65]
7	66603/ current report: III.2	CRC	43	MSS	Normal	Van der Klift *et al*, 2010[110]
8	74028	CRC	70	MSI-H	PMS2 absent	Van der Klift *et al*, 2010[110]
9	74055	CRC	54	MSI-H	PMS2 absent	Van der Klift *et al*, 2010[110]

CRC, colorectal carcinoma; IHC, immunohistochemistry; NR, not reported; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSS, microsatellite stable.

MLH1, *MSH2* and *MSH6* revealed no aberrations in father. Nevertheless, other unknown susceptibility genes can not be excluded. In view of this, first degree relatives of the father, who test negative for the familial *PMS2* mutation, should in our opinion still be offered colorectal surveillance.

The results of MSI and IHC analysis of the tissue of the paternal grandmother are in agreement with analysis of sporadic CRC This finding is in concordance with her not being a carrier of the familial *PMS2* mutation.

The index patient and his sister inherited both *PMS2* germline mutations from their parents, explaining their CMMR-D phenotype. However, MSI was found in only one of the two brain tumors. In gastrointestinal tumors MSI analysis seems a reliable tool to diagnose MMR deficiency. In the literature results of molecular analyses in 21 patients with gastrointestinal malignancies and biallelic MMR gene mutations have been reported. Nineteen patients were diagnosed with CRC and two patients with duodenal cancer. In all tumors MSI was detected. Additional IHC analysis showed absent immunostaining of the corresponding affected MMR proteins in 19 of 21 analysed gastrointestinal tumors.[9, 34, 108, 109, 111-123] In addition to the gastrointestinal patients, 43 patients (mean 8 years, range 4-17, 88% male) with biallelic MMR gene mutations and brain cancer have been reported. In eight of these 43 cases, brain tumor specimens were analyzed for MSI and in five of these cases IHC of MMR proteins was performed (Table 3). Germline mutation analysis showed one patient with *MLH1*, one with *MSH2*, four with *MSH6* and two with *PMS2* mutations. In six of the analysed eight cases no MSI was found in brain tumor tissue. A hypothesis to explain the lack of MSI in brain tumors from germline biallelic *PMS2* mutant patients is that in brain tissue a PMS2 deficiency could lead to tumorigenesis through a different mechanism than the mismatch repair pathway.[34, 106, 108] Also, the extend and pattern of MSI may differ between CRCs and brain tumors, making the MSI analysis that is routinely used for CRC less reliable for brain tumors.[34, 106, 124, 125]

IHC analysis showed absent immunostaining of PMS2 in the brain tumor cells as well as in normal cells in the specimens of our index case and his sister. This is in accordance with the absence of expression of the affected MMR protein in all five investigated brain tumors of germline biallelic mutant MMR gene patients described in the literature.[34, 106, 108, 126, 127] From the literature and our own data, it can be concluded that MMR IHC may be more sensitive than MSI analysis to detect MMR deficiency in brain tumors.

The third child in this family (IV.3) is also at risk of being a heterozygous or compound heterozygous carrier of the familial *PMS2* mutations. Because single CAL spots are a frequent finding in the general population and this child is eight years passed the age of onset of the brain tumors in her siblings, we estimate her risk for CMMR-D to be lower than the theoretical 25%. However, her risk is not excluded. No guidelines are available yet for the surveillance of children at risk for CMMR-D. In this family we think regular clinical surveillance by a paediatric oncologist including colonoscopy and possibly brain

Table 3. Results of analysis for microsattelite instability and immunohistochemical analysis of brain cancer patients from families with biallelic mismatch repair mutations.

Family	Case	Gene	Malignancy	Age at diagnosis of malignancy (years)	Signs of NF1	Analysis of MSI	IHC	Reference
1	Patient 1	*PMS2*	Glioblastoma colonic adenomas NHL of the rectum	4	CAL spots	MSI-H	NA	Hamilton *et al*, 1995[114]
2	IV.2	*MSH2*	Glioblastoma	4	NR	MSS	NA	Bougeard *et al*, 2003[126]
3	III.1	*PMS2*	Giant cell glioblastoma duodenal cancer colonic adenomas	17	CAL spots	MSS glioblastoma, MSI-H duodenal cancer	PMS2 absent in glioblastoma. MSH6 and PMS2 absent in duodenal tumor tissue	Agostini *et al*, 2005[108]
4	V.4	*MSH6*	Oligodendroglioma rectosigmoid cancer	10	CAL spots	MSS	MSH6 absent in CRC, MSH6 present in oligodendroglioma	Menko *et al*, 2004[34]
5	IV.3	*MSH6*	Glioblastoma multiforme	8	CAL spots, axillary freckling	MSI-H	NA	Hegde *et al*, 2005[124]
6	pat. 1	*MSH6*	Astrocytoma	9	CAL spots, axillary freckling, IgA deficiency	NA	MSH6 absent	Ostergaard *et al*, 2005[125]
7	I	*MLH1*	Glioblastoma Wilms tumor	4	CAL spots	MSS	*MLH1*/PMS2 absent in brain tissue	Poley *et al*, 2007[106]
8	I.2	*MSH6*	Glioblastoma multiforme	9	Hyper and hypopigmentation skin	MSS	MSH6 absent, MSH2 expression reduced	Etzler *et al*, 2008[127]

NA, not available; CAL, café au lait; MSI, microsatellite instability; MSS, microsatellite stable; MSI-H, microsatellite instability-high; IHC, immunohistochemistry.

MRI can be considered. Because of behavioural and psychological problems of the third child, she and her mother declined genetic testing for the *PMS2* mutations and surveillance at the moment.

In conclusion, the results of molecular analyses in this family display the diagnostic challenges in *PMS2*-mutation families. In case of a clinical phenotype of CMMR-D, it is recommended to routinely combine MSI analysis with IHC for expression of MMR proteins. With inconclusive or conflicting results, mutation analysis of the MMR genes should be considered after thorough counseling of the patients and/or their relatives.

Chapter 3

Somatic aberrations of mismatch repair genes as a cause of microsatellite-unstable cancers

Willemina R.R. Geurts-Giele, Celine H.M. Leenen, Hendrikus J. Dubbink, Isabelle C. Meijssen, Edward Post, Hein F.B.M. Sleddens, Ernst J. Kuipers, Anne Goverde, Ans M.W. van den Ouweland, Margot G.F. van Lier, Ewout W. Steyerberg, Monique E. van Leerdam, Anja Wagner, Winand N.M. Dinjens

The Journal of Pathology 2014;234(4):548-59

ABSTRACT

Lynch syndrome (LS) is caused by germline mutations in mismatch repair (MMR) genes resulting in microsatellite-unstable tumors. Approximately 35% of suspected LS (sLS) patients test negative for germline MMR gene mutations, hampering conclusive LS diagnosis. The aim of this study was to investigate somatic MMR gene aberrations in microsatellite-unstable colorectal and endometrial cancers of sLS patients negative for germline MMR gene mutations. Suspected LS cases were selected from a retrospective clinical genetics diagnostic cohort and from a prospective multicenter population based study on LS in the Netherlands. In total, microsatellite-unstable tumors of 40 sLS patients (M/F 20/20, median age 57) were screened for somatic MMR gene mutations by next generation sequencing. In addition, loss of heterozygosity (LOH) of the affected MMR genes in these tumors as well as in 68 LS-associated tumors and 27 microsatellite-unstable tumors with *MLH1* promoter hypermethylation was studied. Of the sLS cases, 5/40 (13%) tumors had two pathogenic somatic mutations and 16/40 (40%) tumors had a (likely) pathogenic mutation and LOH. Overall, LOH of the affected MMR gene locus was observed in 24/39 (62%) tumors with informative LOH markers. Of the LS cases and the tumors with *MLH1* promoter hypermethylation 39/61 (64%) and 2/21 (10%) tumors, respectively, demonstrated LOH. Half of microsatellite-unstable tumors of sLS patients without germline MMR gene mutations have two (likely) deleterious somatic MMR gene aberrations, indicating their sporadic origin. Therefore, we advocate adding somatic mutation and LOH analysis of the MMR genes to the molecular diagnostic workflow of LS.

INTRODUCTION

Lynch syndrome (LS) is an autosomal dominant hereditary condition that predisposes to various types of cancer and accounts for about 3% of all colorectal cancers (CRC) and about 2% of all endometrial cancers (EC).[66, 92] The increased risk for malignant lesions in LS is due to an inactivating germline mutation in one of four mismatch repair (MMR) genes, *MLH1*, *MSH2*, *MSH6,* and *PMS2*, or a germline deletion in *EPCAM*.[30, 31] The final diagnosis LS is based on the identification of the germline mutation in one of these genes.[128]

MMR genes are classical tumor suppressor genes and biallelic inactivation results in tumorigenesis. The tumors of LS patients are characterized by a microsatellite instability (MSI) phenotype and absence of expression of one or more MMR proteins, both indicating DNA MMR deficiency. As a result of the LS testing algorithm (Figure 1) patients are indicated as suspected of LS (sLS) or non-suspected of LS, after which germline testing of the affected MMR gene(s), as indicated by immunohistochemistry (IHC), is performed in the sLS cases. Germline testing leads to identification of an MMR gene mutation or of a variant of unknown significance (VUS) in about 65% of the sLS patients, as was shown in a prospective multicenter population based study in the Netherlands in which all consecutive CRC and EC patients ≤ 70 years were screened for LS.[129, 130] The lack of identification of mutations in the remaining 35% severely hampers conclusive diagnosis (LS or no LS) for these patients and their relatives. An existing germline mutation could have been missed by germline analysis or they have a sporadic tumor caused by biallelic somatic MMR gene inactivation. The prevalence of somatic mutations in the *MLH1*, *MSH2,* and *MSH6* genes in sporadic CRC is 16%, 10%, and 6%, respectively,[131, 132] however, not all of the tumors included in these analyses showed MSI. More recently, somatic aberrations of the *MLH1* and *MSH2* genes were studied in CRCs and ECs with MSI, but negative for both MMR germline mutations and promoter hypermethylation.[68, 69] Sourrouille *et al*[69] performed mutation analysis of 18 CRCs and detected in four tumors each two somatic mutations. Mensenkamp *et al*[68] combined mutation- and LOH analysis in 25 CRCs or ECs and identified two somatic hits in 13 tumors. Both studies conclude that these double somatic hits indicate biallelic somatic inactivation and sporadic occurrence of the tumors.

Reliable LS diagnosis is important both for patients with malignancies and for their healthy relatives at risk for carrying an MMR gene germline mutation as surveillance and preventive options can provide substantial health benefits in case of a pathogenic MMR germline mutation.[18-20, 22] In addition, the exclusion of LS in patients suspected of LS can also lead to health benefits, since these patients and their relatives may be released from further surveillance, additional genetic testing and emotional distress. The aim of the present study was to improve LS diagnostics by the determination of somatic MMR gene aberrations in microsatellite-unstable tumors of sLS patients tested negative for germline MMR gene mutations.

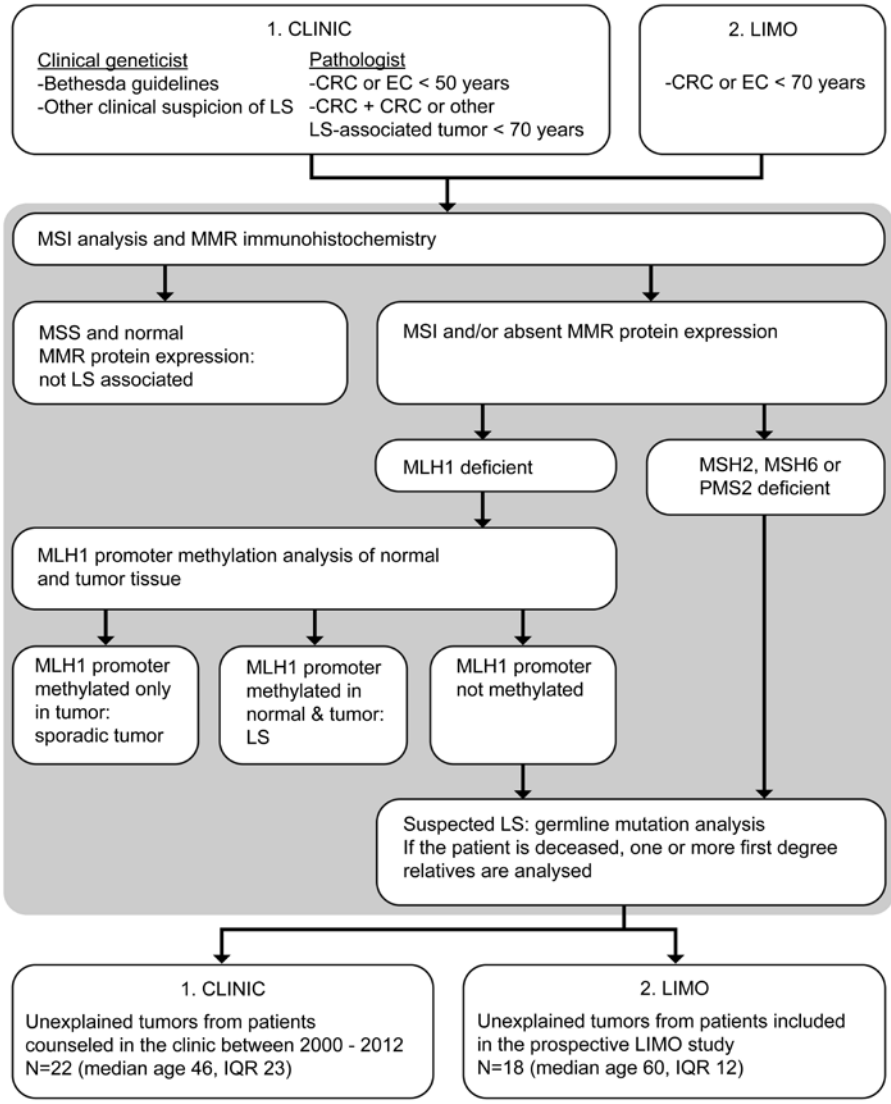


Figure 1. Patient selection flowchart. All unexplained tumors (without germline mutations) from a retrospective series of sLS patients counselled at the Clinical Genetics department of Erasmus MC during 2000–2012 (1. CLINIC) as well as from sLS patients previously involved in a prospective, multicentre, population-based study in The Netherlands (2. LIMO) were included: CRC, colorectal cancer; EC, endometrial cancer; LS, Lynch's syndrome; MMR, mismatch repair; MSI, microsatellite instability; MSS, microsatellite-stable; *n*, number of patients.

METHODS

Patient selection and DNA isolation

Patient selection is described in Figure 1, unexplained tumors from sLS patients were included in the study. sLS patients were defined as patients (i) with microsatellite-unstable CRC, EC or ovarian cancer, (ii) without *MLH1* promoter hypermethylation when *MLH1* was the affected MMR gene as indicated by IHC, and (iii) tested negative for germline mutations and VUS in the affected MMR gene (mutation analysis of entire genes including analysis of large intragenic deletions), and negative for *EPCAM* deletions. If blood was not available as source of constitutional DNA because the patient was deceased at time of germline mutation analysis, one or more first degree relatives were analyzed.

A retrospective series of 22 tumors (including one adenoma) of sLS patients were screened for MMR gene aberrations; these patients or their relatives were counseled at the Clinical Genetics department of Erasmus MC, University Medical Center Rotterdam between 2000 and 2012 (Figure 1). Furthermore, 18 tumors of sLS that were previously involved in a prospective multicenter population based study in the Netherlands (LIMO) were included.[129, 130] As controls, 68 tumors of LS patients with an identified pathogenic MMR gene germline mutation (M/F 35/33, median age 50, IQR 14) and 27 sporadic tumors with *MLH1* promoter hypermethylation (M/F 9/18, median age 64, IQR 8) were analyzed. Of all 135 cases, formalin-fixed and paraffin-embedded (FFPE) normal and tumor tissues were manually microdissected from five to ten haematoxylin stained sections. DNA was extracted using proteinase K and 5% Chelex 100 resin, as previously described.[55]

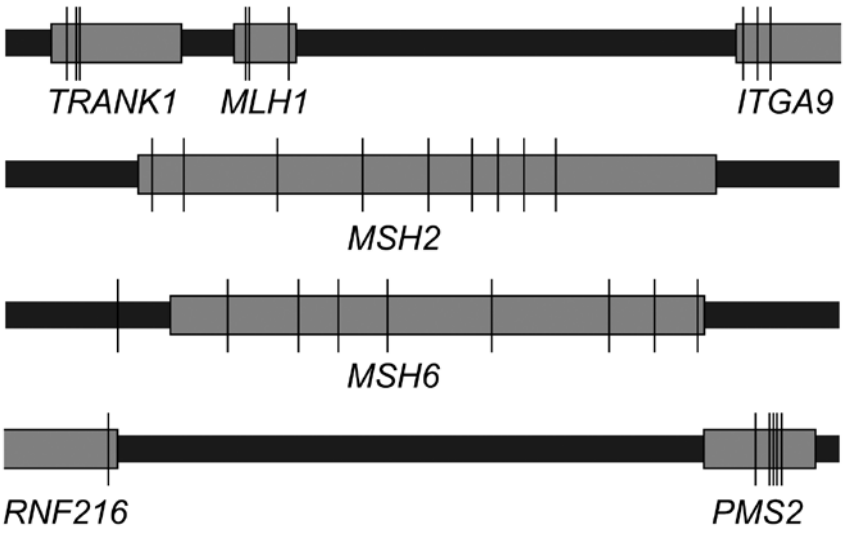


Figure 2. Locations of the SNPs used to screen for LOH of the MMR genes in four multiplex assays: vertical black lines, positions of the SNPs targeted by SNaPshot probes.

MSI analysis, MMR protein IHC and *MLH1*, *MSH2* and *MSH6* promoter hypermethylation assay

MSI, MMR protein IHC and *MLH1*, *MSH2*, and *MSH6* promoter hypermethylation analyses were performed as previously described.[55]

LOH analysis and copy number detection of the MMR genes

LOH analysis was performed for the affected MMR gene (for sLS patients as indicated by IHC) with the SNaPshot multiplex kit (Applied Biosystems, Foster City, CA) on normal and tumor DNA as previously described.[133] Single PCR - and multiplex PCR assays were designed to detect six to nine single nucleotide polymorphisms (SNPs) in or adjacent to each of the MMR genes (Figure 2). Classification of SNP results per gene was as follows: LOH: at least one SNP with LOH, no SNP with retention of heterozygosity (ROH); ROH: at least one SNP with ROH, no SNP with LOH; partial LOH: both SNP(s) with LOH and ROH; NI: all SNPs are non-informative (homozygous). To establish copy number of the affected MMR gene, fluorescent in situ hybridization (FISH) was performed using a commercial probe to detect *MSH2* and custom made probes to detect *MLH1*, *MSH6,* or *PMS2* (all Kreatech, Amsterdam, The Netherlands), according to standard protocols. Control probes targeting the centromere or a locus on the opposite chromosomal arm were included for each gene.

Mutation analysis of the MMR genes and *BRAF*

All tumor samples of sLS patients were screened for somatic mutations of *MLH1*, *MSH2*, *MSH6,* and *PMS2* with the Ion Torrent Personal Genome Machine (PGM) with suppliers materials and protocols (Life Technologies, Carlsbad, CA). A custom primer panel targeting the open reading frame including the exon-intron boundaries of the MMR genes was designed

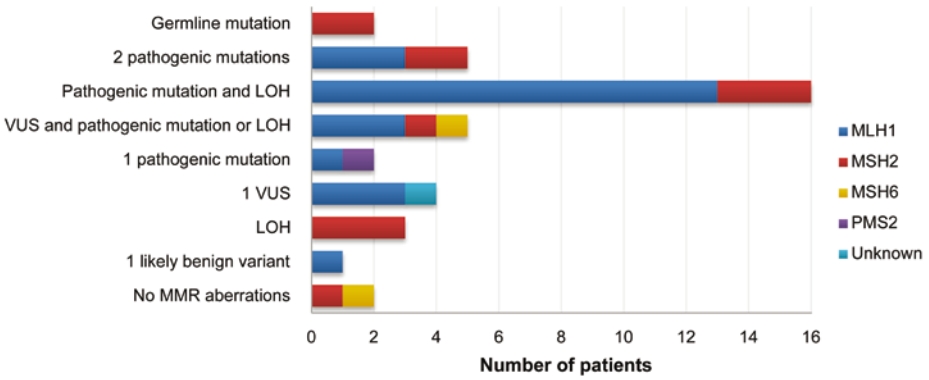


Figure 3. Somatic aberrations for the tumors of 40 suspected Lynch's syndrome patients. The different colours indicate the deficient mismatch repair gene, as shown by immunohistochemistry: LOH, loss of heterozygosity; VUS, variant of unknown significance.

using the Ion AmpliSeq Designer 1.2. This panel consisted of 150 amplicons covering 100%, 92%, 97%, and 79% of *MLH1*, *MSH2*, *MSH6,* and *PMS2*, respectively. All variants in the coding regions and the splice sites were reported, excluding synonymous single nucleotide variants and known bona fide SNPs. All variants detected with the PGM were confirmed by Sanger sequencing in tumor and normal DNA, as previously described.[134] For four MLH1 deficient tumors (sLS-1, sLS-2, sLS-9, and sLS-10) conventional Sanger sequencing of the exonic regions of *MLH1* was performed instead of PGM analysis. All previously identified germline mutations in LS patients were confirmed in normal and tumor tissue if possible. Additionally, all tumor samples were screened for *BRAF* mutations by Sanger sequencing and with mutation specific PCR for *BRAF* V600E and V600K using FAM-labeled primers.

Predicting pathogenicity for somatic MMR variants

Frame-shift, nonsense and splice site mutations were assumed to be pathogenic. For all missense variants and in-frame deletions multiple in silico tools, InSIGHT classification,[135, 136] and a literature search were used to predict pathogenicity. Finally, all variants were classified as: benign (1), likely not pathogenic (2), uncertain (3), likely pathogenic (4), or definitely pathogenic (5).

RESULTS

Somatic MMR aberrations in sLS patients

The tumors of 40 sLS patients negative for MMR gene germline mutations were screened for somatic MMR mutations and for LOH of the affected MMR gene as indicated by IHC (Table 1, Figure 4). This led to detection of 49 somatic MMR gene variants, 31 in *MLH1*, 11 in *MSH2*, six in *MSH6,* and one in *PSM2*. Two of the 40 (5%) patients, both deceased and with first degree relatives negative for germline MMR mutations, showed a pathogenic mutation both in tumor and normal tissues. For all other patients, DNA from normal tissue showed no aberrations. Twenty-one of the 40 (53%) tumors showed either two pathogenic mutations (5) or one (likely) pathogenic mutation and LOH (16) (Figure 5). In 12 of the tumors with a mutation and LOH Sanger sequencing confirmed loss of the wildtype allele. Five of the 40 (13%) tumors showed a VUS combined with a pathogenic mutation or LOH. In 9/40 (23%) tumors only one somatic aberration was detected and 1/40 (3%) tumors showed only a likely benign variant. Two out of 40 (5%) tumors showed no aberrations, including the tumor from patient sLS-38, for which no mutation analysis results were available. In total, two VUS and four likely benign variants, but no pathogenic mutations, were detected in non-affected MMR genes as indicated by IHC.

Table 1 Somatic aberrations for suspected LS patients.

Patient	Cohort	M/F	Age§	Tumor	Germline analysis	Somatic variants	Class (1-5)	% of variant reads with PGM (variant reads/ total reads)	LOH/ ROH Sanger	LOH/ ROH SNaPshot	Copy number FISH	BRAF	Conclustion somatic MMR aberrations
MLH1													
sLS-1	C	F	46	EC	A	c.37delG; p.E13fs*3	5	PGM results ND	ROH	ROH	2	ND	2 pathogenic mutations
						c.1816_1817delGG; p.G606fs*2	5		ROH				
sLS-2	C	M	61	CRC	A	c.790+1G>A	5	PGM results NE	ROH	ROH	2	WT	2 pathogenic mutations
						c.1852_1854delAAG; p.K618del	5		ROH				
sLS-3	C	M	33	CRC	A	c.2059delC; p.R687fs	5	82% (437/536)	LOH	LOH	NE	c.1781A>G; p.D594G	Pathogenic mutation and LOH
sLS-4	C	F	46	CRC	A	c.298C>T; p.R100*	5	86% (730/852)	LOH	LOH	2	WT	Pathogenic mutation and LOH
sLS-5	C	M	46	CRC	B	c.1276delC; p.Q426fs	5	89% (1782/2000)	LOH	LOH	2	WT	Pathogenic mutation and LOH
sLS-6	C	F	59	CRC	A	c.350C>T; p.T117M	5	60% (838/1402)	NE	LOH (1 marker)	2	c.1799T>A; p.V600E	Pathogenic mutation and LOH
sLS-7	C	F	68	CRC	A	c.146T>C; p.V49A	3	44% (2562/5773)	ROH	LOH	2	WT	Pathogenic mutation and LOH
						c.2001delC; p.D667fs	5	90% (751/838)	LOH				

Table 1 (Continued)

Patient	Cohort	M/F	Age§	Tumor	Germline analysis	Somatic variants	Class (1-5)	% of variant reads with PGM (variant reads/ total reads)	LOH/ ROH Sanger	LOH/ ROH SNaPshot	Copy number FISH	BRAF	Conclustion somatic MMR aberrations
sLS-8	C	F	73	CRC	B	c.1A>T; p.M1L	3	81% (520/645)	ROH	LOH	2	WT	Pathogenic mutation and LOH
						c.1852_1854delAAG; p.K618del	5	22% (449/2000)	ROH				
sLS-9	C	M	39	Villous	A	c.453+1G>A	5	PGM results ND	ROH	ROH	2	ND	VUS and pathogenic mutation
				adenoma		c.2270A>T, p.*757L	3		ROH				
sLS-10	C	F	65	CRC	A	c.793C>G; p.R265G	3	PGM results ND	LOH	LOH	2	WT	VUS and LOH
sLS-11	C	F	40	CRC	A	c.638delT; p.V213fs	5	52% (518/1005)	ROH	ROH	2	WT	1 pathogenic mutation
sLS-12	C	F	51	CRC	B	c.1652A>C; p.N551T	3	44% (382/868)	NE	NE	2	NE	1 VUS
sLS-13	C	M	45	CRC	A	c.1270G>A; p.A424T (only PGM)	2	72% (146/202)	ND	ROH (1 marker)	2	c.1799T>A; p.V600E	1 likely benign variant
sLS-14	L	F	65	CRC	B	c.445C>T; p.Q149*	5	33% (366/1123)	ROH	NI	ND	WT	2 pathogenic mutations
						c.676C>T; p.R226*	5	34% (400/1180)	ROH				
						MSH6: c.2914A>T; p.I972F	2	31% (217/689)	ROH				
sLS-15	L	M	64	CRC	A	c.678-1G>A	5	89% (271/304)	LOH	NE	2	WT	Pathogenic mutation and LOH

Table 1 (Continued)

Patient	Cohort	M/F	Age§	Tumor	Germline analysis	Somatic variants	Class (1-5)	% of variant reads with PGM (variant reads/ total reads)	LOH/ ROH Sanger	LOH/ ROH SNaPshot	Copy number FISH	BRAF	Conclustion somatic MMR aberrations
sLS-16	L	M	70	CRC	A	c.638delT; p.V213fs	5	83% (1592/1917)	LOH	LOH	2	c.1801A>G; p.K601E	Pathogenic mutation and LOH
sLS-17	L	F	59	CRC	B	c.1608delT; p.P536fs	5	80% (991/1242)	LOH	LOH	2	WT	Pathogenic mutation and LOH
sLS-18	L	M	61	CRC	A	c.199G>A; p.G67R	5	72% (2831/3907)	LOH	LOH (1 marker)	NE	WT	Pathogenic mutation and LOH
sLS-19	L	F	62	CRC	A	c.350C>T; p.T117M	5	81% (130/161)	NE	LOH	2	WT	Pathogenic mutation and LOH
sLS-20	L	M	65	CRC	A	c.203T>G; p.I68S	3	77% (2618/3406)	LOH	LOH	2	WT	Pathogenic mutation and LOH
						c.350C>T; p.T117M	5	38% (147/382)	ROH				
sLS-21	L	M	71	CRC	A	c.194G>A; p.G65D	4	64% (3064/4823)	LOH	LOH (1 marker)	2	WT	Likely pathogenic mutation and LOH
sLS-22	L	M	52	CRC	A	c.2042C>T; p.A681V	3	81% (438/538)	LOH	LOH	2	WT	VUS and LOH
sLS-23	L	M	58	CRC	A	*MSH6*: c.412C>A; p.P138T	3	32% (664/2066)	ROH	ROH (1 marker) (*MSH6*), ROH (MLH1)	2 (*MLH1* & *MSH6*)	c.1799T>A; p.V600E	1 VUS

Table 1 (Continued)

Patient	Cohort	M/F	Age§	Tumor	Germline analysis	Somatic variants	Class (1-5)	% of variant reads with PGM (variant reads/ total reads)	LOH/ ROH Sanger	LOH/ ROH SNaPshot	Copy number FISH	BRAF	Conclustion somatic MMR aberrations
sLS-24	L	M	27	CRC	A	c.1600-1601delinsAG; p.V534R	3	38% (1410/3727)	ROH	ROH	2	WT	1 VUS
MSH2													
sLS-25	C	F	34	Ovary	B	GL: c.1147C>T; p.R383*	5	86% (7777/9064)	LOH	LOH (1 marker)	2	WT	Germline mutation
						c.965G>A; p.G322D (only PGM)	1	21% (801/3832)	ND				
sLS-26	C	F	39	EC	B	GL: c.1147C>T; p.R383*	5	39% (1126/2868)	ROH	Partial LOH	2	WT	Germline mutation
sLS-27	C	M	61	CRC	A	c.818delT; p.V273fs	5	82% (1644/2000)	LOH	NI	2	NE	Pathogenic mutation and LOH
sLS-28	C	F	36	CRC	A	no variant detected				Partial LOH	2	WT	LOH
sLS-29	C	M	64	CRC	A	no variant detected				LOH	2	WT	LOH
sLS-30	C	F	38	CRC	A	no variant detected				LOH	2	WT	LOH
sLS-31	C	F	47	CRC	A	no variant detected				ROH	2	WT	No MMR aberrations
sLS-32	L	M	66	CRC	A	c.83insG; p.E28fs	5	40% (158/398)	ROH	ROH	2	WT	2 pathogenic mutations
						c.514G>A; p.K172*	5	49% (1091/2209)	ROH				
sLS-33	L	F	53	EC	A	c.255delT; p.N85fs*2	5	42% (842/1996)	NE	ROH	2	WT	2 pathogenic mutations
						c.2145dupT; p.D716*	5	37% (571/1559)	ROH				

Table 1 (Continued)

Patient	Cohort	M/F	Age§	Tumor	Germline analysis	Somatic variants	Class (1-5)	% of variant reads with PGM (variant reads/ total reads)	LOH/ ROH Sanger	LOH/ ROH SNaPshot	Copy number FISH	BRAF	Conclustion somatic MMR aberrations
sLS-34	L	F	58	EC	A	c.1903A>T; p.K635*	5	88% (3650/4150)	LOH	LOH	2	WT	Pathogenic mutation and LOH
sLS-35	L	F	49	CRC	A	c.1601delA; p.N538fs	5	84% (1670/2000)	LOH	NI	ND	WT	Pathogenic mutation and LOH
sLS-36	L	M	55	CRC	A	c.279_281delTCT; p.L94del	1	40% (791/2000)	NE	LOH	2	WT	VUS and LOH
						c.857T>C; p.F286S	3	39% (1186/3078)	ROH				
MSH6													
sLS-37	C	M	79	CRC	B	c.2672_2673delTC; p.I891fs	5	41% (615/1498)	ROH	ROH	2	WT	VUS and pathogenic mutation
						c.3725G>A; p.R1242H	3	41% (3204/7884)	ROH				
sLS-38	C	M	37	CRC	A	no evaluable results		PGM results NE		ROH	NE	NE	No MMR aberrations
PMS2													
sLS-39	L	F	69	EC	A	c.943C>T; p.R315*	5	34% (923/2732)	NE	ROH (low tumor%)	2	WT	1 pathogenic mutation
						MLH1: c.1469T>C; p.M490T	2	26% (495/1905)	ROH				
						MSH2: c.1448A>G; p.E483G	2	43% (211/486)	ROH				

Table 1 (Continued)

Patient	Cohort	M/F	Age§	Tumor	Germline analysis	Somatic variants	Class (1-5)	% of variant reads with PGM (variant reads/ total reads)	LOH/ ROH Sanger	LOH/ ROH SNaPshot	Copy number FISH	BRAF	Conclustion somatic MMR aberrations
						MSH6: c.1054G>A; p.V352I	2	34% (67/200)	ROH				
no deficient MMR protein													
sLS-40	L	M	53	CRC	A	*MSH6*: c.3724C>T; p.R1242C	3	34% (1660/4902)	ROH	ROH (*MSH6*)	2 (all genes)	NE	1 VUS

Patients are categorized according to the affected mismatch repair (MMR) gene as indicated by immunohistochemistry, all somatic aberrations (except for *BRAF*) are detected in the corresponding MMR gene unless specified otherwise. For germline testing patients were classified as: index patient tested for germline MMR gene mutations (A) or index patient deceased, first degree relative(s) tested for germline MMR gene mutations (B). Somatic variants were classified as: benign (1), likely not pathogenic (2), uncertain (3), likely pathogenic (4), or definitely pathogenic (5).[132,133]

§At time of diagnosis C, Clinical Genetics diagnostic cohort; CRC, colorectal cancer; EC, endometrial cancer; F, female; FISH, fluorescent in situ hybridization; L, LIMO study cohort; LOH, loss of heterozygosity; M, male; MMR, mismatch repair; ND, non-determined; NE, non-evaluable; NI, non-informative; ROH, retention of heterozygosity; sLS, suspected Lynch syndrome; VUS, variant of unknown significance; WT, wildtype.

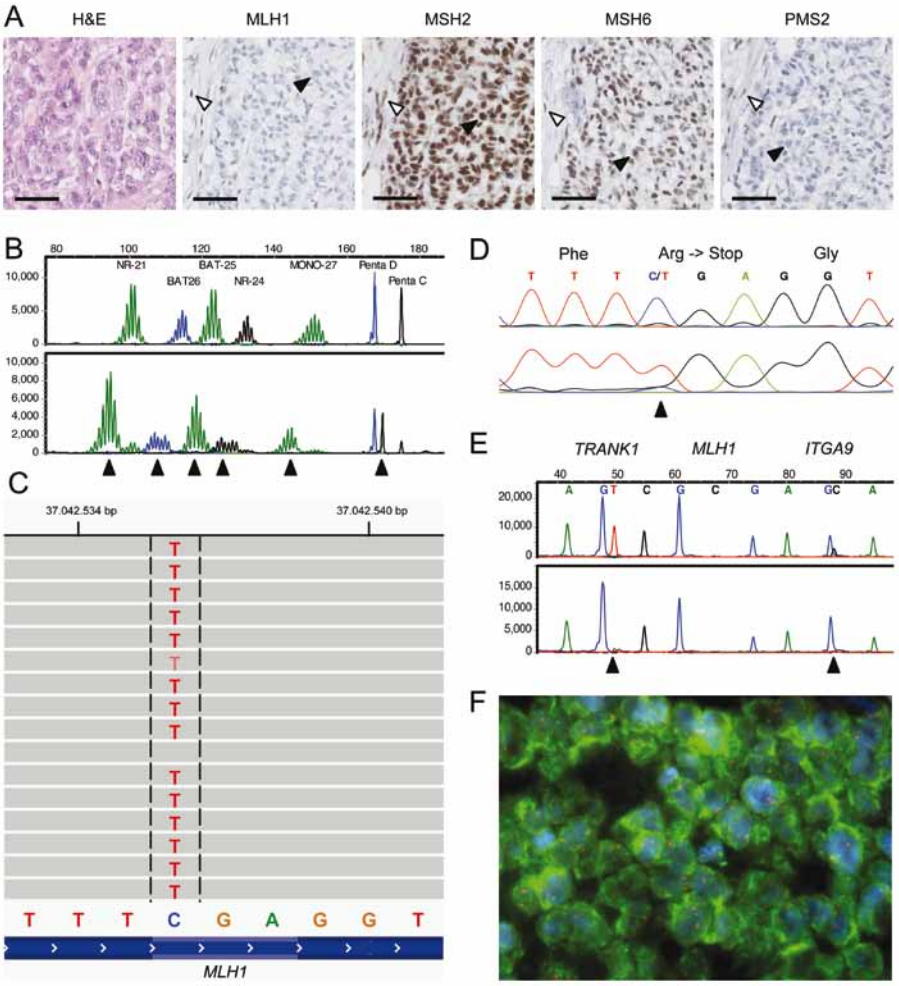


Figure 4. Somatic aberrations for patient sLS-4, who was diagnosed with a moderately–poorly differentiated adenocarcinoma of the colon.

(a) Tumor cells show absence of MLH1 and PMS2 expression and normal MSH2 and MSH6 expression (filled arrowheads), stromal cells show expression of all four proteins (open arrowheads); scale bar=50 µm. (b) Microsatellite instability (MSI) analysis shows MSI of six markers (NR-21, BAT-26, BAT-25, NR-24, MONO-27 and Penta C) in the tumor (lower panel) compared to normal (upper panel); the MSI shifts are indicated by arrowheads. (c) A nonsense mutation (c.298C>T) in *MLH1* was detected with the ion torrent personal genome machine. (d) Sanger sequencing confirmed the presence of the mutation (arrowhead) in tumor tissue (lower panel) and shows the absence of the mutation in normal tissue (upper panel). (e) At the location of the mutation, loss of the wild-type allele was detected, as was confirmed by SNaPshot analysis; one marker in *TRANK1* and one marker in *ITGA9* (arrowheads) are heterozygous in normal tissue (upper panel) and show LOH in tumor tissue (lower panel). (f) Copy number analysis by fluorescence *in situ* hybridization shows two copies of the MLH1 locus (red signal) and two copies of a control locus on the opposite arm of chromosome 3 (green signal) in the tumor cells (open arrowhead).

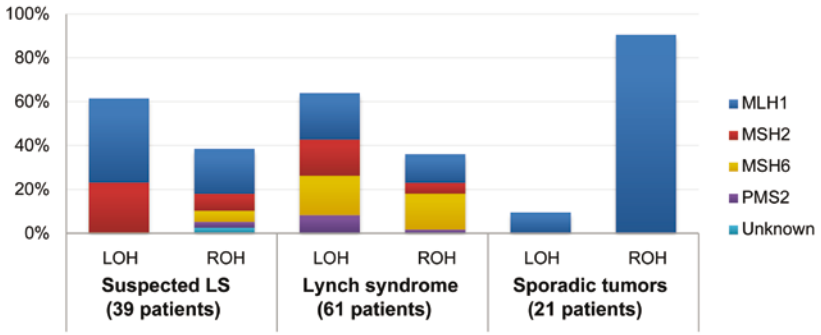


Figure 5. Percentages of tumors with LOH or ROH for suspected Lynch's syndrome (sLS) and LS patients and those with sporadic tumors; different colours indicate the affected mismatch repair genes (for sLS patients) as indicated by immunohistochemistry: LOH, loss of heterozygosity; ROH, retention of heterozygosity.

LOH analysis

All tumors were screened for LOH of the affected MMR gene (for sLS patients as indicated by IHC) by SNaPshot analysis and/or Sanger sequencing. Finally, 39/40 tumors from sLS patients, 61/68 tumors from LS patients, and 21/27 sporadic tumors showed evaluable and informative results. Of those, 24/39 (62%) tumors of sLS patients, 39/61 (64%) LS-associated tumors, and 2/21 (10%) sporadic tumors showed LOH of (part of) the affected gene (Figure 6). For the tumors of sLS patients, LOH was detected in 15/23 (65%) and 9/12 (75%) of the MLH1 and MSH2 deficient tumors respectively. For 36 variants detected in tumors of sLS patients, both PGM data and LOH results by Sanger sequencing were available, for these variants the percentage of variant reads by PGM was compared to LOH results by Sanger sequencing (Table 1). Fifteen out of 36 variants showed LOH both by PGM and Sanger sequencing, 20/36 variants showed ROH both by PGM and Sanger sequencing, and 1 variant showed LOH by PGM (81% variant reads) but ROH by Sanger sequencing. Interestingly, the tumor of this patient (sLS-8) did show LOH by SNaPshot analysis. For the LS-associated tumors, LOH was detected in 13/21 (62%), 10/13 (77%), 11/21 (52%), and in 5/6 (83%) of the tumors of *MLH1*, *MSH2*, *MSH6*, and *PMS2* germline mutation carriers respectively. Overall, seven tumors showed partial LOH by SNaPshot analysis and another eight tumors showed LOH by SNaPshot analysis but ROH by Sanger sequencing with at least one variant.

Copy number analysis

All tumors were screened with FISH to detect MMR gene copy number variations, 121 tumors showed two copies of the affected MMR gene, two tumors showed only one copy, one tumor showed polysomy, nine tumors showed non evaluable results, and for two tumors copy number by FISH could not be determined. Both tumors with only one copy of the affected MMR gene (patients LS-27 and LS-56) showed LOH by SNaPshot analysis.

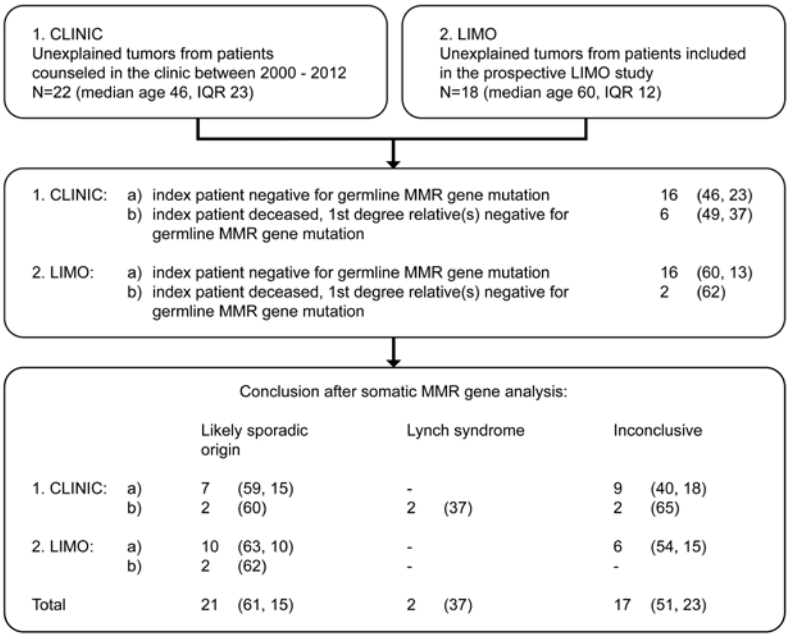


Figure 6. sLS patient details and final conclusions based on tumor analysis; numbers of patients (median age, IQR) are shown for all patient groups: MMR, mismatch repair; *n*, number of patients.

BRAF mutation analysis

All tumors were screened for *BRAF* mutations with a sensitive mutation specific PCR to detect V600E and V600K mutations and with Sanger sequencing. For 127 tumors *BRAF* analysis had evaluable results. Combining the results from both assays, *BRAF* mutations were detected in 5/34 (15%) tumors of sLS patients, 1/67 (1%) LS-associated tumors, and 22/26 (85%) sporadic tumors. V600E mutations were detected in three tumors of sLS patients and in 22 sporadic tumors, a K601E mutation was detected in the tumor of a sLS patient, and a *BRAF* D594G mutation was detected both in a LS-associated tumor and in the tumor of a sLS patient.

DISCUSSION

Somatic MMR gene aberrations were investigated in 40 tumors of sLS patients negative for germline mutations in the affected MMR gene(s) as indicated by IHC; final conclusions are shown in Figure 6. Two somatic and (likely) deleterious aberrations of the affected MMR genes were detected in 21/40 (53%) of these tumors: 16/24 for *MLH1* and 5/12 for *MSH2* (Figure 4). In addition, 5/40 (13%) patients showed a variant of unknown pathogenicity combined with a pathogenic mutation or LOH. No pathogenic mutations were detected in

the non-affected MMR genes as indicated by IHC. This suggests that secondary mutations in the non-affected MMR genes are uncommon. Furthermore, 19/21 (91%) tumors with *MLH1* promoter hypermethylation showed ROH for the *MLH1* gene, which is in accordance with the notions that microsatellite-unstable tumors are generally chromosomal stable[137] and that *MLH1* promoter hypermethylation affects both alleles.[138]

Focusing on the *MLH1* and *MSH2* deficient tumors, 21/36 (58%) tumors had two somatic and (likely) deleterious aberrations. This is comparable to the study of Mensenkamp *et al,*[68] they identified two somatic aberrations in 13/25 (52%) MLH1 or MSH2 deficient tumors. In the current study 5/36 (14%) of the tumors showed 2 pathogenic mutations, and 16/36 (44%) showed a combination of a (likely) pathogenic mutation and LOH. For the study of Mensenkamp *et al*[68] this was 5/25 (20%) and 8/25 (32%) respectively; the slightly lower amount of tumors with a pathogenic mutation and LOH could be explained by the fact that for 10/25 tumors LOH analysis was not informative. We did not observe a different percentage of likely sporadic tumors for CRC (55%; 18/33) compared to EC (60%; 3/5). Mensenkamp *et al*[68] showed that 48% (11/23) of CRC and 100% (2/2) of EC were likely of sporadic origin. Although both studies show that EC can be caused by two somatic aberrations, the numbers of included EC are too low to reliably compare the distribution of somatic aberrations between CRC and EC.

