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PMS2-associated Lynch syndrome : the odd one out

Broeke, S.W. ten

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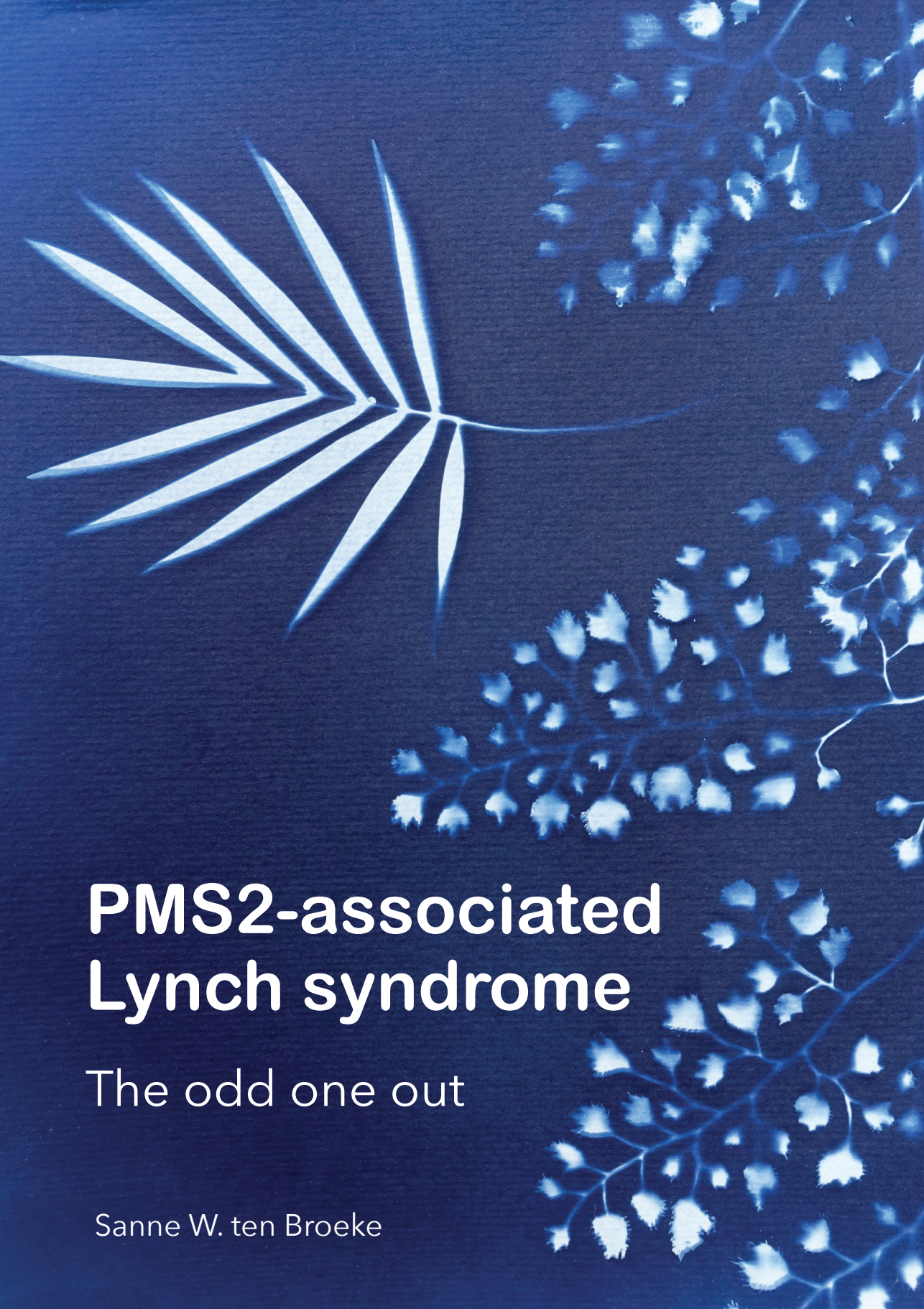

PMS2-associated Lynch syndrome

The odd one out

Sanne W. ten Broeke

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PMS2-associated Lynch syndrome:

the odd one out

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Thesis committee

Promotores

Prof.dr. C.J. van Asperen

Prof.dr. J. Morreau

Co-promotor

Dr. M. Nielsen

Thesis committee

Prof.dr. O.M. Dekkers

Prof. dr. N. Hoogerbrugge, Radboud University Medical Center

Prof. dr. G.H. de Bock, University Medical Center Groningen

Dr. A.M.J. Langers

‘I can’t remember all the times
I tried to tell myself
to hold on to these moments as they pass.’

– Adam Duritz

Lyrics from ‘A Long December’, 1996

CONTENTS

Chapter 1	General introduction	9
Chapter 2	Cancer risk analyses	23
2.1	Lynch Syndrome Caused by Germline *PMS2* Mutations: Delineating the Cancer Risk. *Journal of Clinical Oncology. February 2015.*	25
2.2	Cancer risks for PMS2-associated Lynch syndrome. *In-press at Journal of Clinical Oncology. May 2018.*	51
Chapter 3	Risk modifying factors	79
3.1	SNP association study in PMS2-associated Lynch syndrome. *Familial Cancer. November 2017.*	81
3.2	The risk modifying effect of lifestyle factors in patients with PMS2-associated Lynch syndrome. *Manuscript in preparation.*	121
3.3	The apparent genetic anticipation in PMS2-associated Lynch syndrome patients is explained by birth cohort effect. *In-press at Cancer Epidemiology, Biomarkers & Prevention. July 2018.*	133

Chapter 4 Tumor studies 147

4.1 Coding microsatellite mutation profiles in cancers of *PMS2* mutation carriers. 149
Manuscript in preparation.

4.2 Molecular Background of Colorectal Tumors From Patients With Lynch Syndrome Associated With Germline Variants in *PMS2*. 165
In-press at Gastroenterology. May 2018.

Chapter 5 Clinical guidelines 185

5.1 Incidence of polyps and post-colonoscopy colorectal cancers in patients with PMS2-associated Lynch syndrome: a prospective cohort analysis 187
Manuscript in preparation.

Chapter 6 General discussion 211

Chapter 7 English summary 230
Dutch summary 234
List of publications 242
Acknowledgments 246
Curriculum vitae 248

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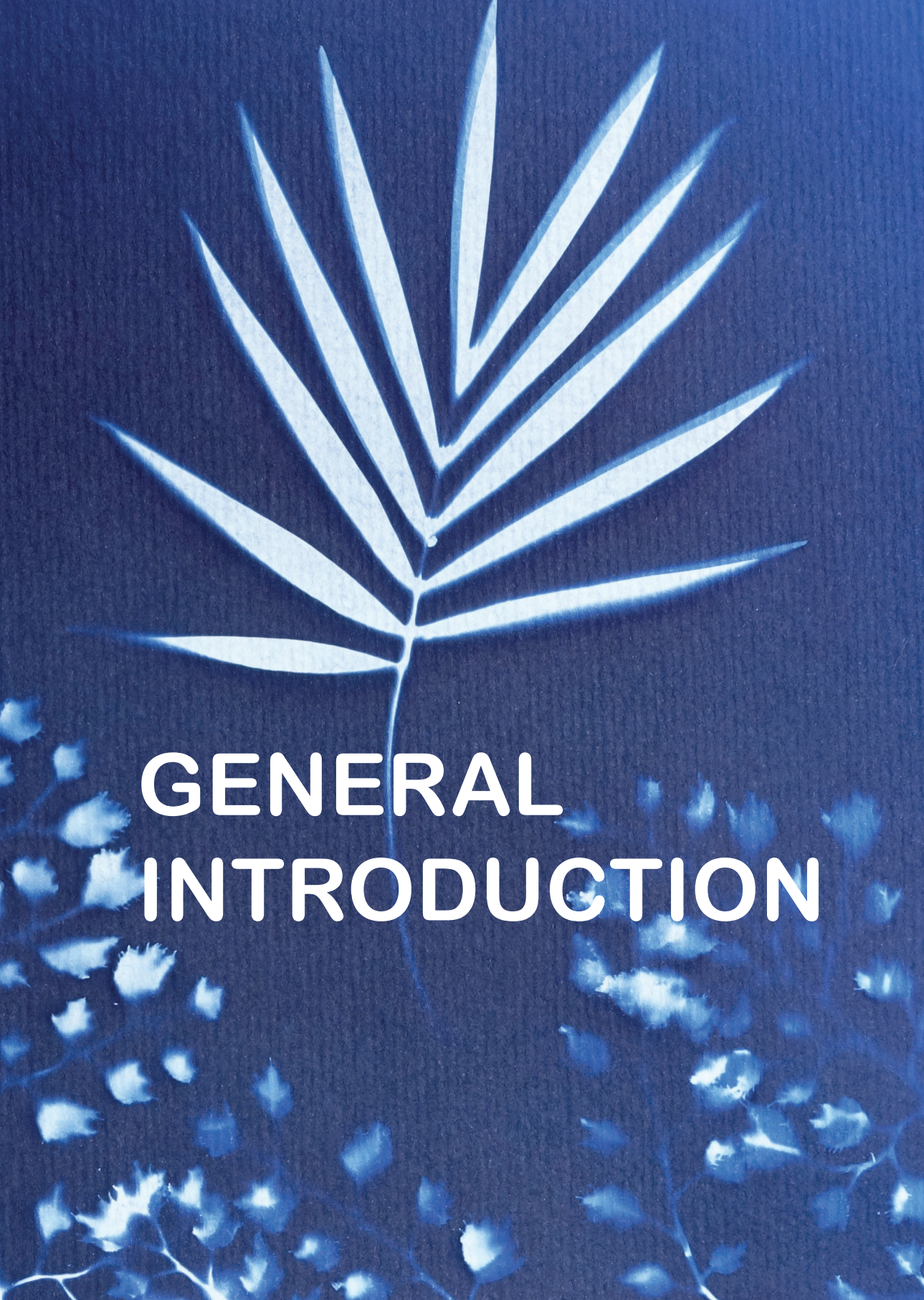

GENERAL INTRODUCTION

INTRODUCTION

Colorectal cancer is the third most frequently identified cancer worldwide and accounted for nearly 1.4 million new cases in 2012. This is expected to rise to 2.4 million new cases diagnosed each year by 2035.[1] The incidence of colorectal cancer in more developed countries is almost three times higher when compared to less developed countries suggesting that unhealthy lifestyle is a major contributor to colorectal cancer development.[1] However, in addition to environmental factors, hereditary factors play a role in the etiology in about 20-30% of colorectal cancer patients.[2] Approximately five percent of all colorectal cancer cases are associated with a highly penetrant inherited syndrome such as Lynch syndrome, familial adenomatous polyposis (FAP) or *MUTYH*-associated polyposis (MAP) (figure 1).[3] This thesis focusses on Lynch syndrome, in particular an underreported and under-investigated subtype, namely that associated with heterozygous pathogenic germline variants in the *PMS2* gene.

Lynch syndrome

Lynch syndrome (MIM 120435; formerly known as Hereditary Non-Polyposis Colorectal Cancer (HNPCC)) is the most common colorectal cancer predisposition syndrome, responsible for almost 3% of colorectal cancer and 2.5% of all endometrial cancer cases.[4,5] This syndrome is characterized by the development of colorectal and endometrial cancer with cumulative risks up to age 70 of 33-55% for colorectal and 10-45% for endometrial cancer.

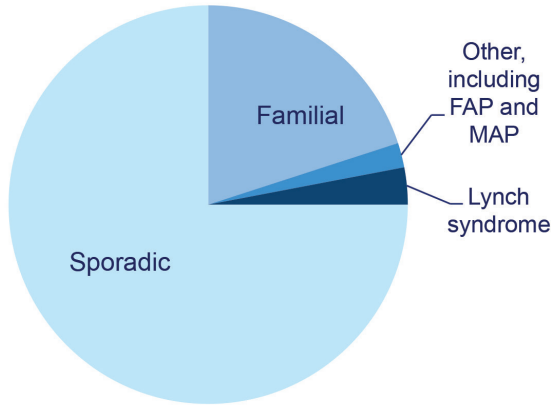

FIGURE 1 Pie-chart representing all diagnosed colorectal cancers.

Moreover, Lynch patients usually present with cancer at a relatively young age compared to the general population.[6] Other malignancies that occur significantly more frequently in Lynch patients than in the general population include ovarian, pancreatic, gastric, urinary tract, prostate and possibly breast cancer.[6-9] Lynch syndrome shows dominant inheritance and the underlying cause is a heterozygous pathogenic germline variant in one of the genes involved in DNA mismatch repair (MMR). These genes include *MLH1* (MIM 120436), *MSH2* (MIM 609309), *MSH6* (MIM 600678) and *PMS2* (MIM 600259). A recent addition to the list of genes causing Lynch syndrome is the epithelial cell adhesion molecule (*EPCAM*) gene, which, when deleted, inactivates *MSH2*.[10,11] Notably, biallelic pathogenic germline variants in the MMR genes cause a recessive form of childhood cancer that has been referred to as constitutional mismatch repair deficiency (CMMR-D) syndrome.[12,13]

In healthy individuals MMR proteins function as heterodimers in two main complexes consisting of MutS homologues MSH2 and either MSH6 or MSH3, and MutL homologues MLH1 binding to PMS2, PMS3, or MLH3. The MutS complex recognizes a mismatch and recruits the MutL complex which then initiates repair. These complexes act together in repairing mismatches and insertion-deletion loops.[14] [15,16] Tumors in Lynch patients arise or progress when the remaining wild type *MLH1*, *MSH2*, *MSH6* or *PMS2* allele is deactived because of a second hit or another truncating event, in line with Knudson's two hit hypothesis.[17] This leads to impaired MMR and subsequent accumulation of somatic variants in other (cancer) genes, which can eventually lead to uncontrolled cell growth and cancer. Hallmarks of these tumors as a result of faulty MMR are the shortening and lengthening of microsatellite regions, referred to as microsatellite instability (MSI), and the absence of MMR protein expression by immunohistochemistry (IHC). These changes allow patients with Lynch syndrome to be identified by the testing of tumors for these specific events.[3,18]

The exact percentage of individuals with an MMR germline variant in the general population is unknown. It has been estimated that the population incidence of Lynch syndrome could be as high as 1 in 370, based on the 2.8% incidence of Lynch syndrome among newly diagnosed colorectal cancer patients[4] and the 5% lifetime risk for colorectal cancer in the Western world. A recent report even estimated the prevalence to be up to 1 in 279.[19] Identifying individuals with Lynch syndrome is important, since clinical surveillance of this group can reduce colorectal cancer mortality by 70%.[20] Current surveillance protocols are identical for all MMR genes and include colonoscopies starting at age 20-25, every 1-2 years. For female MMR carriers biennial transvaginal ultrasounds with biopsies of the uterus can be considered, however there is no convincing evidence that this leads to improved survival.[21] Indeed, survival for

female Lynch patients with endometrial cancer is already very high. A recent report from the Prospective Lynch Syndrome Database (PLSD) estimated 10-year survival to be up to 93% (95% CI: 85%-97%). This database includes Lynch syndrome patients undergoing regular surveillance. Interestingly, *MLH1*, *MSH2* and *MSH6* carriers still had relatively high colorectal cancer risk (46%, 43% and 15% up to age 75, respectively) despite undergoing regular colonoscopies with polypectomies. It can thus be concluded that these polypectomies do not prevent all colorectal cancers for these MMR carriers, while interestingly, this does appear to be the case for *PMS2* carriers as Møller et al reported a 0% risk for this subset of Lynch patients.[22,23]

PMS2-associated Lynch syndrome

Since the first reports of the clinical involvement of *PMS2* in Lynch syndrome, it has been assumed that *PMS2* variants play only a minor role in Lynch syndrome.[24] This view has changed in recent years, as it has become clear that the role of *PMS2* variants has been underestimated. Even though the role of germline *PMS2* variants was described in 1994, clinical testing of the gene did not become available until 2009.[25-28] The reason for this is that the *PMS2* gene is notoriously difficult to analyze due to the existence of multiple pseudogenes. The gene is located on the short arm of chromosome 7 and spans 15 exons. Multiple regions with over 90% homology have been identified, all on chromosome 7. These pseudogene regions can interfere with sequencing of the *PMS2* gene. A variety of strategies, including the design of long-range amplicons[28] and RNA analysis[26], have helped to overcome this problem and have led to improved variant detection. Another explanation for the underestimation of PMS2-associated Lynch syndrome lies in the selection of families for genetic testing by family history or age of diagnosis (Bethesda and Amsterdam criteria)[29], whereas recent work has shown that *PMS2* variants are predominantly found in families that do not comply with these criteria.[30-32] While the identification of *PMS2* carriers in larger numbers seems to be improving, there are indications that many *PMS2* carriers remain to be identified. As the criteria defining Lynch syndrome are of limited use in the identification of these carriers, it may be necessary to examine population-based colorectal cancer cohorts.[32] Studies using IHC analysis in colorectal cancers from population-based cohorts have shown that isolated *PMS2* loss, indicative of a germline *PMS2* variants, is present in between 0.5-1.5% of unselected colorectal cancers.[31,32] The fraction of isolated PMS2 loss in MSI-high colorectal cancers varies between 1 and 8%.[33-35] One study of population-based colorectal cancers even found a higher percentage of abrogation of PMS2 than of MSH2 (12% versus 11%) in tumors with aberrant MMR staining.[32] More recent studies have also shown that in unselected (population-based) cohorts *PMS2*

and *MSH6* variants are much more prevalent (figure 2). Estimates of population carrier frequency based on statistical approaches are 1 in 714 and 1 in 758 for *PMS2* and *MSH6*, while the prevalence for *MLH1* and *MSH2* is 1 in 1946 and 1 in 2841, respectively.[19] This was confirmed in an unselected study involving the entire Icelandic population where they found an incidence of 1 in 226 for *PMS2* and *MSH6* variants combined.[36] Another indicator that the population frequency of *PMS2* carriers may possibly be even higher than that of *MLH1* and *MSH2* is the finding that biallelic *PMS2* variants comprise more than half of the homozygous or compound heterozygous variants in reported CMMR-D cases (31/57).[37] Although this might also be explained by the lower penetrance of *PMS2* variants, i.e. some biallelic *MLH1* or *MSH2* carriers may not be viable in utero.

The earlier under-representation of *PMS2* cases means that the clinical phenotype of carriers still requires further study. A small number of groups, including our own, studied cancer risk in this specific patient group.[31,38-40] In 2006, Hendriks et al published the first study on the clinical phenotype in *PMS2* carriers from our institution. Seven families with a previously unreported pathogenic *PMS2* variant were described in this study and it was shown that the mean age of colorectal cancer diagnosis for *PMS2* carriers was 52 years, 7-8 years higher than the mean age of diagnosis observed in families associated with *MLH1* and *MSH2* variants.[38] By including all available relatives in the penetrance analysis, irrespective of their mutation status (tested or not) an effort was made to

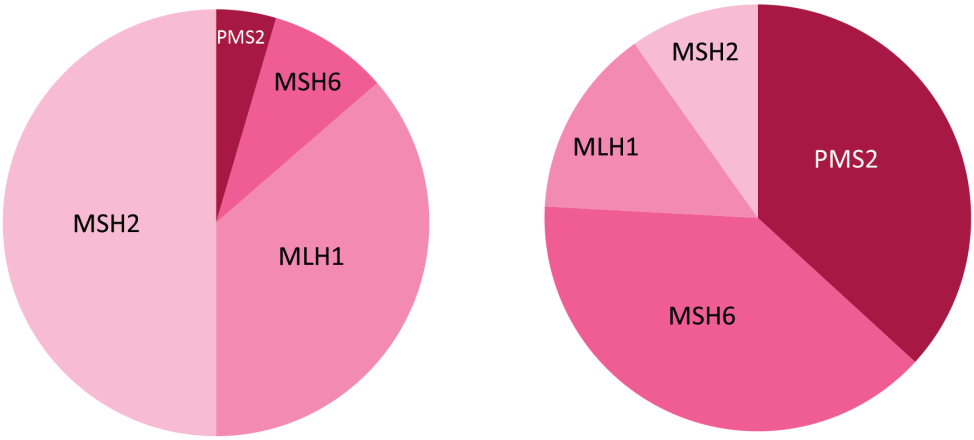

FIGURE 2 Estimated carrier frequencies in clinic- (left) vs. population- (right) based cohorts. Based on data reported by Win et al.[19]

reduce selection bias, but the inclusion of the index cases has likely still led to an overestimation of the cancer risk. Index cases which are almost always relatively young affected patients are unlikely to be representative for *PMS2* carriers in the general population. Therefore, when a low penetrance is to be expected, the inclusion of as many family members as possible on the one hand and the exclusion of index cases on the other, is of the utmost importance in order to limit selection bias. A large cohort of *PMS2* carriers (55 index patients and 55 relatives) was reported by Senter et al in 2008. This study reported a cumulative risk for colorectal cancer at age 70 of 20% (95% confidence interval: 11-34%) for male PMS2 carriers and 15% (95% confidence interval: 8-26%) for female *PMS2* carriers. The cumulative risk at age 70 for endometrial cancer was found to be 15%.[31] These are substantially lower risks than previously reported for *MLH1* and *MSH2* carriers. Reliable estimates on the risk for cancer outside the colon and endometrium will only become possible when larger studies are conducted. Risks estimated in larger studies are furthermore highly relevant for adequate surveillance policies. While Lynch families are currently advised to start surveillance between the ages of 20 and 25 and to continue with intervals of 1-2 years, less stringent surveillance guidelines may also be effective for *PMS2* carriers. The studies described in this thesis provide the basis for such PMS2-specific guidelines.

An essential next step to increase our understanding of the relatively low penetrance of *PMS2* variants is to study the role of this gene in carcinogenesis. Specific hallmarks of the molecular and immunological profile are to be expected and knowing these is relevant, since they can aid in a better identification of cases and might influence treatment and survival outcomes. Indeed, a lower somatic variant load in *PMS2* carriers compared to *MLH1* or *MSH2* carriers can be expected, since the MLH1/MLH3 heterodimer can partially compensate for the loss of the MLH1/PMS2 heterodimer. The high somatic variant load in MMR deficient tumors is thought to be the reason for an increased immune reaction surrounding these cancers, which in turn may explain improved survival in Lynch syndrome patients.[41] If there are indeed fewer somatic variants in PMS2 deficient tumors then this might result in a different involvement of the immune response which may consequently influence survival outcome. Moreover, a future possibility is that prophylactic vaccination of Lynch syndrome might elicit an early T-cell immune response, leading to tumor eradication or impeded tumor progression beyond the early stages.[42-44] Understanding of the role of specific T-cells in Lynch syndrome (including *PMS2*) could thus prove essential for the development of novel immunotherapeutic approaches. Lastly, the mechanism of the second hit in PMS2-asscoaiated cancers is often unknown; deletions, chromosomal loss and

methylation have all been suggested as possibilities.[45] More knowledge on this may also increase possibilities of cancer prevention.

Another clinical challenge is the fact that previous reports on *PMS2* have clearly shown a wide phenotypic variation within and between families. It therefore seems highly plausible that external and/or internal modifiers play a significant role in PMS2-related cancer development. Genome-wide association studies (GWAS) have uncovered numerous robust associations between common variants (single nucleotide polymorphisms, SNPs) and colorectal cancer risk. Researchers from our institution have demonstrated in 2009 that two SNPs, located on 8q23.3 (rs16892766) and 11q23.1 (rs3802842), are associated with an increased colorectal cancer risk in Dutch Lynch syndrome patients. This study revealed that patients homozygous for the minor allele of SNP rs16892766 showed an elevated risk of colorectal cancer in a dose-dependent manner, with a 2.16-fold increased risk of developing colorectal cancer. The CC variant genotype of SNP rs3802842 was also associated with an increased risk of colorectal cancer, but in female carriers only (HR=3.08). In a combined analysis of the two SNPs, risk was significantly associated with the number of risk alleles and the effect was shown to be stronger in female carriers than in male carriers.[46] This effect in Lynch syndrome patients was subsequently confirmed by an independent study in Polish and Australian Lynch syndrome patients.[47] Only one study included *PMS2* carriers, but only a limited number (n=40).[48] They found an increased risk for carriers of the G-allele of rs10795668 (10p14) and rs9929218 (16q22.1). Notably, this was the reversed effect as observed in the GWAS. Larger studies are needed to determine the role of SNPs in PMS2-associated Lynch syndrome.

Other well-known cancer risk factors, such as obesity and smoking might also contribute to the colorectal cancer risk and the development of adenomas.[49] It has been proposed that smoking is involved in epigenetic modification of MMR genes, and can thus serve as a second hit.[50] Indeed, smoking has been found to lead to an increased risk of carcinomas that show microsatellite instability and in a recent prospective study (GEOLynch cohort, consisting of *MLH1*, *MSH2* and *MSH6* carriers), smoking was shown to have a stronger effect on adenoma development in Lynch syndrome subjects than in sporadic cases (HR 7.1 for current smoking and 2.7 for former smokers).[51] A high BMI also leads to a higher tumor development in male Lynch subjects (HR 8.7).[52] Since *PMS2* has a reduced penetrance compared with other MMR genes, and also a still unexplained high clinical variance within families, lifestyle factors might be of even greater importance in this specific group. Since the previously mentioned studies did not include *PMS2* carriers further research on this hypothesis is needed.

Aim of this thesis

Although traditionally *PMS2* carriers were thought to account for only a minority of Lynch syndrome families, recent studies suggest significantly higher carrier frequencies of *PMS2* variants in the population when compared to *MLH1* and *MSH2*. We expect a rise in the identification of PMS2 families with the recent implementation of universal screening for MMR deficiency in all colorectal cancers below age 70. Unfortunately, the phenotype of PMS2-associated Lynch syndrome is poorly defined. This thesis describes epidemiological and molecular studies of PMS2 families and tumors in an attempt to further delineate the PMS2-associated phenotype thus providing enough evidence for gene-specific clinical guidelines.

1

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2

CANCER RISK
ANALYSES

2.1

Lynch syndrome caused by germline *PMS2* mutations; delineating the cancer risk

Journal of Clinical Oncology, 2014

Sanne W. ten Broeke*, Richard M. Brohet*, Carli M. Tops, Heleen M. van der Klift, Mary E. Velthuizen, Inge Bernstein, Gabriel Capellá Munar, Encarna Gomez Garcia, Nicoline Hoogerbrugge, Tom G.W. Letteboer, Fred H. Menko, Annika Lindblom, Arjen R. Mensenkamp, Pål Møller, Theo A. van Os, Nils Rahner, Bert J.W. Redeker, Rolf H. Sijmons, Liesbeth Spruijt, Manon Suerink, Yvonne J. Vos, Anja Wagner, Frederik J. Hes, Hans F. Vasen, M. Nielsen, Juul T. Wijnen

* These authors contributed equally to this work

ABSTRACT

Purpose

The clinical consequences of *PMS2* germline mutations are poorly understood compared to other Lynch-associated mismatch repair gene mutations. The aim of this European cohort study was to define the cancer risk faced by *PMS2* mutation carriers.

Methods

Data were collected from 98 *PMS2* families ascertained from family cancer clinics that included a total of 2548 family members and 377 proven mutation carriers. To adjust for potential ascertainment bias, a modified segregation analysis model was used to calculate colorectal cancer (CRC) and endometrial cancer (EC) risks. Standardized incidence ratios (SIRs) were calculated to estimate risks for other Lynch syndrome associated cancers.

Results

The cumulative risk (CR) of CRC for male mutation carriers by age 70 was 19%. The CR among female carriers was 11% for CRC and 12% for EC. The mean age of CRC development was 52 years and there was a significant difference in mean age of CRC between the probands and other family members with a *PMS2* mutation: 47 (range 26-68) and 58 years (range 31-78) respectively ($p<0,001$). Significant SIRs were observed for cancers of the small bowel, ovaries, breast and renal pelvis.

Conclusion

CRC and EC risks were found to be markedly lower than those previously reported for the other MMR genes. However, these risks embody the isolated risk of carrying a *PMS2* mutation and it should be noted that we observed a substantial variation in cancer phenotype within and between families, suggesting the influence of genetic modifiers and lifestyle factors on cancer risks.

INTRODUCTION

Lynch syndrome (LS) is the most common heritable colorectal carcinoma (CRC) syndrome and is responsible for 2-4 % of all CRC cases in the Western world.[1] The underlying cause for LS is a pathogenic heterozygous germline mutation in *MLH1*, *MSH2*, *MSH6*, *PMS2* or *EPCAM*. Previous clinical studies focused primarily on patients with heterozygous mutations in the *MLH1*, *MSH2* and *MSH6* genes[1-5] and reported high risks for the development of colorectal, endometrial and other cancers including ovarian, small bowel, pancreatic, gastric, urothelial, breast and possibly prostate carcinomas.

Although *PMS2* involvement in LS was described around the same time as that for *MSH2* and *MLH1*[6], technical difficulties in analyzing the *PMS2* gene due to a large number of pseudogenes has possibly led to underreporting of *PMS2* mutations in LS patients. Several strategies to overcome this problem, such as the design of long range amplicons[7, 8] and RNA analysis[9], have led to improvements in *PMS2* mutation detection. As a consequence of the relatively recent development of improved *PMS2* mutation diagnostic procedures clinical reports concerning heterozygous *PMS2* mutation carriers published thus far include quite small cohorts.[10-12] These studies reported a lower *PMS2* mutation penetrance for colorectal and endometrial cancer (EC) compared to *MLH1* and *MSH2* mutation carriers and similar or even lower risks as compared to *MSH6* mutation carriers.[1,5,13] Furthermore, parents and other family members of biallelic *PMS2* mutation carriers rarely develop colorectal or other Lynch-syndrome related cancers[14], indicating a reduced penetrance for cancer in these heterozygous family members. One theory regarding the lower penetrance of *PMS2* mutations is that MLH1/MLH3 and/or MLH1/PMS1 heterodimers partially compensate for the loss of MLH1/PMS2, although it is worth noting that this mechanism has not yet been confirmed by functional studies.[15]

Establishing an accurate cancer risk for mutations in cancer susceptibility genes such as *PMS2* is difficult because families are likely to be ascertained on the basis of the severity of their phenotype and outcomes are therefore variable depending on family selection and methods of data analysis, e.g. correction for ascertainment bias. In this study, using a modified segregation analysis, we aimed to achieve a reliable estimate of the cancer risk for heterozygous *PMS2* germline mutation carriers by including confirmed carriers together with non-tested family members.

METHODS

Data collection

All probands (index cases) included in the study were referred to a cancer family clinic because of a LS associated cancer or because of a suspect family history. They all had a confirmed pathogenic germline mutation in the *PMS2* gene. Available pedigree and patient-specific data were collected from 2009 until 2012, in collaboration with the clinical genetic departments of university hospitals in the Netherlands, Norway, Germany, Sweden, Denmark, Spain and the Leiden-based Netherlands Foundation for the Detection of Hereditary Tumors (table 1). The majority of cases were of white northern European origin.

Mutation screening of the probands was performed between 2007 and 2012. *PMS2* mutation analysis was initiated in most cases on the basis of histological investigations of the tumor suggestive for a *PMS2* germline defect and/or on the basis of a family's compliance with the Bethesda criteria.[16] In addition, eight families were recognized via a proband with biallelic *PMS2* mutations. Patients with biallelic *PMS2* mutations have a very distinct phenotype, with a typical spectrum of tumors at a very young age, so they were excluded from the cancer risk analysis (supplementary table 1).[17-19]

Informed consent was obtained according to protocols approved by local ethical review boards (LUMC Ethics Review Board, No. P01.019). Clinical and pathological data confirming the diagnosis – where available - were obtained from patient records.

Mutation analysis of PMS2

Mutation detection analysis of the *PMS2* gene was performed in multiple laboratories using a variety of methods all aimed at avoiding interference by pseudogenes. These methods included exon-by-exon DNA sequencing of exons 1 to 11 and simultaneous

TABLE 1 Family country of origin

The Netherlands	76
Denmark	6
Spain	6
Sweden	5
Norway	3
Germany	2

RT-PCR (RNA analysis) of the whole coding region of *PMS2* and/or long-range DNA amplicons that avoid pseudogene amplification.[7,8] Multiplex ligation-dependent probe amplification (MLPA) was used to detect large genomic deletions and duplications. *PMS2* mutations were classified as deleterious on the basis of introduction of a premature stop codon, either directly due to a nonsense mutation or as a result of a frameshift mutation, or when a deleterious splicesite mutation was identified. Missense mutations were classified as deleterious based on previous studies.[9,20] Mutations are depicted in supplementary table 2.

Statistical analysis

Cancer risk analysis was based on full pedigree information. A previously described protocol was used for the imputation of unknown dates of birth and death from

TABLE 2 Cohort description of the 98 families

	Total	Male	Female
Total no. family members	2548*	1284	1262
No. mutation carriers	377	172	205
- homozygotes	11	5	6
No. non-mutation carriers	237	108	129
No. of CRC	208	118	90
No. of EC	39	-	39
No. other cancer	218	108	110
– Lip	1	1	0
– Hypopharynx	1	1	0
– Lymphoma of pharynx	1	1	0
– Oesophagus	5	3	2
– Stomach	16	8	8
– Small intestine, including duodenum	8	5	3
– Liver and extra-hepatic bile ducts	3	1	2
– Pancreas	4	1	3
– Other and ill-defined sites within the digestive organs and peritoneum	1	0	1
– Nasal cavities, middle ear, and accessory sinuses	1	1	0

TABLE 2 Cohort description of the 98 families

		Total	Male	Female
–	Trachea, bronchus and lung	29	27	2
–	Bone and articular cartilage	1	0	1
–	Connective and other soft tissue	1	0	1
–	Malignant melanoma of the skin	6	3	3
–	Skin	10	8	2
–	Female breast	44	-	44
–	Kaposi's sarcoma	1	0	1
–	Cervix uteri	7	-	7
–	Ovary and other uterine adnexa	10	-	10
–	Other and unspecified female genital organs	1	-	1
–	Prostate	18	18	-
–	Testis	3	3	-
–	Bladder	5	5	0
–	Kidney and other and unspecified urinary organs	8	4	4
–	Brain	5	3	2
–	Thyroid gland	4	3	1
–	Secondary and unspecified malignant neoplasm of lymph nodes	1	1	0
–	Secondary malignant neoplasm of respiratory and digestive systems	7	1	6
–	Other malignant neoplasms of lymphoid and histiocytic tissue	3	0	3
–	Multiple myeloma and immunoproliferative neoplasms	1	1	0
–	Leukaemia of unspecified cell type	8	6	2
–	Unknown origin	4	3	1

Note: Number of cancers excluding the cancers of the biallelic mutation carriers.
CRC= colorectal carcinoma; EC= endometrial carcinoma
* Gender was unknown for two individuals.

the known dates of their family members. Unknown age at cancer diagnosis was, if possible, imputed from the cohort-, period- and sex specific mean age at cancer diagnosis in the general population.[21] Family members were considered to be at risk from birth until the first occurrence of any of the following events: first CRC diagnosis (N=208); EC diagnosis (N=39); other cancer diagnosis (N=218); death; last contact of a family member with the study center or last DNA test of a family member; and, 70th birthday. CRC and EC risks were estimated using modified segregation analysis implemented in the pedigree analysis software MENDEL as previously described.[12, 22] Restricting the analysis to confirmed carriers would bias the results because affected family members and those with a very strong family history of cancer might be more inclined to pursue mutation testing and deceased individuals would be excluded. The MENDEL program weighs the likelihood contributions of untested individuals according to their probability of being a carrier, which was estimated from their cancer history, age and position in the pedigree. In the analysis, the penetrance function was modelled in terms of the incidence rates in carriers and non-carriers. The incidence rates were assumed to follow a Cox-proportional hazards model in which the non-carriers were assumed to conform to population incidence rates. These population rates were calculated using combined calendar and age-specific incidences from the Netherlands and age-specific rates for the other European countries. All country-specific incidence rates contributed to the mean according to their weight in the total number of families. The relative risk represents the incidence rate in mutation carriers compared to the population incidence rates at age t. A single autosomal dominant model and a mutation frequency of 0.001 for *PMS2* were used. The incidences for each disease at age $\lambda(t)$ were assumed to follow a Cox model: $\lambda(t) = \lambda_0(t) \exp[G(t)]$, where $\lambda_0(t)$ is the age-specific disease incidence rate and exp[G(t)] is the age-specific hazard ratio (HR) or the relative risk in carriers compared to non-carriers.

To estimate the risk of other LS-associated cancers, we calculated the standardized incidence ratio (SIR) in a separate analysis as the ratio of observed cancers in the cohort to the expected cancers derived from the age, sex, calendar period and site-specific Dutch cancer population incidence rates. We restricted the cohort analysis to known Dutch mutation carriers who were alive and free of cancer in 1960, or born after 1960 (n=276). Two-sided statistical significance levels for the SIRs were estimated and 95% confidence intervals (CI) calculated under Poisson distribution of the observed frequencies.

Comparative analyses of mean age of cancer development were done via an independent samples t-test in IBM SPSS Statistics 20.

RESULTS

Our cohort included 98 separate families with 377 proven mutation carriers, of whom 11 were biallelic carriers and 366 heterozygous carriers (see table 2 for a description of the cohort).

CRC and EC

The cumulative CRC risk (table 3, figure 1) calculated using MENDEL was 18.75% (95% CI: 5.60-30.06%) for males at age 70, with a hazard ratio of 6.92 (CI: 2.46-19.42). The CRC risk at age 70 for female carriers was 10.56% (CI: 2.42-18.01%), with a hazard ratio of 4.71 (CI: 1.51-14.72), while the cumulative risk at age 70 for EC (table 3, figure 2) was 11.78% (CI: 2.61-20.09%), with a hazard ratio of 8.74 (CI: 2.14-35.7). The mean age of first CRC development for all cases with a heterozygous *PMS2* mutation was 52 years (range 26-86, table 4). Notably, the age distribution for CRC differed markedly between probands and CRC-affected family members (supplementary figure 1 and 2).

TABLE 3 Age-specific hazard ratios and cumulative cancer risks for CRC and EC in PMS2 mutation carriers

	Age	HR	95% CI	CR (%)	95% CI
CRC male	<40	20.59	3.27-129.60	1.27	0.00-3.51
	40-49	17.06	5.15-56.50	4.63	0.01-9.04
	50-59	3.66	1.29-10.38	7.11	1.76-12.17
	60-69	6.92	2.46-19.42	18.75	5.60-30.06
CRC female	<40	8.82	0.72-107.55	0.46	0.00-1.52
	40-49	2.63	0.27-25.68	0.92	0.00-2.42
	50-59	5.99	1.99-18.00	4.74	0.22-9.05
	60-69	4.71	1.51-14.72	10.56	2.42-18.01
EC	<40	20.03	0.58-688.70	0.19	0.00-0.84
	40-49	7.81	0.81-74.80	0.69	0.00-2.79
	50-59	16.18	5.81-45.06	7.11	0.52-13.26
	60-69	8.74	2.14-35.70	11.78	2.61-20.09

HR = hazard ratio; CR = cumulative risk; CI = confidence interval;
CRC= colorectal carcinoma; EC= endometrial carcinoma

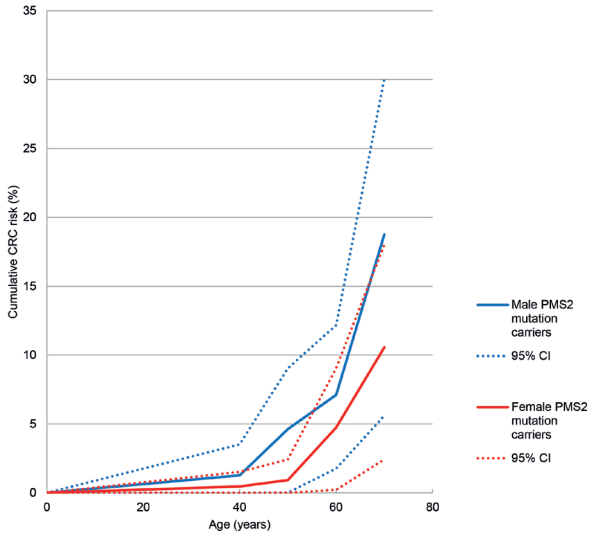

FIGURE 1 Graphic presentation of cumulative cancer risk with 95% confidence intervals (CI) for colorectal carcinoma (CRC).

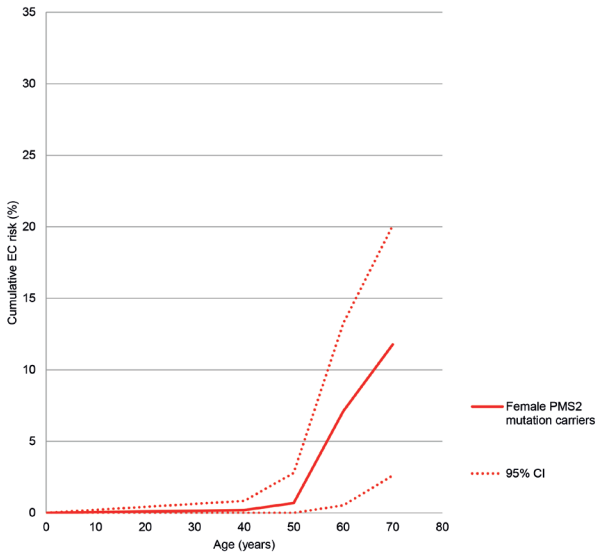

FIGURE 2 Graphic presentation of cumulative cancer risk with 95% confidence intervals (CI) for endometrial cancer (EC).

TABLE 4 Mean age of CRC and EC diagnosis in PMS2 mutation carriers

		No.	Median (years)	Mean age (years)	Range	p-value
CRC	Total group	106	51	52	26-86	0.83
	Male	65	52	51	26-86	
	Female	40	51	52	27-78	
	Probands	62	48	47	26-68	<0.001
	Family members	44	57	58	31-86	
EC	Total group	25	54	55	35-81	
	Probands	15	52	56	42-81	0.76
	Family members	10	58	54	35-68	

CRC= colorectal carcinoma; EC= endometrial carcinoma

TABLE 5 Mean ages at cancer diagnosis of MMR mutation carriers

	PMS2	*MLH1/MSH2* [#4]	*MSH6* [*5]
CRC	52 (26-86) including probands 58 (31-86) excluding probands	47 (15-95)	59 (30-90)
EC	55 (35-81) including probands 54 (35-68) excluding probands	47 (26-75)	54 (32-82)

No probands included
* Including probands
CRC= colorectal carcinoma; EC= endometrial carcinoma MMR: mismatch repair

TABLE 6 Other Lynch syndrome associated cancers

Location of malignancy	Observed	Expected	Standardized Incidence Ratio (95% CI)	p-value	Mean age of diagnosis (range)
Overall cancer *	35	16.9	2.1 (1.4-2.9)	0.00016	57 (18-80)
Small bowel	5	0.042	118.9 (38.6-277.4)	$2.12*10^{-6}$	60 (48-79)
Breast	11	2.9	3.8 (1.9-6.8)	0.0004	55 (36-80)
Ovary	4	0.33	12.0 (3.3-30.7)	0.0008	55 (51-59)
Prostate	2	1.17	1.7 (0.21-6.2)	0.66	56 (42-70)
Renal Pelvis	2	0.040	50.5 (6.1-182.4)	0.0015	78 (77-79)
Brain	1	0.37	2.7 (0.069-15.2)	0.62	55
Leukemia#	1	0.47	2.1 (0.054-11.9)	0.75	45
Stomach	0	0.57	1.7 (0-6.5)	N/A	
Pancreas	0	0.31	0 (0-12)	N/A	
Bladder	1	0.50	2.0 (0.051-11.2)	0.79	49

Note: The standardized incidence ratio is the observed number of cancers divided by the number of expected cancers in the general population; Significant SIRs are highlighted in bold; N/A: not applicable; * Excluding CRC and EC; # Non-Hodgkin Lymphoma
CRC= colorectal carcinoma; EC= endometrial carcinoma

The mean ages of CRC diagnosis in these groups were 47 years (range 26-68) and 58 years (range 31-78), respectively. There was no significant difference in mean age of CRC development between male and female carriers (51 years versus 52 years, p=0.83). The mean age at diagnosis of EC was 55 years (range 35-81), with no significant difference between probands and affected family members (p=0.76, table 4). For both CRC and EC, the mean age at diagnosis of carriers of a pathogenic *PMS2* mutation is higher when compared to carriers of a *MLH1* or *MSH2* mutation, but when compared to *MSH6* the mean age of CRC is lower and for EC similar in our cohort (table 5).

Other LS-associated cancers

Risks for cancers other than CRC or EC among *PMS2* mutation carriers are shown in table 6. Significant SIRs were identified for cancers of the small bowel (118.9 (95% CI: 38.6-277.4)), ovaries (12.0 (CI: 3.3-30.7)), breast (3.8 (CI: 1.9-6.8)) and renal pelvis (50.5 (CI: 6.1-182.4)).

