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Germline variants in the mismatch repair genes: Detection and phenotype

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GERMLINE VARIANTS IN THE MISMATCH REPAIR GENES

Detection and phenotype

Manon Suerink

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Germline variants in the mismatch repair genes: detection and phenotype

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Table of contents

Chapter 1 General introduction 9

Part I – Detection 25

Chapter 2 Constitutional mismatch repair deficiency in a healthy child: On the spot diagnosis? 27
Clinical Genetics. January 2018

Chapter 3 Constitutional mismatch repair deficiency as a differential diagnosis of neurofibromatosis type 1: consensus guidelines for testing a child without malignancy 39
Journal of Medical Genetics. February 2019

Chapter 4 Prevalence of mismatch repair deficiency and Lynch syndrome in a cohort of unselected small bowel adenocarcinomas 65
Journal of Clinical Pathology. Online ahead of print in 2020

Chapter 5 Well documented high-grade serous ovarian cancers should not be tested for mismatch repair deficiency 91
Manuscript in preparation

Part II – Phenotype 107

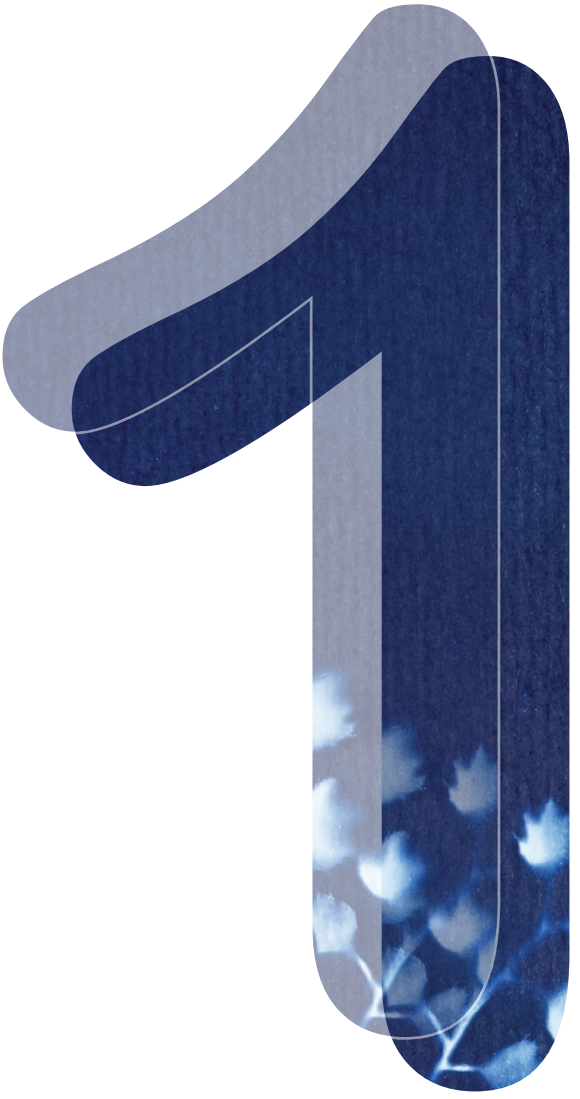
Chapter 6 An alternative approach to establishing unbiased colorectal cancer risk estimation in Lynch syndrome 109
Genetics in Medicine. December 2019

Chapter 7 The effect of genotypes and parent of origin on cancer risk and age of cancer development in PMS2 mutation carriers 139
Genetics in Medicine. April 2016

Chapter 8 Incidence of (adenomatous) polyps and colorectal cancer in patients with PMS2-associated Lynch syndrome undergoing surveillance: a prospective cohort analysis 155
Manuscript in preparation

Chapter 9 General discussion 179

Chapter 10 Dutch summary 200
List of publications 206
Acknowledgments 212
Curriculum vitae 214



Introduction

With 9.6 million estimated deaths in 2018, cancer is the second leading cause of death worldwide[1] and, in the Netherlands, the lifetime risk of developing at least one malignancy is about 1 in 3.[2,3] The most common types of cancer worldwide are lung cancer, breast cancer and colorectal cancer.[1] In some families, clustering of specific cancer subtypes suggests there are factors that increase cancer risk to a level well above population risk. Long before the underlying genes were discovered, it was suggested that a genetic predisposition to the development of cancer may explain the phenotype in at least a proportion of these families.[4-6] One of the most famous examples is Family G, a family that was described for the first time in 1913 by Aldred Scott Warthin, with a clustering of uterine and stomach cancers.[6] This large family intrigued medical professionals and has been described multiple times in the course of history. One of these professionals was Henry T. Lynch, who studied family G in detail and published several families with a similar history.[6] Over the past few decades the genetic basis for many of these syndromes, including the genetic cause in family G, has been unravelled; they are caused by germline pathogenic variants in genes that are important in the maintenance of genomic stability.[6-8]

We now know that the cancer predisposition syndrome responsible for the high cancer risk in family G is Lynch syndrome; an autosomal, dominantly inherited condition caused either by a germline pathogenic variant in one of four mismatch repair (MMR) genes (*MLH1, MSH2, MSH6* or *PMS2*)[9] or, more rarely, by a germline deletion of the ’3 region of the *EPCAM* gene which silences the *MSH2* gene by hypermethylation.[10] In the case of family G, a germline variant in the *MSH2* gene was identified.[11] The MMR system plays a vital role in replication error correction in order to prevent mutations from accumulating. Replication error correction is carried out by MutS and MutL complexes that respectively recognize mismatches and activate downstream activities to initiate repair (Figure 1). MutS exists in two forms: as MSH2 coupled either with MSH6 to form MutSα or with MSH3 to form MutSβ. MutL exists as MutLα (MLH1•PMS2), MutLβ (MLH1•PMS1) and MutLγ (MLH1•MLH3).[12] While variants in *MLH1, MSH2, MSH6* and *PMS2* have been recognized to cause the dominantly inherited Lynch syndrome, variants in *MLH3* and *MSH3* have only been described in recessively inherited cancer syndromes.[13,14] The role of *PMS1* variants as a cause of cancer predisposition seems limited.[15] Homozygous and compound heterozygous pathogenic variants in *MSH2, MSH6, MLH1* and *PMS2* have also been described and result in a rare cancer predisposition syndrome called constitutional mismatch repair deficiency (CMMRD).[16,17]

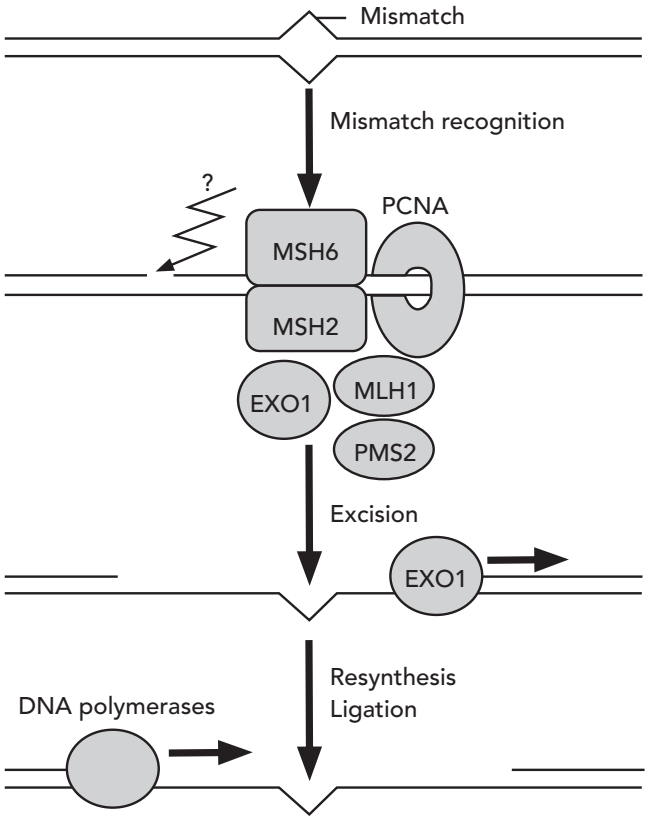


Figure 1 Mismatch recognition and repair by the mismatch repair genes. Reprinted with permission of Springer Nature.[18]

Lynch syndrome

Patients with Lynch syndrome mainly have an increased risk of developing colorectal and endometrial cancer during adulthood, but increased risks of developing cancer of the ovaries, small bowel, stomach, breast, hepatobiliary tract, prostate and urinary tract have also been reported.[19,20]

Previously, clinical criteria (such as the Amsterdam criteria and the (revised) Bethesda guidelines)[21,22] were used to preselect patients before genetic testing was performed. These criteria aimed at preselecting families with a higher a priori chance of a genetic predisposition by incorporating factors such as age at cancer diagnosis, type of cancer and positive family history.[21,23] Over time it became clear that many families do not

meet these criteria despite presence of a pathogenic variant.[24] Therefore, universal screening (sometimes also referred to as reflex testing) for MMR deficiency in colorectal cancer and endometrial cancer is becoming general practice in many countries in order to identify more variant carriers that could benefit from surveillance.[24-26] In the Netherlands all colorectal and endometrial cancers detected before the age of 70 are screened by immunohistochemical staining of the mismatch proteins and/or microsatellite instability analysis for the presence of MMR deficiency. If MLH1 deficiency is detected, *MLH1*-promotor hypermethylation analysis is first performed to rule out this epigenetic event as the somatic, sporadic cause. The presence of *MLH1*-promotor hypermethylation makes an hereditary cause of MMR deficiency highly unlikely, although germline cases have been described.[27-31] In absence of *MLH1*-promotor hypermethylation or when lack of MSH2, MSH6 or PMS2 protein expression is observed in the tumour, patients are referred to a clinical geneticist to further discuss genetic testing. Subsequent genetic testing will then have to determine whether the MMR deficiency is caused by a germline variant or by two somatic hits.

Although this approach facilitates detection of Lynch syndrome in families that do not meet clinical criteria such as the revised Bethesda criteria, it is likely that still many carriers remain unidentified. Recent estimates of carrier frequencies in the general population for pathogenic variants in the MMR genes are 1 in 1,946 for *MLH1*, 1 in 2,841 for *MSH2*, 1 in 758 for *MSH6* and 1 in 714 for *PMS2*, adding up to a total carrier frequency of 1 in 279.[32] This would mean that in a population of 17 million, such as the Netherlands, there should be almost 61.000 carriers. Identification of pathogenic variant carriers is crucial, since colonoscopic surveillance has been proven to be an effective, risk-reducing measure.[33] Of note, estimations of carrier frequencies are largely based on Western populations, in populations with large subpopulations of non-Western immigrants the carrier frequencies may differ.

Currently surveillance is offered in the same manner for all four genes with colonoscopic surveillance starting from age 20-25 years with an interval of 1-2 years,[23] but a plea for gene-specific guidelines is ongoing and will likely be implemented in the near future.[34-36] This was triggered by recent insights that the height of colorectal cancer risk varies depending on the mutated gene. Risks are highest for carriers of a pathogenic *MLH1* or *MSH2* variant with estimations of the colorectal cancer risk up to age 70 varying between 52% and 97%,[19] while these risks estimates are lower for *MSH6* (22-69%)[19] and lowest for *PMS2* (11-20%).[36-38] Prospective data further illustrate the difference in penetrance between the MMR genes: the risk of developing colorectal

cancer whilst being under surveillance, is still substantial (up to 57%) for *MLH1* and *MSH2* pathogenic variant carriers, while it is much lower (20%) for *MSH6* and seems to be very low (0-10.4%) for *PMS2*.[35,39]

The challenge with establishing correct cancer risks for any cancer predisposition syndrome, and Lynch syndrome is no exception, is that retrospective analyses are complicated by the fact that available patient cohorts have been heavily selected on family history and analyses require statistical methods to correct for this ascertainment bias.[40] Nonetheless, statistical methods come with limitations as well. This is nicely illustrated by a study in hereditary breast cancer, showing that much of the variation seen in breast cancer risk estimates can be explained by the use of different bias correction methods.[41] Large initiatives, such as the Prospective Lynch Syndrome Database (PLSD), have therefore been developed to gather prospective data on Lynch syndrome families.[39] Then again, these risk estimations are tricky to use in guideline development; they underestimate true colorectal cancer risk since study participants are undergoing surveillance and are therefore less likely to develop cancer. Further confirmation of previously reported (retrospective) risk estimates is therefore needed.

Gene specific risk stratification is one step in the right direction towards tailored surveillance guidelines, but even then room for improvement remains: large differences in penetrance have been observed between families and individuals with variants in the same gene. Statistical modelling indicates that there is large heterogeneity in cancer risk between *MLH1* and *MSH2* variant carriers with a large proportion (around a quarter) of carriers with a relatively low (0-10%) risk of developing colorectal cancer before the age of 70 and a smaller proportion (10-20%) at extremely high risk (90-100%) (Figure 2).[42] Many mechanisms have been suggested to explain these differences, including lifestyle factors,[43-45] risk modifying SNPs[42,46-48] and genotype-phenotype correlations[42,49-51], but none of these factors have been implemented in clinical practice yet. Further risk stratification would be desirable to reduce the burden of frequent colonoscopies for those with a low risk, while those with a higher risk are adequately targeted. Although there are no similar studies yet to provide evidence for a similar risk distribution in *PMS2* and *MSH6* families, clinical observations suggest similar risk distributions within these families, possibly with an even greater proportion of family members that fall in the lower-risk categories.

CMMRD

In CMMRD, the cancer spectrum is much broader and penetrance is much higher than in Lynch syndrome; cancer penetrance is virtually complete and patients often already present with cancer at very young ages (childhood or adolescence).[17] Apart from a high risk of Lynch syndrome associated cancers at a young age, other cancers risks that are strongly increased in these patients include those for tumours of the central nervous system and haematological malignancies.[17] A non-malignant clinical sign of CMMRD is the presence of café-au-lait macules (CALMs), which is why children with CMMRD are sometimes first suspected of neurofibromatosis type 1 before receiving the correct diagnosis.[52] Diagnostic criteria exist to identify CMMRD in those patients who have already developed cancer [17] and guidelines for surveillance of patients with CMMRD have been published.[53-55] Although more research is needed to definitively prove the efficacy of these surveillance guidelines, preliminary reports in a small series of patients show promising results.[56] Furthermore, the use of aspirin and neo-

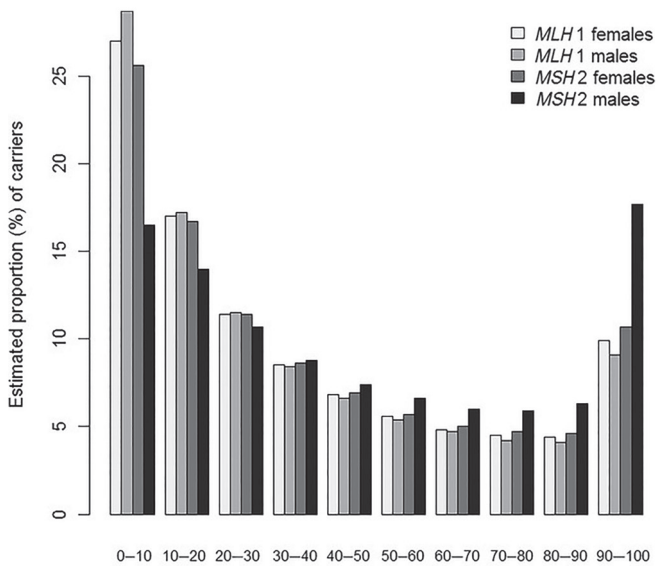


Figure 2 Cumulative cancer risks shows large variation between individuals with a germline pathogenic variant in MLH1 and MSH2.[42] Reprinted with permission of John Wiley and Sons.

antigen based vaccinations have been suggested as potential preventive measures in CMMRD, while treatment with immune checkpoint inhibitors can be effective once cancer has developed.[57,58] Another benefit of an early CMMRD diagnosis is the opportunity to counsel parents on the recurrence risk of 25% for future pregnancies; prenatal diagnostics and preimplantation genetic diagnostics are options that can be offered to parents who wish to have more children, but who want to prevent CMMRD from occurring in future offspring.

Despite its rarity, it may therefore be worthwhile to attempt at diagnosing CMMRD before the development of cancer. Although knowledge and recognition of the syndrome have increased over the years, it is likely that many patients are not diagnosed, particularly if they are a single case within a family and/or if they do not survive the first cancer (and therefore do not develop a second cancer that could raise suspicion of a cancer predisposition syndrome).

Challenges

Due to improved and early detection and removal of adenomas, the incidence of colorectal cancer is expected to decline with the recent introduction of population based screening for faecal blood.[59-62] While this is expected to have a positive effect on colorectal cancer morbidity and mortality,[61] this will mean there are less opportunities to identify Lynch syndrome patients through immunohistochemical staining of colorectal cancers. Immunohistochemical staining for the presence of the MMR proteins in adenomas is not as sensitive as staining of colorectal cancers as it has been shown that not all adenomas in Lynch syndrome patients show MMR deficiency.[63-65] Furthermore, immunohistochemical staining of large cohorts of adenomas resulted in very low MMR deficiency detection rates (0.3-0.4%).[66,67] To compensate for this decline in opportunities to identify carriers of a pathogenic MMR variant, other approaches can be explored. One approach could be the universal screening of cancers, other than colorectal and endometrial cancer, with a relatively high prevalence of Lynch syndrome associated MMR deficiency. One promising candidate for this approach would be small bowel cancer. While small bowel cancers are a relatively rare type of tumour, the prevalence of MMR deficiency in small bowel adenocarcinomas has been reported to be up to 35%, indicating that these tumours may be suitable candidates to perform universal MMR deficiency screening.[68,69] However, these estimations have been based on relatively small cohorts and show a wide range (5-35%),[69] which is why more research is needed to establish a more precise estimation. Furthermore, little is known about the prevalence of Lynch syndrome in these MMR deficient cases.

A second type of tumour with a relatively high penetrance in Lynch syndrome is ovarian cancer. While the association between Lynch syndrome and ovarian cancer has been well established, some discussion remains on the histology of MMR deficient ovarian cancers.[70,71] It has been suggested that standard (i.e. universal) screening for MMR deficiency in ovarian cancer should be limited to specific histological subtypes (*i.e.* endometrioid and clear-cell).[72] Arguing against this is a large meta-analysis which showed that, although less common than in endometrioid and clear-cell tumours, MMR deficiency is still present in 7.9% of high-grade serous ovarian cancers. In addition, 16.7-25% of ovarian cancers identified in Lynch syndrome patients are of high-grade serous histology.[73-75] Based on these numbers, a diagnosis of Lynch syndrome should still be considered when a patient seeks clinical genetic advice. Further research is required to help clinicians determine whether Lynch syndrome should be considered as a differential diagnosis in patients with high-grade serous ovarian cancer.

AIMS AND OUTLINE OF THIS THESIS

The aim of this thesis is 1) to provide insights that may help in the identification of patients with Lynch syndrome and CMMRD, and 2) to further elucidate the phenotype and potential modifying factors that result from carrying a germline pathogenic variant in one of the MMR genes. Both aims are important to further facilitate adequate detection and surveillance of individuals with a germline pathogenic variant in one of the MMR genes.

Part I The detection of patients with Lynch syndrome and CMMRD

In **chapter 2** the first case in literature is described where a diagnosis of CMMRD was made in a healthy child that presented with a neurofibromatosis-type-1-like phenotype. This case description initiated a discussion that resulted in a literature study and guidelines as described in **chapter 3** that indicate when clinicians should be testing for CMMRD in children with CALMs but without an *NF1* mutation. In **chapter 4** the frequency of MMR deficiency and Lynch syndrome in a large cohort of small bowel cancers is described and the implications of these findings for universal testing of MMR deficiency in these tumours are discussed. In **chapter 5**, a case series of serous ovarian cancers that were tested for MMR deficiency is presented and, combined with an overview of recent literature, it is discussed how these results impact on testing for Lynch syndrome in this group of cancer patients.

Part II Cancer penetrance in Lynch syndrome and potential factors of influence

In **chapter 6** a novel approach to estimating cancer risk in *PMS2*- and *MSH6*-associated Lynch syndrome is described. By analysing a large cohort of families where the index patient was diagnosed with CMMRD, the issue of ascertainment bias due to a positive family history is circumvented. In **chapter 7** the influence of genotype and parent-of-origin on the phenotype of *PMS2*-associated Lynch syndrome is analysed. In **chapter 8** the number of polyps and interval cancers in *PMS2* variant carriers is investigated and the implications of our findings in light of the relatively low cancer risks that have been reported for *PMS2* are discussed. In **chapter 9** the main findings of the previous chapters in relation to the most recent literature are discussed and suggestions are made on how to move forward with scientific research in the field of Lynch syndrome and CMMRD.

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Part I

Detection

Constitutional mismatch repair deficiency in a healthy child: On the spot diagnosis?

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ABSTRACT

Constitutional mismatch repair deficiency (CMMRD) is a rare, recessively inherited childhood cancer predisposition syndrome caused by biallelic germline mutations in one of the mismatch repair genes. The CMMRD phenotype overlaps with that of neurofibromatosis type 1 (NF1), since many patients have multiple café-au-lait macules (CALM) and other NF1 signs, but no germline *NF1* mutations. We report of a case of a healthy 6-year-old girl who fulfilled the diagnostic criteria of NF1 with >6 CALM and freckling. Since molecular genetic testing was unable to confirm the diagnosis of NF1 or Legius syndrome and the patient was a child of consanguineous parents, we suspected CMMRD and found a homozygous *PMS2* mutation that impairs MMR function. Current guidelines advise testing for CMMRD only in cancer patients. However, this case illustrates that including CMMRD in the differential diagnosis in suspected sporadic NF1 without causative *NF1* or *SPRED1* mutations may facilitate identification of CMMRD prior to cancer development. We discuss the advantages and potential risks of this CMMRD testing scenario.

INTRODUCTION

Constitutional mismatch repair deficiency (CMMRD; MIM #276300) is a recessively inherited cancer predisposition syndrome caused by homozygous or compound heterozygous mutations in one of the mismatch repair (MMR) genes: *MLH1* (MIM *120436), *MSH2* (MIM *609309), *MSH6* (MIM *600678) and *PMS2* (MIM *600259). In a heterozygous state, MMR mutations lead to Lynch syndrome (LS; MIM #609310, #120435, #614350, #614337), causing a predisposition to develop mainly colorectal and endometrial cancer with an adult age at onset.[1] CMMRD has a more severe phenotype, with an extraordinarily high risk of developing a broad spectrum of different malignancies in childhood or adolescence,[2,3] warranting rigorous surveillance measures.[4-6]

Phenotypically, CMMRD overlaps with neurofibromatosis type 1 (NF1; MIM #162200) and Legius syndrome (MIM #611431). Six or more café-au-lait macules (CALMs) and skinfold freckling, which are included in the NIH diagnostic criteria for NF1 (Table 1),[7,8] are usually the first presenting sign in a child with NF1.[9] At least 91/146 CMMRD patients were reported to have CALMs or hyperpigmented skin areas[3,10] and signs reminiscent of NF1 are highly suggestive of CMMRD when present in a child with a non-NF1-associated malignancy. Therefore, NF1 signs, as well as other non-neoplastic features such as consanguinity of the parents, are included as criteria in a scoring system developed to raise the clinical suspicion of CMMRD among cancer patients.[2]

Table 1. Adapted NIH diagnostic criteria for NF1[a]

Clinical diagnosis based on presence of 2 of the following:
1. Six or more café-au-lait macules, over 5 mm in diameter, in prepubertal individuals and over 15 mm in greatest diameter in postpubertal individuals.
2. Two or more neurofibromas of any type or one plexiform neurofibroma.
3. Freckling in the axillary or inguinal regions.
4. Two or more Lisch nodules (iris hamartomas).
5. Optic glioma.
6. A distinctive osseous lesion such as sphenoid dysplasia or thinning of long bone cortex, with or without pseudarthrosis.
7. A parent or offspring with NF1 by above criteria.[a]

[a] Changed according to the suggestions of Huson.[8] Original diagnostic criteria stated "A first-degree relative (parent, sibling, or offspring) with NF1 by above criteria."[7]

Due to phenotypic overlap, several CMMRD patients have been misdiagnosed with NF1 prior to development of their first malignancy. Earlier diagnosis of CMMRD in these patients might have led to prevention or diagnosis at an earlier stage of the malignancy. However, no guidance is currently available on when to consider CMMRD as a differential diagnosis in a (healthy) child referred for genetic testing due to ≥6 CALMs and/or other signs of NF1 but negative for *NF1* or *SPRED1* mutations. Here we report of a girl, fulfilling the NF1 criteria, without a history of (pre)malignancies. Since she is the offspring of a consanguineous marriage, CMMRD was suspected after *NF1* and *SPRED1* testing rendered negative results. This diagnosis was confirmed by identifying a homozygous *PMS2* mutation.

CASE

A 3-year-old girl, the child of first cousins, was referred by her pediatrician for genetic evaluation. With more than 6 CALMs (size between 1.5 and 2.5 cm) and freckling under the left axilla, she fulfilled the clinical criteria for NF1 (Figure 1). Prior to her referral to our department, analysis of *NF1* and *SPRED1* was performed by Sanger sequencing from genomic DNA and multiplex ligation dependent probe amplification (MLPA), but

Figure 1 Axillary freckling and a café-au-lait macule in the child

no mutations were found. To further rule out any gross chromosomal rearrangements involving the *NF1* locus on chromosome 17 we performed karyotyping. Both parents were referred to a dermatologist and ophthalmologist, but neither showed clinical signs of neurofibromatosis.

Two years later, when the child returned for re-evaluation, we decided to offer testing for CMMRD despite the lack of a personal history of cancer and a 4-generation family history negative for malignancies (Figure 2). Since *PMS2* is the most commonly mutated gene in CMMRD,[3] it was analyzed first and a homozygous mutation (c.2444C>T, p.Ser815Leu) was detected. Both parents proved to be heterozygous for the mutation. This mutation, reported to the Leiden Open Variation Database (http://PMS2.lovd.nl), was previously identified in 3 suspected LS patients with *PMS2*-expression loss in their tumor tissues. It is predicted to be deleterious by aGVGD and SIFT and an in vitro MMR-assay clearly showed loss of MMR-capacity.[11] Hence, it was accepted as the disease-causing mutation in these 3 LS patients, although it should be noted that one of the patients carried an additional variant of unknown significance (VUS) in *PMS2*.[11] To further substantiate that this mutation causes CMMRD when present in a homozygous state, we performed germline MSI (gMSI) analysis in our patient's leucocyte DNA.[12] All analyzed markers showed increased gMSI ratios when compared

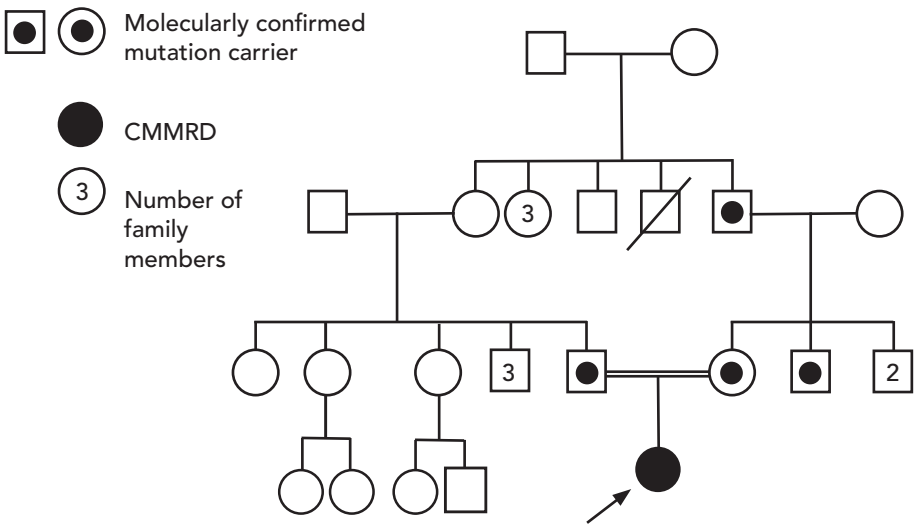

Figure 2 Pedigree of the family

to laboratory-specific thresholds (mean +3 standard deviations of 80-90 control DNAs) supporting the CMMRD diagnosis.

Following diagnosis, our patient was offered screening in accordance with the recommendations of the C4CMMRD consortium.[6] By this time, aged 6, she has undergone brain MRI, ultrasound of the abdomen and a blood count, all without identified abnormalities. Immunology results showed an isolated IgG4 deficiency (<0.01 g/L). IgA, IgG2 and IgG4 deficiency has previously been described in CMMRD patients and is a diagnostic criterion in the C4CMMRD scoring system.[3,13] However, since isolated IgG4 deficiency is found in up to 15% of healthy children,[14] this finding in our patient may be unrelated to CMMRD.

In accordance with the LS surveillance protocol, both parents underwent colonoscopies but no abnormalities were found.

DISCUSSION

This is the first report of CMMRD diagnosis in a child with no personal or family history of malignancies but fulfilling the diagnostic criteria for NF1. This case illustrates that CMMRD syndrome should be included in the differential diagnosis of children suspect for NF1, but without *NF1* or *SPRED1* mutations.

Given that there were no precedents for this particular case, the decision to offer testing for CMMRD was taken after intensive discussion within our team of the benefits and potential problems in the context of pre-symptomatic (with respect to tumor development) testing for CMMRD. A strong motivation to perform testing was the opportunity to offer surveillance. This includes brain MRI (warranting anesthesia in infants) and colonoscopy, and therefore represents a substantial burden to the patient.[4,6] Furthermore, the effectiveness of surveillance has only been evaluated in a small number of CMMRD patients.[4] Given that our current estimates of CMMRD cancer risks may be subject to ascertainment bias, and attenuated forms of CMMRD have been reported,[15] the justification for the proposed surveillance protocols in a case without a personal and family history of cancer can be legitimately questioned. However, even in light of these reservations, we would argue that it is prudent to assume that the cancer risk in CMMRD is very substantial and therefore justifies subjecting the patient to an extensive program of surveillance.

Family planning was another issue that was taken into account when we considered pre-symptomatic testing, since the parents of our patient plan to have more children in the future. Early CMMRD diagnosis enables timely counseling of the parents regarding

the 25% recurrence risk for siblings, thus giving the parents the opportunity to consider prenatal or pre-implantation genetic diagnostics.

A possible outcome of mutation analysis in any gene is the identification of a VUS. Typically in such cases, clinical management would take into account personal or family history of cancer. Due to the absence of a cancer history, predictive testing for CMMRD by mutation analysis can be seen as a special case. In particular, the identification of a homozygous VUS or a heterozygous VUS together with a clearly pathogenic MMR mutation will cause uncertainty regarding the correct diagnosis and, consequently, poses a serious problem in the appropriate management of the patient. *PMS2* variant p.Ser815Leu is still classified as a VUS class 3 under the Insight variant classification system (http://www.insight-database.org/ classifications/index.html). Only the recent functional testing of this variant allowed us to classify it as pathogenic.[11] Parents should be made aware of the possibility of an uncertain outcome before initiating CMMRD diagnostics and the diagnostic lab should be prepared to undertake any measure necessary to definitely confirm or exclude a diagnosis of CMMRD in this situation. To reduce the risk of this problem arising, one option would be to offer MMR mutation analysis only when pre-screening with immunohistochemical staining of skin biopsies (for the presence of the 4 MMR-proteins) and/or gMSI testing (known to be insensitive in biallelic MSH6 mutation carriers) provide substantial support for a diagnosis of CMMRD.[5,12,16]

The diagnosis of CMMRD in a child also entails diagnosing parents and other family members with LS and thus having an increased risk of developing a tumor within the LS spectrum. Extensive investigation of LS surveillance has shown that it is effective.[17] However, absence of a family history of cancer has frequently been observed in CMMRD patients[3] and especially heterozygous *PMS2* mutations may confer a lower cancer risk than mutations identified in classical LS families.[18] LS surveillance protocols might therefore be adapted once more evidence has been gathered on cancer risks for these family members. For the time being, our patient's family members will be offered surveillance according to national guidelines (http://www. oncoline.nl/ erfelijke-darmkanker), which recommend colonoscopy every 2 years from the age of 25, gynecologic surveillance from the age of 40 and, if necessary, eradication of Helicobacter pylori infection.

