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HNPCC, molecular and clinical dilemmas

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CLINICAL–ALIMENTARY TRACT

Cancer Risk in Hereditary Nonpolyposis Colorectal Cancer Due to *MSH6* Mutations: Impact on Counseling and Surveillance

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See editorial on page 334.

Background & Aims: Hereditary nonpolyposis colorectal carcinoma (HNPCC) is caused by a mutated mismatch repair (MMR) gene. The aim of our study was to determine the cumulative risk of developing cancer in a large series of *MSH6* mutation carriers. *Methods:* Mutation analysis was performed in 20 families with a germline mutation in *MSH6*. We compared the cancer risks between *MSH6* and *MLH1/MSH2* mutation carriers. Microsatellite instability (MSI) analysis and immunohistochemistry (IHC) were performed in the available tumors. *Results:* A total of 146 *MSH6* mutation carriers were identified. In these carriers, the cumulative risk for colorectal carcinoma was 69% for men, 30% for women, and 71% for endometrial carcinoma at 70 years of age. The risk for all HNPCC-related tumors was significantly lower in *MSH6* than in *MLH1* or *MSH2* mutation carriers ($P = 0.002$). In female *MSH6* mutation carriers, the risk for colorectal cancer was significantly lower ($P = 0.0049$) and the risk for endometrial cancer significantly higher ($P = 0.02$) than in *MLH1* and *MSH2* mutation carriers. In male carriers, the risk for colorectal cancer was lower in *MSH6* mutation carriers, but the difference was not significant ($P = 0.0854$). MSI analysis in colorectal tumors had a sensitivity of 86% in predicting a MMR defect. IHC in all tumors had a sensitivity of 90% in predicting a mutation in *MSH6*. *Conclusions:* We recommend starting colonoscopic surveillance in female *MSH6* mutation carriers from age 30 years. Prophylactic hysterectomy might be considered in carriers older than 50 years. MSI and IHC analysis are sensitive tools to identify families eligible for *MSH6* mutation analysis.

Colorectal carcinoma is the second most common cause of death due to malignancy in the western world. The cause of colorectal carcinoma is multifactorial, involving both hereditary and environmental factors.[1] A family history of colorectal carcinoma is a clinically significant risk factor and may be found in up to 15% of all patients with colorectal carcinoma.[2] The most common hereditary colorectal carcinoma syndrome is hereditary nonpolyposis colorectal carcinoma (HNPCC), which accounts for 1%–6% of all cases of colorectal carcinoma.[3] HNPCC is an autosomal dominant inherited disorder characterized by the development of colorectal carcinoma, endometrial carcinoma, and various other cancers at an early age. The Amsterdam (I and II) and Bethesda criteria are clinical criteria that can be used to identify families with HNPCC.[4–6] In HNPCC, germline mutations have been found in 4 mismatch repair (MMR) genes: *MSH2,*[7] *MLH1,*[8] *PMS2,*[9] and *MSH6.*[10,11] In 50%–85% of the families fulfilling the Amsterdam criteria, a germline mutation is detected in *MLH1* or *MSH2*.[12–14] The

†Deceased.

Abbreviations used in this paper: HNPCC, hereditary nonpolyposis colorectal carcinoma; IHC, immunohistochemistry; MMR, mismatch repair; MSI, microsatellite instability.

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cumulative lifetime risk of developing any cancer is 85%–90% in carriers of a mutation in *MLH1* or *MSH2*.[15]

The hallmark of HNPCC is microsatellite instability (MSI) in tumor tissue,[16–18] which is caused by a failure of the DNA MMR.[19] MSI is reported in 85%–92% of colorectal carcinomas and in at least 75% of endometrial carcinomas associated with HNPCC, while it occurs in 10%–15% of sporadic colorectal carcinomas[16] and in 17% of sporadic endometrial carcinomas.[20–25] MSI analysis can be used as a prescreening tool to identify families eligible for mutation analysis of the MMR genes. Previous studies have shown that colorectal carcinomas and especially endometrial carcinomas in *MSH6* mutation carriers demonstrate an MSI-high phenotype less frequently using the 5 standard markers.[26–28] Another tool for selecting families for genetic testing is immunohistochemistry (IHC) analysis with monoclonal antibodies directed against the MLH1, MSH2, and MSH6 proteins.[29,30]

