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Discovery of genetic defects in unexplained colorectal cancer syndromes

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Chapter 7

Concluding remarks and future perspectives

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In this thesis, underlying genetic causes of unexplained suspected Lynch Syndrome and unexplained colonic adenomatous polyposis are presented. Furthermore, practical guidelines are presented to curate variants detected in *PMS2* in DNA isolated from formalin-fixed paraffin-embedded (FFPE) tissue. Additionally, an assay determining the functional effect of splice site variants using RNA isolated from FFPE is presented, all with the ultimate goal to determine the underlying genetic cause of colorectal cancer syndromes. New CRC susceptibility genes and new underlying mechanisms currently explain a portion of the previously unexplained cases, but still a large part of the seemingly familial colorectal cancers remains unexplained. The newly discovered causes, current achievements of determining other susceptibility factors and future perspectives are discussed in this chapter.

Unexplained suspected Lynch Syndrome

In 2014, a study described that in up to 60% of mismatch repair (MMR)-deficient and/or microsatellite unstable tumors no *MLH1* methylation or germline MMR variants are detected.¹ These patients are referred to as 'suspected Lynch Syndrome' (sLS)¹ or Lynch-Like Syndrome (LLS)² and clinical management of these patients remains difficult. Little is known about the cancer risk for these patients, although one study showed they have a lower risk of cancer than patients from LS families, but higher than those from families with sporadic CRC.² Still, without an exact genetic diagnosis, determining surveillance strategies for patients and their relatives is difficult, and can lead to over- as well as undertreatment of family members, e.g. intensive cancer screening in those without an increased CRC risk.³ Several theories have been suggested as to what could be the cause of the MMR-deficiency in these tumors. Three potential reasons for MMR-deficient and/or MSI-H tumors in sLS patients are discussed here.

Missed MMR variants

The most obvious explanation of MMR-deficiency in sLS tumors is missed variants in one of the MMR genes. Since most diagnostic and research testing only screen the coding regions of the MMR genes, (intronic) variants can be missed. One study shows a previously missed deep-intronic *MSH2* variant resulting in inclusion of a pseudoexon.⁴ However, as we have shown in Chapter 2, sequencing of intronic regions results in a large number of intronic variants of uncertain significance (VUS), and classification of these variants is challenging.⁵ Furthermore, while some of these intronic variants might affect splicing, this is difficult to predict using most splice-site prediction software, because these depend strongly on the presence of a canonical splice site nearby the variant. More experimental data are needed to optimize existing prediction tools for deep intronic variants.⁶⁻⁸ According to the variant classification guidelines created by the InSiGHT (International Society for Gastrointestinal Hereditary Tumors) variant interpretation committee, intronic variants, but also missense variants, silent variants and promoter variants, are automatically classified as Class 3 (uncertain significance), until proof of pathogenicity is delivered.

Another category of missed variants are large genomic rearrangements. Detecting large insertions/deletions (indel) with next-generation sequencing (NGS) has been shown to be challenging, and these indels might be missed in current molecular tumor diagnostics.⁹ Some studies describe previously missed inversions^{10, 11} and rearrangements^{12, 13} in sLS patients.

Other explanations include the presence of variants for which the impact on MMR protein function is presently not clear. For example, many studies have investigated the effect that promoter variants can have on gene expression. One well studied promoter variant, *MLH1* c.-27C>A, has been described to confer a CRC-risk by dominant inheritance of a constitutional *MLH1* epimutation, to co-segregate with CRC in multiple families affected with CRC and to lead to reduced *MLH1* expression.¹⁴⁻¹⁷ While in the past described as pathogenic, it is currently in the LOVD database classified as a VUS because of 'insufficient evidence'. Other known promoter variants are the *MLH1* c.-28A>G/-7C>T and the *MLH1* c.-93G>A variants described to show partial loss of expression (c.-28)¹⁸ and increased CRC risk due to possible epigenetic silencing (-93).¹⁹⁻²¹ The latter, however, was classified as benign by the InSiGHT group in 2013, because of the high minor allele frequency (MAF) in the general population.