No recommendations are currently available that offer guidance on when to consider CMMRD testing in children with CALMs but lacking *NF1* or *SPRED1* mutations. In around 15% to 20% of sporadic patients meeting NF1 criteria no pathogenic *NF1* or *SPRED1* mutation is identified.[9,19] Hence, CMMRD may be considered in a considerable number of children, even though CMMRD is rarely diagnosed. The estimated carrier

frequencies for mutations in the MMR-genes (1 in 1946 for *MLH1*, 1 in 2841 for *MSH2*, 1 in 758 for *MSH6* and 1 in 714 for *PMS2*)[20] imply that CMMRD incidence should be about 1 per million. True incidence is probably somewhat higher, particularly among children with consanguineous parents.[3,21]

The low incidence of CMMRD combined with the severity of the disease means that a delicate balance must be struck when considering pre-symptomatic testing. In our department we now consider pre-symptomatic testing if there are, in addition to CALMs, other indicators of CMMRD such as consanguinity or a positive family history of cancer. Other features included in the criteria that may raise the suspicion of CMMRD in a cancer patient,[3] for example multiple pilomatricomas, may also be taken into consideration as indicators. With this case report we wish to highlight the need for national and international discussion and consensus on this question.

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2

Constitutional mismatch repair deficiency as a differential diagnosis of neurofibromatosis type 1: consensus guidelines for testing a child without malignancy

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ABSTRACT

Constitutional mismatch repair deficiency (CMMRD) is a rare childhood cancer predisposition syndrome caused by biallelic germline mutations in one of four mismatch- repair genes. Besides very high tumour risks, CMMRD phenotypes are often characterised by the presence of signs reminiscent of neurofibromatosis type 1 (NF1). Because NF1 signs may be present prior to tumour onset, CMMRD is a legitimate differential diagnosis in an otherwise healthy child suspected to have NF1/Legius syndrome without a detectable underlying *NF1*/*SPRED1* germline mutation. However, no guidelines indicate when to counsel and test for CMMRD in this setting. Assuming that CMMRD is rare in these patients and that expected benefits of identifying CMMRD prior to tumour onset should outweigh potential harms associated with CMMRD counselling and testing in this setting, we aimed at elaborating a strategy to preselect, among children suspected to have NF1/Legius syndrome without a causative *NF1*/*SPRED1* mutation and no overt malignancy, those children who have a higher probability of having CMMRD. At an interdisciplinary workshop, we discussed estimations of the frequency of CMMRD as a differential diagnosis of NF1 and potential benefits and harms of CMMRD counselling and testing in a healthy child with no malignancy. Preselection criteria and strategies for counselling and testing were developed and reviewed in two rounds of critical revisions. existing diagnostic CMMRD criteria were adapted to serve as a guideline as to when to consider CMMRD as differential diagnosis of NF1/Legius syndrome. in addition, counselling and testing strategies are suggested to minimise potential harms.

INTRODUCTION

Constitutional mismatch repair deficiency (CMMRD, MIM #276300) is a rare, autosomal-recessively inherited cancer predisposition syndrome caused by biallelic germline mutations in one of four mismatch repair (MMR) genes (*MLH1*, MIM *120436; *MSH2*, MIM *609309; *MSH6*, MIM *600678; *PMS2*, MIM *600259). CMMRD was first described in 1999 in children of consanguineous parents in Lynch syndrome families.[1,2] These children, carrying homozygous *MLH1* mutations, developed early onset tumours and presented with a phenotype reminiscent of neurofibromatosis type 1 (NF1) mainly in the form of multiple café-au-lait macules (CALMs). Since these first reports, well over 200 cancer patients with CMMRD have been described. Through these reports and establishment of initiatives, such as the European consortium 'Care for CMMRD' (C4CMMRD), the international biallelic mismatch repair deficiency (BMMRD) consortium and the European Reference Network for rare genetic tumour risk syndromes (ERN-GENTURIS), awareness of CMMRD and our understanding of the phenotype, the pathophysiological mechanisms of tumour development and potential management options have increased substantially.[3-8]

Individuals with CMMRD are prone to develop a broad spectrum of tumours. The most common are T-cell non-Hodgkin's lymphomas, high-grade gliomas and colorectal cancers or (advanced)colorectal adenomas, and a number of other malignancies are associated with CMMRD.[8-12] Although ascertainment bias cannot be excluded, cancer risks appear to be extremely high, as almost all reported patients are diagnosed with a malignancy and approximately 80% of patients develop their first malignancy before the age of 18 years (median age of onset 10 years).[8-10,13-16] However, attenuated forms of CMMRD with a higher age of tumour onset have also been reported, which are presumably caused by hypomorphic mutations (with reduced penetrance) in at least one allele.[17-19]

Already from the first reports, it became clear that the CMMRD phenotype overlaps with that of NF1 and prior to the onset of CMMRD-associated malignancies, it may be indistinguishable from this condition. Multiple (>5) CALMs (>0.5 cm in diameter) are usually the first diagnostic sign of NF1.[20] In NF1, CALMs generally already appear in the first year of life, followed by skinfold freckling which is present in most children by school age. Neurofibromas usually develop after puberty and in early adulthood.[20] In the past, the majority of NF1 diagnoses were based on clinical criteria from the National Institutes of Health (NIH).[21] However, in young children who have a de novo *NF1* mutation (accounting for almost 50% of NF1 index cases), the NIH criteria are often not fulfilled. Therefore, many NF1 clinics and paediatricians aim for early diagnosis in

children through genetic testing, made possible by the improved sensitivity of *NF1* mutation analysis protocols.[22,23]

The most important differential diagnoses of NF1 in children with multiple CALMs are mosaic NF1 and Legius syndrome.[24,25] From the mutation detection rates in familial and sporadic individuals fulfilling NF1 diagnostic criteria (95% vs 85%),[26] it can be deduced that at least 10% of sporadic NF1 cases have mosaic NF1 caused by postzygotic *NF1* mutations that are undetectable in blood lymphocytes. Mosaic NF1 may present as segmental NF1, with NF1 features confined to one part of the body or as a more generalised form that may be indistinguishable from (mild forms) of NF1 due to a germline mutation.[25] Legius syndrome (MIM #611431), characterised by CALMs and freckling but absence of other diagnostic NF1 features, is caused by germline mutations in *SPRED1* (MIM *609291).[24] About 2.4% of sporadic patients with multiple (>5) CALMs with or without freckling, and in whom no *NF1* mutation can be identified, have Legius syndrome.[26] Other potential differential diagnoses of NF1 include Noonan syndrome, Noonan syndrome with multiple lentigines (previously referred to as LEOPARD syndrome), neurofibromatosis type 2 (NF2), Piebald trait and McCune-Albright syndrome.[27] However, the latter syndromes are often accompanied by other clinical features that can help in differentiating between syndromes.

Since patients with CMMRD with >5 CALMs and other NF1 signs have been described, it is unsurprising that patients with CMMRD occasionally receive an initial clinical diagnosis of NF1 before receiving the correct diagnosis.[1,2,28,29] Although not all patients with CMMRD have sufficient CALMs to meet the NF1 diagnostic criterion of >5 CALMs and some reports emphasise that CALMs in patients with CMMRD often differ from the typical uniformly pigmented and smooth-bordered CALMs associated with NF1,[30-33] the majority of patients with CMMRD have some hyperpigmented macules reminiscent of NF1-associated CALMs.[34] Indeed, Durno et al reported CALMs/hyperpigmented macules in 33 of 34 (97%) patients with CMMRD described by the international BMMRD consortium,[10] and CALMs are present in at least 57 of 76 (75%) patients registered in the C4CMMRD consortium database. The number of CALMs (diameter >1 cm) is known for 35 cases in the latter database, and >5 CALMs >1 cm were found in 26 of 35 (75%) patients (at ages ranging from 0.9 to 21 years) suggesting that at least half of all patients with CMMRD fulfil at least one NIH criterion of NF1 (ie, >5 CALMs).

Awareness that CALMs and occasionally other NF1 signs may be present in a child with CMMRD prior to tumour onset leads to the conclusion that CMMRD is a legitimate differential diagnosis in healthy children with CALMs (with or without other clinical signs of NF1/Legius syndrome) when no causative *NF1* or *SPRED1* mutation is identified,

and no signs of NF1 are found in the parents. Although we can reasonably assume that CMMRD is rare in these patients if the parents are unrelated (see the 'Estimated frequency of CMMRD as a differential diagnosis to NF1 section), a child aged 6 years of consanguineous parents with >5 CALMs and no cancer was recently diagnosed with CMMRD.[28] In this situation, a diagnosis of CMMRD may provide an opportunity for cancer surveillance of a highly penetrant childhood cancer syndrome prior to onset of the first malignancy. It will also allow predictive genetic testing and surveillance in relatives at risk for both CMMRD and Lynch syndrome and may impact family planning. However, it is also important to consider the potential harm associated with CMMRD counselling and testing in this setting, and any harm should be outweighed by expected benefits for both the index patient and his/her at-risk relatives. Therefore, physicians and geneticists have begun to discuss if and when to counsel and test for CMMRD in patients suspected to have NF1.[35]

The C4CMMRD consortium, an interdisciplinary team of international experts in the field, has formulated and published diagnostic criteria for the clinical suspicion of CMMRD in patients with cancer,[8] in addition to surveillance guidelines.[7] At the most recent workshop in Brussels (26 September 2017), the issue of when to test children without malignancy for CMMRD was addressed by presentations covering four main topics: (i) estimations of frequency of CMMRD as a differential diagnosis of NF1, (ii) potential benefit and harm of CMMRD counselling and testing in a child with no malignancy, (iii) testing prerequisites and strategies to preselect children with a high probability of having CMMRD and (iv) counselling and testing strategies to minimise potential harm of testing. These topics were then discussed among the participants of the workshop. MS and KW summarised the presentations and discussion points in a manuscript draft taking all relevant literature into consideration and citing it as comprehensively and completely as possible. Subsequently, all participants of the workshop who contributed to the discussion and had expertise covering the fields of clinical (onco-)genetics, molecular diagnostics of NF1, Legius syndrome and/or CMMRD, paediatric oncology, (paediatric) gastroenterology and CMMRD surveillance commented and discussed the recommendations in two rounds of revisions until all coauthors consented to the content of the manuscript and proposed adaptation of existing diagnostic criteria to serve as a guideline as to when to consider CMMRD counselling and testing as differential diagnosis for NF1 in children with no malignancy.

ESTIMATED FREQUENCY OF CMMRD AS A DIFFERENTIAL DIAGNOSIS OF NF1

The frequency of CMMRD in children suspected to have NF1 or Legius syndrome, but without a causative *NF1* or *SPRED1* mutation and no overt malignancy, is currently unknown. Since knowledge of disease frequency would help in weighing the possible benefits and harm associated with counselling and genetic testing, we attempt to roughly estimate the frequency.

The incidence of CMMRD in the general population depends on the carrier frequency of MMR mutations. Taking, in contrast to previous lower estimations, all four genes into account, the most recent empiric estimation, based on a large North American/Australian registry, calculated carrier frequencies of 1 in 1946 for *MLH1*, 1 in 2841 for *MSH2*, 1 in 758 for *MSH6* and 1 in 714 for *PMS2* mutations.[36] Based on these frequencies, CMMRD incidence was calculated to be about 1:1 000 000 children of unrelated parents (figure 1). The incidence will be substantially higher in populations with founder MMR mutations and in children of consanguineous parents.[15,37,38]

NF1 is much more common, with an estimated incidence of around 1:2000-1:3000.[39-41] Almost half of patients with NF1 are de novo cases.[39] To estimate the frequency of patients suspected to have NF1 or Legius syndrome without an *NF1* or *SPRED1* mutation who are actually affected by CMMRD, we took a number of factors into account. In a study using highly sensitive and comprehensive mutation analysis protocols, with mutation detection rates of 96% in patients with familial NF1, *NF1*/*SPRED1* mutations were identified in 56.4% (764/1354; 751 *NF1* and 13 *SPRED1* mutations) of patients suspected to have sporadic NF1 with >5 CALMs.[26] Therefore, based on the incidence of de novo NF1 of 1:6000 newborns and an *NF1*/*SPRED1* mutation detection rate of 56.4% in patients with >5 CALMs with or without other signs of NF1, we assume that there are 129 patients with >5 CALMs and no *NF1*/*SPRED1* mutation in a population of 1 million individuals (figure 1). Combining this estimate with the estimated frequency of CMMRD, and assuming that half of all patients with CMMRD present as suspected to have NF1 prior to cancer development, we obtain a figure of 1 patient with CMMRD among 258 children suspected to have NF1 without an *NF1*/*SPRED1* mutation (ie, ~0.4%) (figure 1). Given this low estimated frequency, a priori chances of diagnosing CMMRD in this group are low.

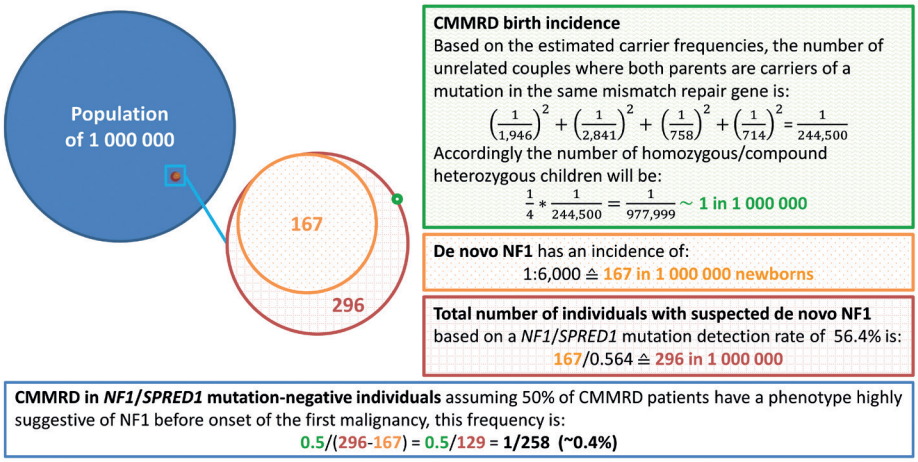

Figure 1 Estimated frequency of CMMRD in children suspected to have sporadic NF1/Legius syndrome but without *NF1/SPRED1* mutations and without malignancy. CMMRD, constitutional mismatch repair deficiency; NF1, neurofibromatosis type 1.

3

POTENTIAL BENEFITS AND HARM OF CMMRD COUNSELLING AND TESTING IN A 'HEALTHY' CHILD

Several factors need to be taken into account when considering CMMRD diagnostics in a child without a (personal history of) malignancy (table 1).

Benefits and their limitations

i. One of the most important benefits of an early CMMRD diagnosis is the possibility to begin surveillance before cancer development and, consequently, potentially detect cancer at an early stage with better treatment options. With regard to colorectal cancer risk, there is even the opportunity to prevent cancer by removal of intestinal polyps prior to malignant transformation, and existing recommendations for CMMRD surveillance provide clinicians with guidance regarding screening programmes.[4,6,7] All available guidelines recommend brain MRI, colonoscopies and video capsule endoscopy (VCE) from a young age, as well as gynaecological and urinary tract analysis from age 10 to 20 years. In addition, whole body MRI5 and preventive measures such as aspirin intake and/or vaccination with neoantigens[42,43] are possible modalities that may have a role in CMMRD management. Preliminary analyses in

a small series of patients showed promising results for surveillance measures.[44] Nevertheless, all recommended programmes are intensive and burdensome and evaluation of the outcome of surveillance protocols in larger studies is yet to be published. Furthermore, when CMMRD is diagnosed in a predictive setting with regard to cancer development, it should be kept in mind that attenuated forms of CMMRD show tumour onset only by the end of the second or in the third decade of life,[17-19,45] and that no evaluated models are available to accurately estimate penetrance of novel MMR mutations or new combinations of mutations. Hence, it is currently unclear whether a less stringent surveillance programme might be sufficient for a subgroup of patients. Despite these reservations, as sufficient evidence points to an overall high cancer risk, the application of intensive, carefully considered screening recommendations to individuals proven to have CMMRD is justified.

ii Another advantage of early diagnosis is the possibility to counsel parents regarding the 25% probability that siblings and subsequent children will also be affected, and to discuss the option of prenatal or preimplantation genetic diagnostics while parents are still in the process of family planning. Once again however, informed decision making is complicated by the fact that current estimates of cancer risk are subject to ascertainment bias and individual cancer risks are difficult to predict.

Potential harms

i Following genetic counselling for CMMRD as a differential diagnosis, parents and children may experience anxiety during genetic testing until the diagnosis is largely excluded. Depending on the diagnostic strategy and performance of the laboratory, this may take several weeks or even months. Moreover, the testing strategy chosen by the laboratory will impact the predictive value of a negative test result (*ie*, the residual risk in the case of a negative test, see the 'Testing strategy' section). This may impact on any remaining anxiety after receiving a negative result. The level of anxiety may also differ depending on the personality and the available coping strategies of the patients/parents and the attitudes of the physicians involved.

ii Test results definitely confirming or refuting CMMRD will be helpful in the management of the patient and his/her family. However, inconclusive test results will pose a challenge for all parties involved. The most important source of inconclusive results will be variants of unknown significance (VUS) in the MMR genes. Although identification of a VUS is an inherent risk of genetic diagnostics, it is important to minimise the number of VUS and the dilemma with regard to diagnosis and appropriate management of the patient that comes along with it. Therefore, laboratories performing CMMRD analysis in a predictive setting should be prepared

to take any measure necessary to reach a less ambiguous classification of a VUS (C3) as either a (likely) pathogenic (C4/C5) mutation or a (likely) benign (C1/C2) variant.[46] Tests assessing hallmarks of MMR deficiency in vivo or the effect of the mutation(s) on mismatch repair protein function in vitro will become important in these situations (see the 'Testing strategy' section).

iii According to Win et al,[36] in the general population one in 279 children tested will be heterozygous for an MMR gene mutation. Particularly in the case of a clearly pathogenic *MLH1* or *MSH2* mutation, this results is the unintentional diagnosis of Lynch syndrome in a minor. Lynch syndrome mainly predisposes to adult-onset colorectal cancer and/or endometrial cancer and surveillance only begins around age 20–25 years.[47,48] Thus, the lack of clinical consequences in children, combined with their right not-to-know, and potential harm due to anxiety and other issues (eg, potential difficulty in acquiring insurance) highlight that a diagnosis of Lynch syndrome is undesirable in a minor.[49] Further considerations on this topic can be found in the study by Bruwer et al, who offered predictive CMMRD testing to children of parents both carrying familial *MLH1* mutations.[50] The situation is more complex for *MSH6* and even more so for *PMS2*. Heterozygous mutations in these genes have a 2–4 times higher prevalence,[36] but a substantially lower penetrance than *MLH1* and *MSH2* mutations.[19,51,52] Hence, in an individual lacking a personal or family history of Lynch syndrome-associated cancer, it is uncertain whether the mutation-associated cancer risk is sufficient to diagnose an individual with a cancer predisposition syndrome that warrants intensive cancer surveillance. This concern also raises the question of whether identifying a mutation in an individual without family history for Lynch syndrome justifies predictive genetic testing in parents and other adult at-risk relatives.

Table 1 Overview of the potential benefits and harms of CMMRD counselling and testing in a child suspected to have sporadic NF1/ Legius syndrome without malignancy and negative outcome of *NF1/SPRED1* germline mutation analysis. CMMRD, constitutional mismatch repair deficiency; VUS, variant of unknown significance.

Potential benefits	Potential harms
• Opportunity to begin surveillance before cancer development. • Parents can be informed of the recurrence risk in a sibling/future child. • Lynch syndrome can be diagnosed in family members and surveillance initiated.	• Risks associated with intensive surveillance while efficacy has not yet been evaluated in a large cohort and attenuated forms of CMMRD exist. • Risk of identifying a VUS, resulting in management dilemmas and potentially inducing anxiety. • Risk of diagnosing Lynch syndrome in a minor.

LIMITING POTENTIAL HARM ASSOCIATED WITH CMMRD COUNSELLING AND TESTING IN A CHILD WITHOUT A MALIGNANCY

Assuming that only a small minority (~0.4%) of all *NF1/SPRED1* mutation-negative children from non-related parents will actually have CMMRD syndrome, it would be desirable to reduce the number of individuals/families with whom the possibility of CMMRD needs to be discussed. Therefore, strategies to preselect children with a high probability of having CMMRD are discussed in the following section.

Testing prerequisites

Three prerequisites for considering testing for CMMRD as a differential diagnosis of NF1/Legius syndrome are defined in box 1: (i) the presence of at least one NF1 diagnostic criterion including multiple hyperpigmented skin patches reminiscent of CALMs. The most prevalent NF1 sign present in a patient with CMMRD is hyperpigmented skin patches reminiscent of NF1-associated CALMs and freckling. Other diagnostic

NF1 features such as neurofibromas, Lisch nodules, tibial pseudarthrosis or optic pathway glioma have so far only been seen in patients with CMMRD who also show CALMs.[1,2,15,53,54] This suggests that CMMRD syndrome is a highly unlikely diagnosis in individuals with only isolated non-pigmentary NF1 features. (ii) *NF1/SPRED1* testing was performed using highly sensitive, comprehensive mutation analysis protocols. The likelihood of identifying CMMRD is of course correlated with the sensitivity of *NF1/SPRED1* mutation analysis performed (further discussed in the 'Testing strategy's section). (iii) The absence of any diagnostic signs of NF1 in either parent. If a parent shows NF1 signs an undetected *NF1/SPRED1* mutation, which might even be present in a mosaic status in the (mildly) affected parent, is probably more likely. NF1 signs might be very subtle in mosaic patients as illustrated by a case of gonosomal mosaicism.[55] In sign of >5 CALMs. However, because this number of CALMs is present in only a very small percentage of individuals in the general population,[56] they might be an indication of familial NF1 or at least familial CALMs when present in a parent of a child with clearly >5 CALMs. Therefore, the physician should use his/her clinical experience to interpret the findings in the parent. It is strongly recommended that both parents undergo a full clinical exam for presence of any (mild) features of NF1, and for this purpose a consultation with an ophthalmologist and dermatologist can be considered. It was decided not to include an age limit in the prerequisites for testing, as in CMMRD a wide variability has been observed in the age of cancer diagnosis.[8,9,17] However, when evaluating a patient who meets the prerequisites it should be kept in mind that the vast majority (around 80%)[9,10,13-16] of patients with CMMRD will have developed a malignancy or intestinal adenomas by the age of 18 years. Hence, absence of a (pre-) malignancy in an older individual decreases the probability of CMMRD substantially.

Preselection strategies

The presence of one or more additional features suggestive of CMMRD substantially increases the likelihood of this differential diagnosis in a child. The European C4CMMRD consortium has previously defined diagnostic criteria based on features that raise suspicion of CMMRD in a patient with cancer.[8] By and large, these features could also be used to select children without cancer who have an increased probability of having CMMRD. Therefore, the list of additional features provided in box 1 largely overlaps with the previously defined diagnostic criteria for CMMRD in a patient with cancer (for further details see Wimmer et al[8]).

A feature listed in the original table in the study by Wimmer et al[8] was 'deficiency/ reduced levels of IgG2/4 and/or IgA'. As a recent study on a cohort of 15 consecutive, unrelated patients was unable to show uniform or specific patterns of laboratory

immunological abnormalities,[57] we did not include this rather unspecific feature in box 1. Two features increasing the likelihood of having CMMRD and not listed in the original table by Wimmer et al7 were added to the current table. The first one is a sibling with diagnostic NF1 signs, in the absence of any diagnostic NF1 signs in both parents when gonadal *NF1/SPRED1* mosaicism in a parent has largely been excluded by mutation analysis in the children. Because not all patients with CMMRD have a sufficient number of CALMs to meet the NF1 diagnostic criterion of >5 CALMs, but at the same time presence of 1–3 CALMs is quite common in the general population (20%–1.2%),[56] we recommend that in this situation >3 CALMs should qualify as an NF1 sign in the sibling. The second new feature is the presence of multiple developmental vascular abnormalities (also known as cerebral venous angiomas) in separate regions of the brain, which were present in 10/10 patients described by Shiran et al,[58] who suggested this feature as additional non-neoplastic sign indicating CMMRD in a patient with cancer.

Furthermore, a number of patients with CMMRD have been reported to have atypical CALMs with irregular borders and different degrees of pigmentation.[30-34] Therefore, atypical macules that might be differentiated from typical NF1-associated macules by an experienced clinician/geneticist (see also the 'Counselling strategy and setting' section), are suggestive of a differential diagnosis such as CMMRD.[30-34] Hence, presence of atypical CALMs is also included as an additional feature in box 1.

Some CMMRD-associated features included in box 1 (eg, brain anomalies) will not be detected by routine clinical examination of a patient suspected to have NF1. Since the prevalence and specificity of these features in patients with CMMRD is not well studied, we do not advocate testing for these features unless clinically indicated.

A thorough family history will help in uncovering family members with Lynch syndrome-associated cancers (box 1). When a Lynch syndrome-associated cancer is present, it may be worthwhile, where possible, to analyse the tumour for signs of mismatch repair deficiency.

A thorough assessment of the family history should include also questions regarding consanguinity of the parents. The risk of having CMMRD based on the allele frequencies of MMR gene mutations36 in for example a child of first cousins is ~1/8849 (using the equation $[p_i f_l)]+[p_i^2(1-f_l)]+[p_j f_l+f_l^2(1-f_l)]+[p_k f_l+p_k^2(1-f_l)]+[p_l f_l+p_l^2(1-f_l)]$, where p_i, p_j, p_k and p_l are the allele frequencies of *MLH1*, *MSH2*, *MSH6* and *PMS2* mutations, respectively, and the consanguinity coefficient fl for first cousins=1/16),[59] which is about 110 times higher than for a child with unrelated parents.

Box 1 Selection strategy for CMMRD counselling and testing in a child suspected to have NF1/Legius syndrome without malignancy and negative outcome of *NF1/SPRED1* germline mutation analysis

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Prerequisites

- Suspicion of NF1 due to the presence of at least one diagnostic NF1 feature[1], including at least two hyperpigmented skin patches reminiscent of CALMs.
- No *NF1* and *SPRED1* germline mutations detected using comprehensive and highly sensitive mutation analysis protocols[2]
- Absence of diagnostic NF1 sign(s) in both parents[#]

Additional features, at least one (either in the family or in the patient) is required

In the family

- Consanguineous parents.
- Genetic diagnosis of Lynch syndrome in one or both of the parental families.
- Sibling with diagnostic NF1 sign(s)[#]
- A (deceased) sibling[3] with any type of childhood malignancy.
- One of the following carcinomas from the Lynch syndrome spectrum[4]: colorectal cancer, endometrial cancer, ovarian cancer, gastric cancer, small bowel cancer, cancer of the bile duct or gall bladder, pancreatic cancer or urothelial cancer before the age of 60 years in first-degree or second-degree relative.

In the patient

- Atypical CALMs (irregular borders and/or pigmentation).
- Hypopigmented skin patches.
- One or more pilomatricoma(s) in the patient.
- Agenesis of the corpus callosum.
- Non-therapy-induced cavernoma.
- Multiple developmental vascular abnormalities (also known as cerebral venous angiomas) in separate regions of the brain.

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[1]Neurofibromatosis conference statement.[21]

[2]See the 'Testing strategy' section.

[#]For further details, please refer to the following sections: 'Testing prerequisites' and 'Preselection strategies'.

[3]This can be expanded to second-degree and third-degree relatives in populations with a high prevalence of founder mutations.

[4]Møller et al.[47]

CALMs, café-au-lait macules; CMMRD, constitutional mismatch repair deficiency; NF1, neurofibromatosis type 1.

Counselling strategy and setting

Since NF1 is a relatively common and often easily recognisable syndrome for which clear management guidelines exist, many paediatricians order molecular analysis of the *NF1* gene directly without involving a clinical genetics specialist. Counselling and management are more challenging for the much rarer and highly penetrant cancer predisposition syndrome CMMRD. We therefore advocate that predictive (with respect to malignancy) CMMRD testing should be ordered by a physician trained in clinical cancer genetics in a centre with specific expertise in NF1 and related disorders in a multidisciplinary setting. As mentioned above, we suggest that CMMRD does not need to be discussed in all suspected NF1 cases without an identified *NF1*/*SPRED1* mutation. Following an interdisciplinary discussion and the decision that counselling for CMMRD is indicated in a child without a malignancy, parents and their affected child, depending on his/her age, should be counselled by an experienced geneticist (or, depending on his/her level of education and experience, a genetic counsellor). To be able to make an informed decision on whether they want their child to be tested, parents should be made aware of the potential benefits, with their limitations, and of the various possible outcomes of genetic testing. Nevertheless, considering the low probability of a CMMRD diagnosis, this information should be provided in a way that minimises risk of inducing a disproportionately high level of anxiety. If parents express the need for psychological support or more information on surveillance protocols or cancer treatment options, consultation with a psycho-oncologist or paediatric oncologist should be offered. Specifically trained clinical geneticists/clinicians may be able to differentiate between typical NF1-associated CALMs and the atypical pigmentations sometimes seen in patients with CMMRD.30–34 Furthermore, he/she can decide whether another syndrome (eg, Noonan syndrome, Noonan syndrome with multiple lentigines, NF2, Piebald trait and McCune-Albright syndrome) within the differential diagnosis of children with CALMs is more likely and should be addressed by genetic testing prior to CMMRD testing. Lastly, we advise that any centre ordering CMMRD diagnostics is able to facilitate the surveillance programme, either in-house or in cooperating centres within reasonable travelling distance.

Testing strategy

A prerequisite for considering CMMRD counselling and testing as a differential diagnosis in patients suspect for NF1/Legius syndrome is the exclusion of the latter diagnoses with high certainty by absence of germline *NF1*/*SPRED1* mutations using highly sensitive mutation analyses. The *NF1* gene is large and has a highly diverse mutational spectrum, with private mutations (ie, not reported in any other patient)

identified in a significant percentage of patients (~25%; LM, personal communication). Furthermore, the NF1 mutation spectrum also includes a large proportion of unusual splice mutations that either completely elude genomic DNA (gDNA)-based mutation analysis protocols (eg, deep intronic mutations are found in 2.5%–3% of all patients with NF1) or defy ready classification as (likely) pathogenic mutations without additional transcript analysis (approximately 20% of patients have a splice mutation NOT affecting the AG/GT dinucleotides, but affect coding nucleotides, nucleotides flanking the exons but further upstream/downstream of the AG/ GT dinucleotides or reside very deep into the introns).[22,60,61] This complicates the classification of novel mutations, especially in the case of silent, missense and intronic variants.[62] Currently, only comprehensive mutation analysis protocols that include NF1 transcript analysis as a primary or complementary assay, such as direct cDNA sequencing,[23] will achieve sufficient sensitivity to exclude a germline mutation with a 96% certainty.[26] Genomic DNA-based mutation analysis methods can achieve high *SPRED1* mutation detection rates (RNA-based mutation analysis performed in >900 patients has not yet identified a *SPRED1* splice mutation that escaped detection in gDNA; LM, unpublished data).

Segmental or mosaic NF1 due to a post-zygotic *NF1* mutation is the most likely differential diagnosis in a child with CALMs, with or without other NF1 signs, and a negative germline *NF1*/*SPRED1* mutation analysis. Confirming mosaic NF1 however requires identification of the same postzygotic mutation in multiple melanocyte or Schwann cell cultures from biopsied CALMs and neurofibromas, respectively.[63] These labour-intensive analyses require specific expertise and therefore are offered only by very few specialised laboratories worldwide. Furthermore, they require invasive procedures. Taken together, this can justify omitting these analyses in children to evaluate mosaic/segmental NF1 prior to CMMRD testing.

In principle, two CMMRD testing strategies can be pursued. The first strategy is direct mutational testing of the MMR genes. The second strategy involves a pre-assay which tests for hallmarks of CMMRD, followed by mutational testing if positive. When opting for direct mutational testing, it should be kept in mind that mutation analysis of *PMS2*, the most commonly mutated gene in CMMRD, is challenging due to the presence of pseudogenes.[64-67] Therefore, appropriate methods should be applied to circumvent potential pitfalls of *PMS2* mutation analysis.[68-74]

An argument in favour of direct mutation analysis using gDNAbased gene panel diagnostics would be that other genes that may mimic the NF1 phenotype (see the 'Introduction' section) can be analysed simultaneously. However, testing a larger number of genes inevitably increases the likelihood of identifying VUS. Therefore, we

advocate a stepwise approach, ruling out other possible differential diagnoses prior to CMMRD testing.