In 1997, Miyaki et al.[10] and Akiyama et al.[11] described 2 families with a truncating germline *MSH6* mutation. Neither of the 2 families fulfilled the Amsterdam I criteria. The family reported by Miyaki et al. was characterized by a high age at onset of cancer and a predominance of endometrial carcinoma. In 1999, Wijnen et al.[26] described 10 kindred with 9 different truncating germline *MSH6* mutations. Most of these families did not fulfill the Amsterdam (I and II) criteria and were characterized by a predominance of endometrial carcinoma and a higher age at diagnosis of cancer compared with families with an *MLH1* or *MSH2* mutation. After this publication, more *MSH6* truncating germline mutations have been reported.[27,28,31–34]

The aims of this study were to (1) evaluate the clinical phenotype of a large series of families with an *MSH6* mutation, (2) evaluate the value of MSI and IHC analysis in the identification of such families, and (3) discuss the appropriate surveillance protocol for *MSH6* mutation carriers.

Patients and Methods

Patients

A total of 20 families with a truncating germline mutation in the *MSH6* gene were included in the study. These families originated from 2 sources. The first is a group of 214 families, tested negatively for pathogenic mutations in *MLH1* or *MSH2,* collected for scientific purposes through The Netherlands Foundation for the Detection of Hereditary Tumours and departments of clinical genetics in The Netherlands and Norway. Most of the families collected by The Netherlands Foundation for the Detection of Hereditary Tumours were selected using the Amsterdam criteria. The families collected by departments of clinical genetics in The Netherlands and Norway were selected on the basis of familial clustering of colorectal carcinoma. The group consists of 71 Amsterdam I–positive and 143 Amsterdam I–negative families. Nine different truncating *MSH6* germline mutations were identified in 10 families, as reported previously by our group.[26,34] Eight of the 10 families agreed to participate in this study.

The second group is composed of 12 families recruited through the departments of clinical genetics of the University Medical Centers of Leiden, Rotterdam, and Amsterdam (VU University Medical Center), The Netherlands, and at the Institute of Medical Genetics of the University Medical Center in Rome, Italy. Most of the referred families do not fulfill the Amsterdam II criteria. The families were referred for genetic analysis because of a positive family history of (colorectal) cancer. Only families with a protein truncating germline mutation in the *MSH6* gene were included in this study.

In the total group of 20 families, 17 different truncating mutations were identified (Table 1). Only 6 families fulfill the Amsterdam II criteria (Table 1).

We collected clinical information, including the age at diagnosis of cancer, site of the tumor, and pathology reports for as many affected individuals as possible. In addition, we collected the results of colonoscopic and gynecologic screening of the high-risk unaffected relatives. Genetic counseling and testing were offered to all relevant relatives. MSI and IHC analyses were performed on all available tumors.

Mutation Analysis

Mutation analysis of the *MSH6* gene was performed by denaturating gradient gel electrophoresis[35] followed by sequence analysis if a variant was identified. A mutation was considered pathogenic when the nucleotide change is predicting truncation of the protein (e.g., nonsense and frameshift mutations) or when it is changing a consensus splice donor or acceptor site, confirmed by testing the mutation in splice site prediction software (Neural Network Splice Site Prediction [http://www.fruitfly.org/seq_tools/splice.html] or CBS NetGene 2 [http://www.cbs.dtu.dk/services/NetGene2]).

Statistical Analysis

Penetrance for age was calculated using the Kaplan–Meier survival analysis method with the SPSS statistical package. Only proven carriers and only cases of cancer that were confirmed by medical records and/or pathology reports were included in the analysis. If more than one tumor developed in the same organ, only the first one diagnosed was included in the analysis. For the analysis of the cumulative risk of all HNPCC-related tumors together, only the first diagnosis was included in the analysis. For the analyses of the cumulative risk of colorectal and endometrial carcinomas, all first diagnoses in the respective organs were included. The observation time was from birth until date of diagnosis of cancer, death, or the end of the study in June 2002. No individuals were lost to follow-up.