DISCUSSION

In an effort to achieve a comprehensive understanding of the cancer risks faced by *PMS2* mutation carriers, we collected and analyzed a cohort of 98 *PMS2* mutation-positive families including over 2500 family members. Analysis of this large cohort revealed that cumulative CRC risk at age 70 is almost 19% for males and 11% for females, while risk for EC is around 12%. Furthermore, we found significant SIRs for cancers of the small bowel, breast, ovaries and renal pelvis. As reported previously for other MMR genes, females appear to have a markedly lower CRC risk compared to males, although this observation was not statistically significant in our study. The calculated CRC and EC risks in our study agree with those reported by Senter *et al.*[12] who used the same statistical methodology to calculate cancer risks in a cohort of 55 *PMS2* mutation-positive families. In contrast, cumulative cancer risks at age 70 reported for *MLH1* and *MSH2* range from 52% to 97% for CRC and 21% to 54% for EC.[1] Far closer to the risks found in our study are the reported risks for carriers of *MSH6* mutations that ranged from 22% to 69% for CRC to 16% to 71% for EC[1], thus *MSH6* and *PMS2* mutations appear to represent significantly lower risks to these carriers.

A striking finding in this study is that cancer risk seems to vary widely between members of the same family and does not appear to be solely dependent on an individual's *PMS2* mutation status. This is illustrated by the wide age range at initial CRC diagnosis (26 to 86-years-old) and the large difference in mean age (10 years) between probands and mutation-positive family members. The observed heterogeneity of risk between mutation carriers agrees with the findings of Dowty *et al.* who also described this phenomenon for *MLH1* and *MSH2* mutation carriers. These authors proposed that lifetime CRC risk for both male and female mutation carriers (from birth to age 70) follows a U-shaped distribution rather than a normal distribution. This means that most MMR carriers have either a high risk or a low risk of developing CRC, with a relatively low proportion of carriers at moderate risk.[4]

One explanation of this high variance may be the presence of internal (e.g. genetic) or external (e.g. lifestyle) modifiers. Indeed, certain single nucleotide polymorphisms (SNPs) associated with a slight increase in CRC risk in the general population, as found by genome-wide association studies (GWAS), are known to significantly influence CRC risk in LS patients with *MLH1* and *MSH2* mutations.[23,24] Lifestyle factors such as BMI and smoking have also been reported to modify CRC risk and adenoma development in LS patients.[25,26] This is possibly explained by a difference in the molecular mechanism underlying carcinogenesis in LS patients that might then result in lifestyle factors having a different effect in these patients as compared to sporadic CRC patients. However,

as previous association studies only included very limited numbers of *PMS2* mutation carriers, further research on this subject is needed.

Due to the increased identification of *PMS2* mutation carriers (and LS patients in general) expected to result from the implementation of next generation sequencing and universal screening programs for CRC and EC patients, there is now a pressing need to establish *PMS2*-specific risk estimates. This increased detection is illustrated by studies using IHC analysis in CRCs from population-based cohorts that showed that isolated PMS2 protein loss in the tumor - indicative of a germline *PMS2* mutation - occurs in 0.5-1.5% of unselected CRC cases.[12,15,27] Previously, identification of LS patients was based on strict Amsterdam and/or Bethesda selection criteria, but recent population-based CRC and EC studies have shown that more than half of patients identified with a LS-like profile do not comply with these criteria and probably would have been missed in the past.[27,28] This problem is even more relevant to *PMS2* families as it is likely that they do not comply with strict selection criteria due to low cancer penetrance and a higher mean age of cancer development (>50 years).[12] Even in our selected cohort only 19% of the families complied with the Amsterdam II criteria and 78% with the revised Bethesda criteria.[16, 29] Almost 22% of the families in our cohort failed to comply with any of the abovementioned criteria and would have been overlooked on the basis of these criteria alone. The possible current underestimation of *PMS2* mutation-positive families is further suggested and illustrated by next generation sequencing studies in which *PMS2* mutations were reported as incidental findings in 0.03-0.4% of individuals not selected on the basis of CRC.[30-32]

Identifying MMR mutation carriers is important because these individuals should be enrolled in surveillance programs. Currently, LS family members are advised to participate in colonic surveillance from around 20-25 years of age, at 1-2 year intervals.[33,34] We now suggest that surveillance in *PMS2* mutation carriers could start at a slightly higher age, e.g. 30 years, similar to the previously suggested age for female *MSH6* carriers - due to the later age of onset and lower cancer risk than that reported for *MLH1* and *MSH2*.[35] It should be noted however that 6 heterozygous probands (6/367, 1.6% of all mutation carriers) developed CRC below or at the age of 30. Interestingly, their mutation-positive family members with CRC had a significantly later age of onset (56 years as opposed to 28 years). Moreover, none of the heterozygous family members in our cohort developed CRC before the age of 30.

The efficacy of surveillance for EC is less well established. However, surveillance is currently still advised and consists of gynecologic examination with transvaginal ultrasound and/or hysteroscopy with aspiration biopsy, starting at the age of 30-35, which may lead to the detection of premalignant disease.[33,34,36] Prophylactic surgery,

i.e. risk reducing salpingo-oophorectomy (RRSO), does not seem to be appropriate for female *PMS2* mutation carriers as mortality from EC is relatively low.[37] Indeed, in our cohort only one out of 25 proven *PMS2* mutation carriers with EC died of this cause, at age 65. Furthermore, OC in LS patients predominantly presents in early stages of carcinogenesis and there is evidence that surveillance (transvaginal ultrasound and CA-125) might have a greater efficacy in these cases than in non-Lynch related OC cases.[38] Therefore, we only advise use of the surveillance protocol described above and not prophylactic surgery.

Additional screening for other LS-associated cancers is currently not advised for LS patients, except in the case of familial clustering of gastric or urinary tract cancer.[33,39] Our cohort of *PMS2* mutation carriers showed significant SIRs for cancers of the small bowel, ovaries, renal pelvis and the breasts (table 6). The first three cancers are accepted as part of the LS tumor spectrum, but the association of breast cancer with germline MMR mutations is currently still a subject of debate.[1] Due to a relatively low incidence and in general a high mean age at diagnosis in our cohort (table 6), additional surveillance other than for CRC and EC does not seem to be indicated. However, in light of a SIR for breast carcinomas of 3.8, mammography from age 40 may be considered, especially in those *PMS2* families that show clustering of breast cancer. The relatively small number of patients in the current study and the low frequency of some LS-associated cancers mean that a larger cohort will be required in order to formulate definitive conclusions and advice.

Besides the implications for surveillance, the risks reported here also have implications for counseling of *PMS2* mutation carriers. Although it is our opinion that gene specific counseling is justified, it should be clearly explained to patients that these risk estimates were corrected for ascertainment and embody the risk of the *PMS2* mutation itself. These unbiased risks are probably most applicable for patients with a *PMS2* mutation identified via population-based screening programs (and not selected on the basis of their family history of cancer) or as an incidental finding of next generation sequencing diagnostics now being introduced in many laboratories. However, given that in daily clinical practice most *PMS2* mutation carriers will still be identified as members of a family severely affected with cancer, it is probable that other genetic or environmental risk factors - that contribute to a higher cancer risk - are present in these families.

In conclusion, the cumulative colorectal and endometrial cancer risks for *PMS2* mutation carriers are markedly lower than the current risk estimates for LS familiar to clinicians and a significant proportion of these families are probably missed due to strict selection criteria. In addition, the wide within-family variance suggests that other risk factors are present in most families. While awaiting the advent of personalized

risk stratification, we suggest a limited modification of surveillance guidelines for *PMS2* mutation carriers similar to that for *MSH6*; begin colorectal surveillance at age 30 instead of the current 25 years of age. Furthermore, prophylactic removal of the uterus and ovaries of female *PMS2* mutation carriers does not appear advisable at the present time.

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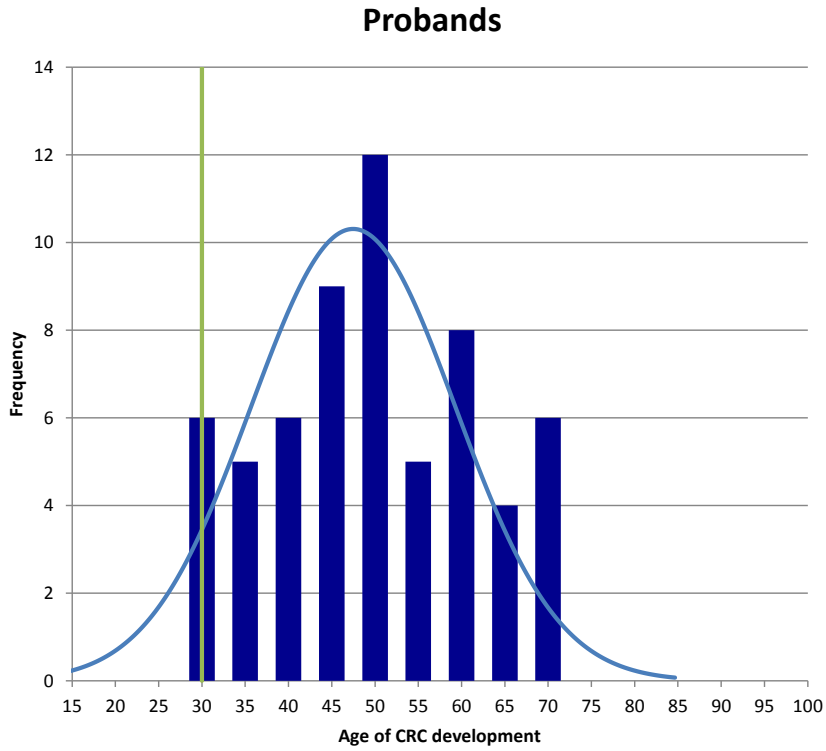
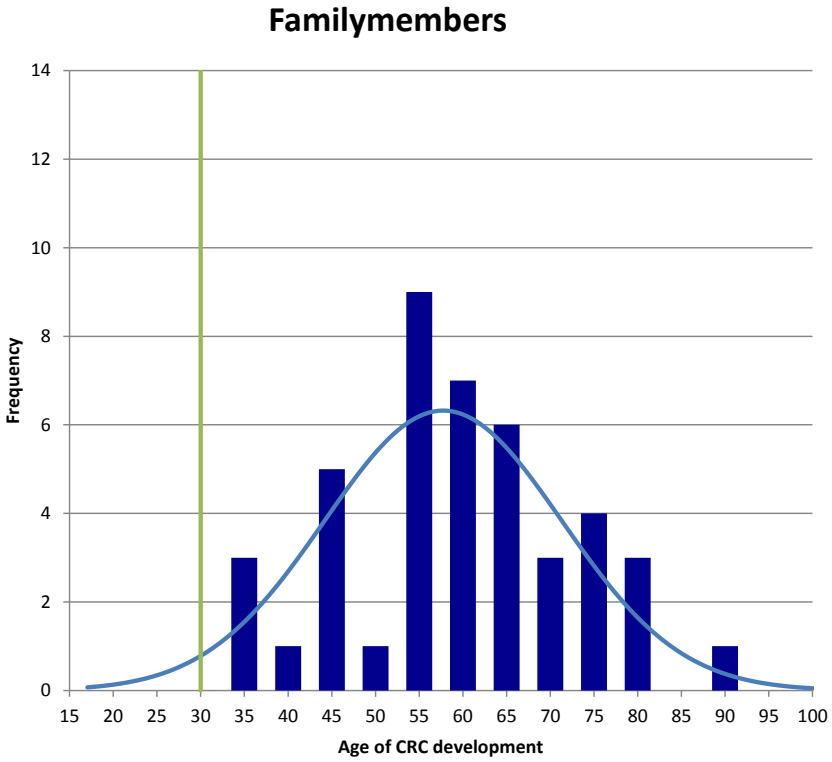
2

SUPPLEMENTAL FIGURES



SUPPLEMENTARY FIGURE 1 Histogram of mean age of CRC development, representing probands with a heterozygous *PMS2* mutation. The green line in figure 4 indicates age 30, which is the suggested start of colorectal surveillance.



SUPPLEMENTARY FIGURE 2 Histogram of mean age of CRC development, representing family members with a heterozygous *PMS2* mutation. The green line in figure 4 indicates age 30, which is the suggested start of colorectal surveillance.

SUPPLEMENTARY TABLE 1 Clinical characteristics of biallelic *PMS2* mutation carriers (N=11)

Sex	CRC	Age CRC	Other cancer	Age other cancer	Other	Mutation
F	Yes	19	Bladder Jejunum	21 Unknown	Multiple adenoma, giant pigmented naevus syndrome, CAL's, hypothyroidism,	c.137G>T AND c.2174+1G>A (compound heterozygous)
M	No		Duodenum Jejunum Acute lymphoid leukaemia (ALL)	17 19 21	Serrated polyp with high grade dysplasia at age 15 Small bowel and rectum loaded with polyps at age 16	c.137G>T AND c.2174+1G>A (compound heterozygous)
M	No		Duodenum	32	Polyposis 20 years, complex cor vitium	Deletion exon 3-7 deletion AND deletion exon 1-11(-15) (compound heterozygous)
M	No		Angiosarcoma	2	CAL	c.736_741delinsTGTGTGTGAAG AND complete gene deletion (compound heterozygous)
F	No		Brain	4	CAL's	c.137G>T AND c.736_741delinsTGTGTGTGAAG (compound heterozygous)
M	No		Brain	8	CAL's	c.137G>T AND c.736_741delinsTGTGTGTGAAG (compound heterozygous)
F	Yes	8	Brain Lymphoma	9 18	-	c.219_220dup (homozygous)
M	No		--	-	Rectum adenoma at 24 years	c.325dup AND c.825A>G (splice mutation) (compound heterozygote)
F	No		Brain	11	-	c.325dup AND c.825A>G (splice mutation) (compound heterozygote)
F	No		Brain	2	1 adenoma at 10, CAL's and freckling	c.2174+1G>A (homozygous)
F	Yes	43	Lymphoma, Endometrial carcinoma	18, 37 and 43 39	Colorectal polyp	c.24-2A>G (splice mutation) (homozygous)

CAL= Café-au-lait maculae; CRC= colorectal carcinoma

SUPPLEMENTARY TABLE 2 *PMS2* mutations

Description of PMS2 mutation[1]	Nationality of index patients[2]	Total nr of families	Family description reported before[3]
c.736_741delinsTGTGTGTGAAG	10x NL; 4x DK; 3x S	17	
c.1882C>T (p.Arg628X)	6x NL	6	Hendriks, 2006 (family 6)
c.697C>T (p.Gln233X)	4x NL	4	
Deletion exon 14	3x NL	3	
Deletion exon 11-15	3x NL	3	Hendriks, 2006 (family 3)
c.137G>T (p.Ser46Ile)	3x NL	3	
c.2192_2196del	3x NL	3	
c.219_220dup	3x NL	3	
c.24-12_107delinsAAAT	2x NL	2	
c.1831dup	2x NL	2	
Deletion exon 3-7	2x NL	2	
Deletion exon 10	2x NL	2	Hendriks, 2006 (family 2)
Whole PMS2 deletion	2x NL	2	Hendriks, 2006 (family 1)
c.2113G>A (p.Glu705Lys)	2x S	2	
c.823C>T (p.Gln275X)	2x NL	2	
unknown	1x NL	1	
c.325dup	1x NL	1	
c.1111_1113delinsTTTA	1x DK	1	
c.2156del	1x N	1	
c.861_864del	1x NL	1	Hendriks, 2006 (family 7)
c.1079_1080del	1x NL	1	
c.2117del	1x NL	1	
c.247_250dup	1x NL	1	
c.904_911del	1x NL	1	
c.658dup	1x NL	1	
c.780del	1x E	1	Borras, 2013 (family D)
Deletion exon 1	1x NL	1	
Deletion exon 2-15	1x NL	1	
Deletion exon (3-)5-15	1x NL	1	
Deletion exon 1-10	1x D	1	
Deletion exon 6-7	1x NL	1	
Deletion exon 11	1x DK	1	
Deletion exon 6	1x E	1	Borras, 2013 (family C)
Duplication exon 11+12	1x N	1	

SUPPLEMENTARY TABLE 2 *PMS2* mutations

Description of PMS2 mutation[1]	Nationality of index patients[2]	Total nr of families	Family description reported before[3]
c.2444C>T (p.Ser815Leu)	1x NL	1	
c.319C>T (p.Arg107Trp and splice effect)	1x NL	1	
c.2404C>T (p.Arg802X)	1x NL	1	
c.2155C>T (p.Gln719X)	1x NL	1	
c.804-60_804-59insJN866832.1 (retrotranspositional insertion of SVA repeat)	1x NL	1	Hendriks, 2006 (family 4); van der Klift, 2012
c.989-1G>T (splice mutation)	1x N	1	Grindedal, 2014
c.989-2A>G (splice mutation)	1x E	1	Borras, 2013 (family G)
c.163+2T>C (splice mutation)	1x NL	1	
c.903G>T (splice mutation)	1x NL	1	
c.2445+1G>T (splice mutation)	1x NL	1	
c.1144+2T>A (splice mutation)	1x NL	1	Hendriks, 2006 (family 5)
c.354-1G>A (splice mutation)	1x NL	1	
c.251-2A>C (splice mutation)	1x NL	1	
c.164-2A>G (splice mutation)	1x E	1	Borras, 2013 (family B)
c.1A>G (variant in start codon)	1x E	1	Borras, 2013 (family A)
c.736_741delinsTGTGTGTGAAG AND complete gene deletion (compound heterozygous)	bi-allelic index (NL)	1	Herkert, 2011
c.137G>T AND c.2174+1G>A (compound heterozygous)	bi-allelic index (NL)	1	Herkert, 2011
c.137G>T AND c.736_741delinsTGTGTGTGAAG (compound heterozygous)	bi-allelic index (NL)	1	Leenen, 2011
c.2174+1G>A (homozygous)	bi-allelic index (NL)	1	Herkert, 2011
Deletion exon 3-7 deletion AND deletion exon 1-11(-15) (compound heterozygous)	bi-allelic index (NL)	1	Herkert, 2011
c.219_220dup (homozygous)	bi-allelic index (NL)	1	
c.325dup AND c.825A>G (splice mutation) (compound heterozygote)	bi-allelic index (NL)	1	Johannesma, 2011
c.24-2A>G (splice mutation) (homozygous)	Bi-allelic index (E)	1	

[1] Nomenclature according to Human Genetic Variation Society (HGVS) approved guidelines (www.hgvs.org/mutnomen) with reference to NM_000535.5, accept for the large deletions or duplications. Large deletions and duplications were in most cases detected with the older MLPA kit P008 (MRC Holland) that lacks reliable probes for PMS2 exon 3, 4, 12-15. Therefore, the exact range of exon deletions was not always established. Although for some large deletions the breakpoints have been characterized, we did not include this information.
[2]D=Germany, DK=Denmark, E=Spain, N=Norway, NL=Netherlands, S=Sweden
[3]references not in main text: Hendriks, 2006; Leenen, 2011; van der Klift, 2012

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2.2

Cancer risks for PMS2-associated Lynch syndrome

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Sanne W ten Broeke, Heleen M. van der Klift, Carli M.J. Tops, Stefan Aretz, Inge Bernstein, Daniel D. Buchanan, Albert de la Chapelle, Gabriel Capella, Mark Clendenning, Christoph Engel, Steven Gallinger, Encarna Gomez Garcia, Jane C. Figueiredo, Robert Haile, Heather L. Hampel, John L. Hopper, Nicoline Hoogerbrugge, Magnus von Knebel Doeberitz, Loic Le Marchand, Tom G.W. Letteboer, Mark A. Jenkins, Annika Lindblom, Noralane M. Lindor, Arjen R. Mensenkamp, Pål Møller, Polly A. Newcomb, Theo A.M. van Os, Rachel Pearlman, Marta Pineda, Nils Rahner, Egbert J.W. Redeker, Maran J.W. Olderode-Berends, Christophe Rosty, Hans K. Schackert, Rodney Scott, Leigha Senter, Liesbeth Spruijt, Verena Steinke-Lange, Manon Suerink, Stephen Thibodeau, Yvonne J. Vos, Anja Wagner, Ingrid Winship, Frederik J. Hes, Hans F.A. Vasen, Juul T. Wijnen, Maartje Nielsen, and Aung Ko Win

ABSTRACT

Purpose

Lynch syndrome due to pathogenic variants in the DNA mismatch repair genes *MLH1*, *MSH2* and *MSH6* is predominantly associated with colorectal and endometrial cancer, although extra-colonic cancers have been described within the Lynch tumor spectrum. However, the age-specific cumulative risk (penetrance) of these cancers is still poorly defined for *PMS2*-associated Lynch syndrome. Using a large dataset from a worldwide collaboration, our aim was to determine accurate penetrance measures of cancers for carriers of heterozygous pathogenic *PMS2* variants.

Patients and methods

A modified segregation analysis was conducted that incorporated both genotyped and non-genotyped relatives, with conditioning for ascertainment to estimates corrected for bias. Hazard ratios (HR) and corresponding 95% confidence intervals (CIs) were estimated for each cancer site for mutation carriers compared with the general population, followed by estimation of penetrance.

Results

In total, 284 families consisting of 4878 first- and second-degree family members were included in the analysis. *PMS2* mutation carriers were at increased risk for colorectal cancer (cumulative risk to age 80 of 13% (95% CI: 7.9-22%) for males and 12% (95% CI: 6.7-21%) for women); and endometrial cancer (13% (95% CI: 7.0-24%)), compared with the general population (6.6%, 4.7% and 2.4%, respectively). There was no clear evidence of an increased risk of ovarian, gastric, hepatobiliary, bladder, renal, brain, breast, prostate or small bowel cancer.

Conclusion

Heterozygous *PMS2* mutation carriers were at small increased risk for colorectal and endometrial cancer but not for any other Lynch syndrome-associated cancer. This finding justifies that *PMS2*-specific screening protocols could be restricted to colonoscopies. The role of risk-reducing hysterectomy and bilateral salpingo-oophorectomy for *PMS2* mutation carriers needs further discussion.

INTRODUCTION

Lynch syndrome is most commonly associated with colorectal cancer and endometrial cancer. However, when first described in 1913, the observation of the co-occurrence of gastric cancer and endometrial cancer led to the initial identification of these families, underlining the apparently diverse phenotype.[1] The genetic background of Lynch syndrome is now known and it is caused by heterozygous germline mutations in one of the four mismatch repair (MMR) genes, *MLH1*, *MSH2*, *MSH6* and *PMS2*, or *EPCAM* deletions. The broad Lynch syndrome-associated tumor spectrum includes not only colorectal cancer and endometrial cancer but also gastric, ovarian, small bowel, brain, urothelial cell, skin, pancreas, prostate and biliary tract cancers.[2, 3] The involvement of germline MMR mutations in the development of breast cancer is still a subject of debate.[4-7] Although the reported cumulative risk (penetrance) to age 70 years for these non-colorectal, non-endometrial cancers in MMR gene mutation carriers is generally below 10%, mutation carriers still have a higher risk relative to the general population.[3] The Lynch syndrome-associated tumor phenotypes and their penetrance could depend on the type of MMR gene mutated or the specific variant.[8, 9]

For heterozygous *PMS2* mutation carriers, accurate estimation of penetrance, especially for extra-colonic cancers, has been hampered both by difficulties in variant analysis related to the existence of multiple pseudogenes, and perhaps more importantly, by problems in identifying *PMS2* mutation carriers due to a markedly lower penetrance.[10-12] Our previous study of penetrance for *PMS2* mutation carriers, using 98 *PMS2* families ascertained through family cancer clinics in several European countries, reported standardized incidence ratios for extra-colonic cancers and found an increased *PMS2*-related risk of cancer of the small bowel, ovaries, renal pelvis and – most notably – of the breast.[11] Although that study presented the largest dataset then available, we were unable to generate reliable estimates of penetrance for these cancers due to their infrequency. In addition, there was an ascertainment bias in this cohort due to the recruitment via family cancer clinics. Another study from Iceland reported significant increases in the risk of colorectal, endometrial and ovarian cancer for two pathogenic *PMS2* founder variants.[13] This study and others have reported relatively high prevalence of *PMS2* variants in the population.[13-15] Thus underlining the need for *PMS2*-specific cancer risks.

In the current study, we have expanded the previous study database to 284 families, including several that were identified through a population-based ascertainment, with

the aim of generating accurate penetrance estimates of colorectal, endometrial and other cancers for *PMS2*-associated Lynch syndrome patients.

METHODS

Data collection

European dataset

Pedigree data on families with a segregating pathogenic variant were originally collected between 2009 and 2012, as previously described.[11] These data were supplemented with *PMS2* families identified between 2012 and 2017. Briefly, data were collected in collaboration with the Netherlands Foundation for the Detection of Hereditary Tumors and with clinical genetic departments in the Netherlands, Norway, Germany, Sweden, Denmark and Spain. Data collection from patient records included demographic data, family pedigrees, age and location of cancer diagnosis, polypectomy, and hysterectomy if applicable. When available, clinical and pathological diagnoses were confirmed using patient records. Data collection and subsequent analysis protocols were approved by the local ethical review board (Leiden University Medical Center Ethics Review Board, protocol ID: P01.019).

Ohio State datasets

For the Ohio State datasets, the first set of patients included both population-based colorectal and endometrial cancer patients from Columbus, Ohio as described elsewhere[10, 16-19] and cancer patients identified at family cancer clinics with absence of PMS2 only on IHC. The second set of patients from Ohio included only population-based colorectal and endometrial cancer patients from 50 hospitals throughout the state of Ohio as described previously.[20] All patients provided informed consent (Ohio State University Biomedical Sciences Institutional Review Board protocol IDs: 1999C0051, 1999C0245 and 2012C0123).

CCFR dataset

The study cohort from the Colon Cancer Family Registry has been described in detail elsewhere[21, 22,] and at www.coloncfr.org. Between 1998 and 2012, the Colon Cancer Family Registry recruited families via population-based probands, recently diagnosed with colorectal cancer, in state or regional population cancer registries in the USA (Washington, California, Arizona, Minnesota, Colorado, New Hampshire, North Carolina, and Hawaii), Australia (Victoria) and Canada (Ontario). In addition, clinic-

based probands were enrolled from multiple-case families referred to family cancer clinics in the USA (Mayo Clinic, Rochester, Minnesota, and Cleveland Clinic, Cleveland, Ohio), Canada (Ontario), Australia (Melbourne, Adelaide, Perth, Brisbane, Sydney and Newcastle) and New Zealand (Auckland). Probands were asked for permission to contact their relatives to seek their enrolment in the Cancer Family Registry (detailed in Newcomb *et al.*[21]). Informed consent was obtained from all study participants, and the study protocol was approved by the institutional research ethics review board at each registry. Information on demographics, personal characteristics, personal and detailed family history of cancer in first- and second-degree relatives, cancer-screening history, history of polyps, polypectomy, and other surgeries was obtained by questionnaires from all probands and participating relatives. Participants were followed approximately every 5 years after baseline to update this information. For the current study, each individual's lifetime cancer history was based on the most recent data (baseline or most recent follow-up). Reported cancer diagnoses and age at diagnosis were confirmed using pathology reports, medical records, cancer registry reports, and death certificates, where possible.

Mutation analysis and clinical variant classification

Probands included in the cohorts were screened for point mutations as well as large genomic rearrangements in the *PMS2* gene (see supplemental methods). Relatives of probands were tested for the specific family mutation. A detailed description of specific variants detected and their classification can be found in Supplementary Table 1 and 2

Statistical analysis

For estimation of the hazard ratios (HRs) and age-specific cumulative risks (penetrance), we used a modified segregation analysis.[23] This analytical method is not subject to population stratification, can rigorously adjust for ascertainment, and uses data on all study participants, whether genotyped or not, thereby maximizing statistical power. Models were fitted by the method of maximum likelihood with the statistical package MENDEL 3.2.[24] Estimates were appropriately adjusted for the ascertainment of families using a combination of retrospective likelihood and ascertainment-corrected joint likelihood. A conditional likelihood was maximized in which each pedigree's data were conditioned on the proband's *PMS2* mutation status, cancer history and ages of cancer diagnoses (for population-based families) or on the proband's *PMS2* mutation status and the cancer history and ages of cancer diagnoses of all family members (for clinic-based families).

For the purposes of analysis, we restricted included subjects to the first- and second-degree relatives of the probands. Observation time started at birth and stopped at age at diagnosis of cancer for affected, and last known age or age at death for unaffected family members. Because age information for each family member was required for the pedigree analysis, missing values were estimated using a defined protocol as follows. If an exact age was unknown but an age range was provided, age was estimated as the midpoint of that range. If age at diagnosis was unknown, it was assumed to be the same as age at death (if the relative was deceased) or the mean age at diagnosis for the specific cancer (if the relative was alive and older than the mean age at diagnosis). For relatives for whom last known age was unknown, ages were censored at the time they were last known to be alive (e.g., at the age at a cancer diagnosis). In the absence of any age information, it was assumed that both parents of the proband were born in the same year, that years of birth differed by 25 years in each generation (e.g., at birth of proband, parents were aged 25 years and grandparents were aged 50 years), and the ages of the siblings were the same. As a sensitivity analysis, we conducted analyses with and without imputing missing age, the results did not differ materially and therefore results from the non-imputed analysis were not shown in detail.

To calculate HRs, we used a likelihood-based approach in which age-specific incidence for *PMS2* mutation carriers was divided by that for non-carriers. Incidence rates for non-carriers were assumed to be the same as age-, sex- and country-specific population incidence rates (Australia, Canada, USA, The Netherlands, Germany) for the period 1998-2002, as obtained from Cancer Incidence in Five Continents.[25] The period of 1998-2002 was selected for analysis because it was the closest available dataset to the mean calendar year of cancer diagnoses in the sample. For each cancer, the age at cancer diagnosis was modeled as a random variable whose hazard was the relevant population incidence multiplied by a cancer-specific HR. For colorectal cancer, HRs for carriers were assumed to be continuous, piece-wise linear functions of age which are constant before age 40 years, linear in the intervals 40-50, 50-60, 60-70 and constant after age 70 years. For all other cancer sites, HRs were assumed to be independent of age. HRs for colorectal cancer, endometrial cancer, and other cancers were estimated simultaneously to allow proper adjustment for colorectal cancer-based ascertainment schemes when estimating the risks of non-colorectal cancers and to increase power (by helping the model identify likely carriers from the placement of Lynch syndrome-associated cancers within each family). HRs were assumed to be independent of country of recruitment.

Age-specific cumulative risks (penetrance) of each cancer site for *PMS2* mutation carriers were calculated separately for males and females, using the formula:

1 - exp $\int_0^{80} 0\lambda(t)\,dt)$ is the HR multiplied by the US population incidence.[26] Corresponding confidence intervals (CIs) were calculated using a parametric bootstrap. More specifically, 5,000 draws were taken from the multivariate normal distribution that the maximum likelihood estimates would be expected to follow under asymptotic likelihood theory. For each age, corresponding values of the cumulative risk were calculated and the 95% CI for the cumulative risks to that age were taken to be the 2.5th and 97.5th percentile of this sample.

TABLE 1 The study dataset description

	No. of family members		
	Total	Male	Female
Probands (= no. of families)	284	149	136
FDR	1904	953	951
SDR	2974	1487	1487
Confirmed *PMS2* mutation carriers	513	209	304
FDR	339	128	211
SDR	174	81	93
Confirmed *PMS2* non-carriers	404	167	237
FDR	230	100	130
SDR	174	67	107

FDR: first-degree relative. SDR: second-degree relative.

RESULTS

The final analysis included 284 families (211 from the European, 19 from the Ohio State and 54 from the CCFR dataset), with 1904 first- and 2974 second-degree family members, in which 513 were confirmed carriers (Table 1). The numbers and mean ages at diagnosis of each cancer site in first- and second-degree relatives are depicted in Table 2.

Colorectal cancer

PMS2 mutation carriers were at increased risk of developing colorectal cancer, with a HR depending on age and sex of the mutation carrier; 6.51 (95% CI: 2.03-20.9) for males aged <40, 1.70 (95% CI: 0.89-3.24) for males aged >70, 6.48 (95% CI: 2.24-18.8) for females aged <40, and 2.23 (95% CI: 1.21-4.12) for females aged >70). Estimated cumulative risks of colorectal cancer to age 80 for PMS2 mutation carriers were approximately 13% (95% CI: 7.9-22%) for male carriers and 12% (95% CI: 6.7-21%) for female carriers (general population 6.6% and 4.7%, respectively) (Figure 1A).

TABLE 2 The number and mean ages at diagnosis of each cancer site in the first- and second-degree relatives of probands

	FDR (n=1904)		SDR (n=2974)	
Cancer	No.	Mean age at diagnosis, years (SD)	No.	Mean age at diagnosis, years (SD)
Colorectal	116	59.6 (14.7)	112	62.7 (3.0)
Endometrial	33	55.7 (9.04)	21	54.8 (13.7)
Ovarian	9	52.2 (14.8)	5	41.6 (22.8)
Brain	18	42.3 (26.9)	10	56.3 (26.0)
Hepatobiliary	5	56.2 (13.6)	3	60.7 (9.87)
Gastric	14	57.8 (8.72)	11	57.3 (11.0)
Bladder	7	71.7 (14.5)	5	70.0 (14.3)
Breast	47	58.1 (12.0)	50	59.2 (13.5)
Prostate	19	70.7 (12.2)	24	69.8 (14.1)
Renal	7	65 (13.7)	5	61.2 (10.8)
Small bowel	4	45.0 (9.6)	1	38

FDR: first-degree relative. SDR: second-degree relative. SD: standard deviation

Gynecological cancers

PMS2 mutation carriers were also at small increased risk of endometrial cancer, with a HR of 5.73 (95% CI: 2.98-11.0) and estimated cumulative risk to age 80 of approximately 13% (95% CI: 7.0-24%), compared with females from the general population (2.4%) (Figure 1B). There was no clear evidence of increase in the risk of ovarian cancer (HR: 1.52; 95% CI: 0.45-5.05) (Figure 2).

Other cancers

There was no clear increase in risk of gastric, hepatobiliary, bladder, renal, brain, breast or prostate cancer for PMS2 mutation carriers (HR for each cancer shown in Figure 2). There were too few occurrences of small bowel cancer (n=5) to generate a HR.

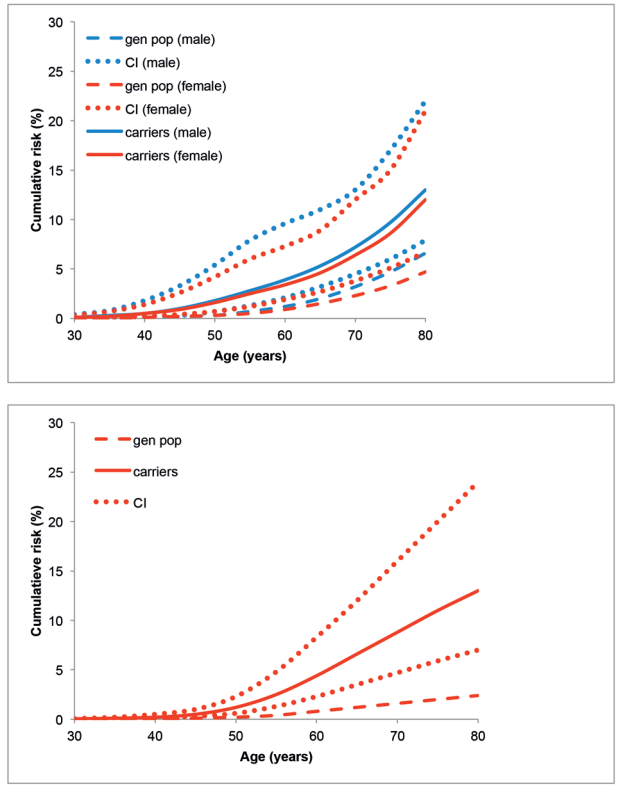

FIGURE 1 Cumulative risks (unbroken lines) and corresponding 95% confidence intervals (dotted lines) of (A) colorectal cancer and (B) endometrial cancer for heterozygous PMS2 mutation carriers, and for the US general population ('gen pop', dashed lines). Blue and red represent males and females, respectively.

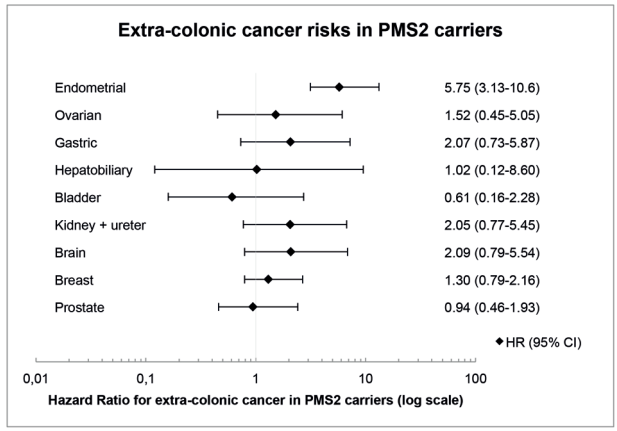

FIGURE 2 Hazard ratios and corresponding 95% confidence intervals of extra-colonic cancers for PMS2 mutation carriers

DISCUSSION

Based on the results from this large, international study of heterozygous *PMS2* mutation carriers, the *PMS2*-associated Lynch syndrome spectrum appears to be restricted to colorectal and endometrial cancer only, underlining the distinct phenotype for *PMS2* mutation carriers. We have also shown that *PMS2* mutation carriers have much lower cancer risks compared with other MMR gene mutation carriers.

The previous two studies of *PMS2* mutation carriers have estimated cumulative risks to age 70 years of 11-20% for colorectal cancer and 12-15% for endometrial cancer.[10, 11] Our current analysis has confirmed that *PMS2* carriers are at small increased risk of colorectal and endometrial cancer. These penetrance estimates are considerably lower than those for other MMR gene mutation carriers, which have been estimated at 35-55% for colorectal cancer and 10-45% for endometrial cancer.[3] A recent report from the Prospective Lynch Syndrome Database (PLSD) described cancer risk and survival for all Lynch syndrome patients, irrespective of the underlying gene variant.[7] This report included 124 *PMS2* mutation carriers, with 524 observation years. The findings support our study data in that endometrial cancer was the sole cancer type observed. Notably, colorectal cancer did not occur in any of the *PMS2* mutation carriers undergoing regular colonoscopic screening. This, together with our penetrance estimates, could justify consideration of less frequent colonoscopy screening for PMS2 mutation carriers. This, together with our low penetrance estimates (Figure 1) could justify modification of the colonoscopy surveillance protocol, for example starting at age 35-40 years, every two-three years, similar to what has been proposed in the NCCN guidelines.[27]

The PLSD database further showed that endometrial cancer survival for all MMR pathogenic variant carriers was excellent, with a 10-year survival of 93% (95% CI: 85-97%). The reported survival for ovarian cancer in Lynch syndrome patients was lower, at 74% (95% CI: 44-90%), but still better than that for sporadic ovarian cancer cases. Current surveillance guidelines advise that risk-reducing hysterectomy and bilateral salpingo-oophorectomy should be considered in women with Lynch syndrome, because transvaginal ultrasound with or without biopsies are ineffective in reducing risk of endometrial cancer and ovarian cancer, and might not have a strong influence on survival.[28] The good survival rates for endometrial cancer, combined with the data presented in the current study showing no evidence of a clinically relevant increase in ovarian cancer risk for *PMS2* mutation carriers, raises questions about the justification of risk-reducing hysterectomy and bilateral salpingo-oophorectomy, which may be too rigorous in carriers of heterozygous pathogenic *PMS2* mutations.

In our previous study of *PMS2*-associated Lynch syndrome patients, we found increased

standardized incidence ratios (SIRs) for cancer of the small bowel, ovary, renal pelvis and of the breast.[11] However, that study was limited by inclusion of confirmed mutation carriers identified through family cancer clinics and a limited number of cancer events. The first factor in particular could have been a potential source of ascertainment bias as a strong family history of cancer and/or early-onset disease increases the likelihood of inclusion and *PMS2* testing. In that report, we did not adjust for this potential ascertainment bias when estimating SIRs for extra-colonic cancers. Traditionally, a strong family history of colorectal and endometrial cancers prompted suspicion of Lynch syndrome and consequently patients were tested for tumor MMR deficiency followed by MMR gene mutation testing. Currently, family histories of *other* cancers are increasingly being ascertained by clinical genetic centers for further evaluation as possible Lynch syndrome. Therefore, it is important to take into account and adjust for ascertainment bias when estimating risks of cancers other than colorectal or endometrial cancer. Furthermore, pathogenic *PMS2* variants are relatively frequently observed using extensive gene panel testing for women with hereditary breast and ovarian cancer.[29-32] Nevertheless, we could not confirm an increased SIR for breast cancer in the present study (HR: 1.30; 95% CI: 0.79-2.16) and the discrepancy with earlier reports can probably be attributed to a high prevalence of *PMS2* (and *MSH6*) mutations in the general population. Conversely, the relative infrequency of *PMS2* variants among Lynch syndrome patients can be explained by the milder phenotype, which makes ascertainment by family cancer clinics less likely.

The current study is the largest to date in estimation of cancer risks for heterozygous *PMS2* mutation carriers. Previous studies have shown that analyses of retrospective data from clinic-based families i.e., ascertained due to family history of cancer, without (statistical) adjustment can lead to overestimation of cancer risks for mutation carriers.[33-35] In the current study, we used a high-level statistical approach to properly adjust for such ascertainment bias. The modified segregation method used data on all family members, regardless of whether they were genotyped, thereby maximizing statistical power while avoiding survival bias.

A potential limitation of the current study was the use of unverified cancer diagnoses that were self- or proband-reported, thus potentially affecting the accuracy of estimates. However, previous studies showed a high probability of agreement between proband-reported cancer status in first-degree relatives and the validated report (for example, 95.4% (95% CI: 92.6-98.3) for breast cancer, 83.3% (95% CI: 72.8-93.8) for ovarian cancer; and 79.3% (95% CI: 70.0-88.6) for prostate cancer).[36] A further possible limitation is that our analysis did not take into account a potential role for genetic or

environmental modifiers of risk. The existence of such modifiers is plausible, as a high degree of variability in penetrance and phenotype has been observed[23], and modifiers of cancer risk such as lifestyle, genetic modifiers and phenotype-genotype correlations have been identified previously.[37-40] Our study estimated cancer risks of all variants combined, however it is plausible that not all *PMS2* variants confer the same risk. A previous study in a selection of the currently analyzed cohort investigated genotype-phenotype correlations and found no difference in risk between the group of variants with retained vs. loss of RNA expression.[40] However, this study did report that those carrying a variant with loss of RNA expression were diagnosed with colorectal cancer on average 9 years younger than those with retained expression. The influence of these modifiers is still not well understood, especially for *PMS2* mutation carriers, although efforts are currently on-going to better define such factors and their potential role in modifying disease risk. Our study results highlight that studies of penetrance modifiers should take the specific MMR gene mutated into account.

In the current study, we analyzed the first dataset large enough to generate the unbiased estimates for the risk of each extra-colonic cancer for *PMS2* mutation carriers. Our results show that *PMS2* carriers are only at small increased risk of colorectal and endometrial cancer. This underlines the importance of gene-specific genetic counseling of Lynch syndrome patients and the development of appropriate clinical guidelines.

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2

SUPPLEMENTAL METHODS

PMS2 mutation analysis

European cohort

This dataset consisted of clinically ascertained families where variant analysis was initiated due to (histological) pre-screening by immunohistochemistry and/or microsatellite instability, usually because a family met Bethesda criteria.[1] *PMS2* mutation screening was performed using Sanger sequencing of PCR or RT-PCR products, and was limited to exons and exon-intron boundaries. Large deletions and duplications were mainly detected by multiplex ligation-dependent probe amplification (MLPA). Comprehensive strategies were applied to avoid unreliable mutation detection caused by interference from pseudogene sequences and frequent gene conversion events.[2]

Ohio State cohort

In the Ohio State cohorts, testing for germline mutations in *MLH1*, *MSH2*, *EPCAM*, *MSH6*, and *PMS2* was performed for all population-based probands who had a colorectal tumor that showed impaired MMR function, as evidenced by tumor microsatellite instability (MSI) or absence of MMR protein expression on immunohistochemical analysis after ruling out MLH1 promoter methylation. In the first cohort, PMS2 testing was performed as published previously.[3] In the second cohort, PMS2 testing was performed by one of two commercial laboratories.[4]

CCFR cohort

Testing for germline mutations in *MLH1*, *MSH2*, *MSH6*, and *PMS2* was performed for all population-based probands who had a colorectal tumor that showed impaired MMR function, as evidenced by tumor microsatellite instability (MSI) or absence of MMR protein expression on immunohistochemical analysis. Testing was also performed for colorectal cancer-affected participants from clinic-based families regardless of tumor MSI or MMR-protein expression status. Sanger sequencing or denaturing high performance liquid chromatography, followed by confirmatory DNA sequencing, was performed to screen for mutations in the *MLH1*, *MSH2*, and *MSH6* genes. Large duplication and deletion mutations were detected by Multiplex Ligation Dependent Probe Amplification (MLPA) according to the manufacturer's instructions (MRC Holland, Amsterdam, The Netherlands).[5-7] *PMS2* mutation testing involved a modified protocol from Senter et al [8], in which exons 1 to 5, 9, and 11 to 15 were amplified in 3 long-range polymerase chain reactions (PCRs), followed by nested exon-specific PCR and sequencing. The remaining exons (7, 8, and 10) were amplified and sequenced directly

from genomic DNA. Large-scale deletions in *PMS2* were detected using the P008-A1 MLPA kit (MRC Holland, Amsterdam, The Netherlands).[9] Relatives of probands with a pathogenic MMR germline mutation[10] who provided a blood sample were tested for the specific mutation identified in the proband.

