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The diverse molecular profiles of lynch syndrome-associated colorectal cancers are (highly) dependent on underlying germline mismatch repair mutations

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ABSTRACT

Lynch syndrome (LS) is a hereditary cancer syndrome that accounts for 3% of all new colorectal cancer (CRC) cases. Patients carry a germline pathogenic variant in one of the mismatch repair (MMR) genes (*MLH1, MSH2, MSH6* or *PMS2*), which encode proteins involved in a post-replicative proofreading and editing mechanism. The clinical presentation of LS is highly heterogeneous, showing high variability in age at onset and penetrance of cancer, which may be partly attributable to the molecular profiles of carcinomas. This review discusses the frequency of alterations in the WNT/B-CATENIN, RAF/MEK/ERK and PI3K/PTEN/AKT pathways identified in all four LS subgroups and how these changes may relate to the 'three pathway model' of carcinogenesis, in which LS CRCs develop from MMR-proficient adenomas, MMR-deficient adenomas or directly from MMR-deficient crypts. Understanding the specific differences in carcinogenesis for each LS subgroup will aid in the further optimization of guidelines for diagnosis, surveillance and treatment.

1. Introduction

Colorectal cancer (CRC) is the third most common type of cancer worldwide and the second leading cause of cancer-related death (Tiwari et al., 2016; Sinicrope, 2018; Siegel et al., 2019). Of all new CRC cases, 3% are attributable to Lynch syndrome (LS) (Tiwari et al., 2016; Sinicrope, 2018). The LS phenotype is mainly characterized by young-onset CRC, and amongst women, endometrial cancer (EC), but other extracolonic cancers may also develop (Lynch et al., 2015; Tamura et al., 2019). People with this syndrome carry a germline variant in one of the mismatch repair (MMR) genes, including *MLH1* (located at 3p22.2), *MSH2* (2p21), *MSH6* (2p16.3) and *PMS2* (7p22.2), or more rarely an *EPCAM* deletion in the *a* gene that lies upstream of *MSH2* (for the extensive forms of all gene acronyms used within this article is referred to Supplementary Table 1) (Tiwari et al., 2016; Sinicrope, 2018; Lynch et al., 2015; Tamura et al., 2019; Peltomaki, 2016; Stojic et al., 2004).

Acting as a post-replicative proofreading and editing system, MMR prevents the accumulation of mutations that are generated by the slippage of DNA polymerases and otherwise overlooked by proofreading mechanisms (Tamura et al., 2019; Peltomaki, 2016; Weinberg, 2014). In addition, MMR recognizes and repairs diverse types of endogenous and exogenous damage, such as damage induced by oxidation (Bridge et al., 2014) or alkylation (Stojic et al., 2004). In short, MSH2 dimerizes either with MSH6 to form hMutSα, which scans DNA for base mismatches and

Abbreviations: COSM, Catalogue of Somatic Mutations in Cancer; COX-2, Cyclooxygenase 2; CRC, Colorectal cancer; EC, Endometrial cancer; EGFR, Epidermal growth factor receptor; EHTG, European Hereditary Tumour Group; FAP, Familial adenomatous polyposis; GSK-3B, Glycogen synthase kinase-3β; IDL, Insertion-deletion loop; LS, Lynch syndrome; MAP, MUTYH-associated polyposis; MMR(d), Mismatch repair(deficient); MMR-DCF, MMR-deficient crypt foci; MSI, Microsatellite instability; MSS, Microsatellite stability; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NGS, Next generation sequencing; NTHL1, Nth like DNA glycosylase 1; PGE_2, Prostaglandin E2; PI3K, Phosphatidylinositol 3-kinase; PIP2, Phosphatidylinositol-4,5-diphosphate; PIP3, Phosphatidylinositol-3,4,5-triphosphate; SCF, Skp1-Cdc53/Cullin-F-box-protein; SWI/SNF, SWItch/Sucrose Non-Fermentable; TSG, Tumour suppressor gene; WTX, APC membrane recruitment protein 1.