Table 1. *MSH6* Mutations and Family Characteristics

Family	Mutation	Amsterdam II criteria	Confirmed tumors with age of diagnosis in **proven carriers** and individuals with unknown mutation status
1[a,b]	1784delT, L594fsX, exon 4	−	**Py77 + Py79, Py76, C84, Py59, C55 + E55, C49 + B49, E57, E60, O50, E53, E50, C50 + O51, C32,** C74
2[a]	467C→G, S156X, exon 3	+	**C67, C45, C47**
3[a]	742C→T, R248X, exon 4	+	**C61, C58 + C59, C59, C26**
4[a]	2191C→T, Q731X, exon 4	−	**C48, C49,** C51
5	2731C→T, R911X, exon 4	−	**C56 + E56 + C57 + C70 + Py69 + Lu70, C59**
6	3103C→T, R1035X, exon 4	−	**O49 + E49,** Ut58
7[a]	467C→G, S156X exon 3	+	**C62 + Py73, C44,** O78
8	1267delT, C426fsX, exon 4	−	**E62 + C65, C63, C56, C62,** E54 + C87, C85
9[a,c]	4001G→A, R1334Q, splice donor defect	−	**C69, E57, C45 + E53 + C66, C64, E50**
10	1784delT, L594fsX, exon 4	−	**E58, E60, E53**
11[d]	2984delA, 996fsX, exon 4	−	**C48 + C67, C54,** C54
12[d]	1960–1961insGTGA, fsX, exon 4	+	**C37 + St56,** C61, C51
13[a]	3261delC, P1087fsX, exon 5	−	**B78, C54 + E56, E51 + St73, E57, E49**
14[a]	IVS7-2A→C, 3647-2A→C, splice acceptor defect	+	**E58, E50, E56,** C50, E54
15	3182delT, 1061fsX exon 5	−	**E50,** C48
16	3987–3988insGTCA, S1329fsX, exon 9	−	**E43, E50**
17	1444C→T, R482X, exon 4	+	**E53 + C78 + Bl80 + Py82, C49, E49**
18	1614–1615delTCinsG, Y538X, exon 4	−	**E65 + C81, E55, O45**
19	651–652insT, K218X, exon 4	−	**E57, C52**
20	651–652insT, K218X, exon 4	−	**C61, C41**

NOTE. Boldface indicates proven carriers.
Py, transitional cell carcinoma of the renal pyelum; C, colorectal carcinoma; E, endometrial carcinoma; B, breast carcinoma; O, ovarian carcinoma; Lu, lung cancer; St, stomach cancer; Bl, bladder cancer.
[a]Previously published.[26]
[b]Previously published.[34]
[c]Previously published.[26,48,49]
[d]Previously published.[50]

A Kaplan–Meier analysis was also performed in 30 families with an *MLH1* mutation and 37 families with an *MSH2* mutation, previously described by Vasen et al. in 2001,[15] in which the same detailed data were available. To evaluate whether the cancer risk differed between the 3 groups of mutation carriers, we used the Wald test criterion of the Cox proportional hazards regression model. $P < 0.05$ was considered statistically significant.

MSI Analysis

MSI analysis was performed on paired tumor DNA and DNA from normal tissue using the Bethesda panel of microsatellite markers D2S123, D5S346, D17S250, BAT25, and BAT26 [19] with the additional BAT40 marker.[36] Tumors were regarded as MSI high if at least 30% of the markers showed instability, MSI low if <30% showed instability, or microsatellite stable if none of the markers showed instability.

IHC

IHC staining was performed on 4-μm sections of formalin-fixed, paraffin-embedded tissues. Slides were stained with antibodies against MLH1 (clone 14; Calbiochem, Cambridge, MA), MSH2 (clone GB12; Calbiochem), and MSH6 (clone 44; Transduction Laboratories/Becton Dickinson, Lexington, KY) in a Dako Techmate 500+ automated tissue stainer using standard protocols[36] and procedures as indicated by the manufacturer. Staining patterns of MMR proteins were evaluated using normal epithelial, stromal, or inflammatory cells or the centers of lymphoid follicles as internal controls. Stained slides were scored as either positive (showing nuclear staining in at least some tumor cells) or negative.