Variant classification

The InSiGHT Colon Cancer Gene Variant Database (https://insight-database.org/variants/PMS2; last update 20 November 2017) was consulted for presence and clinical classification of the 106 *PMS2* variants reported as disease causing in the families included in this study (supplementary table S1). Fifty-four variants were present with clinical classification in the database including 42 pathogenic (class 5), 10 likely pathogenic (class 4) and 2 variants of uncertain significance (VUS; class 3). Sixteen variants were present but not classified; thirty-six variants were not reported to the Insight database at time of consultation (14 December 2017). Most of these variants could be classified as (likely) pathogenic by applying the variant classification criteria formulated by the InSiGHT Variant Interpretation Committee (VIC).[11] For 9 variants that could not a priori be classified immediately as pathogenic (including two missense variants classified as VUS by the Insight VIC) we provide additional evidence that suggests pathogenicity in supplementary table S2. Variants found in the *PMS2* gene were classified for pathogenicity as reported by the InSiGHT Colon Cancer Gene Variant Database (http://insight-group.org/variants/classifications/) or by applying their classification criteria.[11] The majority of the variants were classified as (likely) pathogenic. Three missense variants (NM_000535.5: c.319C>T p.Arg107Trp, c.2113G>A p.Glu705Lys, and c.2444C>T p.Ser815Leu), not yet classified or classified as a variant of uncertain significance (VUS), were included because additional evidence suggested likely pathogenicity.[2] See Supplementary Table 1 for a description of *PMS2* variants.

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2

SUPPLEMENTARY TABLE 1 PMS2 variants reported as disease-causing in the families included in this study

exon/ intron	PMS2 variant[a]	predicted protein effect	type of variant	InSiGHT class[b]	Nr of families[c]
1	c.1A>G	p.Met1?	start codon	4	2
2	c.137G>T	p.Ser46Ile	missense	4	15
intron 2	c.163+2T>C		canonical splice variant	4	1
intron 2	c.164-2A>G		canonical splice variant	4	1
2	c.24-12_107delinsAAAT	p.Ser8Argfs*5	frameshift	5	3
3	c.219_220dup	p.Gly74Valfs*3	frameshift	5	4
intron 4	c.354-1G>A		canonical splice variant	4	1
5	c.400C>T	p.Arg134*	nonsense	5	2
6	c.697C>T	p.Gln233*	nonsense	5	5
intron 6	c.705+1G>T		canonical splice variant	4	1
7	c.736_741delinsTGTGTGTGAAG	p.Pro246Cysfs*3	frameshift	5	39
7	c.780del	p.Asp261Metfs*46	frameshift	5	1
7	c.802dup	p.Tyr268Leufs*31	frameshift	5	1
intron 7	c.804-60_804-59insJN866832.1		retrotransposal SVA insertion	5	3
8	c.861_864del	p.Arg287Serfs*19	frameshift	5	4
8	c.862_863del	p.Gln288Valfs*10	frameshift	5	1
8	c.903G>T	r.804_903del; p.Tyr268*	exonic splice variant	4	4
9	c.943C>T	p.Arg315*	nonsense	5	4
9	c.949C>T	p.Gln317*	nonsense	5	1
intron 9	c.989-1G>T		canonical splice variant	5	1
intron 9	c.989-2A>G		canonical splice variant	4	3
10	c.1021del	p.Arg341Glyfs*15	frameshift	5	1
10	c.1076dup	p.Leu359Phefs*6	frameshift	5	2
10	c.1079_1080del	p.Ile360Argfs*4	frameshift	5	1
intron 10	c.1144+2T>A	p.Glu330_Glu381del	canonical splice variant	4	1
11	c.1261C>T	p.Arg421*	nonsense	5	1
11	c.1738A>T	p.Lys580*	nonsense	5	1
11	c.1831dup	p.Ile611Asnfs*2	frameshift	5	11
11	c.1840A>T	p.Lys614*	nonsense	5	3
11	c.1882C>T	p.Arg628*	nonsense	5	10
11	c.1927C>T	p.Gln643*	nonsense	5	3
11	c.1939A>T	p.Lys647*	nonsense	5	6
intron 11	c.2007−1G>A		canonical splice variant	4	1
12	c.2113G>A	p.Glu705Lys	missense	3 (see supp tbl S2)	7
intron 12	c.2174+1G>A		canonical splice variant	5	4
13	c.2192_2196del	p.Leu731Cysfs*3	frameshift	5	7
14	c.2404C>T ;	p.Arg802*	nonsense	5	3
14	c.2444C>T	p.Ser815Leu	missense	3 (see supp tbl S2)	1
intron 2	c.163+5G>A		intronic splice variant	present, not classified (see supp tbl S2)	1
3	c.247_250dup	p.Thr84Ilefs*9	frameshift	present, not classified (class 5)	1

SUPPLEMENTARY TABLE 1 PMS2 variants reported as disease-causing in the families included in this study

exon/ intron	PMS2 variant[a]	predicted protein effect	type of variant	InSiGHT class[b]	Nr of families[c]
3	c.247_250dup	p.Thr84Ilefs*9	frameshift	present, not classified (class 5)	1
intron 3	c.251-2A>C		canonical splice variant	present, not classified (class 5)	1
4	c.319C>T	p.Arg107Trp	missense	present, not classified (see supp tbl S2)	1
4	c.325dup	p.Glu109Glyfs*30	frameshift	present, not classified (class 5)	4
7	c.746_753del	p.Asp249Valfs*2	frameshift	present, not classified (class 5)	1
8	c.823C>T	p.Gln275*	nonsense	present, not classified (class 5)	1
8	c.825A>G	r.804_825del, p.Ile269Alafs*31	exonic splice variant	present, not classified (see supp tbl S2)	1
8	c.856_857del	p.Asp286Glnfs*12	frameshift	present, not classified class 5)	1
9	c.904_911del	p.Val302Thrfs*4	frameshift	present, not classified (class 5)	1
11	c.1214C>A	p.Ser405*	nonsense	present, not classified (class 5)	1
12	c.2117del	p.Lys706Serfs*19	frameshift	present, not classified (class 5)	3
12	c.2155C>T	p.Gln719*	nonsense	present, not classified class 5)	2
intron 14	c.2445+1G>T		canonical splice variant	present, not classified (class 4)	5
15	c.2500_2501delinsG	p.Met834Glyfs*17	frameshift	present, not classified (see supp tbl S2)	1
intron 1	c.24-2A>G		canonical splice variant	not present (class 4)	1
intron 2	c.164-1G>C		canonical splice variant	not present (class 4)	1
3	c.215G>A	p.Gly72Glu	missense	not present (see supp tbl S2)	1
intron 4	c.354-2A>G		canonical splice variant	not present (class 4)	2
6	c.613C>T;	p.Gln205*	nonsense	not present (class 5)	1
6	c.658dup	p.Ser220Lysfs*29	frameshift	not present (class 5)	1

SUPPLEMENTARY TABLE 1 PMS2 variants reported as disease-causing in the families included in this study

exon/ intron	PMS2 variant[a]	predicted protein effect	type of variant	InSiGHT class[b]	Nr of families[c]
intron 6	c.705+2T>C		canonical splice variant	not present (class 4)	1
7	c.765C>A	p.Tyr255*	nonsense	not present (class 5)	2
7	c.781del	p.Asp261Metfs*46	frameshift	not present (class 5)	1
8	c.809C>G ;	p.Ser270*	nonsense	not present (class 5)	1
10	c.1067del	p.Lys356Argfs*4	frameshift	not present (class 5)	1
10	c.1107del	p.Lys369Asnfs*2	frameshift	not present (class 5)	2
10	c.1111_1113delinsTTTA	p.Asn371Phefs*11	frameshift	not present (class 5)	1
11	c.1151T>G	p.Leu384*	nonsense	not present (class 5)	1
11	c.1237_1238delinsT	p.Lys413*	frameshift	not present (class 5)	1
11	c.1239dup	p.Asp414Argfs*44	frameshift	not present (class 5)	2
11	c.1281del	p.His428Thrfs*20	frameshift	not present (class 5)	1
11	c.1492_1502del	p.Ser498Glyfs*3	frameshift	not present (class 5)	2
11	c.1687C>T	p.Arg563*	nonsense	not present (class 5)	1
11	c.1874del	p.Leu625*	frameshift	not present (class 5)	1
12	c.2156del	p.Gln719Argfs*6	frameshift	not present (class 5)	1
12	c.2161C>T	p.Gln721*	nonsense	not present (class 5)	1
13	c.2182_2184delinsG	p.Thr728Alafs*7	frameshift	not present (class 5)	1
14	c.2413C>T	p.Gln805*	nonsense	not present (class 5)	1
15	c.2520dup	p.Trp841Leufs*47	frameshift	not present (see supp tbl S2)	1
15	c.2521del	p.Trp841Glyfs*10	frameshift	not present (see supp tbl S2)	1
1	genomic deletion including exon 1		large genomic deletion	5	3
2	genomic deletion including exon 2		large genomic deletion	5	3
6	genomic deletion including exon 6		large genomic deletion	5	1
7	genomic deletion including exon 7		large genomic deletion	5	3
8	genomic deletion including exon 8		large genomic deletion	5	1
9	genomic deletion including exon 9		large genomic deletion	5	2
10	genomic deletion including exon 10		large genomic deletion	5	8
14	genomic deletion including exon 14		large genomic deletion	5	5
1_10	genomic deletion including exons 1-10		large genomic deletion	5	7
1_15	genomic deletion whole gene		large genomic deletion	5	6

SUPPLEMENTARY TABLE 1 PMS2 variants reported as disease-causing in the families included in this study

exon/ intron	PMS2 variant[a]	predicted protein effect	type of variant	InSiGHT class[b]	Nr of families[c]
10	genomic deletion including exon 10		large genomic deletion	5	8
14	genomic deletion including exon 14		large genomic deletion	5	5
1_10	genomic deletion including exons 1-10		large genomic deletion	5	7
1_15	genomic deletion whole gene (exons 1-15)		large genomic deletion	5	6
11_12	genomic deletion including exons 11-12		large genomic deletion	5	2
11_15	genomic deletion including exons 11-15		large genomic deletion	5	5
3_7	genomic deletion including exons 3-7		large genomic deletion	5	4
5_15	genomic deletion including exons 5-15		large genomic deletion	5	3
5_7	genomic deletion including exons 5-7		large genomic deletion	5	3
11_12	genomic duplication including exons 11-12		large genomic in tandem duplication	5	1
1_12	genomic deletion including exons 1-12		large genomic deletion	present, not classified (class 5)	2
2_4	genomic deletion including exons 2-4		large genomic deletion (in frame)	not present (class 4)	1
5_6	genomic deletion including exons 5-6		large genomic deletion (in frame)	not present (class 4)	1
6_8	genomic deletion including exons 6-8		large genomic deletion (in frame)	not present (class 4)	1
11	genomic deletion including exon 11		large genomic deletion	not present (class 5)	3
1_7	genomic deletion including exons 1-7		large genomic deletion	not present (class 5)	1
12_15	genomic deletion including exons 12-15		large genomic deletion	not present (class 5)	1
6_12	genomic deletion including exons 6-12		large genomic deletion	not present (class 5)	1
6_7	genomic deletion including exons 6-7		large genomic deletion	not present (class 5)	1
9_12	genomic deletion including exons 9-12		large genomic deletion	not present (class 5)	1

[a] Variant nomenclature according to HGVS guidelines (http://varnomen.hgvs.org/) with reference to NM_000535.5 for PMS2 accept for the large deletions or duplications. Large deletions and duplications were in some cases detected with the older MLPA kit P008 (MRC Holland) that lacks reliable probes for PMS2 exon 3, 4, 12-15. Therefore, the exact range of exon deletions was not always established. Although for some large deletions the breakpoints have been characterized, we did not include this information.

[b] Clinical variant class as reported on https://insight-database.org/variants/PMS2; last accessed on 14 December 2017; 5 = pathogenic, 4 = likely pathogenic, 3 = variant of uncertain significance. Classification of the variants not present or present but not yet classified in the InSiGHT database is given between brackets, using guidelines provided by https://www.insight-group.org/criteria/. Nonsense and frameshift mutations including large genomic deletions were classified as pathogenic (class 5). Canonical splice variants and large in-frame genomic deletions were classified as likely pathogenic (class 4). Additional evidence that suggests pathogenicity for variants that could not be classified a priori as (likely) pathogenic is provided in supplementary table S2.

[c] Six of the 284 families included index patients that were bi-allelic *PMS2* mutation carriers, of which four were compound heterozygous. For one family the description of the specific *PMS2* variant was not provided.

SUPPLEMENTARY TABLE 2 Additional evidence that suggests pathogenicity for nine PMS2 variants

location	PMS2 variant[a]	type of variant	number of families (this study)	Evidence suggestive for pathogenicity[b]
Intron 2	c.163+5G>A	intronic splice variant	1 (Denmark)	• Predicted decrease of splice site strength of canonical splice donor (-32.8%, Alamut Visual) • Minigene splicing assay shows skip of exon 2 (unpublished data) • Not in control population (ExAC, ESP, 1000G)
Exon 3	c.215G>A p.Gly72Glu	missense	1 (United States)	• Predicted as pathogenic by functional prediction software programs (PolyPhen-2, SIFT, aGVGD, MutationTaster) • Highly conserved on nucleotide and amino acid level. Located in ATP-binding domain • Not in control population (ExAC, ESP, 1000G) • Reported as likely pathogenic (Pearlman et al., 2017)
Exon 4	c.319C>T p.Arg107Trp	missense	1 (Netherlands)	• MMR-deficiency shown by in vitro mmr assay (van der Klift et al., 2016) • Incomplete aberrant splicing (van der Klift et al., 2015) • In trans with pathogenic PMS2 variant in a CMMRD patient (van der Klift et al., 2016)
Exon 8	c.825A>G r.804_825del p.Ile269Alafs*31	exonic splice variant	1 (Netherlands)	• Splice analysis (patient RNA and minigene splicing assay) showed activation of cryptic splice acceptor resulting in a skip of the first 22 basepairs of exon 8 (van der Klift et al., 2015) • In trans with pathogenic PMS2 variant in a CMMRD patient (Johannesma et al., 2011)
Exon 12	c.2113G>A p.Glu705Lys	missense	7 (5x Sweden; 2x United States 2x)	• Predicted as pathogenic by functional prediction software programs (PolyPhen-2, SIFT, aGVGD, MutationTaster) • Highly conserved on nucleotide and amino acid level. Located in metal-binding motif essential for PMS2 endonuclease function • Heterozygous in CMMRD patient (Miyaki et al., 1997) • Abrogated repair function demonstrated in Deschênes et al., 2007 (complementation assay) and in Drost et al., 2013 (in vitro mismatch repair assay) • In a mouse model associated with strong cancer predisposition (van Oers et al., 2010) • Recurrent in Swedish Lynch syndrome population (Lagerstedt-Robinson et al., 2016)
Exon 14	c.2444C>T p.Ser815Leu	missense	1 (Netherlands)	• Predicted as pathogenic by functional prediction software programs (PolyPhen-2, SIFT, aGVGD, MutationTaster) • Highly conserved on nucleotide and amino acid level. Located in MutL dimerisation domain • Not reported in large population cohorts (ExAC, ESP, 1000G) • MMR-deficiency shown by 2 different assays in 2 laboratories (van der Klift et al., 2016; González-Acosta et al., 2017). Diminished protein expression (González-Acosta et al., 2017) • Reported in 3 independent Lynch syndrome patients (van der Klift et al., 2016; González-Acosta et al., 2017) • MLA (performed for 1 family) showed LR of 1.83:1, leading to a posterior probability of pathogenicity of 0.993 (González-Acosta et al., 2017) • Homozygous in CMMRD patient (Suerink et al., 2018)

SUPPLEMENTARY TABLE 2 Additional evidence that suggests pathogenicity for nine PMS2 variants

location	PMS2 variant[a]	type of variant	number of families (this study)	Evidence suggestive for pathogenicity[b]
Exon 14	c.2444C>T p.Ser815Leu	missense	1 (Netherlands)	• Predicted as pathogenic by functional prediction software programs (PolyPhen-2, SIFT, aGVGD, MutationTaster) • Highly conserved on nucleotide and amino acid level. Located in MutL dimerisation domain • Not reported in large population cohorts (ExAC, ESP, 1000G) • MMR-deficiency shown by 2 different assays in 2 laboratories (van der Klift et al., 2016; González-Acosta et al., 2017). Diminished protein expression (González-Acosta et al., 2017) • Reported in 3 independent Lynch syndrome patients (van der Klift et al., 2016; González-Acosta et al., 2017) • MLA (performed for 1 family) showed LR of 1.83:1, leading to a posterior probability of pathogenicity of 0.993 (González-Acosta et al., 2017) • Homozygous in CMMRD patient (Suerink et al., 2018)
Exon 15	c.2500_2501 delinsG p.Met834Glyfs*17	Frameshift in last exon	1 (Canada)	• Frameshift from 29 AA upstream of canonical stop codon. The new reading frame ends in a STOP codon 16 positions downstream. • 2x reported in ClinVar as pathogenic (1 * evidence) • disrupts a highly conserved region in the C-terminal domain of PMS2 possibly disabling heterodimerization with MLH1 (Guerrette et al., 1999; Gueneau et al., 2013)
Exon 15	c.2520dup p.Trp841Leufs*47	Frameshift in last exon	1 (Sweden)	• Frameshift from 22 AA upstream of canonical stop codon. The new reading frame ends in a STOP codon 46 positions downstream. • disrupts a highly conserved region in the C-terminal domain of PMS2 possibly disabling heterodimerization with MLH1 (Guerrette et al., 1999; Gueneau et al., 2013)
Exon 15	c.2521del p.Trp841Glyfs*10	Frameshift in last exon	1 (Germany)	• Frameshift from 22 AA upstream of canonical stop codon. The new reading frame ends in a STOP codon 9 positions downstream. • 1x reported in ClinVar as likely pathogenic (1*evidence) • disrupts a highly conserved region in the C-terminal domain of PMS2 possibly disabling heterodimerization with MLH1 (Guerrette et al., 1999; Gueneau et al., 2013)

[a] Variant nomenclature according to HGVS guidelines (http://varnomen.hgvs.org/) with reference to NM_000535.5 for PMS2.
[b] data on conservation, splice prediction, functional prediction (PolyPhen-2, SIFT, aGVGD, MutationTaster), presence in contr population databases
(ExAC, ESP, 1000G) and in the ClinVar archive were obtained through Alamut Visual v.2.6 last accessed on 23-12-2017
Abbreviations: MMR = mismatch repair; CMMRD = constitutional mismatch repair deficiency;
MLA = multifactorial likelihood analysis; LR = likelihood ratio;
AA = amino acid

References:
Pearlman et al., 2017, JAMA Oncol 3: 464
van der Klift et al., 2015, Mol Genet Genomic Med 3:327–345
van der Klift et al., 2016, Hum Mutat 37:1162–1179

3

RISK MODIFYING FACTORS

3.1

SNP association study in PMS2-associated Lynch syndrome

Familial Cancer, 2017

Sanne W. ten Broeke, Fadwa A. Elsayed, Lisa Pagan, Maran J.W. Olderode-Berends, Encarna Gomez Garcia, Hans J.P. Gille, Liselot P. van Hest, Tom G.W. Letteboer, Lizet E. van der Kolk , Arjen R. Mensenkamp, Theo A. van Os, Liesbeth Spruijt, Bert J.W. Redeker, Manon Suerink, Yvonne J. Vos, Anja Wagner, Juul T. Wijnen, E.W. Steyerberg, Carli M.J. Tops, Tom van Wezel, Maartje Nielsen

ABSTRACT

Objective

Lynch syndrome (LS) patients are at high risk of developing colorectal cancer (CRC). Phenotypic variability might in part be explained by common susceptibility loci identified in Genome Wide Association Studies (GWAS). Previous studies focused mostly on *MLH1*, *MSH2* and *MSH6* carriers, with conflicting results. We aimed to determine the role of GWAS SNPs in *PMS2* mutation carriers.

Methods

A cohort study was performed in 507 *PMS2* carriers (124 CRC cases), genotyped for 24 GWAS SNPs, including SNPs at 11q23.1 and 8q23.3. Hazard ratios (HRs) were calculated using a weighted Cox regression analysis to correct for ascertainment bias. Discrimination was assessed with a concordance statistic in a bootstrap cross-validation procedure.

Results

Individual SNPs only had non-significant associations with CRC occurrence with HRs lower than 2, although male carriers of allele A at rs1321311 (6p21.31) may have increased risk of CRC (HR=2.1, 95%CI: 1.2-3.0). A polygenic risk score (PRS) based on 24 HRs had an HR of 2.6 (95%CI:1.5-4.6) for the highest compared to the lowest quartile, but had no discriminative ability (c statistic 0.52).

Conclusion

Previously suggested SNPs do not modify CRC risk in *PMS2* carriers. Future large studies are needed for improved risk stratification among Lynch syndrome patients.

INTRODUCTION

Lynch syndrome (LS) accounts for 2-4% of all CRCs and is characterized by a high risk for developing malignancies, most notably colorectal cancer (CRC) and endometrial cancer (EC). The underlying cause is a germline mutation in one of the mismatch repair (MMR) genes: *MLH1*, *MSH2* (*EPCAM*), *MSH6* or *PMS2*. Mutations in all MMR genes are associated with a significantly increased cancer risk compared to the general population, although *MSH6* and *PMS2* carriers show lower penetrance compared to *MLH1* and *MSH2* carriers.[1,3] Within and between family variability is commonly observed and a range of theories have been proposed to explain the phenomenon, such as genotype-phenotype correlations, parent-of-origin effects, lifestyle factors and the influence of common susceptibility loci. The latter, mainly single nucleotide polymorphisms (SNPs), were identified in genome wide association studies (GWAS) in large cohorts consisting of sporadic CRC cases.[4] Among these candidate SNPs, previous studies have identified statistically significant effects of multiple SNPs in LS patients, and independent studies replicated the effect of SNPs rs3802842 (11q23.1) and rs16892766 (8q23.3) among *MLH1* carriers.[5, 6] It should be noted, however, that others have failed to replicate these findings.[7, 8] Although the latter studies analyzed cohorts of similar size to our own, few or no *PMS2* carriers were included.[7] Due to a relatively low penetrance and high phenotypic variability, this specific subset of LS patients might be of particular interest.[9] In a previous study among 377 *PMS2* carriers, we found age at CRC diagnosis to vary widely (range 26-86 years) and mean age of index carriers and mutation-positive family members differed by 10 years.[3] In the current study, we aim to determine whether these SNPs modify CRC risk in a large cohort of *PMS2* mutation carriers.

MATERIAL AND METHODS

Sample collection

DNA extracted from leucocyte DNA was collected from 8 Dutch family cancer clinics. Index carriers included in this study were sent in between 2007 and 2016 to the Clinical Genetics department, because of a clinical suspicion of LS, e.g. LS-associated cancer at a young age and/or a positive family history. Mutation analysis was initiated based on the presence of histological hallmarks (microsatellite instability (MSI) or loss of PMS2 expression in the tumor) and/or when the family complied with the Bethesda Criteria.[10] Participating clinics provided DNA samples and clinical data on CRC, age at diagnosis,

other cancer development and polypectomy. Controls were defined as carriers that were tested pre-symptomatically, after a pathogenic mutation was identified in the index carrier of the family. All carriers are referred to gastroenterology departments after the diagnosis has been established, which then adhere to international surveillance guidelines, i.e. colonoscopies every 1-2 years from 25 years of age.[11] Data was analyzed anonymously. The study was approved by the medical ethical committee of Leiden University Medical Centre, protocol ID P01-019.

Genotyping

PMS2 genotyping in this cohort was carried out as previously described.[3] SNP genotyping was done at the LUMC laboratory using a KASp genotyping assay (LGC Genomics, Hoddesdon, UK). Primers were designed using Primerpicker (KBioscience, Hoddesdon, UK) and are available upon request. All oligonucleotides were obtained from Eurofins Genomics (Ebersberg, Germany). Genotypes were called using the CFX manager software v3.0 (Bio-Rad, Veenendaal, the Netherlands).

Statistical analysis

PMS2 carriers were analyzed as a birth cohort. A Cox-proportional hazards regression model was fitted to estimate hazard ratios (HRs), with age at CRC as endpoint and SNP genotype as independent variable. Patients without CRC were censored at the last age known to be alive. The HR was calculated separately for heterozygous and homozygous carriers of the risk allele, with homozygotes of the non-risk allele as reference category. We also calculated the per allele HR (additive model). Cox-regression analyses were also stratified for gender. These sub-analyses only includes a per allele (additive) model, due to multiple testing risks. Missing age at CRC diagnosis (n=3) was imputed using median age of CRC in the general population (age 70, n=2) or set at one year before death (n=1). The proportional hazards assumption was investigated by examining the scaled Schoenfeld residuals with a formal statistical test and by visual inspection.

Previous studies have described the oversampling of cases in clinic-based cohorts. Moreover, affected family members are more likely to be tested for the mutation and this too results in oversampling of cases. To adjust for this non-random sampling, we used a weighted cohort approach as previously described.[12] Standard errors were corrected for familial clustering of risk by using the Huber-White sandwich estimator.[13] We also calculated two polygenic risk scores (PRS)[14] based on 1) the odds ratios (ORs) reported in the meta-analysis by Ma et al (Supplemental Table 1) and 2) based on our HRs from the current study.[4] Kaplan Meier (KM) and Cox regression analysis

were concurrently performed. A concordance statistic was calculated to assess the discriminative value of the 24 SNP model. The optimism in the concordance statistic was estimated by fitting the model in each of 500 bootstrap samples (drawn with replacement), and validation in the original sample. Analyses were initially performed for patients with complete data. We also performed imputation of missing values based on the correlation structure between SNPs and with the outcome (transcan function in R software, version 3.2). Since results were similar, we only present complete case results.

Lastly, a post hoc power analysis was performed to assess the chance of finding significantly increased risks using the collected cohort, which contains all currently known *PMS2* mutation carriers in the participating centers (Supplemental Figure 1). We had at least 80% power to find an HR of 1.5 for the majority of SNPs. A more detailed description of the statistical analysis is available in the supplementary methods (Supplemental File 1). Statistical analyses were performed using STATA version 14 (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP) and R software (version 3.2, using the rms library).

RESULTS

Cohort

In total, 521 samples from carriers with a germline *PMS2* mutation were genotyped, derived from 152 families. Fourteen patients were excluded because 1) they were younger than 25 years at censoring and were therefore not yet at risk of developing CRC (n=11) or 2) insufficient clinical data was available (n=3, including one CRC case). The analyzed cohort consisted of 124 cases (*PMS2* carriers with CRC) and 383 controls (*PMS2* carriers without CRC), with attributed person years of 6527 and 19549, respectively. Person-years were calculated until age of CRC for cases (*PMS2* carriers with CRC, n=125), and age at polypectomy, age of death, or last known age alive (whichever occurred first) for controls (*PMS2* carriers without CRC, n=1, n=1 and n=381 respectively). The mean age was 52.5 for CRC cases and 51.0 for non-cases (Table 1). For a detailed description of the families including genotypes see Supplemental Tables 2a+2b.

TABLE 1 Cohort description

	No CRC – controls (n=383)	CRC - cases (n=124)	All (n=507)
Sex			
Male	133 (34%)	60 (48%)	193 (38%)
Female	250 (65%)	64 (52%)	314 (62%)
Age (CRC or censoring)			
Mean (s.d.)	51.0 (14.2)	52.5 (12.7)	
Range	25-88	27-88	
Index carrier			
Yes	38 (10%)	89 (72%)	127 (25%)
No, family member	345 (90%)	35 (28%)	380 (75%)
Other cancers (no. of carriers)			
Endometrial cancer#	30	9	39
Ovarian#	4	0	4
Duodenal cancer#	4	2	6
Breast#	10	3	13
Urothelial#	4	2	6
Esophagus	1	0	1
Leukemia	0	3	3
Testis	2	0	2
Prostate	1	1	2
Vagina	0	1	1
Mesothelioma	0	1	1

'Index carrier' means the first person to be tested. Incidence of cancer in the group of index carriers without CRC: 20 endometrial cancers, 4 ovarian cancers, 3 breast cancers, 3 cancers of the small intestine, 1 testis cancer and 1 carcinoid. Ten of these index carriers had not developed any cancer at the time of DNA diagnostics; they were tested because of polyps at an early age or because they had an (affected) deceased family member. # Lynch syndrome-associated tumor.

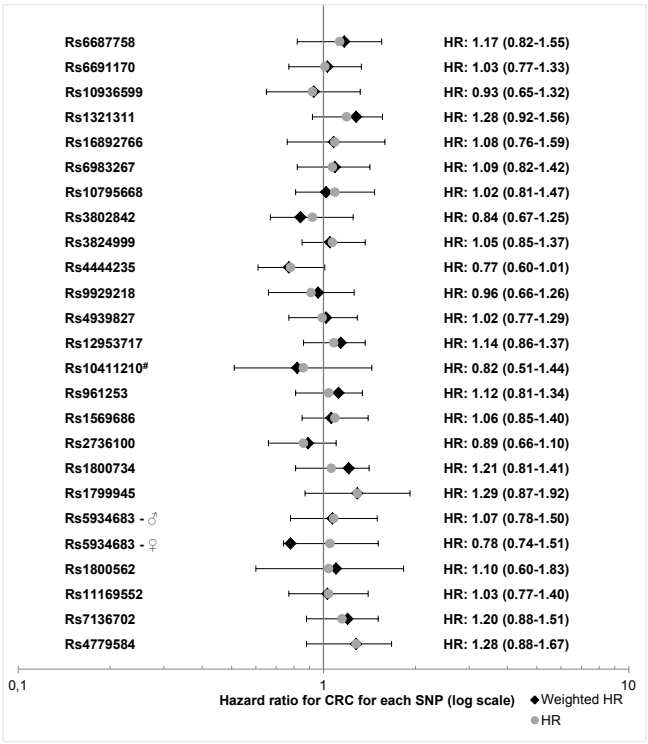

FIGURE 1 Forest plot of HRs for all SNPs. Note: rs5934683 lies on the X chromosome and was therefore stratified for gender. *SNPs previously associated with increased risk in MLH1 mutation carriers. #Reference category: homozygous for risk allele (due to low number of homozygous carriers of the non-risk allele). HR: Hazard Ratio.

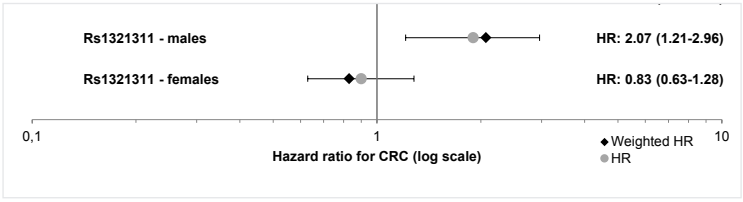

FIGURE 2 Forest plot of HRs for rs1321311. Note: p=.005 for males. HR: Hazard Ratio.

Hardy-Weinberg equilibrium

Two SNPs, rs1048943 (15q24.1) and rs4925386 (20q13), were not in Hardy Weinberg equilibrium (HWE). Violation of the HWE was present in both cases and controls and as this might be the result of a genotyping error, these SNPs were removed from the analysis. Ultimately, 24 SNPs were included in the final analysis.

Risk of colorectal cancer

None of the SNPs individually showed a clear risk modifying effect (Figure 1, Supplemental Table 1). There was a difference in HR between male and female *PMS2* carriers for rs1321311 (6p21.31), with an HR for the each additional A allele of 2.07 (95%CI: 1.21-2.96, p=0.005) and 0.83 (95%CI:0.63-1.28, p=0.56) for males and females, respectively (Figure 2: Forest plot, Supplemental Figure 2: KM curve).

Combination of rs3802842 & rs16892766

A previous meta-analysis reported a significant pairwise effect on CRC risk of rs3802842 (11q23.1) and rs16892766 (8q23.3) in *MLH1* mutation carriers.[6] The HR in the additive model for this combination in our *PMS2* cohort was 0.95 (95%CI:0.80-1.25, p=0.99). For carriers of more than three risk alleles the HR was 1.58 (95%CI: 0.55-3.39) compared to patients with no risk alleles, see Figure 3 for a comparison of previously publishes HRs and results from this study. The mean age at CRC diagnosis for 0, 1, and more than 1 risk alleles was 52.8; 52.9 and 50.4 respectively. The corresponding median ages were 54 (interquartile range (IQR): 43-62), 51 (IQR:43-63) and 47 (IQR:39-63). There was a statistically non-significant difference between the median age of CRC diagnosis between male and female carriers of two or more risk alleles, namely 53 (IQR:39-64, n=7) and 43.5 years to age (IQR:38-63, n=10, p=0.56, Mann-Whitney test).

Polygenic risk score

The polygenic risk score was calculated for 444 *PMS2* carriers with complete genotyping. The medians for PRS1 (meta-analysis derived ORs) were -0.12 (interquartile range (IQR):-0.48-0.30) for controls and -0.03 (IQR:-0.39-0.40) for CRC cases. The HRs for group 2 (second and third quartile) and 3 (fourth quartile) were 1.33 (95%CI:0.76-2.33) and 1.50 (95%CI:0.82-2.72) respectively (Table 2). The medians for PRS2 (based on HRs from our own data) were 0.30 (IQR:-0.057-0.55) for controls and 0.51 (IQR:0.068-0.75) for CRC cases. The corresponding HRs for group 2 and 3 were 1.05 (95%CI:0.59-1.89) and 2.62 (95%CI:1.49-4.60) respectively (Table 2). The KM curves for PRS1 and 2 are shown in and Supplemental Figure 3 and Figure 4, respectively. The difference between survival curves was highly significant for PRS2 (p<0.0001). The optimism-corrected c statistic was only 0.52, indicating no discriminatory value.

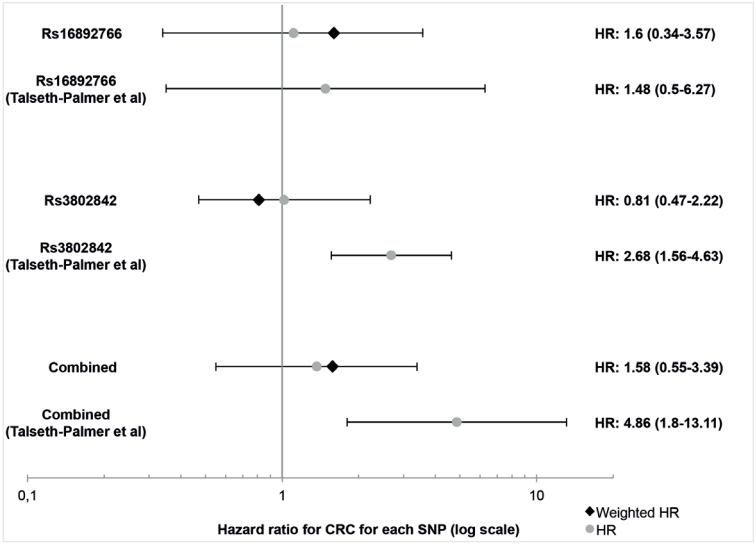

FIGURE 3 Forest plot of HRs for rs3802842 and rs16892766. Note: For the combination of the two SNPs, the plotted HR represents a comparison for carriers of three vs. no risk alleles. HR: Hazard Ratio.

TABLE 2 Polygenic risk scores

	PRS category	Controls	CRC	Expected events	HR	p for HR
PRS1	Median (IQR)	-0.12 (-0.48-0.30)	-0.03 (-0.39-0.40)			
	1st quartile	84	22	28	ref	0.41
	2d & 3d quartile	167	54	52	1.33 (0.76-2.33)	0.31
	4th quartile	84	33	28	1.50 (0.82-2.72)	0.19
PRS2	Median (IQR)	0.30 (-0.057-0.55)	0.51 (0.068-0.75)			
	1st quartile	84	18	26	ref	<0.0001
	2d & 3d quartile	165	38	53	1.05 (0.59-1.89)	0.86
	4th quartile	84	53	30	2.62 (1.49-4.60)	0.001

PRS: polygenic risk score. IQR: interquartile range.
PRS1: Weighted on odds ratios from general population, i.e. in sporadic CRC cases.
PRS2: Weighted on hazard ratios from this study
Log rank survival curves PRS1: p=0.32
Log rank survival curves PRS2: p<0.0001

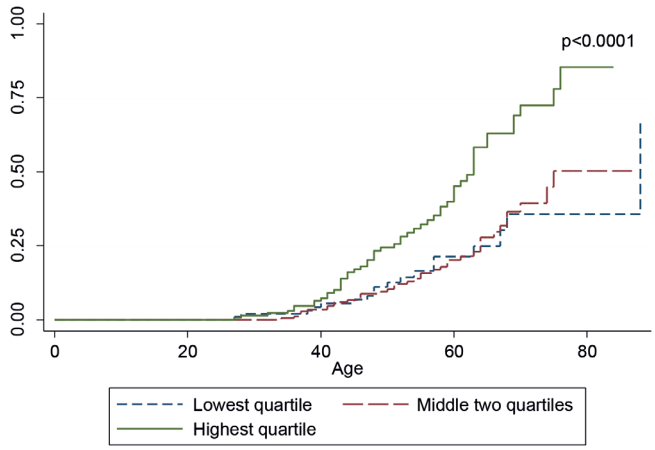

FIGURE 4 Kaplan Meier survival curve with endpoint colorectal cancer for PRS2 . Note: This plot compares curves for the lowest, the two middle and the highest quartile of the PRS. PRS2 is based on hazard ratios from the current study. HR: Hazard Ratio. PRS: polygenic risk score.

DISCUSSION

PMS2 carriers currently represent a relatively small proportion of LS patients. However, the number of *PMS2*-associated LS cases is expected to rise with the implementation of population-based screening protocols for all CRC below age 70. Identification of *PMS2* carriers has been challenging in the past due to difficulties in mutation analysis, a milder phenotype and many families not fulfilling clinical selection criteria.[3, 9, 15-17] Obtaining a better understanding of the specific *PMS2*-associated phenotype is particularly relevant, as it appears to differ markedly from phenotypes associated with other MMR mutations. Unfortunately, we were unable to confirm any risk modifying effects of rs3802842 (11q23.1) and rs16892766 (8q23.1), two SNPs previously shown to be associated with enhanced risk in *MLH1* mutation carriers.[5, 6] Studies in *MLH1* mutation carriers reported that a higher number of risk alleles in a carrier is associated with a younger onset of disease (28 years younger for 3 compared to 0 risk alleles).[6] In our cohort, mean ages where 52.8 and 50.4 for 0 compared to more than 1 risk allele, respectively. As such, there seems to be no clinical utility of rs3802842 and rs16892766 in risk stratification for *PMS2* carriers.

Many studies on (genetic) modifiers in LS patients focus on *MLH1* and *MSH2*, or *MSH6* carriers, while *PMS2* is seldom analyzed. The only study to include *PMS2* carriers (n=40) found that carriers of the G-alleles of rs10795668 (10p14) and rs9929218 (16q22.1) were at lower risk of CRC, a notable finding in that this is the opposite effect compared to sporadic CRC.[7] The authors conceded that their results should be confirmed in larger studies. As these findings have not been confirmed in our much larger cohort, we suggest that these previous findings may indeed have been false positives due to the small number of carriers included.

A relevant question is why our study did not confirm reported findings of previous studies of MMR carriers. One explanation might be that although patients have germline mutations in genes with similar functions, carriers are affected by genetic modifiers in different ways. Indeed, comparable studies in *BRCA1* or *BRCA2* mutation carriers have resulted in the identification of SNPs that clearly modify breast cancer risk. However, *BRCA2* carriers appear unaffected by SNPs that confer an increased breast cancer risk in *BRCA1* carriers, even though both genes play a role in homologous recombination.[18] This could also hold for MMR mutation carriers, as illustrated by the observation that while rs3802842 and rs16892766 may increase risk in *MLH1* carriers, they do not appear to have an effect in *MSH2* or *PMS2* carriers.[5, 6] Researchers should therefore concentrate on building cohorts large enough to analyze Lynch patients in a gene-stratified manner.

Gender stratification in our cohort led to the notable finding that male carriers of allele A at locus rs1321311 (6p21.31) show a per allele HR of 2.07 (95%CI. 1.21-2.96), while the HR for females was 0.83 (95%CI: 0.63-1.28). This SNP has been linked to the *CDKN1A* gene that encodes the p21 protein. p21 is involved in several (p53-independent) pathways as a tumor suppressor, although it also has oncogenic characteristics.[19, 20]Interestingly, down-regulation of p21 is inversely associated with MSI, the hallmark of Lynch-associated tumors. One study found that a larger proportion of Lynch-associated CRCs expressed p21 compared to sporadic CRCs (80% vs 31%).[21] However, a recent study reported on expressive Quantitative Trait Loci (eQTL) in colonic tissue based on data from the GTEx project portal (http://www.gtexportal.org/home/) and did not find a statistically significant effect of rs1321311 on CDKN1A expression in sigmoid and transverse colon tissue (p=0.84 and p=1.00 respectively).[22, 23] It is also unclear why this effect only appears to be present in male *PMS2* carriers. Although no gender difference was noted by the meta-analysis that identified the SNP [19], it is possible that gender differences exist, as CRC risk in the general population and in Lynch patients is known to be higher in men compared to women.[24, 25] Indeed, mutations are more often found in males than females when assessed for Lynch syndrome.[26] Another possible explanation for this gender specific effect might lie in the effect of other risk modifiers. It is perceivable that other factors than SNPs have a stronger influence on (colorectal) cancer development in women, such as hormonal factors.[27, 28] Unfortunately data on hormone levels or other factors previously shown to modify cancer risk in LS such as medication use (e.g. aspirin) or environmental factors were unavailable for analysis and as such we were unable to correct for this.[29-33] Similarly, data on smoking and BMI were only available for a small proportion of carriers (n=131, 26%). It should be emphasized that all results after gender stratification should be interpreted with caution because of small sample size and multiple testing. This could have led to false associations. Further studies are needed to validate these findings.

We also investigated the effect of the 24 SNPs on CRC risk in the *PMS2* cohort by means of a polygenic risk score (PRS). While there did not appear to be a significant effect of the PRS based on ORs from sporadic CRC cohorts, there was a difference in the cumulative incidence of CRC for *PMS2* carriers with a PRS2 (based on HRs in this study) in the highest quartile. Bootstrap validation however refuted this promising observation. Further studies are hence needed in other large cohorts.

There were some limitations to this study. Our study consisted exclusively of Dutch *PMS2* carriers and thus had a relatively homogeneous genetic makeup, implying that differences between our results and previous studies might be due to population-specific effects.

A second limitation might be that we did not correct for the specific mutation present in each family, mainly because in the majority of families the segregating *PMS2* mutation is rare or even unique. A previous study by our group did not identify such a correlation with CRC risk in *PMS2* carriers (Supplemental Tables 2a+2b Table: for more details).[34] Unfortunately, we were not able to validate our findings in an external cohort. To our best knowledge this is one of the largest *PMS2* cohorts currently collected, and bootstrap validation is a strong approach to assess discriminative ability of a prediction model.[35] Stratifying our cohort into a discovery and validation cohort was not a viable option as this would have resulted in a substantial decrease in power. Our study might already have been underpowered to detect weak associations. However, while such associations are interesting from a scientific point of view and may be relevant to tumorigenesis, they are not necessarily useful in clinical practice when the effect is small. For the two SNPs previously found to increase risk in *MLH1* mutation carriers, we had 60-80% power to detect an HR of 1.5, which we would consider clinically relevant. The previously reported HR in *MLH1* carriers for rs3802842 was 2.7, an HR for which we have ample power to detect (Supplemental Figure 1).

Families with a segregating *PMS2* mutation show a high degree of phenotypic variability. We were not able to confirm the risk modifying effect of rs3802842 (11q23.1) and s16892766 (8q23.3), which were previously found to increase the risk in *MLH1*-associated LS. This, together with the established lower penetrance, raises the question of whether *PMS2*-associated LS should be considered a separate Lynch disease entity. Additional explanations for phenotypic variability that warrant greater exploration include gene-environment interactions and risk modification by other genetic variants.