For two related sLS patients (sLS-25 and sLS-26; sisters) the same pathogenic *MSH2* mutation was found both in normal and tumor tissues indicating a germline predisposition. From these patients no blood DNA was available as source of constitutional DNA since both patients were deceased at time of germline mutation analysis. Four of their healthy children were tested and no germline mutations in *MLH1*, *MSH2,* or *MSH6* were found, indicating these children did not inherit the *MSH2* germline mutation from their mothers. This exemplifies that mutation analysis of normal and tumor DNA isolated from archival FFPE tissue can be a valuable approach for LS testing in patients from whom no blood DNA is available.

In the tumors of 21 sLS patients two (likely) deleterious somatic aberrations were detected, either two mutations or one mutation and LOH. It is likely that these aberrations are located on different alleles, causing biallelic inactivation of the involved MMR gene. For 12/16 tumors with a mutation and LOH, loss of the wild type allele could indeed be confirmed by Sanger sequencing. These tumors may now be considered not to be associated with LS. As these patients are no longer suspect for LS, extensive colonoscopic surveillance similar to LS patients is no longer required. The starting age and frequency of colonoscopies for these patients and their relatives can now solely be based on family history.

Part of the sLS patients included in the current study were previously involved in the prospective multicenter LIMO study (Figure 1), in which all consecutive CRC and EC patients ≤ 70 years were screened for LS.[129, 130] In total, 1117 CRC and 179 EC were screened and germline mutation analysis was performed for 52 suspected LS patients: 34 (65%) patients had a germline MMR mutation or VUS and for 18 (35%) patients no mutations

were detected. We screened the tumors of these 18 patients without germline MMR mutations for somatic aberrations of the MMR genes: 12 tumors (ten CRC and two EC) had a likely sporadic origin and for six tumors (five CRC and one EC) the results were inconclusive (Figure 6). Thus, 12/52 (23%) patients that were referred to clinical genetics and tested for germline MMR gene mutations actually had (likely) sporadic tumors.

Only two tumors showed absence of one of the MMR alleles by copy number analysis, whereas LOH was found in 65 tumors. This suggests that the LOH detected is due to copy neutral LOH (cnLOH). Previous studies report that cnLOH is an important mutational event in the carcinogenesis of microsatellite-unstable tumors and usually confined to the locus harboring pathogenic MMR gene mutations.[137, 139] Interestingly, cnLOH was less frequently observed in tumors of *MSH6* mutation carriers,[139] which corresponds to our findings in LS patients where LOH is observed in only 11/21 (52%) of tumors of *MSH6* mutation carriers, but in 13/21 (62%), 10/13 (77%), and 5/6 (83%) of tumors of *MLH1*, *MSH2,* and *PMS2* mutation carriers, respectively. This suggests that the second hit in *MSH6* affected tumors is less often loss of the wildtype allele, but may be a second somatic mutation. An alternative explanation for the absence of copy number alterations is that only a small part of the chromosome is lost, which is not detected by our FISH probes. In 15/65 (23%) tumors we indeed found indications for partial LOH of the involved MMR gene.

BRAF mutation status is regularly used to distinguish LS-associated tumors from sporadic microsatellite-unstable colon cancer, as *BRAF* mutations are correlated with *MLH1* methylation and are strong predictors of MMR gene mutation-negative status.[49, 140] In none of the tumors from LS patients a *BRAF* V600E mutation was detected, however, one germline *MSH6* mutation carrier showed a *BRAF* D594G mutation in the tumor. The same mutation was detected in the tumor of a sLS patient. This mutation appears to be a low activity mutant[141] and has been described before in CRC,[142, 143] but the significance of this mutant in the screening for LS is unknown. In total, *BRAF* mutation status was determined in 15 likely sporadic MLH1 deficient tumors of sLS patients, interestingly, 3/15 (20%) showed a *BRAF* mutation (V600E, K601E and D594G). As these tumors showed no *MLH1* promoter hypermethylation, *BRAF* screening could be valuable in this subgroup of patients to predict the sporadic origin of the tumors.

In 12/40 (30%) tumors of sLS patients no or only one somatic mutation was found in the tumor. Obviously, some aberrations escaped detection by our analyses so no final diagnosis with regard to LS could be made for these cases. Other mechanisms, like mutations in untranslated or (deep) intronic regions, large deletions, or alterations in other genes that are involved in regulation or expression of the MMR genes might be involved in these tumors. Recently, the risk of cancer in families of sLS patients without germline mutations was determined by Rodriguez-Soler *et al*.[144] They found that the risk of CRC is lower in families with sLS than among patients with genetically confirmed LS, but significantly higher than in cases of truly sporadic CRC. Therefore, sLS patients should

only be released from cancer surveillance programs when two somatic hits are detected in the tumor, as any undetected hit can be a germline mutation.

The current study also has some limitations. Some somatic aberrations might have escaped our detection methods; therefore, the number of somatic aberrations of the MMR genes could be underestimated. For the LOH analyses, 12/135 patients showed non-evaluable results probably due to the use of DNA extracted from FFPE tissue. Furthermore, 9/135 patients were homozygous for all investigated SNPs. As LOH might be confined to only a small region of the MMR gene, it could have been missed due to insufficient informative markers. Additionally, some somatic mutations might have been missed due to the design of the PGM primer panel, as not all exonic regions were completely covered.

We did not have a sufficient amount of DNA to analyze all tumors of sLS patients for MMR mutations with an alternative method, therefore, we do not know the false negativity rate for the PGM analysis. The tumors of three sLS patients (sLS-2, sLS-13 and sLS-38) had non evaluable PGM results (less than 80% of the target bases were covered more than 100 times). For two of those tumors (sLS-13 and sLS-38) no conventional Sanger sequencing could be performed as an alternative due to a limited amount of DNA. Despite the low coverage, the tumor of patient sLS-13 did show 1 likely benign variant, but Sanger sequencing of this region could not be performed due to low quality DNA. As the PGM coverage for these patients is very low, potential mutations could have been missed.

In 26/40 (65%) tumors of sLS patients two or more somatic MMR gene aberrations were found. For 21 patients this concerned (likely) pathogenic mutations, indicating the sporadic origin of the tumors. This result indicates that LOH and somatic mutation analyses of the MMR genes in tumors of sLS patients adds substantially to the final diagnostics of these patients and their relatives. Therefore, we propose to add somatic molecular analyses of the MMR genes to the routine molecular diagnostic workflow of tumors of sLS patients. To better document the incidence of somatic MMR mutations, intronic regions and regions that were not covered in the current design should be analyzed as well. Implementation of whole genome sequencing might help to identify unknown germline or somatic aberrations associated with LS.

ACKNOWLEDGEMENTS

We thank B. Stam and J.L.M. Uytdewilligen for excellent technical assistance.

Part II:

Population and clinical based studies

Chapter 4

Yield of routine molecular analyses in colorectal cancer patients ≤ 70 years to detect underlying Lynch syndrome

Margot G.F. van Lier, Celine H.M. Leenen, Anja Wagner, Dewkoemar Ramsoekh, Hendrikus J. Dubbink, Ans M.W. van den Ouweland, Pieter J. Westenend, Eelco J.R. de Graaf, Leonieke M.M. Wolters, Wietske W. Vrijland, Ernst J. Kuipers, Monique E. van Leerdam, Ewout W. Steyerberg, Winand N.M. Dinjens

On behalf of the LIMO study group

The Journal of Pathology 2012;226(5):764-74

ABSTRACT

Although early detection of Lynch syndrome (LS) is important, a considerable proportion of patients with LS remains unrecognized. We aimed to study the yield of LS detection by routine molecular analyses in colorectal cancer (CRC) patients until 70 years of age.
We prospectively included consecutive CRC patients ≤ 70 years. Tumor specimens were analyzed for microsatellite instability (MSI), immunohistochemical mismatch-repair protein expression, and *MLH1* promoter methylation. Tumors were classified as either 1) likely caused by LS, 2) sporadic microsatellite unstable (MSI-H), or 3) microsatellite stable (MSS). Predictors of LS were determined by multivariable logistic regression. A total of 1117 CRC patients (57% males, median age 61 years) were included. Fifty patients (4.5%; 95% CI 3.4-5.9) were likely to have LS, and 71 had a sporadic MSI-H tumor (6.4%; 95% CI 5.1-8.0). Thirty-five patients likely to have LS (70%) were > 50 years. A molecular profile compatible with LS was detected in 10% (15/144) of patients ≤ 50, in 4% (15/377) of those aged 51-60, and in 3% (20/596) of patients older than 61 years. Compared to MSS cases, patients likely to have LS were significantly younger (OR 3.9; 95% CI 1.7-8.7) and had more often right-sided CRCs (OR 14; 95% CI 6.0-34). In conclusion, molecular screening for LS in CRC patients ≤ 70 years leads to identification of a molecular profile compatible with LS in 4.5% of patients, with most of them not fulfilling the age-criterion (≤ 50 years) routinely used for LS-assessment. Routine use of MSI-testing may be considered in CRC patients up to the age of 70 years, with a central role for the pathologist in the selection of patients.

INTRODUCTION

Lynch syndrome (LS) is the most common form of hereditary colorectal cancer (CRC), responsible for approximately 3% of all CRCs.[66, 145] LS is caused by a germline mutation in one of the mismatch repair (MMR) genes; *MLH1*, *MSH2*, *MSH6* and *PMS2*. The burden of LS is considerable, as the cancers are generally diagnosed at a young age and synchronous or metachronous malignancies occur in 30% of the patients. Furthermore, extra-colonic LS-associated malignancies frequently occur.[40, 146, 147]

Early detection of LS is important, since colonoscopic surveillance has been proven to reduce CRC morbidity and mortality by 65-70%.[18-20] However, the diagnosis of LS is complicated by the absence of a pre-morbid phenotype and DNA mutation analysis to confirm the diagnosis is time-consuming and expensive. MMR gene mutations lead to microsatellite instability (MSI) in tumor DNA, the molecular hallmark of LS. As MSI can be detected in approximately 95% of all LS-associated cancers, MSI-analysis can be used in the diagnostic approach of LS.[38-40] The revised Bethesda guidelines have been developed to select patients for MSI-analysis, in order to identify patients at high risk for LS.[84]

The combination of the revised Bethesda guidelines and MSI-testing is currently the most widely accepted approach for the identification of LS patients. However, the Bethesda guidelines have been criticized for being too complex for readily use,[78] and it has been shown that these criteria are poorly implemented in clinical practice.[148-150] In addition, several prediction models have been developed to quantitatively estimate the risk of LS on the basis of personal and familial data,[27, 82, 85, 86, 151, 152] but the implementation of these models into clinical practice is limited. Together, this leads to a suboptimal detection of LS and the concern that many if not most mutation-carriers are not being identified.[153, 154]

Therefore clinicians and researchers are searching for new simple strategies to improve the detection of LS. The aim of the present prospective population-based study was to evaluate the yield of routine molecular analyses, including MSI-analysis, in consecutive CRC patients ≤ 70 years and patients with advanced colorectal adenomas ≤ 45 years.

METHODS

In this prospective multicenter population-based study, we included all consecutive patients newly diagnosed with either an invasive colorectal adenocarcinoma ≤ 70 years, or an advanced colorectal adenoma ≤ 45 years. Adenomas were considered advanced when they were either ≥ 10 mm in diameter, showed a villous component or high-grade dysplasia, or when at least 3 synchronous adenomas (regardless of size and histology) were found. Patients were included between May 2007 and September 2009 in 11 Dutch

hospitals including one academic medical center and 10 general hospitals (5 pathology laboratories). Patients were identified by monthly electronic searches in the institutional pathology databases. The following data were anonymously collected from the original pathology reports (i.e. not by re-evaluation of pathological specimen): gender, age at diagnosis, tumor-characteristics including MSI-related histology features (i.e. presence of tumor-infiltrating lymphocytes, Crohn's like lymphocytic reaction, mucinous / signet-ring differentiation, or medullary growth pattern),[84] TNM-stage (5th edition) and localization. Tumors were defined as right-sided if located at or proximal to the splenic flexure, and left-sided when distal to the splenic flexure. Patients previously diagnosed with (attenuated) FAP and MAP were excluded.

Routine formalin-fixed and paraffin-embedded (FFPE) tissue blocks, either from a surgical resection or, if not available, a diagnostic biopsy specimen, were collected from all included patients. Whenever possible, a biopsy specimen as well as a resection specimen were collected from rectal cancer patients treated with neo-adjuvant radiotherapy or chemoradiation (i.e. TNM ≥ T2), to evaluate possible therapy-effects on MSI-status. The collected tissue-samples were analyzed as shown in Figure 1. First, MSI-analysis and evaluation of immunohistochemical MMR protein expression (IHC) were performed in all patients. In case of a microsatellite unstable tumor (MSI-H, see below) with absent

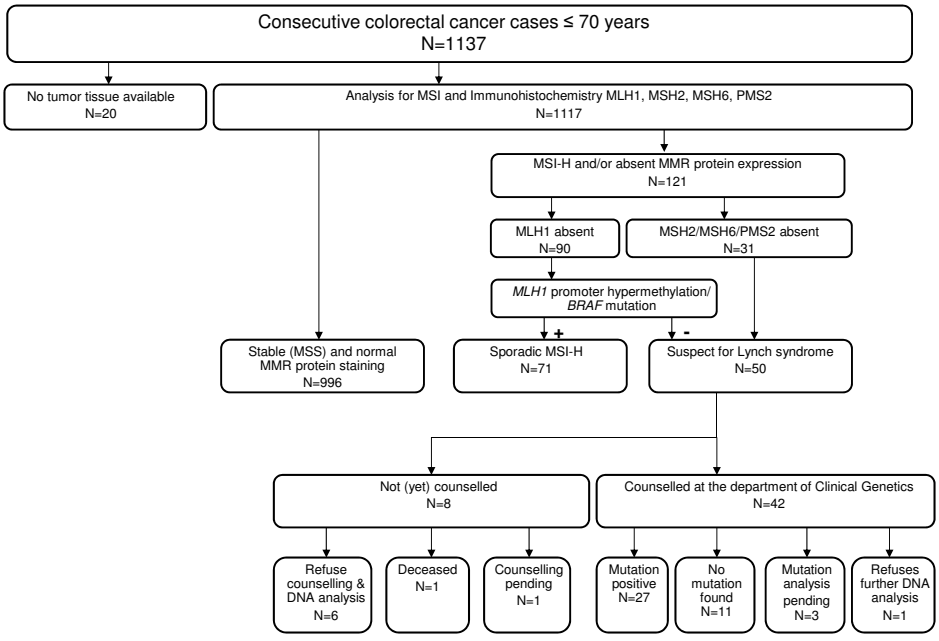


Figure 1. Flow chart of molecular analyses performed on tumor tissue of CRC patients ≤ 70 years.
MSI, microsatellite instability; MMR, mismatch repair; MSS, microsatellite stable; MSI-H, high degree of microsatellite instability; LS, Lynch syndrome.

MLH1 protein expression, we also studied hypermethylation of the *MLH1* promoter and somatic *BRAF* mutations. This was done as MSI can be seen in approximately 15% of sporadic CRCs due to *MLH1* promoter methylation.[155] *MLH1* promoter methylation is in its turn associated with somatic *BRAF* mutations.[156]

MSI-analysis

MSI-analyses were performed on DNA derived from micro-dissected FFPE tumor tissue, using a panel of pentaplex markers as previously described.[55] Tumors with more than one unstable marker were categorized as having a high degree of microsatellite instability (MSI-H). Those with one or no unstable marker were categorized microsatellite stable (MSS).

Immunohistochemistry, *MLH1* promoter hypermethylation assay, & *BRAF* mutation analysis

Immunohistochemistry, *MLH1* promoter hypermethylation assay, and *BRAF* mutation analysis were performed as previously described.[55] IHC analyses were performed for the mismatch repair proteins MLH1, MSH2, MSH6 and PMS2. If there was no MLH1 expression in tumor cells, the methylation status of the *MLH1* promoter was determined by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA), and *BRAF* alterations of mutational hotspot codon V600 were determined by bi-directional cycle-sequencing of PCR-amplified fragments. Additionally, in all MSI-H tumors the methylation status of the other MMR genes (*MSH2*, *MSH6* and *PMS2*) was determined.

Analyzed tumors were classified as either 1) likely caused by LS if MSI-H and simultaneously showing absent MMR protein expression, with exclusion of *MLH1* promoter hypermethylation and/or *BRAF* mutation in the case of absent MLH1 expression, 2) sporadic MSI-H tumors displaying absent MLH1 expression and established *MLH1* promoter hypermethylation and/or *BRAF* mutation, or 3) sporadic, microsatellite stable (MSS) tumors. If difficulties occurred in the interpretation of the MSI or IHC results, the analyses were repeated on biopsy tissue if available.

The study was approved by the Institutional Review Boards of the participating hospitals. Patients were informed about the study by their gastroenterologist or surgeon and received written information. In the written information the relevance of early detection of hereditary CRC was addressed and it was explained that complementary pathological examinations were performed, possibly indicating an elevated risk of hereditary CRC. The information folder allowed patients to lodge an objection to the molecular analyses. If they did object, their archival tissue-blocks were anonymously collected informing neither the patient nor their doctor about the results of the additional analyses. Otherwise the results were discussed with the patient by their doctor, and patients likely to

have LS were referred to the department of Clinical Genetics for counseling eventually followed by germline mutation analysis.

Germline mutation analysis

In DNA isolated from peripheral blood samples all coding regions and intron-exon boundaries of the *MLH1*, *MSH2* and/or *MSH6* gene were completely and systematically analysed using direct sequence analysis. Reaction products were analysed using a capillary automated sequencer (details of method and primer sequences available on request). In addition, MLPA kits P003 and P072 (MRC-Holland) were used to detect large rearrangements. The mutation analysis of *PMS2* was performed as previously described.[157] Germline mutation analyses were guided by the results of the immunohistochemical MMR protein expression, with the most likely affected gene being examined first.[55] In case of an MSI-H tumor with absent MSH2 (and MSH6) expression (and *MSH2* promoter methylation) without a detectable MMR gene mutation, the *TACSTD1* gene was analyzed.[30]

Data were analyzed using the SPSS 15.0 statistical software for Windows, and were reported using descriptive statistics. The incidence of a molecular profile compatible with LS and a sporadic MSI-H phenotype were analyzed, and predictive factors for LS and sporadic microsatellite instability were determined by multivariable logistic regression analyses. Finally, we assessed differences in MSI-status before and after neo-adjuvant therapy in cases with advanced rectal cancers. Two-sided p-values less than 0.05 were considered significant.

RESULTS

Colorectal cancer patients

A total of 1137 CRC patients were eligible for inclusion in this study. Twenty patients had to be excluded as there was either no vital tumor tissue left for the analyses (n = 9), or the tumor specimen could not be collected (n = 11). The 1117 included cases (57% males) had a median age of 61 years (interquartile range 55-66) and the youngest patient was 27 years old. Most CRC patients were older than 50 years and 28% of all CRCs were located in the right colon (Table 1). Only 4 patients (0.4%) lodged an objection to the molecular analyses.

The molecular analyses revealed a profile compatible with LS in 50 of the 1117 CRCs (4.5%; 95% CI 3.4-5.9) and 71 sporadic MSI-H tumors (6.4%; 95% CI 5.1-8.0). Thirty-five of the 50 patients likely to have LS (70%) were older than 50 years at CRC diagnosis. On the basis of immunohistochemical protein expression and *MLH1* promoter methylation

Table 1. Characteristics of 1117 CRC patients and results of molecular analyses.

	MSS	Likely caused by LS	Sporadic MSI-H	Total
	N = 996 (89.2%)	N = 50 (4.5%)	N = 71 (6.4%)	N = 1117 (100%)
Age in years				
Median (IQR)	61 (55-66)	57 (49-65)	64 (61-68)	61 (55-66)
Age ≤ 50 years	128 (13%)	15 (30%)	1 (1.5%)	144 (13%)
Gender				
Males	579 (58%[a])	35 (70%)	19 (27%)	633 (57%)
Localisation	N = 947 (49 NS)	N = 48 (2 NS)	N = 69 (2 NS)	N = 1064 (53 NS)
Right-sided [b]	204 (21.5%)	34 (71%)	62 (90%)	300 (28%)
MSI-H histology [c]	N = 107 (11%)	N = 13 (26%)	N = 22 (31%)	N 142 (13%)
Mucinous	99	12	20	131
Signet ring cells	14	3	2	19
Medullary	-	-	-	-
TIL's [d]	-	-	1	1
Crohn's reaction [e]	-	-	-	-
TNM-stage	N = 660 (336 NS)	N = 38 (12 NS)	N = 58 (13 NS)	N = 756 (361 NS)
I	159	11	8	178
IIA	158	12	23	193
IIB	33	4	6	43
IIIA	53	-	-	52
IIIB	113	6	7	126
IIIC	106	5	11	122
IV	38	-	3	41

CRC, colorectal carcinoma; MSS, microsatellite-stable; LS, Lynch syndrome; MSI-H, high degree of microsatellite instability; NS, not further specified; IQR, interquartile range.

[a] i.e. 58% of all patients with an MSS tumor were male.

[b] CRCs were defined to be right-sided if located at or proximal to the splenic flexure, and left-sided when distal to the splenic flexure.

[c] Several tumors (n = 18) displayed both a mucinous differentiation as well as signet ring cells.

[d] Tumor-infiltrating lymphocytes.

[e] Crohn's-like lymphocytic reaction.

status, 20 patients were suspect for a *MLH1* gene defect, 11 for *MSH2*, 13 for *MSH6* and 6 for a *PMS2* defect.

So far, 42 patients have been referred and counselled. A pathogenic MMR gene mutation has thus far been found in 26 of them (5 *MLH1*, 5 *MSH2*, 11 *MSH6*, and 5 *PMS2* mutations, Table 2), and in one case showing *MSH2* promoter hypermethylation, a deletion in *TACSTD1* (*EPCAM*) was detected. In 11 patients likely to have LS no MMR gene mutation has been detected so far, mutation analyses are still pending in 3 patients, and 1 patient refused DNA analysis. Four cases from known MMR-gene positive families were blindly included for the molecular analyses, and in all these cases the known involved MMR gene was indicated. Thirty-five of all 42 patients (83%) referred for counseling did not have a family history indicative for LS (i.e. fulfilling the Amsterdam criteria II[81]), and 24

Table 2. Characteristics of 27 CRC patients and a germline mutation in an MMR gene or *TACSTD1*.

Gender	Age CRC diagnosis	Histology	MSI-H Histology	Localisation	Criteria		MSI	IHC	Methylation status	Gene	Nucleotide change
					AC	rBC	No. of positive markers	Absent MMR protein			
M	29	Moderately differentiated adenocarcinoma	Mucinous	Transverse	Yes	Yes	5/5	MLH1 / PMS2	*MLH1* unmethylated	*MLH1* (known LS family)	c.1732-?_1896del
M	31	Moderately differentiated adenocarcinoma	Mucinous	Ascending	No	Yes	4/5	MSH6		*MSH6* (UV)	c.1153_1155del
M	33	Moderately differentiated adenocarcinoma		Sigmoid	No	Yes	5/5	MSH2 / MSH6		*MSH2*	c.1226_1227delAG
M	35	Poorly differentiated adenocarcinoma		Colon NFS	No	Yes	5/5	PMS2		*PMS2*	c.2192_2196del5
M	37	Moderately differentiated adenocarcinoma		Hepatic flexure	Yes	Yes	5/5	MLH1 / PMS2	*MLH1* unmethylated	*MLH1* (known LS family)	545+?del
F	41	Well-differentiated adenocarcinoma		Ascending	No	Yes	5/5	MSH2 / MSH6		*MSH2* (UV)	c.2068C>G
F	48	Moderately differentiated adenocarcinoma		Sigmoid	No	Yes	5/5	PMS2		*PMS2*	Deletion exon 6 and 7
M	48	Moderately differentiated adenocarcinoma		Sigmoid	No	Yes	5/5	MSH6		*MSH6*	c.1614_1615delTCinsAG
M	49	Moderately differentiated adenocarcinoma	Mucinous	Rectum	No	Yes	3/5	MSH6		*MSH6*	c.3991C>T p.Arg1331X

Table 2. (Continued)

Gender	Age CRC diagnosis	Histology	MSI-H Histology	Localisation	Criteria		MSI	IHC	Methylation status	Gene	Nucleotide change
					AC	rBC	No. of positive markers	Absent MMR protein			
M	53	Moderately differentiated adenocarcinoma	Mucinous	Cecum	No	No	5/5	MLH1 / PMS2	*MLH1* unmethylated	*MLH1* (UV)	M1T (2T>C)
F	53	Adenocarcinoma NFS	Mucinous	Cecum	No	Yes	5/5	PMS2		*PMS2*	c.736_741del6ins11
F	53	Adenocarcinoma NFS	Mucinous & signet-ring cells	Cecum	Yes	Yes	5/5	MSH2 / MSH6		*MSH6*	c.467C>G
M	55	Adenocarcinoma NFS		Left	Yes	Yes	5/5	MSH2 / MSH6		*MSH2*	c.1511-2A>G
M	57	Moderately differentiated adenocarcinoma		Right	Yes	No	5/5	MSH2 / MSH6		*MSH2* (known LS family)	c.367-?_645+?del
F	57	Moderately differentiated adenocarcinoma		Sigmoid	Yes	Yes	2/5	MSH6		*MSH6*	c.1784delT
M	59	Adenocarcinoma NFS		Transverse	No	No	4/5	MSH6		*MSH6*	c.3477delC
M	59	Well - Moderately differentiated adenocarcinoma		Ascending	No	Yes	5/5	MLH1 / PMS2	*MLH1* unmethylated	*MLH1*	E632E, c.1896G>A
F	61	Moderately differentiated adenocarcinoma		Hepatic flexure	No	Yes	5/5	MLH1 / PMS2	*MLH1* unmethylated	*MLH1*	c.378C>A
M	63	Moderately differentiated adenocarcinoma		Cecum	No	No	5/5	MSH2 / MSH6		*MSH2*	c.1077-?_1386+?del
M	63	Adenocarcinoma NFS	Mucinous & signet-ring cells	Right	No	Yes	5/5	MSH6		*MSH6*	2150delTCAG

Table 2. (Continued)

Gender	Age CRC diagnosis	Histology	MSI-H Histology	Localisation	Criteria		MSI	IHC	Methylation status	Gene	Nucleotide change
					AC	rBC	No. of positive markers	Absent MMR protein			
F	63	Adenocarcinoma NFS		Rectum	No	No	5/5	MSH6		*MSH6*	c.1991C>G
M	66	Moderately differentiated adenocarcinoma		Sigmoid	No	No	5/5	PMS2		*PMS2*	c.2177delA
F	66	Moderately differentiated adenocarcinoma	Mucinous	Cecum	No	No	5/5	MSH6		*MSH6*	458-3392_627+923delins70
F	66	Moderately differentiated adenocarcinoma		Sigmoid	No	Yes	2/5	MSH6		*MSH6* (known LS family)	c.3519_3522dupGTTT
M	69	Poorly differentiated adenocarcinoma		Cecum	No	Yes	5/5	PMS2		*PMS2*	c.736_741delins11
F	70	Moderately - poorly differentiated adenocarcinoma		Cecum	No	Yes	5/5	MSH6		*MSH6*	c.-11863_457+1919del21672ins8
M	56	Adenocarcinoma NFS		Sigmoid	Yes	Yes	5/5	MSH2 / MSH6	*MSH2* methylated	*TACSTD1*	

rBC, revised Bethesda Guidelines; AC, Amsterdam Criteria II, MSI, microsatellite instability; IHC, immunohistochemistry of MMR genes; MMR, Mismatch Repair; MSS, microsatellite stable; MSI-H, high degree of microsatellite instability; UV, unclassified variant; NA, not available; NFS, not further specified.

patients (57%) fulfilled the criteria of the revised Bethesda guidelines. In addition, six patients refused referral to the clinical geneticist, one patient died before referral, and counseling is pending in another patient.

Multivariable analyses demonstrated that CRC patients likely to have LS were significantly younger at cancer diagnosis (OR 3.9; 95% CI 1.7-8.7), had more often right-sided CRCs (OR 14; 95% CI 6.0-34) and had cancers with a lower TNM-stage at diagnosis (OR 0.44; 95% CI 0.28-0.69) than patients with MSS tumors (Table 1). A profile compatible with LS was detected predominantly in younger patients: in 15 of the 144 patients (10%) ≤ 50 years, in 15 of 377 patients (4%) aged 51-60 years, and in 20 of 596 patients (3%) aged 61 years or older. Conversely, a sporadic MSI-H status was more often detected in older patients; in 1/144 (1%) of those aged ≤ 50 years, in 15/377 (4%) of those aged 51-60 years, and in 55/596 (9%) of the patients aged 61 or older (Figure 2).

Furthermore, compared to MSS-tumors, sporadic MSI-H tumors were significantly more often located right-sided (OR 43; 95% CI 15-125), more often displayed MSI-H histology features (OR 2.4; 95% CI 1.2-4.8) and had a lower TNM-stage at time of diagnosis (OR 0.65; 95% CI 0.44-0.96). In addition, patients with sporadic MSI-H tumors were more often female (OR 0.34; 95% CI 0.17-0.66) and older than 50 years (OR 0.12; 95% CI 0.02-

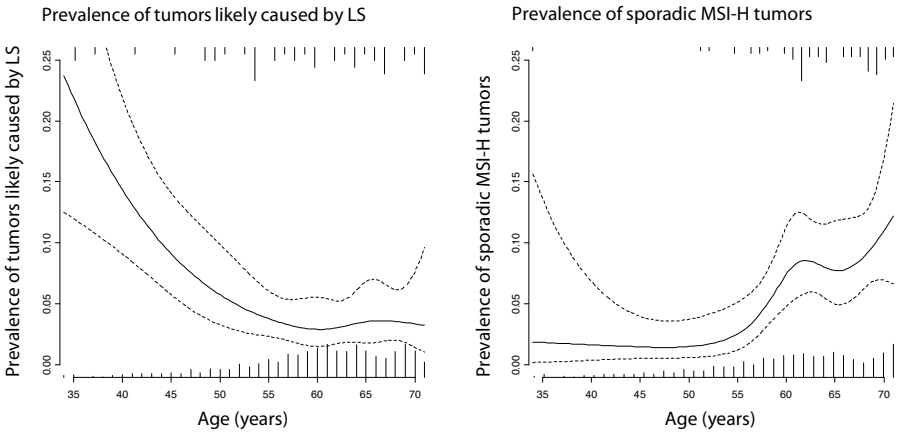


Figure 2. Correlation between age and the results of the molecular analyses. LS, Lynch syndrome; MSI-H, high degree of microsatellite instability. Spline functions with 95% confidence interval. Spikes indicate the ages of individual patients with and without the outcome of interest.
Left panel: Prevalence of tumors likely caused by Lynch syndrome according to age; the chance to detect a patient likely to have Lynch syndrome by molecular analyses clearly decreases with age.
Right panel: Prevalence of sporadic MSI-H tumors according to age; the chance to detect a patient with a sporadic MSI-H tumor by molecular analyses clearly increases with age.

0.92) compared to patients with a MSS tumor. In none of the 50 CRCs likely caused by LS and in 46 of the 71 sporadic MSI-H tumors a V600E *BRAF* mutation was detected.

Of 122 included patients with advanced rectal cancer who were treated by neo-adjuvant chemoradiation prior to resection, both pre-treatment biopsy specimens as well as resection specimens were analyzed. MSI-status did not differ between biopsy and corresponding resection specimen.

Advanced adenoma cases

A total of 130 patients with advanced adenoma were eligible for inclusion in this study. In 125 (96%) of these cases, adenomatous tissue was available for molecular analyses. These 125 subjects (58% males) had a median age of 41 years (interquartile range 37-44, Table 3). Three male adenoma patients (2.4%; 95% CI 0.5-7.1%) aged 34, 41, and 44 years, were likely to have LS. One of these three patients fulfilled the Amsterdam criteria II, and two patients fulfilled the revised Bethesda guidelines. IHC showed lack of MLH1/ PMS2 expression in two of these three patients, and lack of MSH6 expression in one patient. In all three patients a pathogenic MMR gene germline mutation was identified by DNA analysis (two *MLH1* and one *MSH6* mutation, Table 4).

Table 3. Characteristics of 125 patients with advanced adenoma and results of molecular analyses.

	MSS	**Likely caused by LS**	**Sporadic MSI-H**	**Total**
	N= 121 (96.8%)	N= 3 (2.4%)	N= 1 (0.8%)	N= 125
Age in years				
Median (IQR)	41 (37-44)	41 (34-44)	44	41 (37-44)
Gender				
Males	70	3	0	73 (58%)
Localisation				N = 113 (12 NS)
Right-sided #	31	0	0	31
Advanced				
Villous component	77	2	1	80
High grade dysplasia	30	0	0	30
Size ≥ 10 mm	47	1	0	48
≥ 3 synchronous adenomas	14	0	0	14

MSS, microsatellite-stable; LS, Lynch syndrome; MSI-H, high degree of microsatellite instability; NS, not further specified; IQR, interquartile range.
Adenomas were defined to be right-sided if located at or proximal to the splenic flexure, and left-sided when distal to the splenic flexure.

Table 4. Characteristics of three patients with advanced colonic adenomas and a germline mutation in an MMR gene.

Gender	Age	Location	Histology	No. of adenomas	Size of adenoma	Criteria		MSI	IHC	Methylation status	Gene	Nucleotide change
						AC	rBC	No. of pos markers	Absent MMR protein			
M	34	rectum	Tubulovillous Low grade dysplasia	1	< 10 mm	No	Yes	3/5	MLH1/ PMS2	*MLH1* unmethylated	*MLH1* (known LS family)	c.1614del18
M	42	sigmoid	Tubulovillous Low grade dysplasia	1	< 10 mm	Yes	Yes	3/5	MSH6	NA	*MSH6*	deletion exon 3
M	44	rectum	Tubular Low grade dysplasia	1	≥ 10 mm	No	No	3/5	MLH1/ PMS2	*MLH1* unmethylated	*MLH1*	c.676C>T

AC, Amsterdam Criteria II; rBC, revised Bethesda Guidelines; MSI, microsatellite instability; IHC, immunohistochemistry of MMR genes; MMR, Mismatch Repair; MSS, microsatellite stable; MSI-H, high degree of microsatellite instability; known LS, known Lynch syndrome family; NA, not analyzed.

DISCUSSION

This prospective study shows that routine molecular screening for LS in CRC patients ≤ 70 years leads to the identification of a profile compatible with LS in 4.5%. Seventy percent of these patients are older than 50 years at the time of CRC diagnosis and do not meet the age-criterion routinely used for LS assessment. In patients with an advanced adenoma ≤ 45 years a molecular profile compatible with LS was detected in 2.4 %. These adenoma patients would not have been detected by the current screening guidelines such as the revised Bethesda guidelines. The detection of CRC and adenoma patients likely to have LS is of major importance since these patients and their family members at risk (carriers) can enter surveillance programs which have been proven to reduce CRC morbidity and mortality by 65-70%.[18-20] Our analyses furthermore revealed a sporadic MSI-H status in 6.4% of all analyzed CRCs. The establishment of a sporadic MSI-H status by *MLH1* promoter hypermethylation assay and to a lesser extent by *BRAF* mutation analysis considerably reduced the number of patients referred for counseling and germline genetic testing at the Clinical Genetics department. Furthermore, the establishment of a sporadic MSI-H status reduced the number of patients worrying about being an MMR gene mutation carrier.

Of all CRC patients likely to have LS immunohistochemistry indicated *MSH6* or *PMS2* gene involvement in 24% and 12%, respectively. These percentages are higher than expected,[158-160] but in line with a previous report on LS-detection in the Netherlands, describing a high incidence of *MSH6*-mutations.[83] The relatively high incidence of *MSH6* and *PMS2* involved in LS may be explained by the fact that *MSH6* and *PMS2* mutations

have for a long time been underestimated due to the more atypical presentation of disease in *MSH6* and *PMS2* families.[28, 161] In addition, regional differences in germline mutation frequency of *MSH2* and *PMS2* cannot be excluded.

Since we do not have data on family history of all included patients, we cannot compare the yield of our strategy in terms of LS detection to other strategies including the revised Bethesda guidelines,[84] the Amsterdam criteria II[81] and several prediction models.[27, 82, 85, 86, 151, 152] However, the Bethesda guidelines have been criticized for being too complex to use and have been proven to be poorly implemented in clinical practice.[148-150, 162] The Amsterdam criteria II are predominantly hampered by a low sensitivity,[163] and although some of the prediction models to estimate the risk of LS have been validated in population-based cohorts of CRC patients,[87, 89, 164] the implementation of these models in clinical practice is still in its infancy. Therefore, the MIPA criteria (MSI-testing-Indicated-by-a-Pathologist) have been developed.[78] The MIPA criteria simplify the Bethesda guidelines in such a way that pathologists, without knowledge of family history, can select patients for MSI analysis. They resemble our strategy, yet the MIPA criteria recommend MSI analysis in patients newly diagnosed with CRC before age 50, or before age 70 in patients diagnosed with two LS-associated cancers. As we demonstrate that most CRC patients likely to have LS are > 50 years, our strategy may thus help to detect more LS patients.

As neo-adjuvant chemotherapy may alter MMR protein expression in cancer cells[165] and exposure to ionising radiation promotes the development of MSI in mouse tumors,[166] one might hypothesize that neo-adjuvant (chemo)radiation as advocated for advanced rectal cancers,[167] may influence MSI-status. This is an interesting hypothesis, as MSI-analyses are usually performed on surgical resection specimens. Therefore we assessed differences in MSI-status in 122 rectal cancers before and after neo-adjuvant therapy. No differences in MSI-status were found, suggesting that neo-adjuvant (chemo) radiation has no effect on MSI-status and that surgical resection specimens can be used for MSI-analysis.

In contrast to the revised Bethesda guidelines, the original Bethesda guidelines recommended MSI analysis on adenomas of patients < 40 years.[168] Yet, this was found to be ineffective to identify new LS cases.[169, 170] Nevertheless, it has been demonstrated that most adenomas of LS patients show MSI.[171, 172] Furthermore, LS-associated adenomas have been demonstrated to be larger and to have a higher proportion of villous components and/or high grade dysplasia than patients without LS,[26] and MSI-analysis is more reliable in these high risk adenomas.[173] Therefore, we also analysed advanced adenomas of patients younger than 45 years, revealing three patients with LS that would not all have been detected by current screening guidelines. It remains to be established whether or not it is cost-effective to screen advanced adenomas of young patients for LS.

The strength of this study lies in the fact that we performed a prospective population-based study in which we evaluated routine molecular screening for LS in CRC patients using a high age cut-off. The high age cut-off allowed us to gain insight in the correlation between age and diagnostic yield (i.e. LS detection, Figure 2). We chose for an age cut-off of 70 in CRC patients in order to compromise between the feasibility of MSI-analysis in a large number of patients on the one hand, and a reasonable detection of patients likely to have LS on the other hand. In the future, it might be considered to subject all newly diagnosed CRC patients to MSI analysis, as MSI analysis is not only valuable for detection of LS, but also has prognostic and therapeutic implications. Regardless of stage at diagnosis, microsatellite unstable CRCs (including sporadic MSI-H cancers) are associated with a better prognosis than MSS tumors [57, 174] and patients with MSI-H tumors do not seem to benefit from adjuvant chemotherapy with 5-fluorouracil.[57, 61, 63] However, as the optimal strategy for adjuvant therapy of MSI-H cancers still needs to be established, we do not believe that it is justified to screen all CRCs for MSI at this stage.

This study also has some limitations. Since data on family history are lacking, we cannot compare the yield of our strategy in terms of LS detection to other strategies in which family history is one of the cornerstones. However, obtaining a thorough family history is difficult in clinical practice,[79] CRC patients frequently report their family history inaccurately,[175-177] and it may become more difficult to identify LS patients on the basis of family history as family sizes are decreasing. Second, the molecular analyses used in this study also have some drawbacks as previously described.[55] Because we could not perform DNA mutation analysis of the MMR genes in all included subjects, some LS cases may have been missed. LS has not been confirmed by germline mutation analysis in all cases likely to have LS yet as in a few cases analyses are still pending (n=3) and one patient died before he could be referred to the clinical geneticist (in two other deceased cases first degree relatives have been counselled). Furthermore, in a small proportion of patients likely to have LS, no germline MMR gene mutation could be detected by DNA mutation analysis (n=11). Yet, these patients might still suffer from an inherited MMR defect that escaped detection by the used DNA sequencing strategy. A final limitation is that some clinicians may have ethical objections against molecular screening for LS prior to genetic counseling, since the results of the molecular analyses, especially the IHC results, can be very suggestive for LS. However the molecular analyses do not establish the diagnosis of LS, but make underlying LS at best likely. Therefore, we believe that the benefits of accurate LS diagnosis outweigh potential negative effects, especially when the clinician informs the patient that the diagnostic pathological examinations not only give information about the nature of the tumor, but may also indicate an elevated risk of an underlying hereditary disorder. Yet, we underscore that germline analysis for LS should exclusively be performed after genetic counseling.

In conclusion, this study demonstrates that routine molecular screening for LS in CRC patients ≤ 70 years with a central role for the pathologist in the selection of patients, leads to the identification of a profile compatible with LS in 4.5%. For advanced adenoma patients ≤ 45 years a molecular profile compatible with LS was detected in 2.5%. Identification of LS is of major relevance for these patients as well as their affected family members, as CRC-related morbidity and mortality can be reduced by colonoscopic surveillance. As most CRC patients likely to have LS were older than 50 years and do not meet the age-criterion routinely used for LS assessment, and because routine molecular screening is easy to implement in clinical practice, our strategy may help to increase the detection of LS. However, the cost-effectiveness of this approach as well as the optimal age cut-off for molecular screening remain to be established.

Chapter 5

Prospective evaluation of molecular screening for Lynch syndrome in patients with endometrial cancer ≤ 70 years

Celine H.M. Leenen, Margot G.F. van Lier, Helena C. van Doorn, Monique E. van Leerdam, Sjarlot G. Kooi, Judith de Waard, Robert F. Hoedemaeker, Ans M.W. van den Ouweland, Sanne M. Hulspas, Hendrikus J. Dubbink, Ernst J. Kuipers, Anja Wagner, Winand N.M. Dinjens, Ewout W. Steyerberg

On behalf of the LIMO study group

Gynecologic Oncology 2012;125(2):414-20

ABSTRACT

Objective: Lynch syndrome (LS) is a hereditary syndrome that predisposes to multiple malignancies including endometrial cancer (EC). We aimed to evaluate a diagnostic strategy for LS based on routine analysis of microsatellite instability (MSI) and immunohistochemical (IHC) staining for mismatch repair (MMR) proteins in tumor tissue of all newly diagnosed EC patients ≤ 70 years.

Methods: Consecutive EC patients ≤ 70 years were included prospectively in eight Dutch centers. EC specimens were analyzed for MSI, IHC of four MMR proteins, MMR gene methylation status and *BRAF* mutations. Tumors were classified as; 1) likely to be caused by LS, 2) sporadic MSI-H, or 3) microsatellite stable (MSS).

Results: Tumor specimens of 179 patients (median age 61 years, IQR 57-66) were analyzed. In our study 92% of included patients were over 50 years of age. Eleven EC patients were found likely to have LS (6%; 95% CI 3-11%), including 1 patient suspected of an *MLH1*, 2 of an *MSH2*, 6 of an *MSH6* and 2 of a *PMS2* gene defect. Germline mutation analyses revealed 7 MMR gene germline mutations. Ten patients likely to have LS (92%) were older than 50 years. In addition, 31 sporadic MSI-H tumors with *MLH1* promoter hypermethylation (17%; 95% CI 13-24%) were identified.

Conclusions: Molecular screening for LS in patients with EC diagnosed ≤ 70 years, leads to identification of a profile likely to have LS in 6% of cases. New screening guidelines for LS are needed, including recommendations for EC patients older than 50 years of age.