If a VUS is identified in one of the MMR genes, additional analyses should be performed to assist with the interpretation of the variant, such as ex vivo functional assays of the mutated gene[75-81] and/or assays that determine the presence of MMRD in non-neoplastic tissue of the patient. The latter assays could also be used as pre-assays before or in parallel with mutation analysis. This second strategy reduces the risk of VUS identification by providing functional evidence for or against CMMRD, and at the same time increases diagnostic sensitivity by applying two complementary methods.

Microsatellite instability (MSI), defined as a change in the number of mononucleotide or dinucleotide repeats and detectable by alterations in microsatellite fragment length,[82] is a well-established hallmark of somatic MMRD and is frequently assessed in cancer tissues during testing for Lynch syndrome. MSI is not restricted to neoplastic cells in patients with CMMRD and assays have been developed to detect low levels of MSI in leucocyte DNA of these patients.[83] Although highly sensitive and specific in patients with biallelic *PMS2*, *MLH1* and *MSH2* mutations, in patients with biallelic *MSH6* mutations the currently available germline microsatellite instability (gMSI) assays regularly yield normal results.[83] This limitation renders this gMSI assay unsuitable for pre–test selection. However, this simple, fast and inexpensive assay can increase diagnostic sensitivity and accuracy by confirming the pathogenicity of *PMS2*, *MLH1* and *MSH2* VUS.[28] In the near future, more sensitive, simple and reliable gMSI assays may become available, which could potentially be used for pre–test selection. Recently, a highly sensitive and reliable method for the detection of low levels of MSI was developed, with potential applications in the analysis of MSI in non-neoplastic tissue of patients with CMMRD.[84] Another assay, which tests for MSI in EBV-immortalised lymphocytes and in parallel for cell tolerance to methylating agents (another functional consequence of CMMRD), has been specifically developed for CMMRD diagnosis.[85] As this assay is both highly ensitive and specific, it may allow a diagnosis of CMMRD to be definitively confirmed or refuted in cases where mutation analysis and other assays are inconclusive (eg, when only one MMR gene mutation or a homozygous MMR gene VUS has been identified).[85,86] However, the assay is lengthy, labour intensive and requires expertise, making it ill-suited as a pre–test. Immunohistochemistry (IHC) to detect loss of expression of one or more MMR protein(s) in non-neoplastic tissue, such as small skin biopsies, has also been proposed as a diagnostic assay for CMMRD.[10,14] However, as taking a skin biopsy is an invasive procedure that can be unpleasant for a young child, IHC should be avoided as a pre–test. Furthermore, IHC may also be insensitive if antigenic but non-functional mutations are present.[86-88]

Taken together, reliable diagnostics of CMMRD may at times be challenging. Choosing an appropriate testing strategy may depend on the facilities that are most readily available in the centre. Hopefully, more assays will become available that may facilitate simple and reliable selective pretesting for CMMRD.

CONCLUSION

We discussed here the potential benefits and harm (table 1) associated with CMMRD counselling and testing in children suspected to have sporadic NF1/Legius syndrome but without a malignancy and lacking an *NF1* or *SPRED1* germline mutation. After carefully considering all available literature and our own experiences, we arrived at recommendations as to when to counsel and offer CMMRD testing, which are summarised in box 1. We also note that uncertainties exist regarding the incidence of CMMRD and the prevalence of CMMRD-associated features both in the general population and in unselected patients with CMMRD. To evaluate sensitivity and specificity of the proposed selection strategy, it will be important for centres applying these recommendations to systematically record the analysed cases and their outcome. For the evaluation of these prospective data, especially with respect to the sensitivity of the proposed strategy, it will also be important to know the true prevalence of CMMRD among unselected children suspected to have NF1/Legius syndrome, but without a causative *NF1*/*SPRED1* mutation. Large retrospective studies on anonymised samples are needed to answer this question. Clearly, more data are also needed to further support our recommendations, particularly since published CMMRD cases may be biased towards a more severe phenotype. Therefore, we strongly recommend that the clinical course of all patients with CMMRD who are identified before cancer development is meticulously recorded and submitted to a database. In addition, future studies should also evaluate the psychosocial impact of our recommendations to learn more about the perceived benefits and harms of the strategy proposed. Overall, we believe that with the application of the suggested counselling and testing prerequisites an acceptable balance can be achieved between adequate testing of patients at risk of CMMRD, while avoiding exposing an unnecessarily large number of children and families to any harm that might ensue from counselling and genetic testing for CMMRD.

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3

Prevalence of mismatch repair deficiency and Lynch syndrome in a cohort of unselected small bowel adenocarcinomas

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ABSTRACT

Aims

Previous estimates of the prevalence of mismatch repair (MMR) deficiency and Lynch syndrome in small bowel cancer have varied widely. The aim of this study was to establish the prevalence of MMR deficiency and Lynch syndrome in a large group of small bowel adenocarcinomas.

Methods

To this end, a total of 400 small bowel adenocarcinomas (332 resections, 68 biopsies) were collected through PALGA (Dutch Pathology Registry). No preselection criteria, such as family history, were applied, thus avoiding (ascertainment) bias. MMR deficiency status was determined by immunohistochemical staining of MMR proteins, supplemented by *MLH1* promoter hypermethylation analysis and Next Generation Sequencing (NGS) of the MMR genes.

Results

MMR deficiency was observed in 22.3% of resected and 4.4% of biopsied small bowel carcinomas. Prevalence of Lynch syndrome was 6.2% in resections and 0.0% in biopsy samples. Patients with Lynch syndrome-associated small bowel cancer were significantly younger at the time of diagnosis than patients with MMR-proficient and sporadic MMR-deficient cancers (mean age of 54.6 years versus 66.6 years and 68.8 years, respectively, $p<0.000$).

Conclusions

The prevalence of MMR deficiency and Lynch syndrome in resected small bowel adenocarcinomas is at least comparable to prevalence in colorectal cancers, a finding relevant both for treatment (immunotherapy) and family management. We recommend that all small bowel adenocarcinomas should be screened for MMR deficiency.

INTRODUCTION

Small bowel cancer is a rare form of cancer, with an incidence of less than 1.0 per 100,000,[1] and little is known about the risk factors for development of this rare disease. However, monogenic cancer predisposition syndromes, such as familial adenomatous polyposis (FAP) and Lynch syndrome, are known to be responsible for a proportion of small bowel adenocarcinomas.[2] While FAP, which is caused by a germline pathogenic variant in the *APC* gene, is characterized by the presence of polyposis coli, Lynch syndrome may be harder to recognize.[3,4]

Lynch syndrome is caused by germline pathogenic variants in one of four mismatch repair (MMR) genes (*MLH1, MSH2 (EPCAM), MSH6* and *PMS2*) and predisposes carriers to the development of mainly colorectal and endometrial cancer.[4] In addition, risk for several other malignancies is increased, including risk for small bowel adenocarcinomas, currently estimated to be between 0.4% and 12% for *MLH1* and *MSH2* variant carriers.[5] Unlike FAP, there are no overt clinical characteristics that distinguish a small bowel malignancy in a Lynch syndrome patient from a sporadic case, although a personal or family history of a Lynch syndrome-associated cancer may be suggestive. Surveillance of the duodenum is generally not recommended in Lynch syndrome due to lack of evidence supporting its effectiveness.[6] Nonetheless, identification of a Lynch syndrome family via a small bowel cancer case may provide the patient and other family members with the opportunity for surveillance of the colon, which has proven value as a screening strategy [7,8].

A hallmark of Lynch syndrome-related tumours is the presence of MMR deficiency, which results from biallelic inactivation of one of the MMR genes and can be demonstrated by immunohistochemical staining of tumour tissue for the MMR proteins, and/or microsatellite instability (MSI) analysis.[9,10] Lack of nuclear staining of neoplastic cells or presence of MSI are indicative of MMR deficiency. MMR deficiency in Lynch syndrome occurs due to a second somatic hit in neoplastic cells, in addition to a germline variant. MMR deficiency may also occur in sporadic cases due to somatic inactivation of both alleles.[11] The presence of MMR deficiency might also be relevant to patient treatment, given that PDL1-blockers produce a good response in MMR-deficient (colorectal) cancers regardless of sporadic or hereditary aetiology.[11,12] Universal screening for MMR deficiency in small bowel cancers, as introduced for colorectal cancer and endometrial cancer in many countries,[13,14] may therefore be warranted. The potential benefit of a comparable screening strategy can only be accurately assessed if the prevalence of MMR deficiency and Lynch syndrome in unselected small bowel cancer is first reliably estimated. Previous estimates of the prevalence of MMR deficiency were

based on small cohorts and consequently showed wide variability (0-35%).[2,15] Few data are available on the prevalence of Lynch syndrome in these cohorts. In this study, a large, unbiased collection of small bowel cancers was used to reliably establish the prevalence of MMR deficiency and Lynch syndrome in this rare tumour group.

METHODS

Cohort

The nationwide network and registry of histo- and cytopathology in the Netherlands, known as PALGA, was consulted in 2017 in a nationwide search of tumour samples from small bowel cancer patients.[16] All excerpts labelled by the reporting pathologist as a neoplasm of the small bowel were extracted for the five-year period 2012-2016. The conclusions of the resulting pathology reports were then screened for:

1. All resected primary small bowel adenocarcinomas within the five-year time frame. This resulted in the selection of 411 eligible tumour specimens.
2. The hundred most recent samples that included a biopsy of an adenocarcinoma with a (possible) primary origin in the small bowel. This second category of samples was added to ensure inclusion of unresectable cases (some duodenal adenocarcinomas present at an advanced stage and are not resectable due to the high morbidity of surgery).

Formalin-fixed paraffin-embedded (FFPE) material representative of these adenocarcinomas was then requested. Material from 332 resection specimens and 68 biopsy samples was obtained. A favourable ethical opinion was received from the Medical Ethical Review Board of Leiden University Medical Centre (reference number P16.313). Due to the anonymous nature of the samples and the rules and regulations of the PALGA-network, obtaining consent was not possible or required.

Study procedures

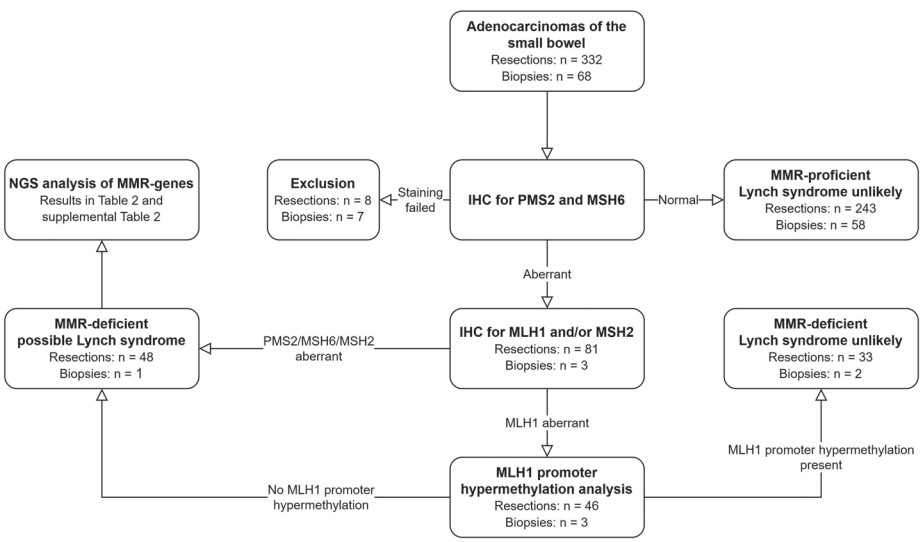
The study flow is visualized in Figure 1. Upon receipt, 4μm sections were taken from the FFPE blocks and subjected to haematoxylin and eosin staining (H&E) and immunohistochemical staining of the MMR proteins. Additionally, depending on tumour size and histology, 10μm sections or punches from the tumour were taken for later DNA isolation. Guided by a matching H&E slide, the 10μm sections were micro-dissected to enrich for tumour. All samples were coded for complete anonymity

according to Dutch guidelines. Anonymous basic personal data (age at diagnosis and gender) was available for each patient, in addition to historical pathology reports. No other clinical data were available.

All adenocarcinomas were initially immunohistochemically stained for PMS2 and MSH6 protein expression.[17] Subsequent immunohistochemical staining for MLH1 and/or MSH2 was performed if the tumour was PMS2- or MSH6-deficient. This approach is more cost-effective than using a four-antibody panel and has good sensitivity. The rationale for this approach is that functionally, MLH1 forms a heterodimer with PMS2, while MSH2 forms a heterodimer with MSH6, and mutations in *MLH1* or *MSH2* result in degradation of their heterodimer partners. Hence, use of PMS2 and MSH6 antibodies as a first screening step will generally identify loss of protein expression of MLH1 or MSH2.[17,18] In cases with MLH1 deficiency, *MLH1* promoter hypermethylation analysis was performed. In cases with loss of expression of MLH1 in the absence of *MLH1* promoter hypermethylation or in cases with MSH2, MSH6 and solitary PMS2 expression loss, the MMR genes were further analysed using Next Generation Sequencing (NGS). If NGS identified a variant with an allele frequency of >40%, DNA from matching non-neoplastic tissue (when available) was isolated to determine whether the variant was germline or somatic in origin.

4

Figure 1 Study procedures. IHC = immunohistochemistry. MMR = mismatch repair. NGS = next generation sequencing



Immunohistochemical staining

Details on the immunohistochemical staining procedures can be found in the Supplemental Methods. The immunohistochemically stained samples were examined by an experienced pathologist (HM or AFS) using light microscopy to evaluate MMR status. MMR proficiency was defined as the presence of nuclear staining within neoplastic cells, as well as within adjacent non-neoplastic cells. MMR deficiency was defined as an absence of nuclear staining within neoplastic cells, together with positive expression in non-neoplastic cells. A third category, subclonal loss of protein expression, was defined for those adenocarcinomas harbouring a subpopulation of cancer cells with loss of expression together with cells retaining expression of an MMR protein.

DNA isolation using the Tissue Preparation System

DNA was isolated using the Tissue Preparation System with VERSANT Tissue Preparation Reagents (Siemens Healthcare Diagnostics, Tarrytown, NY), as previously described.[19]

MLH1 promoter hypermethylation analysis

Cases with loss of MLH1 expression were analysed for *MLH1* promoter hypermethylation by methylation-specific PCR (MSP).[20,21] Bisulphite conversion was carried out using the EZ DNA Methylation-Lightning Kit (D5031; Zymo Research) according to manufacturer's instructions.

Targeted Next Generation Sequencing

Adenocarcinomas with aberrant expression of at least one of the MMR proteins in the absence of *MLH1* promoter hypermethylation underwent DNA variant analysis using an NGS panel. This panel consists of 20 colorectal cancer- and polyposis-associated genes, and hotspot regions of the *CTNNB1* gene (see Supplemental Table 1 for all genes and panel coverage). For the purposes of this study, analysis of NGS results was restricted to *MLH1*, *MSH2*, *MSH6* and *PMS2*. Sequencing was performed using the Ion Torrent platform according to the manufacturer's recommendations. Details can be found in the Supplemental Methods.

The unaligned sequence reads generated by the sequencer were mapped against a human reference genome (hg19) using the Burrows-Wheeler aligner (BWA). VarScan and ANNOVAR software were used for variant calling and annotation, respectively, and Integrative Genomics Viewer (IGV) software was used to visualize the read alignment and presence of variants. Additionally, the Leiden Open Variant Database (LOVD),

ClinVar and Alamut software were used whenever additional variant interpretation was needed.

Statistical analysis

Using IBM SPSS Statistics 24, the chi-square test and one-way ANOVA test were performed as appropriate to compare patient and tumour characteristics of MMR-proficient cases with sporadic MMR-deficient cases and Lynch syndrome-associated cases. A *p*-value <0.05 was considered to be statistically significant. Cases with subclonal loss of one of the MMR proteins were excluded from these analyses.

RESULTS

Immunohistochemistry

The prevalence of MMR deficiency, as determined by immunohistochemical staining, was 22.3% in resected small bowel adenocarcinomas and 4.4% in biopsies (Table 1). Additionally, seven (2.1%) resected samples showed subclonal loss of at least one MMR protein. Eight resected adenocarcinomas and seven adenocarcinoma biopsy samples had to be excluded from further analysis because no (representative) tumour tissue was present in the available FFPE blocks.

Table 1. Prevalence of mismatch repair (MMR) deficiency and immunohistochemical staining patterns in resected and biopsied adenocarcinoma samples

Immunohistochemistry results	Resections *N (%)*	Biopsies *N (%)*
MMR-proficient	**243 (73.2)**	**58 (85.3)**
MMR deficiency - complete tumor	**74 (22.3)**	**3 (4.4)**
- MLH1/PMS2	42	3
- PMS2 only	7	0
- MSH2/MSH6	19	0
- MSH6 only	6	0
Subclonal MMR deficiency	**7 (2.1)**	**0 (0)**
- MLH1/PMS2	4	
- MSH6 only	1	
- All four deficient	2	
No tumor, excluded from further analysis	8 (2.4)	7 (10.3)
Total	332	68

The most common cause of MMR deficiency was *MLH1* promoter hypermethylation (40.5% of MMR-deficient resections and 66.7% of MMR-deficient biopsies, Table 2). In more than a quarter of MMR-deficient resection samples the MMR deficiency was related to Lynch syndrome (27%, Table 2 and Supplemental Table 2). The prevalence of Lynch syndrome within the total resection cohort was therefore at least 20/324 (6.2%). The true number might in fact be higher, because in six cases an MMR gene variant with a high allele frequency (>40% of reads) was identified within the tumour, but matched normal tissue was not available to confirm or refute germline origin of the variant.
A comparison of patient and tumour characteristics of MMR-proficient, (apparently) sporadic MMR-deficient and Lynch syndrome-associated cases included only the resected adenocarcinoma cases, as they represent the largest subcohort and have a documented primary tumour location within the small bowel. The six cases carrying a high allele frequency variant but without available matched normal tissue were excluded due to uncertainty regarding their status as Lynch syndrome or sporadic MMR-deficient cases. Cases with an unexplained MMR deficiency and those with subclonal MMR deficiencies were also excluded from this analysis.

Table 2. Causes of mismatch repair (MMR) deficiency

	MMR-deficient tumors		Subclonal loss
	Resections *N (%)*	Biopsies *N (%)*	Resections *N (%)*
MLH1 promoter hypermethylation	30 (40.5)	2 (66.7)	3 (42.9)
Two somatic hits	10 (13.5)	0	1 (14.3)
Lynch syndrome - MLH1 variant - MSH2 variant - PMS2 variant - MSH6 variant	20 (27.0) 6 7 2 5	0	0
MMR variants identified in tumor, normal tissue not available, but high variant allele frequency	6 (8.1)	0	0
MMR deficiency molecularly unexplained (no or only one somatic hit identified)	8 (10.8)	1 (33.3)	3 (42.9)
Total	74	3	7

Table 3. Cohort characteristics for Lynch syndrome versus mismatch repair (MMR) proficient versus MMR-deficient cases

		MMR-proficient *N*=243	Sporadic MMR-deficient carcinomas *N*= 44	Lynch syndrome *N*=20	*P*-value
Gender – male		126 (51.9%)	23 (52.3%)	13 (65.0%)	0.525
Mean age at diagnosis in years (range)		66.6 (27-91)	68.8 (43-90)	54.6 (35-77)	<0.000
Location (%)	Duodenum	126 (51.9%)	26 (59.1%)	12 (60.0%)	0.893
	Jejunum	51 (21.0%)	7 (15.9%)	3 (15.0%)	
	Ileum	33 (13.6%)	4 (9.1%)	3 (15.0%)	
	Small bowel not otherwise specified	33 (13.6%)	7 (15.9%)	2 (10.0%)	
Previous history of Lynch syndrome-associated* cancer		28 (11.5%)	8 (18.2%)	13 (65.0%)	<0.000
Previous history of other cancer type(s)# (non-Lynch)		27 (11.1%)	6 (13.6%)	6 (30.0%)	0.050
Crohn's disease - yes		8 (3.3%)	0 (0%)	0 (0%)	0.339
Coeliac disease - yes		3 (1.2%)	3 (6.8%)	0 (0%)	0.039

** Lynch syndrome-associated cancers: colorectal cancer, endometrial cancer, ovarian cancer, gastric cancer, cancer of the bile duct or gallbladder, pancreatic cancer or urothelial cancer (Moller et al. 2018). # Excluding basal cell cancer of the skin*

Mean age at cancer diagnosis was significantly lower in the Lynch syndrome patients (Table 3), and a previous history of a Lynch syndrome-associated cancer was significantly elevated in Lynch syndrome patients. Interestingly, coeliac disease (diagnosed based on pathology reports of small bowel biopsies unconnected to the small bowel cancer diagnosis) was significantly more common in sporadic MMR-deficient cases. No other significant associations were identified (e.g. location, gender, other cancer history,[22] Crohn's disease).

DISCUSSION

In a large group of resected primary small bowel adenocarcinomas, we found complete MMR deficiency in 22.3% and subclonal deficiency in 2.1% of cases, while biopsied small bowel adenocarcinomas showed a lower prevalence of MMR deficiency (4.4%). To the best of our knowledge, this is the first study to systematically screen a large, consecutive group of small bowel adenocarcinomas for the prevalence of MMR deficiency. Previous studies were either smaller and/or used selected cases with a higher a priori chance of being related to Lynch syndrome. Furthermore, many of these studies did not include molecular analysis to verify whether MMR deficiency was Lynch syndrome-related or sporadic.[2,15,23]
A recently published French study by Aparicio *et al.*[24] reported a Lynch syndrome prevalence of 6.9% in a large cohort of small bowel adenocarcinomas, in line with a prevalence of at least 6.2% in our cohort. MMR deficiency prevalence could not be compared because this French cohort was not systematically screened for MMR deficiency.

Of particular note, the prevalence of MMR deficiency in our study differed considerably between the resected and biopsied specimens. A higher prevalence of MMR deficiency in resected versus biopsied samples might be related to the association of MMR deficiency with a better prognosis in other cancers,[25] so resections may represent cancer patients with a relatively good prognosis, whereas biopsies may represent patients with a poor prognosis who are less likely to undergo resection. Interestingly, the prevalence of MMR deficiency identified in biopsied samples, 4.4%, is close to the 5.0% prevalence identified in a metastatic colorectal cancer cohort.[26] However, as no further clinical data were available to verify that a biopsied sample was a confirmed primary small bowel cancer, our cohort may also have included cancers with a different primary location (where MMR deficiency prevalence is lower). Further validation of

the prevalence of MMR deficiency in a cohort of small bowel cancers that were not resected is therefore required.
The relevance of subclonal loss of MMR protein expression is still poorly understood. While it seems unlikely that these patients have Lynch syndrome, the relevance of subclonal loss for prognosis and/or therapy will require further investigation.[18,27]

A significant overrepresentation of patients with coeliac disease was noted amongst cases with sporadic MMR deficiency. An association of coeliac disease with sporadic MMR deficiency (particularly with *MLH1* promoter hypermethylation) has been described previously,[28,29] and two out of three MMR-deficient cases from our cohort also showed *MLH1* promoter hypermethylation. A limitation of our study was the lack of accompanying clinical data, which meant that we had no information on treatment/diet and could not verify whether the pathological signs of coeliac disease correlated with patient symptoms. These results should therefore be interpreted with caution, because there are other conditions that mimic the histological signs of coeliac disease.[30] Another drawback of anonymous data is that it precludes verification of the number of Lynch syndrome cases, knowledge that might otherwise be used to establish how many patients are missed using current practices. Nevertheless, from pathology reports we could deduce that thirteen out of twenty Lynch patients were likely already identified, either because MSI and/or immunohistochemical testing was described (in the small bowel tumour or a previous tumour) or a previous diagnosis of Lynch syndrome was mentioned (Supplemental Table 3).

There is an ongoing discussion whether a two-antibody panel for immunohistochemical staining of the MMR proteins has sufficient sensitivity to detect MMR deficient cases. Although a small number of MMR deficient cases may be missed with a two-antibody panel, it is not expected that the results of a four-antibody approach would alter our conclusions.

A molecular cause of MMR deficiency could not always be identified (n=12). This is likely partly explained by the fact that we did not perform multiplex ligation-dependent probe amplification (MPLA) analysis to screen samples for deletions and/or insertions (germline or somatic) of the MMR genes or *EPCAM* (Table 2 and Supplemental Table 2). Nonetheless, NGS data was manually checked using the Integrative Genomics Viewer (IGV) for evidence suggesting a deletion, which led to the identification of deletions in three samples (Supplemental Table 2, e.g. study ID 33). Although this approach lowers the risk of missing copy number variants, not all deletions/insertions

will be identified. As *EPCAM* was not sequenced, deletions of this gene will have been missed by definition. However, as only 1-3% of all Lynch syndrome families carry an *EPCAM* deletion and deletions/insertions of the MMR genes explain a minority of Lynch syndrome families,[4,31] MLPA analysis is unlikely to have altered our conclusions and recommendations. Another possible explanation for the failure of NGS results to resolve all MMR deficiency cases is that some cases lacked the informative single nucleotide polymorphisms (SNPs) required to determine whether loss of heterozygosity has occurred.

The analysis of *PMS2* is complicated by the presence of pseudogenes. Nevertheless, researchers from our group have shown that it is possible to reliably detect variants in *PMS2*, even when using DNA isolated from FFPE material, as long as the correct amplicons are selected.[32] Exceptions include variants in exon 12-15 due to gene conversion. The two germline variants identified in our cohort are found in exons 1-11. In our cohort, the prevalence of MMR deficiency in resected cases (22.3%) was higher than the reported prevalence of MMR deficiency in colorectal cancer (15%).[33] This finding has implications for daily clinical practice in relation to three important issues: prognosis, treatment and surveillance. In (early-stage) colorectal cancer, MMR deficiency has been linked to a better prognosis,[25,34,35] an association that may also hold true for MMR-deficient small bowel cancers. Indeed, the aforementioned study by Aparicio *et al*. reported a trend towards better prognosis for Lynch-associated small bowel adenocarcinomas versus those related to Crohn's disease.[24] Furthermore, with the advent of immunoblockade therapy and its proven efficacy in MMR-deficient cancers,[36] MMR status is relevant when formulating treatment strategies regardless of germline or sporadic status. Finally, due to the high prevalence of Lynch syndrome, small bowel cancer as an entity may facilitate the identification of new Lynch syndrome families and consequently allow surveillance measures to be offered.

In light of the high prevalence of MMR deficiency and Lynch syndrome, together with associated relevance and benefits, we recommend the implementation of universal screening of all primary small bowel adenocarcinomas for the presence of MMR deficiency. An age limit of 70 years is often used in the universal screening of colorectal cancers for mismatch repair deficiency. However, as the Lynch syndrome-associated cases included in our study showed a very broad age range (35-77 years, table 3) at diagnosis, we suggest that age limits on universal screening for small bowel cancer may be detrimental.

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SUPPLEMENTAL METHODS + SUPPLEMENTAL TABLE 1 - 3

Immunohistochemical staining

4µm FFPE sections were deparaffinized with xylene and rehydrated in ethanol. A 0.3% H2O2-solution was used to block endogenous peroxidase, and microwave-mediated antigen retrieval was performed in Tris-EDTA, pH 9.0. Sections were incubated overnight with primary antibodies against MLH1 (clone ES05, 1:50; Agilent, USA), MSH2 (clone FE11, 1:200, Agilent, USA), MSH6 (clone EPR3945, 1:200, Genetex, USA) or PMS2 (clone EP51, 1:40, Agilent, USA) at 4°C. After washing, they were then incubated for 30 minutes with poly-HRP (VWRKDPVM110HRP, ImmunoLogic), visualised using a DAB+ substrate chromogen system (K3468; Agilent) and counterstained with haematoxylin. Finally, the sections were dehydrated and mounted with coverslips.

Targeted Next Generation Sequencing (NGS)

Sequencing was performed using the Ion Torrent platform according to the manufacturer's recommendations. In brief, 21 ng/14 µl isolated DNA was used to prepare two primer pools. After the first PCR, the pools were combined and a new PCR run was performed to digest the primers. A third PCR was then performed to add barcodes to the samples. After purification using AMPureXP beads (A63882; Beckman Coulter), the NGS libraries were pooled, diluted to 60 pmol/L and loaded on a chip using the Ion Chef. Sequencing was subsequently performed in an Ion GeneStudio S5 Series sequencer.