Variants of uncertain significance remain a concern and functional assays are needed to assess pathogenicity, especially for rare variants found in only a few families or moderately penetrant variants which do not show complete co-segregation in a family. Laboratory efforts capable of assessing the effect of a VUS on various aspects of MMR protein function are cell-free assays determining mismatch repair activity²²⁻²⁴, cell-based assays showing (lack of) expression in CRC cell lines²⁵⁻²⁷, nuclear localization assays and RNA splicing assays using patient RNA^{28, 29} or minigene splicing assays.^{7, 30, 31} Consensus about pathogenicity of a variant is important for proper clinical management.

Somatic inactivation

A quite prevalent cause of MMR-deficiency in sLS patients appears to be biallelic somatic inactivation of the MMR-genes. Multiple studies (Table 1) have shown somatic inactivation in 11% to 100% of the tested sLS cohort.³²⁻³⁷ Of the 68 patients described with biallelic somatic inactivation of one of the MMR genes in these six studies, 37 tumors (54%) had one somatic variant and additional loss of heterozygosity. Patients presented with colorectal, endometrial or small bowel cancer with a mean age of 54.6 years (range 27 - 81 years). Two studies also tested for variants in the exonuclease domain of *POLE* and *POLD1*^{34, 35}, in the other four an underlying variant in one of these genes cannot be excluded. We, and others, have detected

Table 1: Studies on biallelic somatic inactivation in sLS patients

	No. of sLS patients†	No. of biallelic som. MMR	2 MMR	1MMR/ LOH	Average age of onset (years)
Chika et al ³²	2	2	2	0	75.0
Geurts-Giele et al ³³	40	21	5	16	58.9
Haraldsdottir et al ³⁴	27*	19	11	8	53.8
Jansen et al ³⁵	53*	10	5	5	48.1
Mensenkamp et al ³⁶	25	13	5	8	47.7
Sourrouille et al ³⁷	27	3	3	0	55.0
Total	174	68	31	37	54.6

†Only patients without germline MMR variants and without *MLH1* promoter hypermethylation are shown

*sLS patients were tested for *POLE* and *POLD1* variants, patients with a variant were excluded. 2MMR; two somatic variants, 1MMR/LOH; one variant and loss of heterozygosity (LOH) of the WT-allele.

two somatic variants in patients with a family history of CRC, showing that biallelic somatic inactivation does indeed explain the occurrence of a tumor. However, it cannot explain other occurrences of colon cancer in the family and it could be that another underlying genetic cause was missed.^{35, 36} In some cases, the somatic MMR variants are secondary to another (germline) defect that results in a higher mutational load. This has previously been described in patients with MMR-deficient/MSI-H tumors, found to carry biallelic *MUTYH* variants impairing base excision repair (BER).³⁸⁻⁴² In the studies where further testing was performed on the *MUTYH* mutated/MMR-deficient tumors, somatic MMR variants³⁸ or *MLH1* promoter hypermethylation⁴⁰ explained the MMR-deficiency. The somatic MMR variants were MAP-specific G>T variants, indicating that the impaired BER was the primary defect followed by MMR-deficiency.³⁸ This mechanism of secondary MMR-deficiency is also seen in *POLE/POLD1* tumors. These tumors have a high mutational load, leading to many variants that could possibly inactivate genes. These findings underline the importance of screening for variants in other CRC susceptibility genes in patients with biallelic somatic inactivation of the MMR genes but with a positive family history of CRC, especially if the other family members do not show MMR-deficiency/MSI in the tumor. Another possibility is that these somatic variants are present as mosaic variants in the leukocyte DNA, something that has been (albeit very rarely) described before. Sourrouille et al describes one patient where a somatic *MSH2* variant found in the tumor of the mother (but not in blood) was also detected in leukocyte- and tumor DNA of the affected son.³⁷ No other germinal mosaic MMR variants have been described.