INTRODUCTION

Lynch syndrome (LS) is an autosomal dominant inherited syndrome that predisposes to multiple malignancies including endometrial cancer (EC). The lifetime risk of women with LS to develop EC is 40 to 60%. In addition, patients with LS carry a lifetime risk of 50 to 85% to develop colorectal cancer (CRC) and also an increased risk of up to 15% to develop other malignancies including gastric, ovarian, small bowel and urinary tract cancers.[14-16, 178]

LS is caused by a mutation in one of the mismatch repair (MMR) genes; *MLH1*, *MSH2*, *MSH6* or *PMS2*. As a consequence, LS tumors are MMR deficient and phenotypically characterized by DNA microsatellite instability (MSI). MSI can be detected in more than 90% of all ECs in LS mutation carriers.[179] Therefore, MSI analysis can be used in the diagnostic approach of LS.[40] However, MSI can also be detected in 17 to 23% of sporadic ECs,[180, 181] which is mostly caused by transcriptional silencing of the *MLH1* gene by promoter hypermethylation.[182, 183]

In addition to MSI analysis, immunohistochemical (IHC) analyses can be performed to evaluate the expression of the four MMR proteins. Tumors showing absent MLH1 expression can be selected for *MLH1* promoter methylation assay to identify sporadic MSI-H tumors. In case of MSI and loss of MMR protein expression, with exclusion of *MLH1* promoter hypermethylation in MLH1 negative tumors, further germline DNA testing is indicated for LS associated MMR genes to make the final diagnosis LS.

Early detection of LS in EC patients is of great importance, since LS carriers are at risk of other cancers, especially CRC, and this risk can significantly be reduced by colonoscopic surveillance.[18, 20, 184] Selection of patients for molecular testing for LS is currently based on clinical criteria, in particular the Amsterdam criteria II and the revised Bethesda guidelines.[81, 185] In the Amsterdam criteria II, EC is included as a diagnostic parameter. However, the Amsterdam criteria II lack sensitivity, particularly in cases of small families or when extensive family history information is not available.[83, 186] The revised Bethesda guidelines focus primarily on patients with CRC and not with EC. Furthermore, current clinical guidelines advise molecular testing for LS for patients with EC below the age of 50 years. This contributes to the concern that LS in EC patients remains undetected.

Therefore, the aim of this prospective multicenter study was to evaluate the feasibility and the yield of large scale molecular analyses in patients newly diagnosed with EC aged 70 years and younger.

METHODS

Endometrial Cancer Patient Population

All consecutive patients ≤ 70 years newly diagnosed with invasive EC of epithelial origin were included in eight Dutch hospitals, including seven regional hospitals and one academic medical center, between May 2007 and September 2009. Patients were identified by monthly electronic searches in the pathology databases of the participating centers. Data were collected on age at diagnosis and tumor-characteristics including histological subtype and pathological tumor (T) stage.

The study was approved by the Institutional Review Boards of the participating hospitals. Prior to cancer treatment the patients were informed about the study by their gynaecologist and they received an information folder about the research project. This folder also enabled patients to lodge an objection to receive the results of the molecular analyses. In case patients did not want to receive the results of molecular analyses but did want to be included in the study, their archival tissue-blocks were collected anonymously without informing the patient or their doctor about the results of the molecular analyses. The treating gynaecologists discussed the test results with their patients. Patients, who wanted to be informed and were likely to have LS based on the molecular profile in their tumor, were referred to the department of Clinical Genetics for counseling and germline mutation analysis.

Routine formalin-fixed, paraffin-embedded (FFPE) tissue blocks were collected from all included EC patients. The collected tissue-samples were analyzed for MSI and IHC of MMR-protein expression was performed (Figure 1). In microsatellite unstable tumors (MSI-H) with loss of MLH1 expression, hypermethylation of the *MLH1* promoter was investigated to identify sporadic MSI-H tumors.

Tumors were classified as either 1) sporadic, microsatellite stable (MSS) tumors, 2) likely caused by LS, or 3) sporadic MSI-H tumors. Tumors likely caused by LS were defined as MSI-H tumor tissue in combination with absent MMR protein expression, with exclusion of *MLH1* promoter hypermethylation in the case of absent MLH1 expression. Sporadic MSI-H tumors were defined as tumor tissues with absent MLH1 expression and established *MLH1* promoter hypermethylation.

Analysis for MSI

Analysis for MSI was performed on FFPE tumor and normal tissue, using a panel of five mononucleotide microsatellite markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27; Promega pentaplex assay) as previously described. [55] In addition, two pentanucleotide markers (Penta C and Penta D) were added for detection of sample mix-up or contamination. For MSI analysis, a fluorescent multiplex PCR-based assay was used (Promega, Madison, WI, USA). The PCR products were separated by capillary electrophoresis using

an ABI PRISM 3130xl genetic analyser (Applied Biosystems, Foster City, CA, USA). PCR was performed according to the kit instructions. The output data were analysed with GeneMarker software (SoftGenetics, State College, PA, USA) to determine MSI status of EC samples. Tumors with more than one unstable marker were categorized as having a high degree of microsatellite instability (MSI-H). Tumors with one or no unstable markers were categorized as being microsatellite stable (MSS).

Immunohistochemistry, *MLH1* promoter hypermethylation assay, & *BRAF* mutation analysis

IHC analysis was performed for four mismatch repair proteins: MLH1, MSH2, MSH6 and PMS2, according to the standard procedure.[55] If there was no MLH1 expression in tumor cells, the methylation status of the *MLH1* promoter was determined by methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA). MS-MLPA was performed with the SALSA MSMLPA Kit ME011-A1 for MMR genes (MRC-Holland, Amsterdam, the Netherlands), as previously described.[55] In all MSI-H tumor specimens additionally the methylation status of the other MMR genes: *MSH2*, *MSH6* and *PMS2,* was ascertained using the same MS-MLPA kit. *BRAF* sequence analysis of the mutation hotspot codon V600 was performed by bi-directional cycle-sequencing of PCR-amplified fragments, using a previously described method.[55]

Germline mutation analysis

Analyses of MSI in combination with IHC revealed patients likely to have LS, i.e. those with an MSI-H tumor tissue profile in combination with absent MMR protein expression, with exclusion of *MLH1* promoter hypermethylation in the case of absent MLH1 expression. To all patients likely to have LS and referred to the department of Clinical Genetics, germline mutation analysis was offered. In DNA isolated from peripheral blood samples all coding regions and intron-exon boundaries of the *MLH1*, *MSH2* and *MSH6* genes were completely and systematically analysed using direct sequence analysis. Reaction products were analysed using a capillary automated sequencer (details of method and primer sequences available on request). In addition, MLPA kits P003 and P072 (MRC-Holland) were used to detect large genomic rearrangements. The mutation analysis of *PMS2* was performed as previously described.[157]

Statistical analysis

Data were analyzed using SPSS 17.0 statistical software for Windows, and were reported using descriptive statistics. The prevalence of a molecular pattern compatible with LS and a sporadic MSI-H phenotype were analyzed. The Mann-Whitney U test was used to compare the different groups. Two-sided p-values less than 0.05 were considered statistically significant.

RESULTS

Endometrial Cancer Patient Population

A total of 183 EC patients were eligible to participate in the study (Figure 1). In four cases no tumor tissue was available for molecular analyses; therefore 179 EC patients were included. The median age at EC diagnosis was 61 years (IQR 57-66). Fifteen patients were ≤ 50 years (8%) at time of diagnosis. One hundred forty-six of 179 ECs (82%) showed endometrioid type histology. The majority of included EC tumor tissues were found to be grade 1 (73 patients, 41%). Histology showed tumor confined to the uterus in 126 tumor specimens (70%, i.e. T1) (Table 1).

Molecular analyses

Overall, 137 tumors were found to be MSS and 42 tumors displayed an MSI-H phenotype with absence of at least one of the MMR proteins. Promoter methylation status of the MMR genes was determined by MS-MLPA in the 42 MSI-H tumors. Thirty-one tissues were methylated at all 5*hMLH1* sites and were concluded to be sporadic MSI-H tumors

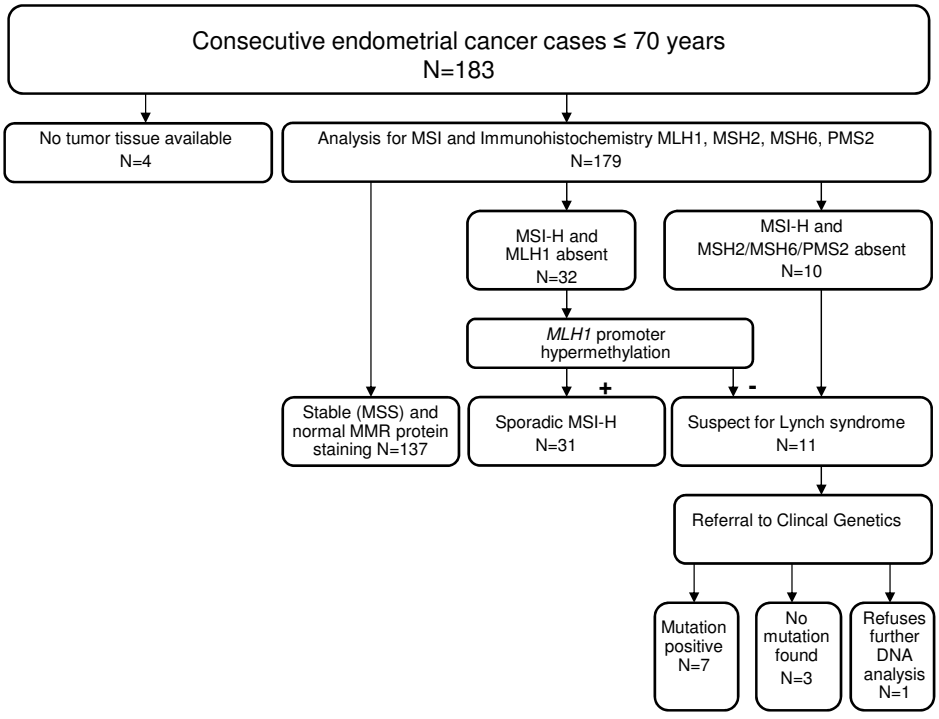


Figure 1. Flow diagram of the diagnostic strategy and results of molecular analyses.
MSI, microsatellite instability; MMR, mismatch repair; MSS, microsatellite stable; MSI-H, high degree of microsatellite instability.

Table 1: Endometrial cancer patient characteristics and results of molecular analyses.

	MSS N= 137 (76%)	Sporadic MSI-H N= 31 (17%)	Likely to have LS N=11 (6%)	Total N= 179 (100%)
Median Age (IQR)	61 (57-66)	62 (57-69)	59 (53-66)	61 (57-66)
Histology				
Endometrioid adenocarcinoma	107 (78%)	30 (97%)	9 (82%)	146 (82%)
Adenosquamous carcinoma	1 (1%)	0	0	1 (1%)
Serous adenocarcinoma	7 (5%)	0	0	7 (4%)
Mixed adenocarcinoma	7 (5%)	0	1 (18%)	8 (4%)
Clear cell carcinoma	1 (1%)	0	1 (9%)	2 (1%)
Squamous cell carcinoma	1 (1%)	0	0	1 (1%)
Adenocarcinoma not further specified	13 (9%)	1 (3%)	0	14 (7%)
Tumor grade				
1	60 (44%)	11 (35%)	2 (18%)	73 (41%)
2	28 (21%)	11 (35%)	4 (36%)	43 (24%)
3	18 (13%)	7 (23%)	4 (36%)	29 (16%)
Grade unknown	31 (22%)	2 (7%)	1 (10%)	34 (19%)
Pathological tumor (T) stage				
Tis	1 (1%)	0 (0%)	0 (0%)	1 (1%)
T1 Confined to corpus	92 (82%)	24 (77%)	10 (91%)	126 (70%)
T2 – T4 Expansion beyond uterus	20 (15%)	4 (12%)	0 (0%)	24 (13%)
T stage unknown	24 (17%)	3 (10%)	1 (9%)	28 (16%)

EC, endometrial carcinoma; MSI, microsatellite instability; MSS, microsatellite stable; LS, Lynch syndrome; Tis, endometrial carcinoma in situ.

(17%; 95% CI 13-24%). In eleven MSI-H tumors with absent MMR protein expression, all MMR genes were unmethylated and thus were classified as likely to have LS (6%; 95% CI 3-11%). On the basis of immunohistochemical MMR protein expression, one of these patients was suspected of an *MLH1* gene defect, two for *MSH2*, six for *MSH6* and two for a *PMS2* defect. There was 100% concordance between IHC results and MSI status in analysed EC tumor tissues.

BRAF mutation analysis was performed in all 42 MSI-H tumors, but no *BRAF* mutations were detected. No difference in age at EC diagnosis between patients with an MSS tumor and patients with a sporadic MSI-H tumor was found.

Patients likely to have Lynch syndrome

Eleven patients were likely to have LS on the basis of the molecular and MMR IHC analyses. The median age of these patients was 59 years (IQR 53-66), and ten patients were older than 50 years at time of EC diagnosis (Table 2). There was no difference in age at EC diagnosis between patients likely to have LS and patients with either sporadic MSI-H tumors ($p = 0.19$) or patients with MSS tumors ($p = 0.46$). The correlation between age and the results of the molecular analyses is shown in figure 2. Comparing tumor grade and pathological tumor (T) stage, there were also no differences between patients likely

to have LS and patients with sporadic MSI-H tumors (p=0.25; p=0.44). Furthermore, no differences in age and pathological tumor (T) stage at EC diagnosis, between patients likely to have LS and patients with MSS tumors were observed (p=0.46; p=0.20; p=0.10). However tumor tissues of patients likely to have LS were of higher tumor grade than MSS tumor tissues (p= 0.02, Table 1).

The gynaecologists of the eleven patients, who were likely to have LS based on their EC cancer phenotype, were advised to refer their patients to the department of Clinical Genetics for counseling and germline mutation analysis (Table 2). All eleven patients

Table 2. Characteristics of the eleven EC patients likely to have LS.

Patient no.	Age at diagnosis	Histology	Grade	Criteria		MSI	IHC	Germline analysis	Mutation
				AC	rBC	No. of positive markers	Absent MMR protein		
1	58	endometrioid adenocarcinoma	2	No	No	5/5	MSH2/ MSH6	Yes	No mutation in *MSH2* and *MSH6*
2	53	endometrioid adenocarcinoma	3	No	No	5/5	MSH2/ MSH6	Yes	No mutation in *MSH2* and *MSH6*
3	62	endometrioid adenocarcinoma	2	No	No	5/5	MSH6	Yes	c.3173-2A>C p.? (*MSH6*, exon 5)
4	59	endometrioid adenocarcinoma	NA	No	Yes	5/5	MSH6	Yes	Known LS family: c.467C>G p.Ser156X (*MSH6* exon 3)
5	62	endometrioid adenocarcinoma	3	No	No	5/5	MSH6	Yes	c.467C>G p.Ser156X (*MSH6* exon 3)
6	69	mixed adenocarcinoma	3	No	No	3/5	PMS2	Yes	No mutation in *PMS2*
7	52	endometrioid adenocarcinoma	1	No	No	3/5	MSH6	Yes	c.3173-452_3556+209del2269 (*MSH6* exon 5-6)
8	53	endometrioid adenocarcinoma	2	Yes	Yes [a]	4/5	MSH6	Yes	c.467C>G p.Ser156X (*MSH6*)
9	66	endometrioid adenocarcinoma	1	No	No	3/5	MLH1/ PMS2 [b]	ND: declines DNA analysis	NA
10	69	endometrioid adenocarcinoma	2	No	No	5/5	PMS2	Yes	c.219_220dup, p.Gly74ValfsX3 (*PMS2*)
11	48	clear cell adenocarcinoma	3	Yes	Yes	4/5	MSH6	Yes	Known LS family: c.1784delT p.Leu595fs (*MSH6*, exon 4)

[a] Patient was diagnosed with colorectal cancer in the same year she developed endometrial cancer.
[b] Additional analysis showed absence of *MLH1* promoter hypermethylation.
rBC, revised Bethesda Guidelines; AC, Amsterdam Criteria II; MSI, analysis of microsatellite instability; IHC, immunohistochemistry; NA, not available; ND, not done.

were referred to the department of Clinical Genetics and ten patients underwent germline analyses. One patient refused germline DNA mutation analysis, due to religious considerations.

Germline mutation analyses revealed in six patients an *MSH6* mutation and in one patient a *PMS2* mutation. Of these, two families were already known to have LS and were blindly included for the molecular analyses. In both cases the MMR protein of the known involved MMR gene mutation was absent in IHC. In three patients whose tumor tissues showed MSI and absence of one or more MMR proteins, DNA mutation analysis of the corresponding genes in these patients was negative.

Nine of eleven referred patients (82%) did not fulfill the Amsterdam criteria II. Three patients (27%) did fulfill the revised Bethesda guidelines (Table 2).

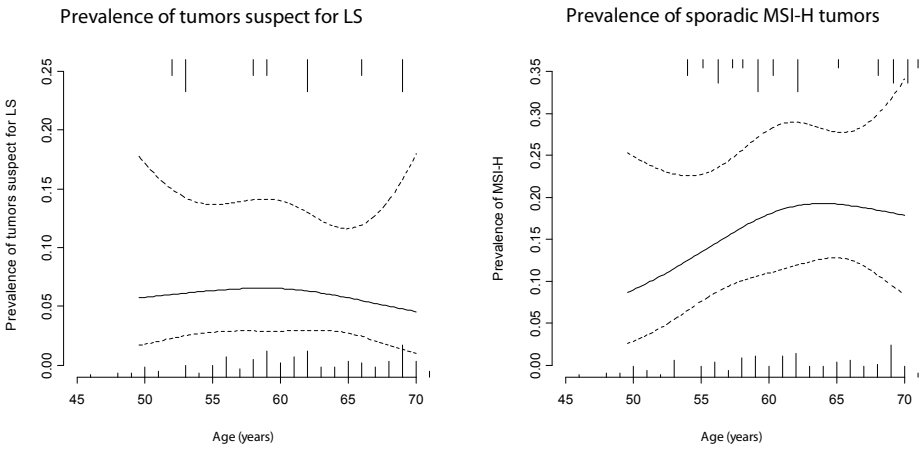


Figure 2. Correlation between age and the results of the molecular analyses.
Spline functions with 95% confidence interval. Spikes indicate the ages of individual patients with and without the outcome of interest.
Left panel: Prevalence of tumors likely caused by Lynch syndrome according to age; the chance to detect a patient likely to have LS by molecular analyses is not related to age.
Right panel: Prevalence of sporadic MSI-H tumors according to age; the chance to detect a patient with a sporadic MSI-H tumor by molecular analyses clearly increases to the age of 63 years.

DISCUSSION

In this prospective multicenter population-based study, we found that routine molecular analyses lead to the identification of 6% (95% CI 3-11%) of all newly diagnosed EC patients aged 70 years and younger as likely to have LS. Seven germline mutations in an MMR gene have been identified in eleven patients likely to have LS. The detection of LS in EC patients is of great importance since these patients are at high risk for synchronous carcinomas, especially CRC. Moreover, the diagnosis of LS is of importance to relatives. EC

patients with LS and their family members harbouring an MMR gene mutation can enter surveillance programs which have been proven to reduce CRC morbidity and mortality by 65-70%.[18, 20, 184] For female LS carriers gynaecologic surveillance programs, including endometrial biopsy and transvaginal ultrasound, are available. However, gynaecological surveillance is currently based on expert opinion since no controlled trials have been published on the effectiveness of surveillance.[187] Instead of surveillance, prophylactic surgery can be considered after childbearing has been completed, as this may prevent endometrial and ovarian carcinoma effectively.[22]

In our study we performed both MSI and IHC for four MMR proteins in all 179 tumors to select patients at high risk for LS. Furthermore, in case of MSI-H MMR gene MS-MLPA and *BRAF* V600 mutation analyses were performed to identify sporadic MSI-H tumors. Since the concordance rate in our study between MSI analysis and IHC analysis was 100%, these results support the use of IHC analyses in combination with MS-MLPA of the MMR genes as molecular strategy to detect patients at high risk for LS in EC patients aged 70 years and younger. However it should be taken into consideration that deleterious missense mutations can escape detection by IHC and that the interpretation of mismatch repair protein IHC is subject to large interobserver variation (45%-83%).[188]

Additionally to MSI and IHC analyses we performed *MLH1* promoter methylation assay (MS-MLPA) in MSI-H tumors with absent MLH1 expression, revealing a sporadic MSI-H status in 17% of all analyzed ECs. This finding is in concordance with findings in previous studies.[182, 183, 189] The establishment of a sporadic MSI-H status considerably reduced the number of patients referred for counseling and germline genetic testing. A recent retrospective study recommended the combination of MSI testing and DNA methylation to detect EC patients at high risk for LS.[189] A disadvantage of this strategy is that methylation status does not provide information on the gene most likely to be affected. A combination with IHC testing is hence needed for selecting patients for further germline mutation analysis.

In all MSI-H tumors we also did *BRAF* mutation analysis. In contrast to *BRAF* mutations in colorectal cancer, previous studies found *BRAF* mutations in only 2-21% of EC cases.[190, 191] Furthermore, in a recent study with MSI-H EC cases, no *BRAF* mutations were detected.[192] However, numbers of investigated ECs were too small to make a statement with regard to the presence of *BRAF* mutations in MSI-H tumors, without MLH1 protein expression and *MLH1* promoter hypermethylation. We did not detect the *BRAF* V600E mutation in any of the MSI-H EC cases in our study. Therefore, *BRAF* mutation analysis showed to be irrelevant for the identification of sporadic MSI-H ECs *MLH1* promoter hypermethylation analysis is more suitable to identify sporadic MSI-H EC cases.

We chose for an age cut-off of 70 years to compromise between the feasibility of the study and an optimal detection of patients likely to have LS. Previous studies have been conducted to evaluate the yield of molecular screening for LS. In the largest unselected

cohort of EC patients (N=543), 1.8% of newly diagnosed patients, aged 39-69 years, was diagnosed with an MMR germline mutation, of whom none above 70 years of age.[92] However, this study may have been biased by the fact that almost half of the eligible patients declined molecular screening of their tumor tissue. Our strategy appears effective in detecting patients at high risk of LS (6%). One can debate whether patients are sufficiently aware of the possible consequences of molecular tumor screening for LS, being informed by their gynaecologist and a folder. In a recent prospective study on routine IHC staining for MMR proteins in EC patients at high risk of LS, low acceptance of genetic consultation (20%) was noted.[193] In our experience, substantial explanation to gynaecologists and their patients was needed to clarify the relevance of referral to a Clinical Genetics department. These findings are supported by the results of a recent questionnaire study among EC patients about the risk for LS in which it was concluded that most patients underestimate their risk of LS. Therefore, physicians should pay more attention to family history and to explaining the results of tumor analysis for LS.[194] All eleven patients likely to have LS in the present study were referred for genetic counseling. Of these patients, one patient declined further DNA analysis, due to religious considerations. In our strategy we experienced that logistic and communication issues were the main causes for delay in informing patients about the molecular test results. This indicates that better implementation of genetic services in hospitals is needed to improve uptake of genetic counseling and testing.

Our current study, as well as previous studies,[92, 193, 195-199] demonstrates the urge to implement EC in diagnostic criteria for LS. In the Netherlands the MIPA criteria and also the international SGO guidelines recognise the importance to advice EC patients on molecular analysis for LS.[200, 201] However, these guidelines include only EC patients diagnosed under the age of 50 years or patients with two or more LS associated tumors. In our study 92% of patients likely to have LS were over 50 years of age. On the basis of these data and data from the literature,[92, 189, 196] molecular testing for LS should not be limited to EC patients under the age of 50 years. A recent study proposed routine molecular screening for LS in tumor tissue of EC patients until 60 years of age.[193] In our study we detected five patients (45%) likely to have LS between 60 and 70 years of age. Therefore, it should be considered to include EC patients until the age of 70 for LS screening in tumor tissue. More studies on the optimal age criterion for molecular testing and cost-benefit analysis data are desirable. Recently, the cost-effectiveness of IHC screening for LS was assessed by a Markov Monte Carlo simulation model, indicating that IHC triage of women with EC at any age having at least one first degree relative with a LS-associated cancer is a cost-effective strategy for detecting LS. The input data for this model came from a variety of studies. An important finding in our study is that only two of eleven patients likely to have LS had a first degree relative with a LS-associated malignancy. This finding contradicts the conclusion that IHC screening can be limited to

women with at least one positive first degree relative and suggests IHC screening in all EC patients under the age of 70, regardless family history.[202]

A limitation of our study is the lack of data on family history in women not referred for genetic counseling. Therefore, we can not compare our strategy to the Amsterdam criteria II, the Bethesda guidelines, or predictive models for LS.[185, 203] However, 82% of patients likely to have LS and referred for counseling did not fulfill the Amsterdam criteria II and 73% did not fulfill the revised Bethesda guidelines. In the population based study by Hampel *et al*, the families of seven of ten families (70%) of EC patients, who were likely to have LS, did not fulfill Amsterdam criteria II or the Bethesda guidelines.[92] This indicates that these guidelines may not be suitable to detect LS in EC patients. Recently, LS predictive models including family history and outcome of molecular analyses for patients with EC were evaluated, and it was concluded that these models worked reasonably well to identify EC patients at high risk for LS. Further research is needed to develop specific LS predictive models for EC patients.[203]

Another limitation is that we did not perform germline testing on all patients in this study. From previous studies among patients with LS-associated EC is known that the sensitivity of MSI detection in EC patients is higher with mononucleotide repeats.[204] In this study a panel of five mononucleotide markers was used to detect MSI. In case of an MSI-H tumor with absent MLH1 protein expression and hypermethylation of the *MLH1* promoter we concluded this tumor to be sporadic MSI-H. This could have lead to underestimation of LS in our study population. However, previous studies indicate that *MLH1* promoter hypermethylation by MS-MLPA is a sufficient tool to detect sporadic tumors.[182, 183, 205]

In conclusion, routine molecular screening for LS by analysis of MSI, immunohistochemical MMR protein expression and *MLH1* promoter hypermethylation in EC patients aged 70 years and younger helps to detect more patients at high risk for LS. Therefore, new screening guidelines for LS are needed for LS detection in EC patients. However, the optimal age-criterion for this strategy needs to be determined, in combination with information on family history. Furthermore, both physicians and patients have to be educated about the importance of identifying LS in EC patients, and should be informed about genetic counseling and surveillance programs for LS mutation carriers and their relatives.

Chapter 6

Age-targeted Lynch syndrome screening in colorectal cancer patients: cost-effectiveness in a population-based setting

Celine H.M. Leenen, Anne Goverde, Esther W. de Bekker-Grob, Anja Wagner, Margot G.F. van Lier, Manon C. Spaander, Marco J. Bruno, Carli M. Tops, Ans M.W. van den Ouweland, Hendrik J. Dubbink, Ernst J. Kuipers, Winand N.M. Dinjens, Monique E. van Leerdam, Ewout W. Steyerberg

Submitted for publication

ABSTRACT

Objective: To assess cost-effectiveness of routine screening for Lynch syndrome (LS) in colorectal cancer (CRC) patients ≤ 70 years of age.

Methods: A population-based series of CRC patients aged ≤ 70 years was routinely screened for LS by analysis for microsatellite instability, immunohistochemistry and *MLH1* hypermethylation, followed by germline mutation analysis. Effectiveness of screening was based on the number of LS carriers detected among CRC patients and their relatives. We calculated life years gained (LYG) and incremental cost-effectiveness ratios (ICERs) comparing different age cut-offs and comparing age-targeted screening to the revised Bethesda guidelines.

Results: Screening amongst 1117 CRC patients identified 23 LS carriers, of whom 7 were ≤ 50, 7 were 51-60 and 9 were 61-70 years of age. Additionally, 67 LS carriers were identified among relatives (12, 42 and 13 per age category respectively). Overall, screening amounted to 74.7 LYG or 13.7, 46.0 and 15.0 LYG per age category. ICERs were €9,494/LYG for LS screening in CRC patients ≤ 60 years compared to ≤ 50 years and €19,833/LYG for screening CRC patients ≤ 70 years compared to ≤ 60 years. The revised Bethesda guidelines identified 17/23 (74%) LS carriers and 50/67 (75%) relatives. The ICER for LS screening in CRC patients ≤ 70 years was €19,695/LYG compared to LS screening according to the revised Bethesda guidelines. All ICERs remained < €29,000/LYG in sensitivity analyses.

Conclusion: Routine screening for LS by analysis for microsatellite instability, immunohistochemistry and MLH1 hypermethylation in CRC patients up to 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 3-5% of all CRC cases.[40, 145] This syndrome is characterized by early onset of CRC, endometrial cancer and other extracolonic cancers.[40, 147] Mutations in one of the four mismatch repair (MMR) genes *MLH1, MSH2, MSH6* and *PMS2* or the 3' region of the *EPCAM* gene are the underlying defect in LS.[17, 23, 24, 102, 103] Detection of LS in CRC patients is of great importance, since treatment with subtotal colectomy can prevent development of metachronous tumors.[206] Moreover, affected family members can benefit from LS surveillance programs, which reduce CRC incidence and mortality by 56-70%.[18, 19, 207]

Molecular diagnostics on tumor 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.[55, 145, 208] MSI and loss of MMR protein expression are markers for MMR deficiency. However, loss of *MLH1* protein expression can also occur in sporadic tumors as a result of somatic MLH1 promoter hypermethylation. Therefore, sporadic MLH1 deficient tumors can be distinguished from LS-associated tumors by *MLH1* hypermethylation analysis.[55]

The revised Bethesda guidelines have been developed to select patients eligible for MSI testing and IHC analysis based on a set of diagnostic criteria.[84] These guidelines are poorly applied in clinical practice and may miss a substantial number of LS patients because of limited sensitivity.[150, 209] Routine analysis of MSI and IHC is recommended in CRC patients under the age of 50 years.[78] 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.[9, 70] Routine molecular screening has been proposed to improve LS detection, but age cut-offs are still under debate.[93, 94, 210]

We previously reported that routine MSI analysis and IHC for MMR proteins revealed a profile compatible with LS in 4.5% of CRC patients ≤ 70 years of age.[130] A majority of these patients were over 50 years of age.[130] The current study aimed to assess the cost-effectiveness of routine screening for LS by MSI and IHC analysis in CRC patients ≤ 70 years of age. We compared costs and health benefits for age-targeted LS screening and compared age-targeted screening with LS screening based on the revised Bethesda guidelines.

METHODS

We performed a cost-effectiveness analysis of routine screening for LS by MSI and IHC analysis in CRC patients ≤ 70 years of age in a population based setting. The analysis es-

timated healthcare costs in euros and health benefits in life years gained for LS patients identified among CRC patients in different age groups and their relatives.

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.[130] 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 tumor analyses and germline mutation analyses have been described in detail elsewhere.[130] In summary, both MSI analysis and IHC for MLH1, MSH2, MSH6 and PMS2 protein expression were performed in tumor tissue of CRC patients ≤ 70 years of age. *MLH1* hypermethylation analysis was performed in cases with loss of MLH1 protein expression. In case tumors showed a high degree of MSI (MSI-H and/or absence of MMR protein without *MLH1* promoter hypermethylation, patients were suspect of having LS. These patients were offered genetic counseling 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 counseling was offered to their first degree relatives.

If a pathogenic germline mutation was identified in one of the MMR genes (*MLH1, MSH2, MSH6, PMS2*) or the *EPCAM* gene, patients were labelled index patients. Patients identified with a variant of unknown significance (VUS) were not considered to be LS index patients. Relatives were contacted by index patients and were offered genetic counseling and germline mutation analysis. We collected data on the number of relatives counselled, the number of relatives accepting germline 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 patients detected among CRC patients and their relatives and using estimations from literature (Table 1).

In previous studies LS surveillance was associated with 0.15-2.5 LYG for index patients and 1.07 to 32.76 LYG for relatives (Table 1).[78, 93-96, 211-213] We used the lowest estimations in our base case scenario, biasing the analysis against routine LS screening. In case the index patient had deceased, only relatives were considered to benefit from surveillance.

Costs

Detailed 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.[214] Cost data included the costs of employment, material, equipment and

overhead, which were obtained from the Department of Pathology and the Department of Clinical Genetics of the Erasmus Medical Centre Rotterdam (Appendix). 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 analysed. The costs for germline mutation analysis in index patients were calculated using the total number of genes analysed. LS surveillance costs for index patients and relatives were estimated from previous literature including costs for colonoscopy and treatment of complications (Table 1 and Appendix).[215] Surveillance was defined as colonoscopy with polypectomy every two years, starting at the age patients were diagnosed with LS. Median age of index patients at time of diagnosis was 57 years.[130] For relatives, surveillance was assumed to start when they turned 25 years of age, or at time of LS diagnosis for relatives over 25 years of age. For index patients as well as relatives surveillance was assumed to be continued until 80 years of age. For cost savings by prevention of CRC in surveillance programs, the most conservative estimate

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

Parameter	Base case value	Range	Source
LS surveillance[†]			
Life years gained			
Index patients	0.15	0.15-2.5	[78, 93, 95, 96, 213]
Relatives	1.07	1.07-32.76	[78, 93, 94, 95, 96, 211, 212]
Median age of LS diagnosis			
Index patients	57	IQR 49-63	[130]
Relatives	42	IQR 32-56	Current study
Number of colonoscopies			
Index patients	12	IQR 8-16	[†]
Relatives	19	IQR 12-24	[†]
Complication rate of colonoscopy	0.0024		[215]
CRC risk and risk reduction			
Lifetime risk of developing CRC for LS carriers	0.25	0.25-0.70	[19, 207, 211, 212]
Reduction in CRC risk by LS surveillance	0.56	0.56-0.70	[19, 20, 207]
Revised Bethesda guidelines			
Proportion of CRC patients fulfilling the revised Bethesda guidelines in an unselected CRC population	0.26	0.26-0.50	[89, 216, 217, 218]

LS, Lynch syndrome; CRC, colorectal cancer.

[†] Lynch syndrome surveillance by colonoscopy with polypectomy every two years, starting from the age of Lynch syndrome diagnosis or at age 25 and continued until 80 years of age.

i.e. only treatment costs for the first 12 months of stage I CRC was used (Appendix). Costs and LYG were not discounted, since we did not use a set time horizon.

Cost-effectiveness analyses

We evaluated cost-effectiveness of our diagnostic work-up to identify LS patients using age cut-offs of 50, 60, and 70 years. 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 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).

Fulfillment of the revised Bethesda guidelines

The proportion of CRC patients fulfilling the revised Bethesda guidelines was based on literature (Table 1). In an unselected population, 26-50% of CRC patients fulfill the revised Bethesda guidelines.[89, 216-218] Assuming that only a small percentage of CRC patients fulfill the revised Bethesda guidelines, reduces the number of patients needed to be screened for LS. Since this approach is favourable for the cost-effectiveness of a strategy using the revised Bethesda guidelines, we assumed only 26% of the CRC patients in our cohort fulfilled the revised Bethesda guidelines.

For all index patients, a detailed family history was obtained during genetic counseling. Fulfillment of revised Bethesda guidelines was assessed by one clinical geneticist (AW).

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 germline mutation analyses 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 germline mutation analysis, this patient was not considered an index patient.

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 was highest in the age category ≤ 50 years where a pathogenic MMR mutation was found in 4.9% (7/144) of the CRC patients. The prevalence of LS decreased

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).

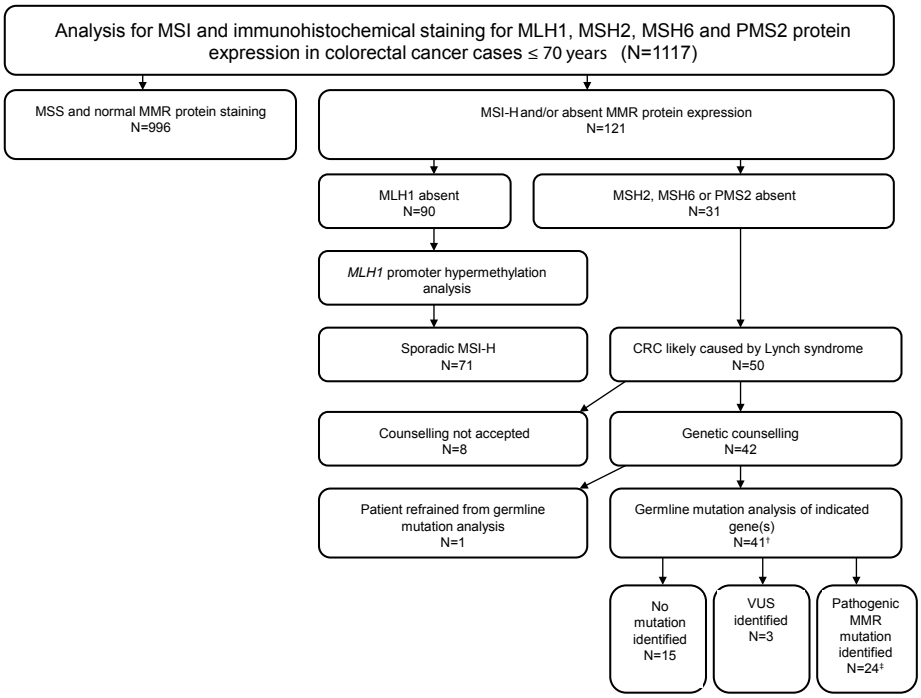


Figure 1. Diagnostic work-up to detect Lynch syndrome among colorectal cancer patients ≤ 70 years.
MSI, microsatellite instability; MSS, microsatellite stable; MMR, mismatch repair; MSI-H, high degree of microsatellite instability; CRC, colorectal cancer; VUS, variant of unknown significance.
† In 4/41 cases the index patient was not available for germline mutation analysis and 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.

For index patients up to 50 years of age a total of 29 first degree relatives were eligible for germline mutation analysis, compared to 44 and 40 first degree relatives in the age categories 51-60 years and 61-70 years respectively. Genetic counseling and germline mutation analysis was offered to these relatives and cascaded to further relatives if indicated. For each index patient a median of 3 (IQR 1-8) relatives finally accepted counseling and germline mutation analysis. There was a wide range in the number of relatives tested for LS. In all families germline mutation analysis was accepted by at least 1 relative and in some cases as many as 20 or 37 relatives were tested. In total, 137 relatives were tested, identifying 67 additional LS carriers. Notably, over three times as many LS carriers were identified among relatives of CRC patients 51-60 years of age compared

to the other age categories (Table 2). In the 51-60 age category a median of 6.0 (IQR 2-8) LS carriers were detected among relatives for each index patient, compared to 1 (IQR 1-2 and IQR 0-2.5 respectively) LS carrier among relatives from index patients ≤ 50 years of age and 61-70 years of age. The high number of LS carriers detected among relatives from CRC patients 51-60 years of age was partly attributable to one index patient with 37 relatives tested and 16 LS carriers identified.

Based on estimated benefit of LS surveillance, a total of 74.7 life years were gained by LS screening in CRC patients up to 70 years of age. Surveillance of relatives led to the highest benefit with a total of 71.7 LYG compared to a total of 3.0 LYG for index patients. Total benefit from LS screening in the age category 51-60 years (46.0 LYG) exceeded the other age categories, due to the high number of LS carriers identified among relatives.

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**	**≤ 70 years**
LS diagnostics in CRC patients				
Analysis for microsatellite instability and IHC testing for MMR protein expression	144	377	596	1117
MLH1 hypermethylation analysis	6	21	65	92
CRC patients suspected of having LS	15	15	20	50
CRC patients or first degree relatives accepting genetic counseling	12	13	17	42
CRC patients or first degree relatives accepting germline mutation analysis	11	13	17	41
Genes tested in CRC patients suspected of having LS or first degree relatives	18	22	30	70
LS index patients identified	7	7[†]	9	23[†]
LS diagnostics in relatives				
Relatives of index patients accepting genetic counseling	23	78	37	138
Relatives of index patients accepting germline mutation analysis	23	77	37	137
LS carriers identified among relatives	12	42	13	67
Life years gained				
Life years gained by index patients	0.9	1.1	1.1	3.0
Life years gained by relatives	12.8	44.9	13.9	71.7
Total life years gained (index patients and relatives together)	13.7	46.0	15.0	74.7

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

LS, Lynch syndrome; CRC, colorectal cancer; IHC, immunohistochemistry; MMR, mismatch repair

[†] 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 screening and surveillance increased from €162,304 (€8,542 per LS carrier detected) for CRC patients ≤ 50 years of age to €895,619 (€9,951 per LS carrier detected) for CRC patients ≤ 70 years of age (Figure 2).

LS screening for CRC patients ≤ 60 years of age had an ICER of €9,494/LYG compared to screening patients up to 50 years of age. The ICER of LS screening in CRC patients up to 70 years of age compared to screening CRC patients ≤ 60 years of age was €19,833 per LYG (Table 3).

In one-way sensitivity analysis ICERs were most sensitive to assumed LYG by relatives and the costs for surveillance colonoscopy. The ICER for screening CRC patients ≤ 60 years of age compared to screening patients with CRC diagnosed ≤ 50 years of age never exceeded €18,000/LYG. The ICER for screening CRC patients ≤ 70 years of age compared to screening patients with CRC diagnosed ≤ 60 years of age remained < €29,000/LYG under all assumptions (Figure 3). Assuming LYG by relatives over 2,20 years would decrease this ICER to < €10,000/LYG. Using the highest number of LYG by relatives from the literature would decrease both ICERs to below €1,000/LYG.

Fulfillment of the revised Bethesda guidelines

In our cohort, application of the revised Bethesda guidelines would have identified 17/23 LS carriers among CRC patients, thereby missing 26%. Among relatives 50/67 (75%) LS carriers would be detected, resulting in a total of 55.8 LYG compared to 74.7

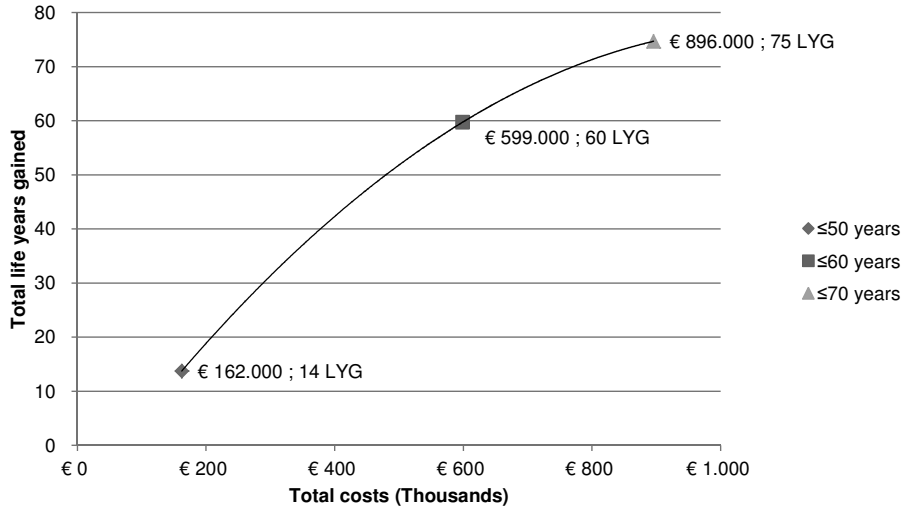


Figure 2. Total costs and life years gained (LYG) for Lynch syndrome screening in colorectal cancer patients ≤ 50 years of age, ≤ 60 years of age and ≤ 70 years of age.

Table 3. Incremental costs in 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	≤ 70 years
Lynch syndrome diagnostics				
CRC patients				
Analysis for microsatellite instability, IHC testing for MMR protein expression and *MLH1* hypermethylation analysis	€ 32,850	€ 86,527	€ 139,939	€ 259,316
Genetic counseling	€ 4,668	€ 5,057	€ 6,613	€ 16,338
Germline mutation analysis	€ 9,684	€ 11,836	€ 16,140	€ 37,660
Relatives				
Genetic counseling	€ 5,632	€ 19,968	€ 9,216	€ 34,816
Germline mutation analysis	€ 6,688	€ 23,408	€ 10,944	€ 41,040
Lynch syndrome surveillance				
Index patients	€ 29,963	€ 34,957	€ 34,957	€ 99,876
Relatives	€ 94,882	€ 332,089	€ 102,789	€ 529,760
Savings by prevention of CRC	- € 22,063	- € 77,222	- € 23,902	- € 123,188
Total costs (minus savings)	€ 162,304	€ 436,619	€ 296,696	€ 895,619
Total life years gained	13.7	46.0	15.0	74.7
Costs per life year gained	reference	€ 9,494	€ 19,833	-

Numbers may not add up due to rounding.
CRC, colorectal cancer; IHC, immunohistochemistry; MMR, mismatch repair.