Results

Mutation Analysis

Mutation analysis was performed in 240 individuals (95 men and 145 women). Of the individuals tested, 55 were affected, 150 were first-degree relatives, and 35 were second-degree relatives. A mutation was identified in 119 individuals. Twenty-seven individuals were obligate carriers (13 affected and 14 not affected), based on the results of mutation analyses in their family members, and were not tested. Therefore, a total of 146 carriers were identified.

Of the 55 affected individuals who have been tested, 4 were proven not to be carriers of the *MSH6* mutation segregating in their respective families and are thus considered phenocopies. Two of these individuals developed colorectal carcinoma at 46 and 75 years of age, respectively, one woman was diagnosed with endometrial carcinoma at 45 years of age, and another women devel-

Table 2. Mean Percentage Cancer Risks at Age 30, 50, and 70 Years for Carriers of a Mutation in *MLH1, MSH2,* or *MSH6*

	Age (*yr*) (95% confidence intervals)		
Gene	30	50	70
All HNPCC-related tumours			
MLH1	4.2 (1.6-6.8)	34 (27-41)	76 (62-85)
MSH2	1.1 (0-2.3)	50 (42-57)	80 (70-86)
MSH6	0.7 (0-2.1)	22 (13-29)	73 (60-82)
Colorectal carcinoma in men			
MLH1	4.1 (0.1-7.9)	31 (19-41)	65 (39-80)
MSH2	2.0 (0-4.4)	39 (28-48)	63 (49-73)
MSH6	1.7 (0-5.0)	17 (4.4-28)	69 (42-83)
Colorectal carcinoma in women			
MLH1	4.3 (0.9-7.7)	26 (17-34)	53 (33-66)
MSH2	0	30 (18-40)	68 (43-82)
MSH6	0	10 (2.4-17)	30 (12-44)
Endometrial carcinoma			
MLH1	0	7.2 (1.4-13)	27 (14-38)
MSH2	0	23 (12-32)	40 (21-54)
MSH6	0	13 (5.3-22)	71 (50-83)

oped colorectal carcinoma at 71 years of age (and breast cancer at 50 years of age).

Statistical Analysis

The 146 proven carriers of a pathogenic *MSH6* mutation (59 men and 87 women) were included in the Kaplan–Meier analysis. Sixty-four affected carriers were identified (22 men and 42 women).

Table 2 shows the mean risks of cancer (percentages) for all HNPCC-related tumors, for colorectal carcinoma in men and women separately, and for endometrial carcinoma as well as the 95% confidence intervals for the ages of 30, 50, and 70 years for *MLH1, MSH2,* and *MSH6* carriers. The respective cumulative risk curves are shown in Figures 1–4. For all HNPCC-related tumors, the cumulative risks in *MSH6* carriers, men and women together, differed statistically significantly from the risk of *MLH1* and *MSH2* ($P = 0.002$) (Figure 1). This is because of the higher mean age at onset. However, the cumulative risks at 70 years of age were similar for the 3 genes.

In Figure 2, the age-related cumulative risk for colorectal carcinoma is shown for men only for *MLH1, MSH2,* and *MSH6*. The risks were lower in *MSH6* mutation carriers, but the difference was not significantly different ($P = 0.0854$). The mean age at diagnosis for colorectal carcinoma in male *MSH6* mutation carriers was 55 years (n = 21; range, 26–84 years) versus 43 and 44 years in *MLH1* and *MSH2* mutation carriers, respectively.

In Figure 3, the age-related cumulative risk for colorectal carcinoma is shown for women only for *MLH1, MSH2,* and *MSH6*. The age-related cumulative risk was significantly lower in *MSH6* mutation carriers ($P = 0.0049$). The mean age at diagnosis for colorectal carcinoma in female *MSH6* mutation carriers was 57 years (n = 15; range, 41–81 years) versus 43 and 44 years in *MLH1* and *MSH2* mutation carriers, respectively.

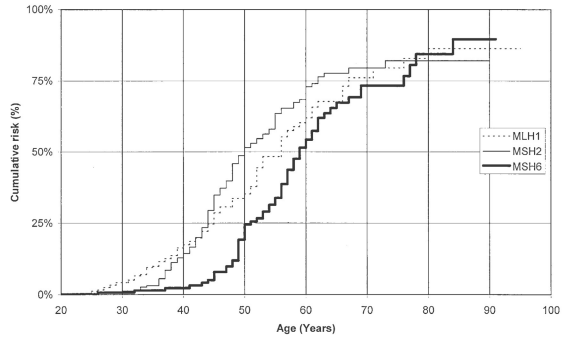


Figure 1. All HNPCC-related tumors; cumulative risks for *MLH1, MSH2,* and *MSH6* mutation carriers.