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short insertion-deletion loops (IDLs), or with MSH3 to form hMutSB, which recognizes longer IDLs (Tamura et al., 2019; Peltomaki, 2016). Upon localization of a mismatch, either hMutLα or hMutLɣ is recruited, consisting of MLH1 and PMS2 or MLH1 and MLH3, respectively. The hMutS-hMutL complex then triggers degradation of the strand containing the mismatch, followed by synthesis of a new strand (Tamura et al., 2019; Peltomaki, 2016; Weinberg, 2014). A deficient MMR (MMRd) system, as found in cells of LS patients who acquire a somatic hit in the remaining wild-type MMR allele, leads to a mutator phenotype and the development of microsatellite instability (MSI) (Peltomaki, 2003). The presence of MSI can be detected using DNA analysis, which together with immunohistochemical staining of the MMR proteins forms the basis for the identification of MMR deficiency. These two strategies show near-perfect concordance and have proven to be effective (Shia, 2008).

Genes that encode proteins with important cellular functions, including growth factor receptor genes (*TGFBRII, IGFIIR, ACVR2A*), genes involved in apoptosis (*BAX, E2F4*) and transcription factors (*TCF4*) may harbour repetitive sequences that are known targets in MMRd tumours (Miyaki et al., 2001; Yamaguchi et al., 2006; Pinheiro et al., 2015). Mutations that disrupt the normal function of these genes may lead to abnormal differentiation, proliferation and cell survival. In addition, a MMRd system leads to an increased frequency of point mutations in non-repetitive sequences, involving a wide variety of oncogenes and tumour suppressor genes (TSGs) (Oliveira et al., 2004). Three signalling pathways frequently affected in LS CRCs are the WNT/B-CATENIN, the RAF/MEK/ERK and the PI3K/PTEN/AKT pathways, all of which aid in a cell's road to malignancy when in a deregulated state (Weinberg, 2014; White et al., 2012; Fearon, 2011).

The phenotype of LS is highly heterogeneous, an example of which is the wide variation in the penetrance of cancer between *MLH1, MSH2, MSH6* and *PMS2* variants. A recent prospective study reported that the cumulative risk to develop CRC by age 70 years is 46 % for *MLH1* carriers, compared to only 35 % for *MSH2* and 20 % for *MSH6* carriers (Moller et al., 2017). Several retrospective studies have reported that PMS2 carrier risk is even lower (Suerink et al., 2019; Ten Broeke et al., 2018a). Furthermore, approximately 45 % of *MLH1*- and *MSH2*-associated CRCs occurring before the age of 75 develop as interval CRCs, whereas only 15 % of *MSH6*-associated CRCs and no *PMS2*-associated CRCs are reported interval cancers (Moller et al., 2018).

Despite this significant variation in phenotype, the need for gene-specific guidelines was only recently acknowledged, with the introduction of the European Hereditary Tumour Group (EHTG) (Seppala et al., 2020) and the guidelines published by the European Society of Gastrointestinal Endoscopy (ESGE) (van Leerdam et al., 2019). Meanwhile, the introduction of population-based LS screening has revealed even greater phenotypic heterogeneity, as population-based screening leads to an increase in the relative proportion of more mildly-affected LS families compared to LS testing based on stringent Amsterdam (Vasen et al., 1999) or Bethesda (Umar et al., 2004) criteria. Since *MSH6* and *PMS2* patients show a lower cancer penetrance, these patients were less likely to fulfil the earlier clinical criteria and therefore a large proportion of these patients were previously overlooked (Sinicrope, 2018). Another issue is that despite the known phenotypic heterogeneity of MMR genes, all diagnosed LS patients are screened for CRC every 1–2 years (Kohlmann and Gruber, 1993). Clearly, in view of the major differences between LS patient groups in the incidence of interval CRCs, optimal surveillance intervals are likely to differ between subgroups.