Variants in other CRC susceptibility genes

Recent advances in next-generation sequencing (NGS) and whole exome sequencing (WES) or even whole genome sequencing (WGS) resulted in the detection of additional genes possibly involved in tumorigenesis of sLS tumors. As mentioned before, we (Chapter 3), and others, have shown germline and somatic variants in *POLE* or *POLD1* in MMR-deficient tumors.^{34, 35, 43} Variants in the exonuclease domain (EDM) of these genes encoding for polymerase ϵ and δ respectively, have been shown to result in a high mutational burden, often with a high number of C>A transversions.⁴⁴ In sLS tumors it has been hypothesized that a somatic or germline *POLE/POLD1* variant can result in somatic MMR variants, subsequently resulting in microsatellite instability.^{35, 43} Notably, a recent study shows synergistic increase in mutation rate when a pathogenic *POLD1* variant (*POLD1* R689W) was combined with MMR-deficiency, indicating that the *POLD1* mutator effect results from a high rate of replication errors.⁴⁵ This variant is not located in the *POLD1*-EDM but does show impaired nucleotide selectivity, showing that even variants outside the *POLE/POLD1*-EDM might confer an increased CRC susceptibility.^{45, 46}

Other genes that have been implicated as the underlying cause of suspected Lynch Syndrome tumors are *BRCA1*, *BRCA2*, *MUTYH*, *APC*, *STK11*, *MLH3* and *EXO1*.^{38, 47-49} As mentioned before, homozygous and compound-heterozygous *MUTYH* variants have been described in multiple sLS patients, some of which were shown to have secondary MMR-deficiency through somatic MMR variants or *MLH1* promoter hypermethylation.^{38, 40} *BRCA1*, *BRCA2*, *APC* and *STK11* are known dominant cancer predisposition genes previously described to be involved in hereditary breast cancer, familial adenomatous polyposis and Peutz-Jeghers Syndrome respectively.⁵⁰⁻⁵² While the increased risk of CRC for *BRCA1* and *BRCA2* mutation

carriers is still under debate, some studies indicate an up to five fold increased risk of CRC.⁵³⁻⁵⁸ However, the 'suspected Lynch Syndrome' patients described in these studies found to carry these *BRCA1*, *BRCA2*, *APC* and *STK11* variants were selected based on personal and family history of CRC but no IHC of the four MMR genes or MSI was performed.

MLH3 and *EXO1* are both mismatch repair genes. *MLH3* interacts with *MLH1*, is believed to participate in insertion deletion loop (IDL) repair and has been shown to exhibit MSI in cell culture.^{59, 60} The role of the mismatch repair gene *MLH3* in colorectal tumorigenesis is under debate.⁶¹⁻⁶⁴ Whereas one study describes germline *MLH3* variants in sLS patients⁶¹ (with positive *MLH1*, *MSH2* and *MSH6* staining, but with MSI), more evidence is needed before it can be regarded as a causal (possible reduced penetrant) Lynch Syndrome gene. The exonuclease 1 (*EXO1*) gene encodes a 5' → 3' exonuclease that is involved in multiple DNA repair pathways.^{65, 66} In MMR, *EXO1* interacts with *MSH2* and in yeast it shows a mutator phenotype when lost.⁶⁶ *EXO1* has been extensively studied in CRC^{48, 64, 67-71}, and is often found not to be associated with (suspected) Lynch Syndrome.^{67, 70, 71} Single nucleotide polymorphisms (SNPs) in this gene have been described to confer a slightly increased risk of CRC in the general population, but have not been associated with LS.^{68, 69} Wu et al describes germline *EXO1* variants in 14 patients fulfilling Amsterdam criteria, with six of the tumors showing microsatellite instability.⁴⁸ Twelve of thirteen tested tumors showed loss of heterozygosity with retention of the wildtype allele. The authors suggest that complete loss of *EXO1* is lethal to the cell, and that a haploinsufficiency effect can give rise to tumors. Other studies screened for *EXO1* variants in sLS patients, but no other carriers were found.^{72, 73} This, together with the fact that patients were not screened for variants in established LS/CRC susceptibility genes (*PMS2*, *POLE*, *POLD1*), shows little evidence to include *EXO1* as a LS gene, although a role as a low penetrant cancer susceptibility gene cannot be excluded at this point in time.