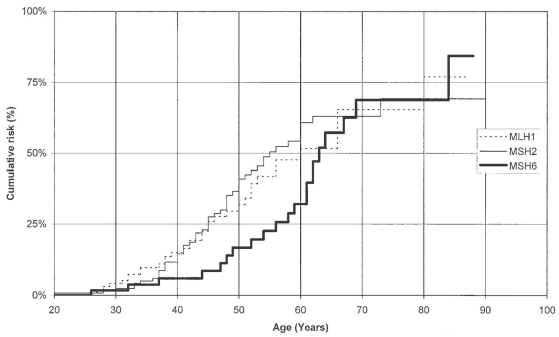


Figure 2. Colorectal carcinoma in men; cumulative risks for *MLH1, MSH2,* and *MSH6* mutation carriers.

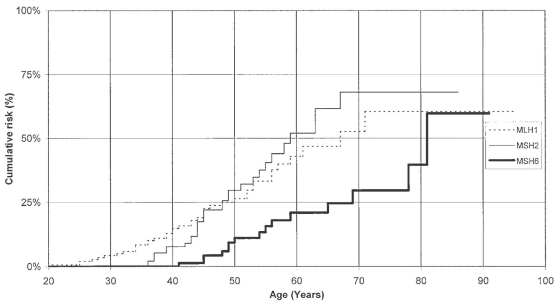


Figure 3. Colorectal carcinoma in women; cumulative risks for *MLH1, MSH2,* and *MSH6* mutation carriers.

Of the colorectal tumors in which the exact localization in the colorectum was known, 13 (39%) were located distally and 20 (61%) were located proximally (proximal to the flexura lienalis).

In Figure 4, the age-related cumulative risk for endometrial carcinoma is shown for *MLH1, MSH2,* and *MSH6*. The cumulative risk was significantly higher in *MSH6* mutation carriers ($P = 0.02$) compared with the risk in *MLH1* and *MSH2* mutation carriers. The mean age at diagnosis of endometrial carcinoma is 54 years (n = 29; range, 43–65 years) versus 48 and 49 years in *MLH1* and *MSH2* mutation carriers, respectively.

For ovarian carcinoma and transitional cell carcinoma of the upper urinary tract, cumulative risks were not calculated because the numbers were too low. The mean age at diagnosis for ovarian carcinoma was 49 years (n = 4; range, 45–51 years), and the mean age at diagnosis for transitional cell carcinoma was 72.5 years (n = 5; range, 59–82 years).

One family (family 1[34]) was substantially more extended than the other families. To exclude the possibility that this large family biased the results, we compared the cumulative risks for the various tumors between this

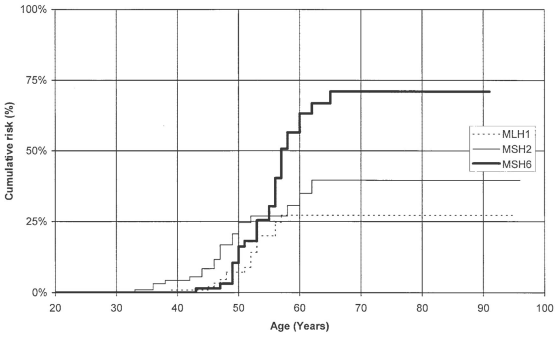


Figure 4. Endometrial carcinoma; cumulative risks for *MLH1, MSH2,* and *MSH6* mutation carriers.