Much of the clinical heterogeneity seen in LS patients, such as wide variability in age of onset and cancer penetrance, can be attributed to the molecular profiles of individual carcinomas, for example due to the presence or absence of alterations in the three previously mentioned signalling pathways, which in part depends on which MMR gene is affected. It is important to stratify LS patients based on these molecular profiles, as this will facilitate the provision of optimal care to each patient.

Following the development of next generation sequencing (NGS), molecular profiles of large numbers of carcinomas can now be rapidly assessed, and this has led to a plethora of studies investigating the molecular profile of LS CRCs. In this review we evaluate available literature on NGS analyses of LS CRCs and analyse similarities and differences between the molecular profiles of *MLH1-*, *MSH2-*, *MSH6-*, and *PMS2*-associated CRCs. The main focus of this analysis is on alterations in the WNT/B-CATENIN, RAF/MEK/ERK and PI3K/PTEN/AKT pathways.

2. Alterations in the WNT/B-CATENIN pathway

The best studied signalling pathway in CRC is WNT/B-CATENIN. This pathway plays an important role in cellular development and differentiation, and was long thought to be the first pathway affected in the development of CRC (Weinberg, 2014; White et al., 2012; Brabletz et al., 2009). At the centre of this pathway is B-CATENIN, encoded by *CTNNB1* (Oliveira et al., 2004; Kohlmann and Gruber, 1993). In the absence of WNT signalling, B-CATENIN is bound to the cytoplasmic component of E-CADHERIN or is captured in a complex consisting of APC, AXIN, APC membrane recruitment protein 1 (WTX) and glycogen synthase kinase-3β (GSK-3B) (Fig. 1A). The latter protein phosphorylates B-CATENIN, tagging it for destruction. However, when WNT binds to its receptors, known as FRIZZLED and LRP, a signalling cascade causes the shutdown of GSK-3B (Fig. 1B). This allows B-CATENIN levels to rise and B-CATENIN will then move to the nucleus, where it mainly interacts with the transcription factors TCF/LEF to influence proliferation and differentiation, in addition to other important processes (Weinberg, 2014; White et al., 2012; Brabletz et al., 2009).

The WNT/B-CATENIN pathway is important in CRC due to the major role of B-CATENIN signalling in confining enterocytes to the colonic crypts (Weinberg, 2014; Brabletz et al., 2009). Normally, enterocytes migrate out of the colonic crypts and die 3–4 days after they form. This prevents enterocytes from acquiring multiple mutations that might possibly lead to (pre-)malignancies. However, when the WNT/B-CATENIN pathway is overactive, high levels of B-CATENIN in the nucleus prevent outmigration of the enterocytes (Weinberg, 2014; White et al., 2012; Brabletz et al., 2009), allowing additional time for enterocytes to acquire mutations that may further stimulate carcinogenesis.

2.1. *APC and CTNNB1*

Overall, 90 % of CRCs have a disordered WNT/B-CATENIN pathway (White et al., 2012). Recent work by Ahadova et al. (2018), 2021), (Engel et al. (2020) and Ten Broeke et al. (2018b) showed that determining the frequencies of *APC* and *CTNNB1* mutations, which were already known to be frequently mutated in LS CRCs, is of particular value (Johnson et al., 2005; Miyaki et al., 1999). *APC* mutations are distributed across the gene and both alleles need to be affected, while *CTNNB1* shows gain-of-function mutations usually located in exon 3, an exon that encodes a regulatory domain normally phosphorylated by GSK-3B (Johnson et al., 2005). Ahadova et al. (2018) proposed that three distinct pathways explain CRC development in Lynch patients, in contrast to the widely accepted idea that mutations in the WNT/B-CATENIN pathway underlie all CRC development in LS. A long-held idea was that LS does not influence the initiation rate of adenomas, but simply accelerates the progression of existing adenomas to invasive cancer. It was thought that an adenoma initially develops independently of MMR deficiency, and at some point a somatic mutation in an MMR gene causes a second hit that initiates carcinogenesis (Fig. 2A). This pathway, also known as the Adenoma–Carcinoma pathway, has been challenged following the discovery of MMR-deficient crypt foci (MMR-DCF) (Shia et al., 2015; Kloor et al., 2012). Two new pathways initiated by MMR deficiency were then proposed Ahadova et al. (2018). MMR-DCFs can either form MMR-deficient adenomas when a second hit in a MMR gene is followed by, for example, two *APC*