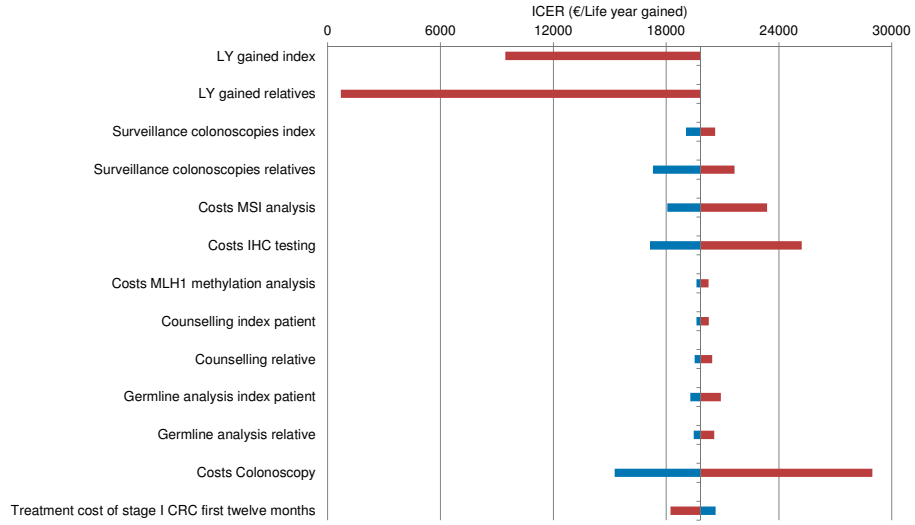


Figure 3. One-way sensitivity analysis for incremental cost-effectiveness ratio (ICER) of routine screening for Lynch syndrome in colorectal cancer patients ≤ 70 years of age compared to routine Lynch syndrome screening in patients with colorectal cancer diagnosed ≤ 60 years.

LYG by screening all CRC patients ≤ 70 years of age. Using assumptions most favourable for the revised Bethesda guidelines i.e. a small number of patients fullfilling the revised Bethesda guidelines and hypermethylation analysis not needed in any cases, total cost for this strategy amounted to €522,601 or €7,800 per LS patient detected. The ICER for routine LS screening in CRC patients ≤ 70 years was €19,695/LYG compared to testing patients fulfilling the revised Bethesda guidelines. In sensitivity analysis this ICER for LS screening in CRC patients up to 70 years compared to the revised Bethesda guidelines did not exceed €29,000/LYG. Assuming LYG by relatives over 2,15 implied an ICER < €10,000/LYG and using the highest number of LYG for relatives from the literature decreased this ICER to €669/LYG.

DISCUSSION

Our economic evaluation indicates that routine screening for LS in CRC patients ≤ 70 years of age by analysis of MSI and IHC is cost-effective according to currently accepted standards. In an one-way sensitivity analysis, expanding routine screening for LS from CRC patients ≤ 50 years of age to CRC patients ≤ 60 years of age never exceeded €18,000/LYG. Subsequently, costs for including CRC patients 61-70 years of age remained < €29,000/LYG. 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.[219] In the UK and US a threshold of $50,000/LYG (approximately €37,000/LYG) is commonly used in cost-effectiveness analyses for cancer screening. Some authors have argued that thresholds over $50,000/LYG could also be justified.[220]

Our sensitivity analysis confirmed the finding of other studies that the assumed benefit (LYG) by LS surveillance has a tremendous effect on ICER, especially LYG assumed for relatives.[78, 93-96] In recent literature benefit of LS surveillance programs for relatives ranges from 1.07 LYG up to 32.76 LYG per relative.[94, 95] 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 study we used conservative estimates. Using the lowest number of LYG from literature for both index patients and relatives, ICERs were well within currently accepted thresholds for cost-effectiveness. Moreover, assuming only a little over 2 LYG per relative led to ICERs < €10.000/LYG, and using the highest number of LYG per relative from literature even decreased all ICERs well below €1.000/LYG.

In our study over three times as many LS carriers were identified among relatives of CRC patients 51-60 years of age compared to the other age categories. In part this dif-

ference can be explained by the fact that 1.5 times as many first degree relatives were eligible for genetic testing in this age group compared to index patients ≤ 50 years of age. The number of adult first degree relatives was similar for the age categories 51-60 years and 61-70 years. The difference between these groups was caused by a higher percentage of relatives from index patients 61-70 years of age testing negative for LS syndrome. A possible explanation for this lower prevalence of LS could be that siblings of these patients are presumably older than siblings from younger CRC patients. As a consequence, the prevalence for LS might be lower in these older relatives, due to the reduced life expectancy of LS carriers. Furthermore, the age category 51-60 years contained a very large familiy with 37 relatives tested and 16 LS carriers identified.

Our results are in line with previous studies using Markov models, in which LS screening by IHC testing for CRC patients > 50 years of age was found to be cost-effective.[93, 94, 213] In one study LS screening of CRC patients up to 60 years of age led to an ICER of $33,800/LYG (€24,875/LYG) compared to 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 $44,000/LYG (€32,381/LYG).[93] The considerably smaller ICERs found in our study might be due to the large number of family members that accepted germline mutation analysis. Ladabaum *et al* assumed that 4 relatives per index patient accept germline mutation analysis.[93] Among our 23 index patients this would lead to a total of 92 relatives tested. We did find 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 137 relatives. Nonetheless, our study may still underestimate the number of LS patients ultimately detected among relatives. Relatives who currently refrain from genetic counseling and germline mutation analysis, could request genetic testing at a later time. Moreover, minors not yet eligible for genetic testing can also be offered germline mutation analysis and can be enrolled in surveillance programs from the age of 25 years. Another reason for the difference in ICERs could be that other studies discounted costs and LYG with 3% per year annually. Since we did not use a set time horizon, costs and LYG were not discounted.

In contrast, a recent Dutch study found an ICER of only €2,703 for LS screening in CRC patients ≤ 70 years of age compared to screening CRC patients ≤ 50 years of age.[213] However, LYG for relatives in their study was 6.9 to 7.22 years. Furthermore, a direct comparison was made for LS screening in CRC patients with an age cut-off of 70 years to 50 years, whereas we used a stepwise approach and compared an age cut-off of 70 years to an age cut-off of 60 years. Assuming 6.9 LYG for relatives in our study and directly comparing LS screening in CRC patients up to 70 years of age to screening in CRC patients up to 50 years of age would lead to a similarly low ICER (€1,922/LYG).

LS screening for CRC patients up to 70 years of age identified over 4.5 times as many LS carriers among index patients and relatives combined, compared to only screening

CRC patients up to 50 years of age. LS screening without any age cut-off is expected to further increase benefit for LS carriers. The AGA recently recommended LS screening of all CRC patients by IHC or MSI analysis as a possible screening strategy.[221] However, it is unclear whether the benefit of LS screening in all CRC patients will come at acceptable costs. In our population-based cohort, the prevalence of LS decreased with increasing age of CRC diagnosis. There are conflicting results on cost-effectiveness for universal LS screening by molecular diagnostics. LS screening by IHC without an upper age limit compared to age-targeted screening was found to be < €30,000/LYG in one study and > €60,000/LYG in another, while using equal discount rates.[93, 94]

The revised Bethesda guidelines were developed to select patients eligible for MSI analysis and IHC testing.[84] Between 26% and 50% of all CRC patients are expected to fulfill these guidelines. To assess cost-effectiveness of age-targeted LS screening we assumed only 26% of CRC patients in our cohort would fulfill the revised Bethesda guidelines. Despite this favourable assumption for the revised Bethesda guidelines, our cost analysis indicates that age-targeted LS screening is cost-effective with ICER remaining < €29,000/LYG in one-way sensitivity analysis. Moreover, we assumed 100% adherence to these guidelines, while in clinical practice molecular diagnostics for LS may be performed in only 11-14% of the patients fullfilling the revised Bethesda guidelines.[150, 209] In a previous study, low rates of failure to apply the revised Bethesda guidelines made LS screening by molecular diagnostics the preferred strategy.[93] We expect that routine screening for LS using molecular diagnostics in CRC patients based on age would be much easier to use in clinical practice compared to clinical criteria based on family history.

Strengths of this study are the use of real life data of index patients and their relatives, our detailed analysis of costs and inclusion of hypermethylation analysis in the diagnostic work-up. 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 using modelling techniques, our approach limits the potentially undesirable effects of assumptions as compared to more reliable data based on observational studies. If needed, conservative assumptions were used, i.e. a low number of LY gained, minimal cost savings by CRC prevention and relatively low lifetime risk of developing CRC.

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.[222, 223] However, two previous cost-effectiveness analyses of LS screening did find an impact on the ICER by including quality of life.[94, 224] Secondly, costs and benefit from surveillance for extracolonic cancers were not included in our analyses. Third, we did not perform a full probabilistic sensitivity analysis. Since we used conservative assumptions, such an analysis is expected to lead

to more favourable ICERs. Our study could also underestimate the benefit of routine LS screening, as patients refraining from genetic counseling and testing at this time might opt for germline mutation analysis at a later stage. Furthermore, in this study we did not analyse cost-effectiveness of MSI and IHC alone. In previous studies, LS screening by IHC alone was found to be more cost-effective compared to LS screening by MSI analysis or MSI analysis and IHC combined.[93, 94] 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 en $PREMM_{1,2,6}$ have been proposed as prescreening tools for LS.[82, 85, 152] It has been suggested that a combined strategy using IHC and prediction models among CRC patients < 70 years of age increases LS detection.[225] Also, prediction models may improve cost-effectiveness of LS screening by excluding 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 with important clinical benefits. 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.[221, 226]

APPENDIX

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
Germline mutation analysis for relatives (per gene)	€ 538	Dept. of Clinical Genetics, Erasmus MC
Germline mutation analysis for relatives	€ 304	Dept. of Clinical Genetics, Erasmus MC
Lynch syndrome surveillance		
Surveillance colonoscopy including polypectomy	€ 413	Wilschut *et al* 2011[215]
Treatment of complications after colonoscopy	€ 1,313	Wilschut *et al* 2011[215]
Treatment cost of stage I CRC (first 12 months)	€ 13,133	Wilschut *et al* 2011[215]

IHC, immunohistochemistry; MMR, mismatch repair; CRC, colorectal cancer.

[†] Costs were determined following the microcosting method and included costs of employment, material, equipment and overhead, obtained from the Department of Pathology and the Department of Clinical Genetics of the Erasmus Medical Centre Rotterdam.

Chapter 7

Prediction models for Lynch syndrome: an international validation study among individuals with colorectal cancer

Fay Kastrinos, Rohit P. Ojha, Celine H.M. Leenen, Carmelita Alvero, Rowena C. Mercado, Judith Balmana, Irene Valenzuela, Francesc Balaguer, Roger Green, Noralane M. Lindor, Stephen N. Thibodeau, Polly Newcomb, Aung Ko Win, Mark Jenkins, Daniel Buchanan, Lucio Bertario, Paola Sala, Heather Hampel, Sapna Syngal, Ewout W. Steyerberg

On behalf of the Lynch syndrome prediction model validation study group

Submitted for publication

ABSTRACT

Background: Lynch syndrome (LS) is the most common inherited colorectal cancer (CRC) syndrome and is caused by mismatch repair (MMR) gene mutations.

Objective: To compare the currently recommended LS prediction models, MMRpredict, MMRpro, and $PREMM_{1,2,6}$, to identify mutation carriers.

Design/Setting/Patients: Pedigree data were obtained from 11 North American, European, and Australian cohorts including CRC patients from 6 clinic and 5 population-based settings. We calculated predicted probabilities of any pathogenic *MLH1*, *MSH2*, and *MSH6* gene mutation by each model and gene-specific predictions by MMRpro and $PREMM_{1,2,6}$.

Measurements: We examined discrimination using the area under the receiver operator curve (AUC), calibration using the observed to expected (O/E) ratio, and clinical usefulness using decision curve analysis to select subjects for further evaluation.

Results: Mutations were detected in 539/2304 (23%) individuals from the clinic-based cohorts (238 *MLH1*, 250 *MSH2*, 51 *MSH6*) and 150/3451 (4.4%) individuals from the population-based cohorts (47 *MLH1*, 71 *MSH2*, 32 *MSH6)*. Discrimination was similar for clinic and population-based cohorts: AUCs of 0.76 vs 0.77 (P=0.77) for MMRpredict, 0.82 vs 0.85 (P=0.27) for MMRpro, and 0.85 vs 0.88 (P=0.06) for $PREMM_{1,2,6}$. For clinic and population-based cohorts, O/E deviated from 1 for MMRpredict (0.38 and 0.31, respectively) and MMRpro (0.62 and 0.36) but were more satisfactory for $PREMM_{1,2,6}$ (1.0 and 0.70). MMRpro or $PREMM_{1,2,6}$ predictions were clinically useful at thresholds ≥ 5% and in particular, at ≥ 15%.

Conclusions: MMRpro and $PREMM_{1,2,6}$ can well be used to select CRC patients from genetics clinics or population-based settings for tumor and/or germline testing at a ≥5% cut-off. Other genetic etiologies should be considered when risk estimates exceed 15% and no MMR deficiency is detected.

INTRODUCTION

Lynch syndrome (LS) is the most prevalent inherited colorectal cancer (CRC) syndrome. It accounts for approximately 3% of CRCs and is caused by germline mutations in the DNA mismatch repair (MMR) system involving the *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM* gene.[14, 227] The diagnosis of LS in a patient with CRC indicates a need for more intensive post-treatment colonoscopic surveillance, consideration for more extensive surgery, and management of extracolonic cancer risks. Furthermore, identification of a LS mutation in a patient with CRC has implications for their family since mutation carriers have a 35-75% lifetime risk of developing CRC and other cancers, often at young ages.[3, 12] Early identification of these individuals allows for implementation of cancer prevention strategies such as intensified surveillance, prophylactic surgery, and/or chemoprevention to reduce the cancer risk and improve survival.[163]

The consideration of a diagnosis of LS has traditionally relied on screening via clinical criteria such as the Amsterdam criteria or Revised Bethesda guidelines.[84, 168, 228] Systematic molecular tumor testing is increasingly supported for newly diagnosed patients with CRC, either as "reflex testing" (*all* patients undergo microsatellite instability [MSI] and/or immunohistochemistry [IHC] testing for protein expression of the MMR genes related to LS), or based on age.[14, 130, 229] The most recent National Comprehensive Cancer Network (NCCN) guidelines for risk assessment and management of LS recommend genetic testing if the predicted risk of carrying an MMR mutation is $\geq$ 5% using one of three recently developed risk prediction models, MMRpro, MMRpredict, or $PREMM_{1,2,6}$.[82, 85, 152, 230] These prediction models quantify an individual's risk for carrying an MMR gene mutation and aim to support decision making regarding genetic evaluation, including germline testing or molecular tumor testing.[225] Their performance in diverse populations (i.e. external validity) has not systematically been compared. We aimed to externally validate and assess the potential clinical usefulness of MMRpro, MMRpredict, and $PREMM_{1,2,6}$ for selecting patients with MMR gene mutations in a multi-center international study.

METHODS

Data sources and subject eligibility

Individual-level data were obtained from eleven international cohorts of subjects with CRC: six were considered clinic-based and five population-based. Additional data on each cohort is provided in the Appendix. Subjects with CRC and available molecular tumor testing and/or MMR gene mutational analyses results were eligible. Only one individual per family (referred to as the proband) was included for analysis and subjects with any polyposis syndrome were excluded.

The clinic-based cohorts recruited subjects through genetics clinics and/or family cancer registries and included the: (1) Medical Genetics Program of Newfoundland (Newfoundland, Canada), (2) Colon Cancer Family Registry (CCFR; http://epi.grants.cancer.gov/CFR/),[231] (3) Dana Farber Cancer Institute Gastrointestinal Cancer Genetics and Prevention Program (Boston, Massachusetts), (4) participating centers in the Hereditary Cancer Group of the Spanish Medical Oncology Society (SEOM), (5) Erasmus MC Genetic Registry (Rotterdam, the Netherlands),[88] and (6) participating centers in the Fondazione IRCCS Istituto Nazionale Tumori (Milan, Italy). The population-based cohorts included the: (1) Newfoundland Colorectal Cancer Registry,[89] (2) CCFR,[231] (3) EPICOLON Consortium (Spain),[232] (4) LIMO Study group (the Netherlands),[130] and the (5) Ohio State University.[14] Information regarding the evaluation process for DNA mutational analysis and/or molecular tumor testing by specific site is as previously described. This study was reviewed and approved by the Dana-Farber/Harvard Cancer Center and Columbia University Medical Center Institutional Review Boards.

Variables for Risk Prediction models

The primary outcome was MMR gene mutation carrier status based on germline testing results for the most common genes, *MLH1*, *MSH2*, and *MSH6*. *PMS2* gene mutation carriers were not included as only a few sites conducted germline testing for *PMS2* mutations. Subjects without germline testing results were classified as noncarriers if CRC tumor testing showed no evidence of MMR deficiency. Each participating site provided deidentified datasets to Dana-Farber/Harvard Cancer Center and Columbia University investigators for analysis (RO, RM, CA, FK). Data for probands included demographic information, cancer history (including ages of cancer diagnoses and date of last follow-up), tumor testing results, and results of germline testing. Family history of cancer was limited to first-degree relatives (FDR) or second-degree relatives (SDR) affected with LS-associated cancers (colon, endometrial, stomach, ovaries, urinary tract, small intestine, pancreas, bile ducts, brain, sebaceous glands), including their ages of diagnosis and/or date of last follow-up. For relatives unaffected by cancer the age, gender, and date of last follow-up were included.

For every subject, predicted probabilities were estimated for carrying an MMR gene mutation using the MMRpredict, $PREMM_{1,2,6}$, and MMRpro models.[82, 85, 152] The MMRpredict and $PREMM_{1,2,6}$ predictions were generated using published formulas and for MMRpro, probabilities were derived using software provided by the developing investigators. Predictions were verified by comparison of probabilities from our calculations with those from web-based calculators for a sample of at least 10 patients. Due to limited available tumor data, model comparisons were based on pedigree data alone and did not incorporate tumor testing information.

Data analysis

We compared predicted probabilities from each model with observed frequencies of mutations (*MLH1*, *MSH2*, *MSH6*, or any of these) in each cohort. We stratified by cohort type (population versus clinic) and considered cohort-specific results before pooling. We tested for differences in predicted probabilities by cohort in the pooled data by modeling an interaction term (cohort*logit of predicted probability) in logistic regression with any one of the mutations as the outcome. An interaction term with $P<0.05$ indicates the relation between predicted probabilities and mutation status varied by cohort.

Discrimination and calibration

Discrimination is the model's ability to differentiate between a mutation carrier and non-carrier. It was assessed using the area under the receiver operating characteristic curve (AUC). An AUC of 0.5 indicates the model does not discriminate any better than random selection for diagnostic work-up. Calibration refers to the agreement between the observed and predicted probabilities and can be depicted graphically.[233] Systematic under- or overestimation ('calibration-in-the-large') was quantified by the intercept in a logistic regression model with the log odds of the predictions as an offset variable: y ~ offset (logit of predicted probability), with *y* the presence of a genetic mutation as a binary outcome. For ease of interpretation, we converted the intercept estimates to Observed to Expected (O/E) ratios: O/E = exp(intercept). We also estimated a calibration slope to indicate the agreement with the 45 degree line in the validation plot by logistic regression analysis: y ~ logit of predicted probability. An intercept of zero (O/E=1) and calibration slope of 1 indicate perfect calibration.[233]

Clinical usefulness

When models are used to guide decisions, decision curve analysis has been advocated to quantify the potential clinical usefulness, considering both true positive classifications and false positive classifications.[234-237] A decision curve shows the net benefit of using a model for a range of potential decision thresholds. The net benefit is the sum of the number of true positive mutation carriers (for whom benefit is obtained) minus a weighted number of false-positive classifications (who should not have been tested): NB = (TP – wFP)/n, where *n* is the total sample size, and *w* is the relative weighted of the harm of missing a mutation carrier versus unnecessary testing. The weight *w* is defined by the threshold probability that is applied to define at-risk subjects that need genetic testing. We calculated the net benefit of each prediction model and two reference strategies: select none or select all for testing. We considered threshold probabilities between 0% (very liberal testing) and 30% (very restricted testing of high-risk probands). The model with the highest net benefit is most clinically useful. Discrimination, calibration and

decision curve analyses were conducted in R version 2.8.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

We studied 5755 individuals including 2304 from clinic-based and 3451 from population-based CRC registries. The median age of CRC diagnosis in the clinic-based cohorts was between 45 and 50 years, while subjects in the population-based cohorts were older (median age between 60 to 70 years, Tables 1a and 1b). The prevalence of MMR gene mutations in the clinic-based cohorts was 23% (539/2304), with similar numbers of *MLH1* and *MSH2* mutations (237 and 251 respectively) and fewer *MSH6* mutations (51/2304, 2.2%). The prevalence of any MMR gene mutation was lower in the population-based cohorts (150/3451, 4.4%, Table 1b) with more *MSH2* than *MLH1* and *MSH6* mutations (71 vs 47 and 32 respectively).

Prediction of any MMR gene mutation

Clinic-based cohorts: The AUCs for any MMR gene mutation prediction for the pooled data were 0.76 (95% CI: 0.74-0.79), 0.82 (95% CI: 0.80-0.84) and 0.85 (95% CI: 0.83-0.87) for MMRpredict, MMRpro, and $PREMM_{1,2,6}$ respectively (Table 2a). The O/E ratio of predicted risk for $PREMM_{1,2,6}$ was 1.0 (95% CI: 0.89-1.2) compared with 0.38 (95% CI: 0.32-0.45) for MMRpro and 0.62 (95% CI: 0.53-0.74) for MMRpredict (Table 2a). The calibration slope for $PREMM_{1,2,6}$ was 0.81 versus 0.42 and 0.28 for MMRpro and MMRpredict respectively (Figures 1a-c). MMRpro and $PREMM_{1,2,6}$ identified a similar percentage of carriers (95%

Table 1a. Characteristics of subject participants: clinic based cohort.

	Total	CCFR	DFCI	Milan, Italy	Newfoundland, Canada	Rotterdam, Netherlands	Spanish Consortium
	N=2304	N=529	N=229	N=232	N=120	N=514	N=680
Male (%)	1136 (49.3)	263 (49.7)	99 (43.3)	118 (50.9)	65 (54.2)	243 (47.3)	348 (51.2)
Median age of CRC diagnosis (y, IQR)	46 (39-55)	45 (38-51)	43 (35-50)	44 (37-53)	53 (43-62)	51 (42-61)	45 (39-55)
Mutation Carriers							
Any mutation (%)	539 (23.4)	166 (31.4)	62 (27.1)	99 (42.7)	19 (15.8)	58 (11.3)	135 (19.9)
MLH1 (%)	237/539 (44)	71/166 (42)	30/62 (48)	44/99 (44)	3/19 (16)	19/58 (33)	70/135 (52)
MSH2 (%)	251/539 (47)	84/166 (51)	26/62 (42)	47/99 (47)	15/19 (79)	21/58 (36)	58/135 (43)
MSH6 (%)	51/539 (9)	11/166 (7)	6/62 (10)	8/99 (9)	1/19 (5)	18/58 (31)	7/135 (5)

Table 1b. Characteristics of subject participants: population based cohort.

	Total	CCFR	Newfoundland, Canada	OSU	Rotterdam, Netherlands	Spanish Consortium
	N= 3451	N=1196	N= 731	N= 191	N= 196	N= 1137
Male (%)	1,905 (55.2)	581 (48.6)	443 (60.6)	89 (46.6)	117 (59.7)	675 (59.4)
Median age of CRC diagnosis (y, IQR)	64 (54-72)	58(48-67)	64(56-71)	63(51-72)	59(52-64)	71(63-78)
Mutation Carriers						
Any mutation	150 (4.4)	78 (6.5)	14 (1.9)	30 (2.5)	18 (9.2)	10 (0.9)
MLH1 (%)	47/150 (31)	30/78 (38)	2/14 (14)	8/30 (27)	4/18 (22)	3/10 (30)
MSH2 (%)	71/150 (47)	36/78 (46)	10/14 (71)	15/30 (50)	4/18 (22)	6/10 (60)
MSH6 (%)	32/150 (21)	12/78 (15)	2/14 (14)	7/30 (23)	10/18 (56)	1/10 (10)

CCFR, Colon Cancer Family Registries; DFCI, Dana Farber Cancer Institute; OSU, Ohio State University; IQR, interquartile range; Y, years of age.

and 96%, respectively) at a cutoff of ≥ 5% (Table 3). Above a cutoff of 10%, $PREMM_{1,2,6}$ identified a higher proportion of known carriers among identified high-risk subjects while MMRpro overestimated the number of mutation carriers.

Population-based cohorts: The pooled AUCs for any MMR gene mutation prediction were slightly higher for population-based than clinic-based cohorts (Table 2b). Predictions were too high for all models, with O/E ratios of 0.70 (95% CI: 0.58-0.84) for $PREMM_{1,2,6}$, 0.36 (95% CI: 0.27-0.47) for MMRpro and 0.31 (95% CI: 0.24-0.39) for MMRpredict. Predictions were too extreme for MMRpro (slope 0.43, 95% CI: 0.37-0.48) and for MMRpredict (slope 0.37, 95% CI: 0.31-0.42, Figures 2a-c, Table 2b). MMRpro and $PREMM_{1,2,6}$ identified a similar proportion of high-risk subjects who were mutation carriers at a cut-off of 5% to identify high risk individuals (13% versus 15% respectively; Table 3). All analyses were also performed by cohort and confirmed the patterns noted for the pooled data sets (Appendix Tables 1a and b).

Gene-Specific Predictions

The $PREMM_{1,2,6}$ and MMRpro models provide gene-specific predictions for *MLH1*, *MSH2*, and *MSH6*. In both clinic- and population-based cohorts, the models performed similarly in discrimination of *MLH1* and *MSH2* mutations (Tables 2a and b). However, discrimination of *MSH6* mutations from no mutation carriers was more difficult. Gene-specific O/E ratios and the calibration slopes were better for $PREMM_{1,2,6}$ than MMRpro for each gene in both types of cohorts (Table 2). Cohort-specific analyses confirmed these patterns.

Table 2a. Pooled performance characteristics of MMRpro, $PREMM_{1,2,6}$, and MMRpredict for prediction of MMR gene mutations associated with colorectal cancer cases in international clinical based cohorts (n=2304).

	MMRpro[a]	$PREMM_{1,2,6}$[b]	MMRpredict[c*]
DISCRIMINATION AUC (95% CL)			
Any mutation[d]	0.82 (0.80, 0.84)	0.85 (0.83, 0.87)	0.76 (0.74, 0.79)
MLH1	0.87 (0.84, 0.89)	0.88 (0.86, 0.90)	
MSH2	0.83 (0.80, 0.86)	0.87 (0.84, 0.89)	
MSH6	0.57 (0.49, 0.65)	0.67 (0.60, 0.75)	
CALIBRATION O/E ratio (95% CL)			
Any mutation[d]	0.62 (0.53, 0.74)	1.0 (0.89, 1.2)	0.38 (0.32, 0.45)
MLH1	0.49 (0.39, 0.62)	0.97 (0.82, 1.2)	
MSH2	0.51 (0.41, 0.64)	0.93 (0.78, 1.1)	
MSH6	0.26 (0.18, 0.37)	1.1 (0.84, 1.5)	
Slope (95% CL)			
Any mutation[d]	0.42 (0.38, 0.45)	0.81 (0.73, 0.88)	0.28 (0.25, 0.32)
MLH1	0.47 (0.42, 0.53)	0.81 (0.72, 0.90)	
MSH2	0.42 (0.37, 0.47)	0.77 (0.68, 0.86)	
MSH6	0.09 (-0.01, 0.20)	0.69 (0.44, 0.93)	

[a] Test of statistical interaction between cohorts and predicted probabilities: $p < 0.001$
[b] Test of statistical interaction between cohorts and predicted probabilities: $p = 0.03$
[c] Test of statistical interaction between cohorts and predicted probabilities: $p < 0.001$
For a-c: an interaction term with $p < 0.05$ indicated that the relation between predicted probabilities and mutation status varies by cohort.
* MMRpredict does not generate gene-specific probabilities
[d] *MLH1*, *MSH2,* or *MSH6* mutation
AUC, area under the receiver operating characteristic curve; CL, confidence limits; O/E, observed/expected.

Table 2b. Pooled performance characteristics of MMRpro, $PREMM_{1,2,6}$, and MMRpredict for prediction of MMR gene mutations associated with colorectal cancer cases in international population based cohorts (n=3451).

	MMRpro[a]	$PREMM_{1,2,6}$[b]	MMRpredict[c]
DISCRIMINATION AUC (95% CL)			
Any mutation[d]	0.85 (0.81, 0.88)	0.88 (0.85, 0.92)	0.77 (0.73, 0.82)
MLH1	0.84 (0.77, 0.91)	0.90 (0.85, 0.96)	
MSH2	0.92 (0.90, 0.95)	0.92 (0.88, 0.96)	
MSH6	0.68 (0.61, 0.76)	0.75 (0.66, 0.84)	
CALIBRATION O/E ratio (95% CL)			
Any mutation[d]	0.36 (0.27, 0.47)	0.70 (0.58, 0.84)	0.31 (0.24, 0.39)
MLH1	0.21 (0.13, 0.32)	0.61 (0.44, 0.85)	
MSH2	0.27 (0.18, 0.39)	0.71 (0.54, 0.94)	
MSH6	0.28 (0.18, 0.45)	0.70 (0.49, 1.00)	
Slope (95% CL)			
Any mutation[d]	0.43(0.37, 0.48)	1.07 (0.94, 1.19)	0.37 (0.31, 0.42)
MLH1	0.42 (0.34, 0.51)	0.93 (0.77, 1.09)	
MSH2	0.52 (0.44, 0.61)	1.03 (0.88, 1.18)	
MSH6	0.21 (0.10, 0.32)	1.12 (0.82, 1.43)	

[a] Test of heterogeneity between cohorts: $P = 0.47$; MMRpro results do not include data from the Spanish cohort.
[b] Test of heterogeneity between cohorts: $P < 0.001$
[c] Test of heterogeneity between cohorts: $P < 0.001$; MMRpredict does not generate gene-specific probabilities
[d] *MLH1*, *MSH2*, or *MSH6* mutation
AUC, area under the receiver operating characteristic curve; CL, confidence limits; O/E, observed/expected.

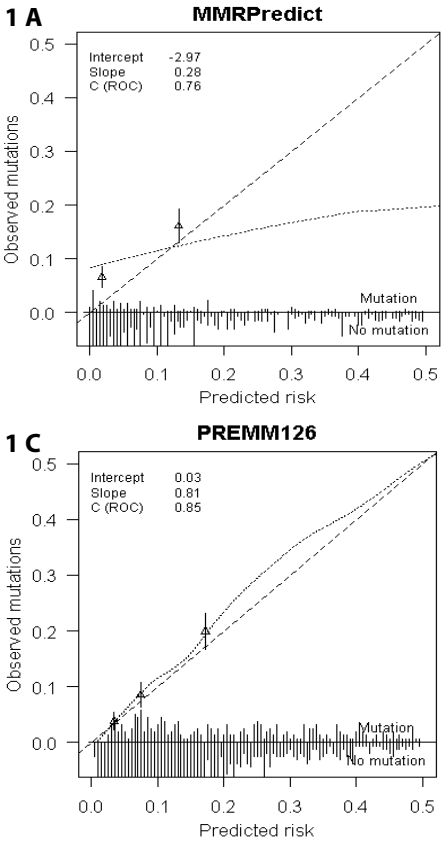


Figure 1a-c. Calibration plots for MMRpredict, MMRpro, and PREMM$_{1,2,6}$ models: clinic-based cohort. Figures 1a-c display calibration plots for external validation of a) MMRpredict, b) MMRpro, and c) PREMM$_{1,2,6}$ for predicting MMR mutations for individuals in clinic-based settings. The X-axis represents predicted probabilities, the Y-axis represents the observed proportion of MMR mutations, and the dashed diagonal line represents the ideal model with perfect prediction. The triangles represent observed frequencies by quantiles of predicted probability with corresponding 95% confidence limits (vertical lines). The population distribution (histogram) of predicted probabilities by mutation status is displayed in the lower portion of the Figure.

Clinical usefulness

In clinic-based cohorts, the decision to recommend genetic testing based on PREMM$_{1,2,6}$ or MMRpro estimates at any threshold ≥ 5% provided a higher net benefit compared to MMRpredict (Figure 3a). There was no net benefit in using MMRpredict to select subjects for testing at a threshold up to 10% compared to a strategy of testing all subjects. The PREMM$_{1,2,6}$ model provided the highest net benefit at thresholds ≥ 15% where more true positive carriers were identified compared to MMRpro. At liberal thresholds (< 5%), none of the models provide clinical usefulness beyond that of testing all subjects.

In population-based cohorts, using any of the models to determine who should undergo genetic testing was superior to testing all subjects for thresholds ≥ 5%. More carriers were identified with PREMM$_{1,2,6}$ than MMRpro and MMRpredict at higher thresholds (Figure 3b).

The net benefit for PREMM$_{1,2,6}$ was higher for gene-specific testing for *MLH1* and *MSH2* compared with MMRpro for both clinic and population-based cohorts while the net

benefit for *MSH6* gene testing was limited. For $PREMM_{1,2,6}$, the net benefit was limited to risk thresholds from 5% to 15% and only in clinic-based cohorts.

Table 3. Proportion of subjects identified as gene mutation carriers by MMRpro and $PREMM_{1,2,6}$ at different decision thresholds.

Model and risk score category	High Risk Subjects * N (%)	Identified gene mutation carriers N (%)
Clinic-based cohorts		
$PREMM_{1,2,6}$		
> 0%	2294 (100)	536 (100)
> 5%	1754 (76)	516 (96)
> 10%	1156 (50)	467 (87)
> 20%	744 (32)	403 (75)
> 40%	449 (20)	304 (57)
MMRpro		
> 0%	2304 (100)	539 (100)
> 5%	1595 (69)	510 (95)
> 10%	1338 (58)	482 (89)
> 20%	990 (43)	422 (78)
> 40%	701 (30)	365 (68)
Population-based cohort		
$PREMM_{1,2,6}$		
> 0%	3451 (100%)	150 (100%)
> 5%	887(26)	130 (87)
> 10%	375 (11)	102 (68)
> 20%	179 (5)	81 (54)
> 40%	91 (3)	52 (35)
MMRpro		
> 0%	2314 (100%)	150 (100%)
> 5%	906 (39)	119 (79)
> 10%	730 (32)	111 (74)
> 20%	532 (23)	102 (68)
> 40%	293 (13)	85 (57)

High Risk= number of subjects within each designated risk score category.

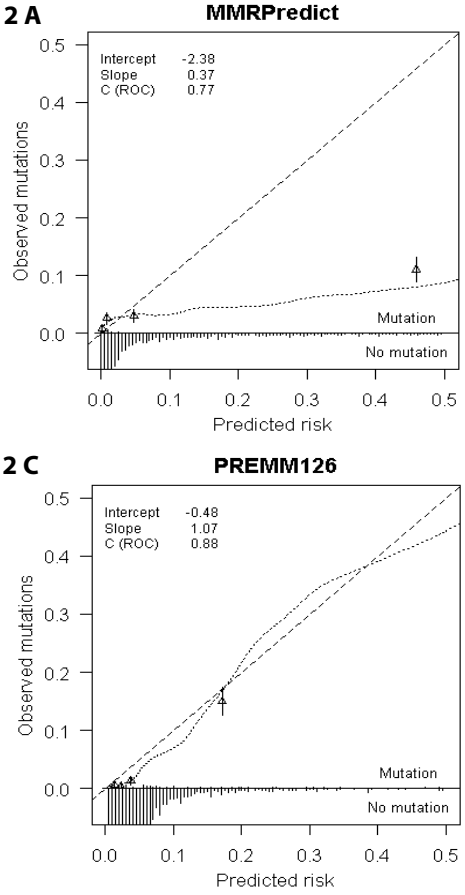


Figure 2a-c. Calibration plots for MMRpredict, MMRpro, and PREMM$_{1,2,6}$ models: population-based cohort. Figures 2a-c display calibration plots for external validation of a) MMRpredict, b) MMRpro, and c) PREMM$_{1,2,6}$ for predicting MMR mutations for individuals in population-based settings. The X-axis represents predicted probabilities, the Y-axis represents the observed proportion of MMR mutations, and the dashed diagonal line represents the ideal model with perfect prediction. The triangles represent observed frequencies by quantiles of predicted probability with corresponding 95% confidence limits (vertical lines). The population distribution (histogram) of predicted probabilities by mutation status is displayed in the lower portion of the Figure.

DISCUSSION

Both MMRpro and PREMM$_{1,2,6}$ better discriminated MMR gene mutation carriers from noncarriers than MMRpredict in this large dataset of international cohorts of individuals diagnosed with CRC in both clinic- and population-based settings. These models were clinically useful at the 5% threshold as recommended in the recent NCCN guideline for consideration of predictive genetic testing.[230] Highest usefulness was seen with PREMM$_{1,2,6}$ at thresholds above 15%, balancing the identification of mutation carriers without unnecessary extra testing. The PREMM$_{1,2,6}$ model also identified more carriers among population-based cases at thresholds ≥ 5%.

Our study provides important insight about the models' clinical usefulness through decision curve analyses which offers information beyond the standard performance metrics that indicate discrimination and calibration.[233-238] This methodology allowed

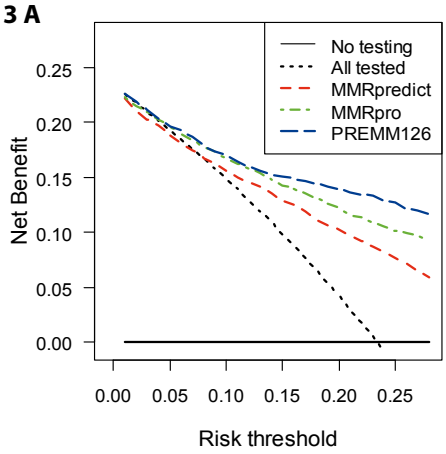


Figure 3a. Net benefit analyses comparing MMRpredict, MMRpro, and $PREMM_{1,2,6}$: clinic-based cohort. Figure 3a displays the net benefit curves comparing the three prediction models among the clinic-based cohort. The y-axis measures net benefit which is calculated by summing the benefits (true positives) and subtracting the harms (false positives), where the latter are weighted by a factor related to the relative harm of a missed mutation carrier compared with the harm of unnecessary genetic testing. A model is considered of clinical value if it has the highest net benefit compared with other models and simple strategies such as performing genetic testing in all patients (dashed black line) or no patients (horizontal black line) across the full range of threshold probabilities at which a patient would choose to undergo genetic testing. For example, at the threshold of 10% used to designate an individual at risk of having a gene mutation, the net benefit of $PREMM_{1,2,6}$ is interpreted as the identification of 18 mutation carriers per 100 screened individuals in a clinic-based setting for whom germline testing would confirm a mutation without increasing the number tested unnecessarily.

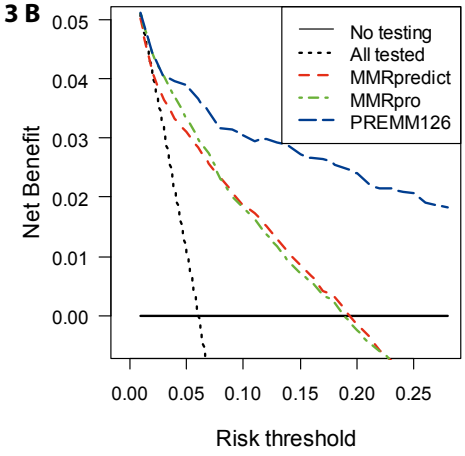


Figure 3b. Net benefit analyses comparing MMRpredict, MMRpro, and $PREMM_{1,2,6}$: population-based cohort. Figure 3b displays the net benefit curves for the three models among the population-based cohort. For example, at the threshold of 10% used to designate an individual at risk of having a gene mutation, the net benefit of $PREMM_{1,2,6}$ is interpreted as the identification of 2 mutation carriers per 100 screened individuals in a population-based setting for whom germline testing would confirm a mutation without increasing the number tested unnecessarily.

us to estimate the net number of carriers identified by each model over different risk thresholds to select cases for further testing, penalizing for the number of patients having unnecessary testing. There was no net benefit of any of the models at relatively low risk thresholds (< 5%), where testing all individuals would be optimal. With thresholds of 5% or greater, both MMRpro and $PREMM_{1,2,6}$ are clinically useful in the clinic-based cohorts, where the selection of those recommended genetic testing is improved with use of the models compared to without them. $PREMM_{1,2,6}$ also had an appreciable net benefit in the population-based cohorts, despite being originally developed using clinic-based CRC cases. This is explained by the better calibration of $PREMM_{1,2,6}$ (i.e. the predicted probabilities are more consistent with the observed probabilities of mutations) than MMRpro and MMRpredict. While all models overestimated the probability of being a carrier among population-based cases, they most often deviated in their predictions thresholds of < 5%, where the predicted number of carriers far exceeded those observed. While this affects overall calibration, it has limited clinical significance since germline testing has not been recommended in subjects with predicted probabilities < 5%.

The geographic diversity of the cohorts provides a more comprehensive assessment of external validity than previous analyses,[87-89, 151, 239-242] in addition to a recent meta-analysis of studies that have validated the prediction models.[243] The clinic-based sample included individuals with CRC evaluated at cancer genetics clinics where personal and family histories of cancers were well characterized. We addressed the potential utility of the models in general medical settings by the inclusion of population-based series. Our results also provide new information about the ability of MMRpro and $PREMM_{1,2,6}$ to predict gene-specific risk estimates. Both models performed equally well in identifying *MLH1* and *MSH2* gene mutation carriers but had low overall discrimination for *MSH6* gene mutations. *MSH6* gene mutation carriers are challenging to identify as the age of CRC diagnosis is commonly higher in comparison to *MLH1* and *MSH2* and cases may be missed as they appear as "sporadic CRC". In addition, endometrial cancer may present as the sentinel malignancy in female *MSH6* carriers.

Several limitations of our study should be considered. The mechanisms of case identification at each site may have contributed to site-specific variation in model performance, although the overall patterns of the validity of the prediction models were rather consistent. Differences in the prevalence of carriers between sites could be attributable to heterogeneity in standard assessments between sites (i.e. the referral filter).[244] Another possible limitation is that some sites screened individuals for MMR deficiency based on tumor testing results and did not pursue germline testing when tumor testing was normal. This partial verification bias may misclassify some individuals as noncarriers[245] and may be more relevant for individuals with *MSH6* gene mutations, whose tumors are not always microsatellite unstable and where certain pathogenic missense muta-

tions do not completely abrogate protein expression yielding false negative IHC results. Therefore, partial verification bias may have contributed to the low discriminatory accuracy for *MSH6* mutations. Lastly, the current models do not predict *PMS2* and *EPCAM* mutations. Since the majority of sites did not perform these mutational analyses, the models' performance for these genes could not be assessed.

The results of our study have several implications for individuals with CRC. Assessment of family history of cancer by the Amsterdam criteria or Bethesda guidelines[84, 168] has been the cornerstone for the diagnosis of LS, but multiple studies have demonstrated limited sensitivity and specificity for both tools.[246, 247] In addition, the performance of $PREMM_{1,2,6}$ and MMRpro has been shown to exceed that of existing clinical criteria to identify mutation carriers[82, 85, 87, 89, 151, 164] and it has been suggested that prediction models replace clinical criteria as prescreening tools in the risk assessment process for LS.[89, 239, 242] For such an application, the $PREMM_{1,2,6}$ model has the advantage of being simpler to apply than MMRpro.[239] Another approach that has been advocated is systematic tumor testing for MMR deficiency through MSI or IHC for *all* newly diagnosed individuals with CRC.[14, 246] This approach may be feasible for some centers and superior to the use of any prediction model if we accept a high rate of unnecessary testing. However, false-positive tumor results may occur due to somatic causes of MSI via *MLH1* gene promoter hypermethylation, particularly in older patients. Among 10,206 population-based CRC cases, selective tumor testing in those diagnosed at ≤ 70 years and in patients > 70 years who met at least one criterion of the revised guidelines, achieved a similar yield to the universal strategy of testing all patients.[248] Our group has also found improved identification of gene mutation carriers though a combined strategies approach using IHC and prediction model risk estimates with the same age cutoff of 70 years.[225] The higher rates of false-positive results on tumor testing in older patients were offset by the increased performance of $PREMM_{1,2,6}$ with every 10-year increase in age of CRC diagnosis. In light of these considerations, the $PREMM_{1,2,6}$ or MMRpro models can be used to direct germline testing or genetic referral when ≥ 5% if tumor testing is unavailable or when resources are limited and the universal tumor testing approach cannot be adopted. These prediction models can also complement results from tumor testing since false-negative results are possible with IHC testing.[225] Individuals with high risk, i.e. ≥ 15%, may need to be considered for inherited cancer syndromes other than LS. Screening patients with CRC for LS based on molecular tumor testing alone may miss the opportunity to identify other familial cancer syndromes. For those patients without a germline MMR mutation but with high prediction scores, intensive surveillance may be considered and additional genetic testing in the future may be warranted as novel genes associated with familial CRC are discovered.