Supplemental table 1 – msCRC panel genes and coverage

Name	Chromosome	Exons	Coverage (%)
APC	5	16	100
BMPR1A	10	11	94.3
BRCA1	17	23	100
BRCA2	13	26	100
ENG	9	15	100
MLH1	3	21	100
MSH2	2	17	100
MSH3	5	24	99.8
MSH6	2	12	100
MUTYH	1	16	100
NTHL1	16	6	100
PALB2	16	42	100
PMS2	7	15	96.8
POLD1	19	26	100
POLE	12	40	100
PTEN	10	10	98.9
RNF43	17	9	99.9
SMAD4	18	11	98.5
STK11	19	9	100
TP53	17	12	100
KRAS	12	2,3,4	Hotspots
HRAS	11	2,3	Hotspots
NRAS	1	2,3,4	Hotspots
BRAF	7	11,15	Hotspots
CTNNB1	3	8	Hotspots
MYC	8	CNV	Hotspots

4

Supplementary Table 2 – Next Generation Sequencing (NGS) result of MMR-deficient cases (excluding cases with MLH1 promoter hypermethylation)
Variants are either likely pathogenic (class 4) or pathogenic (class 5) unless otherwise specified.
Abbreviations: VAF = Variant allele frequency. LOH = Loss of heterozygosity. SNP = Single Nucleotide Polymorphism. NP = not performed.
VAF = Variant allele frequency. Immunohistochemistry results: + = normal nuclear staining. - = loss of staining in neoplastic cells with positive internal controls.
+/++ = weak staining in neoplastic cells compared to internal controls.
* since germline variants may be unique to a family/person, only a general description of the germline variant type is given to protect privacy and maintain data anonymity

Study ID	Resection or biopsy	Immunohistochemistry pattern				NGS results neoplastic tissue				Variant detected in non-neoplastic tissue
		PMS2	MLH1	MSH6	MSH2	Gene	Variant	VAF : coverage	LOH	
3	Resection	+	np	+/++	-	MSH2	Nonsense variant*	0.779:715	Probable based on 1 SNP and VAF	Yes
18	Biopsy	-	-	+	np		No relevant variants detected			
33	Resection	+	np	-	+/++	MSH2	NM_000251.2:c.1777C>T	0.480:125	No	No
							Deletion exon 1			No
46	Resection	-	+	+	np		NGS data of insufficient quality			
48	Resection	-	+	+	+	MLH1	NM_000249.3:c.112A>C	0.48:448	No informative SNPs	Normal tissue not available
71	Resection	+	np	-	+/++		No relevant variants detected			
85	Resection	Failed	np	-	+	MLH1	Missense variant classified as pathogenic by InSiGHT	0.479:1308	No informative SNPs	Yes
							NM_000249.3:c.1513_1520dup	0.168:1985		No
						MSH6	C-deletion			
94	Resection	-	-	+	np	MLH1	NM_000249.3:c.676C>T		yes	Not performed
98	Resection	+	np	-	+/++	MSH6	Frameshift variant*	0.483:1989	No	Yes
							NM_000179.2:c.3743del	0.329:1989		No

118	Resection	+	np	-	-		NGS data of insufficient quality			
119	Resection	-	+	+	np	PMS2	Frameshift variant*	0.498:1933	yes	Yes
						MSH2	NM_000251.2:c.187dup	0.204:1967		No
124	Resection	+	np	-	failed		No relevant variants detected			According to PA-report this is a Lynch syndrome patient
156	Resection	+	np	-	-	MSH2	NM_000251.2:c.2027C>G	0.219:283	No informative SNPs	Not performed
206	Resection	-	-	+	np	MLH1	Frameshift variant*	0.429:1919	No informative SNPs	Yes
211	Resection	-	-	+	np	MLH1	NM_000249.3:c.454-13A>G	0.918:244	No informative SNPs, VAF is however suggestive	Normal tissue not available
214	Resection	subclonal -	+	+	np	MSH2	NM_000251.2:c.1414C>T (class 3 VUS)	0.156:257		Not performed
236	Resection	+	np	-	-	MSH6	Frameshift variant*	0.511:1621		Yes
							NM_000179.2:c.3172G>T (class 3 VUS)	0.313:1995		No
249	Resection	-	+	+	np	PMS2	NM_000535.5:c.2287G>T	0.159:1233	No informative SNPs	Normal tissue not available
							NM_000535.5:c.1882C>T	0.397:315		
316	Resection	+	np	+/++	-	MSH2	Exon deletion*	Not applicable	Yes	Yes
325	Resection	+	np	-	-		No relevant variants detected			
333	Resection	+	np	-	-	MSH6	NM_000179.2:c.3128del	0.185:352	No informative SNPs LOH probable	Not performed

335	Resection	+	np	-	-	MSH2	Missense variant classified as likely pathogenic by InSiGHT*	0.520:1997	based on 1 informative SNP	Yes
344	Resection	-	-	+	np	MLH1	NM_000249.3:c.94_110del	0.341:1510	No informative SNPs	Not performed
363	Resection	+	np	-	-	MSH2	Frameshift variant*	0.499:914	No based on 1 SNP	Yes
379	Resection	-	+	+	np	PMS2	Nonsense variant*	0.500:1225	No	Yes
							NM_000535.5:c.1802C>G	0.421:680		No
414	Resection	+	np	-	+/++	MSH6	Frameshift variant*	0.481:1795	No based on 1 SNP	Yes
							NM_000179.2:c.3533del	0.239:1980		No
426	Resection	-	-	+	np	MLH1	Frameshift variant*	0.539:1990	No informative SNPs	Yes
453	Resection	-	-	+	np	MLH1	NM_000249.3:c.791-2A>C (class 3 VUS)	0.634:1994	No informative SNPs	No
460	Resection	+	np	+/++	-	MSH2	NM_000251.2:c.2557G>T	0.241:1312	Unlikely based on 1 SNP	Not performed
466	Resection	-	-	+	np		No relevant variants detected			
474	Resection	+	np	-	-	MSH2	Frameshift variant*	0.691:676	Probable based on 1 SNP	Yes
480	Resection	-	-	Subclonal	np	MLH1	Nonsense variant*	0.744:1999	yes	Yes
				-		MSH6	No relevant variants detected		yes	
526	Resection	+	np	-	-		No relevant variants detected			
551	Resection	-	-	+	Np	MLH1	NM_000249.3:c.2145_2168del	0.578:211	No informative SNPs	Normal tissue not available
									Possibly	

558	Resection	-	+	Subclonal -	+/++	PMS2	NM_000535.5:c.638del	0.378:1995	based on 3 SNPs	No
568	Resection	-	+/++	+	np	PMS2	NM_000251.2:c.2458+1G>A	0.381:1998	No	Not performed
595	Resection	+	np	-	+/++	MSH6	Frameshift variant*	0.453:1190	No	Yes
							NM_000179.2:c.2232G>T (class 3 VUS)	0.169:349		No
596	Resection	+	np	-	-	MSH2	NM_000251.2:c.1861C>T	0.491:1611	No	Normal tissue not available
							NM_000251.2:c.2458+1G>A	0.271:399		
601	Resection	+	np	-	-	MSH2	NM_000251.2:c.1601G>A	0.346:619	No informative SNPs	Not performed
						MSH6	NM_000179.2:c.1436_1440del	0.35:1980	Yes	
687	Resection	-	np	-	-		NGS data of insufficient quality			
698	Resection	+	np	Subclonal	+		NGS data of insufficient quality			
710	Resection	-	+	+	np	PMS2	NM_000535.5:c.1405A>T	0.522:1994	No	Normal tissue not available
720	Resection	+	np	-	-	MSH2	Nonsense variant*	0.678:1772	No	Yes
722	Resection	+	np	-	+/++	MSH6	Frameshift variant*	0.532:342	no	Yes
							NM_000179.2:c.1444C>T	0.285:895		No
723	Resection	-	-	+	np	MLH1	Frameshift variant*	0.937:1449	Probable based on one SNP and VAF of variant	Yes
746	Resection	-	-	+	np	MLH1	Frameshift variant*	0.594:1721	Yes	Yes
748	Resection	+	np	-	-		NGS data of insufficient quality			
760	Resection	-	-	+	np	MLH1	NM_000249.3:c.252del	0.498:601	yes	No

Supplemental Table 3 - clinical details of Lynch syndrome patients
Abbreviations: n.o.s. = not otherwise specified

Study ID	Gene	Sex	Age decade at small bowel cancer diagnosis (years)	Location of tumour	Differentiation grade as reported in PA-report	Aberrent IHC, MSI or Lynch diagnosis in pathology report	History of Lynch-associated malignancy	Number of Lynch-associated malignancies (excluding small bowel)	History of other malignancy (non-Lynch associated)	Number of other malignancies (non-Lynch associated)
3	MSH2	m	40-49	small bowel n.o.s.	moderate	yes	yes	1	no	-
85	MLH1	m	30-39	ileum	moderate	yes	yes	1	no	-
98	MSH6	m	70-79	duodenum	moderate	no	no	-	yes	1
119	PMS2	m	70-79	duodenum	moderate	yes	no	-	no	-
124	MSH2	v	60-69	jejunum	moderate	yes	yes	4	no	-
206	MLH1	f	30-39	duodenum	moderate	no	no	-	no	-
236	MSH6	m	40-49	duodenum	moderate	yes	yes	1	no	-
316	MSH2	f	50-59	jejunum	could not be assessed	yes	yes	2	no	-
335	MSH2	f	60-69	ileum	moderate	yes	yes	1	yes	1

363	MSH2	m	40-49	duodenum	moderate to high	yes	no	-	no	-
379	PMS2	m	50-59	duodenum	moderate	no	no	-	no	-
414	MSH6	f	50-59	duodenum	moderate	yes	yes	1	yes	1
426	MLH1	m	40-49	duodenum	poorly/high grade	yes	no	-	no	-
474	MSH2	f	50-59	duodenum	moderate	no	yes	1	no	-
480	MLH1	m	60-69	duodenum	moderate	yes	yes	2	no	-
595	MSH6	m	50-59	jejunum	poorly/high grade	no	no	-	no	-
720	MSH2	m	50-59	duodenum	moderate	no	yes	1	no	-
722	MSH6	f	50-59	ileum	could not be assessed	no	yes	2	yes	1
723	MLH1	m	50-59	small bowel n.o.s.	moderate	yes	yes	1	yes	1
746	MLH1	m	50-59	duodenum	moderate	yes	yes	3	yes	1

Well documented high-grade serous ovarian cancers should not be tested for mismatch repair deficiency

Manuscript in preparation

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ABSTRACT

High-grade serous ovarian cancer (HGSOC) is the most common histological subtype of ovarian cancer. Prevalence of cancer predisposition syndrome in this specific subtype is high (up to 24%, mainly *BRCA1* and *BRCA2* pathogenic variants). Whether mismatch repair (MMR) deficiency and Lynch syndrome are associated with HGSOC is still a topic of discussion.

Immunohistochemical staining of the MMR proteins was performed in 54 HGSOC to determine MMR deficiency status. Histopathological review was performed on all included cases to confirm histological subtype. Furthermore, a systematic PubMed search was performed to identify and evaluate recent literature on this topic.

All analysed HGSOC in our case series were MMR proficient. This observation was further strengthened by literature, where we found a prevalence of MMR deficiency and Lynch syndrome of 0 – 0.4%, with the notable exception of one outlier (15.2% MMR deficiency). However, the cases included in the latter study did not undergo central pathology review according to current standards.

There was no association in our cohort between HGSOC and MMR deficiency. This finding is corroborated by a review of recent literature, indicating that well documented HGSOC should not be tested for MMR deficiency.

INTRODUCTION

Ranking 7th in the list of most common cancers in females, ovarian cancer is not one of the most frequent types of cancer.[1] However, if ovarian cancer develops, mortality rates are high (<45% 5-year survival).[2] A substantial proportion (up to 24%) of ovarian cancers is caused by genetic predisposition syndromes, most commonly mutations in *BRCA1* and *BRCA2*.[3] Another genetic predisposition for ovarian cancer is Lynch syndrome, caused by heterozygous pathogenic variants in one of four mismatch repair (MMR) genes (*MLH1, MSH2, MSH6* or *PMS2).*[4]

Lynch syndrome-associated cancers are characterized by MMR deficiency, which can either be demonstrated by expression loss of the MMR proteins through immunohistochemical (IHC) staining or by determining microsatellite instability (MSI) status of the tumour.[5] These two techniques can be used as a pre-screening method to identify patients with a high chance of having Lynch syndrome. If MMR deficiency is present in the tumour, Lynch syndrome can be demonstrated (or ruled out) by subsequent sequencing of the MMR genes in DNA isolated from non-neoplastic tissue. It should be noted that the majority of MMR deficiency tumours (around two-thirds in colorectal and endometrial cancer) is caused by epigenetic silencing of both *MLH1* alleles or two somatic mutations in one of the MMR genes as a sporadic, non-hereditary event.[5,6]

Identifying women with a higher risk of developing ovarian cancer is pursued so they can be offered prophylactic bilateral salpingectomy.[7,8] It is therefore recommended that all women with high-grade serous ovarian cancer (HGSOC) are offered molecular testing of the *BRCA*-genes.[9,10] Universal screening for Lynch syndrome in ovarian cancers is more controversial. Although prevalence of MMR deficiency in unselected ovarian cancer was around 10% in a systematic review published in 2011 by Murphy and Wentzensen, there was high heterogeneity between included studies.[11] Furthermore, after this review new classification guidelines have been published in 2014, which have increased reproducibility of histopathological subtyping in ovarian cancer. This is particularly relevant since it has been suggested that, similar to Lynch syndrome-associated endometrial cancer, there is a predominance of endometroid and clear-cell histological subtypes in Lynch syndrome-associated ovarian cancer.[12] Some therefore recommend universal testing for Lynch syndrome of only these histological subtypes of ovarian cancer.[12-14]

Nonetheless, some controversy remains on whether these recommendations can be justified based on currently available literature.[15,16] In a systematic review, 22% of Lynch syndrome-associated ovarian cancers are reported to be of serous histology[4]

and 25% of ovarian cancers from a Dutch cohort of ovarian cancers in patients with Lynch syndrome was reported to be of high-grade serous histology.[17] Additionally, in the aforementioned systematic review of Murphy and Wentzensen, prevalence of MSI for serous ovarian cancers was 7.9%.[11] No distinction was made yet between low-grade and high-grade ovarian cancers in this review.

Some say the reasons for finding MMR deficiency in serous ovarian cancer and, vice versa, serous ovarian cancers in Lynch syndrome patients are 1) misclassification of histological subtypes and 2) the occurrence of incidental serous tumours in patients with Lynch syndrome.[14,15] Misclassification of histological subtypes is not uncommon in ovarian cancer, particularly if histological sub classification is not up to current standards (i.e. supported by biomarker analysis such as immunohistochemical analysis of TP53 and WT1).[14,18,19] This is particularly relevant in (research) cohorts that include historical cases. Central pathology review by a dedicated gynaecology pathologist to confirm histological subtyping, preferably by applying the world health organisation guidelines of 2014 and supported by biomarker analysis, is therefore important in such cohorts.

We present a series of centrally reviewed HGSOCs (n=54), which were immuno-histochemically stained for the MMR-proteins. Additionally, recent literature was searched for unselected HGSOC cohorts that were screened for MMR deficiency and/or Lynch syndrome.

METHODS

LUMC case series

Our cohort consists of prospectively included ovarian cancer patients from seven hospitals in the Netherlands and was described before as the COBRA cohort by de Jonge *et al.*[20] Sixty-six women with ovarian cancer consented to the study and were included without any preselection criteria (such as family history), 54 of these women had HGSOC. Immunohistochemical staining of formalin-fixed paraffin embedded (FFPE) sections was performed as described before [21] to determine MMR deficiency status. MMR deficiency was defined as absent nuclear staining of at least one of the MMR proteins. A two-antibody approach to immunohistochemical staining was applied (staining PMS2 and MSH6 as a first step, followed by reflex staining of the protein within the same heterodimer if either PMS2 or MSH6 showed aberrant staining).[22]

Histopathology slides from all cases were centrally revised by an expert gynaeco-pathologist (TB) according to the most recent (2014) World Health Organization classification system.
The study was approved by the medical ethics committee of the LUMC (reference number: P16.009).

Literature review

PubMed was searched for publications that report on unselected (i.e. no preselection was made based on family history or other criteria that increase mutation detection rates) serous ovarian cancer cohorts in which screening for MMR deficiency and/or Lynch syndrome was carried out. Data from publications that report on the prevalence of MMR deficiency in serous ovarian cancer and that were published after the release of the latest WHO guidelines in 2014 extracted and summarised. Furthermore, because histological subtyping is prone to interobserver variation, it was assessed whether central pathology review was performed on the cohorts in included publications.
Additionally, data from all publications that report on DNA panel sequencing to detect germline MMR variants were extracted.
The PubMed-search-strategy can be found in the supplementary materials and resulted in 265 hits on April 1st 2020. Titles of publications were screened for relevance. Subsequently, abstracts and, if necessary, content of possibly relevant manuscripts were read to decide whether they contained relevant data.

RESULTS

LUMC case series

Immunohistochemical staining was performed on all 54 HGSOCs (mean age at diagnosis: 65.2 years, age range 46 – 89 years). None of the analysed samples showed expression loss of any of the MMR proteins.

Literature review

Our literature search identified three relevant publications that screened serous ovarian cancers for MMR deficiency, either through immunohistochemical staining of the MMR proteins or through microsatellite instability analysis. Two of these publications performed central pathology review to confirm the diagnosis of HGSOC. Prevalence of MMR deficiency varied was 0% in two studies and 15.2% in the one study that did not perform central pathology review (table 1). This latter study also did

not differentiate between high-grade and low-grade serous ovarian cancer. Our own cohort was included in the table as well (0% MMR deficiency).

Furthermore, three publications were identified that report on the prevalence of Lynch syndrome as analysed by germline gene panel analysis in a cohort of serous ovarian cancers. All three publications were published before or around the time of the release of the WHO guidelines for histological subtyping. Only one of these publications mentions central pathology review. Two out of three studies did not specify whether their serous ovarian cancer cases were high-grade or low-grade. Regardless, the prevalence of Lynch syndrome is very low in all three publications (0 – 0.4%, table 2).
In addition a publication by Chui et al.[12] was identified as being of relevance. In this publication 20 ovarian cancers from Lynch syndrome patients are revised. After expert review, none of the twenty cases was of serous histology. Before review there was one serous carcinoma and two carcinomas of mixed histology with also a serous component, two of these tumours were classified as endometroid and in one mixed tumour there was no serous component after revision, although it was still classified as a mixed type.[12]

Table 1. Mismatch repair (MMR) deficiency as determined through immunohistochemical staining or microsatellite instability analysis in serous ovarian cancer.

Publication	High-grade serous versus serous not specified	Central pathology review	Method of pathology review	Method of MMR deficiency analysis	Number of included cases	MMR deficient
					n	n (%)
Rambau (2016)[29]	High-grade	Yes	Biomarker expression analysis using IHC, panel not clearly specified	Immunohistochemistry on tissue microarrays	149	0 (0)
Leskela (2020)[30]	High-grade	Yes	According to 2014 WHO guidelines (including IHC of WT1, PR, p53, and Napsin A)	Immunohistochemistry on tissue microarrays	124	0 (0)
This manuscript	High-grade	Yes	According to 2014 WHO guidelines	Immunohistochemistry on whole sections	54	0 (0)
Akbari (2017)[25]	Not specified	No		MSI analysis (5 marker panel as recommended by the National Cancer Institute)	389	59 (15.2)
	Subtotal with central pathology review				327	0 (0)
	Total				716	59 (8.2)

Table 2. Prevalence of Lynch syndrome as determined through sequencing of germline DNA in patients with serous ovarian cancer. MMR = mismatch repair.

Publication	Central pathology review	High-grade serous versus serous not specified	Method of central review	No. of cases	Germline MMR-mutation (DNA sequencing)					Total with Lynch syndrome
					MLH1	PMS2	MSH2	MSH6	Not specified	
				n	n	n	n	n	n	n (%)
Walsh (2011)[31]	No	Serous not further specified		242	0	0	0	0	-	0 (0.0)
Pal (2012)[32]	No	Serous not further specified		933	-	-	-	-	2	2 (0.2)
Norquist (2014)[33]	Yes	High-grade serous	centrally reviewed by gynecologic pathologists, unsure cases were resolved by consensus	1118	0	4	0	1	-	5 (0.4)
		Total		2293						7 (0.3)

DISCUSSION

In our cohort of centrally revised HGSOCs, no cases with MMR deficiency were identified. Furthermore, the prevalence of MMR deficiency in recently published serous ovarian cancer cohorts that underwent central pathology review was extremely low (0 – 0.4%, Table 1 and 2).

There are several good arguments in favour of implementing universal MMR deficiency screening in ovarian cancer. First of all, a Lynch syndrome diagnosis benefits the patient and her family as it offers them the opportunity to begin colonoscopy surveillance and/or undergo preventive surgery of the uterus and ovaries. Furthermore, MMR deficiency, regardless of whether it has a sporadic or hereditary cause, is relevant for treatment (immunotherapy) [23,24] and prognosis (MMR deficient tumours have been associated with better survival).[17] Additionally, MMR deficiency status might aid in histological subtyping (*e.g.* when discerning HGSOC from high-grade endometroid ovarian cancer). Nonetheless, health funding should be spent wisely and efficiently and screening for MMR deficiency should be reserved for those histological subtypes with a reasonable a priori chance of a relevant outcome. Furthermore, it is of interest for patients who already have a Lynch syndrome diagnosis to know whether they have an increased risk of HGSOC, since this subtype has a relatively poor prognosis. It is therefore important to establish whether or not an association exists between MMR deficiency/Lynch syndrome and HGSOC.

Our cohort with centrally revised, HGSOCs adds further evidence to the existing literature that the link between MMR deficiency and HGSOC is weak at best. These results corroborate guidelines that suggest not to perform universal MMR deficiency screening in HGSOC.[12-14] Recent literature on MMR deficiency prevalence in unselected serous ovarian cohorts, as summarized in table 1 and 2, supports these guidelines as well. Only one recent publication reports a high prevalence of MMR deficiency in serous ovarian cancer. Considering that the results of this study are such an extreme outlier, we believe that these results are incorrect due to lack of central pathology review, possibly in combination with other factors that cannot be derived from the manuscript.[25] The fact that no differentiation is made between high-grade and low-grade tumours within this study strongly suggests that the 2014 WHO guidelines for histological subtyping are not followed.

An additional argument against a link between HGSOC and Lynch syndrome is the lack of serous tubal intraepithelial carcinomas (STICs) in prophylactic gynaecologic specimens from Lynch syndrome patients.[26] As the majority of HGSOC originate in the fallopian tubes, presence of a precursor lesions in the form of STICs would be

expected in individuals with an increased risk of HGSOC (as observed in *BRCA1/2*-mutation carriers).[27,28]

The most important source of caution regarding subtype specific MMR deficiency screening are publications of case series with ovarian cancer patients from Lynch syndrome families where serous ovarian cancer is quite prevalent.[4,15] This is likely explained by the fact that high-grade endometroid ovarian cancer and HGSOC can be hard to discern and, thus, histological misclassification. Another explanation could be the coincidental occurrence of sporadic serous ovarian cancer within a Lynch syndrome patient (in a minority of cases).[14,15]

As mentioned above, Chui *et al.*[12] already published evidence suggesting that misclassification of histological subtypes is at least part of the explanation. Unfortunately, their cohort is the only publication to thoroughly revise a cohort of Lynch syndrome-associated ovarian cancers. Future research efforts should therefore focus on gathering larger cohorts of ovarian cancers from molecularly confirmed Lynch syndrome patients and perform histological subtyping according to current standards. If there are truly HGSOC cases in Lynch syndrome patients, then these should be analysed for signs of MMR deficiency (*i.e.* loss of MMR staining, presence of MSI and/or a second, somatic hit of the affected MMR protein) to see whether tumour development was a consequence of the germline mutation.

Based on our finding of 0% MMR deficiency in centrally revised HGSOC, the low prevalence of MMR deficiency in well-characterised HGSOC cohorts as published in literature and the argumentation as provided in the discussion, an association between HGSOC and MMR deficiency/Lynch syndrome is unlikely. These findings stress the relevance of careful histological subtyping for pathologists and imply that universal MMR deficiency testing is not required in HGSOC. Clinical geneticists can refrain from requesting MMR deficiency analysis in well-documented (recently diagnosed) HGSOC. In older cases histopathological review should be considered.

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SUPPLEMENTAL INFORMATION

Pubmed Search strategy:
("Colorectal Neoplasms, Hereditary Nonpolyposis"[mesh] OR "Hereditary Nonpolyposis Colorectal Carcinoma"[ti] OR "Hereditary Nonpolyposis Colorectal Cancer"[ti] OR "Lynch syndrome"[ti] OR "Lynch"[ti] OR "Lynch syndrome I (site-specific colonic cancer)" [Supplementary Concept] OR "MLH1 protein, human" [Supplementary Concept] OR "MLH1"[ti] OR "MSH2"[ti] OR "MLH 1"[ti] OR "MSH 2"[ti] OR "PMS2"[ti] OR "MSH6"[ti] OR "MSH 6"[ti] OR "LS"[ti] OR "HNPCC"[ti] OR "MutL Proteins"[mesh] OR "MutL"[ti] OR "MutS Homolog 2 Protein"[mesh] OR "MutS"[ti] OR "MMR genes"[ti] OR "MMR gene"[ti]) AND ("Early Detection of Cancer"[Mesh] OR "screening"[tw] OR "screened"[tw] OR "detecting"[tw] OR "detection"[tw] OR "detected"[tw] OR "identification"[tw] OR "identifying"[tw] OR "identified"[tw] OR "identify"[tw] OR "IHC"[tiab] OR "Immunohistochemistry"[tw] OR "immunocytochemistry"[tw] OR "immunofluorescence"[tw] OR "mismatch repair proteins"[tw] OR "MMR"[tiab] OR "Microsatellite Instability"[Mesh] OR "microsatellite instability"[tw] OR "MSI"[tiab] OR "panel"[tw] OR "panels"[tw] OR "Genetic Testing"[Mesh] OR "Genetic Testing"[tw] OR "Genetic Test"[tw] OR "Genetic Tests"[tw] OR "Microsatellite Repeats"[Mesh] OR "Microsatellite Repeat"[tw] OR "Microsatellite Repeats"[tw] OR "histology"[tw] OR "histological"[tw] OR "Histology"[Mesh]) AND ("Ovarian Neoplasms"[Mesh] OR (("Neoplasms"[Mesh:NoExp] OR "Neoplasm"[tw] OR "Neoplasms"[tw] OR "tumor"[tw] OR "tumors"[tw] OR "tumour"[tw] OR "tumours"[tw] OR "cancer"[tw] OR "cancers"[tw] OR "Carcinoma"[Mesh:NoExp] OR "carcinoma"[tw] OR "carcinomas"[tw]) AND ("Ovary"[Mesh] OR "Ovary"[tw] OR "ovaries"[tw] OR "ovarian"[tw]))) AND ("2011"[Date - Publication] : "3000"[Date - Publication]) AND English[Language]

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Part II

Pheno
type

An alternative approach to establishing unbiased colorectal cancer risk estimation in Lynch syndrome

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ABSTRACT

Purpose

Biallelic pathogenic variants in the mismatch repair (MMR) genes cause a recessive childhood cancer predisposition syndrome known as constitutional mismatch repair deficiency (CMMRD). Family members with a heterozygous MMR variant have Lynch syndrome. We aimed at estimating cancer risk in these heterozygous carriers as a novel approach to avoid complicated statistical methods to correct for ascertainment bias.

Methods

Cumulative colorectal cancer incidence was estimated in a cohort of *PMS2*- and *MSH6*-associated families, ascertained by the CMMRD phenotype of the index, by using mutation probabilities based on kinship coefficients as analytical weights in a proportional hazard regression on the cause-specific hazards. Confidence intervals (CIs) were obtained by bootstrapping at the family level.

Results

The estimated cumulative colorectal cancer risk at age 70 years for heterozygous *PMS2* variant carriers was 8.7% (95% CI 4.3–12.7%) for both sexes combined, and 9.9% (95% CI 4.9–15.3%) for men and 5.9% (95% CI 1.6–11.1%) for women separately. For heterozygous *MSH6* variant carriers these estimates are 11.8% (95% CI 4.5–22.7%) for both sexes combined, 10.0% (95% CI 1.83–24.5%) for men and 11.7% (95% CI 2.10–26.5%) for women.

Conclusion

Our findings are consistent with previous reports that used more complex statistical methods to correct for ascertainment bias. These results underline the need for MMR gene–specific surveillance protocols for Lynch syndrome.

INTRODUCTION

Lynch syndrome (MIM 120435) is an inherited autosomal dominant condition predisposing to the development of primarily colorectal and endometrial cancer. It is caused by pathogenic variants in the mismatch repair (MMR) genes *MLH1* (MIM *120436), *MSH2* (MIM *609309), *MSH6* (MIM *600678), and *PMS2* (MIM *600259). Estimation of Lynch syndrome–associated cancer risk is challenging because until recently, testing for Lynch syndrome was based on clinical or family history criteria such as the Amsterdam II criteria and the (revised) Bethesda guidelines.[1,2] Consequently the majority of known Lynch syndrome families were ascertained based on familial cancer history. In recent years there has been a shift toward universal screening of all colorectal and endometrial cancer patients for tumor hallmarks of Lynch syndrome.[3,4] These hallmarks include aberrant immunohistochemistry for the MMR proteins and the presence of microsatellite instability.[5,6] Furthermore, panel testing of cancer genes, including the MMR genes, is becoming standard practice and is also performed in families with a cancer history that does not necessarily include Lynch syndrome–associated cancers.[7] Families identified through universal screening or panel testing may show lower penetrance for Lynch syndrome–associated malignancies, and Hampel et al. were among the first to notice that Lynch syndrome cancer risks are not as high as previously estimated based on analyses of families ascertained using existing guidelines.[8] Appropriate surveillance measures for these newly identified families can only be established if risks can be estimated accurately.

Based on retrospective cohorts, current estimates of lifetime colorectal cancer risks for carriers of pathogenic variants in *MLH1* and *MSH2* are between 52% and 97%.[9] Colorectal cancer risk estimates are lower for carriers of a pathogenic variant in *MSH6* (22–36%) and lowest of all for *PMS2* (11–20%).[9-12] A recent study of a prospective cohort of pathogenic MMR variant carriers undergoing surveillance reported even lower risks, with colorectal cancer risks of 12% for *MSH6* and 0% for *PMS2*, respectively.[13] As in the general population, men with Lynch syndrome appear to have a higher colorectal cancer risk than women.[14] In most studies, statistical approaches such as modified segregation analysis, exclusion of index cases, and genotype-restricted likelihood estimates have been used to correct for ascertainment bias, but these methods are complex and rely on specific assumptions, and it is difficult to prove that they do not lead to either under- or overestimation of true risk.[14] Indeed, Vos et al. showed that a substantial proportion of the variation found in cancer risk estimation in selected hereditary breast cancer families, who show similar ascertainment patterns to Lynch syndrome families, can be explained by the different ascertainment correction method used.[15]

An alternative approach that minimizes the need for ascertainment bias correction is the selection of families in which the index patient has constitutional mismatch repair deficiency (CMMRD). This childhood cancer predisposition syndrome is caused by biallelic pathogenic variants in one of the MMR genes, most commonly in *PMS2*. The syndrome is characterized by the development of a broad spectrum of cancers, including hematological, central nervous system, and gastrointestinal neoplasia at a very young age. CMMRD patients may also show signs suggestive of neurofibromatosis type 1, most commonly café au lait macules.[16] The CMMRD phenotype is so striking that the diagnosis is often suspected regardless of family history and in one report only 6 of 23 CMMRD patients (26%) had a family history of Lynch syndrome–associated cancers.[17] Identification of a child with CMMRD means that both parents are likely to be heterozygous for a pathogenic MMR variant and are at risk for Lynch syndrome–associated malignancies; other family members may similarly be at risk. Because these families were identified due to the CMMRD phenotype rather than family history, they likely represent a near random sample of Lynch syndrome families.

Pathogenic variants in *PMS2* were once considered rare and were thought to account for less than 5% of all Lynch syndrome cases.[18,19] Nevertheless, germline pathogenic variants in PMS2 were found in a small yet significant proportion (at least 0.57%) of universally screened colorectal cancer cases,[20] and recent insights suggest that the carrier frequency for pathogenic variants in *PMS2* and *MSH6* in the general population is actually much higher than for *MLH1* and *MSH2*.[21] The majority of CMMRD patients carry variants in *PMS2*, followed by *MSH6*, while *MLH1* and *MSH2* variants are rarely associated with CMMRD.[16] One explanation for this phenomenon is that biallelic pathogenic variants in *MLH1* and *MSH2* may be embryonically lethal.[22,23] However, a higher carrier frequency for variants in *PMS2* and *MSH6* may also (partly) explain differences in the frequency of pathogenic variants in the MMR genes among patients with CMMRD.

Here we report cumulative cancer risks in family members of CMMRD patients with variants in the *PMS2* or *MSH6* genes. This study will not only help in the counseling of family members of CMMRD patients, but also represents a novel approach to determining cancer risk in Lynch syndrome.

MATERIALS AND METHODS

Data collection

Families were collected through international collaborations with clinical genetics departments and consortia and by following up CMMRD families described in literature. Corresponding authors were contacted to collect (more) family data. Family structure was recorded and information was collected on each family member regarding gender, variant status, cancer status and age at cancer diagnosis, and last contact or death. A diagnosis of CMMRD was considered confirmed if pathogenic variants were identified or if strong indicators of CMMRD were identified (i.e., phenotype and inheritance pattern plus aberrant immunohistochemistry and/ or microsatellite instability in non-neoplastic tissue and/or abnormal functional tests).[24]

As classified in the InSiGHT database (http://www.insight-database.org/classifications/), 31 unique class 4/5 pathogenic variants in *PMS2* and 19 class 4/5 pathogenic variants in *MSH6* were found in our cohort.[25] Another 30 variants in *PMS2* and 8 variants in *MSH6* have not been officially classified to date, but were deemed either class 4 or 5 (i.e., [likely] pathogenic) by an expert in the field (H.M.v. d.K.) according to InSiGHT variant classification criteria. Twenty variants of uncertain significance (VUS), distributed over 18 families, were identified and included in the analyses (Tables S1–S4). Seven of the VUS were identified in trans with a (likely) pathogenic variant. Since the patients carrying these VUS displayed a CMMRD phenotype this argues in favor of a functional impact of the variants on protein function. Furthermore, six of the VUS were identified in previously published CMMRD patients (Tables S3 and S4) and as such these variants were considered the most probable cause of the phenotype in these patients. The remaining seven variants were all identified in patients with a CMMRD phenotype and were considered a probable cause of the phenotype by the reporting laboratory and clinicians.

Statistical analysis

Eligible first- and second-degree family members for the risk analysis were defined based on complete data describing gender, age at cancer diagnosis, last contact or death, and status as a (possible) carrier of the *PMS2*/*MSH6* variant. Proven and obligate carriers as well as untested family members were included, whereas noncarriers, as confirmed by DNA analysis, were excluded. Known CMMRD patients were excluded from the analysis, as were (deceased) siblings of a CMMRD patient when they had a cancer within the CMMRD spectrum. In consanguineous families, family members with an unknown variant status, but a cancer diagnosis within the CMMRD cancer spectrum

at a young age (i.e., <25 years of age) were considered to be homozygous carriers and were thus excluded from the risk analysis. The total number of colorectal and endometrial cancers is described for the total cohort as well as for the part of the cohort included in the risk analysis. To avoid a reporting bias due to distant relatives (distant family members may be more likely to be included in the pedigree if they were affected, while unaffected distant family members may go unreported), only first- and second-degree relatives of the index patients were included in the risk analyses. This approach was supported by both visual inspection of the pedigrees and by an otherwise unexplained increase in colorectal cancer frequency among more distant family members (data not shown, available upon request).

Colorectal cancer risk is reported as cumulative incidence at age 70, accounting for death and other cancer diagnoses as competing risks.[26] Age at removal of a colon polyp was included as a censoring event because the likelihood of developing colorectal cancer is probably reduced after this preventive measure. Likewise, family members were censored at the development of any type of cancer, excluding basal cell carcinoma, because treatment of a cancer (e.g., by radiotherapy or chemotherapy) might influence future cancer risk.