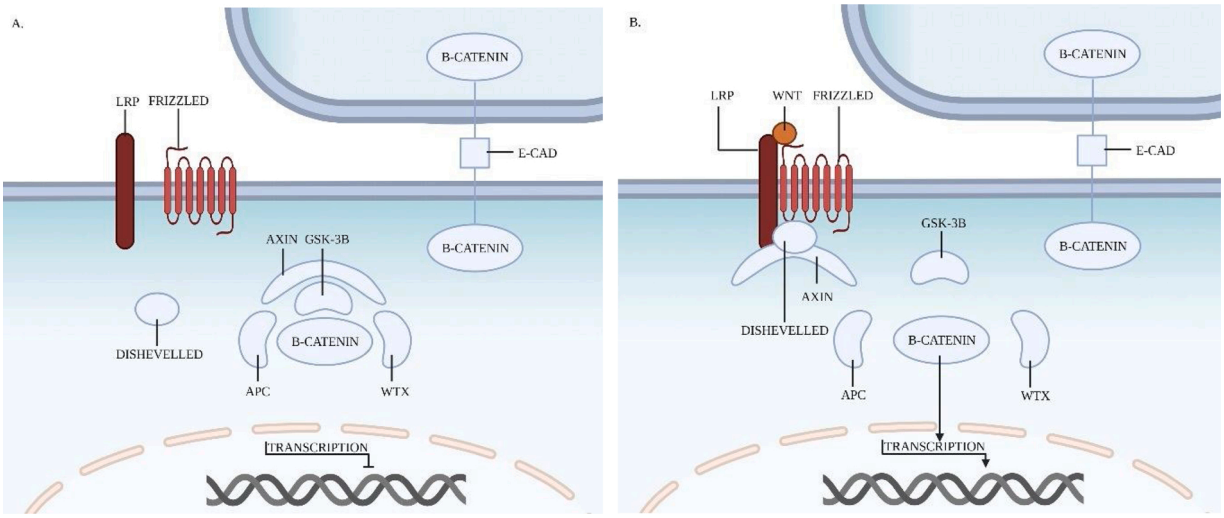

Fig. 1. WNT/B-CATENIN signaling pathway. (A) In the absence of a WNT signal, B-CATENIN is captured in a destruction complex consisting of APC, AXIN, WTX and GSK-3B. GSK-3B, in its active form, phosphorylates and thereby tags B-CATENIN for destruction. This mechanism prevents B-CATENIN from entering the nucleus and influencing transcription. In addition, B-CATENIN is bound to the cytoplasmic component of E-CADHERIN where it acts as an intermediary protein that links E-CADHERIN to the cytoskeleton. (B) When WNT proteins bind to their receptors, LRP and FRIZZLED, the DISHEVELLED and AXIN proteins are recruited to the membrane. This disrupts the destruction complex and inactivates GSK-3B, thereby giving B-CATENIN the opportunity to migrate to the nucleus and regulate the expression of genes involved in cell proliferation and differentiation.