In summary, the MMRpro and $PREMM_{1,2,6}$ models are clinically useful tools to assess patients who are newly diagnosed with CRC for LS. We note that the $PREMM_{1,2,6}$ model

may be preferred for clinical use since it does not require information on unaffected family members and its simple, web-based platform.[239] A threshold of $\geq$ 15% may assist in the clinical management of individuals when MMR deficiency is undetected by indicating a high-risk for familial CRC despite negative tumor and/or genetic test results. These patients likely need continued modified cancer surveillance tailored to their specific cancer spectrum as well as consideration of other genetic etiologies for the cause of cancer in their families.

ACKNOWLEDGMENTS

We would like to acknowledge the following Colon Cancer Family Registries that provided data for the analysis: Australasian Colorectal Cancer Family Registry (U01 CA097735), the University of Southern California (USC) Familial Colorectal Neoplasia Collaborative Group (U01 CA074799), Mayo Clinic Cooperative Family Registry for Colon Cancer Studies (U01 CA074800), Ontario Registry for Studies of Familial Colorectal Cancer (U01 CA074783), Seattle Colorectal Cancer Family Registry (U01 CA074794), and University of Hawaii Colorectal Cancer Family Registry (U01 CA074806).

We would also like to acknowledge the following contributors for providing data for the analyses: the LIMO (Lynch syndrome Identification through Molecular analysis) investigators from Erasmus MC, the Netherlands including van Lier MG, Wagner A, Dubbink HJ, Dinjens WN, Ramsoekh D, van Leerdam ME, Kuipers EJ; members of the Hereditary Cancer Group of the Spanish Medical Oncology Society (SEOM): Blanco I, Torres A, Ramón y Cajal T, Chirivella I, Guillén C, Brunet J, Martínez-Bouzas C, Alonso A, Pérez Segura P, Robles L, Graña B, Urioste M, Lastra E; and the EpiCOLON Consortium (Gastrointestinal Oncology Group of the Spanish Gastroenterological Association): National coordinators: Andreu M, Carracedo A, Castells A, Jover R, and Llor X; Members: Hospital Clínic, Barcelona: Castells A (local coordinator), Piñol V, Castellví-Bel S, Gonzalo V, Ocaña T, Giráldez MD, Pellisé M, Serradesanferm A, Moreira L, Cuatrecasas M, Piqué JM; Hospital 12 de Octubre, Madrid: Morillas JD (local coordinator), Muñoz R, Manzano M, Colina F, Díaz J, Ibarrola C, López G, Ibáñez A; Hospital Clínico Universitario, Zaragoza: Lanas A (local coordinator), Alcedo J, Ortego J; Complexo Hospitalario de Ourense: Cubiella J(local coordinator), Mª Díez A, Salgado M, Sánchez E, Vega M; Hospital del Mar, Barcelona: Andreu M (local coordinator), Abuli A, Bessa X, Iglesias M, Seoane A, Bory F, Navarro G, Bellosillo B; Dedeu J, Álvarez C, Gonzalez B; Hospital San Eloy, Baracaldo and Hospital Donostia, San Sebastián: Bujanda L(local coordinator) Cosme A, Gil I, Larzabal M, Placer C, del Mar Ramírez M, Hijona E, Enríquez-Navascués JM, Elosegui JL; Hospital General Universitario de Alicante: Payá A (EPICOLON I local coordinator), Jover R (EPICOLON II local coordinator), Alenda C, Sempere L, Acame N, Rojas E, Pérez-Carbonell L; Hospital General de Granollers: Rigau J (local

coordinator), Serrano A, Giménez A; Hospital General de Vic: Saló J (local coordinator), Batiste-Alentorn E, Autonell J, Barniol R; Hospital General Universitario de Guadalajara and Fundación para la Formación e Investigación Sanitarias Murcia: García AM (local coordinator), Carballo F, Bienvenido A, Sanz E, González F, Sánchez J, Ono A; Hospital General Universitario de Valencia: Latorre M (local coordinator), Medina E, Cuquerella J, Canelles P, Martorell M, García JA, Quiles F, Orti E; Hospital Meixoeiro, Vigo: Clofent J (EPICOLON I local coordinator), Mª de Castro L (EPICOLON II local coordinator), Seoane J, Tardío A, Sanchez E, Hernández V; Hospital Universitari Germans Trias i Pujol, Badalona and University of Illinois at Chicago, IL: Llor X (local coordinator), Xicola RM, Piñol M, Rosinach M, Roca A, Pons E, Hernández JM, Gassull MA; Hospital Universitari Mútua de Terrassa: Fernández-Bañares F(local coordinator), Viver JM, Salas A, Espinós J, Forné M, Esteve M; Hospital Universitari Arnau de Vilanova, Lleida: Reñé JM (local coordinator), Piñol C, Buenestado J, Viñas J; Hospital Universitario de Canarias, Tenerife: Quintero E (local coordinator), Nicolás D, Parra A, Martín A; Hospital Universitario La Fe, Valencia: Argüello L (local coordinator), Pons V, Pertejo V, Sala T; Hospital de Sant Pau, Barcelona: Gonzalez D (local coordinator) Roman E, Ramon T, Poca M, Mª Concepción M, Martin M, Pétriz L; Hospital Xeral Cies, Vigo: Martinez D (local coordinator); Fundacion Publica Galega de Medicina Xenomica, Santiago de Compostela: Carracedo A (local coordinator), Ruiz-Ponte C, Fernández-Rozadilla C, Mª Castro M; Hospital Universitario Central de Asturias, Oviedo: Riestra S (local coordinator), Rodrigo L; Hospital de Galdácano, Vizcaya: Fernández J (local coordinator), Cabriada JL; Fundación Hospital de Calahorra: Carreño L (local coordinator), Oquiñena S, Bolado F; Hospital Royo Villanova, Zaragoza: Peña E (local coordinator), Blas JM, Ceña G, Sebastián JJ; Hospital Universitario Reina Sofía, Córdoba: Naranjo A (local coordinator).

APPENDIX

Table 1a. Site-specific performance characteristics of MMRpro, $PREMM_{1,2,6}$, and MMRpredict for prediction of any MMR gene mutation among colorectal cancer cases: clinic-based cohort.

	MMRpro	**$PREMM_{1,2,6}$**	**MMRpredict**
DISCRIMINATION AUC (95% CL)			
CCFR	0.85 (0.82, 0.89)	0.84 (0.81, 0.88)	0.78 (0.73, 0.82)
DFCI	0.83 (0.77, 0.89)	0.85 (0.79, 0.90)	0.78 (0.71, 0.85)
Milan, Italy	0.72 (0.65, 0.78)	0.79 (0.73, 0.85)	0.72 (0.65, 0.78)
Newfoundland, Canada	0.87 (0.76, 0.98)	0.91 (0.84, 0.98)	0.86 (0.76, 0.96)
Rotterdam, Netherlands	0.77 (0.70, 0.84)	0.79 (0.73, 0.86)	0.65 (0.57, 0.73)
Spanish Consortium	0.85 (0.81, 0.89)	0.85 (0.82, 0.89)	0.84 (0.79, 0.88)
CALIBRATION O/E ratio (95% CL)			
CCFR	0.61 (0.43, 0.86)	1.0 (0.79, 1.3)	0.29 (0.21, 0.39)
DFCI	0.57 (0.33, 1.0)	1.1 (0.73, 1.6)	0.30 (0.18, 0.49)
Milan, Italy	1.8 (1.3, 2.5)	1.2 (0.82, 1.7)	0.51 (0.31, 0.84)
Newfoundland, Canada	0.35 (0.14, 0.86)	0.74 (0.40, 1.4)	0.13 (0.07, 0.26)
Rotterdam, Netherlands	0.51 (0.35, 0.76)	0.69 (0.50, 0.94)	0.20 (0.14, 0.29)
Spanish Consortium	0.48 (0.35, 0.67)	1.2 (0.96, 1.5)	0.83 (0.63, 1.1)
Slope (95% CL)			
CCFR	0.39 (0.32, 0.46)	0.71 (0.57, 0.84)	0.28 (0.22, 0.34)
DFCI	0.39 (0.28, 0.50)	0.73 (0.52, 0.94)	0.28 (0.19, 0.38)
Milan, Italy	0.38 (0.23, 0.52)	0.52 (0.36, 0.68)	0.20 (0.12, 2.7)
Newfoundland, Canada	0.50 (0.29, 0.71)	1.35 (0.79, 1.9)	0.68 (0.35, 1.0)
Rotterdam, Netherlands	0.42 (0.31, 0.54)	0.91 (0.67, 1.1)	0.17 (0.09, 0.26)
Spanish Consortium	0.53 (0.44, 0.62)	1.0 (0.86, 1.2)	0.61 (0.5, 0.72)

AUC, area under the receiver operating characteristic curve; CL, confidence limits;
O/E, observed/expected; CCFR, Colon Cancer Family Registries; DFCI, Dana Farber Cancer Institute.

Table 1b. Site-specific performance characteristics of MMRpro, PREMM$_{1,2,6}$, and MMRpredict for prediction of any MMR gene mutation among colorectal cancer cases: population-based cohort.

	MMRpro	**PREMM$_{1,2,6}$**	**MMRpredict**
DISCRIMINATION AUC (95% CL)			
CCFR	0.83 (0.78, 0.88)	0.87 (0.82, 0.92)	0.86 (0.81, 0.90)
OSU	0.97 (0.93, 1.00)	0.95 (0.87, 1.00)	0.94 (0.87, 1.00)
Newfoundland, Canada	0.86 (0.80, 0.92)	0.88 (0.81, 0.94)	0.76 (0.66, 0.85)
Rotterdam, Netherlands	0.75 (0.62, 0.88)	0.80 (0.69, 0.91)	0.75 (0.64, 0.85)
Spanish Consortium	-	0.74 (0.53, 0.95)	0.78 (0.60, 0.95)
CALIBRATION O/E ratio (95% CL)			
CCFR	0.26 (0.18, 0.37)	0.79 (0.60, 1.04)	1.01 (0.73, 1.40)
OSU	0.29 (0.12, 0.68)	0.32 (0.17, 0.59)	0.09 (0.05, 0.17)
Newfoundland, Canada	1.09 (0.50, 2.36)	1.51 (0.91, 2.49)	0.56 (0.26, 1.21)
Rotterdam, Netherlands	0.71 (0.36, 1.42)	1.13 (0.67, 1.93)	0.52 (0.23, 1.19)
Spanish Consortium	-	0.28 (0.15, 0.54)	0.06 (0.03, 0.12)
Slope (95% CL)			
CCFR	0.45 (0.36, 0.53)	0.99 (0.82, 1.16)	0.55 (0.45, 0.65)
OSU	0.78 (0.52, 1.04)	1.73 (1.14, 2.32)	0.78 (0.48, 1.09)
Newfoundland, Canada	0.30 (0.19, 0.41)	0.73 (0.46, 1.01)	0.23 (0.14, 0.33)
Rotterdam, Netherlands	0.44 (0.24, 0.64)	1.01 (0.55, 1.47)	0.21 (0.09, 0.34)
Spanish Consortium	-	0.97 (0.55, 1.39)	0.50 (0.27, 0.74)

AUC, area under the receiver operating characteristic curve; CL, confidence limits;
O/E, observed/expected; CCFR, Colon Cancer Family Registries; OSU, Ohio State University.

Chapter 8

Genetic testing for Lynch syndrome: family communication and motivation

Celine H.M. Leenen, Mariska den Heijer, Conny A. van der Meer, Ernst J. Kuipers, Monique E. van Leerdam, Anja Wagner

Submitted for publication

ABSTRACT

Current genetic counseling practice for Lynch syndrome (LS) relies on diagnosed index patients to inform their biological family about LS, referred to as the family-mediated approach.

The objective of this study was to evaluate this approach and to identify factors influencing the uptake of genetic testing for LS. In 59 mutation carriers, 70 non carriers and 16 non-tested relatives socio-demographic characteristics, family communication regarding LS, experiences and attitudes towards the family-mediated approach and motivations for genetic testing, were assessed.

The majority of all respondents (73%) were satisfied with the family-mediated approach. Nevertheless, 59% of the respondents experienced informing a family member and 57% being informed by a family member as burdensome. Non-tested differed from tested respondents, in that they were younger, less closely related to the index patient and a lower proportion had children. The most important reasons for declining genetic testing were 1) anticipating problems with life insurance and mortgage, 2) being content with life as it is, and 3) not experiencing any physical complaints.

In conclusion, the majority of respondents consider the current family-mediated information procedure acceptable, although the provision of information on LS by relatives may be burdensome. Special attention should be paid to communication of LS to more distant relatives.

INTRODUCTION

Lynch syndrome (LS) is a hereditary condition which predisposes to colorectal cancer, endometrial cancer and other cancers.[249, 250] It is caused by inherited germline mutations in mismatch repair (MMR) genes, particularly *MLH1, MSH2, MSH6* and *PMS2* or the 3' region of the *EPCAM* gene.[17, 23-25, 102, 103] LS carriers have an increased cumulative lifetime risk for colorectal cancer of 25 to 70%, while women with LS carry a lifetime risk to develop EC of 13 to 65%.[1-12] In addition, LS carriers have an increased risk for cancers of the stomach, ovaries, small bowel, urinary tract, skin and brain.[15, 16, 54, 251]

Genetic testing for LS is available to all family members of a mutation carrier. Genetic testing can have medical and psychological advantages, irrespective of the outcome in an individual subject. Non-carriers may avoid unnecessary surveillance programs for LS and experience relief from worries about developing cancer both for themselves and their children. For carriers, genetic testing can lead to relief from uncertainty and guide screening recommendations, improving survival through early detection.[54, 252] Despite the potential benefits of genetic testing, a Dutch study on the interest in genetic testing for hereditary colorectal cancer syndromes showed that almost half of the subjects in this cohort of family members at risk did not opt for genetic testing for LS at a median follow-up time after identification of the family specific mutation of 82 months, ranging 10-140 months.[97]

In the Netherlands the communication regarding presence of an MMR gene mutation within a family occurs by means of the family-mediated approach. When a pathogenic mutation is detected the counselee is asked to inform all at risk relatives. During the counseling process, communication strategies to inform relatives are discussed with the counselee. Furthermore a letter to inform relatives is supplied. This approach implies that family members are responsible to inform their relatives on the diagnosis of LS and the possibility of genetic testing. Currently, little is known about patients' experiences with and attitudes towards this family-mediated approach.[99] Knowledge on the challenges with regards to informing family members may help to improve counseling procedures. A previous study on family communication of LS genetic test results showed that most individuals who undergo genetic testing for LS share their test result with first degree relatives, while more distant relatives are reached less often.[253] Interestingly, another study on family communication of LS-genetic testing results showed a significant gender difference. Men were less likely to communicate the diagnosis of LS to their relatives, yet disclosed this result significantly more often via a support person such as a spouse.[254] A previous qualitative study in the Netherlands among 30 individuals from LS families showed that motivation to disclose seemed to increase if there were more cancer cases in the family. Disrupted family relations were found to be an important reason for non-disclosure. The way family members communicate about LS may also influence whether

or not at-risk family members decide to opt for genetic testing.[100, 101] It would be of clinical interest to gain more insight into the factors influencing the decision whether or not to opt for genetic testing. However, clinical information about the group of non-tested individuals for LS is scarce, since individuals who do not opt for genetic testing often do not apply for genetic counseling.

The aims of this study were to 1) evaluate experiences and attitudes towards a family-mediated approach in an LS cohort, 2) compare tested (mutation carriers and non-carriers) and non-tested individuals on demographic characteristics, anxiety, cancer worry, medical history, family communication, experiences and attitudes towards the family mediated approach, and 3) explore the motivations for uptake or decline of genetic testing for LS.

METHODS

Subjects and procedure

We conducted a cross-sectional survey among individuals with a personal or family history of LS. The study was performed at the Department of Clinical Genetics of the Erasmus University Medical Center. Subjects were recruited from a cohort of 40 LS families with a proven LS mutation. All individuals were 25 years or older, since it is recommended to undergo genetic testing after this age. The tested individuals had received their genetic test result between 1995 and 2009. For each individual a family pedigree was available with detailed medical information.

Two hundred ninety seven tested individuals ≥ 25 years of age, including index patients, from the above described LS cohort were notified about the start of the research project by an advanced notification letter. Individuals who were interested in participating were asked to respond via a reply card and were subsequently contacted by de study coordinator. The study coordinator informed the individual about the study and asked the individual to participate in this survey. In addition, the study coordinator specifically asked the tested individuals if they knew family members who had refrained from genetic testing for LS. The tested individuals were asked to contact these non-tested family members, in order to obtain consent for being approached for research purposes. A questionnaire was sent to all individuals who consented to participate. Individuals who did not return the questionnaire after two follow-up telephone calls and two additional mailings were considered non-responders.

This study was approved by the Institutional Review Board of the Erasmus MC, and written informed consent was obtained from all respondents.

Measures

The self-reported questionnaire addressed socio-demographic characteristics including age, gender, marital status, number of children, level of education, employment and medical characteristics.

In addition, respondents were asked whether they, themselves or their relatives had ever been diagnosed with cancer, and to indicate the degree of relatedness to the closest relative affected by cancer. Medical data of tested respondents was cross-checked with their family pedigree at the Department of Clinical Genetics.

Family communication regarding LS was evaluated by a list of questions developed by the authors after a literature search.[100, 255-257] Respondents were asked who informed them about LS, when they were informed, in which way and how the contact was before en after disclosure of the LS diagnosis. Furthermore we asked if it was burdensome to be informed and/or informing relatives on LS using a five-point Likert scale with response options ranging from 1 '*very burdensome*' to 5 '*not burdensome*'.

Attitude towards the family mediated approach was measured by a self-developed questionnaire with two statements regarding moral duty to disclose LS diagnosis. In two other questions we asked the respondents if they were satisfied with the current family mediated approach using a five-point Likert scale.

Anxiety and depression were measured by the Hospital Anxiety and Depression scale (HADS). Seven items of the HADS reflect anxiety and seven reflect depression. Response options range from 0 to 3.[258] The sum on each subscale indicates the overall anxiety and depression score (between 0 and 21). A sum score of 11 or more is the threshold for clinical anxiety.

We assessed concerns regarding cancer by means of the cancer worry scale (CWS).[259] The CWS is a four-item scale that measures worries about the risk of developing cancer and the impact of worries on daily functioning (frequency of thoughts of developing cancer, impact of thoughts about cancer on mood, impact of thoughts about cancer on daily activities, and level of concern for developing cancer). Each item has four possible responses (from 1 *'not at all'*, to 4 *'almost all the time/very concerned'*), which are summed to create a CWS between 4 and 16. A higher score indicates more concerns regarding cancer.

Motivation for genetic testing was evaluated using a list of 15 reasons for non-participation, which was adapted from literature.[260, 261] Non-tested respondents were asked to rate to what extent they agreed with these reasons for non-participation in genetic testing on a five-point Likert scale with response options ranging from 1 *'totally disagree'* to 5 '*totally agree*'. An open field was included to add another reason for non-participation. The questionnaire was pilot tested among ten LS carriers visiting the outpatient clinic.

Statistical analysis

Categorical variables were used to calculate proportions and interquartile ranges. The association between categorical variables was examined by means of the Chi-squared test or Fisher's exact test. For ordered categorical variables, the Mann-Whitney U test was used. Scores from the HADS and cancer worry were treated as continuous variables. For continuous variables the mean and standard deviation was calculated. These variables were tested using the independent sample T-test. Respondents with missing data were omitted from the respective analyses.

Mutation carriers, non-carriers and non-tested respondents were compared on socio-demographic characteristics, anxiety, cancer worry, medical history, family communication, experiences and attitudes towards the family mediated approach. SPSS 17.0 statistical package was used to analyse data. All p-values are two-sided and a p-value of < 0.05 was considered significant.

RESULTS

Subject characteristics

Two hundred ninety seven eligible individuals were approached for enrolment by an advanced notification letter with reply card. Of these, 215 (72%) agreed to be contacted by phone (Figure 1). Of the 215 subjects who agreed to be contacted, 177 (60%) accepted to receive the questionnaire. One-hundred and twenty-nine (43%) tested individuals from 33 LS families returned the questionnaire. A total of 41 non-tested individuals were contacted via the tested individuals and 18/41 (44%) non-tested individuals returned the questionnaire. Two non-tested individuals were excluded, since they underwent genetic testing before completing the questionnaire. There was no difference in age and gender between non-participants, non-responders and responders in the tested and non-tested group (data not shown).

Baseline characteristics of all 145 respondents are shown in Table 1. Of all 129 tested respondents, 59 (46%) were mutation carriers and 70 (54%) had no LS mutation. The mean age of mutation carriers was 52 years (SD 14) and for non-carriers 67 years (SD 13). Both mutation carriers and non-carriers were older than non-tested respondents with a mean age of 42 years (SD 17, p=0.007). Twelve respondents from the 33 LS families were index patients.

LS mutation carriers and non-carriers compared with non-tested respondents

Demographic and family characteristics of mutation carriers, non-carriers and non-tested respondents are shown in Table 1. Non-tested respondents differed from LS mutation carriers and non-carriers in age, number of children, degree of relatedness to the index

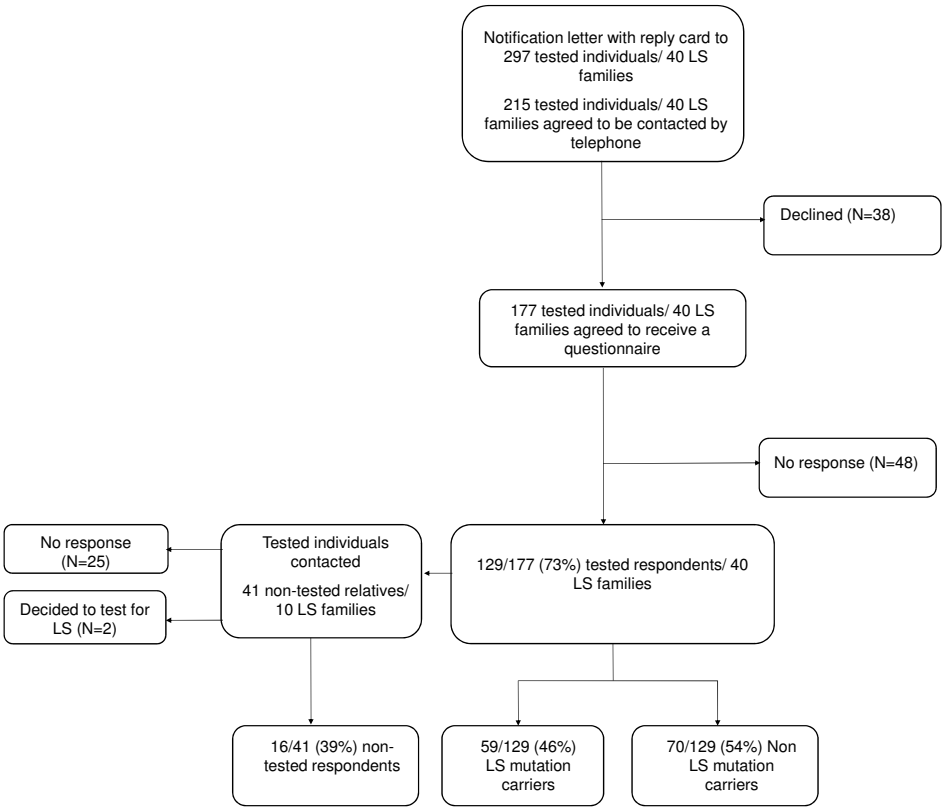


Figure 1. Flowchart of the study procedure.

patient and cancer diagnosis. Of non-tested respondents 44% did not have children, compared to 14% of mutation carriers (p=0.013) and 7% of non-carriers (p=0.02).

Twelve (8%) respondents had been index patients within their family and thus the first informed on LS in the family. Fifty-four respondents (37%) were first-degree relatives of the index patients and 78 (53%) were second or third-degree relative of the index patient. More non-tested respondents (63%) were second-degree relatives of the index patient, compared to mutation carriers (42%, p=0.03) and non-carriers (47%, p=0.02). A minority of total respondents (N=10) were third-degree relatives (Table 1). None of the non-tested respondents reported to be diagnosed with cancer, while 19 (32%; p=0.004) of the mutation carriers and 11 (16%; p=0.116) of the non-carriers reported to be diagnosed with cancer. Furthermore, non-tested family members reported to have a median of one relative with LS-associated cancer, while tested relatives had a median of two relatives with cancer (p=0.01).

HADS scores did not differ between non-tested respondents and LS mutation carriers and non-carriers (mean HADS respectively 4.0; 4.1; 4.7, Table 1) and are comparable with the mean HADS scores of the Dutch general population between 18 and 65 years

Table 1. Characteristics of the respondents.

	Mutation carriers	%	Non-carriers	%	Non-tested respondents	%	Total respondents	%
Number of respondents	59		70		16		145	100%
Male	26	44%	24	34%	6	38%	56	39%
Mean age (±SD)*	52 (14)		67 (13)		42 (17)		55 (15)	
Marital status								
Single	5	8%	7	10%	3	19%	15	10%
(As) married	46	78%	53	76%	9	56%	108	74%
Divorced / separated / widowed	7	12%	8	11%	4	25%	19	13%
Missing	2	3%	1	1%	0	0%	3	2%
Number of children								
None*	8	14%	5	7%	7	44%	20	14%
One or more children*	51	86%	65	93%	9	56%	125	86%
Employed								
Yes	38	64%	30	43%	10	63%	78	54%
Retired*	12	20%	27	39%	2	13%	41	28%
Student	1	2%	1	1%	1	6%	3	2%
Missing	2	3%	2	3%	0	0%	4	3%
Education								
High educational level	24	41%	20	29%	6	38%	50	34%
Low educational level	32	54%	47	67%	10	63%	89	61%
Missing	3	5%	3	4%	0	0%	6	4%
Relation to index								
Index patient	9	15%	3	4%	0	0%	12	8%
First degree relative*	24	41%	27	39%	3	19%	54	37%
Second degree relative*	25	42%	33	47%	10	63%	68	47%
Third degree relative	1	2%	7	10%	2	13%	10	7%
Cancer diagnosis								
Yes*	19	32%	11	16%	0	0%	30	21%

Table 1. (Continued)

	Mutation carriers	%	Non-carriers	%	Non-tested respondents	%	Total respondents	%
Anxiety and cancer worry								
Mean cancer worry (±SD)	5.3 (1.4)		5.2 (1.5)		5.1 (1.2)		5.1 (1.4)	
HADS anxiety (±SD)	4.1 (3.5)		4.7 (3.6)		4.0 (3.3)		4.5 (3.5)	
Median number of relatives with LS cancers*	2		2		1*		2	

* P = <0.05, non-tested respondents vs LS mutation carriers and non-carriers

of age.[262] Fourteen respondents (10%, six mutation carriers, seven non-carriers, one non tested respondent) had an anxiety score ≥11 and two other respondents (1%, one non-carrier, 1 non-tested) had a depression score ≥11. Mean worry about cancer did not differ among mutation carriers, non-carriers and non-tested respondents (Table 1).

Experiences with the family mediated approach

Table 2 shows the experiences with the family-mediated approach. A total of 115 of the 145 (79%) respondents were informed by a family member about the diagnosis LS mostly by means of a personal explanation (70/145; 48%) and/or the letter provided by the Genetics department to the index patient (63/145 43%). Interestingly, five of sixteen non-tested respondents reported to be informed on LS diagnosis by a genetic counsellor. In three cases it was confirmed in our institutional LS database that these cases were counselled but refrained from genetic testing.

The majority of the respondents, who were informed by a family member about the presence of LS in their family, were informed by a first degree family member (81/115; 70%) and most of them (74/115; 64%) reported to have good contact with this family member. For most respondents the LS disclosure did not change their contact with the family member. The majority of respondents informed by a family member about LS (65/115; 57%) reported that they had experienced the process of being informed by a family member as (moderately) burdensome. Significant more mutation carriers than non-carriers reported burden due to being informed on the LS diagnosis by a family member (p= 0.002). Furthermore, more mutation-carriers than non-tested respondents experienced burden while informing other family members about LS, but this difference was not significant (p=0.07).

Table 2. Experiences with the family-mediated approach.

	Mutation carriers	%	Non-carriers	%	Non-tested respondents	%	Total respondents	%
2a. Communication within the family, answered by all respondents								
Number of respondents	59	100%	70	100%	16	100%	145	100%
When were you informed about LS in your family?								
< 1 week after diagnosing LS in a family member	20	34%	18	26%	3	19%	41	28%
< 1month after diagnosing LS in a family member	8	14%	13	19%	3	19%	24	17%
< 6 months after diagnosing LS in a family member	3	5%	11	16%	1	6%	15	10%
< 1 year after diagnosing LS in a family member	3	5%	10	14%	0	0%	13	9%
< 5 years after diagnosing LS in a family member	6	10%	3	4%	3	19%	12	8%
> 5 years after diagnosing LS in a family member	2	3%	1	1%	0	0%	3	2%
Missing	17	29%	14	20%	6	37%	37	26%
Informed on the diagnosis of LS in the family by…(multiple answers)								
Family member	41	-	61	-	13		115	-
Clinical geneticist/ counsellor	31	-	17	-	5		53	-
Missing	7	-	0	-	0		7	-
Communication tools within the family (multiple answers)								
Family information letter genetics	24	-	34	-	5		63	-
Personal letter from a family member	6	-	12	-	0		18	-
Personal explanation from a family member	25	-	34	-	11		70	-
Missing	4	-	2	-	0		6	-
2b. Experiences on being informed by a relative about LS.								
Questions are answered by family members who answered to be informed by a relative about LS.								
Number of respondents	41	69%	61	87%	13	81%	115	79%
Which family member informed you about LS?**								
First degree family member	31	75%	38	62%	12	92%	81	70%
Second degree family member	6	15%	6	10%	1	8%	13	11%
Third degree relative	4	10%	17	28%	0	0%	21	18%
Missing	0	0%	0	0%	0	0%	0	0%

Table 2. (Continued)

	Mutation carriers	%	Non-carriers	%	Non-tested respondents	%	Total respondents	%
Contact with the informing family member **								
Poor	6	15%	13	21%	3	23%	22	19%
Neutral	5	12%	12	20%	0	0%	17	15%
Good	29	71%	35	57%	10	77%	74	64%
Missing	1	2%	1	2%	0	0%	2	2%
Effect on family relations**								
Family relations improved	4	10%	3	5%	1	8%	8	7%
Family relations worsened	2	5%	0	0%	1	8%	3	3%
No change in family relations	34	83%	55	90%	9	69%	98	85%
Missing	1	2%	3	5%	2	15%	6	5%
Burdensome being informed by family members[a]**								
Burdensome*	14	34%	4	7%	1	8%	19	17%
Moderately burdensome	16	39%	24	39%	6	46%	46	40%
Not burdensome	11	27%	30	49%	6	46%	47	40%
Missing	0	0%	3	5%	0	0%	3	3%
2c. Experiences on informing relatives about LS.								
Questions are answered by respondents who answered to have informed a relative or relatives about LS.								
Did you inform a family member about the diagnosis of LS in your family								
Yes	35	56%	35	47%	4	25%	74	51%
No	24	41%	32	46%	12	75%	68	47%
Missing	0	0%	3	7%	0	0%	3	2%
Number of respondents	35	59%	35	50%	4	25%	74	51%
Burdensome to inform family members*[a]**								
Burdensome	10	28%	5	14%	1	25%	16	22%
Moderately burdensome	16	46%	11	32%	1	25%	28	38%
Not burdensome	9	26%	19	54%	2	50%	30	40%
Missing	0	0%	0	0%	0	0%	0	0%

[a] Converted to 3-point Likert scale

* P = < 0.05, LS mutation carriers vs. non-carriers

** Answered by respondents who answered to be informed by a relative about LS

*** Answered by respondents who answered to have informed a relative about LS

Seventy-four respondents (51%) answered they had informed a relative about LS themselves. The majority (44/74; 59%) of these had experienced this as (moderately) burdensome.

Attitudes towards the family mediated approach

Most respondents (106/145; 73%) reported to be satisfied with the current family-mediated approach of communicating LS diagnosis within the family (Table 3). Of the

Table 3. Attitudes towards the family-mediated approach.

Do you think another way of informing relatives on Lynch syndrome is needed?	**Mutation carriers**	**%**	**Non - carriers**	**%**	**Non-tested respondents**	**%**	**Total respondents**	**%**
No, current procedure is sufficient	41	69%	54	77%	11	69%	106	73%
Yes	15	25%	12	17%	3	19%	30	21%
I would have liked to receive no information about LS	1	2%	1	1%	1	6%	3	2%
Missing	2	3%	3	4%	1	6%	6	4%
Respondents who did not agree with the current procedure, suggested to be informed by:	**15**	**100%**	**12**	**100%**	**3**	**100%**	**30**	**100%**
Medical specialist at the hospital	12	80%	9	75%	2	67%	23	77%
General practitioner	1	7%	1	8%	1	33%	3	10%
Family meeting	2	13%	2	17%	0	0%	4	13%
Opinion of all respondents towards statement I:								
It is the personal duty of LS mutation carriers to inform one's family members								
Disagree*	1	2%	2	3%	2	13%	5	3%
Neutral	7	12%	7	10%	5	31%	19	13%
Agree*	51	86%	59	84%	9	56%	119	82%
Missing	0	0%	2	3%	0	0%	2	1%
Opinion of all respondents towards statement II:								
It is the moral duty of physicians to inform patients in case of Lynch syndrome in their family								
Disagree	6	10%	12	17%	4	25%	22	15%
Neutral	14	24%	9	13%	1	6%	24	17%
Agree	35	59%	46	66%	11	69%	92	63%
Missing	4	7%	3	4%	0	0%	7	5%

* P = <0.05, non-tested respondents vs. LS mutation carriers and non-carriers

30 respondents (21%; 15 mutation carriers; 12 non- carriers; 3 non-tested) who did not agree with the current family mediated approach, 23 (77%) respondents preferred being informed by a medical specialist. The 30 respondents, disagreeing with current family mediated approach belonged to sixteen LS families. In these sixteen families, two till four family members per family shared the opinion that not family members but health professionals should inform relatives about LS diagnosis. Women more often than men reported that health professionals should inform relatives (28% vs 14%).

Furthermore, the majority of the respondents agreed with the statement that it is the moral duty of healthcare specialists to inform individuals about LS in their family (63%). Also, most respondents agreed that it is the personal duty of LS carriers to inform relatives about LS (82%). However, significantly more of the non-tested respondents did not agree that it is the personal duty of tested individuals to inform the family about the LS diagnosis in their family compared to tested respondents (13% of non-tested respondents vs 2% of mutation carriers and 3% of non- carriers, p=0.004).

Motivation for genetic testing for LS

The most important reasons for genetic testing were: 1) availability of surveillance programs for LS (61%), 2) preference to end insecurity regarding LS diagnosis (34%), and 3) fear for cancer (14%, Table 4). The three most important reasons for declining genetic testing by non-tested respondents were: 1) worry that testing would lead to problems with life insurance and mortgage (50%), 2) being content with life as it is (44%), and 3) not experiencing any physical complaints (37%, Figure 2). Fear for surveillance programs was reported in 19% of non-tested respondents.

Table 4. Motivations for uptake of genetic testing for LS (N=129), > 100%.

Motivation tested respondents	Mutation carriers	%	Non-carriers	%
Fear for cancer	8	14%	10	14%
Availability of surveillance programs for LS	36	61%	21	30%
To end insecurity regarding LS diagnosis	20	34%	31	44%
Other	11	19%	9	13%

DISCUSSION

In this cross-sectional survey among 145 individuals from LS families, we evaluated the current family-mediated procedure for informing at risk relatives about the identified familial LS mutation. Although the majority of the respondents were satisfied with the current family-mediated approach of communicating LS diagnosis within the families,

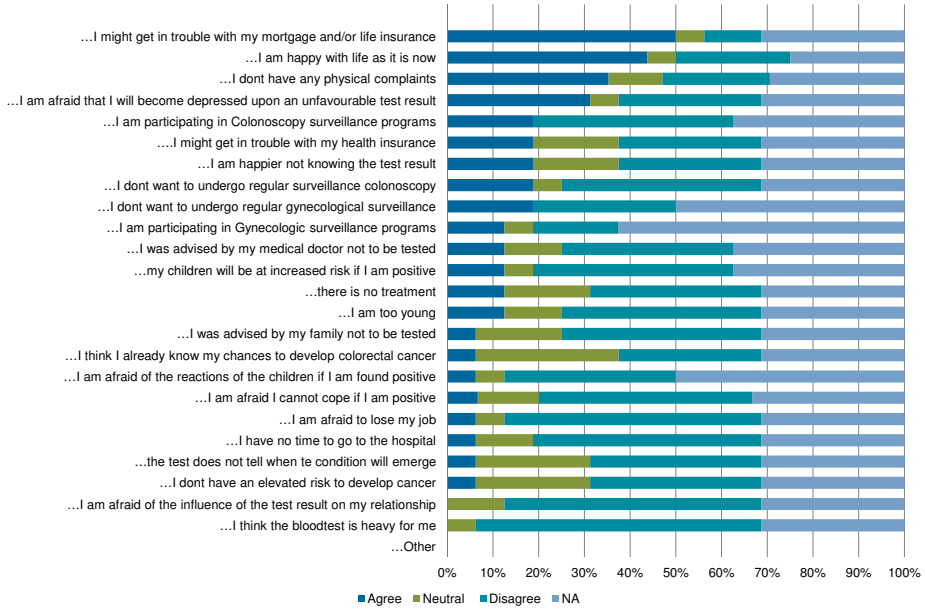


Figure 2. Reported reasons for refraining from genetic testing (N=16). NA, not available.

we found that a majority of the respondents (57%) experienced being informed by a family member as (moderately) burdensome. Moreover, approximately half of the respondents experienced informing a family member about the LS diagnosis as (moderately) burdensome as well. Fortunately, for the vast majority of respondents being informed by a family member did not have an adverse impact on the relationship with that family member.

A minority, 21% of the respondents would prefer another way of informing relatives on LS. Most of these respondents thought family members should be informed directly by a medical specialist. This percentage is in agreement with previous results of Aktan-Collan *et al*, who reported that 25% would prefer another way of informing relatives and 29% of respondents in a study by Pentz *et al*.[254, 263]

We found that half of the respondents who preferred another way of informing reported that more members in their family shared this opinion. It may be that family culture plays a role in person's preferred method of informing relatives. Families in which communication is less open or with less intimate family relationships may experience informing relatives about LS as more burdening. In line with Aktan-Collan *et al*, we also observed gender differences in attitude towards informing relatives. We found that women more often than men reported that health professionals should inform relatives (28% vs 14%).

Informing all at-risk relatives about LS is of great importance, in order to enable each family member to make an informed decision about genetic testing, in particular because surveillance has proven to reduce morbidity and mortality from colorectal cancer.[207] Although all non-tested respondents in the current study were informed about LS in the family, it has been observed in a recent study that the LS diagnosis was less likely to be communicated to distant relatives.[253] Therefore, it is important to conduct further research on optimal methods to inform all at-risk family members, including more distant relatives. Decision aids are an innovative strategy for patient education and proposed to help optimally inform at-risk relatives and support them in their decision about genetic testing for LS. Currently, only one study has evaluated a paper-based decision aid for genetic testing for LS.[264] The results of this randomized trial were promising, since it has been found that the decision aid, in comparison with a control pamphlet, lead to lower decision conflict and increased informed decision making.

In the current study, all non-tested respondents were informed about LS in the family and, consequently not being aware of LS diagnosis was not a reason for refraining from genetic testing. Reported reasons for refraining from genetic testing included problems with life insurance and mortgage, being happy with life as it is and not experiencing any physical complaints. The first two reasons are in agreement with previous studies on other hereditary cancer syndromes.[261] In the Netherlands, insurance companies are restricted in the use of genetic information of their clients by the Medical Examination Act., nevertheless, some people encounter problems when applying for insurance. Although this subject is included in the genetic counseling procedure, there is more need for clear information for the counselees on this topic. Furthermore, not experiencing any physical complaints was a common reason to refrain from testing in our study, which underlines the importance of counseling about LS in order to improve understanding on LS and available surveillance programs.

Non-tested respondents differed from tested respondents on several demographic, medical and family characteristics. We found that non-tested respondents were younger and were less likely to have children than tested respondents. Consistent with this finding, it has been reported that knowledge about the risk for children is one of the main reasons for testing.[265] Furthermore, none of the non-tested respondents were diagnosed with cancer themselves. Compared to tested respondents, non-tested respondents had less family members with LS-associated cancers and were less closely related to the index patient. These factors might influence how one experiences the threat of cancer and, subsequently, the urge to participate in genetic testing for LS. Genetic test decliners may benefit from information and counseling, even if they decide not to have a predictive genetic test. Fortunately, non-tested respondents were not found to be more vulnerable in terms of anxiety or cancer worries as compared to tested respondents.

Our study had a few limitations. First, the response rate among tested individuals was high (73%), however the response rate among non-tested individuals was only 39%. As in other studies, it is very difficult to include non-tested relatives.[266] Nevertheless, this is the first study focussing on the specific group of non-tested relatives. It provides new insight in the characteristics and motivations of non-tested relatives. Second, further qualitative research should be done in order to gain a deeper understanding of family interactions and communication and decision making about genetic testing for LS.

In conclusion, the current family-mediated procedure is accepted by the majority of LS family members, although a substantial proportion experienced burden informing relatives or being informed by relatives about LS. Healthcare workers should therefore carefully explore how index patients would experience communicating the LS diagnosis to family member and whether a patient would prefer more involvement of the healthcare workers in informing relatives about LS, genetic testing and available surveillance programs. Special attention should be paid to communication of LS to more distant relatives. It is important that family members who refrain from genetic testing are optimal and adequately informed about their own risks. They should be aware of the risks for LS, cancer and absence of symptoms in early stage cancer. Future studies should clarify risk perception of individuals who do not reach genetic services and the information and support needs of these individuals should be explored, including (online) decision aids.

Chapter 9

Summary and general discussion

SUMMARY

Lynch syndrome (LS) is an autosomal dominant inherited syndrome that predisposes to multiple malignancies, mainly colorectal cancer (CRC) and endometrial cancer (EC). LS is caused by germline mutations in the mismatch repair (MMR) genes or deletion of the 3' end of the *TACSTD1* gene leading to hypermethylation of the *MSH2* gene promoter. Since colonoscopic surveillance has been proven to reduce CRC morbidity and mortality by 65-70%, recognition of LS is of great importance.[18-20] Current LS detection consists of a combination of clinical criteria (revised Bethesda guidelines) and tumor tissue analysis for microsatellite instability (MSI), immunohistochemical (IHC) staining for MMR proteins and *MLH1* promoter hypermethylation. The final diagnosis LS is by a proven germline mutation in one of the MMR genes or deletion of the 3' end of the *TACSTD1* gene.

An important diagnostic challenge is that germline mutation analyses for LS should be performed in a selected group of patients with MSI-high (MSI-H) tumors and/or loss of protein expression by IHC without *MLH1* hypermethylation. The overall aim of this thesis was to evaluate whether routine molecular testing for LS can improve early LS detection. In a large population based study named LIMO (Lynch Immunohistochemisch en MSI Onderzoek) routine molecular analysis for LS was studied in a prospective cohort of 1117 CRC patients, 125 patients with advanced adenomas and 179 patients with EC. Furthermore, cost-effectiveness and prediction models were evaluated in this cohort. Uptake of genetic testing is far from complete and therefore barriers to genetic testing and family communication were studied in the Erasmus MC clinical cohort of LS families. The main findings are summarized in this section.