Table 3. Results of MSI Analyses in Tumors of *MSH6* Mutation Carriers

Tumor	MSI high	MSI low	Microsatellite stable	Total
Colorectal carcinoma (%)	18 (86)	3 (14)	0	21
Endometrial carcinoma (%)	11 (69)	4 (25)	1 (6)	16
Transitional cell carcinoma (%)	5 (71)	2 (29)	0 (0)	7
Ovarian carcinoma	2	0	0	2
Breast carcinoma	1	0	0	1
Stomach carcinoma	0	0	1	1
Adenocarcinoma of the cervix	0	1	0	1
Total	35	9	5	49

family and the total group. There were no substantial differences. In addition, we examined whether the degree of participation in the families influenced the results. No considerable differences in cumulative risk were found between the families with a higher and lower degree of participation. To avoid bias toward affected individuals, we performed the Kaplan–Meier analyses both with and without index patients. Because these results did not differ, we decided to include the index patients.

MSI Analysis

As shown in Table 3, 49 tumors, all from mutation carriers, have been tested for MSI. Eighteen of 21 (86%) of the colorectal tumors showed an MSI-high phenotype. Two of the 3 tumors with an MSI-low phenotype would have been considered microsatellite stable if the BAT40 marker had not been tested. The third MSI-low tumor showed instability of a dinucleotide marker. If MSI-low tumors are also considered, the sensitivity for MSI analysis in colorectal tumors is 100%. Of the 16 endometrial tumors tested, 11 were MSI high (69%), 4 MSI low (25%), and one microsatellite stable (6%). Two of the MSI-high tumors and 1 of the MSI-low tumors would have been considered MSI low and microsatellite stable, respectively, if the BAT40 marker had not been tested. All MSI-low endometrial tumors showed instability of one of the mononucleotide markers. Five of the 7 (71%) transitional cell carcinomas tested showed an MSI-high phenotype. The other 2 were MSI low (29%). Two ovarian tumors were MSI high. The gastric carcinoma was microsatellite stable. The breast tumor, diagnosed in a proven carrier, showed an MSI-high phenotype. One adenocarcinoma of the cervix was MSI low. MSI in all HNPCC-related tumors together has a sensitivity of 71% and 90%, respectively, if MSI-high and both MSI-high and MSI-low tumors are considered.

IHC

As shown in Table 4, 40 tumors, all from mutation carriers, have been tested for MMR protein expres-

Table 4. Results of IHC in Tumors of *MSH6* Mutation Carriers

IHC pattern	No. of tumors (%)
MLH1+, MSH2+, MSH6−	36 (90)
MLH1+, MSH2−, MSH6−	2 (5)
MLH1−, MSH2+, MSH6−	1 (2.5)
MLH1+, MSH2+, MSH6+	1 (2.5)
Total	40

+, positive staining for protein; −, negative staining for protein.

sion by IHC: 18 colorectal tumors, 15 endometrial tumors, 4 transitional cell tumors, 1 ovarian tumor, 1 breast tumor, 1 gastric tumor, and 1 adenocarcinoma of the cervix. Thirty-six of the 40 tumors (90%) showed the expected pattern of absent staining for the MSH6 protein and retained staining for both the MLH1 and MSH2 proteins.

One of the MSI-low colorectal tumors previously mentioned showed absent MSH6 staining in IHC, indicating an MMR (*MSH6*) mutation. Another MSI-low tumor (diagnosed at age 78 years) showed positive staining for the MLH1, MSH2, and MSH6 proteins. In the same patient, bilateral transitional cell carcinoma showed an MSI-high phenotype and absent staining for the MSH6 protein. The colon tumor in this patient is likely to have been a sporadic tumor that did not develop because of defective MMR. All endometrial and transitional cell carcinomas showed negative staining for MSH6. Two colorectal tumors from different individuals showed absent staining not only for MSH6 but also for MSH2. One of these individuals also developed an endometrial carcinoma that showed negative staining for MSH6 in combination with positive staining for MLH1 and MSH2. Another colorectal tumor showed absent staining for both MLH1 and MSH6. In 98% (39 of 40) of the tested tumors, staining for the MSH6 protein was negative. In 90% (36 of 40) of the tumors, IHC specifically indicated a mutation in the *MSH6* gene by an IHC pattern with positive staining for MLH1 and MSH2 and negative staining for MSH6.