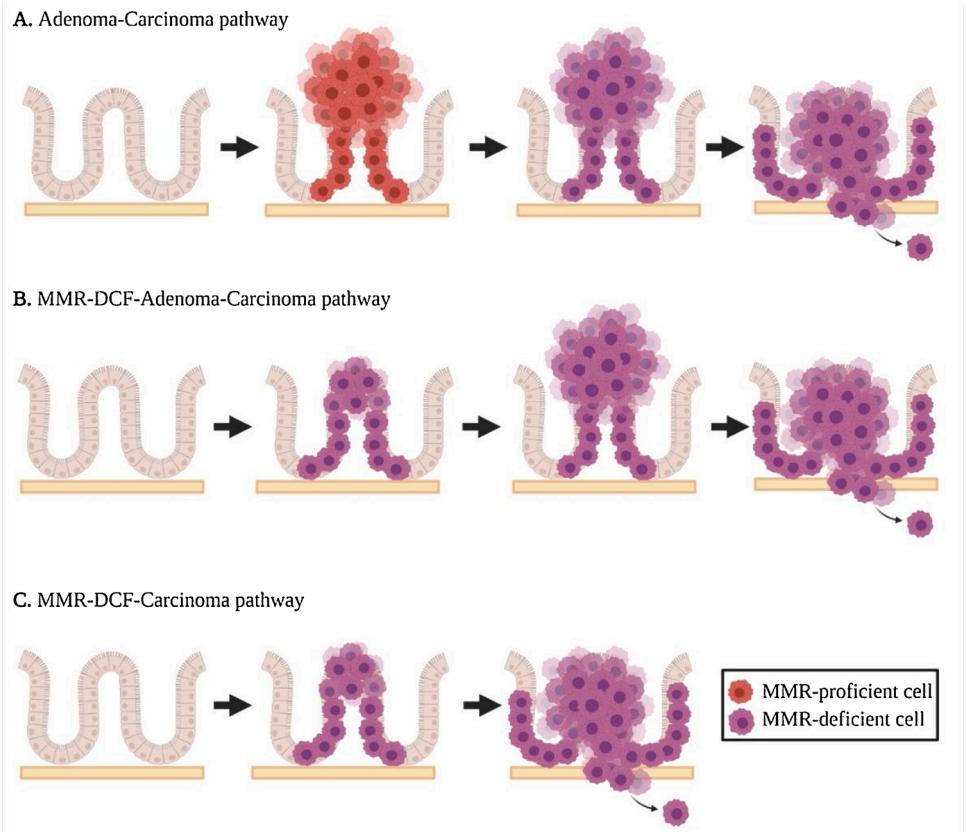

Fig. 2. Schematic overview of the three-pathway carcinogenesis model in LS. As hypothesized by Ahadova et al. (23), LS CRCs develop via three distinct pathways. In the Adenoma-Carcinoma pathway (A), adenomas develop independently of MMR deficiency. In contrast, in the MMR-DCF–Adenoma–Carcinoma pathway (B) and the MMR-DCF–Carcinoma pathway (C), tumour formation starts with MMR deficiency and is either followed by adenoma formation or results directly in a carcinoma.

mutations, which then progress to carcinomas (MMR-DCF–Adenoma–Carcinoma pathway), or they may form carcinomas directly (MMR-DCF–Carcinoma pathway), which show immediate invasive growth without a precursor lesion (Fig. 2B & C). The latter pathway is presumed to result in nonpolypous cancer formation, which is difficult to detect using colonoscopic surveillance.

Tumours arising via the MMR-DCF–Carcinoma pathway pose a serious risk to patients since they can form rapidly and unnoticed and