Part I: Challenges and pitfalls in molecular analyses

Tumor tissue analysis in CMMR-D

CMMR-D is a rare cancer syndrome caused by biallelic mutations in MMR genes. This syndrome is characterized by the development of haematological malignancies, brain tumors and gastrointestinal tumors in early childhood. Previously, more than 100 cases of children with CMMR-D have been reported in the literature.[32-37] MSI and absent MMR protein staining have been described in gastrointestinal tumors of these CMMR-D patients.[35] In contrast, tumor tissue of most reported CMMR-D patients with brain tumors did not show MSI.[34, 106] In **Chapter 2**, we report a family from our clinical research cohort with childhood brain tumors and early onset CRC with biallelic germline mutations in the *PMS2* gene.[267] In only one of five analysed tumors MSI was found. However, IHC analysis showed absent immunostaining of PMS2 in the brain tumor cells as well as in normal cells in the specimens of the index case and his sister. From the literature and our own findings can be concluded that MMR IHC may be more sensitive than MSI analysis

to detect MMR deficiency in brain tumors.[34, 106, 108, 126, 127] Therefore, in case of a clinical phenotype of CMMR-D, we recommended to routinely examine tumor tissues for MSI analysis with IHC. As pointed out by a recent publication by the European Consortium "Care for CMMR-D", a part from screening strategies for CMMR-D, also surveillance strategies for this rare cancer syndrome remain to be evaluated.[268]

Suspected LS patients

From our recent prospective cohort study it is known that by routine molecular testing for LS, 35% of suspected LS (sLS) patients test negative for germline MMR gene mutations.[130] It is possible a germline mutation has been missed, however also somatic mutations in MMR genes appear to occur.[68] Recently, by Mensenkamp *et al* 25 MSI-positive tumors were screened for somatic mutations and loss of heterozygosity (LOH) in mutL homolog 1 (*MLH1*) and mutS homolog 2 (*MSH2*). In 13 of 25 tumors (52%; 8 MLH1-deficient and 5 MSH2-deficient tumors), two somatic mutations were identified in *MLH1* or *MSH2*. We tested 40 tumors (EC and CRC of sLS patients for somatic MMR gene (*MLH1, MSH2, MSH6, PMS2*) aberrations by next generation sequencing and LOH analysis **(Chapter 3**). In half (21/40) of the MSI tumors of sLS patients two (likely) deleterious somatic MMR gene aberrations were found. We could conclude that in these cases the tumor was not associated with LS. Therefore, we advise to add somatic mutation and LOH analyses to the current diagnostic tests for LS in tumor tissue.[269]

Part II: Population and clinical based studies

Routine molecular testing for LS in colorectal cancer, endometrial cancer and advanced adenoma

From a previous study by van Lier *et al* at our institute it is known that the revised Bethesda guidelines are poorly used in clinical setting. This is one of the reasons that LS is under-diagnosed.[150] In 2008 the MIPA criteria were introduced in the Netherlands with a central role for the pathologist (Table 3, page 11). However, this strategy predominantly fails to detect *MSH6* and *PMS2* mutation carriers, since the mean age of CRC diagnosis in these subjects is above the age of 50 years.[9, 70]

In the prospective LIMO study routine molecular screening by MSI testing in combination with IHC and *MLH1* hypermethylation in 1117 CRC patients ≤ 70 years of age is evaluated (**Chapter 4**). Screening showed a profile compatible with LS in 4.5% of CRC patients ≤ 70 years of age and revealed a total of 24 LS carriers (2,1%).[130] Twenty patients suspected of LS (74%) fulfilled the revised Bethesda guidelines. Interestingly, a large majority of sixteen LS carriers (70%) were over 50 years of age and would not fulfill the currently used age criterion for LS screening. On the basis of these results we advised

to expand the age limit for LS screening among CRC patients to 70 years. This result is supported by other international prospective cohort studies.[66, 91]

MSI may be an early event and even detectable in advanced adenoma. For this reason, 125 patients with an advanced adenoma ≤ 45 years were routinely screened for LS and a molecular profile compatible with LS was detected in three patients (2.4 %). Adenomas were considered advanced when they were either > 10 mm in diameter, showed a villous component or high-grade dysplasia, or when at least three synchronous adenomas (regardless of size and histology) were found in one patient. In all three patients suspected of LS in our cohort of patients with advanced adenomas an MMR germline mutation was confirmed. These patients with advanced adenomas would not have been detected by the revised Bethesda guidelines or the current national guidelines. The detection of LS in advanced adenoma patients is of major importance since apart from relatives these patients themselves can enter surveillance programs that have been proven to reduce CRC morbidity and mortality by 65-70%.[18-20]

In addition we evaluated routine screening for LS in EC patients up to the age of 70 years (**Chapter 5**). In the Netherlands, the MIPA criteria and the international guidelines of the Society of Gynaecologic Oncology advice to include EC patients diagnosed under the age of 50 years or patients with two or more LS associated tumors.[200, 201] In our study, we evaluated routine MSI and IHC testing in 179 EC patients up to 70 years of age. We found eleven patients (6%) likely to have LS and in seven patients (4%) an MMR mutation was confirmed. A large majority, 92% of patients likely to have LS were over 50 years of age. Furthermore, 82% of patients likely to have LS and referred for counseling did not fulfill the Amsterdam criteria II and 73% did not fulfill the revised Bethesda guidelines. This indicates that current guidelines may not be suitable to detect LS in EC patients. On the basis of these data, molecular testing for LS should not be limited to EC patients under the age of 50 years.

Cost-effectiveness of routine molecular screening

Since population based screening recommendations followed from our prospective study, cost-effectiveness remained to be evaluated. In previous studies Markov models were used to calculate cost-effectiveness of LS detection. In **Chapter 6** cost-effectiveness analyses of the prospective cohort with CRC patients ≤ 70 years of age was evaluated. Additional 67 LS carriers were identified among relatives of the detected LS index cases by the LIMO study. Cost analyses revealed an incremental cost-effectiveness ratio for LS screening in CRC patients ≤ 70 years of €19,695 per life year gained compared to LS screening according to the revised Bethesda guidelines. We concluded that routine screening for LS by analysis of MSI and IHC in CRC patients up to 70 years is a cost-effective strategy with important clinical benefits for CRC patients and their relatives.

Prediction models

Prediction models for LS have been developed to predict the likelihood of carrying a germline mutation.[27, 82, 85, 86] A major advantage of prediction models is that they quantify the risk of germline MMR mutations. Furthermore, these models can be used for individuals for whom tumor samples are not available and for individuals in whom germline testing finds no mutation. However, large validation of LS prediction models was lacking. Therefore, we joined a large international validation study of prediction models (**Chapter 7**). A total of six clinical based and five population based cohorts were included; resulting in 5755 individuals respectively 2304 from clinic-based and 3451 from population-based CRC registries. In this cohort three prediction models were studied: MMRpredict, MMRpro and $PREMM_{1,2,6}$. Predicted probabilities of any pathogenic *MLH1*, *MSH2*, and *MSH6* gene mutation were calculated by each model and gene-specific predictions by MMRpro and $PREMM_{1,2,6}$. Mutations were detected in 539/2304 (23%) individuals from the clinic-based cohorts and 150/3451 (4.4%) individuals from the population-based cohorts. Discrimination was similar for clinic and population-based cohorts for all three tested prediction models. MMRpro and $PREMM_{1,2,6}$ predictions were clinically useful at thresholds ≥ 5% and in particular, at ≥15%. When focusing on clinical Rotterdam and population based LIMO data alone, included in this international cohort study, we found a better discrimination performance of $PREMM_{1,2,6}$ compared to the other two prediction models. Prediction of gene-specific MMR gene mutations was lowest for *MSH6* in the Rotterdam clinical and population based cohorts compared to other cohorts, possibly due to the known founder effect of *MSH6*.[83]

Family communication

Once LS is detected in a family, genetic testing for the familial mutation can be offered to relatives at risk. From previous research by Ramsoekh *et al* at our institute is known that the uptake of genetic testing for LS is far from complete.[97] One explanation for this could be insufficient information for relatives, by the current family-mediated approach. This approach implicates that relatives are informed by a family member. We evaluated the satisfaction of patients with the current procedure (**Chapter 8**). Although the majority of the respondents were satisfied with the current family-mediated approach of communicating LS diagnosis within the families, we found that a majority of the respondents (57%) experienced being informed by a family member as (moderately) burdensome. Moreover, approximately half of the respondents experienced informing a family member about the LS diagnosis as (moderately) burdensome as well. Fortunately, for the vast majority of the respondents being informed by a family member did not have an adverse impact on the relationship with that family member. A minority, 21% of the respondents would prefer another way of informing relatives on LS. Most of these respondents thought family members should be informed directly by a medical specialist.

Additionally, a small group of sixteen non-tested at risk LS relatives was asked for what reasons they refrained from genetic testing. Reported reasons for refraining from genetic testing included problems with life insurance and mortgage, being happy with life as it is and not experiencing any physical complaints. The first two reasons are in agreement with previous studies on other hereditary cancer syndromes.[261] In the Netherlands, insurance companies are restricted in the use of genetic information of their clients by the Medical Examination Act., nevertheless, some people encounter problems when applying for insurance. Furthermore, not experiencing any physical complaints was a common reason to refrain from testing in our study, which underlines the importance of counseling about LS in order to improve understanding on LS and available surveillance programs.

DISCUSSION

Early detection of LS remains challenging and the optimal LS diagnostic strategy is still under debate. Routine molecular screening for LS showed a profile compatible with LS in 4.5% of CRC patients ≤ 70 years of age amongst 1117 CRC patients and revealed a total of 24 LS carriers (2,1%).[130] This strategy identified more than 3 times as many LS index patients and more than 4.5 times as many LS carriers when including relatives, compared to molecular screening in CRC patients up to 50 years of age. These results are in agreement with two international large prospective cohort studies.[66, 91] Furthermore, the performed economic evaluation in CRC patients up to 70 years revealed an incremental cost-effectiveness ratio for LS screening of €19,695 per life years gained compared to LS screening according to the revised Bethesda guidelines. This result is in line with previous published cost-effectiveness analyses using Markov models.[78, 93, 94] However, our study used real life data and an interesting finding was that in our cohort the uptake of genetic testing was higher than assumed by previous studies.[78, 93, 94] On the basis of our results, routine molecular screening for LS should include CRC patients up to 70 years. The recently published American Gastroenterological Association guidelines on LS advise to perform IHC and/or MSI testing in all CRC patients, irrespectively of age.[270] However, cost-effectiveness of universal molecular evaluation especially by IHC analyses alone with inclusion of quality of life data and data on participation in surveillance programs, remains to be determined by future prospective cost-effectiveness analyses in order to determine the most effective age cut off. [93, 94, 210, 270]

In the 125 included patients with advanced adenomas, LS was detected in three patients (2.4 %). The detection of LS in advanced adenoma patients is valuable since apart from their relatives, these patients themselves can enter surveillance programs which have been proven to reduce CRC morbidity and mortality by 65-70%.[18-20] Previous studies

showed various results regarding effectiveness to identify new LS cases in patients with advanced adenomas.[169-173] Although we detected three LS patients, who would not have been diagnosed with LS by current LS screening guidelines, a limitation of this study was the small number of included patients. Before routine testing for LS in advanced adenomas can be added to the current LS diagnostic strategy, cost-effectiveness remains to be evaluated in a larger cohort. It needs to be emphasized that early detection of LS in patients with advanced adenomas has important benefits since detection is at a very early stage. In case 2% or more LS detection in advanced adenomas is confirmed in a larger cohort of patients, molecular screening for LS in advanced adenomas should therefore be included in LS guidelines.

In our prospective EC cohort with 179 patients up to 70 years, seven LS carriers (4%) were found by routine molecular testing.[129] Six LS carriers were over 50 years of age and would not have been found by current used age criterion for LS testing in EC patients. On the basis of these data and previous published literature,[92, 189, 196] molecular testing for LS should in concordance with CRC patients, not be limited to EC patients under the age of 50 years. LS screening guidelines should be updated to 70 years of age or without an age limit. To answer this question cost-effectiveness of unselected EC patients remains to be evaluated by prospective cohort studies. In a previous study by Kwon *et al* using a Markov Monte Carlo simulation model to evaluate cost-effectiveness for LS screening in EC patients was found that IHC testing of EC patients at any age having at least one first degree relative with LS associated cancer is cost-effective.[271]

There are several potential pitfalls and challenges in routine molecular screening for LS. First, in a small number (1,5%) of CRC and EC cases we found discordant results in our prospective cohort, including discordance between results of MSI and IHC analyses, heterogeneity of tumor tissue and the use of additional hypermethylation assays.[272] These discordant results underscore the importance of correct interpretation of IHC and/ or MSI results by an expert panel in molecular genetics. Forthcoming national screening guidelines for LS advise only IHC analysis in tumor tissue as suggested in two large cohort studies based on costs analysis and improvement of IHC results.[66, 91] In view of the above mentioned pitfalls, additional MSI testing and an expert team should be consulted in case of inconclusive results from IHC.

Second, an important issue in LS diagnostics is testing for *MLH1* promoter hypermethylation. The establishment of a sporadic MSI-H status considerably reduces the number of patients referred for genetic testing and relieve patients from worrying for LS. *MLH1* promoter hypermethylation and thus sporadic MSI can be confirmed by different methods. One of these methods is Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA) as performed in our prospective studies. Furthermore, *BRAF* mutation status is used to distinguish LS-associated tumors from sporadic MSI

colon cancer, as *BRAF* mutations correlate with *MLH1* methylation and are strong predictors of MMR gene mutation-negative status. In our prospective cohort study, in none of the 50 CRCs likely caused by LS and in 65% sporadic MSI-H tumors a V600E *BRAF* mutation was detected. We concluded that MS-MLPA of the MMR genes is the preferred molecular strategy to test *MLH1* promoter hypermethylation in MSI-H CRC with absence of MLH1 staining, instead of *BRAF* mutation testing. In EC the role of *BRAF* mutation status is not as clear as in CRC. In the prospective EC cohort, no *BRAF* V600 mutations were detected and showed to be irrelevant for the identification of sporadic MSI-H EC cases in our study. A recent systematic review evaluated the use of *BRAF* mutation status to determine *MLH1* promoter methylation status and showed that *BRAF* mutations occur infrequently in EC.[273] However, in the additional tumor samples included in the study described in chapter 3, 3/15 (20%) MSI-H CRC cases without *MLH1* promoter hypermethylation showed a *BRAF* mutation (V600E, K601E and D594G). By LOH and somatic mutation analysis these tumors appeared to be all sporadic. *BRAF* screening could therefore be a valuable test in this subgroup of patients to predict the sporadic origin of the tumors.

A last important challenge in molecular testing for LS addressed in this thesis is the fact that a substantial part (35%) of patients with a MSI-H tumor suspected of LS remains without a germline mutation.[130] In half (21/40) of the MSI-H tumors of sLS patients included in our study (chapter 3), two (likely) deleterious somatic MMR gene aberrations were found, indicating the sporadic origin of these tumors.[269] These findings are of great importance for the families involved, since patients are no longer suspect for LS and can be discharged from burdensome LS surveillance programs. The number of somatic aberrations may be underestimated due to the design of the PGM primer panel and the fact that not all exonic regions were completely covered. On the basis of our results and recent literature, we advise to add somatic mutation and LOH analyses to the current diagnostic tests for LS in tumor tissue as shown in Figure 1.[68, 269, 274]

In case no tumor tissue is available or MSI and/or IHC testing is not conclusive prediction models are a promising additional strategy to detect LS. In the international validation study (chapter 6) it was found that both MMRpro and $PREMM_{1,2,6}$ better discriminated MMR gene mutation carriers from non-carriers than MMRpredict in both clinic- and population-based settings. However, for *MSH6* mutation specific predictions were lowest and currently prediction models are lacking for *PMS2*. Most important is that the value of prediction models for LS in clinical practice is still limited, due to the complexity of data entry and interpretation of results. Recently, easy-to-use online referral tools for patients with a high familial risk of CRC have been developed.[275, 276] This may be a promising clinical tool based on prediction models to improve LS detection; however

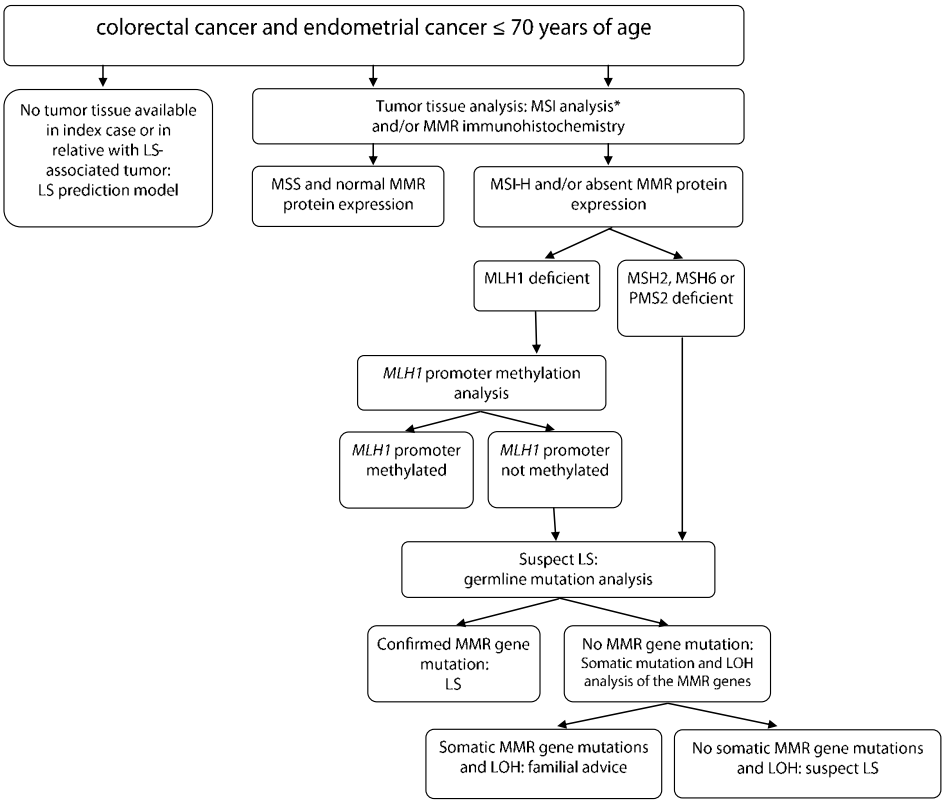


Figure 1. Proposed diagnostic strategy for Lynch syndrome based on this thesis.
*In patients with stage II/III colorectal cancer MSI testing is also done for therapeutic purposes.
MSI, microsatellite instability; MMR, mismatch repair; MSS, microsatellite stable; MSI-H, high degree of microsatellite instability; LS, Lynch syndrome

implementation in the clinical setting is still far from optimal and will probably not replace routine molecular screening in LS.

Another important clinical challenge is the information procedure before and after molecular tissue testing for LS. The molecular analyses do not establish the diagnosis of LS directly as shown by our studies. Clear information form clinicians to patients not only on the nature of the tumor, but also about additional molecular testing for LS is important. In view of the complexity of possible test results and implications for healthy relatives and possible social consequences, germline analysis for LS should exclusively be performed after genetic counseling by a clinical geneticist. In this way, the patient can make an informed decision about genetic testing for LS.

Apart from the use of routine MSI testing for LS diagnostics, a topic not investigated in this thesis but clinical relevant is the use of MSI testing as predictive marker in CRC patients. Several studies have shown that MSI-H tumors do not benefit from 5-fluorouracil

adjuvant chemotherapy.[61-63] As a consequence, routine MSI is tested in CRC patients for therapeutic indications as well and subsequently more patients at high risk for LS will be detected in this way, emphasizing the importance of patient information about MSI testing in tumor tissue by their clinician.

Despite the known benefits from LS surveillance programs, not all relatives of LS carriers participate in genetic counseling, germline mutation analysis and available surveillance programs.[97] One of the known factors of influence in the uptake of genetic testing is insufficient communication by the family-mediated approach. This approach implies that family members are responsible to inform their relatives on the diagnosis of LS and the possibility of genetic testing. We evaluated the satisfaction of patients with the current procedure in a cross-sectional survey among LS families and found that a minority of the respondents (21%) would prefer another way of informing relatives about LS. Most of these respondents thought family members should be informed directly by a medical specialist, in line with previously published literature.[254, 263] In the Netherlands there is no legal responsibility for the physician to inform relatives. It is very challenging for physicians to reach relatives at risk for LS. In recent literature a more active role for geneticist is proposed including a guideline with suggestions to improve the information regarding family communication and a review of ethical and legal considerations.[277] However, the effect of this novel approach remains to be evaluated in patients as well as geneticists. We found that a majority of the respondents experienced being informed by a family member as (moderately) burdensome, indicating more help in this process is needed.

The reported reasons we found for refraining from genetic testing included problems with life insurance and mortgage, being happy with life as it is and not experiencing any physical complaints. These reasons indicate the need for additional information aids for LS relatives, including supportive information about genetic counseling and testing. Further qualitative (in depth interviews) and quantitative research in a larger cohort of non-tested individuals is needed to optimize methods to inform all at-risk family members. Decision aids are an innovative strategy for patient education and proposed to help in order to optimally inform at-risk relatives and to support in their decision about genetic testing for LS.[264]

Chapter 10

APPENDIX

NEDERLANDSE SAMENVATTING

Lynch syndroom (LS) is een autosomaal dominant overervend kankersyndroom dat wordt gekenmerkt door het ontstaan van verschillende kwaadaardige tumoren, voornamelijk dikke darmkanker en baarmoeder kanker. LS wordt veroorzaakt door kiembaanmutaties in de DNA herstelgenen *MLH1, MSH2, MSH6, PMS2* of deletie in 3' exonen van het *TACSTD1* gen. Aangezien surveillance door middel van colonoscopie heeft bewezen de morbiditeit en mortaliteit van darmkanker met 65-70% terug te brengen, is vroege opsporing van LS belangrijk.[18-20] De huidige diagnostiek van LS bestaat uit een combinatie van klinische criteria (gereviseerde Bethesda richtlijnen) en tumorweefselanalyse. Moleculaire tumorweefselanalyse bestaat uit microsatelliet instabiliteit (MSI) analyse in combinatie met immunohistochemische kleuring van DNA hersteleiwitten en methyleringsonderzoek van de *MLH1* promoter. De definitieve diagnose LS wordt gesteld bij een bewezen kiembaanmutatie in één van de DNA herstelgenen of deletie in 3' exonen van het *TACSTD1* gen.

Het doel van dit proefschrift was om te onderzoeken of routine moleculaire screening voor LS kan bijdragen aan vroegtijdige opsporing van LS. Het eerste deel van dit proefschrift beschrijft uitdagingen en valkuilen van tumorweefselanalyse voor LS en het meer zeldzame 'constitutionele mismatch repair-deficiëntie' (CMMR-D). In het tweede deel van dit proefschrift worden de resultaten van een grote populatie studie genaamd LIMO (Lynch syndroom Immunohistochemisch en MSI Onderzoek) beschreven. In dit prospectieve cohort bestaande uit 1117 patiënten met darmkanker ≤ 70 jaar, 125 patiënten met advanced adenomen (darmpoliepen) ≤ 45 jaar en 179 patiënten met baarmoeder kanker ≤ 70 jaar, werd routine moleculaire analyse voor LS uitgevoerd en de opbrengst geëvalueerd. Aanvullend werd de kosteneffectiviteit van deze strategie berekend. Een andere diagnostische strategie voor LS om de kans op een mutatie in een van de DNA herstelgenen te voorspellen is het gebruik van predictiemodellen. Een aantal van deze predictiemodellen voor LS werd in internationaal onderzoeksverband gevalideerd. De belangrijkste resultaten van deze studies zijn samengevat in dit hoofdstuk.

Deel I: Uitdagingen en valkuilen van moleculaire onderzoeken

Tumorweefselanalyse in CMMR-D

Constitutionele mismatch repair-deficiëntie (CMMR-D) is een zeldzaam kankersyndroom veroorzaakt door biallelische (beide allelen) mutaties in de DNA herstelgenen. Dit syndroom wordt gekenmerkt door de ontwikkeling van hematologische maligniteiten, hersentumoren en tumoren in het maagdarmstelsel op jonge leeftijd. Meer dan 100 kinderen met CMMR-D zijn beschreven in de literatuur.[32-36, 106] Bij tumorweefselanalyse van maag- en darmtumoren van deze patiënten werd, in overeenstemming met LS, MSI

en afwezigheid van DNA herstel eiwitexpressie gevonden.[35] Echter, MSI bleek niet detecteerbaar in hersentumorweefsel van de meeste gerapporteerde CMMR-D patiënten.[34, 106] In **hoofdstuk 2,** beschrijven wij een familie met biallelische kiembaanmutaties in het *PMS2* gen uit het Erasmus MC klinisch onderzoekscohort van de afdeling Klinische Genetica. Er werd onder andere bij twee kinderen behorend tot deze familie hersenkanker en bij hun vader darmkanker op jonge leeftijd vastgesteld. Tumorweefselanalyse van tumoren uit deze familie toont de valkuilen van de huidige diagnostische strategie voor CMMR-D. MSI analyse van in totaal vijf tumoren uit deze familie toont slechts één tumor met MSI. Echter, immunohistochemische analyse van DNA herstel eiwitten toont afwezige expressie van PMS2 in de hersentumoren van twee familieleden. Deze bevinding is in overeenstemming met vijf eerder onderzochte hersentumoren van CMMR-D patiënten beschreven in de literatuur.[34, 106, 108, 126] Uit de literatuur en de bevindingen bij deze familie kan worden geconcludeerd dat immunohistochemisch onderzoek van de DNA hersteleiwitten gevoeliger is dan MSI analyse in hersentumoren van CMMR-D patiënten. Om deze reden is het aan te bevelen in geval van een klinisch fenotype passend bij CMMR-D, routinematig MSI analyse te combineren met immunohistochemisch onderzoek van de DNA hersteleiwitten. In geval van onduidelijke of tegenstrijdige resultaten van deze onderzoeken, zal kiembaanmutatie-analyse moeten worden overwogen na counseling van de patiënten en hun familieleden. Daarnaast zal toekomstig onderzoek in Europees verband verder richting geven aan surveillance adviezen voor dit zeldzame kankersyndroom.[268]

Patiënten verdacht voor LS zonder aangetoonde kiembaanmutatie

Vanuit onze recente prospectieve cohortstudies onder darmkanker en baarmoeder kankerpatiënten is bekend dat 35% van de patiënten verdacht voor LS op basis van tumorweefselanalyse, geen kiembaanmutatie blijkt te hebben in één van de DNA herstelgenen.[129, 130] Het is mogelijk dat kiembaanmutaties, die op dit moment nog niet in verband staan met LS, een rol spelen. Daarnaast kunnen somatische mutaties in DNA herstelgenen optreden.[68] Onlangs, werden door Mensenkamp *et al* 25 MSI-positieve tumoren gescreend op het optreden van deze somatische mutaties en verlies van heterozygotie (LOH) in mutL homoloog 1 (*MLH1*) en mutS homoloog 2 (*MSH2*). In 13 van de 25 tumoren (52%, 8 MLH1 deficiënte en 5 MSH2 deficiënte tumoren), werden twee somatische mutaties geïdentificeerd in *MLH1* en *MSH2*. Wij testten 40 tumoren (baarmoederkanker en darmkanker) van patiënten verdacht voor LS voor somatische afwijkingen in de DNA herstelgenen (*MLH1, MSH2, MSH6, PMS2*) door middel van next generation sequencing en LOH analyse (**hoofdstuk 3**). In de helft (21/40) van deze MSI tumoren werden twee somatische afwijkingen in de DNA herstelgenen gevonden. Geconcludeerd werd dat in deze gevallen de tumoren niet geassocieerd zijn met een kiembaanmutatie in het kader van LS. Hieruit volgend hoeven familieleden veelal geen

LS surveillance programma te volgen. Vanwege deze belangrijke gevolgen, adviseren wij om analyse naar somatische DNA afwijkingen en LOH analyse toe te voegen aan de huidige diagnostische tests voor LS in tumorweefsel.

Deel II: Klinische en populatie studies

Routine moleculair onderzoek

Uit een eerdere studie door Van Lier *et al* is bekend dat de gereviseerde Bethesda richtlijnen onvoldoende worden toegepast in de klinische praktijk. Dit is een van de redenen dat er sprake is van onderdiagnostiek van LS.[150] In 2008 werden de MIPA criteria in Nederland geïntroduceerd met een centrale rol voor de patholoog (Tabel 3, Hoofdstuk 1). Echter, deze strategie detecteert slechts een beperkt deel van de mutaties en mist met name mutaties in de genen *MSH6* en *PMS2* aangezien de gemiddelde leeftijd van de diagnose darmkanker bij deze mutatiedragers boven de leeftijd van 50 jaar is.[9, 70]

In de prospectieve LIMO studie werd routine moleculaire screening door middel van MSI analyse in combinatie met immunohistochemisch onderzoek van DNA hersteleiwitten en *MLH1* hypermethylering in 1117 dikke darmkanker patiënten ≤ 70 jaar geëvalueerd (**hoofdstuk 4**). Screening leverde een moleculair profiel verdacht voor LS in 4,5% van de darmkanker patiënten ≤ 70 jaar op. In totaal werd in 2% van de geïncludeerde darmkanker patiënten LS vastgesteld. Twintig patiënten verdacht voor LS (74%) voldeden aan de gereviseerde Bethesda richtlijnen. Interessant is dat een grote meerderheid van de zestien LS mutatiedragers (70%) ouder dan 50 jaar was en dus niet zou worden opgespoord via het momenteel gebruikte leeftijdscriterium voor LS screening. Op basis van deze resultaten adviseren we om de leeftijdsgrens voor LS screening bij darmkanker patiënten uit te breiden naar 70 jaar. Dit resultaat wordt ondersteund door twee internationale prospectieve studies.[66, 91]

MSI kan zelfs al worden aangetoond in advanced adenomen (darmpoliepen). Om deze reden werden 125 patiënten ≤ 45 jaar met advanced adenomen routinematig gescreend voor LS. Adenomen werden als advanced geclassificeerd wanneer: 1) de diameter van het adenoom groter dan 10 mm was, 2) het adenoom een villeuze component bevatte (≥ 25%) of hooggradige dysplasie, of 3) wanneer minimaal drie synchrone adenomen (ongeacht grootte en histologie) werden gevonden bij één patiënt. In alle drie de patiënten (2.4%) met advanced adenomen verdacht voor LS werd een kiembaanmutatie vastgesteld. Deze patiënten met advanced adenomen zouden niet zijn ontdekt via de gereviseerde Bethesda richtlijnen of de huidige nationale richtlijnen voor opsporing van LS. De opsporing van LS in patiënten met advanced adenomen is van groot belang, omdat behalve familieleden, ook deze patiënten zelf mee kunnen doen aan surveillance programma's die morbiditeit en mortaliteit van dikke darmkanker verminderen met 65-70%.[18-20]

Daarnaast evalueerden we volgens dezelfde methode routine moleculair onderzoek in baarmoeder kankerpatiënten tot en met de leeftijd van 70 jaar (**hoofdstuk 5).** In Nederland adviseren de MIPA criteria en de internationale richtlijnen van de *Society of Gynaecologic Oncology* LS screening bij baarmoederkanker patiënten onder de leeftijd van 50 jaar of bij patiënten met twee of meer LS geassocieerde tumoren.[200, 201] In onze studie hebben we routine moleculaire screening voor LS onderzocht in 179 baarmoederkanker patiënten tot en met 70 jaar. Elf patiënten (6%) werden verdacht voor LS en in zeven patiënten (4%) werd de diagnose LS vastgesteld met kiembaanmutatie-analyse. Een belangrijke meerderheid, namelijk 92% van de patiënten verdacht voor LS was ouder dan 50 jaar op het moment van diagnose. Bovendien, vonden we dat 82% van de patiënten verdacht voor LS niet aan de Amsterdam criteria II voldeden en 73% niet aan de gereviseerde Bethesda richtlijnen voldeden. Deze bevindingen geven aan dat de huidige richtlijnen niet geschikt zijn om LS te detecteren in baarmoederkanker patiënten. Op basis van deze gegevens dient routine moleculair onderzoek voor LS niet beperkt te blijven tot baarmoeder kankerpatiënten jonger dan 50 jaar.

Kosteneffectiviteit van routine moleculaire screening

In eerder gepubliceerde kostenstudies werden Markov modellen gebruikt om de kosteneffectiviteit van de opsporing van LS te berekenen. In **hoofdstuk 6** werd kosteneffectiviteit van het prospectieve cohort met darmkanker patiënten ≤ 70 jaar geëvalueerd. Belangrijk is dat via gedetecteerde LS patiënten, 67 LS mutatiedragers gevonden werden onder de familieleden. Bij berekening van de kosteneffectiviteit van moleculaire screening voor LS bij darmkanker patiënten ≤ 70 jaar bleek een incrementele kosteneffectiviteitsratio van €19.695 per gewonnen levensjaar in vergelijking met LS screening volgens de gereviseerde Bethesda richtlijnen. Geconcludeerd werd dat routine screening voor LS door middel van analyse van MSI, immunohistochemisch onderzoek van de DNA hersteleiwitten en *MLH1* hypermethylering in tumorweefsel van darmkanker patiënten tot en met 70 jaar een kosteneffectieve strategie is, met belangrijke klinische voordelen voor darmkanker patiënten en hun familieleden.

Predictiemodellen

Predictiemodellen voor LS zijn ontwikkeld om de kans op een mutatie in een van de DNA herstelgenen te voorspellen. Een groot voordeel van predictiemodellen is dat het risico op LS mutaties wordt gekwantificeerd. Bovendien kunnen deze modellen gebruikt worden voor individuen zonder beschikbaar tumorweefsel of wanneer geen kiembaanmutatie wordt gevonden. Echter, grootschalige validatie van LS predictiemodellen in verschillende darmkanker populaties was nog niet gedaan. Om deze reden hebben we meegewerkt aan een grote internationale validatie studie voor predictiemodellen (**hoofdstuk 7**). Zes klinische en vijf populatiecohorten bestaande uit dikke darmkanker

patiënten werden geïncludeerd in deze validatie studie. Dit resulteerde in een cohort bestaande uit 5755 darmkanker patiënten in totaal, respectievelijk 2304 patiënten uit klinische cohorten en 3451 patiënten uit populatie cohorten. Vanuit het Erasmus MC werden zowel het klinische cohort van de afdeling Klinische Genetica met verwezen dikke darmkanker patiënten op basis van klinische criteria, als het LIMO populatie cohort met dikke darmkanker patiënten die routinematig gescreend waren voor LS, geïncludeerd. In het totale cohort werden drie predictiemodellen bestudeerd: MMRpredict, MMRpro en $PREMM_{1,2,6}$. De voorspelde kans op een pathogene mutatie in de genen *MLH1*, *MSH2* en *MSH6* werd berekend door elk model afzonderlijk. Genspecifieke voorspellingen werden berekend door MMRpro en $PREMM_{1,2,6}$. Bij 539/2304 (23%) patiënten uit de klinische cohorten en bij 150/3451 (4,4%) patiënten uit de populatie cohorten werden mutaties vastgesteld. Discriminatie van alle drie de geteste predictiemodellen was vergelijkbaar voor de klinische en populatie cohorten. Mutatie specifieke voorspellingen door de modellen MMRpro en $PREMM_{1,2,6}$ werden klinisch bruikbaar bevonden. De resultaten voor het klinische Erasmus MC cohort en populatie cohort LIMO, lieten een betere prestatie van $PREMM_{1,2,6}$ in vergelijking met de andere twee predictiemodellen zien. Het voorspellen van de kans op DNA herstel genmutaties bleek het minst nauwkeurig voor *MSH6* in het Erasmus MC klinische en het LIMO populatie cohort in vergelijking met andere cohorten, mogelijk vanwege het bekende founder effect van *MSH6*.[83]

Familiecommunicatie

Zodra LS in een familie wordt vastgesteld, kan genetisch onderzoek binnen de gehele familie worden aangeboden via de vastgestelde LS mutatiedrager (index patiënt). Uit eerder onderzoek door Ramsoekh *et al* is bekend dat slechts de helft van familieleden deelneemt aan genetisch onderzoek voor LS.[97] Een verklaring hiervoor zou kunnen zijn dat er een tekort aan beschikbare informatie is voor familieleden, vanwege de huidige familie-gemedieerde aanpak. Deze aanpak impliceert dat familieleden worden geïnformeerd door een familielid over LS. We evalueerden de tevredenheid van patiënten met deze huidige familie communicatie procedure in **hoofdstuk 8**. Hoewel de meerderheid van de respondenten aangaf tevreden te zijn met de huidige familie communicatie procedure binnen de onderzochte families, gaf een meerderheid van de respondenten (57%) aan het worden geïnformeerd door een familielid als (matig) belastend te ervaren. Echter, een minderheid (21%) van de respondenten gaf de voorkeur aan een andere manier om familieleden te informeren over LS. De meesten van deze respondenten waren van mening dat familieleden rechtstreeks zouden moeten worden geïnformeerd door een medisch specialist.

Tenslotte hebben we onderzocht om welke redenen LS familieleden afzien van counseling en genetisch onderzoek voor LS. De belangrijkste redenen die door zestien niet-geteste LS familieleden werden gerapporteerd om af te zien van genetisch onder-

zoek, betroffen problemen met levensverzekeringen en hypotheken, gelukkig zijn met het leven zoals het is en afwezigheid van lichamelijke klachten. De genoemde redenen onderstrepen vooral het belang van volledige informatie over genetisch onderzoek en de beschikbare surveillance programma's voor LS.

REFERENCES

1. Baglietto L, Lindor NM, Dowty JG, White DM, Wagner A, Gomez Garcia EB, *et al*. Risks of Lynch syndrome cancers for MSH6 mutation carriers. J Natl Cancer Inst. 2010;102(3):193-201.
2. Barrow E, Robinson L, Alduaij W, Shenton A, Clancy T, Lalloo F, *et al*. Cumulative lifetime incidence of extracolonic cancers in Lynch syndrome: a report of 121 families with proven mutations. Clin Genet. 2009;75(2):141-9.
3. Bonadona V, Bonaiti B, Olschwang S, Grandjouan S, Huiart L, Longy M, *et al*. Cancer risks associated with germline mutations in MLH1, MSH2, and MSH6 genes in Lynch syndrome. JAMA. 2011;305(22):2304-10.
4. Dowty JG, Win AK, Buchanan DD, Lindor NM, Macrae FA, Clendenning M, *et al*. Cancer risks for MLH1 and MSH2 mutation carriers. Human mutation. 2013;34(3):490-7.
5. Kempers MJ, Kuiper RP, Ockeloen CW, Chappuis PO, Hutter P, Rahner N, *et al*. Risk of colorectal and endometrial cancers in EPCAM deletion-positive Lynch syndrome: a cohort study. Lancet Oncol. 2011;12(1):49-55.
6. Kopciuk KA, Choi YH, Parkhomenko E, Parfrey P, McLaughlin J, Green J, *et al*. Penetrance of HNPCC-related cancers in a retrolective cohort of 12 large Newfoundland families carrying a MSH2 founder mutation: an evaluation using modified segregation models. Hereditary cancer in clinical practice. 2009;7(1):16.
7. Mukherjee B, Rennert G, Ahn J, Dishon S, Lejbkowicz F, Rennert HS, *et al*. High risk of colorectal and endometrial cancer in Ashkenazi families with the MSH2 A636P founder mutation. Gastroenterology. 2011;140(7):1919-26.
8. Ramsoekh D, Wagner A, van Leerdam ME, Dooijes D, Tops CM, Steyerberg EW, *et al*. Cancer risk in MLH1, MSH2 and MSH6 mutation carriers; different risk profiles may influence clinical management. Hereditary cancer in clinical practice. 2009;7(1):17.
9. Senter L, Clendenning M, Sotamaa K, Hampel H, Green J, Potter JD, *et al*. The clinical phenotype of Lynch syndrome due to germ-line PMS2 mutations. Gastroenterology. 2008;135(2):419-28.
10. Talseth-Palmer BA, Wijnen JT, Brenne IS, Jagmohan-Changur S, Barker D, Ashton KA, *et al*. Combined analysis of three Lynch syndrome cohorts confirms the modifying effects of 8q23.3 and 11q23.1 in MLH1 mutation carriers. Int J Cancer. 2013;132(7):1556-64.
11. van der Post RS, Kiemeney LA, Ligtenberg MJ, Witjes JA, Hulsbergen-van de Kaa CA, Bodmer D, *et al*. Risk of urothelial bladder cancer in Lynch syndrome is increased, in particular among MSH2 mutation carriers. J Med Genet. 2010;47(7):464-70.
12. Stoffel E, Mukherjee B, Raymond VM, Tayob N, Kastrinos F, Sparr J, *et al*. Calculation of risk of colorectal and endometrial cancer among patients with Lynch syndrome. Gastroenterology. 2009;137(5):1621-7.
13. Vasen HF, Stormorken A, Menko FH, Nagengast FM, Kleibeuker JH, Griffioen G, *et al*. MSH2 mutation carriers are at higher risk of cancer than MLH1 mutation carriers: a study of hereditary nonpolyposis colorectal cancer families. J Clin Oncol. 2001;19(20):4074-80.
14. Hampel H, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, *et al*. Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). N Engl J Med. 2005;352(18):1851-60.
15. Koornstra JJ, Mourits MJ, Sijmons RH, Leliveld AM, Hollema H, Kleibeuker JH. Management of extracolonic tumours in patients with Lynch syndrome. Lancet Oncol. 2009;10(4):400-8.

16. Capelle LG, Van Grieken NC, Lingsma HF, Steyerberg EW, Klokman WJ, Bruno MJ, *et al*. Risk and epidemiological time trends of gastric cancer in Lynch syndrome carriers in the Netherlands. Gastroenterology. 2010;138(2):487-92.
17. Niessen RC, Hofstra RM, Westers H, Ligtenberg MJ, Kooi K, Jager PO, *et al*. Germline hypermethylation of MLH1 and EPCAM deletions are a frequent cause of Lynch syndrome. Genes, chromosomes & cancer. 2009;48(8):737-44.
18. de Jong AE, Hendriks YM, Kleibeuker JH, de Boer SY, Cats A, Griffioen G, *et al*. Decrease in mortality in Lynch syndrome families because of surveillance. Gastroenterology. 2006;130(3):665-71.
19. Jarvinen HJ, Aarnio M, Mustonen H, Aktan-Collan K, Aaltonen LA, Peltomaki P, *et al*. Controlled 15-year trial on screening for colorectal cancer in families with hereditary nonpolyposis colorectal cancer. Gastroenterology. 2000;118(5):829-34.
20. Stupart DA, Goldberg PA, Algar U, Ramesar R. Surveillance colonoscopy improves survival in a cohort of subjects with a single mismatch repair gene mutation. Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland. 2009;11(2):126-30.
21. Dove-Edwin I, Boks D, Goff S, Kenter GG, Carpenter R, Vasen HF, *et al*. The outcome of endometrial carcinoma surveillance by ultrasound scan in women at risk of hereditary nonpolyposis colorectal carcinoma and familial colorectal carcinoma. Cancer. 2002;94(6):1708-12.
22. Schmeler KM, Lynch HT, Chen LM, Munsell MF, Soliman PT, Clark MB, *et al*. Prophylactic surgery to reduce the risk of gynecologic cancers in the Lynch syndrome. N Engl J Med. 2006;354(3):261-9.
23. Bronner CE, Baker SM, Morrison PT, Warren G, Smith LG, Lescoe MK, *et al*. Mutation in the DNA mismatch repair gene homologue hMLH1 is associated with hereditary non-polyposis colon cancer. Nature. 1994;368(6468):258-61.
24. Nicolaides NC, Papadopoulos N, Liu B, Wei YF, Carter KC, Ruben SM, *et al*. Mutations of two PMS homologues in hereditary nonpolyposis colon cancer. Nature. 1994;371(6492):75-80.
25. Miyaki M, Konishi M, Tanaka K, Kikuchi-Yanoshita R, Muraoka M, Yasuno M, *et al*. Germline mutation of MSH6 as the cause of hereditary nonpolyposis colorectal cancer. Nat Genet. 1997;17(3):271-2.
26. De Jong AE, Morreau H, Van Puijenbroek M, Eilers PH, Wijnen J, Nagengast FM, *et al*. The role of mismatch repair gene defects in the development of adenomas in patients with HNPCC. Gastroenterology. 2004;126(1):42-8.
27. Wijnen JT, Vasen HF, Khan PM, Zwinderman AH, van der Klift H, Mulder A, *et al*. Clinical findings with implications for genetic testing in families with clustering of colorectal cancer. N Engl J Med. 1998;339(8):511-8.
28. Boland CR, Koi M, Chang DK, Carethers JM. The biochemical basis of microsatellite instability and abnormal immunohistochemistry and clinical behavior in Lynch syndrome: from bench to bedside. Fam Cancer. 2008;7(1):41-52.
29. Jiricny J. Mediating mismatch repair. Nat Genet. 2000;24(1):6-8.
30. Ligtenberg MJ, Kuiper RP, Chan TL, Goossens M, Hebeda KM, Voorendt M, *et al*. Heritable somatic methylation and inactivation of MSH2 in families with Lynch syndrome due to deletion of the 3′ exons of TACSTD1. Nat Genet. 2009;41(1):112-7.
31. Ligtenberg MJ, Kuiper RP, Geurts van Kessel A, Hoogerbrugge N. EPCAM deletion carriers constitute a unique subgroup of Lynch syndrome patients. Fam Cancer. 2013;12(2):169-74.
32. Felton KE, Gilchrist DM, Andrew SE. Constitutive deficiency in DNA mismatch repair: is it time for Lynch III? Clin Genet. 2007;71(6):499-500.
33. Bandipalliam P. Syndrome of early onset colon cancers, hematologic malignancies & features of neurofibromatosis in HNPCC families with homozygous mismatch repair gene mutations. Fam Cancer. 2005;4(4):323-33.