Discussion

We studied 20 families with a truncating germline *MSH6* mutation to determine the age-related cumulative risk of developing cancer and to develop a tailor-made surveillance protocol. We found that the cumulative risk of all HNPCC-related tumors in *MSH6* mutation carriers was significantly lower than the risk in carriers of a truncating *MLH1* or *MSH2* mutation. In women, the cumulative risk of colorectal cancer was significantly lower ($P = 0.0049$) when compared with carriers of a mutation in *MLH1* or *MSH2,* whereas the risk of endometrial cancer was more than twice as high ($P = 0.02$). For both colorectal carcinoma (54 years) and endometrial carcinoma (55 years), the mean age at diagnosis was higher in female *MSH6* mutation carriers compared with carriers of a mutation in *MLH1* or *MSH2*. In men, the risk of colorectal carcinoma was also lower than in *MLH1* and *MSH2* mutation carriers, but the difference was not statistically significant ($P = 0.084$). The mean age at diagnosis (58.5 years) was more than 10 years higher in *MSH6* compared with *MLH1* and *MSH2* mutation carriers.

Previous studies from The Netherlands and Finland on cancer risks in carriers of an *MLH1* or *MSH2* mutation were possibly biased toward overestimation of the risk because most of the families were selected by using the Amsterdam criteria or on the basis of familial clustering of colorectal cancer.[15,27,37] However, the only population-based study (from Scotland) reported similar risks for colorectal carcinoma in men,[38] although the risk for developing colorectal carcinoma in women was lower compared with the findings in the Dutch and Finnish studies. Carayol et al.[39] discussed the fact that the current risks are probably overestimated in HNPCC because of the statistical method used and proposed a novel statistical approach. We have chosen the Kaplan–Meier analysis because all previous studies eligible for comparison with our data used the Kaplan–Meier analysis as well.[15,27,28]

The general finding of a higher age at diagnosis in *MSH6* mutation carriers when compared with carriers of a mutation in *MLH1* or *MSH2* could be explained from the functional level of the MMR proteins. MLH1 and MSH2 are involved in MMR of both single-base mismatches and insertion-deletion loops, and repair is impaired in the absence of MLH1 or MSH2. Likewise, the MSH6 protein is involved in the repair of both single-base mismatches and insertion-deletion loops. However, in the absence of MSH6, MSH3 can partially replace its repair function and such redundancy might represent a protecting factor against accumulation of DNA damage.[40–42]

A striking finding in this study is the difference in cumulative lifetime risk of colorectal carcinoma between men and women. The same trend is described in *MSH2* mutation carriers.[15,28] This cannot be explained by early death caused by endometrial carcinoma, before a colorectal carcinoma can develop, because endometrial carcinoma is not often the cause of death in these families.

The current surveillance protocol used in carriers of a mutation in one of the MMR genes is colonoscopy every

1–2 years starting at the age of 20–25 years and a yearly gynecologic examination, transvaginal ultrasound examination, and blood test for assessment of CA125 levels starting at the age of 30–35 years. If transitional cell carcinoma of the upper urinary tract or stomach cancer occurs in at least 2 individuals in a family, urine cytology yearly or gastroscopy every 1–2 years, respectively, from the age of 30–35 years is recommended.[43] In the present study, we found a mean age at diagnosis of colorectal carcinoma more than 10 years higher than found in *MLH1* and *MSH2;* the youngest age at diagnosis of colorectal cancer was 26 years in male *MSH6* carriers and 41 years in female *MSH6* carriers. We recommend the same colonoscopic surveillance protocol in male carriers of an *MSH6* mutation as recommended in *MLH1* and *MSH2* mutation carriers because the cumulative risks did not differ significantly from the risk in *MLH1* and *MSH2* carriers. However, although this might further complicate the already-complex surveillance protocol, we recommend that female carriers of an *MSH6* mutation start colonoscopy at the age of 30 years because the cumulative risk of colorectal carcinoma was significantly lower compared with carriers of a mutation in *MLH1* and *MSH2* and because the youngest age at diagnosis was 41 years.

Similar to observations in *MLH1* and *MSH2* mutation carriers, the majority (66%) of the colon carcinomas in the families we examined were located in the proximal colon. A previous study reported that 30% of the colon carcinomas associated with *MSH6* mutations were located proximally.[28] The reason for the difference between these studies is unclear.

