



Prospective evaluation of molecular screening for Lynch syndrome in patients with endometrial cancer ≤ 70 years ^{☆, ☆, ☆, ☆}

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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 tumour tissue of all newly diagnosed EC patients ≤ 70 years.

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

Results. Tumour specimens of 179 patients (median age 61 years, IQR 57–66) were analysed. 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 tumours with *MLH1* promoter hypermethylation (17%; 95% CI 13–24%) were identified.

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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.

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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 [1–4].

LS is caused by a mutation in one of the mismatch repair (MMR) genes; *MLH1*, *MSH2*, *MSH6* or *PMS2*. As a consequence, LS tumours are MMR deficient and phenotypically characterised by DNA microsatellite instability (MSI). MSI can be detected in more than 90% of all ECs in LS mutation carriers [5]. Therefore, MSI analysis can be used in the diagnostic approach of LS [6]. However, MSI can also be detected in 17 to 23% of sporadic ECs [7,8], which is mostly caused by transcriptional silencing of the *MLH1* gene by promoter hypermethylation [9,10].

In addition to MSI analysis, immunohistochemical (IHC) analyses can be performed to evaluate the expression of the four MMR proteins. Tumours showing absent MLH1 expression can be selected for *MLH1* promoter methylation assay to identify sporadic MSI-H tumours. In case of MSI and loss of MMR protein expression, with exclusion of *MLH1*-promoter hypermethylation in *MLH1* negative tumours, 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 [11–13]. Selection of patients for molecular testing for LS is currently based on clinical criteria, in particular the Amsterdam II criteria and the revised Bethesda guidelines [14,15]. In the Amsterdam II criteria EC is included as a diagnostic parameter. However, the Amsterdam II criteria lack sensitivity, particularly in cases of small families or when extensive family history information is not available [16,17]. The revised Bethesda criteria 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 multicentre 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 centre, between May 2007 and September 2009. Patients were identified by monthly electronic searches in the pathology databases of the participating centres. Data were collected on age at diagnosis and tumour characteristics including histological subtype and pathological tumour (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 tumour, were referred to the department of clinical genetics for counselling and germline mutation analysis.

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

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

Analysis for MSI

Analysis for MSI was performed on FFPE tumour 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 [18]. 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. Tumours with more than one unstable marker were categorised as having a high degree of microsatellite instability (MSI-H). Tumours with one or no unstable markers were categorised as being microsatellite stable (MSS).

Immunohistochemistry, *MLH1* promoter hypermethylation assay, and *BRAF*-mutation analysis

IHC analysis was performed for four mismatch repair proteins: MLH1, MSH2, MSH6 and PMS2, according to the standard procedure [18]. If there was no MLH1 expression in tumour 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 [18]. In all MSI-H tumour 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