To avoid testing bias, which may arise when the decision to undergo genetic testing is related to cancer status, we included untested family members in our study, weighted according to their genetic distance to confirmed carriers. Specifically, variant probabilities based on kinship coefficients were used as analytical weights in a Cox proportional hazard regression to model the hazard of developing colorectal cancer in the presence of competing events (death and other cancer diagnosis), and including sex as a covariate (for details see "Statistical Methods" in the Supplemental Data). For example, first-degree relatives of a confirmed carrier who were not tested were given a weight of 0.50, whereas second-degree relatives had a weight of 0.25. Confidence intervals (CIs) were obtained by bootstrapping at family level (1000 repetitions).

Medical ethical approval for this study was obtained through the ethics committee of Leiden University Medical Centre (reference number P14.090). Informed consent was not required because all data was collected anonymously.

RESULTS

After exclusion of the CMMRD cases, the *PMS2* cohort included 1809 family members from 77 families and the *MSH6* cohort consisted of 561 family members from 26 families.

Age at colorectal and endometrial cancer diagnosis

Sixty patients from 31 families were diagnosed with colorectal cancer in the total *PMS2* cohort, and 16 women from 14 families were diagnosed with endometrial cancer after excluding the CMMRD cases. Age of colorectal cancer diagnosis within this cohort ranged from 36 to 80 years, with a median age of 60 years. Age at diagnosis was unknown for 17 colorectal cancer cases (Table 1). For the 16 endometrial cancer cases, the age at diagnosis ranged from 40 to 85, with a median of 61 years. Age was missing for only one of these cases.

Seventeen patients from 12 families were diagnosed with colorectal cancer in the total *MSH6* cohort after exclusion of CMMRD cases. Age of colorectal cancer diagnosis in this cohort ranged from 42 to 58 years, with a median of 48 years (Table 1). There were five cases of endometrial cancer distributed over four families, with a median age at diagnosis of 54 years and an age range of 47 to 59 years.

Table 1. Cohort description, CMMRD patients excluded. CRC = colorectal cancer, EC = endometrial cancer

gene			total cohort	in risk analysis
PMS2	number of family members		1809	549
	gender	male female unknown	858 (47.4%) 728 (40.2%) 223 (12.3%)	299 (51.7%) 283 (48.3%) -
	carrier status	carrier unknown	369 1440	212 337
	age (years)	median (range) missing (n)	43.0 (0-94) 1235	49.0 (0-93) -
	CRC	n	60	21
	age at CRC diagnosis (years)	median (range) missing (n)	60.0 (36-80) 17	60.0 (36-80) -
	competing events (right censoring)			
	EC	n	16	6
	age at EC diagnosis (years)	median (range) missing (n)	61.0 (40-85) 1	61.5 (50-80) -
	other cancer or polypectomy/ hysterectomy	n	85	6
	age at other cancer diagnosis or removal of first polyp or uterus (years)	median (range) missing (n)	55.0 (5-85) 11	54 (5-84) -
	death	n	112	44
	age at death (years)	median (range) missing (n)	69.0 (0-94) 55	68.5 (0-93) -
MSH6	number of family members		561	148
	gender	male female unknown	299 (53.3%) 252 (44.9%) 10 (1.8%)	76 (51.4%) 72 (48.6%) -
	carrier status	carrier unknown	146 415	69 79
	age (years)	median (range) missing (n)	43.0 (3-86) 336	45.0 (1-85) -
	CRC	n	17	8
	age at CRC diagnosis (years)	median (range) missing (n)	48.0 (42-58) 4	47.5 (42-58) -
	competing events (right censoring)			
	EC	n	5	0
	age at EC diagnosis (years)	median (range) missing (n)	54.0 (47-59) 0	Not applicable
	other cancer or polypectomy/ hysterectomy	n	40	25
	age at other cancer diagnosis or removal of first polyp (years)	median (range) missing (n)	52.0 (7-78) 3	57.0 (23-78) -
	death	n	37	11
	age at death (years)	median (range) missing (n)	38.5 (1-81) 1	25.0 (1-73) -

Other cancers

While a range of other cancer types were reported in both the *PMS2* and *MSH6* cohort, low numbers did not allow risk analyses to be performed. The most commonly reported cancers were breast cancer, lung cancer, leukemia, and prostate cancer (Table 1 and Table S5).

Colorectal cancer risk

For individuals with CMMRD and variants in *PMS2*, 549 family members from 64 families were eligible for risk analysis; of these, 212 were confirmed or obligate carriers and the rest potential carriers. The estimated cumulative colorectal cancer risk at age 70 for heterozygous *PMS2* variant carriers was 8.7% (95% CI 4.3–12.7%, Fig. 1) for both sexes combined, and was 9.9% (95% CI 4.9–15.3%) for men and 5.9% (95% CI 1.6–11.1%) for women. Endometrial cancer risk could not be estimated due to the low number of events (n = 8).

For *MSH6*, 148 family members from 24 families were eligible for risk analysis; of these 69 were confirmed or obligate carriers and the rest potential carriers. The cumulative colorectal cancer risk at age 70 for heterozygous *MSH6* gene variant carriers was 11.8% (95% CI 4.5–22.7%, Fig. 2) for both sexes, and 10.0% (95% CI 1.8–24.5%) and 11.7% (95% CI 2.1–26.5%) for men and women, respectively. There were no cases of endometrial cancer that could be included in the risk analysis.

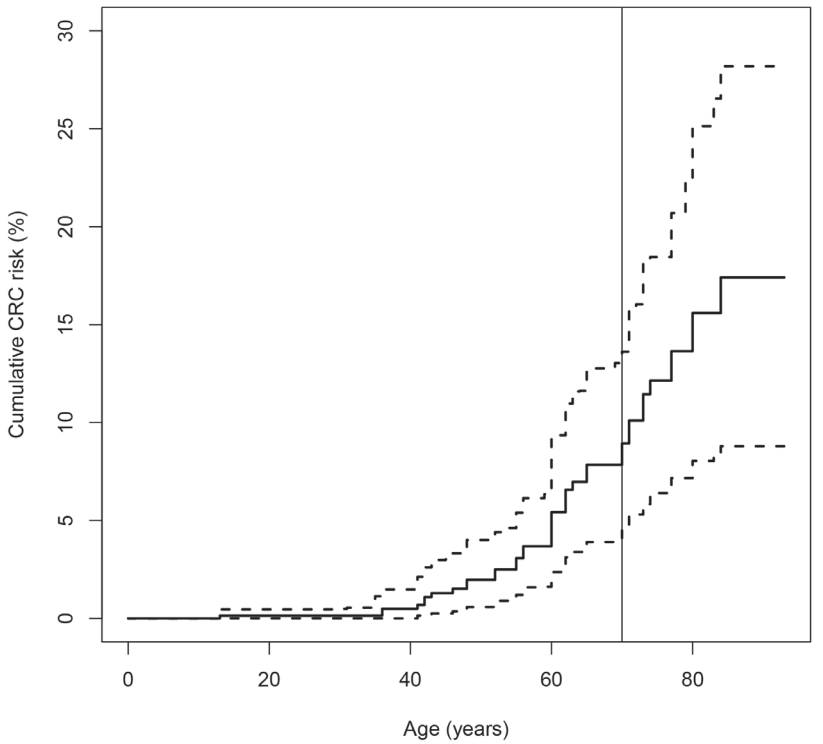

Figure 1 Cumulative colorectal cancer risk for carriers of a pathogenic PMS2 variant, men and women together, with 95% confidence intervals shown as dashed lines. CRC = colorectal cancer.

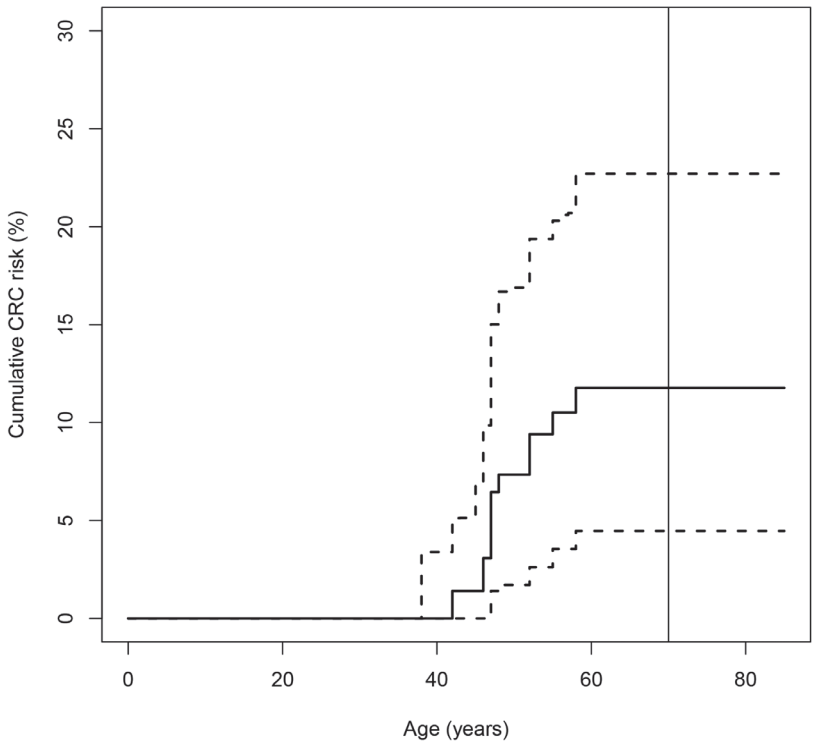

Figure 2 Cumulative colorectal cancer risk for carriers of a pathogenic MSH6 variant, men and women together, with 95% confidence intervals shown as dashed lines. CRC = colorectal cancer.

DISCUSSION

Using a new approach to establishing cancer risks in Lynch syndrome, we can confirm the low *PMS2*- and *MSH6*-associated colorectal cancer risks reported in previous studies that used ascertainment bias correction methods[10-12,14] or prospective data.[13,27] The main strengths of our approach were the reduction in clinical ascertainment bias by analyzing family members of CMMRD patients and the use of a competing risk analysis approach to avoid bias due to informative right censoring. Our results further indicate that gene-specific surveillance guidelines are needed to avoid subjecting carriers at low cancer risk to the invasive processes of surveillance, in some cases from an unnecessarily young age. The earliest age of colorectal cancer diagnosis was 36 and 42 years for *PMS2* and *MSH6*, respectively, well above the age (20–25 years) at which surveillance is usually started for individuals with Lynch syndrome.[28] This suggests that, in heterozygous carriers of *PMS2* or *MSH6* variants from families that do not meet clinical selection criteria for Lynch syndrome, surveillance could be started at a later age, e.g., at 35–40 years. Although current lifetime risk estimates are only slightly (2–3 times) elevated above the population risk of ~4%,[29] there are indications (e.g., from the median age at diagnosis) that risk is elevated at younger ages, and a faster progression from precursor lesion to carcinoma cannot be excluded. Therefore, we do not recommend that surveillance be omitted based on the current data. Furthermore, large variation in penetrance has been observed in clinically ascertained families, indicating that other risk factors may influence risk. Together these considerations suggest that our risk estimates remain useful when counseling families who were not ascertained based on criteria such as the Amsterdam II criteria and the (revised) Bethesda guidelines, e.g., families with a CMMRD proband or with a pathogenic MMR variant identified as an incidental finding through exome sequencing. However, they should be used with caution in more severely affected families, for example when a family history fulfills the Amsterdam criteria.[2]

Unfortunately, both cohorts were too small to provide risk estimations for endometrial cancer. It is striking that there were only some cases of endometrial cancer in the total *MSH6* cohort and none that could be included in the risk analysis, while the risk of endometrial cancer in *MSH6* has been reported to be high.[27] This may be partly due to the relatively low median age of 45 years (Table 1) of the cohort, while the youngest age at diagnosis of endometrial cancer was 47 years (Table 1).

There are some limitations to the current study. Firstly, genotype–phenotype correlations in Lynch syndrome and CMMRD have been proposed (although thus far no conclusive evidence has been yielded and some studies even show contradictory results).[30-35] If

correlations exist, variants with a milder phenotype might be overrepresented in a CMMRD cohort. For *PMS2*, age at cancer diagnosis and risk estimates were within the range of previous retrospective studies that corrected for ascertainment bias, indicating that we have not selected a cohort of (solely) low-risk PMS2 alleles.[10-12] Cancer risk estimates and age at cancer diagnosis for *MSH6* are similar to a study by Bonadona et al.,[36] but risk estimates are slightly lower than those reported by Baglietto et al.[37]

A possible mechanism for a genotype–phenotype correlation could be nonsense-mediated messenger RNA (mRNA) decay. Nonsense-mediated decay (NMD) detects mRNAs with premature termination codons and initiates their degradation, preventing potential dominant negative effects from truncated proteins.[38] Some variants, e.g., missense variants, are likely to escape NMD. To assess a possible role for NMD, we performed a stratified risk analysis that divided family members into groups based on whether their risk variant is expected to result in NMD, as described previously (Suerink et al.[30]). Family members were excluded from this analysis when no reliable prediction of NMD was available for the variant or if it was not known which variant segregated in which half of the family (maternal or paternal). This analysis produced no clear genotype–phenotype correlations and for both genes cases of colorectal cancer were seen in the NMD group as well as in the group with predicted retention of RNA expression. However, it should be noted that wide confidence intervals excluded detection of small differences (data available upon request). Whether risk stratification is possible based on genotype will require further study.

It could also be argued that a bias toward a milder phenotype is inherent to our cohort because those who die of cancer at a young age cannot have children with CMMRD. However, because both the parents and more distant relatives were included in the current analyses, it seems unlikely that this possible bias could have a major impact, particularly because the youngest age at colorectal cancer diagnosis within the total cohort was 36 years. Another potential problem was testing bias, which arises because family members with cancer are more inclined to undergo genetic testing. We therefore used variant probabilities based on genetic distance to confirmed carriers as analytical weights in our statistical analysis, which also enabled inclusion of untested family members. By including obligate carriers in the analysis there is a risk of misidentifying someone as a possible carrier because the CMMRD patient may have had a de novo variant. However, de novo variants are rarely reported in Lynch syndrome (2.3% in a cohort described by Win et al.[39]) and a large proportion (55% and 50% for *PMS2* and *MSH6*, respectively) of CMMRD index patients were homozygous for one variant and/or were from consanguineous families. Moreover, a major testing bias was not expected due to

a low overall cancer risk and because a relatively large proportion of confirmed carriers were obligate carriers (45/212 [21%] for the *PMS2* cohort and 21/69 [30%] for the *MSH6* cohort) whose testing status is by definition uninfluenced by their phenotype. It is worth mentioning that while our approach avoids clinical ascertainment bias, the selection strategy results in a relatively young cohort, which implies large uncertainty in the incidence estimation at older ages, as reflected by the broad confidence intervals in Figs. 1 and 2.

A final limitation of our study that could impact the reliability of data is the fact that most cancer diagnoses in this cohort were based on the proband's knowledge of family history rather than on medical records. Reassuringly, a 2011 study showed that the accuracy of reported colorectal cancer for first-degree family members was over 90%.[40] Because we included only first and second-degree family members, with family history reported by the parents in most cases, we expect a comparable accuracy rate in our risk analysis.

To complement and confirm the data presented here, we suggest a similar risk analysis should be performed in *PMS2* and *MSH6* families detected through universal screening of colorectal cancers for mismatch repair deficiency. These families will also be less affected with ascertainment bias.

In summary, we used an alternative approach to establish colorectal cancer risk in Lynch syndrome patients with *PMS2* and *MSH6* variants in CMMRD families. We confirmed this relatively low cancer risk relative to earlier, biased estimates of risk. These results underline the need for gene-specific surveillance protocols for *PMS2*- and *MSH6*-related Lynch syndrome families. Further investigations will be required to estimate the cancer risk for other Lynch syndrome–associated malignancies for *PMS2* and *MSH6*, as well as estimating unbiased cancer risks estimates for carriers of pathogenic variants in *MLH1* and *MSH2*.

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SUPPLEMENTAL MATERIAL

Supplemental table 1. *PMS2* variants

PMS2 variant[a]	Change at RNA and/ or protein level[b]	type of variant	classification[c]	Number of homozygous CMMRD index cases (families included in risk analysis)	Number of compound heterozygous CMMRD index cases (families included in risk analysis)
c.219T>A	p.(Cys73*)	nonsense	[5]	1 (0)	
c.400C>T	p.(Arg134*)	nonsense	5	1(1)	1 (1)
c.823C>T	r.823c>u, p.(Gln275*)	nonsense	[5]		1 (1)
c.862C>T	p.(Gln288*)	nonsense	[5]	1 (1)	
c.943C>T	p.(Arg315*)	nonsense	5	1 (1)	
c.949C>T	p.(Gln317*)	nonsense	5	1 (1)	
c.1840A>T	p.(Lys614*)	nonsense	5	1 (1)	
c.1882C>T	r.1882c>u, p.(Arg628*)	nonsense	5		1 (1)
c.1927C>T	p.(Gln643*)	nonsense	5		1 (1)
c.2192T>G	p.(Leu731*)	nonsense	[5]	1 (1)	
c.2404C>T	p.(Arg802*)	nonsense	5	2 (1)	
c.219_220dup	r.219_220dup, p.(Gly74Valfs*3)	frameshift	5	1 (1)	
c.247_250dup	r.247_250dup, p.(Thr84Ilefs*9)	frameshift	[5]		1 (1)
c.325dup	r.[325dup, 301_353del, 251_353del], p.([Glu109Glyfs*30, ?, ?])	frameshift	[5]		1 (1)
c.686_687del	p.(Ser229Cysfs*19)	frameshift	[5]	1 (1)	
c.736_741delinsTGTGTGTGAAG	r.736_741delinsugugugugaag, p.(Pro246Cysfs*3)	frameshift	5		3 (3)
c.794del	p.(Asn265Ilefs*42)	frameshift	[5]		1 (1)
c.904_911del	p.(Val302Thrfs*4)	frameshift	[5]		1 (1)
c.1020_1021del	p.(Arg341Alafs*23)	frameshift	[5]		1 (1)
c.1164del	p.(His388Glnfs*10)	frameshift	[5]	1 (0)	
c.1169_1170ins(20)	p.?	frameshift	5	1(1)	
c.1221del	p.(Thr408Leufs*40)	frameshift	5		1 (1)
c.1306dup	p.(Ser436Lysfs*22)	frameshift	5	1 (0)	
c.1486del	p.(His496Thrfs*99)	frameshift	[5]	1 (1)	
c.1500del	p.(Val501Trpfs*94)	frameshift	[5]	2 (1)	
c.1571dup	p.(Gly525Argfs*17)	frameshift	[5]	1 (1)	

PMS2 variant[a]	Change at RNA and/ or protein level[b]	type of variant	classification[c]	Number of homozygous CMMRD index cases (families included in risk analysis)	Number of compound heterozygous CMMRD index cases (families included in risk analysis)
c.1579del	p.(Arg527Glyfs*68)	frameshift	[5]		1 (1)
c.1730dup	p.(Arg578Alafs*3)	frameshift	5		2 (2)
c.1768del	p.(Ile590Phefs*5)	frameshift	5	1 (1)	
c.1831dup	p.(Ile611Asnfs*2)	frameshift	5		2 (2)
c.2117del	r.2117del; p.(Lys706Serfs*19)	frameshift	[5]		1 (1)
c.2361_2364del	p.(Phe788Cysfs*2)	frameshift	5		1 (1)
c.137G>T	r.137g>u, p.(Ser46Ile)	missense	4	5 (5)	10 (10)
c.319C>T	r.[c>u, 301_353del, 251_353del], p.([Arg107Trp, ?, ?])	missense	[3]		1 (1)
c.505C>G	p.(Arg169Gly)	missense	[3]		1 (1)
c.614A>C	r.614a>c, p.(Gln205Pro)	missense	3		1 (1)
c.812G>T	p.(Gly271Val)	missense	[3]	1 (1)	
c.917T>A	p.(Val306Glu)	missense	[3]	1 (1)	
c.2113G>A	p.(Glu705Lys)	missense	3		1 (1)
c.2249G>A	p.(Gly750Asp)	missense	3		2 (2)
c.2444C>T	r.2444c>u, p.(Ser815Leu)	missense	3	1 (1)	
c.2531C>A	p.(Pro844His)	missense	[3]	1 (1)	
c.1A>G	p.?	variant in initiation codon	4		3 (3)
c.1A>T	p.?	variant in initiation codon	[4]		1 (1)
c.24-2A>G	p.?	canonical splice variant	[4]	1 (1)	
c.251-2A>C	p.?	canonical splice variant	[4]		1 (1)
c.803+2T>G	p.?	canonical splice variant	[4]		1 (1)

PMS2 variant[a]	Change at RNA and/ or protein level[b]	type of variant	classification[c]	Number of homozygous CMMRD index cases (families included in risk analysis)	Number of compound heterozygous CMMRD index cases (families included in risk analysis)
c.804-2A>G	p.?	canonical splice variant	[4]		1 (1)
c.989-1G>T	p.?	canonical splice variant	5	1 (1)	
c.2007-2A>G	p.?	canonical splice variant	[4]	4 (1)	
c.2174+1G>A	p.?	canonical splice variant	5	1 (1)	1 (1)
c.2445+1G>T	r.2445_2446ins2445+1_2445+85, p.?	canonical splice variant	[4]	1 (1)	
c.825A>G	r.804_825del, p.(Ile269Alafs*31)	exonic splice variant	[3]		1 (1)
c.903G>T	r.804_903del; p.(Tyr268*)	exonic splice variant	4		1 (0)
c.24-12_107delinsAAAT	r.24_163del, p.(Ser8Argfs*5)	genomic deletion across canonical splice acceptor, resulting in skip of exon 2	5		2 (1)
genomic deletion including exon 1		large genomic deletion	5		1 (0)
genomic deletion including exon 7		large genomic deletion	5	3 (2)	
genomic deletion including exon 8		large genomic deletion	5		1 (1)
genomic deletion including exon 10		large genomic deletion	5		4(4)
genomic deletion whole gene (exons 1-15)		large genomic deletion	5		2 (2)
genomic deletion including exons 1-11		large genomic deletion	[5]		1 (1)

PMS2 variant[a]	Change at RNA and/ or protein level[b]	type of variant	classification[c]	Number of homozygous CMMRD index cases (families included in risk analysis)	Number of compound heterozygous CMMRD index cases (families included in risk analysis)
genomic deletion including exons 5-15		large genomic deletion	5		1 (1)
genomic deletion including exons 5-7		large genomic deletion	5		1 (1)
genomic deletion including exons 6-15		large genomic deletion	[5]	1 (0)	
genomic deletion including exon 7-8		large genomic deletion (in frame)	[4]		2 (1)
genomic deletion including exon 8-9		large genomic deletion	[5]		1 (1)
genomic deletion including exon 9-15		large genomic deletion	5		1 (0)
genomic deletion including exons 12-14		large genomic deletion	[5]	1 (1)	
genomic deletion including exons 13-15		large genomic deletion	[5]		1 (1)
genomic deletion including exons 14-15		large genomic deletion	[5]	1 (1)	1 (1)
mutation(s) not identified				2 (0)	1 (0)

[a.] Variant nomenclature according to HGVS guidelines (http://varnomen.hgvs.org/) with reference to NM_000535.5 for *PMS2* except 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* 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.] As recommended by HGVS, protein changes are presented in parentheses (predicted consequences, i.e. without experimental evidence from protein sequence analysis); RNA changes are provided if experimental RNA analyses are performed (information on RNA analysis extracted from supplemental tables of Van der Klift *et al*. 2015 *Mol Genet Genomic Med 3(4):327-45*, and van der Klift *et al*. 2016 *Hum Mutat 37(11):1162-1179*). [c.] Clinical variant class as reported on https://insight-database.org/variants/PMS2, last accessed on July 14th, 2018; 5 = pathogenic, 4 = likely pathogenic, 3 = variant of uncertain significance. Variants not present or present but not yet classified in the InSiGHT database were classified by us using guidelines provided by https://www.insight-group.org/criteria/. Suggested classes are given in square brackets. Nonsense and frameshift mutations, including large genomic deletions, were classified as pathogenic (class 5). Variants in the initiation codon, canonical splice variants and large in-frame genomic deletions were classified as likely pathogenic (class 4). Information on the class 3 variants that could not be classified *a priori* as (likely) pathogenic (the missense variants and the exonic splice variant) is provided in supplemental table 3.

Supplemental table 2. *MSH6* variants

MSH6 variant[a]	(predicted) protein variant[b]	type of variant	classification[c]	Number of homozygous CMMRD index cases (families included in risk analysis)	Number of compound heterozygous CMMRD index cases (families included in risk analysis)
c.642C>G	p.(Tyr214*)	nonsense	5		1 (1)
c.892C>T	p.(Arg298*)	nonsense	5	1 (1)	
c.2731C>T	p.(Arg911*)	nonsense	5	1 (1)	
c.2815C>T	p.(Gln939*)	nonsense	5		1 (1)
c.3020G>A	p.(Trp1007*)	nonsense	5		1 (1)
c.2653A>T	p.(Lys885*)	nonsense	[5]	1 (1)	
c.3450_3453del	p.(Ala1151*)	nonsense	[5]	1 (1)	
c.651dup	p.(Lys218*)	frameshift	5		1 (1)
c.1421_1422dup	p.(Gln475Cysfs*7)	frameshift	5		1 (1)
c.1596dup	p.(Glu533*)	frameshift	5		1 (1)
c.691del	p.(Val231Tyrfs*15)	frameshift	[5]	1 (1)	
c.1634_1635del	p.(Lys545Argfs*17)	frameshift	[5]		1 (1)
c.1800_1813dup	p.(Thr605Ilefs*10)	frameshift	[5]	1 (1)	
c.1998dup	p.(Asp667*)	frameshift	[5]		1 (1)
c.3261del	p.(Phe1088Serfs*2)	frameshift	5	1 (0)	2 (2)
c.3514dup	p.(Arg1172Lysfs*5)	frameshift	5		1 (1)
c.3482_3510del	p.(Pro1161Argfs*2)	frameshift	[5]	1 (1)	
c.3609_3612del	p.(His1203Glnfs*12)	frameshift	5		1 (1)
c.3635dup	p.(Asp1213Glyfs*2)	frameshift	5	1 (1)	
c.3939_3957dup	p.(Ala1320Serfs*5)	frameshift	5		1 (1)
c.3957dup	p.(Ala1320Serfs*5)	frameshift	5		1 (1)
c.3959_3962del	p.(Ala1320Glufs*6)	frameshift	5		1 (0)
c.3984_3987dup	p.(Leu1330Valfs*12)	frameshift	5		1 (0)
c.1763_1771dup	p.(His588_Pro590dup)	in-frame duplication	[3]	1 (1)	
c.2561_2563del	p.(Lys854del)	In-frame deletion	[3]		1 (1)

Supplemental table 2. *MSH6* variants

MSH6 variant[a]	(predicted) protein variant[b]	type of variant	classification[c]	Number of homozygous CMMRD index cases (families included in risk analysis)	Number of compound heterozygous CMMRD index cases (families included in risk analysis)
c.3386_3388del	p.(Cys1129_ Val1130delinsLeu)	in-frame deletion	3	1 (1)	
c.2098C>T	p.(Leu700Phe)	missense	3		1 (1)
c.1196C>T	p.(Pro399Leu)	missense	[3]		1 (1)
c.2061T>G	p.(Cys687Trp)	missense	[3]		1 (1)
c.2087T>C / c.3163G>A (on one allele)	p.(Ile696Thr)/p.(Ala1055Thr)	missense/missense	both 3		1 (1)
c.2216C>T	p.(Thr739Ile)	missense	[3]	1 (1)	
c.3226C>T	r.3226c>u, p.(Arg1076Cys)	missense	4		2 (2)
c.3725G>A	p.(Arg1242His)	missense	[3]	1 (1)	
c.458-1G>A	p.?	canonical splice variant	4		1 (1)
c.3801+1_3801+5del	r.3647_3801del, p.(Arg1217Metfs*6)	canonical splice variant	[4]		1 (1)
c.3991C>T	r.[3991c>u, 3802_4001del], p.([Arg1331*, Ala1268Glyfs*6])	nonsense + exonic splice variant (partial skip exon 9)	5		1 (1)

[a.] Variant nomenclature according to HGVS guidelines (http://varnomen.hgvs.org/) with reference to NM_000179.2 for *MSH6*. [b.] As recommended by HGVS, protein changes are presented in parentheses (predicted consequences, i.e. without experimental evidence from protein sequence analysis); RNA changes are provided if experimental RNA analyses are performed. RNA analysis reports for NM_000179.2: c.3226C>T in Thompson *et al.* 2013 *Hum Mutat 34(1): 200–209*, for NM_000179.2: c.3991C>T in *Plaschke et al. 2006 Eur J Hum Genet 14(5):561-566*. For NM_000179.2: c.3801+1_3801+5del, unpublished RNA analysis data shows a skip of exon 8 and diminished expression of the mutant transcript through nonsense-mediated mRNA decay (NMD); absence of normal mRNA transcribed from mutated allele not tested therefore classified by us as a likely pathogenic variant class 4 (personal communication HM van der Klift). [c.] Clinical variant class as reported on https://insight-database.org/variants/ MSH6, last accessed on July 14th, 2018; 5 = pathogenic, 4 = likely pathogenic, 3 = variant of uncertain significance. Variants not present or present but not yet classified in the InSiGHT database were classified by us using guidelines provided by https://www.insight-group.org/criteria/. Suggested classes are given in square brackets. Nonsense and frameshift mutations were classified as pathogenic (class 5). Information on the class 3 variants that could not be classified *a priori* as (likely) pathogenic (the missense variants and the small in frame deletion or duplication), is provided in supplemental table 4.

Supplemental table 3. *PMS2* variants of uncertain significance

PMS2 variant[a]	Type of variant	homozygous/compound heterozygous (index included in this study)	CMMRD phenotype or reference describing CMMRD phenotype
c.319C>T r.[c>u, 301_353del, 251_353del] p.([Arg107Trp, ?]) (exon 4)	missense (+altered expression ratio of transcripts)	*in trans* with genomic deletion of exon 10	No information.
c.505C>G p.(Arg169Gly) (exon 5)	missense	*in trans* with c.1831dup p.(Ile611Asnfs*2)	Mork *et al*. Fam Cancer 2016:15(4):587-591
c.614A>C r.614a>c p.(Gln205Pro) (exon 6)	missense	*in trans* with c.1A>G	Senter *et al*. Gastroenterology 2008:135(2):419-28
c.812G>T p.(Gly271Val) (exon 8)	missense	homozygous	Kruger *et al*. Eur J of Hum Genet 2008:16: 62–72
c.825A>G r.804_825del p.(Ile269Alafs*31) (exon 8)	exonic splice variant	*in trans* with c.325dup	Johannesma *et al*. Clin Genet 2011:80: 243–255
c.917T>A p.(Val306Glu) (exon 9)	missense	homozygous	Two siblings with a CMMRD phenotype, details available upon request.
c.2113G>A p.(Glu705Lys) (exon 12)	missense	*in trans* with genomic deletion of exon 7-8	Lavoine *et al*. J Med Genet 2015: 52(11):770-8
c.2249G>A p.(Gly750Asp) (exon 13)	missense	two families: *in trans* with whole gene deletion (Senter 2008) *in trans* with genomic deletion of exon 10 (Lavoine 2015)	Senter *et al*. Gastroenterology 2008:135(2):419-28 Lavoine *et al*. J Med Genet 2015: 52(11):770-8
c.2444C>T r.2444c>u p.(Ser815Leu) (exon 14)	missense	homozygous	Suerink *et al*. Clin Genet 2018:93(1):134-137
c.2531C>A p.(Pro844His) (exon 15)	missense	homozygous	Lavoine *et al*. J Med Genet 2015:52(11):770-8

[a.] Variant nomenclature according to HGVS guidelines (http://varnomen.hgvs.org/) with reference to NM_000535.5 for *PMS2*.