We found that the cumulative risk of endometrial carcinoma increased sharply after the age of 50 years. It is still questionable whether surveillance of the endometrium will lead to the early detection of cancer and improvement of the prognosis.[44] Therefore, based on the substantial risk of developing this type of cancer and the overall mortality from endometrial carcinoma of approximately 14%,[45] we advocate a liberal approach toward prophylactic hysterectomy for women with a truncating *MSH6* mutation who are older than 50 years of age. For surveillance of transitional cell carcinoma, we propose starting from the age of 50 years in families in which this tumor has occurred. However, the value of urine testing for the early detection of cancer is still unknown.[46]

Because DNA analysis is expensive and time consuming, prescreening methods can be of great relevance to increasing the efficiency of genetic testing for the identification of the disease causing mutation. Two prescreening methods currently applied to identify families eligible for mutation analysis of the MMR genes are MSI analysis and IHC. MSI analysis in colorectal tumors caused by an *MSH6* mutation has been reported to show either predominance of an MSI-high phenotype[26,34] or predominance of an MSI-low phenotype.[27,28] We found an MSI-high phenotype in 86% of the *MSH6*-related colorectal carcinomas with a pattern equivalent to that found in *MLH1*- and *MSH2*-related tumors, including instability of both mononucleotide and dinucleotide markers. In the classification of MSI, we included the Bethesda panel of markers[19] as well as the BAT 40 marker because it increases the sensitivity of MSI analysis, as shown in this study and a previous study performed by our group.[36] If the MSI-low tumors are included, the sensitivity of MSI analysis is 100% in colorectal tumors. In endometrial tumors obtained from *MSH6* mutation carriers, MSI analyses have been reported to show predominantly MSI-low phenotypes with mainly instability of mononucleotide repeats.[25–28] Accordingly, in the present study, we found an MSI-low phenotype in a substantial proportion (25% [4 of 16]). An MSI-high phenotype predominated in the other types of carcinoma tested. MSI in all HNPCC-related tumors together has a sensitivity of 71% and 90%, respectively, if MSI-high and both MSI-high and MSI-low tumors are considered. IHC in both colorectal and endometrial tumors has been reported to show positive staining of the MLH1 and MSH2 proteins and absent staining for MSH6.[25,28,34,47] We found an almost 100% sensitivity in predicting an MMR defect, including a mutation in *MSH6*. In 90% of the tumors, IHC specifically predicted a germline mutation in the *MSH6* gene. Two colorectal tumors from different individuals showed absent staining not only for MSH6 but also for MSH2. A possible explanation is that in the colorectal tumor of one of these patients, both the C-8 tract in MSH6 and the A-8 tract of MSH3 were shown to be somatically instable in MSI analysis. As a result, both the MSH2–MSH6 and the MSH2–MSH3 heterodimer might be less frequently formed, which will add to loss of expression of MSH2.

In our clinic, IHC is the first step in prescreening families that fulfill the Amsterdam criteria because the yield of mutation analysis is high and IHC directly indicates which gene to test. If IHC is positive for all tested proteins, MSI analysis is performed. On the other hand, MSI analysis is the first step in prescreening families that do not fulfill the Amsterdam criteria. When an MSI-high or MSI-low phenotype, especially with instability of a mononucleotide marker, is found in an HNPCC-related tumor, IHC of the MMR proteins is the second step. In case of an MSS tumor, IHC of MSH6 is performed. Our results in this study confirm that this

approach has a high sensitivity for identifying families with an *MSH6* mutation.

In conclusion, the present study shows that female *MSH6* mutation carriers develop colorectal carcinoma at a significantly higher age than reported for *MLH1* and *MSH2* mutation carriers and that the cumulative risk is significantly lower. Based on these findings, we recommend starting colonoscopic surveillance from a higher age than recommended in *MLH1* and *MSH2* families in female *MSH6* carriers. Secondly, we found a dramatic increase in the risk of developing endometrial carcinoma after the age of 50 years in female *MSH6* mutation carriers and therefore recommend a liberal approach toward hysterectomy for women above this age. Finally, we show that both MSI analysis and IHC for the MMR proteins are very sensitive prescreening methods for identifying families eligible for mutation analysis of the *MSH6* gene.

This study underscores the distinct phenotype in *MSH6* families and provides guidelines for the identification, counseling, and management of these families.

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