For Lynch Syndrome tumorigenesis, it remains unclear when the MMR-deficient phenotype (e.g. loss of the second allele in germline MMR carriers) is acquired during tumorigenesis. Multiple studies tested adenomas from MMR-mutation carriers for immunohistochemical loss of the MMR proteins or MSI and found a Lynch phenotype in 40-80% of tested adenomas, often associated with adenoma size.⁷⁴⁻⁸⁰ Ahadova et al proposed two pathways in Lynch Syndrome (see Figure 1), one where adenomatous polyps precede MMR-deficiency with an initiating event as *APC* and/or *KRAS* gene variants and one where MMR gene inactivation is the initial event leading to non-polypous precursor lesions and secondary *CTNNB1* hits are needed for tumorigenesis.⁸¹ While it can be debated whether polypous growth precedes MMR gene inactivation in LS tumors, this is probably the preferred pathway in suspected Lynch Syndrome patients with somatic MMR inactivation. In these patients the underlying genetic defects (*MUTYH*, *POLE*, *POLD1*) often result in a mutator phenotype, with the MMR-deficiency acting as a secondary defect resulting in tumorigenesis. It is conceivable that for these patients adenomas can arise before mismatch repair is completely inactivated. Immunohistochemical staining and MSI analysis of these tumors gives a bias to expect an MMR defect, while the true underlying germline defect could be missed. Especially when patients present with early onset colorectal cancer and a positive family history of CRC, tumors should be screened for variants in other CRC susceptibility genes. Whole exome sequencing of unexplained suspected Lynch Syndrome patients might detect CRC susceptibility genes

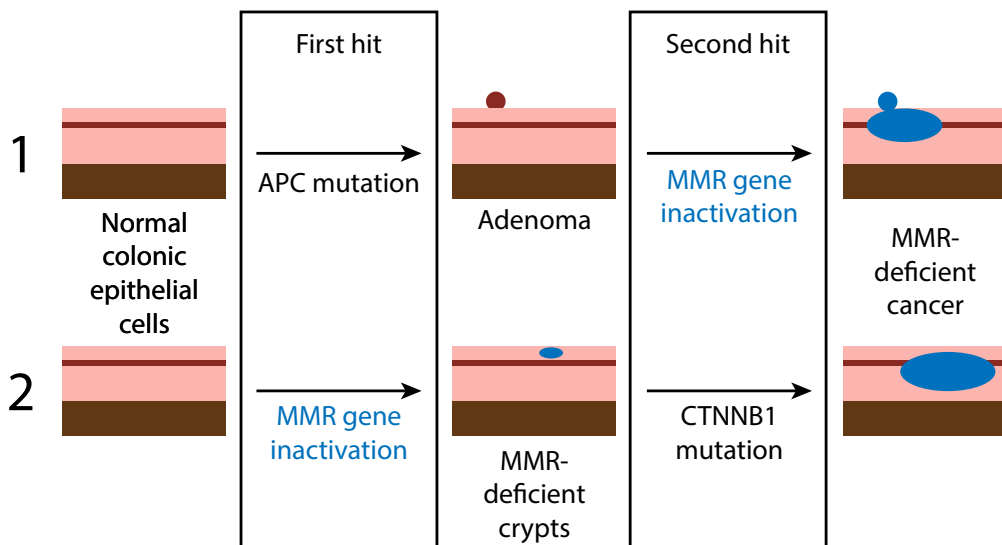


Figure 1: proposed pathways of colorectal tumorigenesis in Lynch Syndrome as described by Ahadova et al, *Familial Cancer*, 2016.⁸¹

or loci which are currently not known, although this may require very large study sizes. The expectation is that through these approaches, rare genetic variants or high-risk combinations of CRC susceptibility SNPs can be detected, an effort that is already being done in MMR-proficient, microsatellite stable tumors.^{82, 83}