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3

SUPPLEMENTAL METHODS - STATISTICAL ANALYSIS

Follow-up and data-collection

For the vast majority of pre-symptomatically tested family members, i.e. controls, last known age was age at DNA diagnosis and thus – in general - age at start of colonoscopic surveillance. This is due to the fact that carriers were ascertained through genetic centres, which made us unable to collect follow-up data, as subsequent surveillance was done at the gastroenterology and/or gynaecology departments.

Ascertainment bias & weighted analysis

All subjects included in this study were derived from family cancer clinics and were therefore not randomly selected with respect to their phenotype. In other words they were selected based on the occurrence of cancer at a young age or due to several family members being affected. Therefore, these carriers usually belong to high-risk families, ascertained as a consequence of their relatively severe phenotype. It is likely that other factors than the germline mutation in PMS2 and the SNPs currently investigated may play a role in the phenotype variability, such as lifestyle or other genetic factors.[1]

Weights were calculated based on incidence rates in the Dutch population. HRs based on a proportion of this cohort have been previously reported and were used to determine age stratum (5 year) specific weights.[3] All calculated weights for cases were smaller than 1, effectively down-weighting cases compared with controls. It is important to note that for hypothesis testing the unweighted p-value and confidence interval are reliable.[12] We therefore report the weighted HRs with both the p-values and the 95% confidence intervals from the unweighted analysis. Both unweighted and weighted HRs are listed in the tables.

Polygenic risk score

PRS1 and 2 included all 24 SNPs that were found to be in Hardy Weinberg equilibrium, however two SNPs in the HFE gene were not taken into account in the PRS1 calculation, because to our knowledge there were no ORs from meta-analysis reported in current literature. The PRS was calculated as previously described[16], by using the following formula for PRS1 and PRS 2 respectively:

$$\sum_{i=1}^{n} a_i \log OR_i$$

$$\sum_{i=1}^{n} a_i \log HR_i$$

where n is the number of SNPs, a is the number of risk alleles for each SNP and the OR the meta-analysis derived OR (supplementary table 1)[4] or the HR from the current study for each SNP for PRS1 and PRS 2 respectively. *PMS2* carriers were categorized into four groups of equal sizes based on the quartiles in the control group.

Multiple testing

Correction for multiple testing was done by calculating the corrected overall critical p-value for all performed tests (four per SNP, including gender stratification, four PRS tests and the combination of rs3802842 and rs16892766) using the Bonferroni method. This leads to a p value 0.05/101= 0.0005.

Other

Mean age at CRC development was examined using one-way Analysis of Variance (ANOVA).

SUPPLEMENTARY TABLE 1 Overview of all tested SNPs, including gender stratification

SNP / OR from literature	Alleles (minor allele in bold)	MAF (dbSNP Overall/ european)	Allele frequencies *PMS2* cohort					Total genotyped for SNP (total n=507)
			Cases (CRC)		Controls			
			Genotype frequency	MAF	Genotype frequency	MAF	p	
rs6687758	AA	1000 genomes: G=0.19	76	0.21	262	0.17	0.36	502
1q41	A**G**	European: 0.22	43		108			
DUSP10	**GG**		3		10			
OR: 1.09 (Ma)	AG+GG							
	Per allele							
rs6691170	GG	1000 genomes: T=0.26	40	0.41	143	0.38	0.61	500
1q41	G**T**	European: 0.40	64		182			
DUSP10	**TT**		17		54			
OR: 1.06 (Ma)	Per allele							
rs10936599	**TT**	ExAC: T=0.28	7	0.25	24	0.26	0.96	495
3q26.6	T**C**	1000 genomes: T=0.27	48		146			
MYNN	CC	European: 0.24	67		203			
OR: 0.93	Per allele							
rs1321311	CC	1000 genomes A=0.28	65	0.26	234	0.23	0.19	503
6p21.2	C**A**	European: 0.22	50		122			
CDKN1A	**AA**		7		25			
OR: 1.1	Per allele							
rs16892766	AA	1000 genomes: C=0.08	88	0.14	290	0.13	0.67	502
8q23.3	**CA**	European: 0.09	30		85			
EIF3H	**CC**		3		6			
OR: 1.25 (Ma)	CA+CC							

Cox regression - overall			Cox regression - Males				Cox regression - females			
HR (95% CI)	wHR	p	PMS2 carriers (cases)	HR (95% CI)	wHR	p	PMS2 carriers (cases)	HR (95% CI)	wHR	p
		0.6								
1.20 (0.82-1.76)	1.27	0.4								
0.98 (0.32-2.97)	0.97	1								
1.18 (0.82-1.72)	1.24	0.4								
1.13 (0.82-1.55)	1.17	0.5	192 (59)	1.21 (0.72-2.03)	1.47	0.5	310 (63)	1.08 (0.71-1.65)	1.07	0.7
		0.7								
1.16 (0.76-1.75)	1.18	0,5								
0.95 (0.51-1.76)	0.97	0.9								
1.01 (0.77-1.33)	1.03	0.9	191 (58)	0.97 (0.66-1.44)	1.05	0.9	309 (63)	1.06 (0.74-1.53)	0.9	0.7
		0.8								
0.73 (0.29-1.88)	0.77	0.5								
0.72 (0.27-1.91)	0.76	0.5								
0.92 (0.65-1.32)	0.93	0.7	189 (60)	0.79 (0.44-1.41)	0.7	0.4	306 (62)	1.03 (0.69-1.54)	1.38	0.9
		0.1								
1.45 (1.00-2.12)	1.57	0.1								
1.02 (0.50-2.05)	1.13	1								
1.19 (0.92-1.56)	1.28	0.2	193 (60)	1.90 (1.21-2.96)	2.07	0	310 (62)	0.90 (0.63-1.28)	0.83	0.6
		0.9								
1.12 (0.73-1.72)	0.99	0.6								
1.11 (0.34-3.57)	1.6	0.9								
1.12 (0.73-1.71)	1.04	0.6								

SUPPLEMENTARY TABLE 1 Overview of all tested SNPs, including gender stratification

SNP / OR from literature	Alleles (minor allele in bold)	MAF (dbSNP Overall/ european)	Allele frequencies *PMS2* cohort					Total genotyped for SNP (total n=507)
			Cases (CRC)		Controls			
			Genotype frequency	MAF	Genotype frequency	MAF	p	
	Per allele							
rs6983267	**TT**	1000 genomes: T=0.39	31	0.48	108	0.53	0.27	485
8q24.21	**TG**	European: 0.50	49		174			
MYC	GG		36		87			
OR: 1.21 (Ma)	Per allele							
rs10795668	**AA**	1000 genomes: A=0.23	12	0.29	31	0.29	0.82	502
10p14	**AG**	European: 0.32	49		160			
FLJ3802842	GG		61		189			
OR: 0.89 (Ma)	Per allele							
rs3802842	AA	1000 genomes: C=0.29	67	0.25	190	0.28	0.79	495
11q23	**AC**	European: 0.27	49		159			
COLCA1/ COLCA2	**CC**		6		24			
OR: 1.11 (Ma)	Per allele							
rs3824999	**AA**	1000 genomes: G=0.33	24	0.47	89	0.49	0.55	498
11q13.4	**AC**	European: 0.52	67		187			
POLD3	CC		31		100			
OR: 0.93 (Ma)	Per allele							
rs4444235	TT	1000 genomes: C=0.43	44	0.39	105	0.47	0.1	497
14q22.2	**TC**	European: 0.49	59		188			

Cox regression - overall				Cox regression - Males				Cox regression - females			
HR (95% CI)	wHR	p	PMS2 carriers (cases)	HR (95% CI)	wHR	p	PMS2 carriers (cases)	HR (95% CI)	wHR	p	
1.09 (0.76-1.59)	1.08	0.6	191 (58)	0.91 (0.57-1.45)	0.82	0.7	311 (63)	1.19 (0.70-2.01)	0.9	0.5	
		0.8									
1.04 (0.61-1.78)	1.1	0.9									
1.16 (0.67-2.01)	1.19	0.6									
1.07 (0.82-1.42)	1.09	0.6	185 (57)	1.09 (0.74-1.60)	1.03	0.7	300 (59)	1.10 (0.79-1.54)	1.24	0.6	
		0.4									
0.74 (0.34-1.59)	0.66	0.4									
0.95 (0.46-1.97)	0.81	0.9									
1.09 (0.81-1.47)	1.02	0.6	191 (59)	0.97 (0.66-1.43)	0.77	0.9	311 (63)	1.25 (0.78-2.00)	1.15	0.4	
		0.7									
0.85 (0.60-1.22)	0.81	0.4									
1.02 (0.47-2.22)	0.81	1									
0.92 (0.67-1.25)	0.84	0.6	189 (60)	0.73 (0.44-1.22)	0.74	0.2	307 (63)	1.08 (0.68-1.70)	1.18	0.8	
		0.3									
1.41 (0.93-2.15)	1.28	0.1									
1.20 (0.71-2.03)	1.12	0.5									
1.07 (0.85-1.37)	1.05	0.5	192 (60)	1.10 (0.75-1.62)	1.28	0.6	306 (62)	1.04 (0.77-1.39)	1	0.8	
		0.2									
0.82 (0.55-1.25)	0.89	0.4									

SUPPLEMENTARY TABLE 1 Overview of all tested SNPs, including gender stratification

SNP / OR from literature	Alleles (minor allele in bold)	MAF (dbSNP Overall/ european)	Allele frequencies *PMS2* cohort					Total genotyped for SNP (total n=507)
			Cases (CRC)		Controls			
			Genotype frequency	MAF	Genotype frequency	MAF	p	
BMP4	**CC**		18		83			
OR: 1.11 (Ma)	Per allele							
rs9929218	**AA**	1000 genomes: A=0.26	8	0.30	31	0.29	0.54	502
16q22.1	**AG**	European: 0.29	57		155			
CDH1	GG		58		193			
OR: 0.91 (Ma)	Per allele							
rs4939827	**CC**	1000 genomes: T=0.35	22	0.48	82	0.49	0.51	500
18q21.1	**CT**	European: 0.53	73		204			
SMAD7	TT		27		92			
OR: 0.85 (Ma)	Per allele							
rs12953717	CC	1000 genomes: T=0.30	36	0.42	120	0.44	0.83	500
18q21.1	**CT**	European: 0.45	65		196			
SMAD7	**TT**		22		61			
OR: 1.12	Per allele							
rs10411210	**TT**	1000 genomes: T=0.26	1	0.09	3	0.10	0.96	489
19q13.1	**TC**	European: 0.10	20		65			
RHPN2	CC		100		300			
OR: 0.87 (Ma)	CC (ref)							
	CT+TT							
rs961253	CC	1000 genomes: A=0.29	43	0.37	149	0.37	0.49	495
		European:						

Cox regression - overall			Cox regression - Males				Cox regression - females			
HR (95% CI)	wHR	p	PMS2 carriers (cases)	HR (95% CI)	wHR	p	PMS2 carriers (cases)	HR (95% CI)	wHR	p
0.60 (0.35-1.03)	0.54	0.1								
0.78 (0.61-1.01)	0.77	0.1	192 (59)	0.80 (0.51-1.26)	0.76	0.3	305 (62)	0.74 (0.53-1.05)	0.85	0.1
		0.7								
1.07 (0.51-2.30)	1.51	0.9								
0.93 (0.41-2.11)	1.26	0.9								
0.91 (0.66-1.26)	0.96	0.6	193 (60)	0.92 (0.57-1.47)	0.97	0.7	309 (63)	0.97 (0.63-1.50)	0.85	0.9
		0.7								
1.19 (0.73-1.96)	1.31	0.5								
1.00 (0.57-1.78)	1.08	1								
0.99 (0.77-1.29)	1.02	1	192 (59)	0.91 (0.61-1.36)	0.74	0.6	308 (63)	1.02 (0.73-1.44)	1.04	0.9
		0.8								
1.08 (0.73-1.63)	1.15	0.7								
1.17 (0.73-1.88)	1.29	0.5								
1.08 (0.86-1.37)	1.14	0.5	192 (60)	1.07 (0.75-1.54)	1.09	0.7	308 (63)	1.04 (0.74-1.47)	1.03	0.8
		0.2								
0.26 (0.064-1.09)		0.1								
0.32 (0.087-1.18)		0.1								
0.86 (0.51-1.44)	0.82	0.6	190 (60)	1.34 (0.61-2.94)	1.36	0.5	299 (61)	1.00 (0.52-1.94)	1.07	1
		0.9								

SUPPLEMENTARY TABLE 1 Overview of all tested SNPs, including gender stratification

SNP / OR from literature	Alleles (minor allele in bold)	MAF (dbSNP Overall/ european)	Allele frequencies *PMS2* cohort					Total genotyped for SNP (total n=507)
			Cases (CRC)		Controls			
			Genotype frequency	MAF	Genotype frequency	MAF	p	
20p12.3	C**A**	0.36	63		179			
BMP2	**AA**		12		49			
OR: 1.12 (Ma)	Per allele							
rs1569686	GG	1000 genomes: G=0.28	49	0.39	144	0.38	0.38	499
20q11.2	G**T**	European: G=0.63	52		181			
DNMT3B	**TT**		22		51			
OR: 0.57 (Ma)	Per allele							
rs2736100	**GG**	1000 genomes: C=0.48	31	0.50	90	0.46	0.36	498
5p15	G**T**	European: 0.50	60		168			
TERT	TT		30		119			
OR: 1.07 (Ma)	Per allele							
rs1800734	GG	1000 genomes: A=0.32	77	0.22	245	0.20	0.88	501
3p21.3	G**A**	European: 0.27	38		112			
MLH1	**AA**		8		21			
OR: 1.51 (Ma)	Per allele							
rs1799945	CC	ExAC: G=0.11	93	0.13	285	0.13	0.74	502
6p21.3	C**G**	1000 genomes: G=0.07	26		89			
HFE	**GG**	European: 0.17	3		6			
No meta-analysis available	CG+GG							
	Per allele							
Rs5934683	CC	1000 genomes: C=0.34						

Cox regression - overall			Cox regression - Males				Cox regression - females			
HR (95% CI)	wHR	p	PMS2 carriers (cases)	HR (95% CI)	wHR	p	PMS2 carriers (cases)	HR (95% CI)	wHR	p
1.08 (0.73-1.58)	1.09	0.7								
1.06 (0.62-1.83)	1.27	0.8								
1.04 (0.81-1.34)	1.12	0.7	191 (58)	1.20 (0.82-1.75)	1.25	0.3	304 (60)	0.87 (0.60-1.26)	0.88	0.5
		0.5								
0.96 (0.65-1.42)		0.8								
1.26 (0.77-2.06)		0.4								
1.09 (0.85-1.40)	1.06	0.5	191 (60)	1.11 (0.79-1.55)	1.08	0.6	308 (63)	1.04 (0.72-1.49)	1.23	0.8
		0.1								
1.09 (0.68-1.75)	1.26	0.7								
0.73 (0.42-1.26)	0.8	0.3								
0.86 (0.66-1.10)	0.89	0.2	192 (60)	0.69 (0.48-0.97)	0.6	0	306 (61)	0.99 (0.71-1.38)	1.03	1
		0.7								
0.99 (0.69-1.43)	1.14	1								
1.30 (0.67-2.54)	1.62	0.4								
1.06 (0.81-1.41)	1.21	0.7	193 (60)	0.80 (0.51-1.27)	0.81	0.3	308 (63)	1.25 (0.86-1.81)	1.89	0.2
		0.4								
1.27 (0.84-1.93)	1.35	0.3								
1.76 (0.45-6.93)	1.36	0.4								
1.31 (0.86-1.99)	1.35	0.2								
1.29 (0.87-1.92)	1.29	0.2	192 (59)	1.58 (0.92-2.70)	2.07	0.1	310 (63)	1.12 (0.64-1.95)	1.41	0.7
										0.2

SUPPLEMENTARY TABLE 1 Overview of all tested SNPs, including gender stratification

SNP / OR from literature	Alleles (minor allele in bold)	MAF (dbSNP Overall/ european)	Allele frequencies *PMS2* cohort					Total genotyped for SNP (total n=507)
			Cases (CRC)		Controls			
			Genotype frequency	MAF	Genotype frequency	MAF	P	
Xp22.2	**TC**	European: 0.46						
SHROOM2	**TT**	GoNL: C=0.63						
OR: 1.07 (Ma)	Per allele							
Rs1800562	CC	ExAC=0.03	106	0.07	334	0.06	0.82	498
6p21.3	**TC**	1000 genomes: A=0.01	15		40			
HFE	**TT**	European: 0.04	1		2			
No meta-analysis available	TC+TT							
	Per allele							
rs11169552	**TT**	1000 genomes: T=0.25	5	0.25	25	0.26	0.55	499
12q13.3	**TC**	European: 0.25	51		146			
ATF1	CC		66		206			
OR: 0.92 (Ma)	Per allele							
12q13.13	CC	1000 genomes: T=0.50	47	0.39	162	0.34	0.34	501
LARP4/ DIP2B	**TC**	European: 0.35	56		176			
OR: 1.06 (Ma)	**TT**		19		41			
	Per allele							
rs4779584	CC	1000 genomes: T=0.49	75	0.19	246	0.22	0.69	499
15q13.3	**TC**	European: 0.20	41		117			
GREM1/ SCG5	**TT**		6		14			
OR: 1.26 (Ma)	Per allele							

Threshold for multiple testing: p<0.0005. HR=Hazard ratio. OR=Odds ratio. 95%CI= 95% confidence interval.

Cox regression - overall			Cox regression - Males				Cox regression - females			
HR (95% CI)	wHR	p	PMS2 carriers (cases)	HR (95% CI)	wHR	p	PMS2 carriers (cases)	HR (95% CI)	wHR	p
N/A								1.43 (0.87-2.35)	0.69	0.2
								0.62 (1.19-2.03)	0.73	0.4
			191 (59)	1.08 (0.78-1.50)	1.07	0.6	310 (63)	1.05 (0.74-1.51)	0.78	0.8
		1								
1.05 (0.56-1.96)	1.18	0.9								
1.03 (0.093-11.4)	0.8	1								
1.05 (0.57-1.93)	1.14	0.9								
1.04 (0.60-1.83)	1.1	0.9	190 (59)	0.99 (0.49-1.97)	1.36	1	308 (63)	1.04 (0.49-2.21)	0.6	0.9
		0.5								
1.60 (0.69-3.71)	2.4	0.3								
1.48 (0.62-3.57)	1.96	0.4								
1.04 (0.77-1.4)	1.03	0.8	190 (59)	1.20 (0.80-1.82)	1.13	0.4	309 (63)	0.88 (0.58-1.33)	0.75	0.5
		0.6								
1.11 (0.75-1.66)	1.19	0.6								
1.36 (0.78-2.36)	1.46	0.3								
1.15 (0.88-1.51)	1.2	0.3	190 (59)	1.44 (0.99-2.10)	1.4	0.1	311 (63)	0.94 (0.67-1.32)	0.94	0.7
		0.4								
1.12 (0.76-1.64)	1.07	0.6								
1.88 (0.66-5.39)	2.5	0.2								
1.21 (0.88-1.67)	1.28	0.2	191 (60)	1.82 (1.16-2.86)	1.81	0	308 (62)	0.89 (0.57-1.39)	0.83	0.6

MAF=minor allele frequency. wHR: weighted Hazard Ratio. #This meta-analysis uses homozygotes for the major allele as a reference category.

SUPPLEMENTARY TABLE 2A Overview of germline *PMS2* mutations in the cohort. Each row describes 1 family

Genotype molecular	Genotype aminoacid	no crc	crc	Total
c.856_857delGA	p.Asp286Glnfs*12	0	1	1
c.1078_1081dupATAG	p.Gly361Aspfs*5	4	0	4
c.1079_1080del	p.Ile360Argfs*4	3	1	4
c.1144+2T>A	p.(Glu330_Glu381del)	3	0	3
c.1145-?_(*160_?)del	deletion exon 11-15	11	1	12
c.1214C>A	p.Ser405*	3	1	4
c.1261C>T	p.Arg421*	1	1	2
c.137G>T	p.Ser46Ile	5	2	7
c.137G>T	p.Ser46Ile	5	1	6
c.137G>T	p.Ser46Ile	5	0	5
c.137G>T	p.Ser46Ile	3	1	4
c.137G>T	p.Ser46Ile	2	0	2
c.137G>T	p.Ser46Ile	2	0	2
c.137G>T	p.Ser46Ile	0	1	1
c.137G>T	p.Ser46Ile	0	1	1
c.137G>T	p.Ser46Ile	0	1	1
c.137G>T	p.Ser46Ile	0	1	1
c.163+2T>C	p.SerArgfs*5	2	1	3
c.164-?_803+?del	deletion exon 3-7	1	0	1
c.1730dup	p.Arg578Alafs*	1	0	1
c.1831dupA	p.Ile611Asnfs*2	3	1	4
c.1831dupA	p.Ile611Asnfs*2	1	0	1
c.1831dupA	p.Ile611Asnfs*2	0	1	1
c.1831dupA	p.Ile611Asnfs*2	2	1	3
c.1882C>T	p.Arg628*	13	2	15
c.1882C>T	p.Arg628*	7	2	9
c.1882C>T	p.Arg628*	8	1	9
c.1882C>T	p.Arg628*	6	1	7
c.1882C>T	p.Arg628*	4	2	6
c.1882C>T	p.Arg628*	5	0	5
c.1882C>T	p.Arg628*	3	2	5

SUPPLEMENTARY TABLE 2A Overview of germline *PMS2* mutations in the cohort. Each row describes 1 family

Genotype molecular	Genotype aminoacid	no crc	crc	Total
c.1882C>T	p.Arg628*	2	1	3
c.1882C>T	p.Arg628*	2	1	3
c.1882C>T	p.Arg628*	3	0	3
c.1882C>T	p.Arg628*	2	1	3
c.1882C>T	p.Arg628*	2	1	3
c.1882C>T	p.Arg628*	1	1	2
c.1882C>T	p.Arg628*	1	1	2
c.1882C>T	p.Arg628*	1	0	1
c.1882C>T	p.Arg628*	0	1	1
c.1882C>T	p.Arg628*	0	1	1
c.1882C>T	p.Arg628*	0	1	1
c.211_214del	p.Asn71Aspfs*4	1	1	2
c.211_214del	p.Asn71Aspfs*4	0	1	1
c.2117delA	p.Lys706Serfs*19	3	1	4
c.2117delA	p.Lys706Serfs*19	2	0	2
c.2155C>T	p.Gln719*	1	1	2
c.2155C>T	p.Gln719*	0	1	1
c.2174+1G>A	p.?	2	0	2
c.219_220dup	p.Gly74Valfs*3	5	2	7
c.219_220dup	p.Gly74Valfs*3	5	2	7
c.219_220dup	p.Gly74Valfs*3	6	0	6
c.219_220dup	p.Gly74Valfs*3	5	0	5
c.219_220dup	p.Gly74Valfs*3	1	0	1
c.2192_2196delTAACT	p.Leu731Cysfs*3	4	2	6
c.2192_2196delTAACT	p.Leu731Cysfs*3	5	0	5
c.2192_2196delTAACT	p.Leu731Cysfs*3	4	1	5
c.2192_2196delTAACT	p.Leu731Cysfs*3	4	0	4
c.2192_2196delTAACT	p.Leu731Cysfs*3	2	1	3
c.2192_2196delTAACT	p.Leu731Cysfs*3	2	1	3
c.2192_2196delTAACT	p.Leu731Cysfs*3	0	2	2
c.2192_2196delTAACT	p.Leu731Cysfs*3	0	1	1

SUPPLEMENTARY TABLE 2A Overview of germline *PMS2* mutations in the cohort. Each row describes 1 family

Genotype molecular	Genotype aminoacid	no crc	crc	Total
c.2275+1G>A	p.?	4	0	4
c.2275+1G>A	p.?	3	1	4
c.2404C>T	p.Arg802*	0	1	1
c.24-12_107del96bp	intronic	2	0	2
c.24-12_107delinsAAAT	p.?/p.Ser8Argfs*5	4	1	5
c.2444C>T	p.Ser815Leu	4	1	5
c.2445+1G>T	p.?	0	1	1
c.247_250dup	p.Thr84Ilefs*9	2	0	2
c.251-2A>C	p.?	2	0	2
c.251-2A>C	p.?	1	1	2
c.251-2A>C	p.?	0	1	1
c.251-2A>C	p.?	0	1	1
c.2T>A		1	0	1
c.319C>T	p.Arg107Trp	6	1	7
c.325dupG	p.Glu109Glyfs*30	3	1	4
c.325dupG	p.Glu109Glyfs*30	3	0	3
c.325dupG	p.Glu109Glyfs*30	0	1	1
c.325dupG	p.Glu109Glyfs*30	0	1	1
c.658dupG	p.Ser220fs	0	1	1
c.697C>T	p.Gln233*	5	0	5
c.697C>T	p.Gln233*	1	1	2
c.697C>T	p.Gln233*	2	0	2
c.697C>T	p.Gln233*	1	0	1
c.697C>T	p.Gln233*	0	1	1
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	7	1	8
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	3	2	5
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	3	1	4
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	3	1	4
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	3	1	4
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	4	0	4
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	2	2	4

SUPPLEMENTARY TABLE 2A Overview of germline *PMS2* mutations in the cohort. Each row describes 1 family

Genotype molecular	Genotype aminoacid	no crc	crc	Total
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	4	0	4
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	3	0	3
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	2	1	3
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	1	1	2
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	0	2	2
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	1	1	2
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	0	1	1
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	0	1	1
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	1	0	1
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	0	1	1
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	0	1	1
c.736_741delCCCCCTinsTGTGTGTGAAG	p.Pro246Cysfs*3	1	0	1
c.804-?_903+?del	p.Tyr268*	0	1	1
c.804-60_804-59ins2kb	p.?	3	1	4
c.804-60_804-59ins2kb	p.?	0	1	1
c.804-60_804-59ins2kb	p.?	1	0	1
c.804-60_804-59ins2kb	p.?	0	1	1
c.823C>T	p.Gln275*	5	1	6
c.861_864delACAG	p.Arg287Serfs*19	4	0	4
c.903G>T	p.Tyr268*	0	1	1
c.904-?_988+?dup	p.Glu330Glyfs*7	4	0	4
c.904-?_988+?dup	p.Glu330Glyfs*7	4	0	4
c.904_911delGTCTGCAG	p.Val302Thrfs*4	1	0	1
c.943C>T	p.Arg315*	2	0	2
c.989-?_2275+?del		6	2	8
c.989_1144 + 685del		1	0	1
deletion at least exon 1-11		0	1	1
deletion at least exon 9-11		2	0	2
deletion entire PMS2		3	1	4
deletion entire PMS2		2	0	2
deletion exon 10		4	0	4

SUPPLEMENTARY TABLE 2A Overview of germline *PMS2* mutations in the cohort. Each row describes 1 family

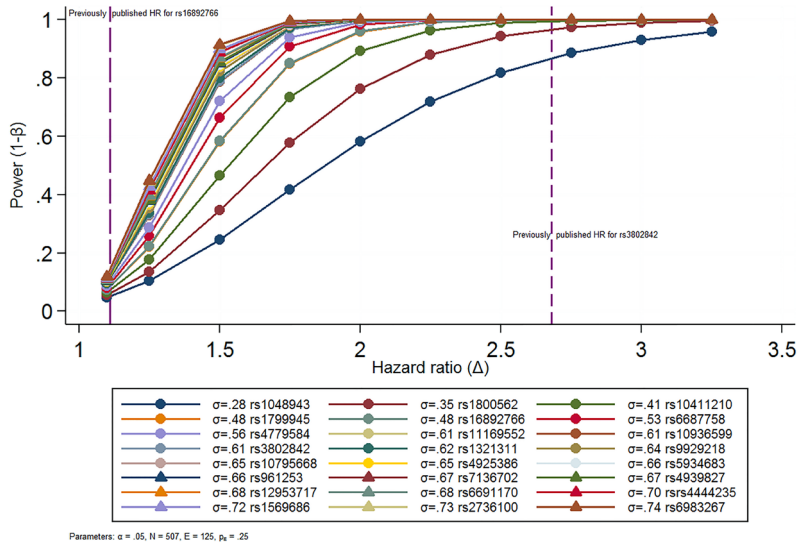
Genotype molecular	Genotype aminoacid	no crc	crc	Total
deletion exon 10		1	1	2
deletion exon 10		0	1	1
deletion exon 11-15		6	1	7
deletion exon 11-15		5	2	7
deletion exon 11-15		4	1	5
deletion exon 11-12		6	0	6
deletion exon 11-12		4	0	4
deletion exon 11-12		2	0	2
deletion exon 11-12		1	1	2
deletion exon 13-15		2	0	2
c.2276-?_c.2445+?del	deletion exon 14	5	0	5
c.2276-?_c.2445+?del		2	2	4
c.2276-?_c.2445+?del		1	1	2
c.2276-?_c.2445+?del		0	1	1
deletion exon 14 and 15		1	0	1
deletion exon 2		10	4	14
deletion exon 2		8	1	9
deletion exon 2		2	1	3
deletion exon 2, 5-11, 13-15		0	1	1
deletion exon 2-4 (c.24-?_353+?del)		3	2	5
deletion exon 5-15		0	1	1
deletion exon 5-7		5	1	6
deletion exon 5-7		4	2	6
deletion exon 5-7		1	1	2
deletion exon 5-7		0	1	1
deletion exon 6-7		0	1	1
duplication of exon 11 + exon 12, inserted in intron 12		0	1	1
Two segregating mutations: deletion PMS2 exon 1 t/m 11 OR deletion exon 5 t/m 7		17	1	18
Total		**383**	**124**	**507**

SUPPLEMENTARY TABLE 2B Most frequent germline *PMS2* mutations in the cohort

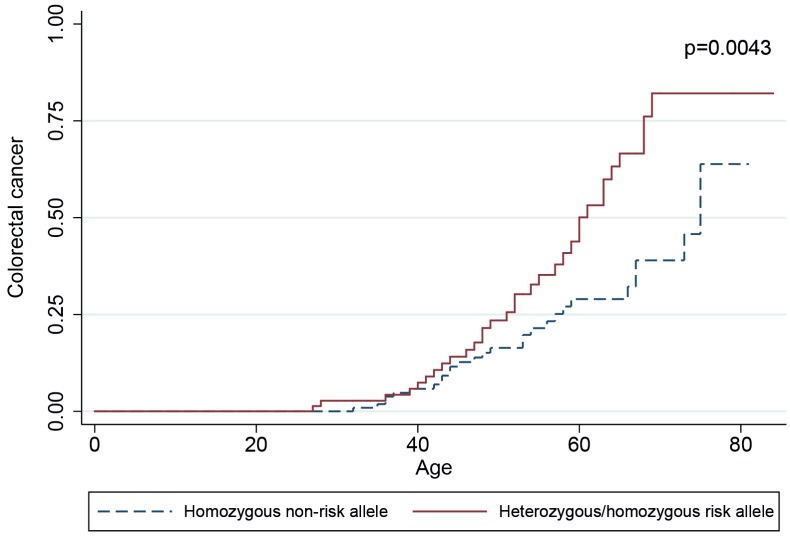
GENOTYPE	No CRC			CRC	Total	Mean members per family	Mean affected members per family	Mean age at CRC for cases	Classification Suerink et al
c.1145-?_(*160_?)del	11	1	12	2 (17%)	1	12,0	1,0	45,0	3
c.137G>T	19	8	27	12 (44%)	10	2,7	0,8	58,7	2
c.1882C>T	60	19	79	24 (30%)	18	4,4	1,1	55,2	1
c.2192_2196delTAACT	21	8	16	12 (41%)	8	2,0	1,0	53,5	1
c.219_220dup	22	4	26	8 (31%)	5	5,2	0,8	65,0	1
c.697C>T	9	2	11	5 (45%)	5	2,2	0,4	51,5	1
c.736_741delCCCCCTinsTGTGTGTGAAG	40	17	57	20 (35%)	20	2,9	0,9	50,3	1
deletion exon 11-15	15	4	19	9 (47%)	3	6,3	1,3	58,8	1
deletion exon 11-12	13	1	14	9 (64%)	5	2,8	0,2	65,0	3
deletion exon 14	8	3	11	6 (55%)	3	3,7	1,0	53,8	3
deletion exon 2	20	6	26	10 (38%)	3	8,7	2,0	57,2	3
deletion exon 5-7	19	6	25	13 (52%)	5	5,0	1,2	55,5	3
Total	**252**	**77**	**316**	**129 (41%)**	86				

CRC: colorectal cancer
A previous study by our group (Suerink et al[1]) showed that mutation carriers with loss of RNA expression (group 1) had lower age at CRC diagnosis (difference 10 years), compared to group 2 mutation carriers. We do not observe marked differences in mean age at CRC diagnosis for this group. Nor did we see some mutations with a high affected family member amount. This together with the results of the study by Suerink et al suggests that genotype did not heavily influence our results.
1=no mRNA expression from mutated allele, 2=normal mRNA expression; 3=RNA expression unknown

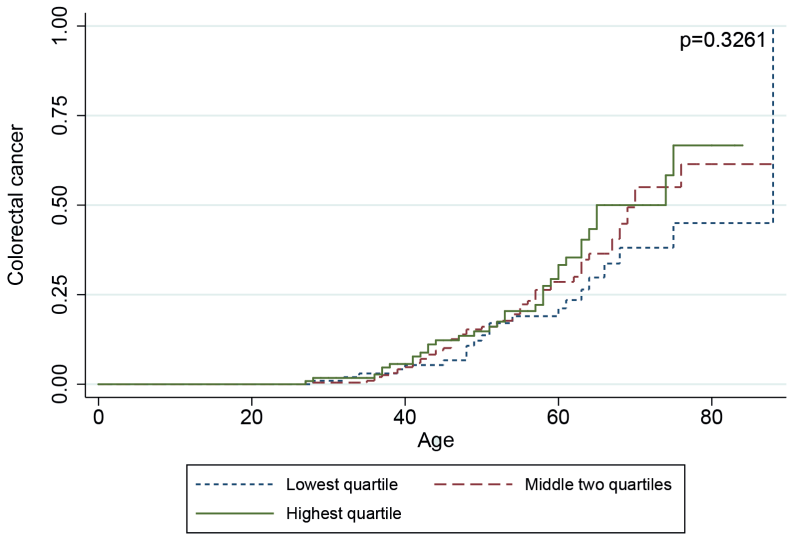
1. Suerink M, van der Klift HM, ten Broeke SW, et al. The effect of genotypes and parent of origin on cancer risk and age of cancer development in PMS2 mutation carriers. Genet Med 2016;18:405-409.



SUPPLEMENTARY FIGURE 1 Post-hoc power analysis.



SUPLEMENTARY FIGURE 2 Kaplan Meier survival curves with endpoint colorectal cancer for Rs1321311. The p-value represents the comparison between PMS2 carriers who are homozygous for the non-risk allele with PMS2 carriers who are heterozygous/homozygous for the risk allele.



SUPPLEMENTARY FIGURE 3 Kaplan Meier survival curves with endpoint colorectal cancer for polygenic risk score (PRS) 2. The p-value represents the comparison for the lowest, the two middle and the highest quartile of the PRS.

3.2

The risk modifying effect of lifestyle factors in patients with PMS2-associated Lynch syndrome

Manuscript in preparation

Sanne W. ten Broeke, Ghazaleh Dashti, Encarna Gomez Garcia, Liselot P. van Hest, Tom G.W. Letteboer, Lizet E. van der Kolk, Maran J.W. Olderode-Berends, Theo A. van Os, Liesbeth Spruijt, Anja Wagner, Ellen Kampman, Franzel van Duijnhoven, Aung Ko Win, Maartje Nielsen

ABSTRACT

Introduction

The clinical phenotype of *MLH1*, *MSH2*, *MSH6* has been thoroughly described. There are, however, still many outstanding questions concerning the clinical phenotype of Lynch Syndrome patients with a *PMS2* variant. Variants in the *PMS2* gene display a lower penetrance compared to *MLH1* and *MSH2* for cancer, and a wide interfamilial variance in clinical phenotype. It is therefore likely that external factors or genetic modifiers are involved in the *PMS2* phenotype. The aim of this retrospective study was to assess whether lifestyle factors influence colorectal cancer risk in this subset of Lynch patients.

Methods

To assess whether lifestyle factors influence colon cancer risk and polyp count, lifestyle questionnaires were sent to 193 *PMS2* carriers. This questionnaire was developed by investigators of Leiden University and Wageningen University and included 7 questions about: sex, height, weight, smoking, alcohol and lastly whether they had used aspirin. Additional data was collected in collaboration with the Colon Cancer Family Registry (CCFR). A weighted cox-proportional hazards regression model was used to estimate hazard ratios.

Results

A total of 270 *PMS2* carriers were included. There was no evidence of a strong association between BMI at age 20, smoking or alcohol consumption and the risk of colorectal cancer. Of note, a possible trend was observed for *PMS2* carriers that were overweight/obese at age 20 (HR 1.32, 95% CI: 0.59-2.97, p= 0.09).

Conclusion

In summary, we found no strong association of smoking, obesity in adolescence, or alcohol use with colorectal cancer risk in our cohort of 270 PMS2-associated Lynch syndrome patients. Future studies should have a prospective design and focus an adenoma occurrence as an endpoint.

INTRODUCTION

Patients with Lynch syndrome have a hereditary predisposition for the development of colorectal cancer, endometrial cancer and several other cancers. These patients carry a heterozygous pathogenic germline variant in one of the mismatch repair (MMR) genes; *MLH1*, *MSH2*, *MSH6* or *PMS2*, or a deletion in *EPCAM* which causes dysfunction of *MSH2*. A second somatic hit results in malfunctioning of the MMR system, which in turn leads to the accumulation of somatic variants in other genes and can ultimately result in cancer. The reported cancer risk varies widely[1] and appears to differ not only between families but also between members of the same family.[2] Multiple theories have been proposed to explain this phenomenon, which include the possible risk modifying effect of lifestyle factors such as cigarette smoking, obesity or alcohol consumption.[3] These factors may have a different effect on the development of colorectal cancer in Lynch syndrome patients, primarily because of the differences in tumorigenesis compared to sporadic colorectal cancer cases. Indeed, it has been suggested that hereditary cancer patients might be more susceptible for lifestyle factors.[3] This increased effect in hereditary colorectal cancer could be due to the fact that only a second hit is needed for a defect in the MMR machinery[4] where sporadic microsatellite instable tumors need two somatic hits within the same MMR gene. In other words, damaging lifestyle factors might increase the likelihood of a second hit in the wild type allele of MMR germline mutated patients.

Campbell et al. found evidence that recent BMI and adult weight gain were associated with the microsatellite stable phenotype of colorectal cancer cases.[5] In line with this finding, Win et al also linked obesity (at age 20 years) to colorectal cancer, but found no difference in the increase in risk of colorectal cancer between carriers and non-carriers.[6] The same group also investigated the effect of BMI on endometrial cancer. Interestingly, only non-MMR mutation carriers were found to be at increased risk at higher BMI, suggesting that other pathways besides the estrogen pathway are important in endometrial carcinogenesis in female Lynch patients.[7] A large study by Pande et al. showed statistically significant increased hazard ratios of colorectal cancer for Lynch patients that smoked cigarettes.[8] However this study did not include patients that carry a variant in *PMS2*.

The cancer risk for *PMS2* carriers is lower compared to carriers of a variant in one of the other MMR genes[9, 10] and recent studies have suggested differences in tumorigenesis which may also result in a different effect of lifestyle factors in this specific subset of Lynch patients.[11] In this study we used a case-control design to investigate the effect

of lifestyle factors on the development of colorectal cancer and endometrial cancer in a combined Dutch, Australasian and Northern-American cohort of 270 *PMS2* carriers.

METHODS

Data collection

Dutch cohort

Available pedigree and patient specific data has been collected from 2009 until 2015 in collaboration with the clinical genetic departments of the university hospitals in the Netherlands. We received informed consent of 193 Dutch *PMS2* carriers. Patient records were screened in an attempt to confirm all clinical and pathological data where possible. Most index patients (probands) were sent in for variant analysis because their phenotype and/or the family history was suspect for Lynch Syndrome. Some people also gave consent to use data on their deceased relatives. To decrease the risk of survival bias, we approached these family members to also fill in a lifestyle questionnaire on their deceased relative. Excluding these cases would mean that carriers that die at a young age (e.g. from a Lynch syndrome associated cancer) are not included in the analyses and could thereby lead to a decrease in the total effect on the outcome measure, i.e. a bias towards null.

All confirmed carriers of *PMS2* variants with informed consent were sent a questionnaire on lifestyle factors. This questionnaire was developed by investigators of Leiden University and Wageningen University and included 7 questions about: sex, height, weight (at age 18 and age 40 years if applicable), smoking (duration, number of cigarettes and when applicable year of cessation), alcohol (duration, number of units per week) and lastly whether they had used aspirin. Carriers were asked specifically for their use just before they were diagnosed with cancer, polyps or upon entering screening. The response rate was 81%.

Colon Cancer Family Registry

Data collection from the Colon Cancer Family Registry (CCFR) has been described previously by Newcomb et al.[12,] and at www.coloncfr.org. In brief, data was collected between 1998 and 2012. The CCFR recruited families through population-based probands that were diagnosed with colorectal cancer. These families originate from the USA (Washington, California, Arizona, Minnesota, Colorado, New Hampshire, North Carolina, and Hawaii), Australia (Victoria) and Canada (Ontario). Clinically ascertained probands were also included and were derived from families referred to family cancer

clinics in the USA (Mayo Clinic, Rochester, Minnesota, and Cleveland Clinic, Cleveland, Ohio), Canada (Ontario), Australia (Melbourne, Adelaide, Perth, Brisbane, Sydney and Newcastle) and New Zealand (Auckland). The process of obtaining informed consent is outlined in Newcomb et al.[12] The study protocol was approved by the institutional research ethics review board of which the family members were derived. Clinical data was obtained through extensive questionnaires. The total cohort of which lifestyle data was available included 100 participants.

Statistical analysis

Previous studies have described the oversampling of cases in clinic-based cohorts, which is the larger part of the currently analyzed cohort. These carriers usually belong to high-risk families, ascertained because of their relatively severe phenotype. Moreover affected family members are more likely to be tested for the variant and this too gives over-sampling of cases. To account for this bias, we used a weighted cohort approach, previously described by Antoniou et al.[13] Weights were calculated based on incidence rates from either the Dutch or the American population (for CCFR patients). Hazard ratios (HRs) based on a proportion of this cohort were previously reported and used to determine age stratum (5 year) specific weights.[10] All calculated weights for cases were smaller than 1, effectively down-weighting cases compared with controls. It is important to note that for hypothesis testing the unweighted p-value and confidence interval are to be used.

A Cox-proportional hazards regression model was used to estimate HRs. The time at risk for every participant was set at age 20 and ended at the age of diagnosis of colorectal cancer (n=93), age of diagnosis of any other cancer (n=42), or age at interview (n=133), whichever occurred first. The rationale behind censoring for other cancers is that this affects the risk of developing colorectal cancer, for example due to (long-term) treatment effects. Estimates were corrected for familial clustering of risk by using the Huber-White sandwich estimator. Some variables were analyzed as time-dependent covariates because not doing so resulted in a violation of the proportional hazards assumption, which was investigated by examining the Schoenfeld residuals with a formal statistical test and by plotting them against time. Time-varying variables were generated for polypectomy, smoking status, and total pack-years smoked by splitting dataset per year and taking into account age at first polypectomy, age at initiation of smoking, age at quitting smoking, and years of smoking. Due to the retrospective design of this study taking into account recent BMI is difficult, therefore we used BMI in early adulthood (18-20 years) as a covariate in the model.

All analysis were performed in Stata, version 20.

RESULTS

A total of 270 *PMS2* carriers were included, results are given in table 1. There was no evidence of a strong association between BMI at age 20, smoking or alcohol consumption and the risk of colorectal cancer. Of note, a possible trend was observed for *PMS2* carriers that were overweight/obese at age 20 (HR 1.32, 95% CI: 0.93-2.83, p= 0.09).

TABLE 1 Hazard ratios for associations between sex, BMI at age 20, polypectomy, smoking, and alcohol consumption and the risk of colorectal cancer for participants with a germline variant in *PMS2*.