Supplemental table 4. MSH6 variants of uncertain significance

MSH6 variant[a]	Type of variant	homozygous/compound heterozygous (index included in this study)	CMMRD phenotype or reference describing CMMRD phenotype
c.1196C>T p.(Pro399Leu) (exon 4)	missense	*in trans* with c.2061T>G p.(Cys687Trp)	Patient with a CMMRD phenotype and loss of MSH6 expression in cancer and normal tissue. Further information available upon request.
c.1763_1771dup p.(His588_Pro590dup) (exon 4)	in-frame duplication of 3 amino acids	homozygous	Lavoine *et al*. J Med Genet 2015:52(11):770-8
c.2061T>G p.(Cys687Trp) (exon 4)	missense	*in trans* with c.1196C>T p.(Pro399Leu)	see c.1196C>T
c.2087T>C/ c.3163G>A (on one allele) p.(Ile696Thr)/p.(Ala1055Thr)	missense/missense	*in trans* with c.2098C>T	Patient with a CMMRD phenotype at age 30, details available upon request.
c.2098C>T p.(Leu700Phe)	missense	*in trans* with c.2087T>C/ c.3163G>A (on one allele)	See c.2087T>C/ c.3163G>A
c.2216C>T p.(Thr739Ile) (exon 4)	missense	homozygous	Lavoine *et al*. J Med Genet 2015:52(11):770-8
c.2561_2563del p.(Lys854del) (exon 4)	in-frame deletion of 1 amino acid	*in trans* with c.3261dup p.(Phe1088Serfs*2)	Bougeard *et al*. Fam Cancer 2014:13(1):131-5 Lavoine *et al*. J Med Genet 2015:52(11):770-8
c.3386_3388del p.(Cys1129_Val1130delinsLeu) (exon 5)	in-frame deletion, 2 amino acids replaced by another amino acid	homozygous	Menko *et al*. Fam Cancer 2004:3(2):123-7
c.3725G>A p.(Arg1242His) (exon 8)	missense	homozygous	Two siblings with a CMMRD phenotype and loss of MSH6 expression in the tumor and normal tissue. Functional testing as described by Bodo *et al*.[b] showed *ex vivo* microsatellite instability and tolerance to methylation. Further details available upon request.

[a.] Variant nomenclature according to HGVS guidelines (http://varnomen.hgvs.org/) with reference to NM_000179.2 for *MSH6*
[b.] Bodo *et al. 2015 Gastroenterology 2015 149(4):1017–1029*

Supplemental table 5. Frequency of other cancers

Cancer type	PMS2 (n=93)	MSH6 (n=34)
Leukaemia	10	3
Acute	2	2
Chronic	1	1
Not specified	7	0
Lymphoma	0	2
Gynaecological	3	2
Ovarian	1	0
Cervical	2	2
Prostate	9	2
Testicular	0	1
Respiratory tract	17	4
Lung	13	3
Upper airway/throat	4	1
Gastrointestinal tract	16	7
Biliary tract	3	0
Hepatic	1	1
Pancreatic	1	0
Duodenal	2	2
Stomach	6	2
Oesophageal	3	1
Not further specified	0	1
Urinary tract	2	4
Kidney	1	0
Bladder/ureters	1	4
Breast	14	2
Eye	0	1
Melanoma	4	1
Mesothelioma	0	1
Brain	4	1
Thyroid	1	1
Bone	1	1
Rhabdomyosarcoma	1	0
Teratoma	1	0
Mullerian tumor	1	0
Tumor of unspecified site	9	1

SUPPLEMENTALS STATISTICAL METHODS

Colorectal cancer (CRC) risk estimates are corrected by the presence of competing risks given by death and other cancer diagnoses, to account for the realistic possibility of the studied mutation affecting other cancer incidences and death. In general, the observed data in a competing risk setting is given by the failure time *T*, and the cause of failure *D (D=1,...k)*. In our case, we denote by *k* the cause of interest, CRC, and the CRC risk at age *t* is estimated by the cumulative incidence:

$$I_k(t|x) = \int_0^t h_k(s|x_i)S(s|x_i)ds \quad (1)$$

In this expression $h_k(t|x) = lim_{\Delta t \to 0} \frac{P(t \leq T < t+\Delta t, D=k|T \geq}{\Delta t}$ is the cause-specific hazard function, the hazard of failing from a given cause (CRC in our case) in the presence of the competing events (death and other cancer diagnosis) and *x* is the covariate sex. h_k is estimated using proportional hazard regression:

$$h_k(t|x) = h_{k,0}(t)\exp(\beta x) \quad (2)$$

In this equation is the baseline cause-specific hazard of cause *k* (CRC) and β is the effect of sex on cause *k*. To deal with the missing carrier status of some of the included individuals, we perform weighted regression, by including mutation probabilities as weights in the score function:

$$U_w(\beta) = \sum_{i=1}^{n} w_i \left[x_i - \frac{\sum_{j \epsilon R_i} w_j x_j \exp(x_j \beta)}{\sum_{j \epsilon R_i} w_j \exp(x_j \beta)} \right] \quad (3)$$

Analytical weight for individual *j*, *wj* = is given by the kinship coefficient between individual *j* and the closest family member with observed mutation. This probability is always positive for all the individuals in the studied cohort given the design based on the identification of at least one member carrying a biallelic mutation in each included family.

Once the cause-specific hazard is estimated using expressions (2) and (3), the cumulative cause-specific hazard can be calculated as $\Lambda_k(t|x) = \int_0^t h_k(s|x_i)ds$ and the marginal survival function, $S(t|x) = exp\left(-\sum_{k=1}^{K} \Lambda_k(t)\right)$ which is plugged in expression (1) to obtain the cumulative incidence of interest.

Confidence intervals (CI) were obtained by bootstrapping at family level (1,000 repetitions) to account for possible dependencies between family members.

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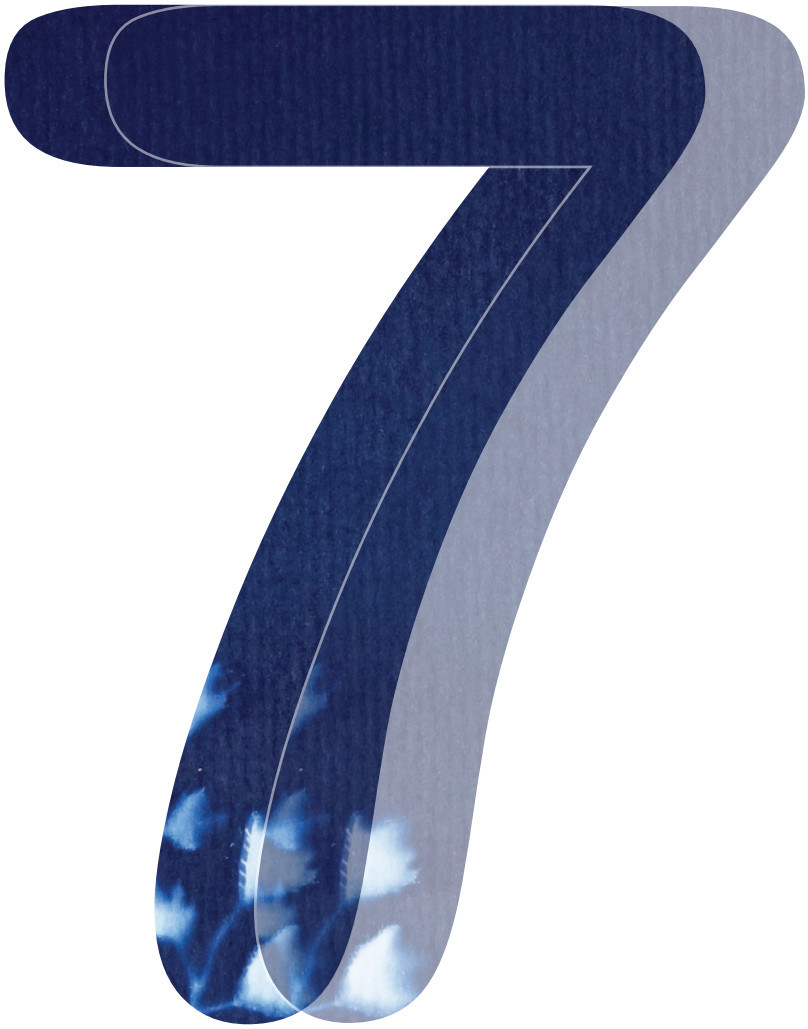

The effect of genotype and parent-of-origin on cancer risk and age of cancer development in PMS2 mutation carriers

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ABSTRACT

Purpose

Lynch syndrome (LS), a heritable disorder with an increased risk of primarily colorectal cancer (CRC) and endometrial cancer (EC), can be caused by mutations in the *PMS2* gene. We wished to establish whether genotype and/or parent-of-origin effects (POE) explain (part of) the reported variability in severity of the phenotype.

Methods

European *PMS2* mutation carriers (n=381) were grouped and compared based on RNA expression and whether the mutation was inherited paternally or maternally.

Results

Mutation carriers with loss of RNA expression (group 1) had a significantly lower age at CRC diagnosis (51.1 years versus 60.0 years, p=0.035) and a lower age at EC diagnosis (55.8 years versus 61.0 years, p=0.2, non-significant) compared to group 2 (retention of RNA expression). Furthermore, group 1 showed slightly higher, but non-significant, hazard ratios (HRs) for both CRC (HR: 1.31, p=0.38) and EC (HR: 1.22, p=0.72). No evidence for a significant parent-of-origin effect was found for either CRC or EC.

Conclusions

PMS2 mutation carriers with retention of RNA expression developed CRC nine years later than those with loss of RNA expression. If confirmed, this finding would justify a delay in surveillance for these cases. Cancer risk was not influenced by a parent-of-origin effect.

INTRODUCTION

A germline mutation in one of the mismatch repair (MMR) genes causes Lynch Syndrome (LS), an autosomal dominant disorder characterized by the clustering of colorectal (CRC) and endometrial cancer (EC) within affected families. Also higher risks have been reported for other cancers such as ovarian and urothelial cell cancer. However, thus far only one study confirmed these risks in *PMS2* mutation carriers.[1]

The MMR proteins normally act together to repair mismatches that occur during cell replication. MSH2 and MSH6 form a heterodimer that recognizes base–base mismatches and insertion/deletion mispairs, whereas MLH1 and PMS2 form a heterodimer that supports initiated repair.[2] A mutation can result in complete loss of protein or a protein with impaired function. Cancer risks associated with *PMS2* are lower than those reported for *MLH1* and *MSH2*.[1,3]

Phenotypes resulting from germ-line MMR gene mutations vary both among and within families.[4] Interfamilial variance might be partly attributable to known genotype–phenotype correlations of MMR genes,[5-7] whereas intrafamilial variance could be due to the influence of parental transmission on penetrance of the disease, a so-called parent-of-origin effect (POE). Although a POE has previously been reported in LS, studies have shown conflicting results.[8,9]

The aims of our study were to investigate genotype–phenotype relationships in *PMS2* mutation carriers and to explore a possible parent-of-origin effect in *PMS2*. Significant results would have implications for the surveillance and management of patients and their families.

MATERIALS AND METHODS

7

Patients

The study cohort included 381 pre-symptomatic and symptomatic mutation carriers (from 130 families) with a heterozygous *PMS2* mutation, and consisted of 120 apparently unrelated index patients and 261 relatives. Index cases with missing clinical data (n=1) or constitutional mismatch repair deficiency (CMMR-D) (n=9)[10] were excluded from analysis (due to a much younger age-of-onset and a different tumor spectrum compared to heterozygous mutation carriers, CMMR-D is considered a separate syndrome). When available, clinical data from participating clinical genetics departments (the Netherlands, Spain, Norway, Denmark, Sweden and Germany) were used to confirm the diagnosis. DNA analysis of patients and family members was conducted between 2007 and 2013, and in the majority of cases the analysis was

indicated due to compliance with the Bethesda criteria[11] or MSI-testing-indicated-by-a-Pathologist (MIPA) criteria[12]. When applicable, informed consent was obtained according to local approved protocols (LUMC Ethics Review Board, P01.019). Information on inheritance was available for 183 *PMS2* mutation carriers.

PMS2 mutations

The *PMS2* mutations included in this study were detected using a range of mutation detection strategies as applied by the diagnostic laboratories connected to the above-mentioned clinical genetic departments. All laboratories aimed at avoiding interference by pseudogenes by applying different methods, see table S1 for more details. Data on RNA splicing and transcript expression were available for around half of all mutations [13-17] The mutations were therefore classified into three genotype groups:

1. Mutations with an observed reduction in mRNA expression or entirely absent expression in RT-PCR screenings assays
2. Mutations without an effect on mRNA expression
3. Mutations for which no or inconclusive data on RNA analysis were available

PMS2 mutations were described according to Human Genetic Variation Society approved guidelines (http://www.hgvs.org/mutnomen/); see table 1 and table S1 for more details. Group 1 included a missense,c.903G>T (p.Tyr268) and a silent mutation,c.825A>G (p.Ile269Alafs*31) with a known effect on splicing, large genomic out-of-frame deletions, and deletions that involve the start and/or the end of the gene. Group 2 consisted of two missense mutations, 137G>T (p.Ser46Ile) and c.2113G>A (p.Glu705Lys), that were shown to be mismatch-repair deficient in a cell-free functional test.[18] Although no functional assay was available for a third missense mutation, c.2444C>T (p.Ser815Leu), analysis with three in-silico prediction programs classified this variant as deleterious (SIFT; score 0.00), probably damaging (Polyphen-2; score 1.00) and likely to interfere with function: (aGVGD; class C65).

Group 3 consisted of all mutations for which protein expression from the mutated allele was uncertain, including large genomic in-frame deletions, splice variants causing in-frame exon skipping (e.g. exon 10 deletion or skip), splice variants inducing multiple aberrant transcripts of which some are in-frame, and nonsense or frame-shift mutations that escape NMD due to their location in the gene.

Statistics

The Chi-square test and the Cox regression analyses were carried out separately for CRC and EC, using IBM SPSS Statistics 20. A p-value of <0.05 was considered to be statistically significant. Because the majority of group 3 mutations probably result in loss of RNA expression, mutation carriers in groups 1 and 3 were combined and then compared with group 2 in a sensitivity analysis.

For the CRC risk analyses, using the Cox regression method, mutation carriers were considered to be informative from birth until complete or partial colectomy, start of surveillance and/or first polyp detection, last contact or death. In the case of the EC risk analyses, mutation carriers were considered to be informative until hysterectomy, last contact or death. The development of CRC or EC was taken as the end point. Mutation carriers could reasonably be considered informative from birth because a very young age of cancer development would prompt genetic testing, resulting in no young index case being missed. These analyses resulted in hazard ratios comparing the two groups.

Generalized estimating equations (GEE) were used to rule out the possibility that Cox regression results were influenced by the coincidental clustering of family characteristics other than the mutation itself.

RESULTS

In 381 mutation carriers from 130 families, a total of 53 different *PMS2* germline mutations were found, with 248 mutation carriers carrying a mutation that results in loss of RNA expression. The most common mutations were c.736_741delCCCCTinsTC TCTCTGAAG, present in 61 mutation carriers, and c.1882C>T, present in 47 mutation carriers. Together, these two mutations accounted for 28.3% of all mutation carriers (table S1).

Genotype groups

Of the 282 mutation carriers in RNA groups 1 and 2, ninety-six developed CRC. a significant difference (p=0.035) was noted in mean ages of CRC development (51.1 versus 60.0 years) (see table 1). Comparing groups 1 and 2 produced no evidence for a significantly elevated risk of CRC development (HR1.31, p=0.38). Of the 155 women included in the analyses, 27 developed EC, with a slightly lower, but non-significant, mean age of EC development in group 1 compared to group 2 (55.8 years versus 61.0, p=0.2).

Evidence that males have a higher risk of developing an MMR-related CRC prompted us to include gender as a co-variate.[8,11] As expected, males had a higher risk (HR 1.72) of developing CRC than females (p=0.012). No statistically significant differences in EC development were found for the 140 women in the various genotype groups (HR 1.22, p=0.72).

Sensitivity analyses

For this analysis, mutation carriers in group 1 were combined with mutation carriers in group 3. This new and larger group of mutation carriers was then compared to mutation carriers in group 2. The HR for CRC development (HR 1.30, p=0.39) was

Table 1. Description of cohort.

		RNA-group		
		Group 1: Loss of RNA expression N = 248 (% or range)	Group 2: Retained RNA expression N=34 (% or range)	p-value*
Sex	male (%)	97 (39.1)	18 (52.9)	0.20
	Female (%)	142 (57.3)	16 (47.0)	
	Unknown (%)	9 (3.6)	0	
Cancer#	CRC (%)	84 (33.9)	12 (29.4)	0.87
	EC (% of females)	23 (16.2)	4 (25.0)	0.40
	other cancer (%)	32 (12.9)	3 (8.8)	0.50
	no cancer (%)	121 (48.7)	18 (52.9)	
Mean age of Cancer	age CRC (age range)	51.1 (25 – 86)	60.0 (43 – 79)	0.035
	age EC (age range)	55.8 (46 – 68)	61.0 (54 – 68)	0.2
Parent-of-Origin	father (%)	54 (21.8)	8 (23.5)	0.96
	mother (%)	64 (25.8)	9 (26.5)	
	unknown (%)	130 (52.4)	17 (50.0)	

CRC=colorectal cancer, EC=endometrial cancer, *Variables were tested for assumed equal distribution using a Pearson-Chi square test.# Some individuals had more than one form of cancer.

similar to the HR for just groups 1 and 2. The HR for EC was slightly lower (HR 1.07, p=0.91). Additional analysis using GEE showed no significant differences for the different genotype groups after clustering the data, although male gender remained a significant risk factor for the development of CRC.

Parent-of-origin

The parent of origin was known for 183 of the 381 mutation carriers. Of these, 39 developed CRC and 9 females developed EC. Inheritance of a mutation via either the paternal or maternal line did not significantly influence the mean age of CRC (46.9 versus 45.6 years, p= 0.68) or EC onset (49.2 versus 55.5 years, p=0.23). Cox regression analysis, in both the group as a whole and following separate analysis of males and females, also produced no evidence for a POE. The colorectal cancer HR associated with paternal inheritance of mutations was comparable to that for maternal inheritance (0.80, p=0.51). For endometrial cancer, the hazard ratio was 1.73 (p=0.46, table 2).

Table 2. Hazard ratios for genotype effect (genotype group 1 vs. group 2) and POE paternally vs. maternally inherited mutations)

			HR	Confidence Interval	P-value
Genotype	CRC	All carriers	1.31	0.71 – 2.42	0.38
		Index cases	1.58	0.67 – 3.71	0.30
		c.2444C>T excluded	1.39	0.74 – 2.61	0.31
	EC	All carriers	1.22	0.42 – 3.56	0.72
		Index cases	0.91	0.21 – 4.05	0.91
		c.2444C>T excluded	1.32	0.39 – 4.47	0.65
POE	CRC	All patients	0.80	0.41 – 1.57	0.51
		Males	0.94	0.39 – 2.24	0.89
		Females	0.68	0.24 – 1.97	0.48
	EC		1.73	0.41 – 7.22	0.46

HR = hazard ratio, CRC = colorectal cancer, EC = endometrial cancer,
POE = parent-of-origin effect.

DISCUSSION

Mutation carriers with a *PMS2* mutation that results in the loss of RNA expression develop CRC, on average, nine years earlier than carriers of mutations that do not effect RNA expression. An explanation for this finding could be that, in the latter group, protein with some residual function is still produced. Indeed, while some (functional) protein expression (~25%)[19] and a (limited) repair function was found for the c.2113G>A mutation, functional studies demonstrated severely impaired repair efficiency,[18,19] perhaps suggesting that a severely impaired protein is still superior to no protein at all.

Further support for the notion that mutations leading to retention of mRNA expression result in a milder phenotype comes from the underreporting of this type of mutation. Both in the present study and that of Senter *et al.*, the majority of *PMS2* mutations found in patients result in loss of RNA expression.[3] On a population basis, there is no obvious reason why fewer group 2 mutations would occur compared to group 1 mutations, suggesting that individuals with group 2 mutations may have less severe phenotypes and/or no family history and are therefore less likely to be referred to a clinical geneticist. This idea has been suggested previously by Beck *et al.*[20], who found a relative overabundance of missense *MLH1* and *MSH2* mutations in 10 families which failed to meet the Amsterdam I criteria, compared to families that meet the criteria.

There are a number of shortcomings of our study. For one, the inclusion of affected family members of index patients might have resulted in bias due to the phenotypes of relatives being more similar than phenotypes of unrelated index cases. We attempted to overcome this problem using GEE analysis. We also repeated the analyses for index patients alone, which resulted in similar HR and p-values (Table 2). Another shortcoming was the relatively small number of patients in group 2 (retained RNA expression), reducing statistical power. This may explain non-significant results and indicates the need for analyses in larger patient groups. Also, in this group, one mutation, the c.2444C>T, found in one family, is classified as VUS/Class 3 in the InSight database (link: http://insight-group.org/variants/database/), and larger cohorts are still needed to prove its pathogenicity. When excluding the family (4 relatives) form the analyses similar HR and p-values were found (Table 2). Lastly, two mutations in the cohort are overrepresented in our cohort (namely the c.736_741delinsTGTGTGTGAAG and the c.1882C>T mutation) might dominate the results. When comparing patients with these mutations and patients with other mutations no significant differences in mean age of CRC or CRC risk (HR) were found though.

We were unable to confirm earlier reports that found a significant POE.[8,9] However, a trend was observed towards a lower HR for CRC in females with a paternally-inherited mutation. This is broadly in line with the results of van Vliet *et al*. for the males in their research population, although their results showed a much higher, and significant, HR of 3.2 (p=0.03) for males when comparing maternally-inherited mutations to paternally-inherited mutations.[9]

A possible explanation for the differences in POE findings could be the fact that van Vliet *et al*. used another statistical approach - a modified segregation analyses.[9] We did not use this broad approach because, to the best of our knowledge, no bias or confounders were present in our cohort that would make a modified segregation analysis necessary. The possible exception would be a POE-dependent selection bias; for example, if mutation carriers with a maternally-inherited mutation were more severely affected, more carriers of a maternally-inherited mutation with CRC would be expected in our database. Using a chi square test, we therefore analyzed whether there was a bias in maternal inheritance for mutation carriers with CRC compared to those without CRC. This was not the case (p-value = 0.12).[8,9]

With the ever-wider adoption of whole genome DNA analysis, more families with *PMS2* mutations will be identified in the near future, including some with no apparent history suggestive of Lynch syndrome. Because many of these families may have milder phenotypes, studies such as ours provide useful advice on surveillance programs for these mutation carriers. Should our results be confirmed in larger studies, the significant age differences in CRC development reported here provide some justification for starting surveillance at a later age for mutation carriers who show retention of *PMS2* RNA expression.

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SUPPLEMENTARY INFORMATION

Table S1

Mutation[a,b]	Predicted protein change	RNA group[d]	references RNA analysis[e]	Number of mutation carriers (families)	Frequency (%)
c.736_741delinsTGTGTGTGAAG	p.Pro246Cysfs*3	1	1	61 (25)	15.9
c.1882C>T	p.Arg628*	1	1	47(14)	12.3
deletion exon 11 - 15 (c.1145-1350_ *20545del)	p.?	(1)	na	23(4)	4.7
c.2192_2196del	p.Leu731Cysfs*3	1	1	18(6)	4.4
c.697C>T	p.Gln233*	1	1	13(5)	3.7
c.1831dup	p.Ile611Asnfs*2	(1)	na	10(3)	2.6
deletion exon 1 – 11[c]	p.?	(1)	na	9(1)	2.3
c.823C>T	p.Gln275*	1	2	8(2)	2.1
deletion of the whole gene	p.0	(1)	na	7(3)	1.8
c.1112_1113delinsTTTA	p.Asn371Ilefs*2	(1)	na	5(1)	1.3
c.325dup	p.Glu109Glyfs*30	1	2	5(3)	1.3
c.1079_1080del	p.Ile360Argfs*4	(1)	na	4(1)	1
c.2117delA	p.Lys706SerfsX19	1	2	4(1)	1
c.861_864del	p.Arg287Serfs*19	1	1	4(1)	1
c.903G>T (skips exon 8)	p.Tyr268*	1	3	3(1)	1
c.1145-?_c.2006-?del (deletion exon 11)[c]	p.?	(1)	na	3(1)	0.8
c.2155C>T	p.Gln719*	1	2	3(2)	0.8
c.804-60_804-59insJN866832.1	p.?	1	4	3(2)	0.8
c.1214C>A	p.Ser405*	(1)	na	2(1)	0.5
c.2156delA	p.Gln719Argfs*6	(1)	na	2(1)	0.5
c.354-1G>A	p.?	(1)	na	2(1)	0.5
c.251-2A>C	p.?	(1)	na	2(2)	0.5
c.856_857del	p.Asp286Glnfs*12	(1)	na	1(1)	0.3
c.1261C>T	p.Arg421*	(1)	na	1(1)	0.3
c.211_214delAATG	p.Asn71Aspfs*4	(1)	na	1(1)	0.3
c.658dup	p.Ser220Lysfs*29	(1)	na	1(1)	0.3
c.904_911delGTCTGCAG	p.Val302Thrfs*4	(1)	na	1(1)	0.3
c.989-?_2275+?del (deletion exon 10-13)[c]	p.?	(1)	na	1(1)	0.3
deletion exon 5 - 15[c]	p.?	(1)	na	1(1)	0.3
deletion exon 9 -11[c]	p.?	(1)	na	1(1)	0.3
c.247_250dupTTAA	p.Thr84Ilefs*9	1	2	1(1)	0.3
c.825A>G (first 22 nucleotides exon 8 spliced out)	p.Ile269Alafs*31	1	5	1(1)	0.3
c.137G>T	p.Ser46Ile	2	1	19(8)	5
c.2113G>A	p.Glu705Lys	(2)	na	11(2)	2.9
c.2444C>T	p.Ser815Leu	2	1	4(1)	1
deletion exon 5 – 7[c]	p.?	3	na	18(5)	4.7
deletion exon 14	p.?	3 (no NMD observed)	1	11(3)	2.9
c.219_220dup	p.Gly74Valfs*3	3 (partial NMD observed)	1	10(3)	2.6

Mutation[a,b]	Predicted protein change	RNA group[d]	references RNA analysis[e]	Number of mutation carriers (families)	Frequency (%)
c.24-12_107delinsAAAT	p.Ser8Argfs*5	3 (no NMD observed)	1	9(2)	2.3
c.989-1G>T	p.?	3 (no NMD observed)	6	9(1)	2.3
c.989-2A>G	p.Glu330_Glu381del	3 (no NMD observed)	7	8(1)	2.1
deletion exon 2[c]	p.?	3	na	7(4)	1.8
c.319C>T	p.Arg107Trp	3 (change in ratio alternative transcripts)	2	7(1)	1.8
c.2404C>T	p.Arg802*	3	na	4(2)	1
c.1144+2T>A	p.Glu330_Glu381del	3 (no NMD observed)	1	4(1)	1
deletion exon 10	p.?	3	na	3(2)	0.8
c.2174+1G>A	p.?	3 (multiple transcripts)	2	3(1)	0.8
c.1A>G	p.?	3	na	1(1)	0.3
c.989-296_1144+706del (deletion exon 10)	p.Glu330_Glu381del	3	na	1(1)	0.3
deletion exon 6 - 7	p.?	3 (multiple transcripts)	2	1(1)	0.3
c.163+2T>C	p.Ser8Argfs*5	3 (no NMD observed)	1	1(1)	0.3
deletion exon 3 - 7	p.?	3 (no NMD observed)	1	1(1)	0.3
c.2445+1G>T	p.?	3 (no NMD observed)	2	1(1)	0.3
Total				381 (134[f])	100

7

[a] Except large genomic deletions, mutations were described according to the Human Genetic Variation Society approved guidelines (http://www.hgvs.org/mutnomen/) with reference to PMS2 GenBank reference sequence NM_000535.5. The large genomic rearrangements, nonsense, frame-shift, and canonical splice site mutations in this study are considered pathogenic or likely pathogenic (class 5 or 4).[1]

[b] To avoid interference of pseudogene sequences using long range PCR, either with cDNA or genomic DNA as template was used for detection of point mutations and small insertions and deletions.[2-5] Mutations were found using different techniques, depending on the involved diagnostic laboratory.

[c] The large deletions were mostly detected using the multiplex ligation-dependent probe amplification (MLPA) kit P008-A1 (MRC-Holland, Amsterdam, the Netherlands). This MLPA kit version lacks (reliable) probes for PMS2 exon 3, 4, 12, 13, 14 and 15. Because the exact extent of these deletions is often not characterized, they are included with an informal description.

[d] 1=no mRNA expression from mutated allele, 2=normal mRNA expression; 3=RNA expression unknown, or mRNA present but with exon(s) skipped

[e] references 1=van der Klift et al 2010[4]; 2=van der Klift, unpublished observations; 3= microattribution Mensenkamp & Ligtenberg in LOVDdb ; 4=van der Klift, 2012[6]; 5=Johannesma et al.2011[7]; 6=Sjursen et al 2009[8]; 7=Borras et al 2013[9]; na=not available

[f] the total number of families in is 134 because four families carry two different segregating mutations.

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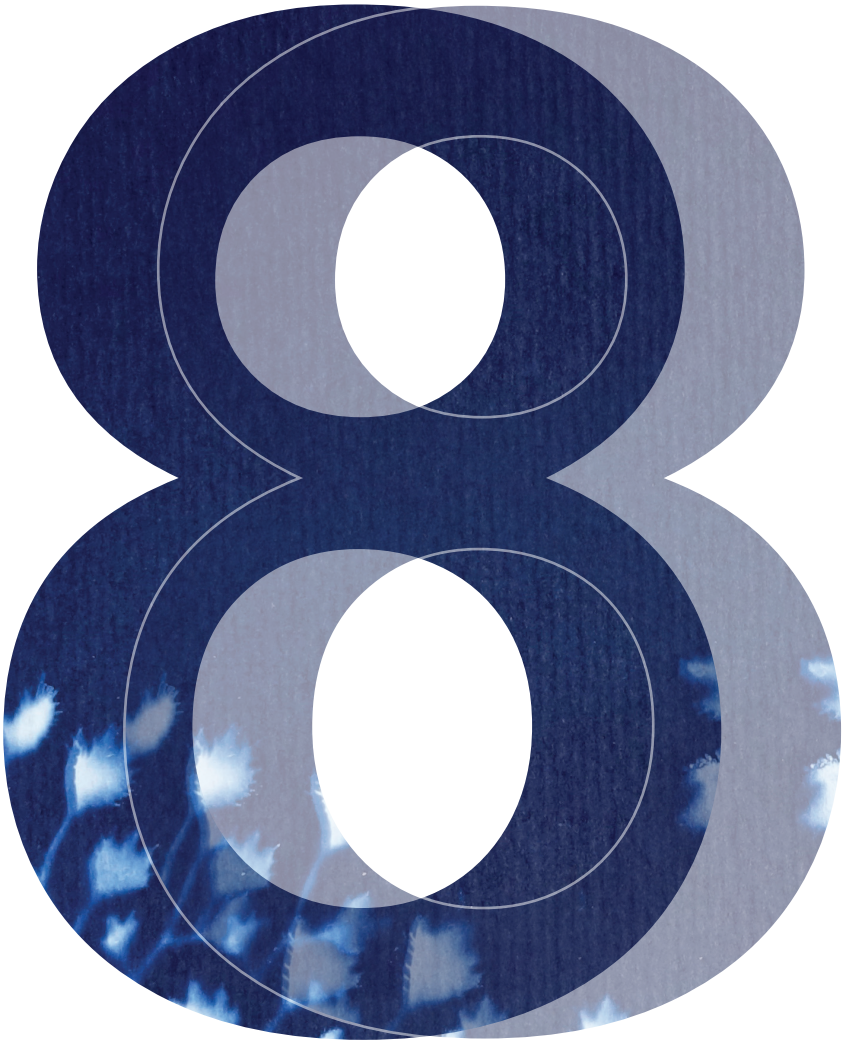

Incidence of (adenomatous) polyps and colorectal cancer in patients with PMS2-associated Lynch syndrome undergoing surveillance: a prospective cohort analysis

Manuscript in preparation

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ABSTRACT

Purpose

Lynch syndrome predisposes carriers of a heterozygous pathogenic germline variant in the *MLH1*, *MSH2*, *MSH6* or *PMS2* genes to the development of mainly colorectal (CRC) and endometrial cancer. Of the four mismatch repair (MMR) genes, *PMS2* variant carriers have 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 incident CRC in *PMS2* variant carriers undergoing regular surveillance.

Methods

We collected a cohort of 171 *PMS2* variant carriers and recorded the occurrence and characteristics of incident adenomas and CRC. 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 (1044 observation years, median surveillance interval 2 years), 435 polyps were removed, of which 237 (54.5%) were adenomatous. Forty-one (16.9%) of those 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 in the pathway to cancer. One incident CRC was reported.