Unexplained polyposis

In approximately 76-82% of the severe and typical FAP patients a pathogenic germline variant in *APC* is found, while the majority of patients with atypical or attenuated polyposis can be explained by *APC* or homozygous/compound-heterozygous germline *MUTYH* variants.⁸⁴⁻⁸⁶ Still, in a small fraction of patients no genetic predisposition can be found.

In recent years three new polyposis syndromes have been described which may account for a part of these unexplained polyposis cases. These syndromes are polymerase proofreading associated polyposis (PPAP)⁸⁷, caused by variants in the proofreading domain of *POLE* and *POLD1*, NTHL1-associated polyposis (NAP)⁸⁸, caused by homozygous or compound-heterozygous variants in the base excision repair gene *NTHL1* and the recently described MSH3-associated polyposis⁸⁹ caused by biallelic germline inactivation of the mismatch repair gene *MSH3*. Both PPAP and NAP have a variable phenotype with polyps but also with the occurrence of colorectal cancers and other extracolonic tumors. Both are also described to confer a specific mutation spectrum, with a *POLE/POLD1* defect resulting in an ultramutated phenotype with an increase of C:T>A:G transversions and *NTHL1* deficient tumors showing an increase in C:G>T:A transitions.^{44, 88} Compound-heterozygous *MSH3* variants have only been described in two unrelated individuals, one female with over 40 polyps and a history of thyroid adenoma (age 35), uterine leiomyoma (age 44) and polyps in corpus uteri and

duodenum, and one female with multiple adenomas at age 32, astrocytoma (age 26), ovarian and dermoid cysts and follicular thyroid adenomas at age 42.⁸⁹ Both also had small intraductal papillomas in the mammary gland. Whether these neoplasms are specific for an *MSH3* defect is unknown, and can only be concluded after more *MSH3* variant carriers are detected.

Of all patients carrying a pathogenic *de novo* *APC* variant, explaining the FAP phenotype, approximately 20% are estimated to have somatic mosaicism.⁹⁰⁻⁹² This phenomenon, in which (*APC*) variants are only present in a fraction of the cells, was already described in 1999 in a study where parents of five *de novo* FAP patients were tested for the *APC* variant found in the index patient.⁹³ Multiple studies have been conducted since then, always focusing on testing leukocyte DNA for variants with a low variant allele frequency, or on analyzing parents of *de novo* patients.^{90-92, 94-99} We (Chapter 5) and others have recently shown a high number of missed mosaic *APC* patients, by sequencing multiple adenomas of the same patients.^{100, 101} If two or more adenomas carry an identical *APC* variant, this might indicate an underlying *APC* mosaicism. This strategy has been proven to be more sensitive and specific than sequencing leukocyte DNA for variants with a low variant frequency, and can detect mosaicism confined to the colon.^{100, 101} Early detection of patients with somatic *APC* mosaicism is important and will help guide clinical management. Patients often show an attenuated FAP phenotype but if the variant is inherited by the patients offspring, they will show a full blown FAP phenotype with possibly 100 to more than 1000 adenomas.^{90, 91, 100}