			Unweighted univariable model		Weighted univariable model	
	No. with CRC	Person-years	HR (95% CI)	*P* value	HR (95% CI)	*P* value
Sex						
Female	43	5064	1 [Reference]		1 [Reference]	
Male	50	3422	1.65 (1.10 – 2.46)	0.02	2.14 (1.21 – 3.77)	0.01
BMI at age 20						
Normal	68	6324	1 [Reference]		1 [Reference]	
Underweight	6	862	0.69 (0.33 – 1.43)	0.32	0.44 (0.16 – 1.21)	0.11
Overweight/ Obese	16	1098	1.63 (0.93 – 2.83)	0.09	1.32 (0.59 – 2.97)	0.5
Any polypectomy						
No	90	8228	1 [Reference]		1 [Reference]	
Yes	3	258	0.84 (0.28 – 2.53)	0.76	0.81 (0.22 – 2.99)	0.75
Smoking status						
Never	39	3281	1 [Reference]		1 [Reference]	
Former	31	2002	0.93 (0.57 – 1.52)	0.78	0.81 (0.38 – 1.70)	0.58
Current	23	3203	0.81 (0.48 – 1.35)	0.42	0.89 (0.43 – 1.84)	0.74
Total pack-year*	62	6762	1.00 (0.97 – 1.03)	0.93	1.01 (0.97 – 1.05)	0.62
Alcohol consumption (units/week)	53	6218	0.97 (0.93 – 1.01)	0.16	0.95 (0.90 – 1.00)	0.07

*Pack-years: Number of cigarettes per day multiplied by the number of years

DISCUSSION

In this study we investigated the association of BMI, smoking and alcohol consumption with the development of colorectal cancer in Lynch syndrome patients carrying a *PMS2* variant. Notably we did not see major effects of any of these lifestyle factors. Interestingly, we did observe a trend towards carriers with overweight in adolescence being at increased risk of colorectal cancer, which is in line with previous observations by Win et al.[14] It should be noted however that previous studies have linked obesity to MSS tumors, whereas Lynch-associated colorectal cancers are usually MSI-high.[5] Indeed, the previous study by Win et al. found no significant difference between MMR carriers compared to non-carriers, which suggests that the effect is not stronger in Lynch syndrome, but similar to the general population.[14] Clinical interference on BMI at adolescence if of course problematic as many carriers only become apparent after age 20, even those that are tested pre-symptomatically, and preventive weight loss might be too late as the damage could have already been done. Therefore advises concerning weight should also focus on children of known carriers, even before variant screening is performed.

In contrast to BMI, smoking has previously been associated with MSI-H colorectal cancer. Paradoxically, cigarette smoking appeared to be associated with colorectal adenomas in most studies, but reports are inconsistent about the association between cigarette smoking and colorectal cancer.[3, 8, 15-18] This is however, most likely due to the fact that cigarette smoking only contributes to the development of a minority of colon cancers, namely those that are MSI-H.[17] This makes it a particularly interesting potential risk modifier in Lynch syndrome, as MSI is a known hallmark of Lynch associated colorectal cancer. Slattery et al. estimated that 21% of MSI in colon tumor tissue might be attributed to cigarette smoking.[16] The biological explanation for this could be that smoking cigarettes causes replication errors in too large a number to be repaired by the MMR system or that it interferes within the MMR system itself. In patients with Lynch syndrome with an already vulnerable MMR system the latter might lead to higher risk of colorectal cancer. Indeed, a previous study reported significant HRs for MMR mutation carriers. This study reported a significant HR (2.15 (1.22-3.8)) for the heterodimer partner PMS2, MLH1.[8] However, we were not able to confirm the risk modifying effects of smoking in this PMS2 cohort. This could have several biological reasons. One explanation could be that the effect of smoking in Lynch syndrome is gene-specific. MLH1 and PMS2 proteins function in a heterodimer within the MMR machinery, as do MSH2 and MSH6. Notably, MLH1 can also form a heterodimer with MLH3 or PMS1, which might in part explain both the lower penetrance but also a

lower damaging effect of smoking, because there is still a partly functioning MMR system even in the absence of PMS2.[19] Conversely, the same study as mentioned above also reported a significantly increased HR (6.02 (1.40-25.87)) for *MSH6* carriers, while the MSH2 protein also has the capacity to bind with another protein, namely MSH3. Hence, this does not seem to be the final answer.[8] Another possibility is that *PMS2* is less susceptible for a second hit caused by lifestyle factors, compared to for example *MSH6*. Indeed, the *MSH6* gene is known to have a microsatellite region which may be a potential target for smoke-related DNA toxicity. This second hit theory has been suggested as an explanation for a higher susceptibility to lifestyle mediated cancer risk increase in hereditary cancer patients.[4] More functionally oriented studies are needed to be able to investigate the underlying biological mechanism further. Lastly, lack of an association might also be caused by (genetic) heterogeneity of the cohort, for example interaction of smoking with SNPs in xenobiotic metabolizing enzymes such as CYP1A1.[3] This might mean that only a proportion of (*PMS2*) carriers is at an increased risk when they smoke, which might have been missed in this crude analysis. Identifying these cases could select carriers that should be counselled more proactively for smoking cessation.

It should be noted however, that the lack of significant results in this study could also be a consequence of a relatively small cohort size, which is a limitation of this study. Another limitation is the retrospective case-control design, which means that there is a chance of selection bias. We attempted to minimize this bias by using a weighted cohort approach. Lastly, the parameters investigated in this study were self-reported which means that recall bias could be present. This is especially relevant for questionnaires of deceased carriers that were filled in by family members. Conversely, these questionnaires are also a strength of this study, as they limit survival bias, i.e. we do not exclude people with a poor clinical outcome.

In summary, we found no strong association of smoking, obesity in adolescence, or alcohol use with colorectal cancer risk in our cohort of 270 PMS2-associated Lynch syndrome patients, although there might be a trend for those *PMS2* carriers that are obese in adolescence. Larger studies are needed to investigate this finding further. As recent work by our group suggested that colorectal cancer in *PMS2* carriers may only develop through the adenoma-to-colorectal cancer pathway (ten Broeke et al, 2018, accepted at Gastroenterology), using the development of adenomas as an endpoint might be of particular interest in this subset of Lynch patients. Future studies should therefore focus on prospectively obtained data.

3

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3.3

The apparent genetic anticipation in PMS2-associated Lynch syndrome families is explained by birth-cohort effect

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Sanne W. ten Broeke, Mar Rodríguez-Girondo, Manon Suerink, Stefan Aretz, Inge Bernstein, Gabriel Capellá, Christoph Engel, Encarna Gomez Garcia, Liselot P. van Hest, Magnus von Knebel Doeberitz, Kristina Lagerstedt-Robinson, Tom G.W. Letteboer, Pål Møller, Theo A. van Os, Marta Pineda, Nils Rahner, Maran J.W. Olderode-Berends, Jenny von Salomé, Hans K. Schackert, Liesbeth Spruijt, Verena Steinke-Lange, Anja Wagner, Carli M.J. Tops, Maartje Nielsen

ABSTRACT

Background

PMS2-associated Lynch syndrome is characterized by a relatively low colorectal cancer (CRC) penetrance compared to other Lynch syndromes. However, age at CRC diagnosis varies widely and a strong genetic anticipation effect has been suggested for *PMS2* families. In this study we examined proposed genetic anticipation in a sample of 152 European *PMS2* families.

Methods

The 152 families (637 family members) that were eligible for analysis were mainly clinically ascertained via clinical genetics centers. We used weighted Cox-type random effects model, adjusted by birth-cohort and sex, to estimate the generational effect on the age of onset of CRC. Probands and young birth-cohorts were excluded from the analyses. Weights represented mutation probabilities based on kinship coefficients, thus avoiding testing bias.

Results

Family data across three generations, including 123 CRCs, were analyzed. When compared to the first generation, the crude Hazard Ratio (HR) for anticipation was 2.242 (95%CI: 1.162-4.328) for the second and 2.644 (95%CI: 1.082-6.464) for the third generation. However, after correction for birth-cohort and sex the effect vanished (HR=1.302 (95%CI: 0.648-2.619) and HR=1.074 (95%CI: 0.406-2.842) for second and third generations, respectively).

Conclusions

Our study did not confirm previous reports of genetic anticipation in *PMS2*-associated Lynch syndrome. Birth-cohort effect seems the most likely explanation for observed younger CRC diagnosis in subsequent generations, particularly since there is currently no commonly accepted biological mechanism that could explain genetic anticipation in Lynch syndrome.

INTRODUCTION

Lynch syndrome is the most common cause of hereditary colorectal cancer, accounting for 3-5% of all colorectal cancers diagnosed annually.[1] The underlying cause is a heterozygous pathogenic germline variant in one of the mismatch repair genes: *MLH1*, *MSH2* (*EPCAM*), *MSH6* or *PMS2*. The latter gene is associated with a lower estimated penetrance and thus a markedly lower incidence of cancer. However, PMS2 families show phenotypic variability, with very wide differences in age at colorectal cancer diagnosis. While the mean age of onset for colorectal cancer for PMS2-associated Lynch syndrome is around 60, some PMS2 carriers develop colorectal cancer as early as 23.[2-4] Several external and internal modifiers have been suggested as possible explanations, one of which, genetic anticipation, has been the subject of much debate.[5-9] The phenomenon of genetic anticipation is clearly defined in genetic disorders involving trinucleotide repeats such as Huntington's disease, where expansion of the repeat in subsequent generations is a clear precursor of disease.[10] However, a mechanism of this type has not been described in Lynch syndrome, which in fact requires a second somatic hit for mismatch repair (MMR) deficiency to occur. A single germline mutation in one of the MMR genes does not confer haploinsufficiency.[11]

Nevertheless, genetic anticipation in Lynch syndrome and other dominantly inherited cancer predisposition syndromes has been reported by several groups. If a genetic anticipation effect could indeed be confirmed it would be of clinical utility in the development of individually-tailored surveillance schemes. The only report of genetic anticipation in PMS2-associated Lynch syndrome families found a very strong effect (anticipation of 7.3 years per subsequent generation).[12] However, sample size in that study was small, including only 12 PMS2 families. In the same study, carriers of pathogenic germline variants in other MMR genes showed only small or absent anticipation effects.[12] By investigating a much larger cohort of 152 families, our aim was to reassess the possibility of genetic anticipation in PMS2-associated Lynch syndrome.

METHODS

Description of the cohort

Pedigree data on European families carrying a segregating pathogenic PMS2 variant were originally collected from clinical genetic departments between 2009 and 2012, as previously described.[4] Further families were collected between 2012 and 2017 and an extensive description is available elsewhere (ten Broeke et al, 2018, in-press

at Journal of Clinical Oncology). The PMS2 families included originated from the Netherlands, Norway, Germany, Sweden, Denmark and Spain. Data collection was approved by the local ethical review board (Leiden University Medical Center Ethics Review Board, protocol ID: P01.019). This dataset consisted of clinically ascertained families where variant analysis was initiated due to (histological) pre-screening by immunohistochemistry and/or microsatellite instability, usually because a family met Bethesda criteria.[13] Data collection from patient records included demographic data, family pedigrees, age and location of cancer diagnosis, polypectomy, and hysterectomy if applicable. When available, clinical and pathological diagnoses were confirmed using patient records.

Statistical analysis

The outcome of interest was age at first diagnosis of colorectal cancer. The follow-up time was defined as the time elapsed from birth till the first colorectal cancer diagnosis or censoring. Censoring occurred on the basis of last known other cancer diagnosis, death or administrative censoring at age of last contact with the family, whichever occurred last. Family members with bi-allelic PMS2 mutations were excluded from the analysis given the severe and markedly different phenotype of these constitutional mismatch repair deficiency (CMMRD) patients. Genetic anticipation was estimated as the effect of generation on a person's hazard for cancer diagnosis, using a shared gamma frailty proportional hazard model:

$$\lambda(t_{ij}) = u_i \lambda_0(t_{ij}) \exp(\boldsymbol{\beta Z}_{ij} + \boldsymbol{\gamma X}_{ij}),$$

where t_{ij} is the age at first diagnosis of colorectal cancer or the age at censoring for member j in family i, x λ_0 (t_{ij}) refers to the baseline hazard, which is left completely unspecified (Cox-type model), $\beta = (\beta_1, \beta_2)$contains the main effects of interest, the regression coefficient of second and third generation $Z = (Z_1, Z_2)$ taking the first oldest generation of each family as reference and $u > 0$ refers to an unobserved random effect (frailty) shared by the members of the same family. This unobserved heterogeneity shared within families was assumed to follow a gamma distribution (normal frailty was also checked as a sensitivity analysis). γ contains the effect of person-specific covariates χ included in a second adjusted analysis, namely sex and year of birth.

Since not all family members were tested for *PMS2* variants, mutation probabilities based on kinship coefficients were used as analytical weights to avoid possible testing bias and increase efficiency. Specifically, the weight for individual j, $w_j = P(mutation \mid family$

history of mutation) is given by the kinship coefficient between individual *j* and the closest family member with observed mutation. Mutation probabilities are included as case weights in the corresponding penalized score function provided in the R package *survival*.[14] Remaining ascertainment bias was controlled by excluding the probands and focusing on individuals born before 1950, so that all included individuals were at risk for at least 65 years, hence avoiding potential bias due to right truncation. Statistical significance was established at 5%.

RESULTS

A description of the cohort is given in tables 1 and 2. The analysis included 637 family members with 123 colorectal cancers (table 1), divided over 3 generations (table 2). After weighting, the estimated number of mutation carriers in the sample is 360. Results of the Cox-type random effects model are given in table 3, which shows increased hazard ratios (HRs) in the crude analysis (HR=2.24, 95% CI=1.16-4.33 for the second generation and HR=2.64, 95% CI=1.08-6.46 for the third generation, respectively). After correction for gender and birth-cohort, HR size decreased (half of the crude effect) and was no longer statistically significant (as the corresponding confidence intervals included 1). The adjusted analysis showed a strong effect of year of birth (HR=1.05, 95% CI = 1.02-1.07), equaling a roughly 5% increase of risk for every year towards the present time. These results suggest that the estimated anticipation effect in the crude analysis is strongly confounded by birth-cohort and that the apparent effect of generation is mainly explained by secular trends in colorectal cancer diagnosis. The use of normal random effects instead of gamma provided very similar results in terms of genetic anticipation, sex and birth-cohort effects (results not shown).

TABLE 1 Cohort description

Number of families	152
Family members included	637
Mutation status	
100%*	176
[50%,100%)	282
[25%,50%)	158
[12.5%,25%)	21
Colorectal cancer	
Number	123
Mean Age (s.d.)	69.58 (12.94)
Median age (IQR)	71 (62-77)

s.d.: Standard deviation; IQR: Interquartile range.
* Confirmed and obligate carriers.
Note: Probands were excluded from the analysis.

TABLE 2 Number of family members for each generation and median year of birth

Generation	Number	Median (IQR)
1	153	1912 (1902-1924)
2	399	1927 (1918-1938)
3	85	1943 (1937-1950)

IQR: Interquartile range

TABLE 3 Results of Cox model

	Crude analysis		Adjusted analysis*	
	HR	95% CI	HR	95% CI
1	*reference*		*reference*	
2	2.24	1.16 to 4.33	1.30	0.65 to 2.62
3	2.64	1.08 to 6.46	1.07	0.41 to 2.84

*Adjusted for: gender and year of birth

DISCUSSION

The occurrence of genetic anticipation in Lynch syndrome has been a subject of considerable debate and gene-specific effects have been offered as an explanation. After correction for birth-cohort, our analysis found no evidence of anticipation in a very large cohort of PMS2-associated Lynch syndrome families. A rise in colorectal cancer incidence as well as lower age at diagnosis in recent decades in the general population has been previously observed.[15-17] Reasons for this might include better detection with more sensitive screening methods, lifestyle factors, population-based screening protocols and increased life-expectancy. These factors could also play a role in Lynch syndrome patients. Other factors that could cause a false genetic anticipation signal that are specific to Lynch syndrome, and other dominantly inherited cancer predisposition syndromes in general, involve the genetic diagnostic process. For example, after identification of the proband, pre-symptomatic family members are tested and subsequently screened if they carry the *PMS2* variant. This might lower age at diagnoses of indolent tumors which might not have presented itself otherwise. An alternative explanation for false genetic anticipation effect may be that colorectal cancer diagnosis in older generations may have been underreported.

Analysis of dominantly inherited cancer predisposition is potentially influenced by several forms of bias. First, clinically ascertained families are accompanied by a selection bias, as they were selected due to their compliance with clinical selection criteria and are therefore often severely affected, i.e. many family members with (colorectal) cancer or an unusually low age at diagnosis. A problem arises when the phenotype is not caused by the pathogenic *PMS2* variant alone but is affected by other modifying factors. This is especially problematic for PMS2, as selection based on, for example, the Bethesda guidelines is influenced by criteria for classic Lynch families involving mainly pathogenic *MLH1* or *MSH2* variants. In the case of *PMS2* variants, it is well documented that variants are at most only moderately penetrant[2, 4], suggesting that *PMS2* families selected on the basis of these criteria alone will include many relatively severely affected members. However, due to universal screening for mismatch repair deficiency in all colorectal cancers below age 70 in most Western countries, a rise in unselected *PMS2* carriers is expected.[18]

A second form of bias that should be considered is testing bias due to the fact that people affected with (colorectal) cancer (at a young age) are more likely to be tested for the presence of a *PMS2* variant. Probands (i.e. the first person in the family with a confirmed pathogenic germline *PMS2* variant) are the most notable example of this, and all probands were therefore excluded from our analysis. Moreover, we also used

analytical weights to model mutation probabilities. For example, first-degree relatives of a confirmed carrier that were not tested were given a weight of 0.5, whereas second-degree relatives had a weight of 0.25. This approach also helped improve the power of the analysis.

Although there is no clear biological rationale for genetic anticipation in Lynch syndrome, alternative explanations besides birth-cohort have been proposed in other studies. It is generally accepted that families with Li-Fraumeni syndrome (which strongly predisposes to several forms of cancer) exhibit anticipation that cannot be explained by a birth-cohort effect.[19, 20] A recent whole genome sequencing study of germline DNA in 13 Li-Fraumeni syndrome cases did not find increased DNA copy-number variations, suggesting that CNVs do not mediate the genetic anticipation effect. The authors proposed an alternative model explaining apparent anticipation in which variants from the non-carrier parent influence tumorigenesis in the offspring of TP53 mutation carriers with late onset of cancer.[21] In other words, parents with relatively late onset might have offspring that are more prone to tumorigenesis due to inheritance of specific risk increasing variants from the non-carrier parent. Similar mechanisms may also influence cancer age of onset and thus explain variability within families and birth-cohorts in Lynch syndrome. Another suggested biological mechanism involves telomeres. Retrospective studies have identified shorter telomeres in colorectal cancer cases vs. controls, arguing that shorter telomeres cause chromosomal instability and might therefore lead to cancer. Indeed, shortening of telomeres was also observed in peripheral blood in Lynch Syndrome patients affected with colorectal cancer, compared to non-affected mutation carriers.[22] This finding has not been replicated in prospective studies, suggesting that the shortening of telomeres might be the result of the cancer process rather than a causative factor.[23]

Ours is not the first study to report bias in anticipation analysis due to birth-cohort effects. Similar results have been found in other genetic syndromes, including a study by Guindalini et al. in BRCA1/2 families.[24] This study corrected for various types of bias by excluding probands, including mutation probabilities and correcting for birth-cohort. Our analysis followed similar principles and incorporated additional flexibility in the specification of the regression model. Our model is semi-parametric, since the baseline hazard is left completely unspecified and is therefore more flexible than the model used by Guindalini et al., which was based on a parametric specification of the underlying time-to-event data distribution.[25] Moreover, we have allowed for a more flexible, non-linear effect of generation, considering two possibly different effects for second and third generations with respect to the first, oldest generation. Previous reports have relied on a linear and perhaps too stringent specification of the

anticipation effect. We also used gamma random effects in our main analyses and checked the impact of random effect specification by also considering normal random effects. The results regarding anticipation and birth-cohort effect remained the same. Normal random effect modeling of hazard was previously used by von Salome et al. in a study in which the authors reported strong genetic anticipation in twelve PMS2 families.[12] However, cohort effects were not considered and a linear specification was assumed for the generation effect. Daugherty et al. also used a Cox-type hazard regression method to study anticipation in lymphoproliferative tumors but adopted a less flexible approach since random effects were not considered and hence family-specific effects could not be captured.[26] Nevertheless, these authors also identified a confounding effect of secular trends on apparent anticipation effects of generation.

Regression strategies have previously been shown to be preferable over hypothesis testing based on parent-child pairs.[9] Since our regression strategy is flexible, it is possible to reasonably reflect the underlying structure of the data while still getting interpretable results and preserving sufficient power. Boonstra et al. have reported genetic anticipation in Lynch syndrome based on an alternative specification that allowed for family-specific anticipation effects (random slopes).[27] Such specification is flexible since it allows for a specific effect of generation in each family, although the effect is linear within families. We have introduced flexibility in a different manner, by allowing for a non-linear fixed anticipation effect, which is less dependent in the chosen parametric family on random effects. Moreover, Boonstra et al. did not directly estimate cohort effects based on the sample, but inferred them from external cancer incidence registries (not specific for Lynch syndrome) on the basis of a piecewise (5-year knots) linear hazard assumption.[27] Misspecification in this step may have introduced bias in the estimated anticipation effect. Despite our efforts to account for possible bias in our analysis strategy, the retrospective nature of our data is still a limitation of our study. Similarly, in an effort to avoid ascertainment bias we excluded some data, leading to a reduction in power. Models which can accommodate right truncated data should be developed and used in this field. A last limitation is that the weights that were used to estimate the probability of carrying the familial *PMS2* mutation only took into account degree of kinship, but not the presence of a cancer phenotype, e.g. colorectal cancer. Including this factor in the weigh calculation is complicated given the complex pedigree structure. Moreover, recent work by our own group suggests that the lifetime risk for colorectal cancer is only 2-3 times increased compared to the general population (ten Broeke et al, 2018, in-press at Journal of Clinical Oncology), which may cause misspecification of PMS2-associated colorectal cancer as this cancer also occurs frequently in the general population.

In conclusion, after correction for birth-cohort, our study did not confirm previous findings of genetic anticipation in PMS2-associated Lynch syndrome patients. Therefore, anticipation cannot be used in individual risk estimation. Given the large phenotypic variability in Lynch syndrome patients, future studies should focus on other potential modifiers.

3

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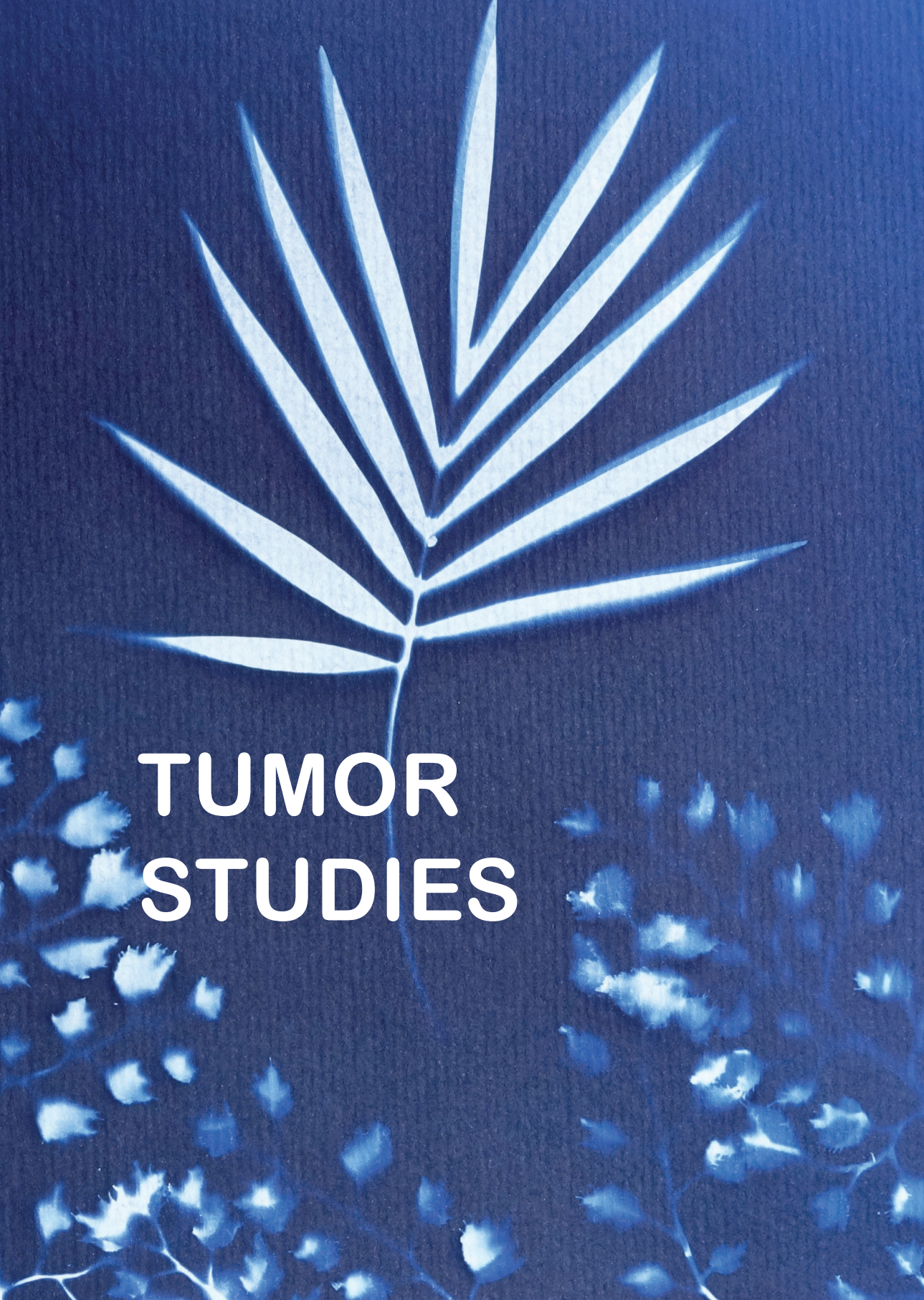
TUMOR
STUDIES

4.1

Coding microsatellite mutation profiles in cancers of *PMS2* mutation carriers

Manuscript in preparation

Sanne W. ten Broeke*, Alexej Ballhausen*, Aysel Ahadova, Manon Suerink, Hans Morreau, Tom van Wezel, Julia Krzykalla, Axel Benner, Noel de Miranda, Magnus von Knebel Doeberitz, Maartje Nielsen, Matthias Kloor

* These authors contributed equally to this work.

ABSTRACT

Introduction

Lynch syndrome is caused by heterozygous pathogenic germline variants in one of the mismatch repair (MMR) genes (*MLH1*, *MSH2*, *MSH6* and *PMS2*). Lynch syndrome cancers are characterized by MMR deficiency and by the accumulation of multiple insertion/deletion mutations at coding microsatellites (cMS). MMR deficiency-induced mutations at defined cMS loci have a driver function and promote tumorigenesis in Lynch syndrome. However, *PMS2* mutation carriers have only a moderately increased risk of developing colorectal cancer (CRC) or other cancers. In the present study we asked whether the lower penetrance of PMS2-associated Lynch syndrome may be reflected by the phenotype of manifest tumors.

Material & Methods

Tumor DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue cores (n=90). The mutation spectrum was analyzed by using fluorescently labeled primers specific for a selected series of 18 cMS previously described as mutational targets in MSI cancer development. Immune cell infiltration was analyzed by immunohistochemical staining of FFPE tissue sections for CD3+ T cells.

Results

The cMS spectrum of PMS2-associated CRCs did not show any significant differences from other MMR gene-associated CRCs. Most commonly mutant target cMS were located in the genes *ACVR2*, *AIM2*, *BANP*, *C4orf6*, and *ZNF294*. However, PMS2 tumors displayed a significantly lower CD3+ infiltration (p=0.0016).

Discussion

Our observation suggests that MMR deficiency plays a similar role in the pathogenesis of PMS2-associated CRCs compared to MMR-deficient cancers lacking functional MLH1 or MSH2. Moreover, our results also imply that the spectrum of cMS mutation-induced frameshift peptide neoantigens of PMS2-associated CRCs is expected to be similar to that from other MMR-deficient CRCs. Studies analyzing the mechanisms underlying the lower immune infiltration in *PMS2*-associated cancers are warranted.

INTRODUCTION

Lynch syndrome is caused by a heterozygous pathogenic germline variant in one of the DNA mismatch repair (MMR) genes: *MLH1*, *MSH2 (EPCAM)*, *MSH6* or *PMS2*. After somatic inactivation of the remaining functional MMR gene allele, MMR deficiency leads to the accumulation of numerous small insertions or deletions at repetitive sequence stretches termed microsatellites (microsatellite instability, MSI). Insertions or deletions affecting microsatellites located in gene-encoding regions can lead to shifts of the translational reading frame and thus to inactivation of the affected genes. Moreover, through shifting of the reading frame, completely new peptide stretches are synthesized that are unknown to the immune system and therefore can elicit strong immune responses of the host.[1, 2] Several coding microsatellite mutations that drive Lynch syndrome cancer progression through the inactivation of tumor suppressor genes have been previously identified.[3, 4] As coding microsatellite mutations can contribute to cancer development, the patterns of coding microsatellite mutations observed in manifest cancers reflect evolutionary selection and therefore the pathogenesis of tumor developments. This is for example illustrated by marked differences in coding microsatellite mutation frequency between colorectal and endometrial cancers.[5, 6]

PMS2-associated Lynch syndrome patients have a markedly lower penetrance and later age of onset of colorectal and endometrial cancer than carriers of *MLH1* or *MSH2* mutations.[7, 8] The reported cumulative risk of CRC is 11-20% for *PMS2* carriers, which is in sharp contrast to a cumulative risk of 35-55% up to age 70 for *MLH1/MSH2* carriers.[9] Notably, prospective studies have now reported that the cumulative risk of colorectal cancer for *PMS2* mutation carriers undergoing colonoscopic surveillance is 0%.[10, 11] Again, this is in contrast to *MLH1/MSH2* carriers with risks of CRCs arising between follow-up colonoscopies to be up to 46% and 43% respectively. Even *MSH6* carriers who also have a milder phenotype are at risk (15%) of such interval cancers.[11] Consequently, the functional significance of *PMS2* mutations during the pathogenesis of cancers has been questioned and it is perceivable that *PMS2* may play a different and a (minor) role in tumorigenesis. In the present study, we compared coding microsatellite mutation patterns in PMS2-associated Lynch syndrome cancers to those observed in colorectal cancers from *MLH1* and *MSH2* mutation carriers.

MATERIAL & METHODS

Tumor specimens

Tumor material from 10 *PMS2* germline mutation carriers was collected within Leiden University Medical Centre (Table 1). Tumor material from 41 *MLH1*, 23 *MSH2*, 12 *MSH6*, and 4 *PMS2* germline mutation carriers was collected within the Department of Applied Tumor Biology, Institute of Pathology, University Hospital Heidelberg, as a center of the German HNPCC Consortium. Informed consent was obtained from all participating patients.

Tumor workup and DNA isolation

Tissue blocks were collected, and DNA was isolated from three tissue cores of variable length (0.3 mm diameter, or 0.7 mm in case of tissue with a low cell count) or from whole tissue sections after manual microdissection using the DNeasy FFPE Kit (Qiagen, Germany).

Microsatellite analysis

For the characterization of cMS patterns, we performed fragment length analysis using fluorescently labeled primers specific for a selected series of 18 coding microsatellites previously described as mutational targets in MSI cancer development.[12] Primer sequences are provided in Table 2. Selection criteria were (1) frequency of mutation in MMR-deficient cancers, (2) evidence of a functional driver role of mutations suggested by a mutation frequency higher than expected from microsatellite length, and (3) potential significance as source of immunogenic frameshift peptide neoantigens supported by epitope prediction algorithms. PCR products were visualized on an ABI3130xl sequencer, and the obtained results were processed using a newly developed algorithm to obtain quantitative estimation of the frequency of the mutant alleles in tumor specimens (qMSI, Ballhausen, Przybilla et al., in preparation).

Immunohistochemical analysis of CD3

From 10 PMS2-associated tumors formalin fixed paraffin embedded (FFPE) tumor blocks were available for further analysis of immune cell infiltration. For immunohistochemical detection of CD3+ cells, 4 µm thick sections of the tumors were stained with an antibody specific for CD3 (DAKO monoclonal antibody, dilution 1:100). For immune cell scoring 4 areas of interest (0.1 mm^2 each) were randomly placed in the tumor center, CD3+ immune cell infiltration was scored as the mean number of CD3+ immune cells of the 4 areas.

TABLE 1 Description of Leiden cohort

Patient ID	Index	Gender	Age at CRC	CRC location	Germline PMS2 mutation	TNM	Differentiation	MSI	IHC	TILs (subjectively scored LUMC)	Mean CD3+ cell count per 0.1mm^2	B2M status
1	Yes	Female	46	Right	c.804-60_804-59in-sJN866832.1	T3N0	Moderate	High	PMS2-/ MSH6-	Not scored	N.A.	wildtype
2	Yes	Female	48	Descending colon (flexura lienalis)	c. 856_857delGA (p.Asp286fs)	T3/4N0Mx	Moderate	Not tested	PMS2-/ MSH6-	Little	35	wildtype
3	Yes	Male	48	Sigmoid	Deletion exon 5-7	T3N2	Poor	High	PMS2-/ MSH6-	Marked	38.25	wildtype
4	No	Female	64	Coecum	c.2192_2196delTAACT (p.Leu731CysfsX3)	T4N0	Poor	High	PMS2-/ MLH1+	Little	10.75	mutated
5	Yes	Female	46	Rectum (recurrence)	Deletion exon 11-15	T2N0	Moderate	High	PMS2-/ MLH1+	Marked	12.5	wildtype
6	Yes	Male	57	Transverse colon	Deletion exon 14	T4N1	Moderate-Poor	High	PMS2-/ MLH1+	Little	N.A.	N.A.
7	No	Male	54	Coecum	Deletion exon 5-7	T2N0	Moderate-Poor	High	PMS2-/ MLH1+	Moderate	47.75	wildtype
8	Yes	Female	41	Transverse colon	c.736_741delCCCCC TinsTGTGTGTGAAG (p.Pro246CysfsX3)	T3N1M1	Poor	Stable	PMS2-/ MLH1+	(very) Little	3	wildtype
9	Yes	Male	47	Transverse colon	c.354-1G>A	T2N0	Well-Moderate	High	PMS2-/ MLH1+	Moderate	13.75	wildtype
10	Yes	Male	27	Coecum	c.1882C>T (p.Arg628X)	T3N2	Poor	High	PMS2- / MLH1+	Little	27.75	wildtype

CRC: Colorectal cancer

TABLE 2 Primer sequences of the analyzed cMS

Gene name	cMS length	Forward primer	Reverse primer
ACVR2A	A8	GTTGCCATTTGAGGAGGAAA	CAGCATGTTTCTGCCAATAATC
AIM2	A10	TTCTCCATCCAGGTTATTAAGGC	TTAGACCAGTTGGCTTGAATTG
ASTE1	A11	ATATGCCCCCGCTGAAATA	TTGGTGTGTGCAGTGGTTCT
BANP	T12	TTCTGTGGAAGCTCTGCCTT	TCAAGTCGCATCAGATCCAG
C4orf6	T10	CCAGAAGCAAATTCACAAGAC	TTTTGCGTGTTCCTTCCTTC
CASP5	A10	CAGAGTTATGTCTTAGGTGAAGG	ACCATGAAGAACATCTTTGCCCAG
ELAVL3	G9	GATGCGACCTGTTATCTCCAG	AGGTTGGTCTTGCTGTCGTC
GLYR1	G8	GCCTCCAGAAGCTGTGACTT	ATCACCAACATCCCGTCATT
LMAN1	A9	CACCCATGTCAGCTTTGCTA	GGAGGAATTTGAGCACTTTCA
MARCKS	A11	GACTTCTTCGCCCAAGGC	GCCGCTCAGCTTGAAAGA
NDUFC2	T9	TGAATTTCAGGTTTGCATCG	AACATTTCACGGTCCCTCAC
PTHLH	A11	TTTCACTTTCAGTACAGCACTTCTG	GAAGTAACAGGGGACTCTTAAATAATG
SLC22A9	A11	GCGCCTACAGTGCCTACTCT	GCATGTGGAGCATTTCACAC
SLC35F5	T10	TGTGGGGAAACTTACTGCAA	TCAAGTTTCAAACATCATATGCAA
TAF1B	A11	ACCCAAATAAAAGCCCTCAAC	CTACTTAAAATTCCATTCCATGTCC
TCF7L2	A9	GCCTCTATTCACAGATAACTC	GTTCACCTTGTATGTAGCGAA
TGFBR2	A10	GCTGCTTCTCCAAAGTGCAT	CAGATCTCAGGTCCCACACC
ZNF294	A11	AAGCCGAAGAGCTCATTGAA	CAGTTGTTAATTCCCAGCCTTC

Statistical analysis

Statistical significance of differences in mutation rates between tumors from *PMS2* mutation carriers and those from *MLH1* and/or *MSH2* mutation carriers was calculated in three steps.

First, all cMS showing a prevalence of at least 15% non-wild type alleles in a certain tumor were classified as mutant. The distribution of mutation rates was compared between tumors from *PMS2* mutation carriers and those from *MLH1* and *MSH2* mutation carriers grouped together using general mutation frequency of cMS in a chi-squared test. Benjamini-Hochberg procedure was used to adjust the raw p-values over all genes. P values smaller than 0.05 were classified as statistically significant.

In a second step, quantitatively analyzed mutant allele ratios were used to test for differences in cMS mutation patterns between tumors from *PMS2* mutation carriers and those from *MLH1* and *MSH2* mutation carriers grouped together. For this, Wilcoxon-

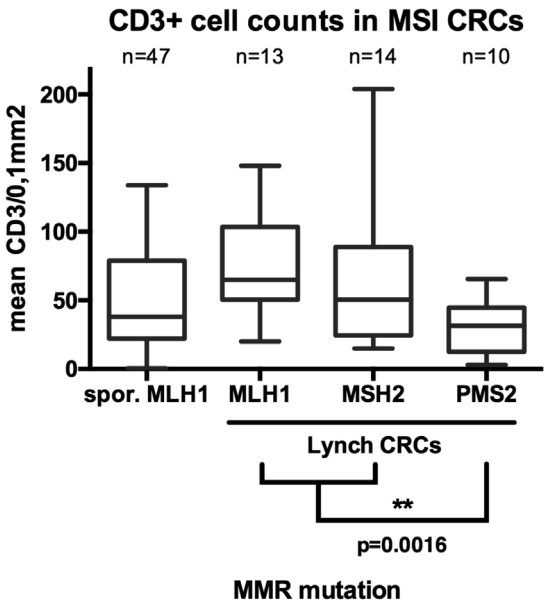

FIGURE 1 Proportion of samples with common hotspot variants G12D and G13D, or other KRAS variants.
Note: p-values represent comparison between PMS2-associated colorectal tumors and MLH1- or MSH2-associated tumors, respectively.

Mann-Whitney test was used and raw p-values were adjusted over all genes using Benjamini-Hochberg procedure.

Finally, quantitatively analyzed cMS patterns of *PMS2* mutation carriers were separately tested against those from *MLH1* and *MSH2* mutation carriers. The global p-value states if there is a significant difference for at least one of the two pairwise comparisons using Wilcoxon-Mann-Whitney test (*PMS2* vs. *MLH1* or *PMS2* vs. *MSH2*). The pairwise p-values give the results for local pairwise comparison. Then, a two-sample Kolmogorov-Smirnov test was applied for the pairwise comparisons in order to test for equal distribution. Pairwise p-values for both approaches were adjusted over all genes using Benjamini-Hochberg procedure. No separate comparison of mutation patterns between tumors from *PMS2* mutation carriers and *MSH6* mutation carriers was performed due to the limited number of *MSH6* mutation carriers available.

Additionally, T-cell infiltration measured by mean number of CD3+ T cells was compared between *PMS2* mutation carriers and *MLH1* and *MSH2* mutation carriers grouped together. Wilcoxon-Mann-Whitney test was applied in order to test the difference in median CD3+ T-cell infiltration between the two groups.

RESULTS

Comparison of cMS mutation frequency between PMS2 CRCs and non-PMS2 CRCs

Tumors from 14 *PMS2*, 41 *MLH1*, 23 *MSH2* and 12 *MSH6* mutation carriers were analyzed for cMS mutation patterns. Five out of 18 analyzed cMS showed mutations in all analyzable PMS2-associated CRCs: *ACVR2* (n=12), *AIM2* (n=12), *BANP* (n=12), *C4orf6* (n=10), *ZNF294* (n=12). Common functionally relevant target cMS presented with similar mutation frequencies in PMS2 vs. non-PMS2-associated CRCs, including *ACVR2* (PMS2: 12/12, 100% vs non-PMS2: 46/51, 90.2%, p=0.59) and *AIM2* (PMS2: 12/12, 100% vs. non-PMS2: 42/48, 87.5%). *TGFBR2*, the most commonly analyzed cMS target in MMR-deficient colorectal cancer, displayed a similar frequency of mutations in the PMS2 vs. the non-PMS2 CRC collection (PMS2: 9/11, 81.8% vs. non-PMS2: 41/44, 93.2%, Table 3).

No significant differences in coding microsatellite mutation frequencies were observed between colorectal cancers from *PMS2* mutation carriers and colorectal cancers from *MLH1* and *MSH2* mutation carriers after adjusting for multiple testing (Table 3).

Quantitative analysis of mutant cMS alleles

The time point of MMR deficiency during CRC pathogenesis has previously shown to be related to mutational signatures.[13] In order to evaluate whether *PMS2*-mutant tumors may show a quantitative difference of cMS mutations compared to MLH1- and MSH2-deficient CRCs, we quantitatively analyzed mutant allele ratio. The analysis did not reveal any significant difference. However, a trend towards a higher proportion of mutant alleles was observed for the cMS of *C4orf6* (PMS2: 0.434, n=10 vs. 0.253, n=47, raw p=0.004, Benjamini-Hochberg corrected p=0.07). For this candidate, a separate comparison of PMS2-associated CRCs with MLH1-associated and with MSH2-associated CRCs revealed a significant difference between mutation rates of *C4orf6* between the PMS2 and MSH2 group (PMS2: 0.434; MSH2: 0.196, adjusted pairwise p= 0.01).

TABLE 3 Percentage of tumors harboring mutations

cMS	Mutation frequency (PMS2)	Sample number (PMS2)	Mutation frequency (MLH1+MSH2)	Sample number (MLH1+MSH2)	p value	Adjusted p value
ACVR2A	100,0%	12	90,2%	51	0,5913	1
AIM2	100,0%	12	87,5%	48	0,4514	1
ASTE1	75,0%	12	89,4%	47	0,4096	1
BANP	100,0%	12	95,3%	43	1	1
C4orf6	100,0%	10	59,6%	47	**0,0363**	0,4198
CASP5	54,5%	11	75,0%	44	0,3346	1
ELAVL3	50,0%	10	64,4%	45	0,6237	1
GLYR1	54,5%	11	58,0%	50	1	1
LMAN1	45,5%	11	54,0%	50	0,8569	1
MARCKS	90,9%	11	88,1%	42	1	1
NDUFC2	76,9%	13	88,6%	44	0,5393	1
PTHLH	66,7%	12	68,0%	50	1	1
SLC22A9	91,7%	12	86,4%	44	1	1
SLC35F5	91,7%	12	55,3%	47	**0,0466**	0,4198
TAF1B	90,9%	11	82,4%	51	0,8043	1
TCF7L2	75,0%	8	69,8%	43	1	1
TGFBR2	81,8%	11	93,2%	44	0,5577	1
ZNF294	100,0%	12	94,3%	53	0,9346	1

Quantitative analysis of intratumoral CD3+ lymphocyte infiltration

As a next step, we analyzed the density of tumor-infiltrating lymphocytes in tumors from *PMS2*, *MLH1* and *MSH2* mutation carriers. The analysis of CD3+ lymphocyte infiltration revealed a significantly lower CD3+ T-cell counts in PMS2-associated CRCs when compared to tumors of *MLH1* and *MSH2* mutation carriers (Mann-Whitney test, p=0.0016, Figure 1). Interestingly, median CD3+ T-cell counts of *PMS2* mutation carriers were closer to those from sporadic MSI CRCs (38 compared to 31.50, Table 4).

TABLE 4 Frequency of mutant alleles

cMS	Mutant allele ratio (PMS2)	Sample number (PMS2)	Mutant allele ratio (MLH1+MSH2)	Sample number (MLH1+MSH2)	p value	Adjusted p value
ACVR2A	0.503	12	0.506	51	0.6193	0.7962
AIM2	0.523	12	0.426	48	**0.0460**	0.1901
ASTE1	0.325	12	0.473	47	0.0402	0.1901
BANP	0.483	12	0.570	43	0.2286	0.6179
C4orf6	0.434	10	0.253	47	**0.0039**	0.0702
CASP5	0.238	11	0.299	44	0.3433	0.6179
ELAVL3	0.191	10	0.246	45	0.3138	0.6179
GLYR1	0.177	11	0.268	50	0.3004	0.6179
LMAN1	0.179	11	0.201	50	0.9779	0.9779
MARCKS	0.341	11	0.414	42	0.2820	0.6179
NDUFC2	0.272	13	0.322	44	0.4571	0.6329
PTHLH	0.315	12	0.334	50	0.9613	0.9779
SLC22A9	0.409	12	0.379	44	0.7007	0.8408
SLC35F5	0.341	12	0.230	47	0.0528	0.1901
TAF1B	0.405	11	0.293	51	**0.0114**	0.1026
TCF7L2	0.381	8	0.318	43	0.4071	0.6329
TGFBR2	0.3944	11	0.466	44	0.5105	0.7068
ZNF294	0.469	12	0.486	53	0.7959	0.8954

DISCUSSION

PMS2 mutation carriers represent a distinct entity among Lynch syndrome patients, denoted mainly by a lower penetrance, making *PMS2* a moderately penetrant gene at most.[7, 8, 14] However, recent work has suggested that tumors with PMS2 deficiency may demonstrate a more aggressive phenotype.[15] These observations suggest that there may be fundamental differences in the pathogenesis of PMS2-associated CRCs that distinguish them from Lynch syndrome CRCs caused by germline variants affecting other MMR genes such as *MLH1* and *MSH2*.