Conclusion

In this large cohort of *PMS2* variant carriers, only one incident CRC were observed. This tumor was preceded by a colonoscopy with insufficient bowel preparation. Further analyses are required to draw firm conclusions about adenoma risk in *PMS2* carriers compared to the other MMR genes.

INTRODUCTION

Lynch syndrome predisposes carriers of a germline heterozygous pathogenic variant in one of the mismatch repair genes (MMR): *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 immunohistochemical staining for the involved MMR protein.[1,2] To prevent the development of colorectal cancer, patients with Lynch syndrome are offered surveillance by colonoscopy every 1-2 years, starting at age 25.[3] Despite these regular surveillance colonoscopies, incident colorectal cancers do occur, particularly in *MLH1* and *MSH2* variant carriers.[4-7] Data on polyps and incident colorectal cancer 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 (1:714).[8] *PMS2* variant carriers display a distinct phenotype, with retrospective cohort studies reporting substantially lower cancer risks compared to carriers of *MLH1* and *MSH2* variants,[9-11] which has resulted in discussion of MMR-gene-specific surveillance protocols.[4] This discussion would be greatly assisted by more prospectively collected gene-specific data. Previously, the prospective Lynch syndrome database (PLSD) consortium has confirmed low cancer risks for carriers of pathogenic variants in the *PMS2* gene.[4,12-14] However, these studies did not include exact data on endoscopic detection of adenomas, which is essential for a better understanding of the role of MMR deficiency in Lynch syndrome associated carcinogenesis. Moreover, data on quality of surveillance is usually also lacking. To this aim, we collected prospective colonoscopy data on a large cohort of *PMS2* variant 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.[15] 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 fourteen *PMS2* variant carriers both the PALGA search and request for colonoscopy reports did not yield any results, therefore these patients most likely are not undergoing regular surveillance and they were excluded from the analyses. Furthermore, one patient was excluded from the analyses, because of an exceptionally severe phenotype (three synchronous colorectal cancers and 18 adenomas at age 26 and an intellectual disability). This extraordinary phenotype is likely not completely explained by his *PMS2* variant alone. The study was approved by the IRB of the LUMC.

PMS2 variant analysis

Our cohort consisted of clinically ascertained families in which variant analysis was initiated because a family met the Bethesda criteria[16] and/or (histological) pre-screening by immunohistochemistry and/or microsatellite instability was indicative of MMR deficiency. Germline *PMS2* variant screening was performed as previously described.[10,11,17] Comprehensive strategies were applied to avoid unreliable variant detection caused by interference from pseudogene sequences and frequent gene conversion events.[17] 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. Timepoint zero was the time at first colonoscopy. Advanced adenomas were defined by a size of ≥1 cm in diameter, a villous component of >25%, and/or the presence of high-grade dysplasia.

Results were compared to data from two studies. One study by Engel et al. which reports the occurrence of incident adenomas and advanced adenomas in a large

cohort of *MLH1*-, *MSH2*-, and *MSH6*-associated Lynch syndrome patients.[18] Forsberg et al. report more detailed data on histological subtypes and numbers of (adenomatous) polyps at first colonoscopy in a cohort of MLH1-, MSH2-, and MSH6-associated Lynch syndrome patients and compare this data to control data from an earlier prospective population-based colonoscopy study by the same group.[19]

RESULTS

Between 1987 and 2017, a total of 675 colonoscopies were performed in this cohort of 171 *PMS2* variant carriers, representing 1044 years of follow-up. The median time between follow-up colonoscopies was 2.0 years. 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.[10,11,17,20] A detailed description of the cohort is provided in table 1.

Polyps

In total, 435 polyps were removed from 171 *PMS2* variant carriers, half of which were adenomatous (54.5%). Figure 1 shows the cumulative risk of developing an adenoma after the first colonoscopy. The risk of developing an adenoma is 54.5% (95% CI 41.4 – 68.8%) after 10 years. This is higher than the risks reported for carriers of a mutation in the other genes as reported by Engel et al (44.2% for *MSH2,* 38.4% for *MSH6* and 32.2% for *MLH1*).[18]

Figure 2 shows the cumulative risk of developing an advanced adenoma after first colonoscopy, which was 23.7% (95% CI 12.3 – 43.0%) after 10 years. This risks appears to be higher than for carriers of a pathogenic variant in the other genes as reported by Engel et al.[18] However, because of a wide confidence interval, no reliable comparison can be made.

When comparing the cumulative proportion of individuals with an adenoma at first colonoscopy as a function of age between our *PMS2* cohort (supplemental figure 1) and the cohorts as published by Forsberg et al.[21], the *PMS2* cohort shows a lower adenoma risk than the Forsberg Lynch cohort, but a higher risk than the Forsberg control cohort. The same can be said for the cumulative proportion of advanced adenomas (supplemental figure 2).

The sixteen adenomas with low-grade dysplasia, two sessile serrated lesions and two hyperplastic polyps stained for PMS2 protein expression showed normal staining (table 2).

Table 1. Cohort characteristics

	PMS2 cohort	MLH1 (Engel et al)	MSH2 (Engel et al)	MSH6 (Engel et al)
Patients	171	1407	986	354
Men	69 (40.4%)	47.8%	49%	45.2%
Follow-up (years)				
Total	1044	12798	7961	2550
Mean (s.d.)	6.1 (5.9)			
Median (IQR)	4.2 (1.7-9.0)	8.5 (4.2-13.2)	7.4 (4.4-11.3)	6.5 (4.1-9.4)
Range	0-28.4			
Colonoscopies				
Total	675	8299	6300	1798
Number per patient				
Mean (s.d.)	3.9 (3.0)			
Median (IQR)	3 (1-5)	5 (3-8)	6 (4-8)	4 (3-6)
Range	1-18			
Time interval (years)$				
Mean (s.d.)	2.1 (1.9)			
Median (IQR)	2.0 (1.1-2.2)			
Range	0.02-22.5			
Mean age first colonoscopy (s.d.)	50.6 (12.9)	42.7 (13.5)	44.0 (12.3)	48.7 (13.7)
Mean age first adenoma detected (s.d.)	55.3 (12.5)			
Mean age first advanced adenoma detected (s.d.)	56.8 (13.1)			
Total polyps	435			
Hyperplastic polyps	181 (41.6%)			
Location				
Right-sided	52 (28.7%)			
Left sided	111 (61.3%)			
Not specified	18 (9.9%)			
Sessile serrated polyps/adenomas*	16 (3.7%)			
Location left-sided				
Right-sided	8 (50%)			
Left sided	8 (50%)			
Not specified	0			
Mixed	1 (0.2%)			
Adenomas	237 (54.5%)			
Histology				
Tubular adenoma	154 (65%)			
Tubulovillous adenoma	23 (9.7%)			
Villous adenoma	1 (0.4%)			
Sessile serrated adenoma with dysplasia	12 (5.1%)			
Adenoma n.o.s.	47 (19.8%)			

	PMS2 cohort	MLH1 (Engel et al)	MSH2 (Engel et al)	MSH6 (Engel et al)
Size (mm)				
0-4	134 (56.5%)			
5-10	50 (21.1%)			
10<	21 (8.9%)			
Not specified	32 (13.5%)			
Location				
Right-sided	92 (38.8%)			
Left sided	120 (50.6%)			
Not specified	25 (10.6%)			
Dysplasia				
None	1 (0.4%)			
High grade	6 (2.5%)			
Low grade	222 (93.7%)			
Not specified	8 (3.4%)			
Advanced	41 (16.9%)			

n.o.s.: not otherwise specified, IQR: Interquartile range; s.d.: Standard deviation
Advanced: adenomas ≥1 cm in diameter, villous component, and/or high-grade dysplasia
$ only if >1 colonoscopy was performed
* Sessile serrated adenomas were listed in this category if there was no dysplasia

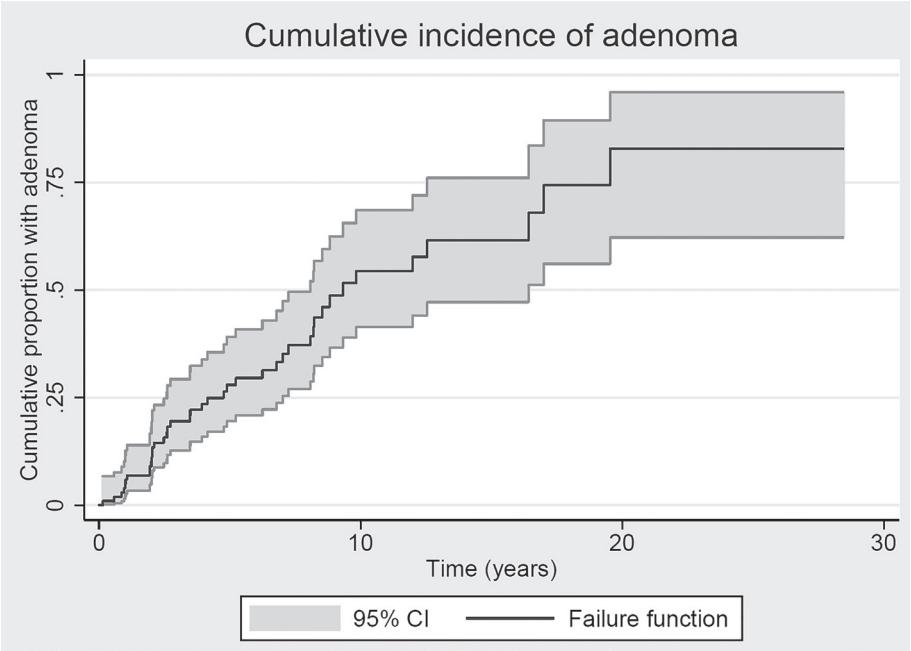

Figure 1 Cumulative proportion of *PMS2* carriers with an adenoma since start of colonoscopy (t=0) with 95% confidence intervals

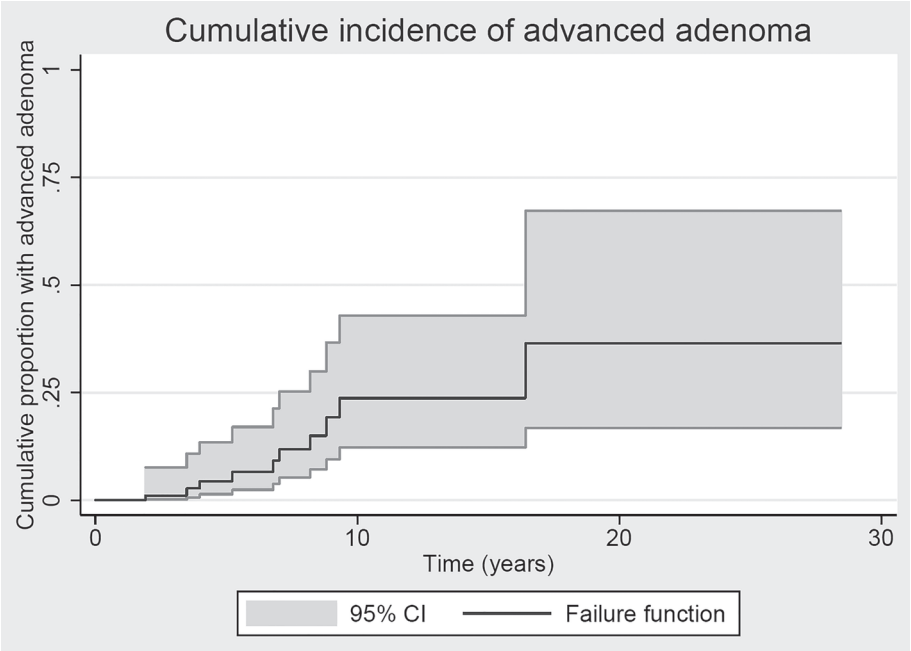

Figure 2 Cumulative incidence of advanced adenomas in *PMS2* carriers with 95% confidence intervals, t=0 is first colonoscopy.

8

Table 2. Polyps stained for PMS2 protein expression
n.a.: not applicable; n.o.s.: not otherwise specified; CRC: colorectal cancer

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	+

One *PMS2* variant carrier developed an incident colorectal cancer despite undergoing biennial regular colonoscopic surveillance (table 3). The patient presented with colorectal cancer at age 65, was diagnosed with Lynch syndrome, and had a subsequent incident colorectal cancer at age 75. However, this patient had a record of incomplete colonoscopies due to insufficient bowel cleansing, including the colonoscopy preceding the colorectal cancer. The tumor was immunohistochemically stained for MMR protein expression which revealed absent PMS2 staining, as did the initial tumor.

Table 3. *PMS2* carrier with an incident CRC

Sex	Male
Surveillance scopies	10
Years of surveillance	11
Time since last scopy before incident CRC	2 years
Findings at last scopy/clinical evaluation before incident CRC	No adenomas were removed during colonoscopy. There was poor bowel preparation. One adenoma with low grade dysplasia was removed from the stoma of the patient.
Initial CRC	
Age	65
Location	Rectum
IHC	PMS2-
MSI	MSI-H
Incident CRC	
Age	75
Location	Transverse colon
IHC PMS2	Absent
MSI	NA

CRC: colorectal cancer; MSI: Microsatellite instability; IHC: Immunohistochemistry

8

DISCUSSION

PMS2-associated Lynch syndrome is characterized by relatively low penetrance of colorectal cancer, both in retrospective cohorts of patients who are not under surveillance, as well as in prospective cohorts where patients receive regular colonoscopies.[10,11,22-24] Our study confirms the very low risk for colorectal cancer in *PMS2* variant carriers who undergo regular colonoscopic surveillance. Recent studies have shown that MMR deficient (MMR-d) colorectal cancer in Lynch syndrome patients may develop not only through the traditional MMR proficient (MMR-p) adenoma-to-colorectal cancer progression pathway, but may also arise from the MMR-d crypt pathway.[25-28] Tumors arising via this latter pathway directly proceed from MMR-d crypt to cancer or can first develop into an MMR-d adenoma before becoming malignant.[26,27] The cancers that develop directly from an MMR-d crypt lack a benign precursor lesion and cannot be prevented by colonoscopies. Clinically, these tumors may appear as incident colorectal cancer (i.e. tumors that develop between protocolized follow-up surveillance colonoscopies).[26] Recent work by our group suggests that the MMR-d crypt pathway may be absent in *PMS2* variant carriers.[29] This finding, combined with previous reports that colorectal cancer in non-PMS2 MMR variant carriers develops through the MMR-d crypt pathway, may explain the low penetrance observed in *PMS2* variant carriers, particularly those under surveillance.[10,11,22,23,27,30] This is in line with our current observation of only one incident cancer. This, combined with normal PMS2 staining in all analyzed adenomas, supports the hypothesis that *PMS2* carriers only develop colorectal cancer through de MMR-p adenoma 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* variant carriers is actually adenoma formation. Indeed, as the *PMS2* variant 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. The observation that the (advanced) adenoma risk at first colonoscopy in our cohort lies between the Forsberg Lynch cohort (which consists of *MLH1*, *MSH2* and *MSH6* carriers) and their control cohort may well be an illustration of this.[21]

When comparing the cumulative 10-year adenoma risk as reported by Engel *et al.*[18], a higher adenoma risk is seen in our cohort compared to the other MMR genes. Engel et al. hypothesize that *MLH1* carriers mainly develop cancer through the MMR-d crypt pathway, *MSH2* carriers through quick progression of an MMR-d adenoma into

a carcinoma and *MSH6* carriers through the MMR-p adenoma-carcinoma pathway. The retained PMS2 expression in the adenomas and previous published data on somatic mutation patterns in *PMS2* associated colorectal cancers,[31] suggest that the predominant pathway to colorectal cancer in *PMS2* carriers is similar to *MSH6* and involves the MMR-p adenoma-carcinoma pathway. However, it is surprising that a high 10-year risk of adenoma development is identified in our cohort. As suggested before, a possible explanation for the relatively high prevalence of adenomas is enrichment for adenoma risk factors in clinically ascertained *PMS2* families. However, interpretation of the comparison of adenoma risks is complicated by the differences in mean age at first colonoscopy between our cohort (50.6 years) compared to the cohort of Engel et al. where it is 42.7 years for *MLH1*, 44.0 years for *MSH2* and *48.7* years in *MSH6*. When factoring in age, a higher risk of adenoma development was noted with increasing age within our own cohort (data not shown), but additional analyses will have to show how much of the difference between the cohorts can be explained by age.

It is striking that different conclusions are drawn when comparing our cohort to two different studies (*i.e.* a relatively low number of adenomas at first colonoscopy compared to the Lynch families as described by Forsberg *et al.*[21] and a relatively high 10-year adenoma risk as compared to the Lynch syndrome patients as reported by Engel *et al.*[18]). Because both studies apply different analyses methods, at this moment it is not possible to find out whether these differences can be attributed to the different approaches in data analysis.

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* variant carriers.[32,33] If indeed colorectal cancer development in *PMS2* variant carriers can mostly be prevented by regular surveillance and polypectomies, we would expect a very low cancer risk in this prospective cohort. Nevertheless, we did observe one incident colorectal cancer in our cohort, a finding that on closer inspection of colonoscopy reports appeared to be related to insufficient bowel preparation in this carrier (table 3), highlighting the need for high quality colonoscopy with good bowel preparation to prevent incident colorectal cancer.[34]

Future studies should include a larger number of both tumors and (advanced) adenomas for immunohistochemical staining. Further studies should also elaborate on molecular analysis of, for example, *APC* and *CTNNB1* variants, as specific variants in these genes can help identify the timing of MMR deficiency, as previously shown in the study by Ahadova et al.[27] and Engel et al.[18] This approach might ultimately provide definitive proof of the late involvement of PMS2 deficiency.

In summary, we confirm that *PMS2* variant carriers undergoing regular surveillance colonoscopies are at very low risk for colorectal cancer. This finding supports previous proposals for a less intensive surveillance protocol in these Lynch patients, for example every 2-3 years, starting at age 35-40 years. Comparison of *PMS2* adenoma risk to the adenoma risk in other MMR gene variant carriers is complicated by differences in cohort characteristics and analyses methods between our study and previous publications and requires further investigation.

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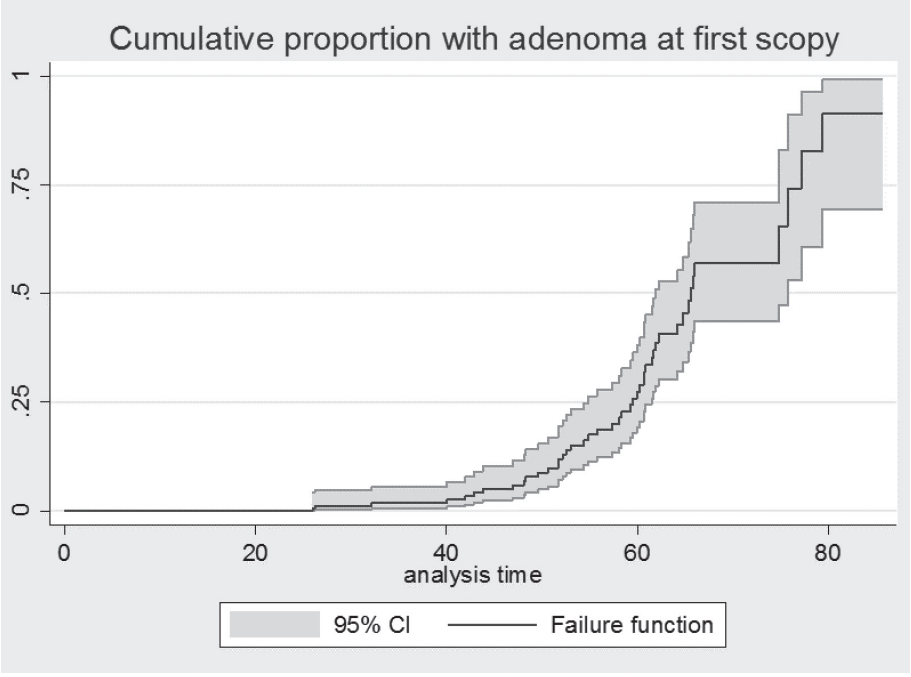
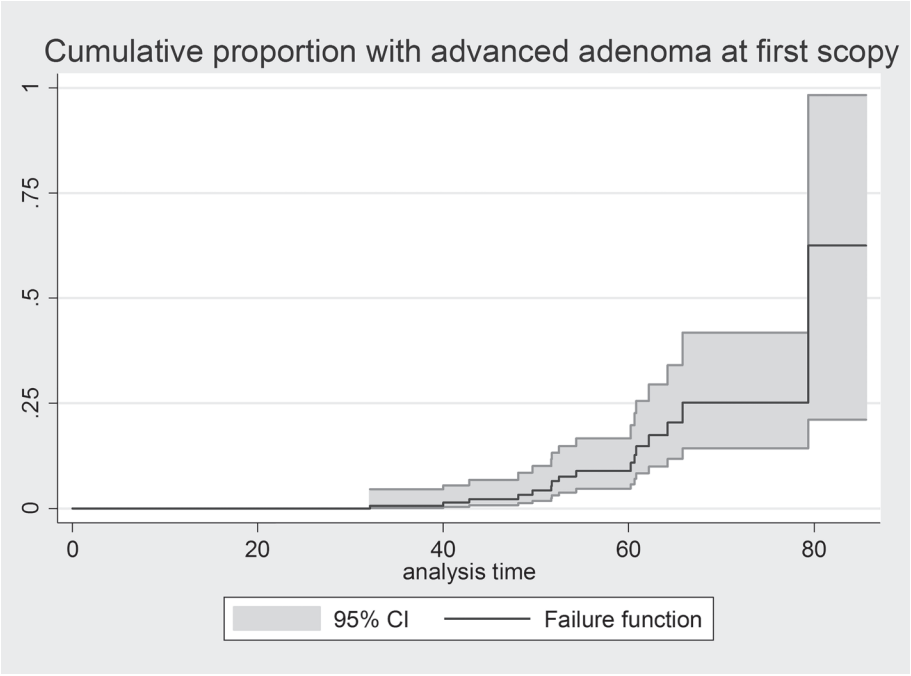
SUPPLEMENTAL INFORMATION



Supplemental Figure 1 Cumulative proportion of *PMS2* carriers with an adenoma at first colonoscopy

8



Supplemental Figure 2 Cumulative proportion of *PMS2* carriers with an advanced adenoma at first colonoscopy

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

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
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.or /criteria/. Nonsense and frameshift mutations, including large genomic deletions, were classified as pathogenic (class 5). Canoni splice variants and large in-frame genomic deletions were classified as likely pathogenic (class 4). Additional evidence that sugg pathogenicity for variants that could not be classified a priori as (likely) pathogenic is provided in supplementary table S2.

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

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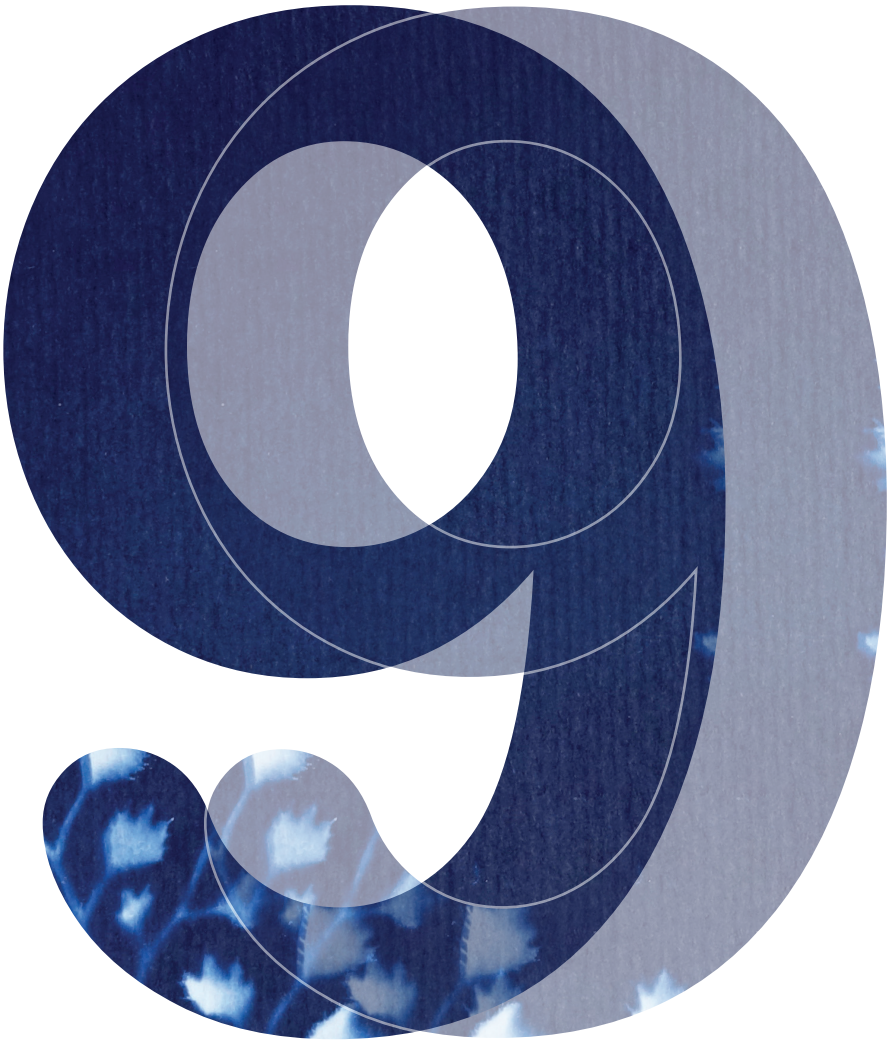

Discussion

ABSTRACT

The studies in this thesis aimed at exploring strategies to improve the detection of pathogenic germline variants in the mismatch repair (MMR) genes (**Part I, chapters 2-5**) as well as elucidating the phenotype of these variants once identified (**Part II, chapters 6-8**). Part I discusses when testing for Constitutional Mismatch Repair Deficiency (CMMRD) should be considered in children without cancer but with an NF1-like phenotype. Furthermore, the prevalence of MMR deficiency and Lynch syndrome in small bowel cancer and serous ovarian cancer is described. Part II explores a new method to establish unbiased colorectal cancer risk for Lynch syndrome patients, which is vital information to define (new) surveillance protocols. In addition, the effect of genotype and parent-of-origin on phenotype is determined in a cohort of *PMS2* variant carriers. Lastly, the prevalence of adenomas and incident colorectal cancers is described for the largest cohort of *PMS2* variant carriers to date.
In the following chapter, the main findings are briefly summarized and further discussed in the context of current literature.

LYNCH SYNDROME

Detection (Part I)

Given the estimated carrier frequency of 1 in 279 (1 in 1,946 for *MLH1*, 1 in 2,841 for *MSH2*, 1 in 758 for *MSH6* and 1 in 714 for *PMS2)* in the general population for pathogenic variants in the MMR genes,[1] it is clear that with current strategies many individuals with Lynch syndrome remain unidentified. This is likely partly due to the relatively mild phenotype associated with *PMS2* and *MSH6* (colorectal cancer risk estimates between 11 and 69%), compared to the phenotype associated with *MLH1* and *MSH2* (colorectal cancer risks estimates between 52 and 97%).[2-6] With the introduction of universal screening for MMR deficiency in all colorectal and endometrial cancers diagnosed before age 70, increasing numbers of these more mildly affected families are identified. Nonetheless, room for improvement in the identification of new Lynch syndrome families remains. In this thesis (chapter 4) we showed that the prevalence of Lynch syndrome (6.2%) and MMR deficiency in general (22.3% and 2.1% for respectively complete and subclonal MMR deficiency) in resected adenocarcinomas of the small bowel is high. Introducing universal (i.e. reflex analysis by pathologists regardless of family history) MMR deficiency screening in these tumors would therefore be an efficient way to identify more Lynch syndrome families. In particular since small

bowel tumors are relatively rare (300 cases in the Netherlands in 2018 according to www.cijfersoverkanker.nl). Hence, performing immunohistochemistry and subsequent molecular diagnostics would be a relatively low burden for pathology departments. Since a large range in age at diagnosis of the Lynch syndrome related tumors was observed (range 35-77 years, *this thesis*) we suggest not to put age restrictions or any other prerequisites on the universal screening of these tumors.

Introducing universal screening for MMR deficiency in small bowel carcinomas and the subsequent identification of Lynch syndrome families through an index patient with small bowel cancer is going to pose questions and challenges during the counselling process. While this strategy is a good opportunity to offer newly identified Lynch syndrome carriers colonoscopic and gynecologic surveillance, family members of an index patient with small bowel cancer may feel worried about developing small bowel cancer themselves. This may be particularly distressing since surveillance of the small bowel is currently not offered due to lack of evidence for its effectiveness.[7] Reassurance of these family members will require knowledge and skills from any clinical geneticist or genetic counsellor that counsels these families. Furthermore, this demonstrates that the prevention and detection of small bowel cancer (in the context of Lynch syndrome) can be improved. To address prevention and treatment of Lynch syndrome-associated small bowel cancer, future research should focus on unraveling the molecular pathways and mechanisms that lead to the development of these rare tumors. Recent research efforts have identified different molecular pathways in the development of colorectal cancer in Lynch syndrome for different MMR genes.[8-10] Evidence is accumulating that carriers of *MLH1* variants can develop colorectal cancer directly from mismatch repair deficient crypts, predisposing them to an increased risk of developing incident colorectal cancer (i.e. cancer in between two surveillance colonoscopies) and is associated with somatic *CTNNB1* variants.[8,10] This direct pathway to cancer without a benign precursor seems to be lacking in *PMS2* carriers. It is still unknown why different (molecular) pathways exist for the different MMR genes. *CTNNB1*-hotspot variants were also analyzed in the small bowel cancer cohort in this thesis. The number of Lynch syndrome related small bowel cancers was too small to power a reliable analysis of the molecular pathways, but the only tumor carrying a *CTNNB1* pathogenic variant was from an *MLH1* patient (unpublished data). Further insights into the pathogenesis of small bowel cancer may provide us with clues to identify those individuals at greater risk who may indeed benefit from surveillance measures. Known risk factors for the development of small bowel cancer in the general population are the presence of Crohn's disease and celiac disease.[11] Lifestyle related

factors that influence the risk of sporadic small bowel cancers are similar to those for cancer of the colon and include alcohol consumption, smoking and the consumption of red meat.[11] Should these lifestyle factors influence Lynch syndrome and the risk of small bowel cancer, they might act as a way to preselect patients with higher a-priori risks. However, currently, it is unknown whether these are also risks factors in Lynch syndrome-related small bowel cancer.

Our findings in small bowel cancer are also relevant for therapy purposes. Since MMR deficient colorectal cancers are highly immunogenic, they are a good target for immune checkpoint inhibition through treatment with PD1/PD-L1 blockers[12-14] and evidence is emerging that this is also the case for other MMR deficient tumors, including small bowel cancer.[15] Hence, patients may benefit from the knowledge that their tumor is MMR deficient, regardless of its etiology (*i.e.* sporadic due to two somatic mutations or Lynch syndrome-related).

While a convincing argument can be made to start universal screening for MMR deficiency in all small bowel carcinomas, this is different for ovarian cancers. In our case series (n=54) of high-grade serous ovarian cancer, there we no cases of MMR deficiency. These results corroborate the guidelines as suggested by, among others, Chui et al.[16] and Zeimet et al.[17] to only perform MMR deficiency screening in specific histological subtypes of ovarian cancer (*i.e.* endometroid and clear-cell ovarian cancer). Although MMR deficiency has been described by others in relatively high frequencies in serous ovarian cancers,[18] this is potentially due to misclassification of the histological subtype of these cancers in the past. Classification of the histological subtypes in ovarian cancer is known to be challenging and inter-observer variability has been described.[19] Over the recent years significant improvements have been made in histological subtyping of ovarian cancers which may influence the conclusions drawn in previous studies.[20,21] The tumors in our cohort have undergone central pathology review according to the latest World Health Organisation (WHO) guidelines, while many of the previously reported cases series have either been published before the most recent guidelines and/or do not specifically state that central pathology review was performed on their samples. This hypothesis also explains why in Lynch syndrome cohorts with ovarian cancer still a relatively large proportion (22% - 36%) of serous ovarian cancers is described.[22-24] An interesting follow-up study to provide further support to restrict screening for MMR deficiency to non-serous ovarian cancers would be to reclassify Lynch syndrome-associated ovarian tumors from previous studies according to current standards. Another angle would be to evaluate MMR deficiency status of serous ovarian cancers

in Lynch syndrome patients, since they could also be sporadic ovarian cancers that occurred by chance in a Lynch syndrome patient and are not related to the germline mutation.[24] Drawing firm conclusions from these results regarding an association with the germline MMR mutation will however be challenging. A similar discussion has been going on for the possible association of breast cancer with Lynch syndrome. Although quite a large proportion (65%) of breast cancers that have occurred in Lynch syndrome patients show MMR deficiency,[25] this has still not put the debate to rest. Presence of MMR deficiency is still not iron-clad proof that it is causally related to the development of the tumour.
In daily clinical practice caution is warranted when excluding Lynch syndrome as a differential diagnosis in a patient with serous ovarian cancer. Particularly if the diagnosis was made several years ago, histopathological review according to current standards should be considered.