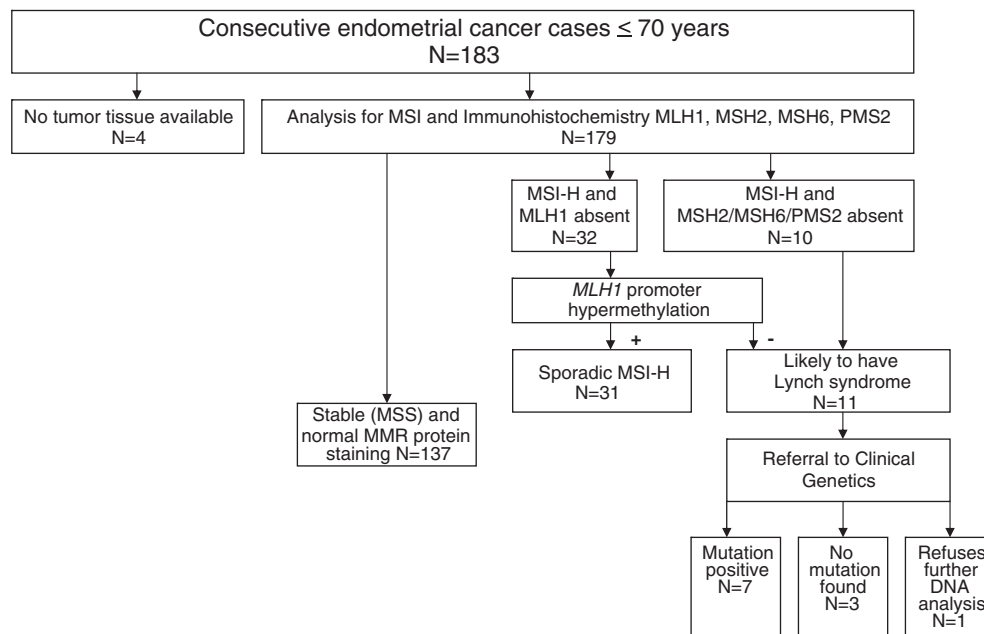


Fig. 1. Flow diagram of the diagnostic strategy and results of molecular analyses. MSI = microsatellite instability, MSI-H = MSI-high, IHC = immunohistochemistry.

performed by bi-directional cycle-sequencing of PCR-amplified fragments, using a previously described method [18].

Germline mutation analysis

Analyses of MSI in combination with IHC revealed patients likely to have LS, i.e. those with an MSI-H tumour 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 [19].

Statistical analysis

Data were analysed 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 analysed. 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 (Fig. 1). In four cases no tumour 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

tumour tissues were found to be grade 1 (73 patients, 41%). Histology showed tumour confined to the uterus in 126 tumour specimens (70%, i.e. T1) (Table 1).

Molecular analyses

Overall, 137 tumours were found to be MSS and 42 tumours 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 tumours. Thirty-one tissues were methylated at all 5*hMLH1* sites and were concluded to be sporadic MSI-H tumours (17%; 95% CI 13–24%). In eleven MSI-H tumours 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 tumour tissues.

BRAF mutation analysis was performed in all 42 MSI-H tumours, but no *BRAF* mutations were detected. No difference in age at EC diagnosis between patients with an MSS tumour and patients with a sporadic MSI-H tumour 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 tumours (*p*=0.19) or patients with MSS tumours (*p*=0.46). The correlation between age and the results of the molecular analyses is shown in Fig. 2. Comparing tumour grade and pathological tumour (T) stage there were also no differences between patients likely to have LS and patients with sporadic MSI-H tumours (*p*=0.25; *p*=0.44). Furthermore, no differences in age and pathological tumour (T) stage at EC diagnosis, between patients likely to have LS and patients with MSS tumours were observed

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%)
Tumour 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 tumour (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.

($p = 0.46$; $p = 0.20$; $p = 0.10$). However tumour tissues of patients likely to have LS were of higher tumour grade than MSS tumour 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 counselling and germline mutation analysis (Table 2). All eleven patients 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 tumour 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 fulfil the Amsterdam II criteria. Three patients (27%) did fulfil the revised Bethesda guidelines (Table 2).

Discussion

In this prospective multicentre 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 programmes which have been proven to reduce CRC morbidity and mortality by 65–70% [11–13]. For female LS carriers gynaecologic surveillance programmes, 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 [20]. Instead of surveillance, prophylactic surgery can be considered after childbearing has been completed, as this may prevent endometrial and ovarian carcinoma effectively [21].

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

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

rBC = revised Bethesda Guidelines, AC = Amsterdam Criteria II, MSI = Analysis of microsatellite instability, IHC = Immunohistochemistry,

NA = not available, ND = not done.

^a Additional analysis showed absence of *MLH1* promoter hypermethylation.