So far, the somatic cMS mutation landscape of PMS2-associated CRCs has been unknown. We observed very similar somatic mutation patterns in PMS2-associated CRCs to those obtained in MLH1- and MSH2-mutant CRCs. This observation suggests that the role of cMS mutations during the development of *PMS2*-mutant cancers is comparable to cMS mutations in MLH1-deficient and MSH2-deficient CRCs. This may imply that *PMS2*-mutant colorectal cancers develop through a similar pathogenetic mechanism, with a similar impact of MMR deficiency, compared to other Lynch syndrome-associated cancers. Our results strongly support the hypothesis that MMR deficiency caused by *PMS2* mutations is a significant driving force of tumor development in these cancers rather than representing merely an epiphenomenon.

However, there are reports that *PMS2* deficiency may occur at a relatively late stage of carcinogenesis as illustrated by the reported relatively low frequency of MMR-associated KRAS hotspot variants G12D and G13D in a study that included CRCs analyzed here as well.[16] The same study also reported a lack of β-catenin variants in PMS2-associated CRCs, while the majority of a *MLH1* control cohort did harbor such variants. β-catenin variants have been suggested to be associated with CRCs that develop from dMMR crypts.[13, 17] CRCs developing through the dMMR crypt pathway and not through the traditional MMR proficient (pMMR) adenoma to CRC pathway may present as CRCs that develop in between surveillance colonoscopies in Lynch syndrome patients. These tumors may develop more rapidly and perhaps for some part also lack a benign (dMMR) adenoma precursor that can be prevented by a polypectomy.[13, 17] Indeed, prospective cohorts report low or even absent risk for *PMS2* carriers undergoing regular surveillance and polypectomies if needed (ten Broeke and Suerink et al, 2018, manuscript in preparation).[10, 11] One of these studies also reported normal PMS2 expression in 16 adenomas stained with immunohistochemistry, again underlining the possibly late timing of PMS2 deficiency (ten Broeke and Suerink et al, 2018, in preparation).

In general Lynch-associated CRCs appear to have better prognosis which is believed

to be a consequence of increased immune activation due to frameshift neo-antigens due to cMS.[1, 18] A recent paper by Alpert and colleagues reported that CRCs with isolated PMS2 loss due to germline *PMS2* mutations showed trends towards presenting with more distant metastasis and higher disease-specific death when compared to tumors due to pathogenic variants in other MMR genes.[15] They also reported that CRCs with isolated PMS2 loss have a lower frequency of MSI-related features such as increased tumor-infiltrating lymphocytes (TILs) and Crohn-like infiltrate, suggesting a lower degree of immune activation, possibly explaining their observation of a worse prognosis for PMS2-associated CRCs. Our results showing significantly lower CD3+ T-cell counts are in line with this observation and may be a result of later occurrence of PMS2 deficiency. However, the explanation suggested by Alpert et al. that these tumors may develop less neoantigen-producing mutations could not be confirmed by our study, as no significant differences between PMS2-associated CRCs and MLH1- or MSH2-associated CRCs were detected. However, due to the limited number of analyzed tumor specimens, more subtle differences may have been missed.

Another limitation of our study is that only certain cMS targets selected according to their high likelihood of representing functionally relevant drivers of tumorigenesis were analyzed, so that the level of "irrelevant" background MSI affecting functionally neutral or less significant cMS is not properly reflected by the analysis. Our panel was therefore not properly suited to asses a general estimation of the quantitative level of MSI in PMS2-associated tumors. A lower overall amount of MSI could still explain the observation of lower immune response by the host surrounding these tumors. Therefore, further studies on larger sample sets are required to validate our observation of a similar cMS spectrum for all MMR carriers. Due to the similar pattern of neoantigen-inducing cMS, we expect that vaccines developed for the prevention of Lynch syndrome-associated cancers should also cover *PMS2*-mutant tumors. In addition, *PMS2*-mutant CRC patients with metastasized disease may likely benefit from immune checkpoint blockade using anti-PD-1 or anti-PD-L1 antibodies that have shown very promising results in MMR-deficient cancer patients.

In conclusion, while we observed a similar cMS spectrum for *PMS2*-associated CRCs when compared to other Lynch associated tumors we did see lower CD3+ infiltration, possibly suggesting a later occurrence of PMS2 deficiency. A lower immune response in *PMS2* carriers that develop CRC may have consequences for metastatic potential and overall prognosis, which should be explored in future studies that also include clinical data.

4

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4.2

Molecular Background of Colorectal Tumors From Patients with Lynch Syndrome Associated With Germline Variants in *PMS2*

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Sanne W. ten Broeke, Tom C. van Bavel, Anne M.L. Jansen, Encarna Gómez-García, Frederik J. Hes, Liselot P. van Hest, Tom G.W. Letteboer, Maran J.W. Olderode-Berends, Dina Ruano, Liesbeth Spruijt, Manon Suerink, Carli M. Tops, Ronald van Eijk, Hans Morreau, Tom van Wezel, Maartje Nielsen

ABSTRACT

Background & Aims

Germline variants in the mismatch repair genes *MLH1*, *MSH2* (*EPCAM*), *MSH6*, or *PMS2* cause Lynch syndrome. Patients with these variants have an increased risk of developing colorectal cancers (CRCs) that differ from sporadic CRCs in genetic and histologic features. It has been a challenge to study CRCs associated with *PMS2* variants (*PMS2*-associated CRCs) because these develop less frequently and in patients of older ages than colorectal tumors with variants in the other mismatch repair genes.

Methods

We analyzed 20 CRCs associated with germline variants in *PMS2*, 22 sporadic CRCs, 18 CRCs with germline variants in *MSH2*, and 24 CRCs from patients with germline variants in *MLH1*. Tumor tissue blocks were collected from Dutch pathology departments in 2017. After extraction of tumor DNA, we used a platform designed to detect approximately 3000 somatic hotspot variants in 55 genes (including *KRAS, APC, CTNNB1*, and *TP53*). Somatic variant frequencies were compared using the Fisher's exact test.

Results

None of the *PMS2*-associated CRCs contained any somatic variants in the catenin beta 1 gene (*CTNNB1*), which encodes β-catenin, whereas 14/24 *MLH1*-associated CRCs (58%) contained variants in *CTNNB1*. Half of PMS2-associated CRCs contained *KRAS* variants, but only 20% of these were in hotspots that encoded G12D or G13D. These hotspot variants occurred more frequently in CRCs associated with variants in *MLH1* (37.5%, *P*=.44) and *MSH2* (and 71.4%, *P*=.035) than with variants in *PMS2*.

Conclusions

In a genetic analysis of 84 colorectal tumors, we found tumors from patients with *PMS2*-associated Lynch syndrome to be distinct from colorectal tumors associated with defects in other mismatch repair genes. This might account for differences in development and less frequent occurrence.

INTRODUCTION

Patients carrying a germline variant in one of the mismatch repair (MMR) genes *MLH1*, *MSH2 (EPCAM)*, *MSH6*, or *PMS2* have Lynch syndrome. These patients have a strongly increased risk of developing colorectal cancer (CRC) and endometrial cancer (EC). In contrast to sporadic tumors, which usually show chromosomal instability, MMR-deficient tumors are characterized by microsatellite instability (MSI) and a high mutational burden. MMR-deficient tumors show different treatment responses and often better patient survival.[1-3] Previous studies also showed notable differences in mutational spectrum between sporadic and Lynch-associated MSI-H tumors, exemplified by a higher degree of activation of the Wnt signaling pathway.[4]

Studies of Lynch-related tumors to date have mainly focused on *MLH1*, *MSH2* and *MSH6*-deficient tumors. Data on tumors associated with *PMS2* variants (*PMS2*-associated) tumors are still relatively sparse, probably due to challenging *PMS2* variant detection.[5] The recognition of *PMS2*-related families can also be challenging, as *PMS2* carriers develop CRCs less frequently and at a later age compared to other MMR variant carriers.[6, 7] One explanation for lower penetrance in *PMS2*-associated Lynch syndrome is that the functional binding partner of PMS2, MLH1, is able to form an alternative heterodimer with MLH3 or PMS1. This alternative heterodimer may then partly rescue MMR function in the absence of PMS2. This redundancy might not only manifest in the clinical phenotype, but might also result in distinct features of these carcinomas. Therefore, our goal was to define the molecular hallmarks of PMS2-associated CRCs compared to other Lynch-associated and sporadic carcinomas.

METHODS

Patients and samples

The cohort consisted of 24 CRCs from Dutch confirmed heterozygous pathogenic *PMS2* variant carriers who had given prior informed consent for use of clinical data and tissue samples. Tissue blocks were retrieved from Dutch pathology centers in 2017. We also collected tissue blocks from *MLH1*- and *MSH2*-associated CRCs from confirmed *MLH1* or *MSH2* carriers that had been previously analyzed for MMR deficiency status at Leiden University Medical Center (n=24 and n=18 respectively). In addition, we retrieved somatic variant data from a cohort of sporadic CRCs which were not suspected of Lynch syndrome (n=22) and were analyzed in the context of routine diagnostics (mainly to assess therapy options) using the same panel as this study, also in the Leiden University Medical Center.

Sample preparation

Tissue samples were extracted from tissue blocks by taking a minimum of three tissue cores of variable length (0.3 mm diameter, or 0.7 mm in case of tissue with a low cell count). Tumor DNA was isolated by use of the Tissue Preparation System with VERSANT Tissue Preparation Reagents (Siemens Healthcare Diagnostics, Tarrytown, NY), as previously described.[8] Briefly, nucleic acid was extracted from paraffin-embedded tissue by use of heat lysis in FFPE buffer and subsequent enzymatic lysis with Proteinase K. The lysate was incubated with silica-coated iron oxide beads, which binds nucleic acid, and subjected to magnetic separation for purification. After being transferred to a new tube, the lysate was again incubated with beads, washed three times and ultimately the nucleic acid was eluted in elution buffer.

Histological and molecular evaluation

Histological hallmarks were scored using the original report, with missing data on specific Lynch-associated hallmarks supplemented by an experienced pathologist when possible. Somatic variant analysis was performed using custom-designed AmpliSeq sequencing panels (Thermo Fisher). Sequencing libraries were prepared according to the manufacturer's recommendations and sequenced on the Ion Torrent Proton Platform (Thermo Fisher). The used cancer hotspot panel is adapted from Ion AmpliSeq™Cancer Hotspot Panel v2 (composition on request) and is designed to detect variations in 207 amplicons covering 21,820 bases of 50 genes with known cancer associations (~3000 COSMIC variants), including well-known somatically mutated genes such as *KRAS*, *APC* and *TP53*. The samples from the PMS2 cohort, were additionally sequenced with a second AmpliSeq panel (custom) covering the coding regions of *MLH1*, *MSH2*, *MSH6*, *PMS2*, *MUTYH* and the exonuclease domain of *POLD1* and *POLE*. In total 19,904 bases were covered spread over 202 amplicons (composition on request). This second panel was also used to detect a second hit (somatic point variants or loss-of-heterozygosity (LOH)) in these tumors). Results for this panel can be found in Table 1 (second hits in *PMS2*) and Supplementary Figure 3 (analysis of *POLE* variants in three *KRAS* mutated CRCs).

The unaligned bam file generated by the Proton sequencer were mapped against the human reference genome (GRCh37/hg19) using the TMAP 5.0.7 software with default parameters (https://github.com/iontorrent/TS). Subsequently variant calling was done using the Ion Torrent specific caller, Torrent Variant Caller (TVC)-5.0.2, using the recommended Variant Caller Parameter for Cancer Hotspot Panel v2. Variant interpretation was done using Genetic Assistant which assigns Functional Prediction, Conservation scores and Disease associated information to each variant

4

TABLE 1 Description of the cohort

Cohort description	PMS2 (n=20)	MLH1 controls (n=24)	MSH2 controls (n=18)	Sporadic controls (n=22)
Gender				
Male	10/20 (50%)	13/24 (54%)	11/18 (61%)	
Unknown	0%	0%		100%
Index	18/20 (90%)	16/23 (70%)	11/18 (61%)	N/A
Unknown		1 (4%)		
Age at CRC				
Median (IQR)	48 (43.5-59.5)	46 (39.5-57)	45.5 (38-58)	65 (61-69)
Mean (s.d.)	50.1 (12.3)	47.3 (10.4)	47.8 (12.0)	63 (8.6)
Range	26-70	30-68	29-67	43-73
T-stage				Not available
Advanced: 3/4	12/20 (60%)	14/22 (64%)	10/14 (71%)	
Missing	0	2 (8%)	4	
N-stage				Not available
1/2	6/20 (30%)	9/22 (41%)	2/13 (15%)	
Missing	0	2 (8%)	5 (28%)	
Location				
Proximal location¥	15/20 (75%)	19/20 (95%)	10/16 (63%)	6/20 (30%)
Cecum	8 (40%)	7 (35%)	5	N/A
Ascending	2 (10%)	3 (15%)	2	N/A
Transverse	5 (25%)	4 (20%)	0	N/A
Descending	3 (15%)	0	0	N/A
Rectosigmoid	2 (10%)	0	5	N/A
Missing	0	4 (17%)	2	2 (9%)
Histological hallmarks				Not available
Poor differentiation	6/20 (30%)	15/24 (68%)	4/11 (36%)	
Lymphocyte infiltration (TILs)		Sparse data**	Sparse data**	
Moderate & marked	5/20 (25%)			
Marked	1/20 (5%)			
Crohn's like infiltrate	9/20 (45%)	Sparse data**	Sparse data**	
Mucinous (>50%)	8/20 (40%)	Sparse data**	Sparse data**	
MSI-H	94% (17/18)	18/21 (86%)	13/13 (100%)	Not available
missing	2	4 (17%)	5 (28%)	
Second hit PMS2 identified?*				
Yes	16/20 (80%)	N/A	N/A	N/A
LOH	9/16 (56%)			

Cohort description	PMS2 (n=20)	MLH1 controls (n=24)			MSH2 controls (n=18)			Sporadic controls (n=22)
CTNNB1 variant detected	0/20 (0%)	14/24 (58%)			1/18 (6%)			0%
[4] c.110C>T (p.Ser37Phe)		2 (8%)			0			
[5] c.121A>G (p.Thr41Ala)		4$ (17%)			0			
[5] c.133T>G (p.Ser45Ala)		1 (4%)			0			
[5] c.133T>C (p.Ser45Pro)		2$$ (8%)			0			
[5] c.134C>T (p.Ser45Phe)		5 (21%)			0			
[4] c.133_135delTCT (p.Ser45del)		0			1 (6%)			
Comparison CTNNB1 variant absent/present	N/A	CTNNB1			CTNNB1			N/A
		Absent	Present	p-value	Absent	Present	p-value	
Index		6/10 (60%)	10/13 (77%)	0.337	0/1 (0%)	11/17 (65%)	N/A	
Male		5/10 (50%)	8/14 (57%)	0.527	0/1 (0%)	11/17 (65%)	N/A	
Age at CRC (median, IQR)		46.5 (35-51)	46 (42-58)	0.66	67	45 (38-57)	N/A	
Proximal location		8/9 (89%)	11/11 (100%)	0.45	1/1 (100%)	9/15 (60%)	N/A	
Advanced T stage		6/9 (67%)	8/13 (62%)	0.584	1/1 (100%)	9/13 (69%)	N/A	
Lymphnode metastasis		4/9 (44%)	5/13 (39%)	0.561	0/1 (0%)	2/12 (17%)	N/A	
Poor differentiation		5/9 (56%)	10/13 (77%)	0.276	1/1 (100%)	3/10 (30%)	N/A	
MSI-H		6/8 (75%)	12/12 (100%)	0.147	1/1 (100%)	12/12 (100%)	N/A	

N/A: Not applicable
IQR: Interquartile range
* See supplementary table 1 for a more detailed description of the second hit
¥ *Proximal location: cecum, ascending colon, transverse colon. Includes* tumours described as 'right-sided'
$One of these tumours also harbored a class 3 APC variant: c.4709A>G (p.Asp1470Gly)
$$One of these tumours also harbored class 5 APC mutation: c.4393_4394delAG (p.Ser1465Trpfs*3)
**For the control cohorts MLH1 and MSH2 only sparse data available, see supplementary table 1 for a description of available data for each tumour

(http://softgenetics.com/GeneticistAssistant_2.php). Variants were called using an in-house developed pipeline and analyzed using the Geneticist Assistant NGS Interpretative Workbench (version 1.1.8, SoftGenetics, State College, PA). All variants were manually curated and visualized in the integrative genomics viewer (IGV).[9, 10] Variants with low coverage (<100 reads) and/or with an allele frequency of <10% were not considered. Although the latter approach is conservative, it was adopted due to the substantial background noise due to FFPE treatment of the tissue samples, as evinced by a high transition/transversion (TsTv) ratio. Four samples of *PMS2*-associated CRCs, showed a high number of variants and a high TsTv ratio and were excluded from the final analysis. The ratio of these samples were considered to be too high for meaningful interpretation, regardless of the known association MMR deficient cancer and C>T transitions (mutational signature 6).[11]
Proportions were compared using the Fisher's exact test in Stata version 14 (Statacorp). Reported p-values are two-tailed.

RESULTS

Cohort description

A description of the cohort is given in Table 1. The analyzed cohorts were comparable in terms of index status, gender and age at CRC diagnosis. A full description of all available histological and molecular characteristics (including germline MMR and somatic variants) for each analyzed CRC can be found in Supplementary Table 1 and Supplementary Figure 1.

Most frequently mutated genes

The most frequently mutated genes were *TP53*, *KRAS*, *FBXW7*, *CTNNB1*, *APC* and *PIK3CA* (Table 2). The relative overrepresentation of C>T transitions observed matched mutation 'signature 6', previously associated with MMR deficiency (Supplementary Figure 2).[11] PMS2-associated CRCs harbored proportionally more variants in *FBXW7* (p=0.043) and *PIK3CA* (p=0.229) compared to sporadic CRCs, and relatively fewer functional variants in *TP53* (p=0.007) and *APC* (p=0.531).

CTNNB1

CTNNB1 variants were identified in 14/24 (58%) *MLH1*-, 1/18 (6%) *MSH2*-, and in none of *PMS2*-associated CRCs. In the *MLH1* tumors, all *CTNNB1* variants were missense while the *CTNNB1* variant in the *MSH2* tumor was a deletion of three base pairs. All

TABLE 2 Proportion of samples with a variant in the most frequently mutated genes

					p-value (Two-sided Fisher's exact test)			
	Sporadic (n=22)	MLH1 (n=24)	PMS2 (n=20)	MSH2 (n=18)	Overall	Sporadic vs. PMS2	MLH1 vs. PMS2	MSH2 vs. PMS2
TP53	15 (68%)	6 (25%)	5 (25%)	5 (28%)	0.007	0.007	1.00	1.00
KRAS	18 (42%)*	7 (29%)	10 (50%)	7 (39%)	0.564	0.594	0.218	0.532
FBXW7	0 (0%)	4 (17%)	4 (20%)	3 (17%)	0.126	0.043	1.00	1.00
CTNNB1	0 (0%)	14 (58%)	0 (0%)	1 (6%)	<0.001	N/A	<0.001	0.474
APC	9 (41%)	3 (13%)	6 (30%)	6 (33%)	0.158	0.531	0.261	1.00
PIK3CA	2 (9%)	8 (33%)	5 (25%)	7 (39%)	0.124	0.229	0.742	0.489

Note:
Class 3-5 variants were included in the analysis.
*Sporadic tumors underwent routine diagnostic pre-screening for several common hotspot variants (e.g. *KRAS* c.35G>T, *BRAF* c.1799T>A) and when found were excluded from full panel analysis. To prevent skewing of the results for variants in KRAS, sporadic tumors with one of these common variants not entered in the full panel analysis have also been included in the comparison. In total, 18/43 carried a *KRAS* variant.

variants but one targeted a Serine at amino acid location 37 or 45, the remaining variant involved a Threonine located at amino acid location 41 (see Table 1 for a full description of the identified variants). No statistically significant differences in index status, gender, age, tumor location, stadium, lymph node metastasis or MSI were identified when *CTNNB1*-mutated CRCs were compared to non-mutated CRCs (see Table 1). *MLH1* tumors with a *CTNNB1* variant appeared to be more likely poorly differentiated (56% vs. 77%), however this finding was not statistically significant (p=0.276). All *CTNNB1*-mutated tumors were microsatellite instable.

KRAS

KRAS variants were more frequently observed in the *PMS2* cohort (11/20 (55%)) compared to the *MLH1* cohort (7/24 (29%), p=0.218) and – to a lesser extent – to *MSH2*-associated CRC (7/18 (39%), p=0.532). Two hotspot variants in *KRAS* are known to occur frequently in CRC in general, but even more frequently in Lynch syndrome CRCs,

namely G12D (c.35G>A) and G13D (c.38G>A).[12] Notably, these hotspots accounted for only a minority of identified *KRAS* variants in PMS2-associated CRCs (20%). The contribution of these variants appears to be lower when compared to *MLH1* tumors (37.5%, p=0.44) and even more so when compared to *MSH2* tumors (71.4%, p=0.035). Sporadic CRCs harbored hotspot and non-hotspot *KRAS* variants to an equal extent (Figure 1).

FBXW7

PMS2-associated CRCs harbored *FBXW7* variants in 20% (4/20), which was similar to what we found in the *MLH1* (4/24, 17%) and *MSH2* (3/18, 17%) cohorts but markedly higher than in sporadic CRCs (0/22, p=0.043). It is worth mentioning that the most common hotspot mutation c.1993C>T (p.R465C) was the only identified variant in the PMS2 cohort, while this was only the case for 1 in 4 *FBXW7* variants in *MLH1*- and 1 in 3 *MSH2*- associated CRCs.

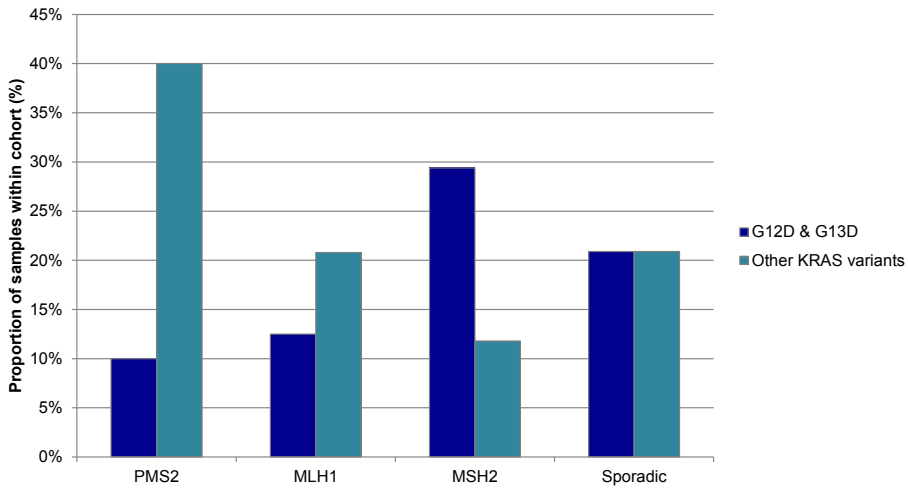

FIGURE 1 Proportion of samples with common hotspot variants G12D and G13D, or other KRAS variants.
Note: p-values represent comparison between PMS2-associated colorectal tumors and MLH1- or MSH2-associated tumors, respectively.

DISCUSSION

This is the first study to investigate molecular hallmarks of *PMS2*-associated CRCs. Overall, the variant spectrum of *MLH1*-, *MSH2*- and *PMS2*-associated CRCs look very similar. The molecular profile of *PMS2*-associated CRC is characterized by *KRAS* and *FBXW7* variants. However, the most notable difference ($p<0.001$) was in the relative frequencies of the catenin beta 1 gene (*CTNNB1*, which encodes β-catenin) variants (all classic exon 3 variants - Table 1), with *CTNNB1* variants entirely absent in all *PMS2* tumors but (very) frequent in *MLH1*-associated CRC (58%, 14/24). β-catenin is involved in the Wnt signaling pathway which plays a key role in colorectal carcinogenesis, exemplified by the high frequency of *APC* variants in CRCs.[13] Briefly summarized, Wnt/β-catenin signaling inhibits the degradation of β-catenin in the cytoplasm, which leads to accumulation of the protein. β-catenin then translocates to the nucleus where it trans-activates target genes involved in cell maintenance, differentiation and proliferation. To prevent over-accumulation of β-catenin, a destruction complex that includes *APC* degrades β-catenin by phosphorylation before it can translocate to the nucleus. A tumor cell can exploit Wnt signaling by disabling both *APC* alleles or through an activating variant in the *CTNNB1* gene. Activating variants in exon 3 of *CTNNB1* are relatively frequent in *MLH1*-associated tumors[4, 14], and the combined previously reported prevalence of 65% for *APC* and *CTNNB1* variants in Lynch-associated CRC[14] is very similar to our *MLH1* cohort (71%), further underlining the remarkable absence of these variants in *PMS2* tumors.

Interestingly, it had been reported that *CTNNB1* variants seem to occur at a later stage of adenoma-to-CRC progression in Lynch syndrome patients, with 18.2% of CRCs harboring *CTNNB1* variants compared to only 1.2% of adenomas.[4] This observation suggests that these variants are not initiating, in contrast to *APC* variants for example, and may therefore play a role in enhancing tumor cell survival, a conclusion also supported by another observation from the same report that *CTNNB1* variants are more prevalent at higher tumor stages (i.e. the majority were found in Dukes' tumor stages C and D).[4] It was also suggested that *CTNNB1* variants may not be a direct consequence of general MMR deficiency per se, because no *CTNNB1* variants were found in a sporadic MSI-H CRC cohort.[14]

A recent paper, by Ahadova et al., suggested that *CTNNB1* variants characterize a distinct type of CRC, with an aggressive and invasive growth pattern, that develops from MMR-deficient (dMMR) crypts instead of through the more commonly accepted adenoma-to-CRC pathway.[15] These tumors are suggested to present as interval CRCs, i.e. tumors that cannot be prevented by colonoscopies due to absence of a benign

precursor stage, i.e. adenomas. In the adenoma-to-CRC pathway, the second hit in a germline-mutated MMR gene occurs at a later stage of tumor progression and results in a significantly decreased time to CRC development. Thus, the lack of *CTNNB1* variants in *PMS2*-associated CRCs suggests that the dMMR crypt model might not apply to *PMS2* variant carriers (see Figure 2). This is supported by our detection of relatively frequent deleterious variants in the *APC* gene which are typical for adenoma development and may represent the adenoma-to-CRC progression pathway, although this finding was not statistically significant. Moreover, a recent paper by the same group showed that *KRAS* variants likely occur frequently only after loss of the wildtype MMR allele, which is in line with other work showing that *KRAS* variants are involved in a later stage of tumorigenesis.[12, 16] While the two known hotspot variants in the *KRAS*

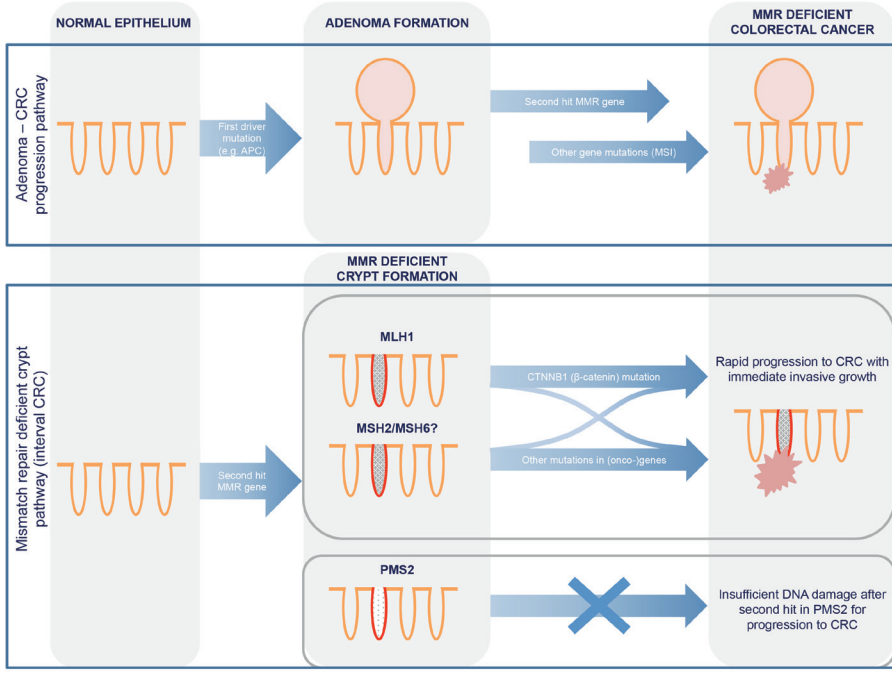

FIGURE 2 Proposed gene-specific development of CRC in Lynch syndrome patients
CRC: colorectal carcinoma. MMR: mismatch repair
Note: Figure inspired by Figure 4 from the paper by Ahadova et al[15]. At this moment it is unsure whether MMR deficient (dMMR) crypts exist in PMS2 carriers. Further studies should elucidate this.

gene, G12D and G13D, occur frequently in CRC overall, they are even more prevalent in MSI-H CRC.[12] The prevalence of these two variants was relatively sparse in the *PMS2* cohort (Figure 1) as compared to the *MLH1* and *MSH2* cohort, which may suggest a later chronological timing of PMS2 deficiency in tumor progression, i.e. the *KRAS* variants in PMS2 deficient tumors may occur before loss of the *PMS2* wildtype allele. Indeed, a recent paper by Alpert et al. may further corroborate the late occurrence of PMS2 deficiency in tumorigenesis as they reported that CRCs showing isolated loss of PMS2 display significantly less histological features associated with MSI, including immune-related hallmarks.[17] Larger studies are needed to more accurately determine timing of PMS2 deficiency in adenoma-to-CRC progression.

The results of our study may (partly) explain clinical observations in PMS2 carriers such as lower penetrance and the absence of surveillance interval CRCs in *PMS2* carriers, observed in prospective cohort studies.[6, 7, 18] Indeed, in contrast to *PMS2* carriers, both *MLH1* and *MSH2* carriers develop such interval CRCs between surveillance colonoscopies, with cumulative risks reported to be up to 46% and 43% to age 75 years, respectively.[18] The high risk of interval CRCs for *MSH2* carriers is interesting in light of the markedly lower prevalence of *CTNNB1* variants observed in our cohort (1/16, 6%) and a previous report (2/27, 7%) when compared to *MLH1*-associated CRCs, which may be explained by their different role in the MMR machinery.[15] In mammalian cells MMR proteins function as heterodimers in two main complexes existing of MutS homologues MSH2 with either MSH6 or MSH3, or MutL homologues MLH1 binding to PMS2, PMS3, or MLH3.[19] *PMS2* and *MLH1* carriers may therefore follow similar tumorigenesis as they function within the same heterodimer, making the difference in *CTNNB1* variant prevalence all the more striking. A possible reason for a difference in carcinogenesis of *MLH1* and *PMS2* carriers may be the ability of the MLH1 protein to form a heterodimer with MLH3 or PMS1 in the absence of PMS2, which means that in the absence of PMS2 some MMR function might still remain.[20] Our finding of low *CTNNB1* variant prevalence in *MSH2*-associated CRC despite observed high interval CRC risk suggests that CRC development from dMMR crypts in *MSH2* carriers not only utilizes *CTNNB1* variants but might require or utilize variants in other (onco-)genes as well (Figure 2). However, this is only speculation at this point and further research is needed to shed more light on the precise tumorigenesis in *PMS2*- and other Lynch syndrome-associated CRCs, as our study is limited by the number of analyzed tumors. A further limitation was the use of a hotspot panel which means that we may have missed Lynch specific somatic variants which could play an essential role in progression from either adenoma or dMMR crypt to CRC. This is especially relevant for the latter pathway as we did not identify specific genes that were mutated more frequently in the

MSH2 cohort. A possible candidate gene that has been proposed in CRC development where MMR deficiency occurs at very early stages is *RNF43*.[21] Subsequent studies should therefore focus on more extensive (onco-)panels or preferentially whole exome sequencing. This may also allow for more thorough analysis of MMR deficiency timing by looking into Lynch syndrome specific variants in for example *KRAS* and *APC*, similar to what has been done in a recent study by Ahadova et al and the preliminary results from our *KRAS* analysis.[12] Lastly, future studies should have a prospective design in which interval carcinomas with aggressive, non-polypous growth patterns could be sequenced and compared to CRCs identified in index carriers. However, based on our data, the existence of such tumors in *PMS2*-associated Lynch syndrome is doubtful.

In conclusion, this study supports the hypothesis that *PMS2* carriers represent a distinct entity among Lynch syndrome patients. If confirmed, the possible absence of CRC formation through the dMMR crypt pathway in *PMS2* carriers could have major implications for surveillance guidelines and might justify a longer colonoscopic surveillance interval in PMS2-associated Lynch patients, for example every 2-3 years. The current biennial surveillance interval of 1-2 years was selected primarily on the basis of data from *MLH1* and *MSH2* patient cohorts and therefore might be too rigorous for PMS2 carriers.[22, 23]

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SUPPLEMENTARY FIGURE 1 Schematic overview of variants in most commonly mutated genes for each tumor sample
N/A: Not available. * Patient ID from supplementary table 1.

PMS2

Patient ID*	4	13	6	14	1	11	9	16	5	7	17	8	12	20	18	15	2	3	10	19
TP53																				
KRAS																				
FBXW7																				
CTNNB1																				
APC																				
PIK3CA																				
Tumorstage	3	2	4	2	3	4	4	2	3	3	4	2	2	3	1	3	2	2	1	4

MLH1

Patient ID*	9	19	23	18	3	15	1	21	5	12	22	4	13	20	7	11	10	6	24	2	16	14	8	17
TP53																								
KRAS																								
FBXW7																								
CTNNB1																								
APC																								
PIK3CA																								
Tumorstage	4	2	N/A	3	1	1	3	4	3	3	3	3	4	2	2	2	3	2	N/A	4	1	3	4	3

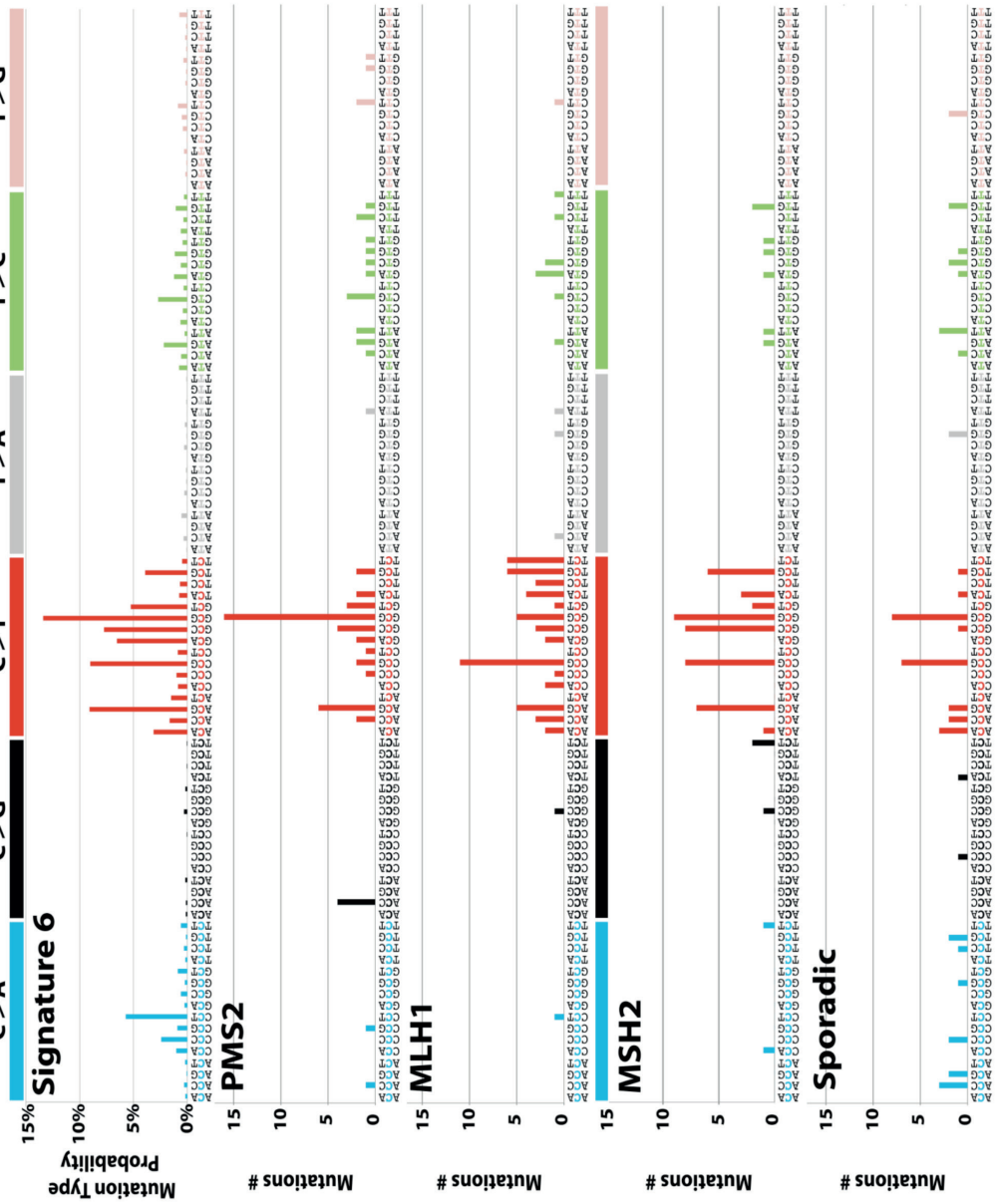
MSH2

Patient ID*	1	11	14	6	3	2	9	10	7	8	5	12	13	17	15	16	18	4
TP53																		
KRAS																		
FBXW7																		
CTNNB1																		
APC																		
PIK3CA																		
Tumorstage	3	2	N/A	3	2	3	3	N/A	3	3	N/A	N/A	3	2	4	3	Intra mucosal	3

Sporadic

TP53																						
KRAS																						
FBXW7																						
CTNNB1																						
APC																						
PIK3CA																						
Tumorstage	N/A	N/A	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3	N/A

SUPPLEMENTARY FIGURE 2 Mutational signatures
Note: The PMS2, MLH1, MSH2-associated colorectal cancer cohorts show overrepresentation of C>T transitions, similar to what is found in signature 6 (1), which has been associated with mismatch repair deficient tumors. The PMS2 cohort appears to have relatively few transitions targeting CCG trinucleotides. However, the panel used in this study is not well suited for mutational signature analysis, therefore results should be interpreted carefully.
Reference : (1) Alexandrov, L.B. et al. Nature 500, 415-21 (2013).



SUPPLEMENTARY FIGURE 3 Proportion of samples with specific KRAS mutation
Note: PMS2 associated CRCs frequently display KRAS mutations. Which is important to know for therapy options, e.g. KRAS mutated tumors have reduced sensitivity to anti-EGFR treatments (such as cetuximab).(1) It is worth noting that in 3/20 CRCs the specific KRAS mutation was c.436G>A (p.A146T), which only accounts for 0.6-1.2% of KRAS mutations in sporadic CRCs.(2,3) Strikingly, this mutation was found in a mother and son, as well as in a non-related PMS2 carrier, with different germline PMS2 mutations (c.2192_2196delTAACT (p.Leu731CysfsX3) and c.804-60_804-59ins2kb respectively). This specific KRAS mutation in codon 146 has previously been associated with the POLE associated phenotype. (4) Therefore we hypothesized that these PMS2 carriers might also carry a (germline) variant in POLE. Pathogenic mutations in this gene reside in the exonuclease domain, which was covered by DNA repair panel. However, there was no overlapping POLE variant in the mother and son. Moreover, only the mother had a somatic nonsynonymous POLE variant (44% reads, NM_006231:exon7:c.659A>G, p.D220G), which lies somewhat before the exonuclease domain and has not been previously described. Consequently underlying POLE mutations do not appear to explain the association of PMS2 CRCs with this specific KRAS variant. Although it should be noted that we also identified this KRAS mutation in one of the MLH1 associated CRCs. Hence this could also represent an association of this variant with MMR deficient CRCs in general, as opposed to only PMS2.
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SUPPLEMENTARY TABLE 1 is available online: S.W. ten Broeke et al, Gastroenterology 2018, doi: 10.1053/j.gastro.2018.05.020

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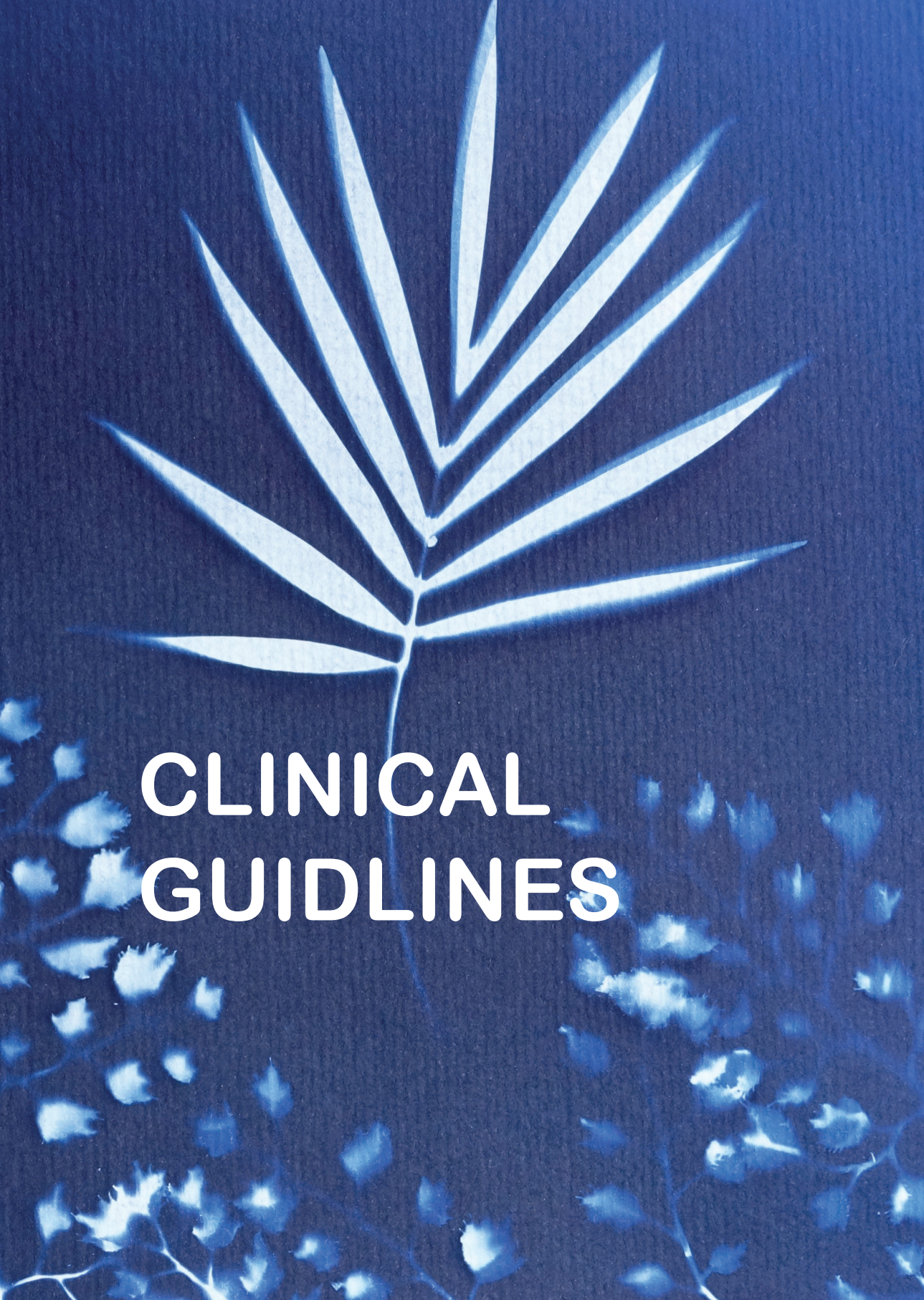

CLINICAL
GUIDLINES

5.1

Incidence of polyps and post-colonoscopy colorectal cancers in patients with PMS2-associated Lynch syndrome: a prospective cohort analysis

Manuscript in preparation

Sanne W. ten Broeke*, Manon Suerink*, Diantha Terlouw, Alexandra M.J. Langers, Evelien Dekker, Carli M.J. Tops, Hans F.A. Vasen, Tom van Wezel, Hans Morreau, Maartje Nielsen,
on behalf of the PALGA-group and the Dutch working group for clinical oncogenetics

* These authors contributed equally to this work

ABSTRACT

Purpose

Lynch syndrome predisposes carriers of a heterozygous pathogenic germline variant in the *MLH1*, *MSH2* (*EPCAM*), *MSH6* or *PMS2* genes to the development of mainly colorectal and endometrial cancer. Of the four mismatch repair genes, *PMS2* carries the lowest cancer risk, yet surveillance protocols are identical for all Lynch syndrome patients. The aim of this study was to determine the characteristics and incidence of polyps and post-colonoscopy colorectal cancers (PCCRCs) in *PMS2* carriers undergoing regular surveillance.