Phenotype (Part II)
While improving the detection of Lynch syndrome through strategies such as universal tumor testing is an important field of investigation, it is equally important to gain further insight in cancer risk, surveillance strategies and molecular pathways that are involved in the development of Lynch syndrome-associated tumors. All these elements are crucial to be able to offer adequate surveillance programs to a newly identified carrier of a pathogenic MMR variant.

The cancer risk analyses as published in chapter 6 of this thesis provide important evidence by using a novel risk estimation approach that supports previous publications on the low cancer risks in *PMS2*- and *MSH6*-associated Lynch syndrome.[2,3,5,6,26-28] In older publications, colorectal cancer risks were estimated to be as high as 70% in Lynch syndrome patients[5] and up until recently the same cancer risks for all four genes were communicated to patients. Recent literature, however, shows that these early studies may have overestimated true cancer risks in general due to bias.[29] Also, cancer risks in *PMS2* and *MSH6* carriers are lower than those in *MLH1* and *MSH2*.[2,5,6,26] The more recent publications on risk estimation for Lynch syndrome, use statistical approaches (such as modified segregation analysis) to correct for ascertainment bias.[2,6,26] The downside of these statistical approaches is potential overcorrection. In chapter 6 of this thesis we describe a cohort of families that were ascertained through the CMMRD phenotype of the index patient instead of a family history suspect for Lynch syndrome, thus circumventing the need for complicated statistical approaches to correct for ascertainment bias. The results from this study show that cumulative

colorectal cancer risk at age 70 lies between 4.3 and 12.7% for PMS2 and between 4.5 and 22.7% for MSH6.[30] Together with previous reports that used statistical approaches, these estimates can be used to adapt surveillance guidelines in Lynch syndrome by making them gene-specific.

To further improve cancer risk estimations, also for the rarer types of Lynch syndrome-associated cancer, initiatives were developed to gather large amounts of data on Lynch syndrome families. In the near future these initiatives, such as the Colon Cancer Family Registry (CCFR, https://www.coloncfr.org/)[31] and the Prospective Lynch Syndrome Database (PLSD, https://www.plsd.eu) are expected to provide us with detailed data and risk estimations and indeed, some of the first results have already been published.[3,27,28,32] Although these initiatives are large enough to stratify risk estimations, not only per gene, gender and other factors such as country, much knowledge is also still to be gained on 1) why cancer risks are so different for the different genes and 2) why cancer risks can be so different even for carriers of mutations within the same gene. The genotype-phenotype study presented in chapter 7 of this thesis suggests that part of the explanation of risk differences within one gene (in this case *PMS2)* may lie within the type of mutation (genotype). Although no significant differences in colorectal cancer risk were identified between the genotype groups (hazard ratio: 1.31, P = 0.38), there was a lower age at colorectal cancer diagnosis in those with a variant that results in loss of RNA expression compared to those with a variant with retained RNA expression (mean age at colorectal cancer diagnosis of 51.1 versus 60.0 years).[33] However, further confirmation of any such correlation is yet needed since our results are limited by the amount of patients that could be included in the analyses at that time. Furthermore, a recent paper on genotype-phenotype associations in *MLH1*-related Lynch syndrome suggested a different type of phenotype-genotype correlation.[34] Based on their data, Ryan *et al*. suggest that the age of onset of endometrial cancer for *MLH1* is later for those with a truncation mutation versus those with a missense mutation, potentially indicating a dominant negative effect of missense *MLH1* mutations.[34] While this is an interesting finding, even leading the authors to propose genotype-specific gynecological surveillance, these data are in striking contrast to the genotype-phenotype correlation that we identified in our cohort for colorectal cancer (with a later age at onset for carriers of a mutation that shows retained RNA expression). A possible explanation for the discrepancy between the results in chapter 7 of this thesis and the study by Ryan et al.[34] could be a bias, due to the analysis of index and non-index patients together.[35] Index patients tend to be patients with the most severe phenotype in the family. If there is a relative overrepresentation of index

patients in either one of the genotype-subgroups as presented by Ryan et al., then this could explain any differences found between genotype-subgroups. Similarly, it can be debated whether the way mutations were grouped according to genotype (missense versus truncation mutations) is the correct method. Careful consideration should be given to categorizing mutations into different genotype groups. Although it may seem logical and intuitive to group mutations according to missense versus truncating variants, the truth is likely more complicated since certain truncating mutations may indeed result in nonsense mediated decay, while others (e.g. in frame exon deletions in *PMS2*) may still result in a protein with potential residual activity. On the other hand, missense variants within a specific domain of the gene or with an effect on splicing may be just as detrimental to protein function (or even cause a dominant negative effect as suggested by Ryan et al)[34] as a truncating mutation. Therefore, to suggest that genotype-phenotype correlations can be implemented in screening guidelines is preliminary. Further evidence is first needed to substantiate any genotype-phenotypes correlations. The aforementioned databases (CCFR and PLSD) may provide a good dataset to perform such studies.

Other mechanisms that have been suggested to explain risk differences within the same MMR gene are parent of origin effect,[36] anticipation,[37] SNPs,[38-40], gut microbiome[41] and lifestyle factors such as smoking and body mass index.[42-45] Since a parent of origin effect could not be identified in our *PMS2* cohort (chapter 7, this thesis) and there is no biological mechanism that could explain such an effect this is a factor that is unlikely to truly influence cancer risk. Anticipation is an unlikely factor for similar reasons; there is lack of a biological explanation for an anticipation effect and, as demonstrated recently by our research group, apparent anticipation effects in previous publications are more likely to be caused by a form of bias or a cohort effect.[46]

The genotype-phenotype manuscript in this thesis focused on any such correlations for the *PMS2* gene, but much knowledge is also still to be gained on genotype-phenotype correlations between the different MMR genes. For a long time these genotype-phenotype studies mainly focused on clinical phenotype (i.e. cancer risk). However, it is now generally accepted that the clinical phenotype is very different for the different genes (with higher cancer risks for *MLH1* and *MSH2*, moderate to low cancer risks for *MSH6* and low risks for *PMS2*). As briefly discussed above in the context of small bowel cancer; an exciting and relatively new research field that focusses more on the etiology behind these risk differences, is the molecular tumor analysis. Data generated by these molecular analyses are being used to understand the different pathways that

can eventually cause a normal colon mucosa crypt to develop into cancer.[8-10,47,48] These pathways are not only studied to understand carcinogenesis for Lynch syndrome in general, but also to attempt to understand why differences in phenotype between the different genes exist, despite the fact that they are all part of the same MMR complex.[8] In chapter 8 of this thesis, we show that the risk of incident colorectal cancer in *PMS2* carriers is very low, particularly compared to the other MMR genes. We also describe the number of adenomas in our cohort and the 10-year cumulative risk of developing an adenoma or advanced adenoma after start of surveillance. These data are however more difficult to compare to previously published data on the other MMR genes, due to differences in cohort characteristics (age at first colonoscopy) and analyses methods. Absence of MMR deficiency in the 16 adenomas in our cohort together with previously published molecular data,[8] suggest that *PMS2*-associated colorectal cancer mainly develops through the MMR proficient adenoma-carcinoma pathway, while colon cancers in *MLH1*- mutation carriers are thought to develop primarily from MMR deficient crypts without going through an adenoma stage.[8,10] However, we also identified a relatively high 10-year adenoma risk compared to the other MMR genes as published by others.[49] If *PMS2* variants predispose to the development of more adenomas, than this would not fit within this molecular pathway hypothesis. Potentially, this relatively high adenoma risk is (partly) explained by a higher age at colonoscopy in our cohort, but further analyses and collaboration initiatives will have to prove this. Additionally, our *PMS2* cohort may be enriched for adenoma risk factors due to ascertainment bias.

Clinical and molecular evidence brings us closer to understanding what the differences in pathogenesis and tumor development are between the genes, but it still does not explain how these differences fundamentally develop. In other words: it does not explain why mutations in genes from the same MMR machinery result in different molecular pathways in the development of a tumor. It has been hypothesized that the function of the PMS2 and MSH6 proteins within the complex can in part be taken over by other proteins such as MSH3 and PMS1, while MLH1 and MSH2 lack such a back-up system.[8,50,51] In line with the theory of a back-up system, Morak et al. suggest that pathogenic variants in *MSH3* might even aggravate the *MSH6* phenotype, even though *MSH3* heterozygous variants are not enough to cause a phenotype by themselves.[52] While a back-up system seems a plausible explanation for the differences between the different genes, further evidence is still needed to support this by showing that the mutation rate and microsatellite instability are indeed lower in cells from *PMS2*- and *MSH6*-variant carriers. For this purpose, we recently analyzed the microsatellite

instability patterns of *PMS2*-associated colon tumors in coding microsatellites and compared them to the patterns in other MMR deficient cancer, but we did not identify any significant differences (ten Broeke et al, unpublished data). This type of research is however challenged by the fact that tumors, once developed, are likely to show similar mutational patterns due to selection pressure (i.e. only those cells with a sufficient number of mutations in the right combination of genes will become clinically evident as tumors). A follow-up study is therefore needed to also analyze non-coding microsatellites. In addition, an interesting field of research would be the analysis of molecular changes in different tissues from Lynch syndrome patients, from normal mucosa, MMR deficient crypts, low- and high-grade adenomas to invasive cancers.

A completely different challenge that lies ahead is the interpretation of variants of unknown significance (VUS). Molecular geneticists and clinicians are faced with these VUSs and their dilemmas all across the different disciplines within the field of clinical genetics. If a VUS is identified the question remains whether the phenotype in the patient and/or family has been explained by this finding. But, even more importantly in the field of oncogenetics, it also poses the question of how to manage these patients and their family members. Can the variant be used to discern those with an increased cancer risk from those with an average risk? Should variant carriers be following surveillance as if they have Lynch syndrome or is a milder regime more appropriate? Luckily, several in vitro analyses have been developed that can aid in the classification of any such VUS.[53-60] While these functional analyses are very useful, there are also some drawbacks. First, these analyses are labor intensive and time-consuming before results can be used in clinical practice. Furthermore, not all functional tests are suited for all different types of variants (e.g. splice variants)[55]. Compared to interpreting VUS in some other genes, the advantage of the MMR genes is that there are also clues from the tumor that can be used to further interpret the variant.[61] Particularly if several family members are affected, segregation of the variant along with a MMR deficient tumor phenotype can provide a strong clue towards pathogenicity. Unfortunately, segregation is not always possible. An additional and relatively new valuable source of data to help give some direction in classifying a VUS is the molecular analyses of the tumor of the index patient. If a second hit has occurred in the tumor on top of the VUS, this may be a clue that the VUS is actually pathogenic, while if molecular analysis of the tumor shows two additional pathogenic somatic hits that explain the MMR deficient phenotype this may be a strong argument against pathogenicity of the VUS.[62] While conceptionally this seems like a straight forward principle, more research is needed to establish how much weight can be given to evidence such as this.[62] For example, while loss of heterozygosity is a common second hit that could explain the

MMR deficiency if it occurs on top of a potentially pathogenic VUS, it could also be a consequence of a more generalized, non-specific chromosomal event.[62] Furthermore, tumor heterogeneity may cause different parts of the tumor to have different second hits, which would mean the presence of three variants (the VUS plus two additional pathogenic variants) in the tumor does not necessarily argue against pathogenicity of the VUS.

CONSTITUTIONAL MISMATCH REPAIR DEFICIENCY (CMMRD)

While it seems logical to improve detection of germline heterozygous MMR variants because of clear consequences for clinical management, much more discussion can be held on improving the detection of individuals with CMMRD. In chapter 2 and 3 of this thesis, relevant considerations and literature are discussed to come to appropriate testing guidelines to improve the detection of CMMRD in healthy individuals. However, as also indicated in these chapters, much of the literature that was used to base these guidelines on is still limited by publication bias and selection bias. Furthermore, surveillance guidelines are yet to be proven to be effective. One step forward to providing more evidence in support of the testing criteria has already been made since their publication. When formulating the guidelines, it was estimated that the prevalence of CMMRD in children suspected of NF1, but without a germline *NF1* pathogenic variant, is 0.4%. Recently, this estimation was confirmed by analyzing the prevalence of CMMRD in a large cohort (n=735) of children suspected of NF1 but lacking an *NF1/SPRED1* pathogenic variant. The prevalence of CMMRD in this cohort was 0.41%.[63]

More research is still needed to evaluate whether these newly diagnosed patients and their parents indeed benefit from such a diagnosis.

There are two large, international research consortia that are focused on CMMRD: the International Biallelic Mismatch Repair Deficiency Consortium, which is in an initiative from Canada, and the European 'Care for CMMRD' (C4CMMRD) consortium. The guidelines as outlined in chapter 3 are supported by the C4CMMRD consortium and at their latest meeting a study proposal was presented to prospectively evaluate the guidelines in order to establish how many CMMRD diagnoses are being made based on these guidelines and whether there is room for improvement of the testing criteria.[64]

Whether an early diagnosis is actually beneficial for the patient and their family members is perhaps more difficult to establish. Any such answer should not only take into

account whether surveillance measures are indeed effective, but should also consider quality of life of the patient and his/her parents. Both the Canadian consortium and the C4CMMRD consortium are evaluating the outcomes of their surveillance programs.[64-68] One of the difficulties is that there are also attenuated forms of CMMRD where cancer does not tend to develop until adulthood.[69] As a consequence, surveillance programs may be appropriate for one patient, while they may be overkill for another and cause unnecessary medicalization and stress. In the future genotype-phenotype correlations, as researched in Lynch syndrome, will hopefully provide clinicians with some guidance to predict phenotype severity also in CMMRD.

An area of study that has not been explored up to now in CMMRD is the psychological impact of the diagnosis and subsequent surveillance measures. While some lessons can be learned from other cancer predisposition syndromes such as Li-Fraumeni syndrome, which is also characterized by high cancer risks and may become manifest through a childhood malignancy,[70,71] the situation is still not completely comparable; CMMRD presents itself predominantly during childhood and has a recessive, rather than a dominant inheritance pattern.[72,73] Future studies should therefore map the psychological burden and quality of life of CMMRD patients with a diagnosis, comparing those diagnosed after they have developed cancer versus those that were diagnosed when they were still healthy. Data from these studies can then be taken into account in testing strategies and surveillance programs.[64]

In addition, while one of the arguments for an early CMMRD diagnosis is the possibility for parents to think about family planning and use the opportunity to use preimplantation genetic diagnostics (PGD), it remains to be seen whether parents will indeed use PGD. While PGD is available for Li-Fraumeni families in the Netherlands, thus far only six couples have gone through the process of using this technique to prevent a germline *TP53* mutation in their offspring.[74]

Concluding remarks

In conclusion, the work described in this thesis explores opportunities to further improve detection of germline pathogenic variants in the MMR genes, both in the setting of Lynch syndrome and CMMRD. Furthermore, an effort has been made to learn more about the phenotype of these germline variants, since identification of germline variants will only be of help to the patient if evidence based surveillance guidelines are available. Future research should focus on providing evidence for further tailoring of surveillance guidelines (ideally on an individual level) and improvement of ways to classify variants of unknown significance.

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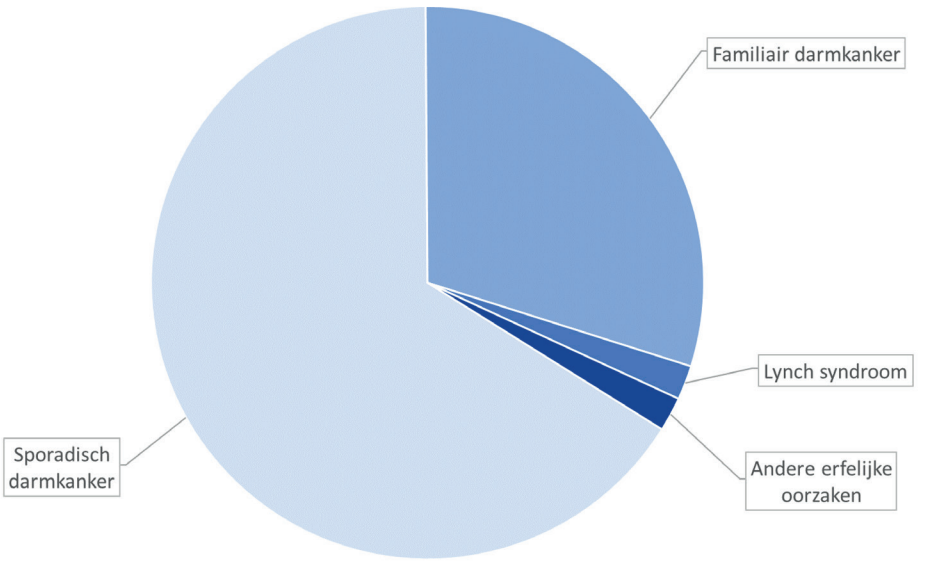
9

10

Nederlandse samenvatting
List of publications
Dankwoord
Curriculum Vitae

NEDERLANDSE SAMENVATTING

Darmkanker is een van de meest voorkomende vormen van kanker. Ieder jaar krijgen ongeveer 12.000 Nederlanders deze diagnose. Bij meer dan 30% van deze patiënten komt darmkanker in de naaste familie voor. In slechts een minderheid van de gevallen wordt een erfelijke aanleg voor darmkanker aangetoond. De meest voorkomende erfelijke aanleg voor darmkanker is het Lynch syndroom (Figuur 1). Patiënten met het Lynch syndroom hebben niet alleen een verhoogd risico op darmkanker, maar ook op andere vormen van kanker zoals baarmoederkanker, eierstokkanker, dunne darmkanker en maagkanker.



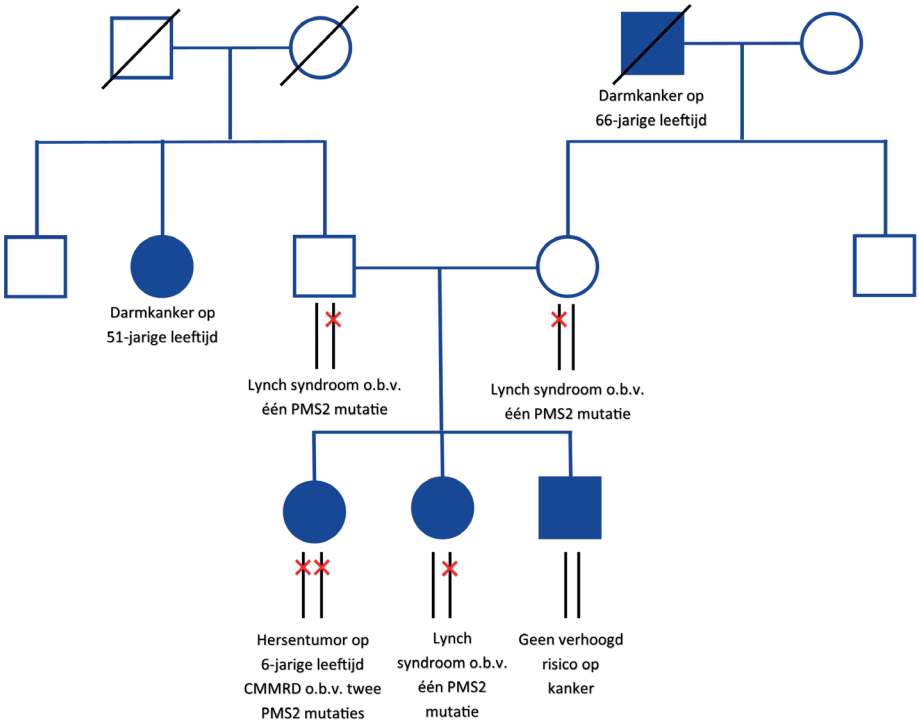
Figuur 1. Ongeveer 4% van alle gevallen van darmkanker wordt veroorzaakt door een erfelijke oorzaak. De helft hiervan betreft het Lynch syndroom. Bij 30% van de patiënten komt darmkanker weliswaar in de familie voor, maar kan geen erfelijke oorzaak aangetoond worden.

Lynch syndroom wordt veroorzaakt door een mutatie in één van de mismatch repair genen, namelijk *MLH1, MSH2, MSH6* of *PMS2.* Patiënten met het Lynch syndroom worden geboren met één "gezonde" kopie en één kopie met een DNA-verandering (mutatie). Als gedurende het leven in één van de lichaamscellen ook een mutatie op de gezonde kopie van dit gen ontstaat, dan is deze cel mismatch repair deficiënt geworden en kan uitgroeien tot kanker doordat mutaties zich opstapelen. Mismatch repair deficiëntie kan ook ontstaan in een tumor als per toeval beide kopieën van een mismatch repair gen gemuteerd raken. In dat geval is er geen sprake van Lynch syndroom, dergelijke mutaties die alleen in de tumor aanwezig zijn, zijn niet erfelijk. Er is een grote spreiding in het kankerrisico bij patiënten met het Lynch syndroom. Zowel tussen patiënten met mutaties in verschillende genen, als onder patiënten met een mutatie in hetzelfde gen.

In zeldzame gevallen komt het voor dat iemand geboren wordt met een mutatie op beide kopieën van een mismatch repair gen. Dit kan gebeuren als beide ouders (vaak zonder het zelf te weten) Lynch syndroom hebben (Figuur 2). In dat geval wordt iemand geboren met een slecht functionerend mismatch repair systeem in alle lichaamscellen. Dit wordt constitutional mismatch repair deficiency (CMMRD) genoemd. CMMRD erft autosomaal recessief over. Patiënten met CMMRD ontwikkelen vaak al kanker op de kinderleeftijd. Daarnaast kunnen zij kenmerken hebben die niet gerelateerd zijn aan kanker, zoals café-au-lait vlekken van de huid (koffie met melk kleurige vlekken).

Als eenmaal vaststaat dat iemand het Lynch syndroom of CMMRD heeft, dan gelden er controle adviezen. Voor Lynch syndroom geldt het advies om vanaf 25-jarige leeftijd de dikke darm eenmaal per 1 a 2 jaar te controleren middels een colonoscopie. Voor vrouwen geldt daarnaast vanaf 40-jarige leeftijd het advies om de baarmoeder regelmatig te laten controleren en eventueel preventief de baarmoeder en eierstokken weg te laten halen. Voor CMMRD gelden intensieve controles vanaf de kinderleeftijd, waaronder regelmatige beeldvorming van de hersenen. Het doel van deze controles is het opsporen en weghalen van poliepen in de darm en het detecteren van kanker in een vroeg en behandelbaar stadium.

Dit proefschrift behandelt twee aspecten rondom Lynch syndroom en CMMRD. Enerzijds worden strategieën onderzocht die de detectie van Lynch syndroom en CMMRD kunnen verbeteren.
Daarnaast wordt in de proefschrift onderzocht hoe hoog het kankerrisico is bij Lynch syndroom en welke factoren mogelijk van invloed zijn op dit kanker risico.



Figuur 2. Overerving van twee PMS2 mutaties (één van beide ouders) leidt tot CMMRD bij het kind. Lynch syndroom wordt veroorzaakt door een mutatie in een mismatch repair gen en erft autosomaal dominant over. CMMRD wordt veroorzaakt door twee mutaties in hetzelfde gen, dit heet autosomaal recessieve overerving.

DEEL 1 – DETECTIE

In **hoofdstuk 2** van dit proefschrift wordt een casus beschreven van een gezond meisje met café-au-lait vlekken van de huid. De meest waarschijnlijke verklaring voor de aanwezigheid van veel van deze vlekken bij een kind, is neurofibromatose type 1 (NF1). Er werd bij haar echter geen mutatie in het *NF1* gen aangetoond. Omdat haar ouders consanguin waren (neef-nicht huwelijk) is er een verhoogde kans op autosomaal recessieve aandoeningen, zoals CMMRD. Om deze reden werd CMMRD diagnostiek ingezet en werd een homozygote mutatie (mutatie op beide kopieën van het gen) aangetoond in het *PMS2* gen. Naar aanleiding van deze casus werd binnen het Europese CMMRD consortium (C4CMMRD) een discussie gestart, wanneer erfelijkheidsonderzoek naar CMMRD geïndiceerd is bij een kind dat (nog) geen kanker heeft ontwikkeld. Zoals beschreven in **hoofdstuk 3**, werd op basis van de literatuur en de expertise binnen het consortium geconcludeerd dat CMMRD diagnostiek overwogen moet worden bij kinderen met een NF1-achtig fenotype, mits er ten minste één additionele aanwijzing is voor CMMRD, zoals consanguine ouders of een familielid met een Lynch syndroom gerelateerde tumor.

Op dit moment wordt er standaard screening naar mismatch repair deficiëntie in tumoren verricht via de pathologie bij patiënten met darmkanker of baarmoederkanker voor de leeftijd van 70. Het is echter de vraag of deze standaard screening ook toegepast moet worden op andere, zeldzamere vormen van kanker die geassocieerd zijn met het Lynch syndroom, zoals dunne darmkanker en/of eierstokkanker. In **hoofdstuk 4** wordt beschreven hoe vaak mismatch repair deficiëntie en Lynch syndroom voorkomen bij dunne darmkanker. Dit is onderzocht door via de Nederlandse pathologie registratie (PALGA) weefsel te verzamelen van een grote groep dunne darmkankers. Middels een kleuring van de mismatch repair eiwitten kan aangetoond worden of een tumor mismatch repair deficiënt is. Als dit het geval was, werd aanvullend DNA onderzoek gedaan om te kijken of de mismatch repair deficiëntie veroorzaakt was door Lynch syndroom (erfelijk) of door twee mutaties in de tumor (niet erfelijk). Hiermee toonden we aan dat 22.3% (74/332) van de geopereerde tumoren mismatch repair deficiënt was. In 20 van deze mismatch repair deficiënte tumoren (6.2% van het totale cohort) konden we aantonen dat het om Lynch syndroom ging. Bij de tumoren waarvan alleen een biopt beschikbaar was, was slechts 4.4% (3/68) mismatch repair deficiënt. Er waren geen klinische of pathologische kenmerken die onderscheidend waren tussen de mismatch repair deficiënte en de mismatch repair proficiënte tumoren. Op basis van deze bevindingen adviseren we om in alle dunne darm tumoren onderzoek te

doen naar mismatch repair deficiëntie. Dit is van belang voor het opsporen van Lynch syndroom, maar ook voor de behandeling van de patiënt. Dit laatste is ongeacht of het om mismatch repair deficiëntie in erfelijke of sporadische setting gaat.

In **hoofdstuk 5** wordt uiteengezet waarom de kans klein is om Lynch syndroom en/of mismatch repair deficiëntie te vinden bij een specifiek subtype eierstokkanker, namelijk het hooggradig sereuze type. Enerzijds baseren we ons hierbij op de gegevens uit ons eigen cohort hooggradig sereuze tumoren (0/54 tumoren was mismatch repair deficiënt). Anderzijds op recente literatuur. Bij ons literatuuronderzoek hebben we specifiek gezocht naar recente publicaties over dit onderwerp omdat de classificatie van ovariumtumoren verbeterd is in de loop der jaren en objectiever geworden is. In de twee artikelen over het voorkomen van mismatch repair deficiëntie in hooggradig sereuze tumoren waarin de huidige standaarden aangehouden worden voor classificatie van de tumoren, worden geen gevallen van mismatch repair deficiëntie beschreven (0/273). Daarnaast wordt er in drie grote studies waarbij direct genetisch onderzoek bij de patiënten werd gedaan, slechts in 7 van de 2293 (0.3%) onderzoeken de diagnose Lynch syndroom gesteld. Mismatch repair deficiëntie was wel aanwezig in 15.2% van de gevallen in een studie waarbij de tumoren niet volgens de huidige richtlijnen werden geclassificeerd. We concluderen dan ook dat onderzoek naar mismatch repair deficiëntie en/of Lynch syndroom achterwege kan blijven bij patiënten met eierstokkanker, mits de tumor geclassificeerd is volgens de richtlijn van de World Health Organisation (WHO) uit 2014.

DEEL 2 – FENOTYPE

Het is belangrijk om voor ieder mismatch repair gen apart te weten wat de hoogte van het darmkanker risico is, zodat controle adviezen hierop aangepast kunnen worden. Een uitdaging bij het bepalen van de hoogte van dit risico, is dat we in de kliniek vaak alleen de patiënten zien die al kanker gehad hebben. De gezonde personen die ook drager zijn van dezelfde mutatie kennen we niet, omdat er bij hen volgens de huidige richtlijnen geen aanleiding is voor genetisch onderzoek. Het gevaar hiervan is dat de kankerrisico's overschat worden. Een manier om hiervoor te corrigeren is door statistische correcties toe te passen op de risico berekeningen, maar ook hier zitten onzekerheden is. In **hoofdstuk 6** wordt een alternatieve aanpak beschreven om het kankerrisico bij PMS2 en MSH6 mutatiedragers te bepalen, zonder ingewikkelde statistische correcties toe te hoeven passen. Middels dit onderzoek konden we bevestigen dat kankerrisico voor MSH6 en PMS2 laag is, namelijk 8.7% tot de leeftijd van 70 voor PMS2 en 11.7% voor MSH6.

In **hoofdstuk 7** wordt onderzoek beschreven naar de invloed van twee factoren op de hoogte van het darmkankerrisico bij patiënten met een *PMS2* mutatie. De eerste factor die we bekeken hebben is of het soort mutatie invloed heeft op de hoogte van het risico en de leeftijd waarop darmkanker zich voordoet. Dit wordt ook wel een genotype-fenotype correlatie genoemd. Hoewel er geen duidelijk effect gezien werd van het genotype op de hoogte van het risico, zagen we wel dat patiënten met een specifiek type mutatie (waarbij er naar verwachting nog wel eiwit tot expressie komt) gemiddeld ouder waren op het moment van darmkankerdiagnose dan patiënten die een variant hebben waarbij geen eiwit meer tot expressie komt. We toonden daarnaast aan dat het niet uitmaakt of je de mutatie van je vader of van je moeder erft voor de hoogte van het kankerrisico.

Ten slotte wordt in **hoofdstuk 8** beschreven hoe vaak er poliepen en darmkanker gevonden worden bij patiënten met een *PMS2* mutatie die regelmatige darmcontroles krijgen. De controles blijken zeer effectief te zijn aangezien er slechts één patiënt was die darmkanker ontwikkelde. Opvallend genoeg vonden we dat patiënten met een *PMS2* mutatie meer poliepen lijken te ontwikkelen dan patiënten met een mutatie in een van de andere Lynch genen. Dit is opvallend omdat het kankerrisico juist lager is bij *PMS2* dan bij de andere genen. Een mogelijke factor die hierbij een rol zou kunnen spelen is dat de patiënten in ons cohort gemiddeld ouder zijn.

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CURRICULUM VITAE

Manon was born in Rijswijk, the Netherlands, on February 11th 1991. In 2010 she graduated cum laude at her high school Gymnasium Juvenaat in Bergen op Zoom.

From July 2007 until May 2008 she participated in a high school exchange program, living with a host family and attending high school in Australia.

Manon started medical school in September 2010 at the Leiden University Medical Centre (LUMC) and graduated in 2016. During her medical education (in 2012) she joined the research group of Maartje Nielsen at the department of clinical genetics at the LUMC, at first as a student assistant to work on database management. Later on this turned into a research internship and her first scientific publication on the effect of genotype and parent-of-origin on the phenotype of *PMS2* carriers. After graduating medical school, she started as a PhD student combined with a part-time appointment as a resident (not in training) of clinical genetics. In January 2018 she started her training to become a clinical geneticist. This training was interrupted for 9 months from April 2018 onwards to work on research related to her PhD as well as research on *APC* mosaicism. Manon is actively involved in the European consortium 'Care for CMMRD' (C4CMMRD), which provides a platform to collaborate on research concerning constitutional mismatch repair deficiency. She will also join the European Reference Network (ERN) Genturis on the topic of CMMRD.