Besides the recently described colorectal cancer syndromes with *POLE*, *POLD1*, *NTHL1* and *MSH3* germline variants, other genes have been implicated in unexplained familial polyposis syndromes. Three recent studies performing whole exome sequencing or genome-wide SNP genotyping in unexplained adenomatous polyposis patients describe variants or copy number variations in the *CNTN6*, *FOCAD*, *HSPH1*, *KIF26B*, *MCM3AP*, *YBEY*, *ARHGAP*, *CTNNB1*, *DSC2*, *PIEZO1*, *ZSWIM7* and *MCM9* genes.¹⁰²⁻¹⁰⁴ Variants in *DSC2*, *PIEZO1* and *ZSWIM7* were first detected in a cohort of 7 unrelated polyposis patients with 20-40 adenomas. Sequencing these three genes in a validation cohort of 191 unexplained polyposis patients resulted in the detection of 4 additional *DSC2* variants and 4 additional *PIEZO1* variants.¹⁰⁴ Copy number variant (CNV) analysis in 221 unexplained polyposis patients showed rare, non-recurrent germline CNVs in 77 proteins.¹⁰³ Targeted NGS found point-mutations in 10 of the 77 investigated genes (*CNTN6*, *FOCAD*, *HSPH1*, *KIF26B*, *MCM3AP*, *YBEY*, *CTNNB1* and three genes from the *ARHGAP* family).¹⁰³ Of these 10 genes, only *FOCAD* and *CTNNB1*, involved in Wnt signaling, have previously been related to CRC or colorectal adenoma predisposition.^{105, 106} The third study shows homozygous variants in the *MCM9* gene in two sisters with multiple polyps and metastatic CRC at young age. The *MCM9* gene encodes a DNA helicase involved in homologous recombination (HR) and mismatch repair (MMR).¹⁰⁷ However, an independent study sequencing *MCM9* in suspected Lynch Syndrome patients only detected variants of uncertain significance.¹⁰⁸ For all newly discovered genes more evidence is needed to establish them as CRC or polyposis susceptibility genes.

Other factors in colorectal cancer

While genetic factors are expected to play an important role in families with multiple affected patients, other factors are known to increase CRC susceptibility. Diet is an important factor, and it has been shown that a Western diet characterized by high intake of meat, refined

sugar and saturated fat, but lacking in fiber, contributes to development of CRC.¹⁰⁹⁻¹¹² Other lifestyle factors contributing to CRC risk are excess body weight, low physical activity, alcohol consumption and smoking.¹¹¹⁻¹¹³ Calcium, fiber, milk and whole grains on the other hand, have been associated with a lower risk of CRC.¹¹²⁻¹¹⁴

Another factor involved in increased CRC is the gut microbiota. The human intestinal tract is inhabited by trillions of microorganisms and the presence of specific bacteria or the dysbiosis of bacteria can aid in development and progression of colorectal cancer.^{115, 116} Bacteria can affect CRC tumorigenesis by secretion of toxins that can induce DNA damage and secretion of metabolites that affect translation, gene regulation and cell proliferation.^{115, 116} The gut microbiome is also shown to affect innate immunity through activation of toll-like receptors and through influencing T-cell differentiation.^{115, 116} Interestingly, diet and alcohol consumption have been shown to be able to dysregulate microbiota, possibly explaining the link between diet and colorectal cancer.¹¹⁵⁻¹¹⁷

Abovementioned non-genetic factors can result in believing multiple cancers in a family are due to an underlying genetic cause, while in fact the affected patients are phenocopies, i.e. displaying characteristics of a certain genotype but produced by environmental factors. This underlines a challenge in determining the underlying genetic cause in unexplained families with CRC. While WES and WGS enable the detection of rare pathogenic variants, it is difficult to determine whether a family with a few affected family members is indeed the result of one dominant DNA variation. Colorectal cancer is a common disease, and many environmental, as well as polygenic, factors can modulate CRC risk. A recent study shows that families with exactly two first-degree relatives only have a moderate probability of being due to segregating familial disease and advises to first focus on families with three or more members to increase the probability of finding genetic factors.¹¹⁸