Methods

We collected a cohort of 171 *PMS2* carriers and recorded the occurrence and characteristics of PCCRCs and polyps. After receiving consent to request clinical data, we obtained information through PALGA, the Dutch nationwide network and registry of histo- and cytopathology, and by requesting colonoscopy reports at gastroenterology departments. Twenty polyps were available for immunohistochemical staining of the PMS2 protein.

Results

During a total of 675 colonoscopies (1039 observation years), 435 polyps were removed, of which 237 (54.5%) were adenomatous. Forty-one (16.9%) adenomas were advanced (i.e. ≥1 cm in diameter, villous component and/or high-grade dysplasia). None of the twenty polyps that were immunohistochemically stained showed loss of PMS2 expression, suggesting late involvement of PMS2 deficiency. One PCCRC was reported.

Conclusion

This large cohort of *PMS2* carriers showed a low incidence of advanced adenomas and only one PCCRCs. The latter was preceded by difficult and possibly incomplete colonoscopy. Based on these results, widening of the colonoscopy interval can be considered in patients without risk factors for suboptimal colonoscopic surveillance.

INTRODUCTION

Lynch syndrome predisposes carriers of a germline heterozygous pathogenic variant in one of the mismatch repair genes (*MLH1*, *MSH2*, *MSH6* or *PMS2*) to the development of mainly colorectal and endometrial cancer. Lynch syndrome-associated cancers are characterized by microsatellite instability (MSI) and negative staining for the involved MMR protein.[1, 2] In order to prevent the development of colorectal cancer in Lynch syndrome patients, patients are offered surveillance by colonoscopy every 1-2 years, starting at the age of 25.[3] Despite these regular surveillance colonoscopies, post-colonoscopy colorectal cancers (PCCRCs) do occur, particularly in *MLH1* and *MSH2* carriers.[4-7] Data on polyp and PCCRC development in PMS2-associated Lynch syndrome is sparse, yet highly clinically relevant since recent studies reported a high prevalence of *PMS2* variants in the general population.[8] *PMS2* carriers display a distinct phenotype, with retrospective cohort studies reporting substantially lower cancer risks than carriers of *MLH1* and *MSH2* variants,[9, 10] which has resulted in discussion on the issue of gene-specific surveillance.[4] This discussion would be greatly assisted by more prospectively collected gene-specific data. The prospective Lynch syndrome database (PLSD) consortium already confirmed low cancer risks associated with variants in the *PMS2* gene.[4, 11, 12] However, these studies did not include data on adenoma incidence, while this may be essential for a better understanding of the role of mismatch repair deficiency in Lynch syndrome associated carcinogenesis. To this aim, we collected prospective data on a large cohort of *PMS2* carriers (n=171) and evaluated PMS2 protein expression in twenty polyps.

MATERIALS & METHODS

Data collection

Consent was obtained to request clinical information and pathology samples for 186 Dutch Lynch syndrome patients with a confirmed pathogenic germline *PMS2* variant diagnosed at Dutch family cancer clinics. Obtaining pathology reports was facilitated by PALGA, the nationwide network and registry of histology and cytopathology in the Netherlands.[13] As PALGA encompasses all pathology laboratories in the Netherlands, all pathology reports on each patient can be obtained, even if a patient attended different hospitals for colonoscopies. Corresponding colonoscopy reports were requested at the respective gastroenterology departments. For fifteen *PMS2* carriers the PALGA search and request for colonoscopy reports came back with no results,

these patients most likely are not undergoing regular surveillance and they were therefore excluded from the analyses.

PMS2 variant analysis

Our cohort consisted of clinically ascertained families in which variant analysis was initiated due to (histological) pre-screening by immunohistochemistry and/or microsatellite instability, usually because a family met the Bethesda criteria.[14] Germline *PMS2* variant screening was performed as previously described.[10, 15] Comprehensive strategies were applied to avoid unreliable variant detection caused by interference from pseudogene sequences and frequent gene conversion events.[15] All variants found in the included *PMS2* carriers are listed in supplemental tables 1 and 2.

Immunohistochemistry

We retrieved formalin-fixed, paraffin-embedded (FFPE) tissue blocks of 16 adenomas with low-grade dysplasia (one of which was scored as advanced because of a villous component), two sessile serrated lesions and two hyperplastic polyps, and performed immunohistochemical analysis of PMS2 expression. In brief, the FFPE material was sectioned at 4 µm and stained with an antibody to PMS2 (Clone EP51, Agilent, Santa Clara, CA, USA). If the staining results showed absence of nuclear staining in the cells of an adenoma or polyp in the presence of positive control cells (e.g. leukocytes) than this was interpreted as PMS2 deficiency.

Statistical analysis

Descriptive results of colonoscopy findings were computed using Stata (Statacorp version 14). A Kaplan Meier analysis was carried out to estimate time to first adenoma or first advanced adenoma. Advanced adenomas were defined by a size of ≥1 cm in diameter, a villous component, and/or the presence of high-grade dysplasia.

Results were compared to data from a study by Forsberg et al, in which colonoscopy findings in MLH1-, MSH2-, and MSH6-associated Lynch syndrome patients were compared to control data from an earlier prospective population-based colonoscopy study by the same group.[16]

RESULTS

A description of the cohort is provided in table 1. Between 1987 and 2017 (median 2012), a total of 677 colonoscopies were performed in this cohort of 171 *PMS2* carriers, representing 1039 years of follow-up. All included PMS2-associated Lynch syndrome patients had a confirmed germline heterozygous pathogenic variant in the *PMS2* gene (supplemental material) and all have been described in previous studies.[10, 15, 17]

TABLE 1 Description of the cohort

Patients	171
Men	69 (40.4%)
Follow-up (years)	
Total	1039
Mean (s.d.)	9.6 (6.3)
Median (IQR)	8.4 (4.4-14.3)
Range	0-25
Colonoscopies	
Total	675
Number per patient	
Mean (s.d.)	3.9 (3.0)
Median (IQR)	3 (2-5)
Range	1-18
Time interval (years)	
Mean (s.d.)	2.1 (1.9)
Median (IQR)	1.9 (1.1-2.2)
Range	0.02-22.5

IQR: Interquartile range; s.d.: Standard deviation

TABLE 2 Characteristics of polyps

	PMS2 cohort	MLH1/MSH2/MSH6 (Forsberg et al)	Control cohort (Forsberg et al)
Patients	171	138	745
Mean age first colonoscopy (s.d.)	50.6 (12.9)	43,8	51,1
Mean age first adenoma detected (s.d.)	55.3 (12.5)	47,2	59,7
Mean age first advanced adenoma detected (s.d.)	56.8 (13.1)	50,8	62
Total polyps	436	223	474
Hyperplastic polyps	181 (41.6%)	110 (49%)	359 (76%)
Location			
Right-sided	52 (28.7%)		
Left sided	111 (61.3%)		
Not specified	18 (9.9%)		
Sessile serrated polyps/ adenomas*	16 (3.7%)	NA	NA
Location left-sided			
Right-sided	8 (50%)		
Left sided	8 (50%)		
Not specified	0		
Mixed	1 (0.2%)	NA	NA
Adenomas	237 (54.5%)	113 (51%)	115 (24%)
Histology			
Tubular adenoma	154 (65%)	93 (82%)	95 (83%)
Tubulovillous adenoma	23 (9.7%)	14 (12%)	15 (13%)
Villous adenoma	1 (0.4%)		
Sessile serrated adenoma with dysplasia	12 (5.1%)	6 (6%)	5 (4%)
Adenoma n.o.s.	47 (19.8%)		
Size (mm)			
0-4	134 (56.5%)	69 (61%)	76 (66%)
5-10	50 (21.1%)	24 (21%)	31 (27%)
10<	21 (8.9%)	9 (8%)	8 (7%)

TABLE 2 Characteristics of polyps

	PMS2 cohort	MLH1/MSH2/MSH6 (Forsberg et al)	Control cohort (Forsberg et al)
Not specified	32 (13.5%)		
Location			
Right-sided	92 (38.8%)	53 (47%)	39 (34%)
Left sided	120 (50.6%)	56 (49%)	73 (63%)
Not specified	25 (10.6%)	4 (4%)	3 (3%)
Dysplasia			
None	1 (0.4%)		
High grade	6 (2.5%)	13 (12%)	8 (7%)
Low grade	222 (93.7%)	91 (80%)	107 (93%)
Not specified	8 (3.4%)	9 (8%)	
Advanced	41 (16.9%)	27 (24%)	22 (19%)

n.o.s. = not otherwise specified
Advanced: adenomas ≥1 cm in diameter, villous component, and/or high-grade dysplasia
*Sessile serrated adenomas were listed in this category if there was no dysplasia

Polyps

In total, 436 polyps were removed from 171 *PMS2* carriers, the majority of which were adenomatous (54.6%). The most notable difference in *PMS2* carriers compared to Lynch patients carrying other MMR gene variants was the very low frequency of adenomas with high-grade dysplasia (2.5% vs. 12% for the Forsberg Lynch syndrome cohort) and, subsequently a low frequency of advanced adenomas (17.2% vs. 24%). This figure was also slightly lower than that reported in the Forsberg control cohort of average-risk individuals (19%). Mean age at first adenoma detection was 55.3 years (table 2). The proportion of carriers with an adenoma at first colonoscopy is depicted in figure 1 (proportion with advanced adenoma can be found in supplementary figure 1). Figure 2 shows the proportion of *PMS2* carriers free of adenomas as a function of age. The sixteen adenomas with low-grade dysplasia, two sessile serrated lesions and two hyperplastic polyps stained for PMS2 protein expression showed normal staining (table 3).

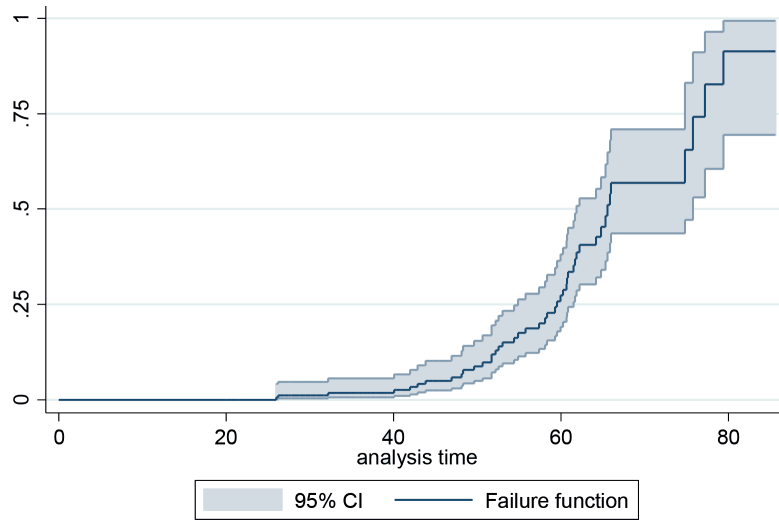


FIGURE 1 Cumulative proportion of PMS2 carriers with an adenoma at first colonoscopy

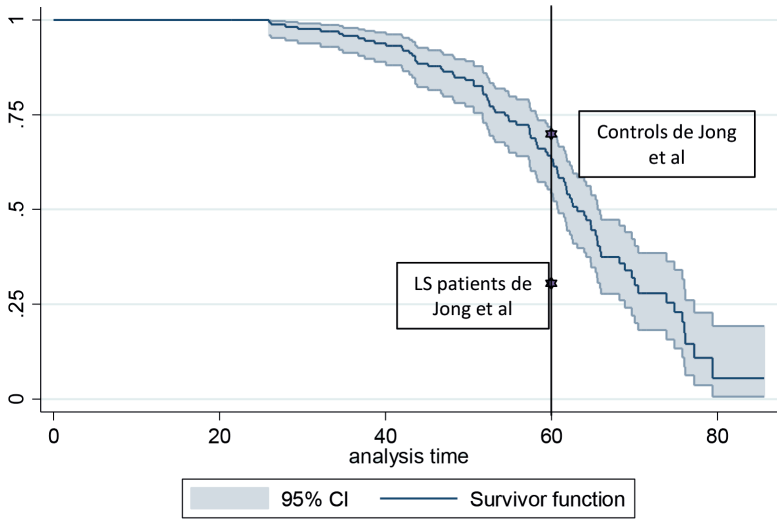


FIGURE 2 Cumulative proportion of PMS2 carriers free from adenomas, compared to the study by de Jong et al [28]

TABLE 3 Polyps stained for PMS2 protein expression

Case ID	Gender	CRC	Cumulative number of adenomas	Age of diagnosis first adenoma (years)	Cumulative number of hyperplastic polyps	Cumulative No of sessile serrated lesions	Polyp ID	Site of adenoma	Histology	Grade of Dysplasia	Size (mm)	PMS2 IHC
1	F	No	3	62	1	0	1.1	Right	Tubulovillous adenoma	Low	5	+
							1.2	Left	Tubular adenoma	Low	3	+
2	F	Yes	2	67	23	5	2.1	Pouch	Sessile serrated adenoma	Low	3	+
							2.2	Left	Mixed adenoma	Low	2	+
							2.3	Right	Tubular adenoma	Low	3	+
							2.4	Colon n.o.s.	Hyperplastic polyp	n.a.	8	+
3	F	No	6	57	2	0	3.1	Right	Tubular adenoma	Low	2	+
							3.2	Right	Adenomatous n.o.s.	Low	2,5	+
4	F	No	3	61	0	1	4.1	Right	Tubular adenoma	Low	2	+
							4.2	Left	Tubular adenoma	Low	3	+
							4.3	Left	Sessile serrated polyp	None	10	+
							4.4	Left	Tubular adenoma	Low	2	+
5	M	Yes	3	54	1	0	5.1	Left	Tubular adenoma	Low	2	+
6	F	No	1	45	0	0	6.1	Right	Adenomatous n.o.s.	Low	2	+
7	F	Yes	2	28	1	0	7.1	Left	Adenomatous n.o.s.	Low	3	+
8	M	Yes	15	64	0	0	8.1	Right	Adenomatous n.o.s.	Low	2	+
							8.2	Right	Tubular adenoma	Low	5	+
							8.3	Right	Adenomatous n.o.s.	Low	3	+
9	F	No	1	42	0	0	9.1	Colon n.o.s.	Tubular adenoma	Low	3	+
10	F	Yes	3	76	5	0	10.1	Colon n.o.s.	Hyperplastic polyp	n.a.	5	+

n.a.: not applicable; n.o.s.: not otherwise specified; CRC: colorectal cancer

TABLE 4 PMS2 carrier with a PCCRC

Sex	Male
Surveillance scopies	10
Years of surveillance	11
Last scopy before interval CRC	2
Initial CRC	
Age	65
Location	Rectum
IHC	PMS2-
MSI	MSI-H
PCCRC	
Age	75
Location	Transverse colon
IHC PMS2	Absent
MSI	NA

CRC: colorectal cancer; PCCRC: Post-colonoscopy colorectal cancer;
MSI: Microsatellite instability;
IHC: Immunohistochemistry

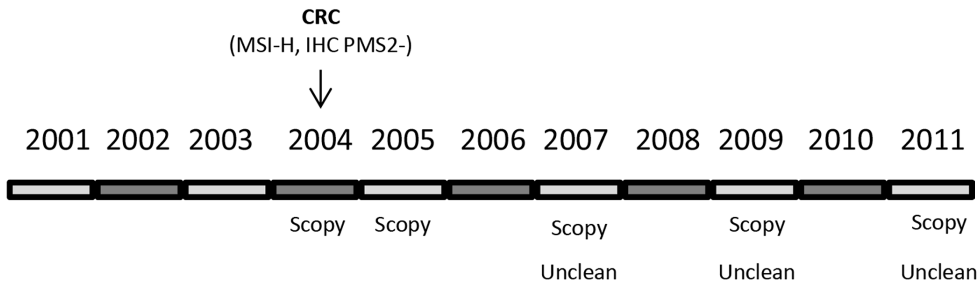
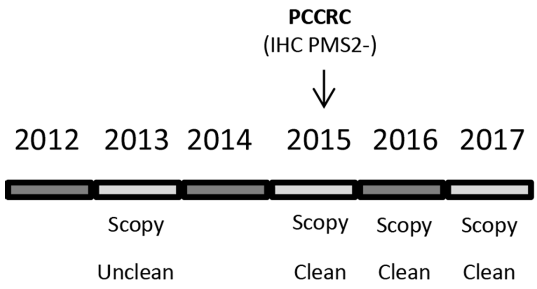


FIGURE 3 Timeline post-colonoscopy colorectal cancer (PCCRC)

Colorectal cancer

One *PMS2* carrier developed a PCCRC despite undergoing biennial regular colonoscopic surveillance (table 4, figure 3). However, this patient had a record of incomplete colonoscopies due to insufficient colon preparation. This patient presented with colorectal cancer at age 65 and had a subsequent PCCRC at age 75. The tumor was immunohistochemically stained for MMR protein expression which revealed absent PMS2 staining, as did the initial tumor.



DISCUSSION

PMS2-associated Lynch syndrome is characterized by relatively low penetrance of colorectal cancer.[10, 18, 19] Our study confirms the very low risk of colorectal cancer development in *PMS2* carriers who undergo regular, complete and good quality colonoscopies and polypectomies. Recent studies have shown that MMR deficient (dMMR) colorectal cancer in Lynch syndrome patients may develop not only through the traditional MMR proficient (pMMR) adenoma-to-colorectal cancer progression pathway, but may also arise from the dMMR crypt pathway.[20-23] Tumors arising via this latter pathway directly proceed from dMMR crypt to cancer or can first develop into an dMMR adenoma before becoming malignant.[21, 22] Clinically, these tumors may appear as PCCRCs (i.e. colorectal cancers that develop between follow-up surveillance colonoscopies and are detected at the next routine colonoscopy).[21] Because the cancers that develop directly from a dMMR crypt lack a benign precursor lesion they cannot be prevented by colonoscopies. Of note, recent work by our group suggests that the dMMR crypt pathway may be absent in *PMS2* carriers.[24] As it has been suggested that dMMR colorectal cancer only rarely arises from pMMR adenomas, this may explain low penetrance in *PMS2* carriers. [10, 18, 19, 25] In other MMR carriers, colorectal cancer is thought to arise mainly from the dMMR crypt pathway, i.e. from adenomas that are dMMR from the beginning of adenoma formation, or even directly from dMMR crypts.[22, 25]

Previous prospective studies in smaller cohorts than the current study have shown that *PMS2* carriers undergoing regular colonoscopies rarely develop colorectal cancer, further supporting the notion that this subset of Lynch syndrome patients may have distinct characteristics.[26, 27] It also underlines the notion that *PMS2* carriers may only develop colorectal cancer through the pMMR adenoma-to-colorectal cancer pathway. In this pathway PMS2 deficiency may occur as a relatively late event in (advanced) adenomas which could then stimulate the malignant transformation. If we assume that this is the only pathway that occurs in these Lynch syndrome patients, it is conceivable that the most important risk factor for colorectal cancer in *PMS2* carriers is actually adenoma formation. Indeed, as the *PMS2* carriers included in this study were members of families ascertained by high-risk family cancer clinics, our cohort may have been enriched for adenoma risk factors. This is illustrated by the similar proportion of *PMS2* carriers where an adenoma is identified at first colonoscopy when compared to the (non-Lynch) familial cancer cohorts described by Forsberg et al. Notably, in their study the proportion of carriers with adenomas at first colonoscopy is much higher for *MLH1* and *MSH2* carriers. The mean age at first adenoma detection in *PMS2* carriers was 55.3 years

(table 2, figure 1), which lies closer to the Forsberg control cohort (59.7 years) than the Forsberg Lynch cohort (47.2 years). Age at first advanced adenoma detection showed a similar pattern (50.8 years for other MMR carriers, 57.6 years for *PMS2* carriers and 62 years for the Forsberg et al. control cohort. Table 2, supplemental figure 1).[16] Another study by de Jong et al found mean age at first adenoma detection in *MLH1* and *MSH2* carries to be even lower, namely 46±9.7 years.[28] It should be noted though that age at adenoma detection is of course related to age at start of colonoscopic surveillance. For our cohort this was comparable to the Forsberg cohorts but higher than the cohorts described by de Jong et al. However, the proportion of carriers that were free from adenomas at age 60 was drastically lower for the PMS2 cohort when compared to the de Jong et al *MLH1*/*MSH2* carrier cohort, but comparable to the MMR variant negative control cohort by the same group (figure 2). This higher adenoma incidence in other MMR carriers may be explained by additional adenoma formation from dMMR crypts.[16] The lack of PMS2 deficient adenomas in this study also provides further evidence for the relatively late involvement of PMS2 deficiency in cancer development, which may be correlated to the later age at first (advanced) adenoma detection (approximately 7-8 years compared to other MMR carriers).[6, 25] Delayed PMS2 deficiency might also be related to the infrequency of advanced adenomas. A recent study suggested that MMR deficiency is often an early and possibly initiating event in tumorigenesis in Lynch patients carrying *MLH1*, *MSH2* or *MSH6* gene variants,[22] and the authors identified an MMR deficiency in 491/640 adenomas (76.7%). This in clear contrast to our data where none of the 16 stained adenomas showed loss of PMS2 expression, suggesting that PMS2 was not involved in the formation of these adenomas.

Future studies should investigate the influence of known adenoma risk factors in PMS2 families, such as obesity and smoking, as this may be important in further decreasing colorectal cancer risk in *PMS2* carriers.[29, 30] If indeed colorectal cancer development in *PMS2* carriers can mostly be prevented by regular polypectomies, then we would expect a very low cancer risk in this prospective cohort. Nevertheless, we did observe one case with a PCCRC, a surprising finding that on closer inspection of colonoscopy reports appeared to be related to frequently insufficient bowel preparation in this carrier(figure 3). This could have complicated early detection of adenoma and/or colorectal cancer formation. This PCCRC did exhibit PMS2 abrogation on immunohistochemistry, suggesting that PMS2 deficiency played a role in tumor progression in this patient. Despite this one case, the risk of developing PCCRC in *PMS2* carriers appears to be low and can probably be prevented by regular surveillance and polypectomy, possibly even at extended intervals (e.g. every 2-3 years) provided that the preceding colonoscopy was complete and of good quality.

A limitation of our study is the lack of good control data on adenoma prevalence in the general population. People with Lynch syndrome start colonoscopic surveillance at a very young age, whereas colonoscopies in the general population are generally performed at later ages and only upon clinical indication. This makes a direct comparison challenging. The only study, to our knowledge, to report adenoma occurrence in an unselected, relatively large and age-stratified cohort is that of Forsberg et al., who performed a prospective colonoscopy study that also reported adenoma prevalence in participants aged below 45 years.[35]

A second limitation of our study was that the adenomas stained for PMS2 expressions were all low-grade dysplastic adenomas (table 3). Future studies should include a larger number of both tumors and advanced adenomas. Further studies should also include molecular analysis of, for example, *APC* and *KRAS* variants, as specific variants in these genes can help identify the timing of MMR deficiency, as previously shown in the same study by Ahadova et al.[22] This approach might ultimately provide definitive proof of the late involvement of PMS2 deficiency.

Finally, the reported adenoma frequency in our cohort may have been an overestimate, as a consequence of the previously mentioned possibility of enrichment for adenoma risk factors in high-risk families. This implies that our findings cannot be easily extrapolated to *PMS2* carriers ascertained from the general population. Indeed, we expect families not selected based on a conspicuous phenotype, i.e. at a very young age and/or a positive family history, to become more numerous due to the universal MMR protein screening now being implemented in many countries.[36] In the Netherlands, for example, all colorectal cancers in patients aged below 70 are now screened by MMR immunohistochemistry. More prospectively gathered population-based data is needed and will form a valuable adjunct to data from traditionally selected clinic-based families.

In summary, we can confirm that *PMS2* carriers undergoing regular surveillance colonoscopies show a low risk of developing colorectal cancer and appear to develop less adenomas than other MMR carriers. These findings support previous proposals for an attenuated surveillance protocol in these Lynch patients, for example every 2-3 years, starting at age 35-40 years.

5

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SUPPLEMENTARY TABLE 1 *PMS2* variants reported as disease-causing in the families included in this study

exon/ intron	PMS2 variant[a]	predicted protein effect	type of variant	InSiGHT class[b]	No of carriers with variant
2	c.137G>T	p.Ser46Ile	missense	4	4
2	c.24-12_107delinsAAAT	p.Ser8Argfs*5	frameshift	5	4
2	c.150delinsAG	p.Ala51Glyfs*3	frameshift	Not present, reported by clinic as pathogenic	1
3	c.219_220dup	p.Gly74Valfs*3	frameshift	5	12
6	c.697C>T	p.Gln233*	nonsense	5	6
7	c.736_741delinsTGTGTGTGAAG	p.Pro246Cysfs*3	frameshift	5	20
intron 7	c.804-60_804-59insJN866832.1		retrotransposal SVA insertion	5	3
8	c.861_864del	p.Arg287Serfs*19	frameshift	5	3
8	c.903G>T	r.804_903del; p.Tyr268*	exonic splice variant	4	2
intron 10	c.1144+2T>A	p.Glu330_Glu381del	canonical splice variant	4	1
11	c.1831dup	p.Ile611Asnfs*2	frameshift	5	5
11	c.1882C>T	p.Arg628*	nonsense	5	21
13	c.2192_2196del	p.Leu731Cysfs*3	frameshift	5	7
14	c.2404C>T ;	p.Arg802*	nonsense	5	1
14	c.2444C>T	p.Ser815Leu	missense	3 (see supp tbl S2)	1
4	c.325dup	p.Glu109Glyfs*30	frameshift	present, not classified (class 5)	5
8	c.823C>T	p.Gln275*	nonsense	present, not classified (class 5)	4
8	c.856_857del	p.Asp286Glnfs*12	frameshift	present, not classified (class 5)	1
11	c.1214C>A	p.Ser405*	nonsense	present, not classified (class 5)	3
12	c.2117del	p.Lys706Serfs*19	frameshift	present, not classified (class 5)	1
intron 4	c.354-2A>G		canonical splice variant	not present (class 4)	2
11	c.1237_1238delinsT	p.Lys413*	frameshift	not present (class 5)	1
Intron 13	c.2275+1G>A			Not present, ClinVar class 4/5	1
2	genomic deletion including exon 2		large genomic deletion	5	5
10	genomic deletion including exon 10		large genomic deletion	5	1
14	genomic deletion including exon 14		large genomic deletion	5	10
1_15	genomic deletion whole gene (exons 1-15)		large genomic deletion	5	3

SUPPLEMENTARY TABLE 1 *PMS2* variants reported as disease-causing in the families included in this study

exon/ intron	PMS2 variant[a]	predicted protein effect	type of variant	InSiGHT class[b]	No of carriers with variant
11	c.1831dup	p.Ile611Asnfs*2	frameshift	5	5
11	c.1882C>T	p.Arg628*	nonsense	5	21
13	c.2192_2196del	p.Leu731Cysfs*3	frameshift	5	7
14	c.2404C>T ;	p.Arg802*	nonsense	5	1
14	c.2444C>T	p.Ser815Leu	missense	3 (see supp tbl S2)	1
4	c.325dup	p.Glu109Glyfs*30	frameshift	present, not classified (class 5)	5
8	c.823C>T	p.Gln275*	nonsense	present, not classified (class 5)	4
8	c.856_857del	p.Asp286Glnfs*12	frameshift	present, not classified (class 5)	1
11	c.1214C>A	p.Ser405*	nonsense	present, not classified (class 5)	3
12	c.2117del	p.Lys706Serfs*19	frameshift	present, not classified (class 5)	1
intron 4	c.354-2A>G		canonical splice variant	not present (class 4)	2
11	c.1237_1238delinsT	p.Lys413*	frameshift	not present (class 5)	1
Intron 13	c.2275+1G>A			Not present, ClinVar class 4/5	1
2	genomic deletion including exon 2		large genomic deletion	5	5
10	genomic deletion including exon 10		large genomic deletion	5	1
14	genomic deletion including exon 14		large genomic deletion	5	10
1_15	genomic deletion whole gene (exons 1-15)		large genomic deletion	5	3
11_12	genomic deletion including exons 11-12		large genomic deletion	5	4
11_15	genomic deletion including exons 11-15		large genomic deletion	5	16
3_7	genomic deletion including exons 3-7		large genomic deletion	5	8
5_15	genomic deletion including exons 5-15		large genomic deletion	5	1
5_7	genomic deletion including exons 5-7		large genomic deletion	5	4
1_11	genomic deletion including exons 1-11		large genomic deletion	5	4
2_4	genomic deletion including exons 2-4		large genomic deletion (in frame)	not present (class 4)	4

[a] Variant nomenclature according to HGVS guidelines (http://varnomen.hgvs.org/) with reference to NM_000535.5 for PMS2, except for large deletions or duplications. Large deletions and duplications were in some cases detected with the older MLPA kit P008 (MRC Holland) that lacks reliable probes for PMS2 exons 3, 4, 12-15. Therefore, the exact range of exon deletions was not always established. Although for some large deletions the breakpoints have been characterized, we did not include this information.
[b] Clinical variant class as reported on https://insight-database.org/variants/PMS2; last accessed on 14 December 2017; 5 = pathogenic, 4 = likely pathogenic, 3 = variant of uncertain significance. Classification of the variants not present or present but not yet classified in the InSiGHT database is given between brackets, using guidelines provided by https://www.insight-group.org/criteria/. Nonsense and frameshift mutations, including large genomic deletions, were classified as pathogenic (class 5). Canonical splice variants and large in-frame genomic deletions were classified as likely pathogenic (class 4). Additional evidence that suggests pathogenicity for variants that could not be classified a priori as (likely) pathogenic is provided in supplementary table S2.

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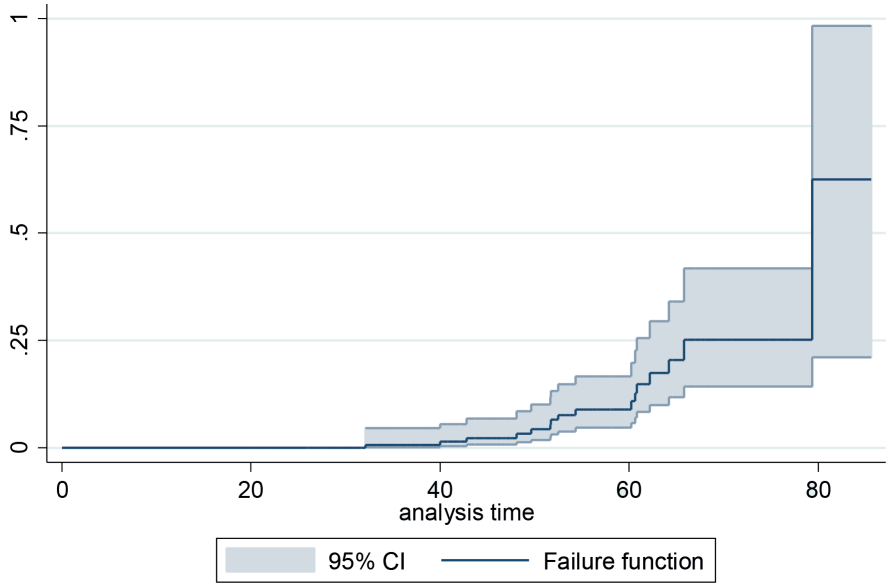
SUPPLEMENTARY TABLE 2 Additional evidence that suggests pathogenicity for one PMS2 variants

location	PMS2 variant[a]	type of variant	number of families (this study)	Evidence suggestive for pathogenicity[b]	
Exon 4	c.319C>T p.Arg107Trp	missense	1 (Netherlands)	• MMR-deficiency shown by in vitro MMR assay (van der Klift et al., 2016) • Incomplete aberrant splicing (van der Klift et al., 2015) • In trans with pathogenic PMS2 variant in a CMMRD patient (van der Klift et al., 2016)	2

[a] Variant nomenclature according to HGVS guidelines (http://varnomen.hgvs.org/), with reference to NM_000535.5 for PMS2.
[b] data on conservation, splice prediction, functional predictions (PolyPhen-2, SIFT, aGVGD, MutationTaster), presence in control population databases (ExAC, ESP, 1000G) and in the ClinVar archive were obtained through Alamut Visual v.2.6, last accessed on 23-12-2017.
Abbreviations: MMR = mismatch repair; CMMRD = constitutional mismatch repair deficiency; MLA = multifactorial likelihood analysis; LR = likelihood ratio; AA = amino acid



SUPPLEMENTARY FIGURE 1 Cumulative proportion of PMS2 carriers with an advanced adenoma at first colonoscopy

6

GENERAL DISCUSSION

The studies described in this thesis were aimed at further delineating the PMS2-associated Lynch syndrome phenotype and to provide sufficient grounds for PMS2-specific surveillance guidelines, including risk estimates which can be used to inform pre-symptomatic carriers about their chances of developing cancer.

In recent years it became clear that the less penetrant Mismatch Repair (MMR) genes, *MSH6* and *PMS2*, are likely much more prevalent than what was previously suspected. This is illustrated by a recent study that shows an over two times higher prevalence of *MSH6* and *PMS2* variants (1 in 758 and 1 in 714 respectively) compared to *MLH1* and *MSH2* (1 in 1946 and 1 in 2814 respectively).[1] This is surprising in light of the vast overrepresentation of known MLH1/MSH2 families.[2] The underreporting of PMS2 families is partly due to the existence of multiple pseudogenes, making DNA variant detection challenging. This has now been solved by a range of solutions, including RNA analyses and long-range PCR amplicons.[3-7] However, the problem in the detection of PMS2 families mainly lies in the mild phenotype, i.e. these families can be easily overlooked. Recently, initiatives have been undertaken to improve detection with prediction models aimed at selecting patients for genetic testing by adding further clinical parameters such as unaffected status at specific ages, tumor location and a lower testing threshold.[8-10] Yet the most significant improvement in detection is most likely the introduction of universal immunohistochemistry for the MMR proteins for all colorectal cancers below the age of 70 years as recommended by the Dutch guideline '*Erfelijke darmkanker*' (www.oncoline.nl). This approach has been shown to be cost-effective and has the added benefit that no additional selection criteria are needed.[11-13] The latter is very important as other selection criteria such as the Amsterdam criteria and Bethesda criteria are not successful in detecting all families with a segregating pathogenic *PMS2* variant.[14] A steep rise in *PMS2* carriers is to be expected in coming years due to these universal screening protocols. Most of these newly detected PMS2 (and MSH6) families will very likely have much milder phenotypes because there is no selection on family history. More studies on PMS2-associated Lynch syndrome are therefore essential.

Not much was known about the associated cancer risks for *PMS2* carriers. Cancer risk analyses are usually biased as families included in such studies are mainly ascertained in family cancer clinics. These families therefore comply with clinical selection criteria such as the Bethesda criteria, meaning that multiple cancers or cancer at a young age have already arisen.[15] Estimating cancer risks in such families results in upwardly biased results.[16] This is especially relevant when estimating the risk for *PMS2* carriers as the first study to report on 55 probands carrying a pathogenic *PMS2* variant estimated cancer risks up to age 70 to be 15-20% for colorectal and 15% for endometrial

cancer.[17] This was a striking difference with previously reported cancer risks for other Lynch syndrome patients in general, i.e. 25-70%.[16, 18-24] This first study in *PMS2* carriers analyzed data using a *modified segregation* analysis.[17, 23, 25] In brief, this method includes all available family members regardless of whether or not they have been tested. It also conditions on the phenotype of the proband thereby correcting for ascertainment through family cancer clinics. The advantage of this approach is that data of large retrospective cohorts can be used to estimate cancer risks. However, the down-side is the possible overcorrection of risk estimates.[26] Indeed, Vos et al. showed that the wide range of reported cancer risks in *BRCA1* and *BRCA2* carriers is largely explained by the statistical method being used. It has been suggested that correcting in a too stringent manner by using for example the modified segregation analysis can result in underestimation of the risk in clinic-based families. However, as mentioned above, with the expected rise of *PMS2* carriers ascertained from the population and the likely selection of relatively severely affected PMS2 families (for example due to the influence of risk modifiers) in the past, rigorous correction for bias in risk estimation in PMS2 cohorts seems vital.

In 2015 we analyzed 98 PMS2 families and found similar cancer risks to what was previously reported, i.e. risks of 11-19% for colorectal and 12% for endometrial cancer up to age 70.[25] Thereby further supporting the notion that PMS2-associated Lynch syndrome patients indeed face significantly lower risks than other Lynch patients. This study was also the first to report on extra-colonic risks. Due to a relatively low number of events we were only able to report standardized incidence ratios (SIRs), which do not account for ascertainment and testing bias as only confirmed carriers were included. SIRs were statistically significantly increased for cancer of the ovaries, small bowel, renal pelvis and – notably – of the breasts. However, the corresponding confidence intervals were too wide for meaningful interpretation in terms of surveillance protocols. Our group therefore set out to enlarge the cohort through global collaboration. Surprisingly, in this much larger cohort, containing 284 families we only found an increased risk for endometrial cancer (ten Broeke et al, 2018, in-press at Journal of Clinical Oncology, chapter 2). Cancer risks for *PMS2* carriers compared to the general population and other Lynch syndrome patients are given in table 1. The lack of an association of *PMS2* variants with extra-colonic, extra-uterine cancer in the larger study underlines the necessity of large global datasets, which is emphasized even more by an even lower cumulative risk for colorectal cancer and endometrial cancer than our first report, namely 13-14% and 14% to age 80, respectively.

TABLE 1 Overview of reported cancer risks

	General population (lifetime)	Lynch syndrome Barrow et al (up to age 70)	PMS2- associated Lynch syndrome			
			Senter et al 2008 (95% CI)	Ten Broeke et al 2015 (95% CI)	Ten Broeke et al 2018	
					Up to age 70 (95% CI)	Up to age 80 (95% CI)
Colorectal	~4-6%	25-75%	♂: 20% (11–34%) ♀: 15% (8–26%)	♂: 19% (6-30%) ♀: 11% (2-18%)	♂: 6% (3-13%) ♀: 6% (3-12%)	♂: 13% (8-22%) ♀: 12% (7-21%)
Endometrial	~3%	30-35%	15% (6–35%)	12% (3-20%)	10% (5-17%)	14% (7-24%)
Ovarian	~1%	6-14 %	N/A	SIR: 12.0 (3.3-30.7)	HR: 1.52 (0.45-5.05)	
Gastric	~1%	0.7-13 %	N/A	SIR: 0.0 (0-6.5)	HR: 2.07 (0.73-5.87)	
Urothelial	~1-2%	1.9-11.2%	N/A	SIR (bladder): 2.0 (0.05-11.2) SIR (renal pelvis): 50.5 (6.1-182.4)	HR: 2.05 (0.77-5.45) (kidney and ureter)	
Small Bowel	~0.1%	0.6-7%	N/A	SIR: 118.9 (38.6-277.4)	Too few events for analysis	
CNS	~0.5%	1.2-3.7%	N/A	SIR: 2.7 (0.069-15.2)	HR: 2.09 (0.79-5.54) (brain)	
Pancreas & biliary tree	~1-2%	0.6-2.1%	N/A	SIR: 0 (0-12) (only pancreas)	HR: 1.02 (0.12-8.60) (hepatobiliary)	
Breast	~12%	Conflicting results of association	N/A	SIR: 3.8 (1.9-6.8)	HR: 1.30 (0.79-2.16)	

SIR: Standardized Incidence Ratio. HR: Hazard Ratio. 95% CI: Confidence Interval

Another example of the importance of large cohorts is the association of breast cancer risk and Lynch syndrome. As mentioned, our first penetrance analysis identified a significant increase in breast cancer risk (SIR: 3.8 (95% CI: 1.9-6.8)).[25] If confirmed, this might have justified additional breast surveillance, for example with mammography from age 40. It may have also justified inclusion of the *PMS2* gene in multigene panels being used in breast cancer cohorts, which we have suggested in response to a study only including specific breast cancer genes.[27] Such panels may miss clinically relevant genes in patients (or families) with cancer at other sites as well. However, our second analysis in a much larger cohort did not yield increased breast cancer risk (HR: 1.30 (95% CI: 0.79-2.16)) and the first analysis was therefore likely hampered by the relatively small cohort and a method that did not correct for (ascertainment) bias. The difficulty with breast cancer is that it is a frequent cancer site, also in the general population. This, together with the likely high prevalence of *PMS2* variants in unselected cohorts, may have led to a recent claim of an association between increased breast cancer risk and *PMS2* and *MSH6* variants.[28] In this study a multigene hereditary cancer panel was performed in breast cancer cohorts, where they observed a relatively high incidence of *PMS2* and *MSH6* variants thus leading to their conclusion that variants in these genes may cause an increased breast cancer risk. We have written a reply to this study suggesting that the high prevalence of breast cancer in the general population in combination with a relatively high prevalence of *PMS2* and *MSH6* variants in unselected (population-based) cohorts, may form a more plausible explanation for the observed (possibly false) association of *PMS2*/*MSH6* variants with breast cancer.[29]

There are of course other ways to deal with ascertainment bias besides using the modified segregation method. The first is to include families which have not been ascertained because of the Lynch syndrome phenotype. Such families include those ascertained through universal immunohistochemistry for the MMR proteins. These families usually exhibit much milder phenotypes compared with clinically ascertained families. However, these families are still relatively sparse and studies using such cohorts will be seriously underpowered. Ascertainment bias may also be circumvented by analyzing families ascertained because of a patient with Constitutional Mismatch Repair Deficiency (CMMR-D). These patients carry homozygous or compound heterozygous variants in one of the MMR genes, usually *PMS2* and *MSH6*. Due to their constitutional MMR deficiency these patients display a very striking phenotype of cancer in childhood. They also present with axillary freckling and café-au-lait maculas.[30, 31] As de novo MMR variants have been reported to be extremely rare, parents are usually carriers of a heterozygous MMR variant and therefore have Lynch syndrome. Notably, these families almost never comply with traditional selection criteria due to a

very mild phenotype. Our group has gathered a large cohort of CMMRD-ascertained Lynch syndrome families and found similar results to those that are described in chapter 2 (unpublished data, Suerink et al). Thereby confirming previous reports of low cancer risks for PMS2-associated Lynch syndrome patients. A third method of estimating cancer risks is to gather prospective data. A global collaboration has now been formed to gather such data. The Prospective Lynch Syndrome Database (PLSD) has currently published two reports on penetrance.[32, 33] The most recent report also included 124 *PMS2* carriers with 524 observation years in which no colorectal cancers developed, i.e. the cumulative risk was zero. The only cancer that did occur in this cohort was endometrial cancer, therefore this form of cancer is not prevented by surveillance protocols currently in place. Although the cohort might be underpowered for other cancers it is still worth noting that the observed carriers also did not develop cancer at other sites. This confirms our findings in retrospective cohorts.