34. Menko FH, Kaspers GL, Meijer GA, Claes K, van Hagen JM, Gille JJ. A homozygous MSH6 mutation in a child with cafe-au-lait spots, oligodendroglioma and rectal cancer. Fam Cancer. 2004;3(2):123-7.
35. Wimmer K, Etzler J. Constitutional mismatch repair-deficiency syndrome: have we so far seen only the tip of an iceberg? Hum Genet. 2008;124(2):105-22.
36. Durno CA, Holter S, Sherman PM, Gallinger S. The Gastrointestinal Phenotype of Germline Biallelic Mismatch Repair Gene Mutations. The American journal of gastroenterology. 2010.
37. Durno CA, Holter S, Sherman PM, Gallinger S. The gastrointestinal phenotype of germline biallelic mismatch repair gene mutations. Am J Gastroenterol. 2010;105(11):2449-56.
38. Aaltonen LA, Peltomaki P, Mecklin JP, Jarvinen H, Jass JR, Green JS, *et al*. Replication errors in benign and malignant tumors from hereditary nonpolyposis colorectal cancer patients. Cancer Res. 1994;54(7):1645-8.
39. Boland CR, Thibodeau SN, Hamilton SR, Sidransky D, Eshleman JR, Burt RW, *et al*. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res. 1998;58(22):5248-57.
40. Lynch HT, de la Chapelle A. Hereditary colorectal cancer. N Engl J Med. 2003;348(10):919-32.
41. Kuismanen SA, Holmberg MT, Salovaara R, de la Chapelle A, Peltomaki P. Genetic and epigenetic modification of MLH1 accounts for a major share of microsatellite-unstable colorectal cancers. The American journal of pathology. 2000;156(5):1773-9.
42. Bettstetter M, Dechant S, Ruemmele P, Grabowski M, Keller G, Holinski-Feder E, *et al*. Distinction of hereditary nonpolyposis colorectal cancer and sporadic microsatellite-unstable colorectal cancer through quantification of MLH1 methylation by real-time PCR. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(11):3221-8.
43. Loughrey MB, Waring PM, Tan A, Trivett M, Kovalenko S, Beshay V, *et al*. Incorporation of somatic BRAF mutation testing into an algorithm for the investigation of hereditary non-polyposis colorectal cancer. Fam Cancer. 2007;6(3):301-10.
44. Poynter JN, Siegmund KD, Weisenberger DJ, Long TI, Thibodeau SN, Lindor N, *et al*. Molecular characterization of MSI-H colorectal cancer by MLHI promoter methylation, immunohistochemistry, and mismatch repair germline mutation screening. Cancer Epidemiol Biomarkers Prev. 2008;17(11):3208-15.
45. Domingo E, Espin E, Armengol M, Oliveira C, Pinto M, Duval A, *et al*. Activated BRAF targets proximal colon tumors with mismatch repair deficiency and MLH1 inactivation. Genes, chromosomes & cancer. 2004;39(2):138-42.
46. Domingo E, Laiho P, Ollikainen M, Pinto M, Wang L, French AJ, *et al*. BRAF screening as a low-cost effective strategy for simplifying HNPCC genetic testing. J Med Genet. 2004;41(9):664-8.
47. Domingo E, Niessen RC, Oliveira C, Alhopuro P, Moutinho C, Espin E, *et al*. BRAF-V600E is not involved in the colorectal tumorigenesis of HNPCC in patients with functional MLH1 and MSH2 genes. Oncogene. 2005;24(24):3995-8.
48. Lubomierski N, Plotz G, Wormek M, Engels K, Kriener S, Trojan J, *et al*. BRAF mutations in colorectal carcinoma suggest two entities of microsatellite-unstable tumors. Cancer. 2005;104(5):952-61.
49. McGivern A, Wynter CV, Whitehall VL, Kambara T, Spring KJ, Walsh MD, *et al*. Promoter hypermethylation frequency and BRAF mutations distinguish hereditary non-polyposis colon cancer from sporadic MSI-H colon cancer. Fam Cancer. 2004;3(2):101-7.

50. Wang L, Cunningham JM, Winters JL, Guenther JC, French AJ, Boardman LA, *et al*. BRAF mutations in colon cancer are not likely attributable to defective DNA mismatch repair. Cancer Res. 2003;63(17):5209-12.
51. Berends MJ, Wu Y, Sijmons RH, Mensink RG, van der Sluis T, Hordijk-Hos JM, *et al*. Molecular and clinical characteristics of MSH6 variants: an analysis of 25 index carriers of a germline variant. Am J Hum Genet. 2002;70(1):26-37.
52. Xicola RM, Llor X, Pons E, Castells A, Alenda C, Pinol V, *et al*. Performance of different microsatellite marker panels for detection of mismatch repair-deficient colorectal tumors. J Natl Cancer Inst. 2007;99(3):244-52.
53. Ferreira AM, Westers H, Sousa S, Wu Y, Niessen RC, Olderode-Berends M, *et al*. Mononucleotide precedes dinucleotide repeat instability during colorectal tumour development in Lynch syndrome patients. The Journal of pathology. 2009;219(1):96-102.
54. Hendriks YM, de Jong AE, Morreau H, Tops CM, Vasen HF, Wijnen JT, *et al*. Diagnostic approach and management of Lynch syndrome (hereditary nonpolyposis colorectal carcinoma): a guide for clinicians. CA Cancer J Clin. 2006;56(4):213-25.
55. van Lier MG, Wagner A, van Leerdam ME, Biermann K, Kuipers EJ, Steyerberg EW, *et al*. A review on the molecular diagnostics of Lynch syndrome: a central role for the pathology laboratory. Journal of cellular and molecular medicine. 2010;14(1-2):181-97.
56. Shia J, Klimstra DS, Nafa K, Offit K, Guillem JG, Markowitz AJ, *et al*. Value of immunohistochemical detection of DNA mismatch repair proteins in predicting germline mutation in hereditary colorectal neoplasms. The American journal of surgical pathology. 2005;29(1):96-104.
57. Popat S, Hubner R, Houlston RS. Systematic review of microsatellite instability and colorectal cancer prognosis. J Clin Oncol. 2005;23(3):609-18.
58. Westra JL, Schaapveld M, Hollema H, de Boer JP, Kraak MM, de Jong D, *et al*. Determination of TP53 mutation is more relevant than microsatellite instability status for the prediction of disease-free survival in adjuvant-treated stage III colon cancer patients. J Clin Oncol. 2005;23(24):5635-43.
59. Sinicrope FA, Rego RL, Foster N, Sargent DJ, Windschitl HE, Burgart LJ, *et al*. Microsatellite instability accounts for tumor site-related differences in clinicopathologic variables and prognosis in human colon cancers. The American journal of gastroenterology. 2006;101(12):2818-25.
60. Soreide K, Slewa A, Stokkeland PJ, van Diermen B, Janssen EA, Soreide JA, *et al*. Microsatellite instability and DNA ploidy in colorectal cancer: potential implications for patients undergoing systematic surveillance after resection. Cancer. 2009;115(2):271-82.
61. Jover R, Zapater P, Castells A, Llor X, Andreu M, Cubiella J, *et al*. Mismatch repair status in the prediction of benefit from adjuvant fluorouracil chemotherapy in colorectal cancer. Gut. 2006;55(6):848-55.
62. Jover R, Zapater P, Castells A, Llor X, Andreu M, Cubiella J, *et al*. The efficacy of adjuvant chemotherapy with 5-fluorouracil in colorectal cancer depends on the mismatch repair status. European journal of cancer. 2009;45(3):365-73.
63. Ribic CM, Sargent DJ, Moore MJ, Thibodeau SN, French AJ, Goldberg RM, *et al*. Tumor microsatellite-instability status as a predictor of benefit from fluorouracil-based adjuvant chemotherapy for colon cancer. N Engl J Med. 2003;349(3):247-57.
64. De Vos M, Hayward BE, Picton S, Sheridan E, Bonthron DT. Novel PMS2 pseudogenes can conceal recessive mutations causing a distinctive childhood cancer syndrome. Am J Hum Genet. 2004;74(5):954-64.
65. Nakagawa H, Lockman JC, Frankel WL, Hampel H, Steenblock K, Burgart LJ, *et al*. Mismatch repair gene PMS2: disease-causing germline mutations are frequent in patients whose tumors stain

negative for PMS2 protein, but paralogous genes obscure mutation detection and interpretation. Cancer Res. 2004;64(14):4721-7.

66. Hampel H, Frankel WL, Martin E, Arnold M, Khanduja K, Kuebler P, *et al*. Feasibility of screening for Lynch syndrome among patients with colorectal cancer. J Clin Oncol. 2008;26(35):5783-8.
67. Julie C, Tresallet C, Brouquet A, Vallot C, Zimmermann U, Mitry E, *et al*. Identification in daily practice of patients with Lynch syndrome (hereditary nonpolyposis colorectal cancer): revised Bethesda guidelines-based approach versus molecular screening. The American journal of gastroenterology. 2008;103(11):2825-35; quiz 36.
68. Mensenkamp AR, Vogelaar IP, van Zelst-Stams WA, Goossens M, Ouchene H, Hendriks-Cornelissen SJ, *et al*. Somatic mutations in MLH1 and MSH2 are a frequent cause of mismatch-repair deficiency in Lynch syndrome-like tumors. Gastroenterology. 2014;146(3):643-6 e8.
69. Sourrouille I, Coulet F, Lefevre JH, Colas C, Eyries M, Svrcek M, *et al*. Somatic mosaicism and double somatic hits can lead to MSI colorectal tumors. Fam Cancer. 2013;12(1):27-33.
70. Hendriks YM, Wagner A, Morreau H, Menko F, Stormorken A, Quehenberger F, *et al*. Cancer risk in hereditary nonpolyposis colorectal cancer due to MSH6 mutations: impact on counseling and surveillance. Gastroenterology. 2004;127(1):17-25.
71. Plaschke J, Engel C, Kruger S, Holinski-Feder E, Pagenstecher C, Mangold E, *et al*. Lower incidence of colorectal cancer and later age of disease onset in 27 families with pathogenic MSH6 germline mutations compared with families with MLH1 or MSH2 mutations: the German Hereditary Nonpolyposis Colorectal Cancer Consortium. J Clin Oncol. 2004;22(22):4486-94.
72. Lynch HT. Hereditary nonpolyposis colorectal cancer (HNPCC). Cytogenetics and cell genetics. 1999;86(2):130-5.
73. Karamurzin Y, Rutgers JK. DNA mismatch repair deficiency in endometrial carcinoma. International journal of gynecological pathology : official journal of the International Society of Gynecological Pathologists. 2009;28(3):239-55.
74. Umar A. Lynch syndrome (HNPCC) and microsatellite instability. Disease markers. 2004;20(4-5):179-80.
75. Jass JR, Stewart SM. Evolution of hereditary non-polyposis colorectal cancer. Gut. 1992;33(6):783-6.
76. Smyrk TC, Watson P, Kaul K, Lynch HT. Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma. Cancer. 2001;91(12):2417-22.
77. Jenkins MA, Hayashi S, O'Shea AM, Burgart LJ, Smyrk TC, Shimizu D, *et al*. Pathology features in Bethesda guidelines predict colorectal cancer microsatellite instability: a population-based study. Gastroenterology. 2007;133(1):48-56.
78. Kievit W, de Bruin JH, Adang EM, Severens JL, Kleibeuker JH, Sijmons RH, *et al*. Cost effectiveness of a new strategy to identify HNPCC patients. Gut. 2005;54(1):97-102.
79. Church J, McGannon E. Family history of colorectal cancer: how often and how accurately is it recorded? Diseases of the colon and rectum. 2000;43(11):1540-4.
80. Vasen HF, Offerhaus GJ, den Hartog Jager FC, Menko FH, Nagengast FM, Griffioen G, *et al*. The tumour spectrum in hereditary non-polyposis colorectal cancer: a study of 24 kindreds in the Netherlands. Int J Cancer. 1990;46(1):31-4.
81. Vasen HF, Watson P, Mecklin JP, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. Gastroenterology. 1999;116(6):1453-6.

82. Barnetson RA, Tenesa A, Farrington SM, Nicholl ID, Cetnarskyj R, Porteous ME, *et al*. Identification and survival of carriers of mutations in DNA mismatch-repair genes in colon cancer. N Engl J Med. 2006;354(26):2751-63.
83. Ramsoekh D, Wagner A, van Leerdam ME, Dinjens WN, Steyerberg EW, Halley DJ, *et al*. A high incidence of MSH6 mutations in Amsterdam criteria II-negative families tested in a diagnostic setting. Gut. 2008;57(11):1539-44.
84. Umar A, Boland CR, Terdiman JP, Syngal S, de la Chapelle A, Ruschoff J, *et al*. Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability. J Natl Cancer Inst. 2004;96(4):261-8.
85. Chen S, Wang W, Lee S, Nafa K, Lee J, Romans K, *et al*. Prediction of germline mutations and cancer risk in the Lynch syndrome. Jama. 2006;296(12):1479-87.
86. Lipton LR, Johnson V, Cummings C, Fisher S, Risby P, Eftekhar Sadat AT, *et al*. Refining the Amsterdam Criteria and Bethesda Guidelines: testing algorithms for the prediction of mismatch repair mutation status in the familial cancer clinic. J Clin Oncol. 2004;22(24):4934-43.
87. Balaguer F, Balmana J, Castellvi-Bel S, Steyerberg EW, Andreu M, Llor X, *et al*. Validation and extension of the PREMM1,2 model in a population-based cohort of colorectal cancer patients. Gastroenterology. 2008;134(1):39-46.
88. Ramsoekh D, van Leerdam ME, Wagner A, Kuipers EJ, Steyerberg EW. Mutation prediction models in Lynch syndrome: evaluation in a clinical genetic setting. J Med Genet. 2009;46(11):745-51.
89. Green RC, Parfrey PS, Woods MO, Younghusband HB. Prediction of Lynch syndrome in consecutive patients with colorectal cancer. J Natl Cancer Inst. 2009;101(5):331-40.
90. Kastrinos F, Balmana J, Syngal S. Prediction models in Lynch syndrome. Fam Cancer. 2013;12(2):217-28.
91. Perez-Carbonell L, Ruiz-Ponte C, Guarinos C, Alenda C, Paya A, Brea A, *et al*. Comparison between universal molecular screening for Lynch syndrome and revised Bethesda guidelines in a large population-based cohort of patients with colorectal cancer. Gut. 2012;61(6):865-72.
92. Hampel H, Frankel W, Panescu J, Lockman J, Sotamaa K, Fix D, *et al*. Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients. Cancer Res. 2006;66(15):7810-7.
93. Ladabaum U, Wang G, Terdiman J, Blanco A, Kuppermann M, Boland CR, *et al*. Strategies to identify the Lynch syndrome among patients with colorectal cancer: a cost-effectiveness analysis. Ann Intern Med. 2011;155(2):69-79.
94. Mvundura M, Grosse SD, Hampel H, Palomaki GE. The cost-effectiveness of genetic testing strategies for Lynch syndrome among newly diagnosed patients with colorectal cancer. Genetics in medicine : official journal of the American College of Medical Genetics. 2010;12(2):93-104.
95. Ramsey SD, Burke W, Clarke L. An economic viewpoint on alternative strategies for identifying persons with hereditary nonpolyposis colorectal cancer. Genetics in medicine : official journal of the American College of Medical Genetics. 2003;5(5):353-63.
96. Ramsey SD, Clarke L, Etzioni R, Higashi M, Berry K, Urban N. Cost-effectiveness of microsatellite instability screening as a method for detecting hereditary nonpolyposis colorectal cancer. Ann Intern Med. 2001;135(8 Pt 1):577-88.
97. Ramsoekh D, van Leerdam ME, Tops CM, Dooijes D, Steyerberg EW, Kuipers EJ, *et al*. The use of genetic testing in hereditary colorectal cancer syndromes: genetic testing in HNPCC, (A)FAP and MAP. Clin Genet. 2007;72(6):562-7.

98. Hadley DW, Jenkins J, Dimond E, Nakahara K, Grogan L, Liewehr DJ, *et al*. Genetic counseling and testing in families with hereditary nonpolyposis colorectal cancer. Archives of internal medicine. 2003;163(5):573-82.
99. Sharaf RN, Myer P, Stave CD, Diamond LC, Ladabaum U. Uptake of genetic testing by relatives of lynch syndrome probands: a systematic review. Clin Gastroenterol Hepatol. 2013;11(9):1093-100.
100. Aktan-Collan K, Haukkala A, Pylvanainen K, Jarvinen HJ, Aaltonen LA, Peltomaki P, *et al*. Direct contact in inviting high-risk members of hereditary colon cancer families to genetic counselling and DNA testing. J Med Genet. 2007;44(11):732-8.
101. Peterson SK, Watts BG, Koehly LM, Vernon SW, Baile WF, Kohlmann WK, *et al*. How families communicate about HNPCC genetic testing: findings from a qualitative study. American journal of medical genetics. 2003;119C(1):78-86.
102. Akiyama Y, Sato H, Yamada T, Nagasaki H, Tsuchiya A, Abe R, *et al*. Germ-line mutation of the hMSH6/GTBP gene in an atypical hereditary nonpolyposis colorectal cancer kindred. Cancer Res. 1997;57(18):3920-3.
103. Fishel R, Lescoe MK, Rao MR, Copeland NG, Jenkins NA, Garber J, *et al*. The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. Cell. 1993;75(5):1027-38.
104. Aaltonen LA, Peltomaki P, Leach FS, Sistonen P, Pylkkanen L, Mecklin JP, *et al*. Clues to the pathogenesis of familial colorectal cancer. Science. 1993;260(5109):812-6.
105. Peltomaki P, Aaltonen LA, Sistonen P, Pylkkanen L, Mecklin JP, Jarvinen H, *et al*. Genetic mapping of a locus predisposing to human colorectal cancer. Science. 1993;260(5109):810-2.
106. Poley JW, Wagner A, Hoogmans MM, Menko FH, Tops C, Kros JM, *et al*. Biallelic germline mutations of mismatch-repair genes: a possible cause for multiple pediatric malignancies. Cancer. 2007;109(11):2349-56.
107. Clendenning M, Senter L, Hampel H, Robinson KL, Sun S, Buchanan D, *et al*. A frame-shift mutation of PMS2 is a widespread cause of Lynch syndrome. J Med Genet. 2008;45(6):340-5.
108. Agostini M, Tibiletti MG, Lucci-Cordisco E, Chiaravalli A, Morreau H, Furlan D, *et al*. Two PMS2 mutations in a Turcot syndrome family with small bowel cancers. The American journal of gastroenterology. 2005;100(8):1886-91.
109. Jackson CC, Holter S, Pollett A, Clendenning M, Chou S, Senter L, *et al*. Cafe-au-lait macules and pediatric malignancy caused by biallelic mutations in the DNA mismatch repair (MMR) gene PMS2. Pediatr Blood Cancer. 2008;50(6):1268-70.
110. van der Klift HM, Tops CM, Bik EC, Boogaard MW, Borgstein AM, Hansson KB, *et al*. Quantification of sequence exchange events between PMS2 and PMS2CL provides a basis for improved mutation scanning of Lynch syndrome patients. Human mutation. 2010;31(5):578-87.
111. Auclair J, Leroux D, Desseigne F, Lasset C, Saurin JC, Joly MO, *et al*. Novel biallelic mutations in MSH6 and PMS2 genes: gene conversion as a likely cause of PMS2 gene inactivation. Hum Mutat. 2007;28(11):1084-90.
112. De Rosa M, Fasano C, Panariello L, Scarano MI, Belli G, Iannelli A, *et al*. Evidence for a recessive inheritance of Turcot's syndrome caused by compound heterozygous mutations within the PMS2 gene. Oncogene. 2000;19(13):1719-23.
113. Gallinger S, Aronson M, Shayan K, Ratcliffe EM, Gerstle JT, Parkin PC, *et al*. Gastrointestinal cancers and neurofibromatosis type 1 features in children with a germline homozygous MLH1 mutation. Gastroenterology. 2004;126(2):576-85.
114. Hamilton SR, Liu B, Parsons RE, Papadopoulos N, Jen J, Powell SM, *et al*. The molecular basis of Turcot's syndrome. N Engl J Med. 1995;332(13):839-47.

115. Kratz CP, Niemeyer CM, Juttner E, Kartal M, Weninger A, Schmitt-Graeff A, *et al*. Childhood T-cell non-Hodgkin's lymphoma, colorectal carcinoma and brain tumor in association with cafe-au-lait spots caused by a novel homozygous PMS2 mutation. Leukemia. 2008;22(5):1078-80.
116. Kruger S, Kinzel M, Walldorf C, Gottschling S, Bier A, Tinschert S, *et al*. Homozygous PMS2 germline mutations in two families with early-onset haematological malignancy, brain tumours, HNPCC-associated tumours, and signs of neurofibromatosis type 1. Eur J Hum Genet. 2008;16(1):62-72.
117. Liu T, Tannergard P, Hackman P, Rubio C, Kressner U, Lindmark G, *et al*. Missense mutations in hMLH1 associated with colorectal cancer. Hum Genet. 1999;105(5):437-41.
118. Muller A, Schackert HK, Lange B, Ruschoff J, Fuzesi L, Willert J, *et al*. A novel MSH2 germline mutation in homozygous state in two brothers with colorectal cancers diagnosed at the age of 11 and 12 years. Am J Med Genet A. 2006;140(3):195-9.
119. Plaschke J, Linnebacher M, Kloor M, Gebert J, Cremer FW, Tinschert S, *et al*. Compound heterozygosity for two MSH6 mutations in a patient with early onset of HNPCC-associated cancers, but without hematological malignancy and brain tumor. Eur J Hum Genet. 2006;14(5):561-6.
120. Rahner N, Hoefler G, Hogenauer C, Lackner C, Steinke V, Sengteller M, *et al*. Compound heterozygosity for two MSH6 mutations in a patient with early onset colorectal cancer, vitiligo and systemic lupus erythematosus. Am J Med Genet A. 2008;146A(10):1314-9.
121. Tan TY, Orme LM, Lynch E, Croxford MA, Dow C, Dewan PA, *et al*. Biallelic PMS2 mutations and a distinctive childhood cancer syndrome. J Pediatr Hematol Oncol. 2008;30(3):254-7.
122. Trimbath JD, Petersen GM, Erdman SH, Ferre M, Luce MC, Giardiello FM. Cafe-au-lait spots and early onset colorectal neoplasia: a variant of HNPCC? Fam Cancer. 2001;1(2):101-5.
123. Will O, Carvajal-Carmona LG, Gorman P, Howarth KM, Jones AM, Polanco-Echeverry GM, *et al*. Homozygous PMS2 deletion causes a severe colorectal cancer and multiple adenoma phenotype without extraintestinal cancer. Gastroenterology. 2007;132(2):527-30.
124. Hegde MR, Chong B, Blazo ME, Chin LH, Ward PA, Chintagumpala MM, *et al*. A homozygous mutation in MSH6 causes Turcot syndrome. Clinical cancer research : an official journal of the American Association for Cancer Research. 2005;11(13):4689-93.
125. Ostergaard JR, Sunde L, Okkels H. Neurofibromatosis von Recklinghausen type I phenotype and early onset of cancers in siblings compound heterozygous for mutations in MSH6. Am J Med Genet A. 2005;139A(2):96-105; discussion 96.
126. Bougeard G, Charbonnier F, Moerman A, Martin C, Ruchoux MM, Drouot N, *et al*. Early onset brain tumor and lymphoma in MSH2-deficient children. Am J Hum Genet. 2003;72(1):213-6.
127. Etzler J, Peyrl A, Zatkova A, Schildhaus HU, Ficek A, Merkelbach-Bruse S, *et al*. RNA-based mutation analysis identifies an unusual MSH6 splicing defect and circumvents PMS2 pseudogene interference. Hum Mutat. 2008;29(2):299-305.
128. Boland CR. Evolution of the nomenclature for the hereditary colorectal cancer syndromes. Fam Cancer. 2005;4(3):211-8.
129. Leenen CH, van Lier MG, van Doorn HC, van Leerdam ME, Kooi SG, de Waard J, *et al*. Prospective evaluation of molecular screening for Lynch syndrome in patients with endometrial cancer ≤ 70 years. Gynecologic oncology. 2012;125(2):414-20.
130. van Lier MG, Leenen CH, Wagner A, Ramsoekh D, Dubbink HJ, van den Ouweland AM, *et al*. Yield of routine molecular analyses in colorectal cancer patients ≤ 70 years to detect underlying Lynch syndrome. The Journal of pathology. 2012;226(5):764-74.
131. Zhang R, Qin W, Xu GL, Zeng FF, Li CX. A meta-analysis of the prevalence of somatic mutations in the hMLH1 and hMSH2 genes in colorectal cancer. Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland. 2012;14(3):e80-9.

132. Zhao YS, Hu FL, Wang F, Han B, Li DD, Li XW, *et al*. Meta-analysis of MSH6 gene mutation frequency in colorectal and endometrial cancers. Journal of toxicology and environmental health Part A. 2009;72(11-12):690-7.
133. Lurkin I, Stoehr R, Hurst CD, van Tilborg AA, Knowles MA, Hartmann A, *et al*. Two multiplex assays that simultaneously identify 22 possible mutation sites in the KRAS, BRAF, NRAS and PIK3CA genes. PloS one. 2010;5(1):e8802.
134. Geurts-Giele WR, Dirkx-van der Velden AW, Bartalits NM, Verhoog LC, Hanselaar WE, Dinjens WN. Molecular diagnostics of a single multifocal non-small cell lung cancer case using targeted next generation sequencing. Virchows Archiv : an international journal of pathology. 2013;462(2):249-54.
135. Plon SE, Eccles DM, Easton D, Foulkes WD, Genuardi M, Greenblatt MS, *et al*. Sequence variant classification and reporting: recommendations for improving the interpretation of cancer susceptibility genetic test results. Human mutation. 2008;29(11):1282-91.
136. Thompson BA, Spurdle AB, Plazzer JP, Greenblatt MS, Akagi K, Al-Mulla F, *et al*. Application of a 5-tiered scheme for standardized classification of 2,360 unique mismatch repair gene variants in the InSiGHT locus-specific database. Nat Genet. 2014;46(2):107-15.
137. Melcher R, Hartmann E, Zopf W, Herterich S, Wilke P, Muller L, *et al*. LOH and copy neutral LOH (cnLOH) act as alternative mechanism in sporadic colorectal cancers with chromosomal and microsatellite instability. Carcinogenesis. 2011;32(4):636-42.
138. Veigl ML, Kasturi L, Olechnowicz J, Ma AH, Lutterbaugh JD, Periyasamy S, *et al*. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(15):8698-702.
139. van Puijenbroek M, Middeldorp A, Tops CM, van Eijk R, van der Klift HM, Vasen HF, *et al*. Genome-wide copy neutral LOH is infrequent in familial and sporadic microsatellite unstable carcinomas. Fam Cancer. 2008;7(4):319-30.
140. Parsons MT, Buchanan DD, Thompson B, Young JP, Spurdle AB. Correlation of tumour BRAF mutations and MLH1 methylation with germline mismatch repair (MMR) gene mutation status: a literature review assessing utility of tumour features for MMR variant classification. J Med Genet. 2012;49(3):151-7.
141. Smalley KS, Xiao M, Villanueva J, Nguyen TK, Flaherty KT, Letrero R, *et al*. CRAF inhibition induces apoptosis in melanoma cells with non-V600E BRAF mutations. Oncogene. 2009;28(1):85-94.
142. Arcila M, Lau C, Nafa K, Ladanyi M. Detection of KRAS and BRAF mutations in colorectal carcinoma roles for high-sensitivity locked nucleic acid-PCR sequencing and broad-spectrum mass spectrometry genotyping. The Journal of molecular diagnostics : JMD. 2011;13(1):64-73.
143. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, *et al*. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. Lancet Oncol. 2010;11(8):753-62.
144. Rodriguez-Soler M, Perez-Carbonell L, Guarinos C, Zapater P, Castillejo A, Barbera VM, *et al*. Risk of cancer in cases of suspected lynch syndrome without germline mutation. Gastroenterology. 2013;144(5):926-32 e1; quiz e13-4.
145. Aaltonen LA, Salovaara R, Kristo P, Canzian F, Hemminki A, Peltomaki P, *et al*. Incidence of hereditary nonpolyposis colorectal cancer and the feasibility of molecular screening for the disease. N Engl J Med. 1998;338(21):1481-7.

146. Lynch HT, Smyrk TC, Watson P, Lanspa SJ, Lynch JF, Lynch PM, *et al*. Genetics, natural history, tumor spectrum, and pathology of hereditary nonpolyposis colorectal cancer: an updated review. Gastroenterology. 1993;104(5):1535-49.
147. Watson P, Lynch HT. Extracolonic cancer in hereditary nonpolyposis colorectal cancer. Cancer. 1993;71(3):677-85.
148. Overbeek LI, Hoogerbrugge N, van Krieken JH, Nagengast FM, Ruers TJ, Ligtenberg MJ, *et al*. Most patients with colorectal tumors at young age do not visit a cancer genetics clinic. Diseases of the colon and rectum. 2008;51(8):1249-54.
149. Singh H, Schiesser R, Anand G, Richardson PA, El-Serag HB. Underdiagnosis of Lynch syndrome involves more than family history criteria. Clin Gastroenterol Hepatol. 2010;8(6):523-9.
150. Van Lier MG, De Wilt JH, Wagemakers JJ, Dinjens WN, Damhuis RA, Wagner A, *et al*. Underutilization of microsatellite instability analysis in colorectal cancer patients at high risk for Lynch syndrome. Scandinavian journal of gastroenterology. 2009;44(5):600-4.
151. Balmana J, Stockwell DH, Steyerberg EW, Stoffel EM, Deffenbaugh AM, Reid JE, *et al*. Prediction of MLH1 and MSH2 mutations in Lynch syndrome. Jama. 2006;296(12):1469-78.
152. Kastrinos F, Steyerberg EW, Mercado R, Balmana J, Holter S, Gallinger S, *et al*. The PREMM(1,2,6) model predicts risk of MLH1, MSH2, and MSH6 germline mutations based on cancer history. Gastroenterology. 2011;140(1):73-81.
153. Lynch HT, Riley BD, Weissman SM, Coronel SM, Kinarsky Y, Lynch JF, *et al*. Hereditary nonpolyposis colorectal carcinoma (HNPCC) and HNPCC-like families: Problems in diagnosis, surveillance, and management. Cancer. 2004;100(1):53-64.
154. Terdiman JP. It is time to get serious about diagnosing Lynch syndrome (hereditary nonpolyposis colorectal cancer with defective DNA mismatch repair) in the general population. Gastroenterology. 2005;129(2):741-4.
155. Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, *et al*. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proceedings of the National Academy of Sciences of the United States of America. 1998;95(12):6870-5.
156. Grady WM, Carethers JM. Genomic and epigenetic instability in colorectal cancer pathogenesis. Gastroenterology. 2008;135(4):1079-99.
157. van der Klift HM, Tops CM, Bik EC, Boogaard MW, Borgstein AM, Hansson KB, *et al*. Quantification of sequence exchange events between PMS2 and PMS2CL provides a basis for improved mutation scanning of Lynch syndrome patients. Human mutation. 2010;31(5):578-87.
158. Peltomaki P, Vasen H. Mutations associated with HNPCC predisposition -- Update of ICG-HNPCC/INSiGHT mutation database. Disease markers. 2004;20(4-5):269-76.
159. Peterlongo P, Nafa K, Lerman GS, Glogowski E, Shia J, Ye TZ, *et al*. MSH6 germline mutations are rare in colorectal cancer families. Int J Cancer. 2003;107(4):571-9.
160. Talseth-Palmer BA, McPhillips M, Groombridge C, Spigelman A, Scott RJ. MSH6 and PMS2 mutation positive Australian Lynch syndrome families: novel mutations, cancer risk and age of diagnosis of colorectal cancer. Hereditary cancer in clinical practice. 2010;8(1):5.
161. Wagner A, Hendriks Y, Meijers-Heijboer EJ, de Leeuw WJ, Morreau H, Hofstra R, *et al*. Atypical HNPCC owing to MSH6 germline mutations: analysis of a large Dutch pedigree. J Med Genet. 2001;38(5):318-22.
162. van Dijk DA, Oostindier MJ, Kloosterman-Boele WM, Krijnen P, Vasen HF, Hereditary Tumor Study Group of the Comprehensive Cancer Centre W. Family history is neglected in the work-up of patients with colorectal cancer: a quality assessment using cancer registry data. Fam Cancer. 2007;6(1):131-4.

163. Lindor NM, Petersen GM, Hadley DW, Kinney AY, Miesfeldt S, Lu KH, *et al*. Recommendations for the care of individuals with an inherited predisposition to Lynch syndrome: a systematic review. JAMA. 2006;296(12):1507-17.
164. Balmana J, Balaguer F, Castellvi-Bel S, Steyerberg EW, Andreu M, Llor X, *et al*. Comparison of predictive models, clinical criteria and molecular tumour screening for the identification of patients with Lynch syndrome in a population-based cohort of colorectal cancer patients. J Med Genet. 2008;45(9):557-63.
165. Samimi G, Fink D, Varki NM, Husain A, Hoskins WJ, Alberts DS, *et al*. Analysis of MLH1 and MSH2 expression in ovarian cancer before and after platinum drug-based chemotherapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2000;6(4):1415-21.
166. Haines J, Bacher J, Coster M, Huiskamp R, Meijne E, Mancuso M, *et al*. Microsatellite instability in radiation-induced murine tumours; influence of tumour type and radiation quality. International journal of radiation biology. 2010;86(7):555-68.
167. Peeters KC, Marijnen CA, Nagtegaal ID, Kranenbarg EK, Putter H, Wiggers T, *et al*. The TME trial after a median follow-up of 6 years: increased local control but no survival benefit in irradiated patients with resectable rectal carcinoma. Annals of surgery. 2007;246(5):693-701.
168. Rodriguez-Bigas MA, Boland CR, Hamilton SR, Henson DE, Jass JR, Khan PM, *et al*. A National Cancer Institute Workshop on Hereditary Nonpolyposis Colorectal Cancer Syndrome: meeting highlights and Bethesda guidelines. J Natl Cancer Inst. 1997;89(23):1758-62.
169. Ferreira S, Claro I, Lage P, Filipe B, Fonseca R, Sousa R, *et al*. Colorectal adenomas in young patients: microsatellite instability is not a useful marker to detect new cases of Lynch syndrome. Diseases of the colon and rectum. 2008;51(6):909-15.
170. Velayos FS, Allen BA, Conrad PG, Gum J, Jr., Kakar S, Chung DC, *et al*. Low rate of microsatellite instability in young patients with adenomas: reassessing the Bethesda guidelines. The American journal of gastroenterology. 2005;100(5):1143-9.
171. German HC, Muller A, Beckmann C, Westphal G, Bocker Edmonston T, Friedrichs N, *et al*. Prevalence of the mismatch-repair-deficient phenotype in colonic adenomas arising in HNPCC patients: results of a 5-year follow-up study. International journal of colorectal disease. 2006;21(7):632-41.
172. Iino H, Simms L, Young J, Arnold J, Winship IM, Webb SI, *et al*. DNA microsatellite instability and mismatch repair protein loss in adenomas presenting in hereditary non-polyposis colorectal cancer. Gut. 2000;47(1):37-42.
173. Loukola A, Salovaara R, Kristo P, Moisio AL, Kaariainen H, Ahtola H, *et al*. Microsatellite instability in adenomas as a marker for hereditary nonpolyposis colorectal cancer. The American journal of pathology. 1999;155(6):1849-53.
174. Gryfe R, Kim H, Hsieh ET, Aronson MD, Holowaty EJ, Bull SB, *et al*. Tumor microsatellite instability and clinical outcome in young patients with colorectal cancer. N Engl J Med. 2000;342(2):69-77.
175. Katballe N, Juul S, Christensen M, Orntoft TF, Wikman FP, Laurberg S. Patient accuracy of reporting on hereditary non-polyposis colorectal cancer-related malignancy in family members. The British journal of surgery. 2001;88(9):1228-33.
176. Sijmons RH, Boonstra AE, Reefhuis J, Hordijk-Hos JM, de Walle HE, Oosterwijk JC, *et al*. Accuracy of family history of cancer: clinical genetic implications. Eur J Hum Genet. 2000;8(3):181-6.
177. Mitchell RJ, Brewster D, Campbell H, Porteous ME, Wyllie AH, Bird CC, *et al*. Accuracy of reporting of family history of colorectal cancer. Gut. 2004;53(2):291-5.
178. Aarnio M, Sankila R, Pukkala E, Salovaara R, Aaltonen LA, de la Chapelle A, *et al*. Cancer risk in mutation carriers of DNA-mismatch-repair genes. Int J Cancer. 1999;81(2):214-8.

179. Loukola A, Eklin K, Laiho P, Salovaara R, Kristo P, Jarvinen H, *et al*. Microsatellite marker analysis in screening for hereditary nonpolyposis colorectal cancer (HNPCC). Cancer Res. 2001;61(11):4545-9.
180. Burks RT, Kessis TD, Cho KR, Hedrick L. Microsatellite instability in endometrial carcinoma. Oncogene. 1994;9(4):1163-6.
181. Risinger JI, Berchuck A, Kohler MF, Watson P, Lynch HT, Boyd J. Genetic instability of microsatellites in endometrial carcinoma. Cancer Res. 1993;53(21):5100-3.
182. Buttin BM, Powell MA, Mutch DG, Rader JS, Herzog TJ, Gibb RK, *et al*. Increased risk for hereditary nonpolyposis colorectal cancer-associated synchronous and metachronous malignancies in patients with microsatellite instability-positive endometrial carcinoma lacking MLH1 promoter methylation. Clinical cancer research : an official journal of the American Association for Cancer Research. 2004;10(2):481-90.
183. Simpkins SB, Bocker T, Swisher EM, Mutch DG, Gersell DJ, Kovatich AJ, *et al*. MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers. Hum Mol Genet. 1999;8(4):661-6.
184. Jarvinen HJ, Aarnio M. Surveillance on mutation carriers of DNA mismatch repair genes. Annales chirurgiae et gynaecologiae. 2000;89(3):207-10.
185. Umar A, Risinger JI, Hawk ET, Barrett JC. Testing guidelines for hereditary non-polyposis colorectal cancer. Nat Rev Cancer. 2004;4(2):153-8.
186. Syngal S, Fox EA, Eng C, Kolodner RD, Garber JE. Sensitivity and specificity of clinical criteria for hereditary non-polyposis colorectal cancer associated mutations in MSH2 and MLH1. J Med Genet. 2000;37(9):641-5.
187. Burke W, Petersen G, Lynch P, Botkin J, Daly M, Garber J, *et al*. Recommendations for follow-up care of individuals with an inherited predisposition to cancer. I. Hereditary nonpolyposis colon cancer. Cancer Genetics Studies Consortium. JAMA. 1997;277(11):915-9.
188. Klarskov L, Ladelund S, Holck S, Roenlund K, Lindebjerg J, Elebro J, *et al*. Interobserver variability in the evaluation of mismatch repair protein immunostaining. Hum Pathol. 2010;41(10):1387-96.
189. Zauber NP, Denehy TR, Taylor RR, Ongcapin EH, Marotta SP, Sabbath-Solitare M, *et al*. Microsatellite instability and DNA methylation of endometrial tumors and clinical features in young women compared with older women. Int J Gynecol Cancer. 2010;20(9):1549-56.
190. Feng YZ, Shiozawa T, Miyamoto T, Kashima H, Kurai M, Suzuki A, *et al*. BRAF mutation in endometrial carcinoma and hyperplasia: correlation with KRAS and p53 mutations and mismatch repair protein expression. Clinical cancer research : an official journal of the American Association for Cancer Research. 2005;11(17):6133-8.
191. Salvesen HB, Kumar R, Stefansson I, Angelini S, MacDonald N, Smeds J, *et al*. Low frequency of BRAF and CDKN2A mutations in endometrial cancer. Int J Cancer. 2005;115(6):930-4.
192. Kawaguchi M, Yanokura M, Banno K, Kobayashi Y, Kuwabara Y, Kobayashi M, *et al*. Analysis of a correlation between the BRAF V600E mutation and abnormal DNA mismatch repair in patients with sporadic endometrial cancer. Int J Oncol. 2009;34(6):1541-7.
193. Backes FJ, Leon ME, Ivanov I, Suarez A, Frankel WL, Hampel H, *et al*. Prospective evaluation of DNA mismatch repair protein expression in primary endometrial cancer. Gynecologic oncology. 2009;114(3):486-90.
194. Backes FJ, Mitchell E, Hampel H, Cohn DE. Endometrial cancer patients and compliance with genetic counseling: Room for improvement. Gynecologic oncology. 2011.
195. Black D, Soslow RA, Levine DA, Tornos C, Chen SC, Hummer AJ, *et al*. Clinicopathologic significance of defective DNA mismatch repair in endometrial carcinoma. J Clin Oncol. 2006;24(11):1745-53.

196. Goodfellow PJ, Buttin BM, Herzog TJ, Rader JS, Gibb RK, Swisher E, *et al*. Prevalence of defective DNA mismatch repair and MSH6 mutation in an unselected series of endometrial cancers. Proceedings of the National Academy of Sciences of the United States of America. 2003;100(10):5908-13.
197. Lu KH, Schorge JO, Rodabaugh KJ, Daniels MS, Sun CC, Soliman PT, *et al*. Prospective determination of prevalence of lynch syndrome in young women with endometrial cancer. J Clin Oncol. 2007;25(33):5158-64.
198. Modica I, Soslow RA, Black D, Tornos C, Kauff N, Shia J. Utility of immunohistochemistry in predicting microsatellite instability in endometrial carcinoma. The American journal of surgical pathology. 2007;31(5):744-51.
199. Ollikainen M, Abdel-Rahman WM, Moisio AL, Lindroos A, Kariola R, Jarvela I, *et al*. Molecular analysis of familial endometrial carcinoma: a manifestation of hereditary nonpolyposis colorectal cancer or a separate syndrome? J Clin Oncol. 2005;23(21):4609-16.
200. Richtlijn Erfelijke Darmkanker 2008. Vereniging Klinische Genetica Nederland; Kwaliteitsinstituut voor de Gezondheidszorg CBO. 2008.
201. Lancaster JM, Powell CB, Kauff ND, Cass I, Chen LM, Lu KH, *et al*. Society of Gynecologic Oncologists Education Committee statement on risk assessment for inherited gynecologic cancer predispositions. Gynecologic oncology. 2007;107(2):159-62.
202. Kwon JS, Scott JL, Gilks CB, Daniels MS, Sun CC, Lu KH. Testing Women With Endometrial Cancer to Detect Lynch Syndrome. J Clin Oncol. 2011.
203. Backes FJ, Hampel H, Backes KA, Vaccarello L, Lewandowski G, Bell JA, *et al*. Are prediction models for Lynch syndrome valid for probands with endometrial cancer? Fam Cancer. 2009;8(4):483-7.
204. de Leeuw WJ, Dierssen J, Vasen HF, Wijnen JT, Kenter GG, Meijers-Heijboer H, *et al*. Prediction of a mismatch repair gene defect by microsatellite instability and immunohistochemical analysis in endometrial tumours from HNPCC patients. The Journal of pathology. 2000;192(3):328-35.
205. Walsh CS, Blum A, Walts A, Alsabeh R, Tran H, Koeffler HP, *et al*. Lynch syndrome among gynecologic oncology patients meeting Bethesda guidelines for screening. Gynecologic oncology. 2010;116(3):516-21.
206. Van Dalen R, Church J, McGannon E, Fay S, Burke C, Clark B. Patterns of surgery in patients belonging to amsterdam-positive families. Dis Colon Rectum. 2003;46(5):617-20.
207. Jarvinen HJ, Renkonen-Sinisalo L, Aktan-Collan K, Peltomaki P, Aaltonen LA, Mecklin JP. Ten years after mutation testing for Lynch syndrome: cancer incidence and outcome in mutation-positive and mutation-negative family members. J Clin Oncol. 2009;27(28):4793-7.
208. de la Chapelle A. Microsatellite instability. N Engl J Med. 2003;349(3):209-10.
209. Cross DS, Rahm AK, Kauffman TL, Webster J, Le AQ, Spencer Feigelson H, *et al*. Underutilization of Lynch syndrome screening in a multisite study of patients with colorectal cancer. Genet Med. 2013;15(12):933-40.
210. Gudgeon JM, Belnap TW, Williams JL, Williams MS. Impact of age cutoffs on a lynch syndrome screening program. J Oncol Pract. 2013;9(4):175-9.
211. Syngal S, Weeks JC, Schrag D, Garber JE, Kuntz KM. Benefits of colonoscopic surveillance and prophylactic colectomy in patients with hereditary nonpolyposis colorectal cancer mutations. Ann Intern Med. 1998;129(10):787-96.
212. Vasen HF, Abdirahman M, Brohet R, Langers AM, Kleibeuker JH, van Kouwen M, *et al*. One to 2-year surveillance intervals reduce risk of colorectal cancer in families with Lynch syndrome. Gastroenterology. 2010;138(7):2300-6.