can thus result in an interval carcinoma (Ahadova et al., 2018, 2021; Engel et al., 2020). Interestingly, (Ahadova et al., 2018) hypothesized that *CTNNB1* mutations are involved in nonpolypous carcinogenesis. This is in line with their previous work, which showed that LS CRCs harbouring *CTNNB1* mutations lack signs of polypous growth and have a comparable growth pattern, with immediate invasive growth (Ahadova et al., 2021). Although the association between *CTNNB1* mutations and nonpolypous growth was not significant (Ahadova et al., 2018), and the number of analysed *CTNNB1*-mutated LS CRCs was low (Ahadova et al., 2021), this hypothesis provides a logical explanation for the results of other studies (Moller et al., 2018; Ten Broeke et al., 2018b). A study performed by Ten Broeke et al. reported no *CTNNB1* mutations (0/20, 0%) in *PMS2*-associated CRCs (Ten Broeke et al., 2018b), whereas *MLH1*-associated CRCs carried a significant number of *CTNNB1* mutations (14/24, 58 %) (Table 1). This finding is particularly interesting in light of data from the Prospective Lynch Syndrome Database (www.plsd.eu), which demonstrated that *PMS2* carriers do not develop interval CRCs, whereas *MLH1* and *MSH2* carriers have a cumulative risk to develop interval CRCs before the age of 75 of approximately 45 % (Moller et al., 2018). Based on the facts that *PMS2* carriers do not show interval CRCs or carcinomas with *CTNNB1* mutations, while *MLH1* carriers show both, and that *APC* mutations appear to be less frequent in *MLH1*-associated cancers than in those associated with other MMR genes (Ahadova et al., 2018; Ten Broeke et al., 2018b), the following two conclusions can be drawn: first, since a significant percentage of *MLH1*-associated cancers harbour *CTNNB1* mutations, a sizeable proportion of all *MLH1*-associated CRCs must arise via the MMR-DCF–Carcinoma pathway. Secondly, the absence of *CTNNB1* mutations and the presence of *APC* mutations in *PMS2*-associated LS CRCs (Ahadova et al., 2018, 2021; Ten Broeke et al., 2018b) suggests that this pathway is of little importance in *PMS2* carriers, though studies in larger cohorts will be needed to confirm this.

The absence of interval CRCs may in fact contribute to the extremely low cancer penetrance observed in *PMS2* carriers under surveillance (Moller et al., 2017). Because all carcinomas arise from adenomas, which are effectively removed during surveillance colonoscopy, *PMS2* carriers have a low probability of developing interval cancers. As *MSH6* carriers also show a lower cancer penetrance, it is plausible to suppose that the molecular profile of *MSH6*- and *PMS2*-associated CRCs may be comparable. However, due to a paucity of data available on *MSH6*-associated CRCs, this remains a speculation at this time.

An alternative and generally accepted explanation for the lower penetrance of *PMS2* and *MSH6* variants is that MLH1 forms an alternative heterodimer with MLH3 or PMS1 in case of a PMS2 deficiency, and that MSH2 does the same with MSH3 in case of MSH6 deficiency (Peltomaki, 2016). This mechanism would still allow partial damage repair and thus limit the number of mutations that might otherwise lead to cancer. The interplay between the two ideas is interesting and it is possible that mutations accumulate more readily in cells of *MLH1* carriers compared to *PMS2* carriers due to the functional redundancy of *PMS2,* which might explain the difference in *CTNNB1* mutational frequencies (Ten Broeke et al., 2018b). However, due to a lack of studies of the mutational burden in these tumours, this hypothesis cannot yet be proven.

An alternative explanation would be that *CTNNB1* mutations are not a direct consequence of MMRd, but are attributable to another process, for example, resulting from a high selection pressure on WNT/B-CATENIN signalling in MMR-DCF, which at this point do not have an *APC* mutation, leading to loss of other components of the pathway. This idea is in line with a limited contribution of MMRd to *CTNNB1* mutation, as suggested by previous studies (Ahadova et al., 2018; Johnson et al., 2005). However, this probably does not explain the absence of *CTNNB1* mutations in *PMS2*-associated LS CRCs, since this would imply that *PMS2* carriers do not have MMR-DCF, an idea for which no evidence has been found.