Interestingly, the colorectal cancer risks reported in our second study (ten Broeke et al, 2018, in-press at Journal of Clinical Oncology, chapter 2) are only two to three times increased when compared to the general population. PMS2-specific guidelines for colorectal cancer surveillance have been much debated with some groups propagating more conservative approaches, while others (including our group) have suggested less stringent guidelines.[17, 25, 34, 35] General consensus in the Netherlands is that additional screening advices are only given when the colorectal cancer risk exceeds a three times increased threshold. Does it therefore follow that *PMS2* carriers should not undergo any colonoscopic surveillance? We believe that these carriers should still follow regular surveillance as we confirmed the role of PMS2-associated mismatch repair (MMR) deficiency in tumor development which will most likely result in increased adenoma to colorectal cancer transformation (ten Broeke and Ballhausen et al, manuscript in preparation, chapter 4). This conclusion was drawn after analyzing[16] PMS2-associated colorectal cancers for the coding microsatellite instability spectrum. Interestingly, and perhaps surprisingly, the results showed no significant differences when compared to other Lynch-associated colorectal cancers while on theoretical grounds differences might have been expected. Indeed, we started analyzing tumor tissue because the consensus on the lower penetrance of *PMS2* variants inevitably led to the question as to why this is the case. The PMS2 protein is part of the MMR machinery. In mammalian cells MMR proteins function as heterodimers in two main complexes existing of MutS homologues MSH2 with either MSH6 or MSH3, and MutL homologues MLH1 binding to PMS2, PMS1, or MLH3. These complexes act together in repairing mismatches and insertion-deletion loops that occur during DNA replication.[36, 37] Somatic loss of the remaining wild type *MLH1*, *MSH2*, *MSH6* or *PMS2* allele in patients with a germline

variant in one of the MMR genes leads to an impaired mismatch repair system, according to Knudson's two-hit model.[38] The theory regarding the lower penetrance of *PMS2* variants is that MLH1/MLH3 and/or MLH1/PMS1 heterodimers can partially compensate for the loss of the MLH1/PMS2 heterodimer.[39]

Our second study of PMS2-associated colorectal cancers showed that these tumors are characterized by a complete lack of β-catenin (*CTNNB1*) variants (ten Broeke et al, 2018, in-press at Gastroenterology, chapter 4). Previous studies have linked β-catenin variants to cancer formation arising from MMR-deficient (dMMR) crypts. Currently, two main pathways of tumor development in Lynch syndrome patients have been described. The first pathway being the traditional MMR proficient (pMMR) adenoma to colorectal cancer progression pathway, in which MMR deficiency has a secondary role. A regular adenoma is formed through for example inactivating variants in the *APC* gene. When a second hit occurs in the MMR gene in which a Lynch syndrome patient already has a germline variant than the interval between adenoma and colorectal cancer formation shortens dramatically. The normal time path through which an adenoma progresses into colorectal cancer is approximately 15 years. In Lynch syndrome patients, due to the accumulation of DNA damage arising from a faulty mismatch repair system, the interval between normal mucosa to adenoma and subsequently colorectal cancer may be as short as three years.[40] This short interval necessitates regular colonoscopic surveillance. However, clinical studies have observed that patients undergoing surveillance still develop colorectal cancer, with recent prospective studies reporting this risk to be up to 46% in patients with a pathogenic *MLH1* variant.[32, 33] A second pathway might explain this observation, as tumors may also arise directly from dMMR crypts in which the second hit has already occurred in crypt cells.[41] These tumors reportedly have a more rapid and aggressive growth pattern for which surveillance is not a sufficient solution, although recent work has suggested that aspirin might work well in preventing these colorectal cancers.[41] As mentioned above, β-catenin variants have been linked to this type of colorectal cancer in Lynch syndrome patients because they mainly found these variants in colorectal cancers not displaying any sign of an adenoma precursor. It is further supported by the fact that β-catenin variants are only found in dMMR tumors that occurred in the context of Lynch syndrome and not through somatic hits in one of the MMR genes resulting in MSI.[42] The complete lack of these variants in *PMS2* carriers suggests that they do not develop colorectal cancers through the dMMR pathway. Consequently, a re-evaluation of the surveillance protocol seems to be in order, which is discussed in more detail below.

However, a known obstacle for designing surveillance guidelines in dominantly inherited cancer predisposition is observed risk variability both between and within

families. This is illustrated by an analysis by Dowty et al. which showed that cancer risk in MLH1- and MSH2-associated Lynch syndrome patients follows a U-shaped distribution rather than a normal Bell-shaped distribution.[23] In other words the majority of patients are either at low or exceptionally high risk. This makes the existence of risk modifiers very likely. These modifiers can include external as well as internal factors, such as lifestyle and other genetic variants.

An example of internal modifiers are colorectal cancer susceptibility loci (single nucleotide polymorphisms, SNPs) identified in genome wide association studies (GWAS) in the general population.[43-45] Several studies have attempted to investigate the role of these modifiers in phenotype variability in Lynch syndrome patients and found positive associations for several SNPs.[46-49] To investigate the effect of these and other SNPs we gathered the largest cohort of DNA samples from *PMS2* carriers reported to date (n=507). Notably, previous findings in carriers of variants in the heterodimer partner of *PMS2*, *MLH1*, could not be confirmed,[46, 47] nor were the previously reported risk modifying effects of rs10795668 and rs992918, identified in a small cohort of 40 *PMS2* carriers.[48] However, there did appear to be some effect for male carriers of allele A at rs1321311 (6p21.31) (HR=2.1, 95%CI: 1.2-3.0).[50] This result needs to be replicated in other larger studies before it can be of clinical utility. Moreover, the influence of these SNPs seems to be relatively small in *PMS2* carriers and the value of using them in individual risk prediction is doubtful at best. However, incorporating them into larger models with multiple risk modifiers may be of clinical utility.

Another possible modifier that could be included in such a model is lifestyle. Multiple groups have published on the effect of lifestyle factors on colorectal cancer risk in Lynch syndrome patients and identified associations between adenoma or colorectal cancer development and smoking, increased BMI and alcohol consumption.[51-59] Unfortunately, none of these studies included a sufficient number of *PMS2* carriers for meaningful interpretation of the effect of lifestyle factors in this subset of Lynch syndrome patients. We attempted to analyze these factors in *PMS2* carriers and did not find any significant association between colorectal cancer development and smoking, BMI at age 20 and alcohol consumption (unpublished data, results described in chapter 3). However, this study was limited by its retrospective design and limited cohort size. It remains to be seen whether lifestyle factors are of significant influence in *PMS2* carriers. Future studies such as those reporting on the GEOLynch cohort[51, 53, 60, 61], which includes prospectively collected data, will shed more light on this. As a result of the prospective design of these studies, the end-point of the analyses is usually adenoma development. The reason for this being that using colorectal cancer as an endpoint is biased in Lynch syndrome patients undergoing regular colonoscopic

surveillance and if needed polypectomies, because you are effectively preventing the development of colorectal cancer. A prospective design taking adenoma development as an endpoint could actually be very relevant for *PMS2* carriers if we can indeed confirm that colorectal cancer in these patients only develops through the pMMR adenoma to colorectal cancer progression pathway. Meaning that further prevention of adenoma development in *PMS2* carriers may result in an even lower cancer risks. Data on risk factors in adenoma formation from the general population might be consciously extrapolated to *PMS2* carriers as our results indicate that PMS2 deficiency is not expected to play a role in the formation of adenomas. The latter is also supported by our data showing no PMS2 deficiency in 16 collected adenomas from *PMS2* carriers (ten Broeke and Suerink et al, manuscript in preparation, chapter 5). The study describing this data also showed that age at first adenoma detection and proportion of carriers with an adenoma at first colonoscopy is similar to what is seen in non-Lynch syndrome familial colorectal cancer cohorts, but markedly higher and lower, respectively, than seen in *MLH1* or *MSH2* carriers. This suggests that clinically ascertained *PMS2* carriers may have additional risk factors for adenoma development, leading to their selection because of colorectal cancer development with accelerated carcinogenesis due to secondary PMS2 deficiency. The late stage of PMS2 deficiency is also supported by a study by Alpert et al, reporting less Lynch-associated hallmarks (such as immune infiltration) in tumors with isolated PMS2 loss.[62] Further research is needed to provide more evidence for the late involvement of PMS2 as this is mainly speculation at this point.

Another explanation for the high degree of phenotype variability between families is that the type of variant (e.g. truncating or non-truncating, or position within the gene) might predict the severity of the phenotype. While it seems intuitive to think that more deleterious variants could lead to the most striking phenotypes, a recent study surprisingly reported that truncating variants actually lead to a later age of onset of endometrial cancer in female *MLH1* mutation carriers[63], although we have raised questions about the validity of these results.[64] Indeed, most other studies report a milder phenotype associated with non-truncating variants. Our group is the only one to have analyzed genotype-phenotype correlations in a PMS2 cohort.[65] In this study a cohort of 381 European *PMS2* carriers was analyzed. Groups were formed based on RNA expression. Notably, retained RNA expression was associated with an age of colorectal cancer diagnosis 9 years later than those with loss of RNA expression. At the moment these findings have not been replicated and as such it is too soon to integrate genotype into tailor-made surveillance guidelines.

The last possible modifier which will be discussed here is the much debated existence

of genetic anticipation.[66-72] Genetic anticipation would supposedly result in decreasing age of onset of colorectal cancer in Lynch families. A recent study in a Swedish cohort suggested that PMS2 families display anticipation to a notable degree, reporting a 7.3 year decrease of age at colorectal cancer onset for each subsequent generation.[73] This in contrast to *MLH1*, *MSH2* and *MSH6* carriers where (much) smaller anticipation effects were identified. However, this analysis only included 12 PMS2 families. Initially, we could actually confirm the results of anticipation in our much larger cohort of 152 European families. However, after correcting for birth cohort, the effect completely vanished (ten Broeke et al, 2018, in-press at Cancer Epidemiology, Biomarkers & Prevention, chapter 3). This is in line with the observed increased incidence of colorectal cancer in Western-European countries and the fact that no logical biological explanation for genetic anticipation in Lynch families exists. Notably, several studies have reported on different trends of colorectal incidence in for example the USA and Australia, where incidence only seems to be rising in very recent birth-cohorts resulting in increased identification of young onset colorectal cancer.[74, 75] However, this is only after a steady decline up to birth cohorts from approximately 1940.[76, 77] Future studies on genetic anticipation should therefore take geographic origin into consideration.

In conclusion, results from studies presented in this thesis as well as the work of others have made it apparent that PMS2-associated Lynch syndrome patients show an attenuated phenotype when compared to other MMR variant carriers. We therefore suggest that surveillance guidelines can be loosened to integrate these new data in daily practice. First off, we would like to stress the importance of gene-specific risk communication. Based on our results in the Netherlands current risks being communicated for *PMS2* carriers are 5-35% for both colorectal and endometrial cancer. It should also be emphasized that carriers only appear to be at risk of these two cancers and of none of the other Lynch-associated cancers, such as ovarian and gastric cancer. Based on the lack of prospectively observed colorectal cancers in patients undergoing surveillance as well as the corresponding molecular explanation, i.e. no colorectal cancer development through dMMR crypts, we advise colonoscopic follow-up every 2-3 years instead of once every 1-2 years (ten Broeke et al, 2018, in-press at Journal of Clinical Oncology, chapter 2).[33] We would also recommend starting surveillance at age 35-40 due to low cancer risks and later age at onset.[17, 25, 34] Moreover, there seems to be no role for gynecological risk reducing surgery for female carriers, as survival for endometrial cancer is excellent and the risk for ovarian cancer was not increased in our data (ten Broeke et al, 2018, in-press at Journal of Clinical Oncology, chapter 2).[33] Routine transvaginal ultrasounds with biopsies every 1-2 years from age

40-60 in an effort to identify endometrial cancer at an early stage can be considered, but the added value (or lack thereof) should be discussed with female carriers.[21, 78-81] There is currently insufficient evidence for internal and/or external risk modifiers to be included in risk stratification among PMS2-associated Lynch syndrome patients. Future studies incorporating many modifiers in one risk prediction tool should be developed but these have to take gene-specific effects into account.

The clinical implementation of the results reported in this thesis is a good example of translational research that can bring scientific results directly in to the daily practice of physicians charged with the care of Lynch syndrome patients.

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7

ENGLISH SUMMARY
DUTCH SUMMARY
PUBLISHED MANUSCRIPTS
ACKNOWLEDGMENTS
CURRICULUM VITAE

ENGLISH SUMMARY

Colorectal cancer is one of the most frequently diagnosed cancers in the Western world. Both hereditary and genetic factors play a role in its etiology. In approximately 3% of colorectal cancer cases the underlying cause is a hereditary cancer predisposition syndrome called Lynch syndrome (formerly HNPCC, Hereditary Non-Polyposis Colorectal Cancer). This thesis focuses on an important subset of Lynch syndrome patients, namely those carrying a heterozygous pathogenic germline variant in the mismatch repair gene *PMS2*. Lynch syndrome can also be caused by pathogenic variants in one of the other mismatch repair genes *MLH1*, *MSH2* (and *EPCAM*) and *MSH6*. This condition gives an increased risk of developing mainly colorectal and endometrial cancer, but also – to a lesser extent - other forms of cancer.

Relatively little was known about PMS2-associated Lynch syndrome compared to Lynch syndrome caused by other genes. More research on the PMS2-associated Lynch syndrome was necessary because many questions still existed about this condition, such as the risk of developing cancer and the tumorigenesis. A further delineation of the PMS2-associated phenotype is important as the prevalence of *PMS2* variants has been largely underestimated due to stringent selection criteria and challenges in molecular diagnostics due to the existence of multiple pseudogenes. The aim of this thesis is to further define the phenotype associated with a pathogenic variant in the *PMS2* gene, and to work towards gene-specific clinical guidelines. It also describes studies of several external and internal risk modifiers in an attempt to explain the large clinical variation between and within families with *PMS2* variants.

Chapter 1 is an introduction on hereditary colorectal cancer and Lynch syndrome. The first step in the delineation of the PMS2 phenotype is described in **Chapter 2** and includes two studies reporting on the penetrance of pathogenic germline variants in the *PMS2* gene. In the first study, published in 2015, we analyzed 98 European families and reported cumulative risk up to age 70 of 11-19% for colorectal and 12% for endometrial cancer. This confirmed an earlier report from the United States that also stated that the penetrance for *PMS2* variants is significantly lower when compared to other MMR variants, where risks are reported to be up to 25-75%. Our study was the first to attempt risk analysis for other tumor locations associated with PMS2-associated Lynch syndrome as well. Unfortunately this study was limited by the cohort size. After further expansion of the cohort with families from the United States, Australia and Canada we set out to analyze these other cancers in more detail and with proper statistical methods to correct for ascertainment bias. In this second penetrance

analysis we reconfirmed the relatively low risk of colorectal and endometrial cancer, but also found that *PMS2* carriers were not at increased risk of any of the other Lynch-associated cancers, such as ovarian and gastric cancer.

While it is generally accepted that *PMS2* carriers face markedly lower penetrance, a high degree of phenotype variability is observed both within and between families. **Chapter 3** therefore includes three studies aimed at studying risk modifiers previously associated with Lynch syndrome. No risk modifying effects were identified when analyzing single nucleotide polymorphisms (SNPs) selected from Genome Wide Association Studies (GWAS) in sporadic colorectal cancer cohorts. This includes two loci at 11q23.1 and 8q23.3 that have been repeatedly associated with increased risk in (female) MLH1 carriers. We also attempted to estimate the effect of lifestyle factors (smoking, alcohol use and BMI at age 20) in a retrospective study. No clear effects were identified, although it should be noted that this study was relatively small (n=270 *PMS2* carriers) and may have lacked power. Lastly, a genetic anticipation study was undertaken. The existence of this phenomenon has been much debated. While we initially confirmed an anticipation effect of decreasing age at colorectal cancer, we show with the introduction of a new flexible Cox regression model that this effect is completely explained by a birth cohort effect. Thus, anticipation in PMS2 families seems to be due to a general trend in the general population rather than a biological effect that would increase risk with each subsequent generation in which the PMS2 variant is transmitted.

The notably different phenotype of PMS2-associated Lynch syndrome has led to speculation of differences in tumorigenesis. **Chapter 4** describes the role of PMS2 deficiency in tumorigenesis. This chapter includes two papers. The first is a collaboration with the University of Heidelberg on the presence of instability in coding microsatellites (cMS). The results showed that there are no clear differences in cMS profiles between PMS2 and MLH1/MSH2 tumors. This means that it is likely that similar neo-antigens appear on the celmembrane, which in turn makes it probable that vaccines now targeted at these neo-antigens might work for *PMS2* carriers as well. This chapter also describes the analysis of the somatic variant spectrum in PMS2-associated colorectal cancer. The most notable result was a complete lack of *CTNNB1* (β-catenin) variants in PMS2 tumors (0/20) while the majority of a control cohort of 24 MLH1 tumors did harbor such a variant (14/24). It has been suggested that *CTNNB1* mutated colorectal cancers actually represent an aggressive subtype of cancer which presents as post-colonoscopy colorectal cancers, i.e. cancers that arise in between follow-up colonoscopies. These in turn may be derived from mismatch repair deficient (dMMR) crypts that progress to colorectal cancer without a MMR proficient precursor

adenoma stage. Our data suggests that *PMS2* carriers do not develop colorectal cancer through dMMR crypts, thus explaining lower penetrance and the reported lack of post-colonoscopy colorectal cancers in prospectively observed cohorts. The latter observation is in contrast to other Lynch syndrome patients, where the cumulative risk of developing colorectal cancer is still up to 46% despite undergoing regular colonoscopies and polypectomies.

Chapter 5 investigates this further and contains a study describing prospectively collected data on PMS2 families. This data showed that high-grade adenomas were only rarely found and moreover that only two PMS2 carriers undergoing surveillance developed post-colonoscopy colorectal cancers in over a 1000 years of observation. The latter might be seen as conflicting in light of our hypothesis of a lack of tumor development from dMMR crypts. However upon closer inspection of the colonoscopy reports, the preceding colonoscopies proved unreliable due to insufficient cleansing of the colon and obesity. Moreover, one of these tumors was MMR proficient and it was therefore unlikely a consequence of the underlying germline *PMS2* variant. The last relevant observation in this study is that immunohistochemistry of 16 low-grade adenomas showed no abrogation of the PMS2 protein. Moreover, the proportion of carriers free from adenomas was much higher for *PMS2* carriers when compared to *MLH1* and *MSH2* carriers. This confirms the notion that PMS2 deficiency only occurs at a later stage of colorectal cancer development, rather than being the initiating factor as *MLH1/MSH2* carriers may also form dMMR adenomas from dMMR crypts.

In summary, this thesis shows that *PMS2* carriers are a separate entity among Lynch syndrome patients. This is based on both epidemiological analyses of the clinical phenotype and on tumor analyses. Following the results of the studies in this thesis and studies by other groups, we have formulated preliminary PMS2-specific surveillance guidelines. This entails that *PMS2* carriers start with 2-3 yearly colonoscopies beginning at age 35-40. There seems to be no role for risk-reducing gynecological surgery given the low risk of endometrial cancer with a good chance of survival. Routine transvaginal ultrasounds with biopsies every 1-2 years from age 40-60 can be considered, but female carriers should be properly informed that there is currently no conclusive evidence for better survival after early detection of endometrial cancer.

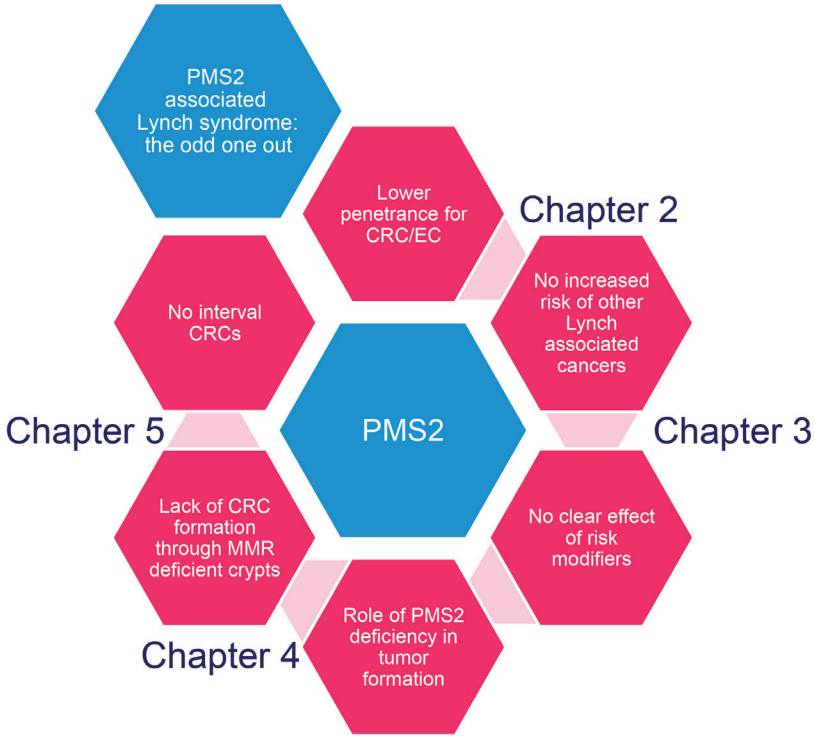


FIGURE 1 Overview of thesis

DUTCH SUMMARY

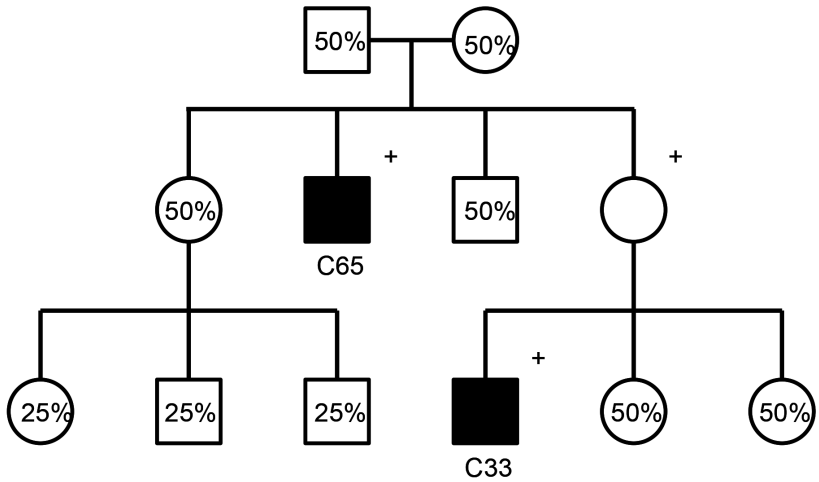
In het Westen is darmkanker een van de meest voorkomende soorten van kanker. Zo wordt in Nederland elk jaar bij ongeveer 11.000 mensen darmkanker gediagnosticeerd. Het risico op deze vorm van kanker hangt af van omgevings-en genetische factoren. Naar schatting is er bij 15-30% van de patiënten sprake van een familiaire of een erfelijke predispositie voor darmkanker. Een erfelijke predispositie voor darmkanker wordt met name veroorzaakt door de volgende drie meest voorkomende syndromen: het Lynch syndroom (vroeger HNPCC genaamd), het familiaire adenomateuze polyposis syndroom (FAP) en het MUTYH-geassocieerde polyposis syndroom (MAP). Deze syndromen verklaren tezamen 4- 5% van de patiënten met darmkanker. In de overige 10-25% is er sprake van zogenaamd familiaire darmkanker, waarbij de precieze reden voor het vaker voorkomen van deze ziekte niet bekend is.
Dit proefschrift richt zich op onderzoek naar een belangrijke deelgroep van Lynch syndroom patiënten, namelijk degenen die een fout (mutatie) hebben in het mismatch reparatie gen *PMS2*. Lynch syndroom kan daarnaast worden veroorzaakt door een mutatie in één van de andere mismatch reparatie genen: *MLH1*, *MSH2* (en *EPCAM*) en *MSH6*. Bij Lynch syndroom patiënten is de mutatie in één van de mismatch reparatie genen aanwezig in alle lichaamscellen, een zogenaamde kiembaanmutatie. Dit geeft een verhoogd risico op het ontwikkelen van met name darmkanker en baarmoederkanker, maar ook andere vormen van kanker kunnen vaker optreden. Het daadwerkelijk transformeren van een gewone cel naar een kankercel gebeurt pas als er naast de reeds bij geboorte aanwezige kiembaanmutatie, lokaal nog een tweede somatische (=spontane) mutatie in een cel ontstaat. Het gevolg van de kiembaan mutatie en de somatische mutatie in beide kopieën van hetzelfde mismatch reparatie gen leidt tot mismatch reparatie deficiëntie. Dit wil zeggen dat het systeem dat zorgt voor de reparatie van mismatches ('foutjes') die ontstaan tijdens de DNA replicatie (verdubbeling) van een delende cel niet goed meer werkt. Dit resulteert in het ontstaan van diverse somatische mutaties in andere genen. De opeenstapeling van mutaties in de (tumor)cellen kunnen er voor zorgen dat een cel ongeremd gaat groeien. Een ander gevolg van mismatch reparatie deficiëntie is het ontstaan van microsatelliet instabiliteit (MSI), waarbij repeterende gebieden van nucleotiden (de letters van het DNA) instabiel worden. Indien een dergelijke instabiliteit in de tumor kan worden aangetoond, dan is dat een sterke aanwijzing voor mismatch reparatie deficiëntie. Deze kennis kan gebruikt worden in de diagnostiek naar Lynch syndroom.
Naar verhouding was er tot voor kort nog maar weinig bekend over de patiëntengroep met PMS2-geassocieerd Lynch syndroom, met name in vergelijking met Lynch

syndroom veroorzaakt door mutaties in de genen *MLH1* en *MSH2*. Meer onderzoek naar PMS2-geassocieerd Lynch syndroom was dan ook nodig. Met name het vaststellen van het precieze risico op het ontwikkelen van kanker was van belang. In het verleden was deze groep patiënten onderbelicht wegens te stringente selectiecriteria en een moeizame mutatie analyse van het *PMS2* gen door de aanwezigheid van zogenaamde pseudogenen. De doelstelling van dit proefschrift is om de risico's op kanker als gevolg van een mutatie in het *PMS2* gen nader te definiëren en toe te werken naar een op maat gemaakte preventie van kanker. Een ander aandachtspunt is de taxatie van de oorzaak van de grote klinische variatie tussen en binnen families met *PMS2* mutaties, waarbij met name de rol van genetische - en leefstijl factoren onderzocht is.

Hoofdstuk 1 van dit proefschrift is een inleiding en beschrijft wat al bekend was over PMS2-geassocieerd Lynch syndroom voorafgaand aan de hierna beschreven studies. Allereerst was een bevestiging nodig van het veronderstelde lagere kankerrisico van *PMS2* mutaties en een analyse van minder voorkomende kanker locaties (zoals bijvoorbeeld eierstok- en maagkanker) die eerder geassocieerd zijn met andere Lynch syndroom mutaties. **Hoofdstuk 2** bevat daarom twee kankerrisico-analyses. In 2015 werd de eerste studie gepubliceerd waarin een analyse in 98 Europese families werd uitgevoerd. Gevonden risico's tot 70 jaar in deze studie waren voor dikke darmkanker respectievelijk 11 en 19% voor vrouwen en mannen, en 12% voor baarmoederkanker. De statistische methode die werd gebruikt in deze studie is de zogenaamde *modified segregation* analysis, een methode die corrigeert voor *bias* (vertekening) van het resultaat in families die werden geselecteerd in de kliniek. Een andere naam voor deze vorm van selectie *bias* is *ascertainment bias*. Het achterliggende idee is dat vanwege het gebruik van klinische selectiecriteria, de meest ernstig aangedane families vanzelfsprekend meer bekend zijn in de kliniek en om die reden terecht komen in onderzoek cohorten. Het volgende voorbeeld is een goede illustratie van *ascertainment bias*: Stel men wil de gemiddelde lengte in de Amerikaanse bevolking bepalen door het nemen van een steekproef met spelers uit de NBA, de Amerikaanse basketbal competitie. Dit zal leiden tot een forse overschatting van de lengte. Daarnaast is het waarschijnlijk dat mensen die al kanker hebben gehad zich eerder laten testen of eerder getest worden voor de aanwezigheid van de genetische aanleg voor Lynch syndroom, waarbij dan een *PMS2* mutatie gevonden kan worden. Dit geldt in ieder geval voor de eerst geteste persoon in een familie. Deze vorm van *bias* wordt *testing bias* genoemd. Risico analyses zonder correcties voor deze twee vormen van *bias* zullen daarom leiden tot een overschatting van het risico op kanker. De *modified segregation* analyse corrigeert op twee belangrijke manieren voor *bias*.

Ten eerste neemt het alle familieleden mee in de berekening, of ze nu getest zijn of niet. Het geeft deze personen allemaal een kans op het hebben van de mutatie. Alle familieleden krijgen op deze manier een gewichtje mee in de analyse. Dit gaat niet alleen *testing* bias tegen, maar vergroot ook het te analyseren cohort. Zie voor een voorbeeld hiervan figuur 1. Voorts wordt de analyse zo aangepast dat het resultaat gecorrigeerd wordt voor de mate waarin een familie is aangedaan, hiermee wordt gecorrigeerd voor de *ascertainment bias*.

De in **hoofdstuk 2** beschreven eerste kankerrisico analyse maakt dus gebruik van de *modified segregation analysis* om het risico op dikke darm- en baarmoeder kanker te schatten. Bekend is echter dat ook andere vormen van kanker vaker voorkomen bij Lynch syndroom patiënten, zoals onder andere eierstok- en maagkanker. In de groep van 98 families in de eerste analyse kwamen deze vormen van kanker te weinig frequent voor om de *modified segregation* methode toe te passen. Daarom is een alternatieve, eenvoudiger, methode gebruikt. Hierin wordt de prevalentie van een bepaalde soort kanker in bevestigde mutatiedragers vergeleken met de algemene bevolking, waarbij rekening gehouden wordt met de leeftijdsverdeling (*standardized incidence ratio*; SIR).

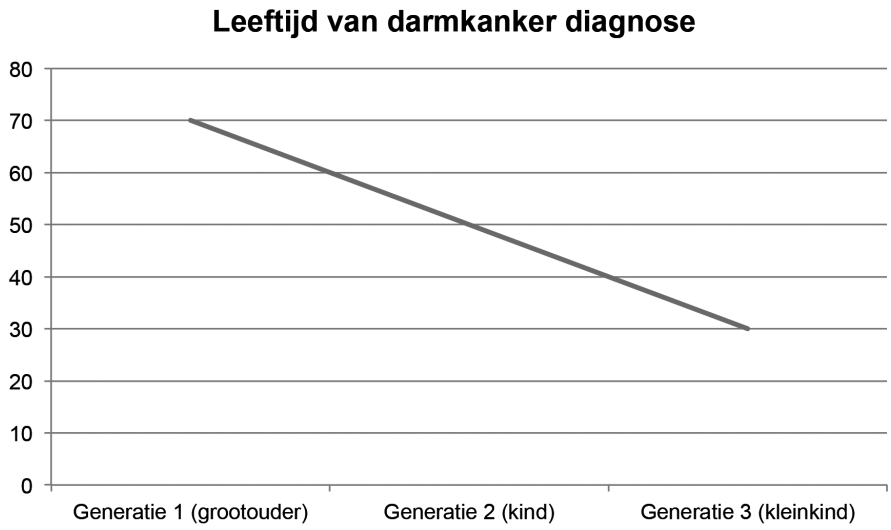


FIGUUR 1 In de modified segregation analyse krijgt ieder ongetest familielid een gewicht mee met de kans op het hebben van de *PMS2* mutatie. 'C' staat voor colorectaal carcinoom oftewel dikke darmkanker. '+' betekent dat de mutatie bevestigd is.

Hieruit kwam naar voren dat er een verhoogd risico zou zijn op eierstok-, dunne darm-, nierbekken- en mogelijk ook borstkanker. Deze analyse was echter zeer waarschijnlijk beïnvloed door de hierboven beschreven vormen van *bias*. Om dat te ondervangen is door het aangaan van nieuwe samenwerkingsverbanden met Amerika, Canada en Australië een veel groter cohort verzameld, bestaande uit 284 families. In deze groep was het mogelijk om een corrigerende analyse uit te voeren. Hieruit bleek dat alleen het risico op dikke darm- en baarmoederkanker verhoogd is in PMS2 families, geen van de andere vormen van kanker kwam vaker voor, vergeleken met de algemene bevolking. Het risico op dikke darmkanker tot de leeftijd van 80 jaar was 12-13% en voor baarmoederkanker 14%. Ten opzichte van het bevolkingsrisico betekent dat een verhoging van respectievelijk twee tot drie maal en vier tot vijf maal. Deze resultaten onderstrepen het belang van het toepassen van adequate statistische methodes en het gebruik van voldoende grote patiëntgroepen om kankerrisico's op een valide manier te berekenen. Bij het interpreteren van bovenstaande geschatte risico's moet benoemd worden dat zowel binnen als tussen families veel variatie bestaat in de frequentie waarin darmkanker gediagnosticeerd wordt en op welke leeftijd. De verklaring daarvoor moet waarschijnlijk gezocht worden in zogenoemde *modifiers* (risico-beïnvloedende factoren). Deze *modifiers* kunnen zowel extern (leefstijl, omgevingsfactoren) als intern zijn (genetische variantie in andere genen dan *PMS2*).

Hoofdstuk 3 beschrijft drie studies waarin potentieel relevante modifiers werden onderzocht in *PMS2* families. De eerste studie is een analyse van het effect van milde risicovarianten in het DNA (*single nucleotide polymorphisms*, ook wel SNPs genoemd). Deze SNPs zijn geïdentificeerd in darmkanker-cohorten uit de algemene bevolking. Uit eerdere studies zou zijn gebleken dat twee SNPs het risico op darmkanker significant beïnvloeden in Lynch syndroom. Deze studies includeerden echter geen of maar weinig PMS2 mutatiedragers. In een cohort van 507 dragers vonden we geen effect van deze en 22 andere SNPs. Vervolgens werd door middel van een vragenlijstonderzoek geprobeerd vast te stellen wat de invloed is van leefstijlfactoren zoals roken, alcoholgebruik en overgewicht op jonge leeftijd op risico om darmkanker te krijgen. Er werden geen duidelijke associaties gevonden. Echter, de onderzochte groep bestond uit 270 PMS2 dragers en was daarmee relatief klein. Meer subtiele effecten kunnen om die reden gemist zijn en grotere studies zijn nodig om tot een meer definitief antwoord te komen. Tot slot is nog gekeken naar het bestaan van genetische anticipatie, dat wil zeggen het steeds jonger worden van de leeftijd van darmkanker diagnose bij elke opvolgende generatie waaraan de *PMS2* mutatie doorgegeven wordt (figuur 2).

Eerdere studies rapporteerden dat over de generaties de leeftijd van diagnose van darmkanker in Lynch syndroom patiënten steeds lager werd. Onze studie beschrijft een nieuw ontwikkeld statistisch model om het genetische anticipatie effect nader te onderzoeken en te kunnen corrigeren voor *ascertainment* en *testing bias*. De resultaten bevestigden dat er inderdaad op het eerste gezicht anticipatie lijkt plaatst te vinden in *PMS2* families, maar als wordt gecorrigeerd voor het geboortecohort (het jaar waarin iemand geboren is) dan verdwijnt dit effect. Dit is deels te verklaren door een algemene stijging van het aantal darmkanker gevallen, ook op jonge leeftijd, beschreven in de algemene bevolking. Redenen hiervoor zijn bijvoorbeeld schadelijke leefstijl en een betere detectie van kanker door bijvoorbeeld het bevolkingsonderzoek. Hiermee lijkt de geobserveerde anticipatie in PMS2 families het gevolg te zijn van een algemene trend in de algemene bevolking in plaats van een specifiek biologisch effect dat versterkt wordt bij elke generatie waarin de *PMS2* mutatie doorgegeven wordt. Samenvattend kan er gezegd worden dat met de huidige studies geen duidelijke *modifiers* die het wisselende klinische beeld tussen PMS2 families kunnen verklaren. Grotere studies zijn noodzakelijk om hier meer duidelijkheid in te scheppen, zodat er toegewerkt kan worden naar individuele risicoschattingen.

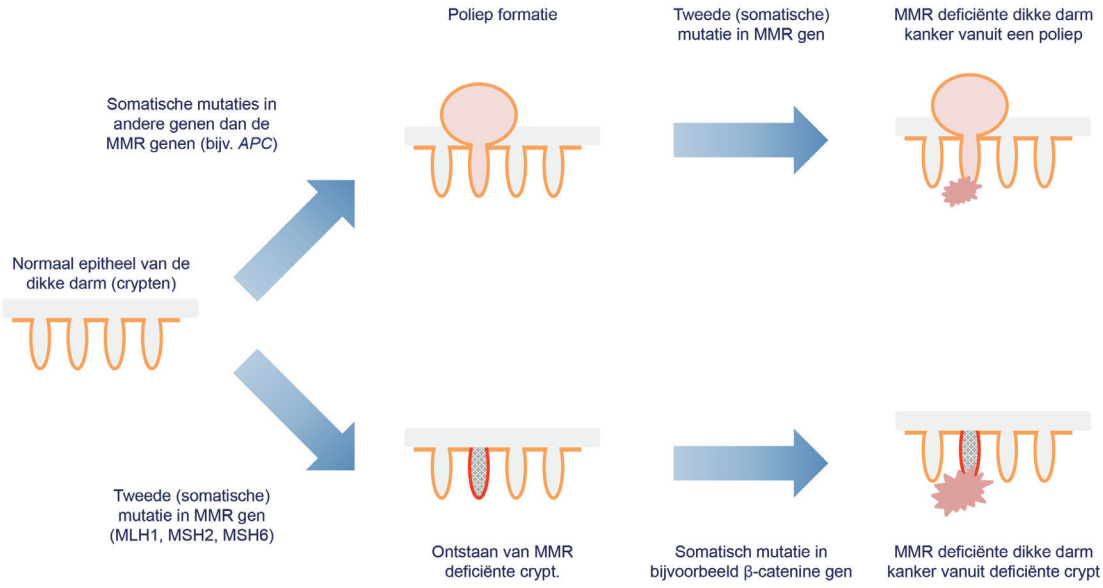


FIGUUR 2 Voorbeeld van genetische anticipatie. Over de generaties kan er verjonging van de leeftijd van darmkanker diagnose optreden, de vraag is echter of dit effect bij PMS2 families wel bestaat.

De verschillen in het klinisch beeld tussen PMS2- en andere Lynch patiënten leidde ook tot de vraag of de tumoren zelf PMS2-specifieke karakteristieken hebben en wat de rol van een *PMS2* mutatie is in het ontstaan van tumoren. **Hoofdstuk 4** beschrijft daarom twee studies betreffende deze twee onderzoeksvragen. De eerste studie is een samenwerking met de universiteit van Heidelberg en bekijkt de rol van kiembaan *PMS2* mutaties in het ontstaan van dikke darm tumoren. Hiervoor is gekeken naar het zogenoemde microsatelliet instabiliteit profiel en werden PMS2 tumoren vergeleken met andere Lynch geassocieerde dikke darm tumoren. Er werd geen verschil gevonden in het microsatelliet instabiliteitspatroon, terwijl er op theoretische gronden wel een andere tumorontwikkeling te verwachten valt. De voorlopige conclusie uit onze eerste data in een relatief kleine groep is dat *PMS2* mutaties wel een rol spelen bij het ontstaan van darmkanker. De tweede studie betreft een analyse van somatische DNA-mutaties in de tumor die secundair ontstaan zijn aan het niet meer werkende mismatch reparatie systeem. Het specifieke profiel van zulke mutaties in onze PMS2 tumoren werd daartoe nader onderzocht. De meest opvallende bevinding is het compleet ontbreken van somatische mutaties in het *CTNNB1* gen in PMS2 tumoren, terwijl in MLH1 tumoren in de meerderheid wel een *CTNNB1* mutatie gevonden werd. Dit gen codeert voor het eiwit β-catenine. Er is door anderen gesuggereerd dat mutaties in dit gen karakteristiek zijn voor een agressief groeiend type darmkanker dat zich niet ontwikkelt vanuit een goedaardig poliep voorstadium, maar direct invasief groeit vanuit zogenoemde mismatch reparatie deficiënte crypten in het darmepitheel. Het gebrek aan β-catenine mutaties in de PMS2 tumoren suggereert dat darmkanker in *PMS2* mutatiedragers niet volgens dit tweede type groeipatroon ontstaat (figuur 3). De conclusie die uit hoofdstuk 4 getrokken kan worden is daarom dat hoewel *PMS2* mutaties wel een rol lijken te spelen in de progressie van darmkanker, ze mogelijk niet de oorzaak zijn van het ontstaat van een tumor. Dit vormt daarmee een mogelijke verklaring voor het relatief lage kankerrisico van *PMS2* mutatiedragers. Deze hypothese wordt verder gestaafd door eerdere observaties dat *PMS2*-mutatiedragers die regelmatige colonoscopieën (dikke darm onderzoeken) en, indien nodig, polypectomieën (verwijderen van weefsel dat het goedaardige voorstadium van darmkanker vormt) ondergaan, geen darmkanker ontwikkelen. Dit in tegenstelling tot andere Lynch patiënten die tot 46% kans hebben om ondanks deze surveillance toch darmkanker ontwikkelen. Dit zou kunnen betekenen dat het tijdig verwijderen van de poliepen bij *PMS2* mutatiedragers het ontstaan van darmkanker voorkomt.

Hoofdstuk 5 gaat nader in op hetgeen in hoofdstuk 4 is besproken en bevat een manuscript dat de samenhang tussen de aanwezigheid van poliepen en het ontstaan van interval darmkanker (kanker die ontstaat tussen de colonocopieën in) beschrijft

in een prospectieve studie opzet. In deze studie werden colonoscopie-verslagen opgevraagd van 171 *PMS2* mutatiedragers. Twee *PMS2* mutatiedragers ontwikkelden darmkanker ondanks regelmatige surveillance. Dit zou gezien kunnen worden als een ontkrachting van onze hypothese. Echter, verdere inspectie van de scopie-verslagen bracht aan het licht dat voorafgaande colonoscopieën suboptimaal waren door onvolledige reiniging van de dikke darm en het bestaan van obesitas bij de desbetreffende patiënten. Daardoor zou een eerdere poliep mogelijk gemist en dus niet verwijderd kunnen zijn, waardoor deze verder kon ontwikkelen tot darmkanker. Daarnaast bleek dat één van deze twee darmtumoren geen kenmerken liet zien passende bij PMS2 deficiënte en dus niet het gevolg kon zijn van het onderliggende Lynch syndroom bij deze patiënt. De andere belangrijke observatie in deze studie is dat *PMS2* mutatiedragers in vergelijking met *MLH1*- en *MSH2* mutatiedragers waarschijnlijk langer poliep vrij blijven. Ook dit kan mogelijk verklaard worden door het feit dat er geen mismatch reparatie deficiënte poliepen groeien uit mismatch reparatie deficiënte crypten (figuur 3).



FIGUUR 3 Een beschrijving van de twee manieren waarop dikke darmkanker zich in Lynch syndroom patiënten ontwikkelt. Onze data suggereert dat alleen de bovenste route daadwerkelijk plaatsvindt bij PMS2 mutatiedragers.

Dit proefschrift toont aan dat *PMS2* mutatiedragers een aparte entiteit zijn onder Lynch syndroom patiënten. Dit is gebaseerd op zowel epidemiologische analyse van het klinische fenotype als op tumor analyses. Naar aanleiding van de resultaten van de studies in dit proefschrift en studies van andere onderzoeksgroepen, hebben wij een voorlopig PMS2-specifiek controle advies geformuleerd. Dit houdt in dat *PMS2* mutatiedragers starten met twee tot drie jaarlijkse colonoscopieën op 35 tot 40-jarige leeftijd. Gezien het lage risico op baarmoederkanker en de goede kans op overleving, dient men terughoudend te zijn ten aanzien van risico-reducerende gynaecologische operaties bij vrouwelijke *PMS2* mutatiedragers. Het verrichten van een tweejaarlijkse transvaginale echo van de baarmoeder, eventueel met het nemen van biopten uit de baarmoeder is een optie die aan deze vrouwen kan worden aangeboden. Niettemin dient dan besproken te worden dat er vooralsnog geen sluitend bewijs is voor een betere overleving na vroege opsporing van baarmoederkanker.

Door de directe klinische toepasbaarheid van de studie-uitkomsten op het screeningsbeleid in PMS2-geassocieerd Lynch syndroom is hetgeen in dit proefschrift is beschreven een goed voorbeeld van translationeel onderzoek dat direct toepasbaar is in de patiëntenzorg.

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Ten Broeke SW
KankerBreed 2014

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CURRICULUM VITAE

Sanne ten Broeke was born in Deventer, the Netherlands on September 15th 1989. In 2007 she graduated high school from Etty Hillesum Lyceum in Deventer. Sanne started medical school in September 2007 at Leiden University Medical Center and graduated in 2013. She conducted her research internship in 2011 with Dr. Maartje Nielsen and Dr. Frederik Hes. During this time at the clinical genetics department she worked on her first cancer risk study in PMS2 families, which was later published in Journal of Clinical Oncology. In 2013 she continued her research as a part-time PhD student, while working as a medical intern at the clinical genetics department in Leiden in 2013 as part of her medical degree. After graduating she continued working full-time as a PhD student under Dr. Maartje Nielsen, Prof. Christi van Asperen and Prof. Hans Morreau. During her PhD, in 2015, Sanne spend 3 months working with Prof. Mark Jenkins and Dr. Aung Ko Win at the University of Melbourne, School of Population and Global Health. She revisited Melbourne in 2017 to work on the second cancer risk study which has also been published in Journal of Clinical Oncology. Sanne participated in many international conferences where she gave multiple oral presentations on her research. In 2018 she was awarded best talk at the Joint Meeting of the Dutch and UK clinical genetics. While finishing her PhD thesis, Sanne started her training as a clinical geneticist in Leiden in April 2017. She has recently transferred to the University Medical Center Groningen where she will continue her training. In the future Sanne hopes to combine her clinical work with her research on PMS2-associated Lynch syndrome.