Final conclusions

The estimated worldwide incidence of colorectal cancer is 746,000 new cases annually.¹¹⁹ Approximately 15% of these colorectal cancers will display MSI, while 3% of these CRCs will carry a germline MMR variant. Routine molecular screening of all early-onset CRCs with immunohistochemical staining of the four MMR proteins and/or MSI analysis yields a high number of suspected Lynch Syndrome cases of which the majority will likely remain suspected after conventional germline mutation screening and MSI analysis. In this thesis we describe possible genetic causes of unexplained suspected Lynch Syndrome (sLS) and unexplained adenomatous polyposis. We hypothesize that unexplained sLS patients could be explained by (1) missed MMR variants (Chapter 2), (2) by biallelic somatic inactivation (Chapter 2 and 3) or (3) by variants in other CRC susceptibility genes (Chapter 3).

We (Chapter 2 and 3), and others, show that biallelic somatic inactivation could possibly explain up to half of all unexplained sLS patients. Screening for somatic MMR variants should be broadly introduced in molecular tumor diagnostics, giving more insight in these sporadic MMR-deficient cases. Families should be critically assessed, so no underlying genetic variants are missed in these seemingly sporadic cases. Recent advances in (whole) exome and targeted next-generation sequencing enable detection of rare variants in previously unknown CRC susceptibility genes, and show that MMR-deficiency could be due to (secondary) somatic events, sometimes with underlying germline gene variants in genes such as *POLE*, *POLD1* or *MUTYH*. In future research it would be interesting to test affected family members of the patients of whom the tumor occurrence is explained by somatic events, to see whether these patients also have MMR-deficient tumors and to find a common underlying genetic cause in these family members.

While missed MMR variants seem a rare event, explaining only a fraction of patients (Chapter 2), it cannot be excluded that the remaining unexplained sLS tumors still carry undetected MMR variants. These variants could be currently misunderstood and classified as uncertain significance (VUS) or could be missed by conventional screening, because they are in intronic regions or in close proximity to the MMR genes. Functional assessment of VUS is critical. In Chapter 4 we show an easy method to detect aberrant splicing in formalin-fixed paraffin-embedded tissue, showing opportunities for the future. However, while this method enables functional assessment of variants predicted to result in splicing, it cannot detect aberrant splicing when the mutation status is unknown. A high-throughput NGS-based assay sequencing all exon-exon boundaries in RNA to detect any possible RNA aberration could possibly detect the cause of the MMR-deficiency. Also, it could indicate missed deep-intronic variants resulting in an aberrant RNA product. Furthermore, the current study relies on *in silico* prediction tools and effects that fall outside the amplification window will not be detected, while a high throughput assay would analyse the RNA in an unbiased manner. Other currently undiscovered mechanisms leading to aberrant RNA could be detected with this unbiased NGS approach.

The introduction of the population-based screening leads to an increasing number of patients with a low number of polyps (5-40) at older age (60+) which would previously go undiscovered. We (Chapter 6), and others, recently showed that many of these patients with

an attenuated FAP (AFAP) phenotype carry somatic mosaic *APC* variants. Current screening consisting of screening leukocyte DNA for variants with a low variant allele frequency is shown to miss many of these patients, because the variant is present with a too low variant allele frequency or because the mosaicism is confined to the colon. In-depth analyses of adenomas of AFAP patients could lead to the detection of more mosaic *APC* carriers, affecting clinical management of their children, who, if the variant is inherited, will show a full blown FAP phenotype.

While the exact incidence of biallelic somatic MMR variants, *POLE/POLD1* variants with secondary MMR-deficiency and somatic mosaicism still needs to be assessed, a large fraction of the previously unexplained sLS patients can now be explained and receive proper clinical management. However, in the remaining, approximately, 20-40% of sLS patients the underlying (familial) cause is still unknown. Whole exome or genome sequencing in the future will possibly lead to the detection of more rare gene variants, variants with moderate increase in CRC susceptibility or variants with moderate penetrance. Joint efforts screening for variants in larger cohorts and data sharing are essential in finding these (low penetrant) CRC susceptibility loci and might enable establishment of a CRC polygenic risk model with a personal cancer risk score.

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