213. Sie AS, Mensenkamp AR, Adang EM, Ligtenberg MJ, Hoogerbrugge N. Fourfold increased detection of Lynch syndrome by raising age limit for tumour genetic testing from 50 to 70 years is cost-effective. Ann Oncol. 2014.
214. Siegel JE, Weinstein MC, Russell LB, Gold MR. Recommendations for reporting cost-effectiveness analyses. Panel on Cost-Effectiveness in Health and Medicine. Jama. 1996;276(16):1339-41.
215. Wilschut JA, Hol L, Dekker E, Jansen JB, Van Leerdam ME, Lansdorp-Vogelaar I, *et al*. Cost-effectiveness analysis of a quantitative immunochemical test for colorectal cancer screening. Gastroenterology. 2011;141(5):1648-55 e1.
216. Koehler-Santos P, Izetti P, Abud J, Pitroski CE, Cossio SL, Camey SA, *et al*. Identification of patients at-risk for Lynch syndrome in a hospital-based colorectal surgery clinic. World J Gastroenterol. 2011;17(6):766-73.
217. Krivokapic Z, Markovic S, Antic J, Dimitrijevic I, Bojic D, Svorcan P, *et al*. Clinical and pathological tools for identifying microsatellite instability in colorectal cancer. Croat Med J. 2012;53(4):328-35.
218. Lamberti C, Mangold E, Pagenstecher C, Jungck M, Schwering D, Bollmann M, *et al*. Frequency of hereditary non-polyposis colorectal cancer among unselected patients with colorectal cancer in Germany. Digestion. 2006;74(1):58-67.
219. CVZ. Het pakketprincipe kosteneffectiviteit achtergrondstudie ten behoeve van de 'appraisal' fase in pakketbeheer. Diemen: 2010.
220. Braithwaite RS, Meltzer DO, King JT, Jr., Leslie D, Roberts MS. What does the value of modern medicine say about the $50,000 per quality-adjusted life-year decision rule? Med Care. 2008;46(4):349-56.
221. Giardiello FM, Allen JI, Axilbund JE, Boland CR, Burke CA, Burt RW, *et al*. Guidelines on Genetic Evaluation and Management of Lynch Syndrome: A Consensus Statement by the US Multi-Society Task Force on Colorectal Cancer. Gastroenterology. 2014;147(2):502-26.
222. Chapman RH, Berger M, Weinstein MC, Weeks JC, Goldie S, Neumann PJ. When does quality-adjusting life-years matter in cost-effectiveness analysis? Health Econ. 2004;13(5):429-36.
223. Ramsey S, Blough D, McDermott C, Clarke L, Bennett R, Burke W, *et al*. Will knowledge of gene-based colorectal cancer disease risk influence quality of life and screening behavior? Findings from a population-based study. Public Health Genomics. 2010;13(1):1-12.
224. Wang G, Kuppermann M, Kim B, Phillips KA, Ladabaum U. Influence of patient preferences on the cost-effectiveness of screening for lynch syndrome. J Oncol Pract. 2012;8(3 Suppl):e24s-30s.
225. Kastrinos F, Steyerberg EW, Balmana J, Mercado R, Gallinger S, Haile R, *et al*. Comparison of the clinical prediction model PREMM(1,2,6) and molecular testing for the systematic identification of Lynch syndrome in colorectal cancer. Gut. 2013;62(2):272-9.
226. Vasen HF, Blanco I, Aktan-Collan K, Gopie JP, Alonso A, Aretz S, *et al*. Revised guidelines for the clinical management of Lynch syndrome (HNPCC): recommendations by a group of European experts. Gut. 2013;62(6):812-23.
227. Jasperson KW, Tuohy TM, Neklason DW, Burt RW. Hereditary and familial colon cancer. Gastroenterology. 2010;138(6):2044-58.
228. Vasen HF, Mecklin JP, Khan PM, Lynch HT. The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). Diseases of the colon and rectum. 1991;34(5):424-5.
229. Palomaki GE, McClain MR, Melillo S, Hampel HL, Thibodeau SN. EGAPP supplementary evidence review: DNA testing strategies aimed at reducing morbidity and mortality from Lynch syndrome. Genetics in medicine : official journal of the American College of Medical Genetics. 2009;11(1):42-65.

230. National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology. Guidelines for detection, prevention, and risk reduction. Genetic/Familial High-Risk Assessment: Colorectal Cancer.
231. Newcomb PA, Baron J, Cotterchio M, Gallinger S, Grove J, Haile R, *et al*. Colon Cancer Family Registry: an international resource for studies of the genetic epidemiology of colon cancer. Cancer Epidemiol Biomarkers Prev. 2007;16(11):2331-43.
232. Pinol V, Castells A, Andreu M, Castellvi-Bel S, Alenda C, Llor X, *et al*. Accuracy of revised Bethesda guidelines, microsatellite instability, and immunohistochemistry for the identification of patients with hereditary nonpolyposis colorectal cancer. JAMA. 2005;293(16):1986-94.
233. Austin PC, Steyerberg EW. Graphical assessment of internal and external calibration of logistic regression models by using loess smoothers. Statistics in medicine. 2014;33(3):517-35.
234. Steyerberg EW, Vergouwe Y. Towards better clinical prediction models: seven steps for development and an ABCD for validation. European heart journal. 2014;35(29):1925-31.
235. Vickers AJ, Elkin EB. Decision curve analysis: a novel method for evaluating prediction models. Medical decision making : an international journal of the Society for Medical Decision Making. 2006;26(6):565-74.
236. Vickers AJ, Cronin AM. Traditional statistical methods for evaluating prediction models are uninformative as to clinical value: towards a decision analytic framework. Seminars in oncology. 2010;37(1):31-8.
237. Steyerberg EW, Vickers AJ, Cook NR, Gerds T, Gonen M, Obuchowski N, *et al*. Assessing the performance of prediction models: a framework for traditional and novel measures. Epidemiology. 2010;21(1):128-38.
238. Localio AR, Goodman S. Beyond the usual prediction accuracy metrics: reporting results for clinical decision making. Ann Intern Med. 2012;157(4):294-5.
239. Khan O, Blanco A, Conrad P, Gulden C, Moss TZ, Olopade OI, *et al*. Performance of Lynch syndrome predictive models in a multi-center US referral population. The American journal of gastroenterology. 2011;106(10):1822-7; quiz 8.
240. Pouchet CJ, Wong N, Chong G, Sheehan MJ, Schneider G, Rosen-Sheidley B, *et al*. A comparison of models used to predict MLH1, MSH2 and MSH6 mutation carriers. Ann Oncol. 2009;20(4):681-8.
241. Monzon JG, Cremin C, Armstrong L, Nuk J, Young S, Horsman DE, *et al*. Validation of predictive models for germline mutations in DNA mismatch repair genes in colorectal cancer. Int J Cancer. 2010;126(4):930-9.
242. Tresallet C, Brouquet A, Julie C, Beauchet A, Vallot C, Menegaux F, *et al*. Evaluation of predictive models in daily practice for the identification of patients with Lynch syndrome. Int J Cancer. 2012;130(6):1367-77.
243. Win AK, Macinnis RJ, Dowty JG, Jenkins MA. Criteria and prediction models for mismatch repair gene mutations: a review. J Med Genet. 2013;50(12):785-93.
244. Leeflang MM, Bossuyt PM, Irwig L. Diagnostic test accuracy may vary with prevalence: implications for evidence-based diagnosis. J Clin Epidemiol. 2009;62(1):5-12.
245. Whiting P, Rutjes AW, Reitsma JB, Glas AS, Bossuyt PM, Kleijnen J. Sources of variation and bias in studies of diagnostic accuracy: a systematic review. Ann Intern Med. 2004;140(3):189-202.
246. Hampel H. Point: justification for Lynch syndrome screening among all patients with newly diagnosed colorectal cancer. Journal of the National Comprehensive Cancer Network : JNCCN. 2010;8(5):597-601.

247. Hampel H, Stephens JA, Pukkala E, Sankila R, Aaltonen LA, Mecklin JP, *et al*. Cancer risk in hereditary nonpolyposis colorectal cancer syndrome: later age of onset. Gastroenterology. 2005;129(2):415-21.
248. Moreira L, Balaguer F, Lindor N, de la Chapelle A, Hampel H, Aaltonen LA, *et al*. Identification of Lynch syndrome among patients with colorectal cancer. JAMA. 2012;308(15):1555-65.
249. Lynch HT, Boland CR, Gong G, Shaw TG, Lynch PM, Fodde R, *et al*. Phenotypic and genotypic heterogeneity in the Lynch syndrome: diagnostic, surveillance and management implications. Eur J Hum Genet. 2006;14(4):390-402.
250. Lynch HT, Krush AJ. Cancer family "G" revisited: 1895-1970. Cancer. 1971;27(6):1505-11.
251. Vasen HF, Wijnen JT, Menko FH, Kleibeuker JH, Taal BG, Griffioen G, *et al*. Cancer risk in families with hereditary nonpolyposis colorectal cancer diagnosed by mutation analysis. Gastroenterology. 1996;110(4):1020-7.
252. Wagner A, van Kessel I, Kriege MG, Tops CM, Wijnen JT, Vasen HF, *et al*. Long term follow-up of HNPCC gene mutation carriers: compliance with screening and satisfaction with counseling and screening procedures. Fam Cancer. 2005;4(4):295-300.
253. Stoffel EM, Ford B, Mercado RC, Punglia D, Kohlmann W, Conrad P, *et al*. Sharing genetic test results in Lynch syndrome: communication with close and distant relatives. Clin Gastroenterol Hepatol. 2008;6(3):333-8.
254. Aktan-Collan KI, Kaariainen HA, Kolttola EM, Pylvanainen K, Jarvinen HJ, Haukkala AH, *et al*. Sharing genetic risk with next generation: mutation-positive parents' communication with their offspring in Lynch Syndrome. Fam Cancer. 2011;10(1):43-50.
255. Lerman C, Hughes C, Trock BJ, Myers RE, Main D, Bonney A, *et al*. Genetic testing in families with hereditary nonpolyposis colon cancer. Jama. 1999;281(17):1618-22.
256. Mesters I, Ausems M, Eichhorn S, Vasen H. Informing one's family about genetic testing for hereditary non-polyposis colorectal cancer (HNPCC): a retrospective exploratory study. Fam Cancer. 2005;4(2):163-7.
257. Aktan-Collan K, Kaariainen H, Jarvinen H, Peltomaki P, Pylvanainen K, Mecklin JP, *et al*. Psychosocial consequences of predictive genetic testing for Lynch syndrome and associations to surveillance behaviour in a 7-year follow-up study. Fam Cancer. 2013;12(4):639-46.
258. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. Acta Psychiatr Scand. 1983;67(6):361-70.
259. Lerman C, Trock B, Rimer BK, Boyce A, Jepson C, Engstrom PF. Psychological and behavioral implications of abnormal mammograms. Ann Intern Med. 1991;114(8):657-61.
260. Foster C, Evans DG, Eeles R, Eccles D, Ashley S, Brooks L, *et al*. Non-uptake of predictive genetic testing for BRCA1/2 among relatives of known carriers: attributes, cancer worry, and barriers to testing in a multicenter clinical cohort. Genet Test. 2004;8(1):23-9.
261. Riedijk SR, de Snoo FA, van Dijk S, Bergman W, van Haeringen A, Silberg S, *et al*. Hereditary melanoma and predictive genetic testing: why not? Psychooncology. 2005;14(9):738-45.
262. Spinhoven P, Ormel J, Sloekers PP, Kempen GI, Speckens AE, Van Hemert AM. A validation study of the Hospital Anxiety and Depression Scale (HADS) in different groups of Dutch subjects. Psychol Med. 1997;27(2):363-70.
263. Pentz RD, Peterson SK, Watts B, Vernon SW, Lynch PM, Koehly LM, *et al*. Hereditary nonpolyposis colorectal cancer family members' perceptions about the duty to inform and health professionals' role in disseminating genetic information. Genet Test. 2005;9(3):261-8.

264. Wakefield CE, Meiser B, Homewood J, Ward R, O'Donnell S, Kirk J, *et al*. Randomized trial of a decision aid for individuals considering genetic testing for hereditary nonpolyposis colorectal cancer risk. Cancer. 2008;113(5):956-65.
265. Esplen MJ, Madlensky L, Aronson M, Rothenmund H, Gallinger S, Butler K, *et al*. Colorectal cancer survivors undergoing genetic testing for hereditary non-polyposis colorectal cancer: motivational factors and psychosocial functioning. Clinical genetics. 2007;72(5):394-401.
266. Aktan-Collan K, Mecklin JP, Jarvinen H, Nystrom-Lahti M, Peltomaki P, Soderling I, *et al*. Predictive genetic testing for hereditary non-polyposis colorectal cancer: uptake and long-term satisfaction. Int J Cancer. 2000;89(1):44-50.
267. Leenen CH, Geurts-Giele WR, Dubbink HJ, Reddingius R, van den Ouweland AM, Tops CM, *et al*. Pitfalls in molecular analysis for mismatch repair deficiency in a family with biallelic pms2 germline mutations. Clin Genet. 2011;80(6):558-65.
268. Vasen HF, Ghorbanoghli Z, Bourdeaut F, Cabaret O, Caron O, Duval A, *et al*. Guidelines for surveillance of individuals with constitutional mismatch repair-deficiency proposed by the European Consortium "Care for CMMR-D" (C4CMMR-D). J Med Genet. 2014;51(5):283-93.
269. Geurts-Giele WR, Leenen CH, Dubbink HJ, Meijssen IC, Post E, Sleddens HF, *et al*. Somatic Aberrations Of Mismatch Repair Genes As A Cause Of Microsatellite-Instable Cancers. The Journal of pathology. 2014.
270. Giardiello FM, Allen JI, Axilbund JE, Boland CR, Burke CA, Burt RW, *et al*. Guidelines on genetic evaluation and management of Lynch syndrome: a consensus statement by the US Multi-Society Task Force on Colorectal Cancer. Diseases of the colon and rectum. 2014;57(8):1025-48.
271. Kwon JS, Scott JL, Gilks CB, Daniels MS, Sun CC, Lu KH. Testing women with endometrial cancer to detect Lynch syndrome. J Clin Oncol. 2011;29(16):2247-52.
272. Leenen CHM, Dubbink HJ, van Lier MGF, Hulspas S, Kuipers EJ, van Leerdam ME, *et al*. Challenges and Pitfalls in Screening for Lynch Syndrome by Molecular Tumor Tissue Analysis. Gastroenterology; May 2011 2011. p. Supplement 1, Pages S-352–S-3.
273. Metcalf AM, Spurdle AB. Endometrial tumour BRAF mutations and MLH1 promoter methylation as predictors of germline mismatch repair gene mutation status: a literature review. Fam Cancer. 2014;13(1):1-12.
274. Haraldsdottir S, Hampel H, Tomsic J, Frankel WL, Pearlman R, de la Chapelle A, *et al*. Colon and Endometrial Cancers with Mismatch Repair Deficiency can Arise from Somatic, Rather Than Germline, Mutations. Gastroenterology. 2014.
275. Sie AS, Brunner HG, Hoogerbrugge N. Easy-to-Use Decision Aids for Improved Cancer Family History Collection and Use Among Oncology Practices. J Clin Oncol. 2014;32(29):3343.
276. Dekker N, Hermens RP, Mensenkamp AR, van Zelst-Stams WA, Hoogerbrugge N. Easy-to-use online referral test detects most patients with a high familial risk of colorectal cancer. Colorectal disease : the official journal of the Association of Coloproctology of Great Britain and Ireland. 2014;16(1):O26-34.
277. Menko FH, Aalfs CM, Henneman L, Stol Y, Wijdenes M, Otten E, *et al*. Informing family members of individuals with Lynch syndrome: a guideline for clinical geneticists. Fam Cancer. 2013;12(2):319-24.

LIST OF CO-AUTHORS AND STUDY GROUPS

In alphabetical order

Affiliations at the time this research was conducted

Esther W. de Bekker-Grob
Department of Public Health, Erasmus University Medical Centre, Rotterdam, the Netherlands

Marco J. Bruno
Department of Gastroenterology & Hepatology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Winand N.M. Dinjens
Department of Pathology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Helena C. van Doorn
Department of Gynaecology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Hendrikus J. Dubbink
Department of Pathology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Willemina R.R. Geurts-Giele
Department of Pathology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Eelco J.R. de Graaf
Department of Surgery, IJsselland Ziekenhuis, Capelle a/d IJssel, the Netherlands

Mariska den Heijer
Departments Clinical Genetics and Medical Psychology and Psychotherapy, Erasmus University Medical Centre, Rotterdam, the Netherlands

Robert F. Hoedemaeker
Pathology Laboratory Pathan, Rotterdam, the Netherlands

Sanne M. Hulspas
Department of Pathology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Heleen M. van de Klift
Department of Human and Clinical Genetics, Leiden University Medical Center, Leiden, the Netherlands

Sjarlot G. Kooi
Department of Gynaecology, Albert Schweitzer Ziekenhuis, Dordrecht, the Netherlands

Ernst J. Kuipers
Departments of Gastroenterology & Hepatology and Internal Medicine, Erasmus University Medical Centre, Rotterdam, the Netherlands

Monique E. van Leerdam
Department of Gastroenterology & Hepatology, Erasmus MC University Medical Centre, Rotterdam, and National Cancer Institute, Amsterdam, the Netherlands

Margot G.F. van Lier
Department of Gastroenterology & Hepatology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Conny A. van der Meer
Department of Clinical Genetics, Family Cancer Clinic, Erasmus University Medical Centre, Rotterdam, the Netherlands

Isabelle C. Meijssen
Department of Pathology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Ans M.W. van den Ouwenland
Department of Clinical Genetics, Erasmus University Medical Centre, Rotterdam, the Netherlands

Edward Post
Department of Pathology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Dewkoemar Ramsoekh
Department of Gastroenterology & Hepatology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Roel E. Reddingius
Department of Paediatrics, Erasmus University Medical Centre, Rotterdam, the Netherlands

Hein F.B.M. Sleddens
Department of Pathology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Manon C. Spaander
Department of Gastroenterology & Hepatology, Erasmus University Medical Centre, Rotterdam, the Netherlands

Ewout W. Steyerberg
Department of Public Health, Erasmus University Medical Centre, Rotterdam, the Netherlands

Carli M.J. Tops
Department of Human and Clinical Genetics, Leiden University Medical Center, Leiden, the Netherlands

Wietske W. Vrijland
Department of Surgery, Sint Franciscus Gasthuis, Rotterdam, the Netherlands

Judith de Waard
Department of Gynaecology, Sint Franciscus Gasthuis, Rotterdam, the Netherlands

Anja Wagner
Department of Clinical Genetics, Family Cancer Clinic, Erasmus University Medical Centre, Rotterdam, the Netherlands

Pieter J. Westenend
Department of Pathology, Albert Schweitzer Ziekenhuis, Dordrecht, the Netherlands

Leonieke M.M. Wolters
Department of Gastroenterology and Hepatology, Albert Schweitzer Ziekenhuis, Dordrecht, the Netherlands

THE LYNCH SYNDROME PREDICTION MODEL VALIDATION STUDY GROUP

Carmelita Alvero
Statistical and Data Analysis Center, Harvard School Public Health, Boston, Massachusetts, USA

Francesc Balaguer
Department of Gastroenterology, University of Barcelona, Barcelona, Spain

Judith Balmana
Department of Oncology, University Hospital Vall d'Hebrón, Barcelona, Spain

Lucio Bertario
Unit of Hereditary Digestive Tract Tumors, Department of Preventive and Predictive Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

Daniel Buchanan
Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, University of Melbourne, Parkville, Victoria, Australia

Roger Green
Discipline of Genetics, Faculty of Medicine, Memorial University of Newfoundland, St John's, NL, Canada

Heather Hampel
Clinical Cancer Genetics Program, Ohio State University Comprehensive Cancer Center, Columbus, Ohio

Mark Jenkins
Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, University of Melbourne, Parkville, Victoria, Australia

Fay Kastrinos
Herbert Irving Comprehensive Cancer Center, Columbia University, Medical Center, New York, New York, USA
Division of Digestive and Liver Diseases, Columbia University Medical Center, New York, New York, USA

Aung Ko Win
Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, University of Melbourne, Parkville, Victoria, Australia

Noralane M. Lindor
Department of Health Sciences Research, Mayo Clinic, Scottsdale, Arizona, USA

Rowena C. Mercado
Population Sciences Division, Dana-Farber Cancer Institute, Boston, Massachusetts, USA

Polly Newcomb
Epidemiology Department, University of Washington; Public Health Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA

Rohit P. Ojha
Department of Epidemiology and Cancer Control, St. Jude Children's Research Hospital, Memphis, Tennessee, USA

Paola Sala
Unit of Hereditary Digestive Tract Tumors, Department of Preventive and Predictive Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

Sapna Syngal
Division of Gastroenterology, Brigham and Women's Hospital, Boston, Massachusetts, Harvard Medical School, Boston, Massachusetts, USA

Stephen N. Thibodeau
Division of Molecular Genetics, Mayo Clinic, Rochester, Minnesota, USA

Irene Valenzuela
Genetics Department, University Hospital Vall d'Hebrón, Barcelona, Spain

THE LIMO COLORECTAL AND ENDOMETRIAL CANCER STUDY GROUP

Erasmus MC, Rotterdam, the Netherlands

Department of Clinical Genetics	Dr. A.M.W. van den Ouwenland
	Dr. A. Wagner
Department of Gastroenterology and Hepatology	Prof.dr. E.J. Kuipers
	Drs. C.H.M. Leenen
	Dr. M.E. van Leerdam
	Dr. M.G.F. van Lier
	Dr. D. Ramsoekh
Department of Gynaecology	Dr. H.C. van Doorn
Department of Pathology	Dr. H.J. Dubbink
	Dr. W.N.M. Dinjens
	Drs. S. Hulspas
	Drs. G. van Tilburg
Department of Surgery	Prof.dr. J.F. Lange
Department of Public Health	Prof.dr. E.W. Steyerberg

Pathan, Rotterdam, the Netherlands

Department of Pathology	Dr. R.F. Hoedemaeker

IJsselland Ziekenhuis, Capelle a/d IJssel, the Netherlands

Department of Gastroenterology and Hepatology	Dr. W.A. Bode
Department of Gynaecology	Drs. P.A.A van Hagen
Department of Surgery	Dr. E.J.R. de Graaf

Havenziekenhuis, Rotterdam, the Netherlands

Department of Gynaecology	Dr. L. Pijpers
Department of Internal Medicine	Dr. P.J. Wismans
Department of Surgery	Dr. J.H. van Dam

Ruwaard van Putten Ziekenhuis, Spijkenisse, the Netherlands

Department of Gynaecology	Dr. P.B von Tongelen
Department of Internal Medicine	Dr. E.J.H.M. van de Weijgert
Department of Surgery	Dr. R. den Toom

Sint Franciscus Gasthuis, Rotterdam, the Netherlands

Department of Gastroenterology and Hepatology	Dr. A.J.P. van Tilburg
Department of Gynaecology	Drs. J. de Waard
Department of Surgery	Dr. W.W. Vrijland

Vlietland Ziekenhuis, Schiedam, the Netherlands

Department of Gastroenterology and Hepatology	Drs. B.W.M. van 't Hoff
Department of Gynaecology	Drs. A.M.G. Huijssoon
Department of Surgery	Drs. H.E. Lont

Albert Schweitzer Ziekenhuis, Dordrecht, the Netherlands

Department of Gastroenterology and Hepatology	Dr. L.M.M. Wolters
Department of Gynaecology	Dr. S.G. Kooi
Department of Pathology	Dr. P.J. Westenend
Department of Surgery	Dr. R.J. Oostenbroek

Beatrixziekenhuis, Gorinchem, the Netherlands

Department of Gastroenterology and Hepatology	Dr. O. van Baalen
Department of Surgery	Dr. F. Logeman

Maasstad ziekenhuis Rotterdam, the Netherlands

Department of Gastroenterology and Hepatology	Dr. F.J.G.M. Kubben
Department of Pathology	Dr. H.F.G.M. van den Ingh
Department of Surgery	Dr. E. van der Harst

Ikazia Ziekenhuis, Rotterdam, the Netherlands

Department of Gastroenterology and Hepatology	Dr. R.J.Th. Ouwendijk
Department of Surgery	Dr. W.F. Weidema

Deventer Ziekenhuis, Deventer, the Netherlands

Department of Gastroenterology and Hepatology	Dr. F. ter Borg
Department of Gynaecology	Dr. A. Bouman
Department of Pathology	Drs. R.H. van Rijssel
Department of Surgery	Dr. E.H. Eddes

PUBLICATIONS

Leenen CHM,* Goverde A,*de Bekker-Grob EW, Wagner A, van Lier MGF, Spaander MC, Bruno MJ, Tops C, van den Ouweland AMW, Dubbink HJ, Kuipers EJ, Dinjens WNM, van Leerdam ME, Steyerberg EW. Age-targeted Lynch syndrome screening in colorectal cancer patients: cost-effectiveness in a population-based setting. *equal contribution. *Submitted for publication*

Kastrinos F, Ojha RP, **Leenen CHM**, Alvero C, Mercado RC, Balmana J, Valenzuela I, Balaguer F, Green R, Lindor NM, Thibodeau SN, Newcomb P, Ko Win A, Jenkins M, Buchanan D, Bertario L, Sala P, Hampel H, Syngal S, Steyerberg EW; for the Lynch syndrome prediction model validation study group. Identification of mismatch repair gene mutations among colorectal cancer patients: an international validation study of prediction models for Lynch syndrome. *Submitted for publication*

Leenen CHM, den Heijer M, van der Meer, C, Kuipers EJ, van Leerdam ME, Wagner A. Genetic testing for Lynch syndrome: family communication and motivation. *Submitted for publication*

Geurts-Giele WRR,* **Leenen CHM**,* Dubbink HJ, Meijssen IC, Post E, Sleddens HFBM, Kuipers EJ, Goverde A, van den Ouweland AMW, van Lier MGF, Steyerberg EW, van Leerdam ME, Wagner A, Dinjens WNM. Somatic aberrations of mismatch repair genes as a cause of microsatellite-unstable cancers. *equal contribution. *The Journal of Pathology 2014;234(4):548-59*

Leenen CHM, van Lier MGF, van Doorn HC, van Leerdam ME, Kooi SG, de Waard J, Hoedemaeker RF, van den Ouweland AMW, Hulspas SM, Dubbink HJ, Kuipers EJ, Wagner A, Dinjens WNM,* Steyerberg EW;* on behalf of the LIMO study group. Prospective evaluation of molecular screening for Lynch syndrome in patients with endometrial cancer ≤ 70 years. *equal contribution. *Gynecologic Oncology 2012;125(2):414-20*

van Lier MGF, **Leenen CHM**, Wagner A, Ramsoekh D, Dubbink HJ, van den Ouweland AMW, Westenend PJ, de Graaf EJR, Wolters LMM, Vrijland WW, Kuipers EJ, van Leerdam ME, Steyerberg EW,* Dinjens WNM;* on behalf of the LIMO study group. *equal contribution. Yield of routine molecular analyses in colorectal cancer patients ≤ 70 years to detect underlying Lynch syndrome. *The Journal of Pathology 2012;226(5):764-74*

Leenen CHM, Geurts-Giele WRR, Dubbink HJ, Reddingius RE, van den Ouweland AMW, Tops CMJ, van de Klift HM, Kuipers EJ, van Leerdam ME, Dinjens WNM, Wagner A. Pitfalls in molecular analysis for mismatch repair deficiency in a family with biallelic *PMS2* Germline. *Clinical Genetics 2011;80(6):558-65*

NOT RELATED TO THIS THESIS

Leenen CHM, Dieleman LA. Inulin and Oligofructose in chronic inflammatory bowel disease. *Journal of Nutrition 2007;137(11):2572S-2575S*

DANKWOORD

Tijdens mijn onderzoeksperiode heb ik de kans gekregen om een groot aantal onderzoekers in het Erasmus MC en daar buiten te leren kennen en van hen te leren. Dit proefschrift is het eindproduct, dat dankzij hun hulp en inspiratie tot stand gekomen is.

Meer dan 1500 patiënten hebben hun familiegegevens en weefsels ter beschikking gesteld voor het onderzoek, dat tot dit proefschrift heeft geleid. Ik ben hen zeer dankbaar hiervoor. Uiteraard gaat mijn dank ook uit naar de LIMO en RIGHT onderzoeksgroepen met alle co-auteurs.

Mijn promotoren dank ik voor hun begeleiding:
Prof.dr. E.J. Kuipers, beste Ernst het is mei 2010 tijdens een tussenstop in Detroit. Ik ging voor het eerst mee naar de *Digestive Disease Week*, gewapend met een goed gevulde posterkoker en mijn rugzakje. We zaten toevallig in hetzelfde vliegtuig en jij nam me tijdens een overstap mee naar de VIP lounge. Je laadde mijn bordje vol met brownies en cookies. Dit zie ik nu als een metafoor voor de hele periode: je zorgde dat het aan niets ontbrak. Een goede onderzoekslijn, experts, veel onderzoeksgegevens; alle ingrediënten waren aanwezig. En niet te vergeten kreeg ieder manuscript nog een finishing touch van jouw hand: veel dank dat ik bij jou mag promoveren.

Prof.dr. E.W. Steyerberg, beste Ewout dank voor alle positieve inbreng, onderzoeksideeën en vooral voor je onuitputtelijke energie om hoofdstuk 6 tot een goed einde te brengen. Jij liet mij meekijken in het internationale Lynch syndroom predictie onderzoeksteam, wat ik als zeer interessant heb ervaren. Dank ook voor de snelle feedback altijd. Zoals het recente mailtje met feedback over de kostenstudie, die jij tussen een kudde Afrikaanse olifanten verzond. Het maakt dat ik geen enkel overlegmoment ben vergeten en af en toe heel erg heb kunnen lachen.

Ook mijn co-promotoren dank ik voor hun begeleiding:
Dr. A. Wagner, beste Anja dank voor het gebruik maken van het door jou prachtig opgezette cohort met Lynch syndroom families. Jouw toewijding aan de zorg voor patiënten vind ik fantastisch en uiterst leerzaam. Dr. M.E. van Leerdam, beste Monique dank voor je begeleiding. Als ik af en toe door de bomen het bos niet meer zag, wist je me meestal weer ergens heen te leiden, veel dank daarvoor. Zonder jouw input en betrokkenheid was het nooit gelukt! Dr. W.N.M. Dinjens, beste Winand, jouw bruisende onderzoeksplannen zijn fantastisch. Dank nogmaals voor het interessante afscheidskado dat ik van je kreeg: het boek 'Ons feilbare denken', waar ik ook veel van heb geleerd.

Mijn dank gaat tevens uit naar de andere commissieleden voor de beoordeling van mijn proefschrift en het plaatsnemen in de promotiecommissie.

In aansluiting hierop dank ik de gehele RIGHT onderzoeksgroep. In het bijzonder, Erik-Jan Dubbink voor alle hulp en vooral bereidheid om altijd mijn talloze vragen te beantwoorden. Ook het complete analistenteam ben ik zeer dankbaar voor al hun werk. Margot van Lier en Dewkoemar Ramsoekh, met zo'n goede voorgangers was het succes verzekerd, dank dat jullie er waren. Het stokje (lees: cohorten met duizenden personen) is inmiddels overgedragen aan Anne Goverde. Dank voor jouw hulp bij de kostenstudie, zonder jou was het nooit op tijd afgekomen. Ina Geurts, dank ik voor alle moleculaire data, en uitleg hierbij. Mariska den Heijer, ben ik zeer dankbaar voor de samenwerking bij de vragenlijststudie, op naar afronding en publicatie! Lena van Doorn dank ik voor alle gynaecologische input voor de LIMO studie. Alle verzamelde data werden zorgvuldig verwerkt door het datateam van de afdeling klinische genetica, onder aanvoering van Anja Nieuwlaat. Zonder hen was er geen onderzoek geweest, ik ben jullie ontzettend dankbaar.

Naast mijn onderzoek, werkte ik mee aan Parelsnoer (PSI biobanken), wat mij een interessante inkijk gaf in biobanking voor gevorderden. Ik dank met name Paula Jansen voor alle hulp die zij mij hierbij bood.

Als een ware werkplek-nomade ben ik door het Erasmus MC getrokken, in volgorde: 20^{e} verdieping (afdeling Klinische Genetica), afdalend naar verdieping 4: laboratorium MDL, vervolgens laboratorium dependance (voor en achter de vriezer), en toen die vriezer terrein won: heel even in het laboratorium van de moleculaire diagnostiek, tot ik het laatste jaar een vaste stek had gevonden met Florine Kastelein, Aria Sana en Lauran Vogelaar op de kamer, met regelmatig bezoek van Renate Massl. Ik denk dat geen enkel onderwerp onbesproken is gebleven in onze werkkamer. Ook al is onze plek inmiddels letterlijk met de grond gelijk gemaakt, onze herinneringen en mooie momenten, die we samen hebben gedeeld, blijven voor altijd. Ik schrijf dit dan ook met een hele brede glimlach. Uiteraard dank ik alle collega's van de afdeling MDL en Klinische Genetica voor de gezelligheid in en buiten het Erasmus MC.

Wendy Holleman, Bernadette Lourens dank ik voor alle ondersteuning en Wendy speciaal voor de gezellige samenwerking bij het organiseren van de MDL dag.

Graag wil ik op deze plaats de mensen bedanken die mijn eerste stappen op het wetenschappelijke pad hebben begeleid en gestimuleerd**:** prof. dr. Chris Mulder en prof.dr. Leo Dieleman. Lieve Chris en lieve Leo, ik denk nog altijd met veel blijdschap terug aan mijn

Canada avontuur wat door jullie beiden mogelijk is gemaakt en hoop nog lang contact te houden!

Inmiddels ben ik alweer enkele jaren werkzaam in het Sint Lucas Andreas Ziekenhuis. Ik wil mijn opleider dr. Carl Siegert, speciaal bedanken. Ik geloof dat ik onder jouw vleugels de eerste tekenen van ware volwassenheid heb laten zien en ben je zeer dankbaar voor je begeleiding daarbij. Dr. Jan Veenstra en dr. Janneke Langendonk (Erasmus MC) dank ik voor ons porfyrie avontuur. Ik hoop er nu snel een mooi artikel van te maken. Tot slot dank ik alle collega's voor de gezellige dagelijkse samenwerking.

Mijn paranimfen: Lidewij van de Mheen en Roosmarijn Luttmer, beiden gynaecoloog in spé, maar vooral vriendinnen en geneeskundemaatjes vanaf het eerste uur: dank voor jullie interesse en vriendschap! Lieve Lidewij, dat jij van promoveren je hobby hebt gemaakt, laat wel zien hoe gedreven jij bent. Ik vind het een grote eer om in maart tijdens jouw promotie aan je zijde te staan. Ik heb daar nu al veel zin in. Lieve Roosmarijn, onlangs ben je de dag voor je zwangerschapsverlof in het vliegtuig naar de USA gesprongen om jouw onderzoek te presenteren. Een van de voorbeelden van jouw grensoverschrijdende dapperheid. Ik vind het een heel geruststellend idee dat jullie tijdens de promotie naast me staan.

Veel mensen die niet direct bij het onderzoek en werk betrokken zijn geweest, maar wel in mijn leven een belangrijke rol spelen wil ik hier ook noemen:

Dank lieve vrienden uit Abcoude, van het Ig, de VU en de rest van Amsterdam (en ook een klein stukje daarbuiten) voor alle feestelijke en mooie momenten, die we afgelopen jaren gedeeld hebben. Lieve RACcers, clubgenootjes, wij delen samen iets heel bijzonders. Dankzij onze projecten afgelopen jaren bleef ik oog houden voor de wereld direct om ons heen. Van tandemfietsen met slechtzienden, bingoën met oma's en koken voor Amsterdamse daklozen, we delen vele bijzondere ervaringen.

"Geneet van 't laeve zolang este kins"…zong mijn lieve oma vaker. Helaas is ze er vandaag niet meer bij. Gelukkig heeft ze mij vele wijsheden en liedjes nagelaten, waar ik altijd en vandaag in het bijzonder aan zal blijven denken. Ook dank ik mijn oom Frans. Je bent nu zelf een moedige strijder geworden als velen in dit proefschrift. Ik hoop dat je nog lang bij ons blijft.
Lieve oom Len, oftewel emeritus hoogleraar Len de Klerk, ruim 30 jaar geleden stond jij aan de zijde van wijlen mijn vader, als zijn paranimf. Het betekent veel voor mij dat je vandaag ook hier aanwezig bent samen met tante Nellie. Veel dank voor al jullie support.

Familie van Wijgerden, Vincents familie: Magda en Dick, Ymke en Coen en opa Albert… van Oegstgeest tot aan Flims en van Rijswijk tot in Cannero, mag ik altijd gezellig bij jullie aanschuiven, veel dank daarvoor.

"Je bereikt alleen het doel, als je weet waar je naartoe wilt." Lieve mamma, net als wijlen mijn vader heb ik mijn promotie afgerond. Ook ik had dit niet zonder jou gekund! Het was jij, die me liet inzien, toen ik dertien jaar oud was, dat je binnen twee weken met alleen een tent en een paar pannetjes in de fietstas best naar Zuid-Frankrijk kunt fietsen. Af en toe een hobbel of een fikse klim onderweg, en een enkele keer geen eten of ijskoude tenen boven op de bergpas, maar aankomen zul je altijd. Mijn dankbaarheid voor jou is niet te beschrijven zo groot.

Tot slot, lieve Vincent, met jouw eigen motto: "*gebakken aardappels met een stukje vlees kunnen we nog ons hele leven eten"*, ontstaan vele (kook) avonturen aan de Uiterwaardenstraat en hebben we samen ontzettend veel plezier. Ik heb jou het allerliefste om mij heen!

Amsterdam, november 2014
Celine

ABOUT THE AUTHOR

Celine Leenen was born in Amsterdam on October 3, 1984. After she received her diploma at the St. Ignatiusgymnasium Amsterdam in 2002, she was selected for medical school at the VU University Medical Centre, Amsterdam. In 2006, as part of her graduate program in Immunology, she started a research internship of six months into the effect of prebiotics in *HLA-B27* transgenic rats under supervision of prof.dr. L.A. Dieleman at the University of Alberta in Edmonton, Canada. In 2007 she was awarded the third prize at the VU University Medical Center Student Research Prize. During her clinical rotations, she participated in the elective program for European interns for two months at the Department of Internal Medicine and Gastroenterology at the University Hospital of Wales in Cardiff under the supervision of dr. G.A. Thomas. After completing her last clinical rotation at the department of Internal Medicine at the Sint Lucas Andreas Hospital, she received her medical degree in 2009. Subsequently, she started her PhD research at the Erasmus University Medical Center under the guidance of prof.dr. E.J. Kuipers, prof.dr. E. W. Steyerberg and co-supervisors dr. M. E. van Leerdam, dr. A. Wagner and dr. W.N.M. Dinjens. Since 2012 she is in training to become an internist via the VU University Medical Center (program director: prof.dr. Y.M. Smulders). At the moment she does the first part of her specialty training at the Sint Lucas Andreas Hospital in Amsterdam (program director: dr. C.E.H. Siegert). Celine lives with Vincent van Wijgerden in the Amsterdam Rivierenbuurt.

PHD PORTFOLIO SUMMARY

Summary of PhD training and teaching activities

Courses

Molecular Medicine Erasmus Postgraduate School, Rotterdam, the Netherlands

2011 Presenting skills for scientists
2010 Research time management
2009 Basic introduction course on SPSS
2009 SNPs and Human Diseases
2009 Biomedical research Techniques VIII

Netherlands Institute for Health Sciences, Rotterdam, the Netherlands

2011 Biostatistics for Clinicians
2010 Regression Analysis for Clinicians
2010 Courses for the Quantitative Researcher
2009 Principles of Research in Medicine and Epidemiology

Erasmus University Medical Centre, Rotterdam, the Netherlands

2010 Biomedical English Writing and Communication

Seminars and workshops

2010 Moleculaire diagnostiek voor dokters
Molecular Medicine Erasmus Postgraduate School, Rotterdam, the Netherlands

2010 Minicursus Methodologie van Patiëntgebonden Onderzoek en Voorbereiding van Subsidieaanvragen
Erasmus MC, Rotterdam, the Netherlands

Oral presentations at (inter)national conferences

2012 Barriers to genetic testing for Lynch syndrome
Dutch Society of Gastroenterology, Veldhoven, the Netherlands

2012 Patients with Lynch-compatible colorectal cancer without identifiable germline mutation do not have a positive family history of Lynch syndrome
Dutch Society of Gastroenterology, Veldhoven, the Netherlands

2011 Improving Lynch syndrome diagnostics by multiplex SNaPshot assays for the detection of mismatch repair gene LOH in MSI-H tumors
Digestive Disease Week, Chicago, USA

2010 Routine MSI-analysis in advanced adenomas in patients younger than 45 years leads to the identification of more patients at high risk for Lynch syndrome
Dutch Society of Gastroenterology, Veldhoven, the Netherlands

Video abstract at international conference

2011 Challenges and pitfalls in screening for Lynch syndrome by molecular tumor tissue analysis.
Digestive Disease Week, Chicago, USA
link: http://youtu.be/jF10Z9VHGuM

Poster presentations at (inter)national conferences

2013 Screening for somatic mismatch repair gene aberrations improves the molecular diagnostics of patients suspected for Lynch syndrome
Nederlandse vereniging voor Humane Genetica symposium, the Netherlands

2012 Patients with Lynch-compatible colorectal cancer without identifiable germline mutation do not have a positive family history of Lynch syndrome
Digestive Disease Week, San Diego, CA, USA

2012 Barriers to genetic testing for Lynch syndrome
Digestive Disease Week, San Diego, CA, USA

2011 Improving Lynch syndrome diagnostics by multiplex SNaPshot assays for the detection of mismatch repair gene LOH in MSI-H tumors
Dutch Experimental Gastroenterology and Hepatology Meeting, Veldhoven, the Netherlands

2011 Challenges and pitfalls in screening for Lynch syndrome by molecular tumor tissue analysis
Digestive Disease Week, Chicago, USA
Poster of distinction

2011 Family communication in Lynch cyndrome families: experiences with the family-linked approach
Digestive Disease Week, Chicago, USA
4th Biennial Meeting International Society for Gastrointestinal Hereditary Tumours (InSiGHT), San Antonio, Texas, USA

2010 Routine MSI-analysis in advanced adenomas in patients younger than 45 years leads to the identification of more patients at high risk for Lynch syndrome
Digestive Disease Week, New Orleans, USA
Poster of distinction

2010 Routine MSI-analysis in endometrial cancer ≤ 70 years increases identification of patients at risk for Lynch syndrome
Digestive Disease Week, New Orleans, USA

2010 Biallelic *PMS2* germline mutations in a family with a microsatellite-stable brain tumor and early onset colorectal cancer
Digestive Disease Week, New Orleans, USA

Grants

2011 Travelgrant Stichting Simonsfonds (€800)

Memberships

Molecular Medicine Postgraduate School Erasmus MC (MolMed)
Dutch Society of Gastroenterology and Experimental Gastroenterology and Hepatology (NVGE)
Netherlands Association of Internal Medicine (NIV)

Tutoring

2010 Student biotechnology S. Gupta, Indian Institute of Technology, Kharagpur
Co-supervision internship on data management and predictive models for Lynch syndrome

Peer review activities

2014 Gynaecologic oncology
Cancer Biology and Medicine

Others

2014 Improving Lynch syndrome diagnostics
Lecture at 'de Industrieele Groote Club', Rotary club Amsterdam-West, the Netherlands

2012 Prospective evaluation of molecular screening for Lynch in patients with endometrial cancer ≤ 70 years
Integraal Kankercentrum Rotterdam, regional meeting workgroup gynecologic tumors, Rotterdam, the Netherlands

2011 Presentation Lynch syndrome research
'Parelsnoer symposium', Radboud University Medical Center, Nijmegen, the Netherlands

2010 Presentation at the Lynch syndrome information day for Lynch syndrome families
Erasmus University Medical Centre, Rotterdam, the Netherlands