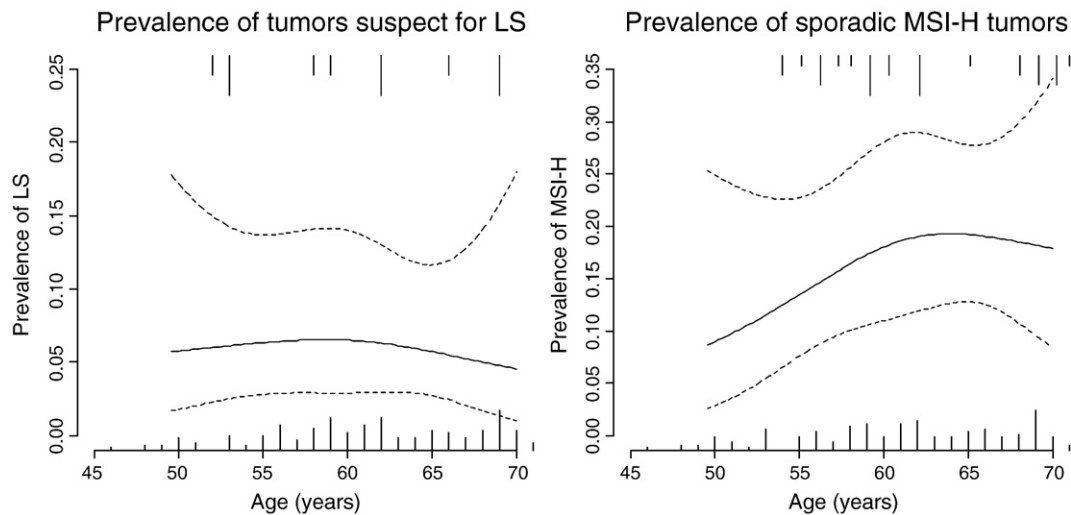


Fig. 2. Correlation between age and the results of the molecular analyses. Spline functions with 95% confidence interval. Spikes indicate the ages of individual patients with and without the outcome of interest. *Left panel:* Prevalence of tumours likely caused by LS according to age; the chance to detect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

In our study we performed both MSI and IHC for four MMR proteins in all 179 tumours 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 tumours. 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%) [22].

Additionally to MSI and IHC analyses we performed *MLH1* promoter methylation assay (MS-MLPA) in MSI-H tumours with absent *MLH1* expression, revealing a sporadic MSI-H status in 17% of all analysed ECs. This finding is in concordance with findings in previous studies [9,10,23]. The establishment of a sporadic MSI-H status considerably reduced the number of patients referred for counselling 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 [23]. 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 tumours 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 [24,25]. Furthermore in a recent study with MSI-H EC cases, no *BRAF* mutations were detected [26]. However, numbers of investigated ECs were too small to make a statement with regard to the presence of *BRAF* mutations in MSI-H tumours, 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 endometrial cancers. *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 [27]. However this study may have been biased by the fact that almost half of the eligible patients declined molecular screening of their tumour 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 tumour 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 [28]. 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 tumour analysis for LS [29]. All eleven patients likely to have LS in the present study were referred for genetic counselling. 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 counselling and testing.

Our current study, as well as previous studies [27,28,30–34], 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 advise EC patients on molecular analysis for LS [35,36]. However, these guidelines include only EC patients diagnosed under the age of 50 years or patients with two or more LS associated tumours. 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 [23,27,31], 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 tumour tissue of EC patients until 60 years of age [28]. 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 tumour 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 [37].

A limitation of our study is the lack of data on family history in women not referred for genetic counselling. Therefore we can not compare our strategy to the Amsterdam II criteria, the Bethesda guidelines, or predictive models for LS [14,38]. However, 82% of patients likely to have LS and referred for counselling did not fulfil the Amsterdam II criteria and 73% did not fulfil 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 fulfil Amsterdam II criteria or the Bethesda guidelines [27]. 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 [38].

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 [39]. In this study a panel of five mononucleotide markers was used to detect MSI. In case of an MSI-H tumour with absent MLH1 protein expression and hypermethylation of the *MLH1*-promoter we concluded this tumour to be sporadic MSI-H. This could have led 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 tumours [9,10,40].

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 counselling and surveillance programmes for LS mutation carriers and their relatives.

Conflicts of interest

- None of the authors have conflicts of interest.
- None of the material in the manuscript is included in another manuscript, has been published previously, or is currently under consideration for publication elsewhere.
- Only people who contributed to the intellectual content, the analysis of data, and the writing of the manuscript are listed as authors and all authors take public responsibility for the research results being reported.
- Ethical guidelines were followed and the approval of the institutional review board has been cited in the Methods section of the text.
- All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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