While the association between *CTNNB1* mutations and interval CRCs appears valid, it does not explain observations in *MSH2* carriers. Various studies have shown that *MSH2*-associated CRCs have relatively few *CTNNB1* mutations compared to *MLH1*-associated cancers (Table 1) (Ahadova et al., 2018, 2021; Engel et al., 2020; Ten Broeke et al., 2018b). Nonetheless, *MSH2*-associated CRCs arise as interval CRCs at a frequency similar to those associated with *MLH1* (Moller et al., 2018). Ten Broeke et al. (2018b) suggested that *MSH2* carriers might require variants in additional genes in order to enter the MMR-DCF–Carcinoma pathway. This, in turn, raises the question of why *MSH2* carriers require contributions from additional genes while *MLH1* carriers do not. An alternative explanation is that the majority of *MSH2*-associated CRCs are formed not by the MMR-DCF–Carcinoma pathway but by the other two pathways, an idea in line with the observation that both adenomas and advanced adenomas are found significantly more frequently in *MSH2* carriers compared to *MLH1* carriers (Engel et al., 2020). One explanation of the high frequency of interval CRCs in *MSH2* carriers is that the MMR-DCF–Adenoma–Carcinoma pathway, in which cells are already genetically unstable early in the process, leads to cancer more rapidly than the Adenoma–Carcinoma pathway, in which the mutator phenotype is acquired at a later timepoint (Engel et al., 2020). According to this mechanism, tumours arising via the MMR-DCF–Adenoma–Carcinoma pathway already have a high mutational burden and might thus develop unnoticed, eventually appearing as interval CRCs. Therefore, in addition to the small percentage of *MSH2*-associated CRCs that acquire *CTNNB1* mutations and follow the MMR-DCF–Carcinoma pathway, a larger number of tumours arise via the MMR-DCF–Adenoma–Carcinoma pathway and contribute to the high number of interval CRCs in *MSH2* carriers.

Table 1
APC, CTNNB1 and *RNF43* mutational frequencies in LS CRCs, stratified for germline variant.

	MLH1	MSH2	MSH6	PMS2	Study
APC	1/9 (11 %)	6/8 (75 %)	3/3 (100 %)	1/1 (100 %)	(Ahadova et al., 2018)
	3/24 (13 %)	6/18 (33 %)	N/A	6/20 (30 %)	(Ten Broeke et al., 2018b)
	8/9 (89 %)	1/8 (13 %)	0/3 (0%)	0/1 (0%)	(Ahadova et al., 2018)
CTNNB1	6/14 (43 %)	2/29 (7%)	0/3 (0%)	0/2 (0%)	(Ahadova et al., 2021)
	8/16 (50 %)	2/29 (7%)	0/3 (0%)	N/A	(Engel et al., 2020)[1]
	14/24 (58 %)	1/18 (6%)	N/A	0/20 (0%)	(Ten Broeke et al., 2018b)
RNF43	10/18 (56 %)	7/17 (41 %)	2/4 (50 %)	0/5 (0%)	(Fennell et al., 2018)

NOTE:
N/A. not applicable.
[1] CRCs analysed in (Engel et al., 2020) were also used in (Ahadova et al., 2018) and (Ahadova et al., 2021).

2.2. *RNF43*

Another gene product involved in the WNT/B-CATENIN pathway and now receiving increasing attention is *RNF43*. *RNF43* is a TSG that encodes RNF43, a protein that negatively regulates WNT signalling by ubiquitinating the WNT receptors, FRIZZLED and LRP (Fennell et al., 2018; Giannakis et al., 2014; Sekine et al., 2017). Numerous mutations in *RNF43* were identified in CRCs only recently (Giannakis et al., 2014), and since most mutations involved small IDLs and the *RNF43* gene contains two mononucleotide repeats, it was hypothesized that these mutations might be related to MSI. This hypothesis was strengthened by the finding that these mutations were more common in MSI CRCs than MSS CRCs (Giannakis et al., 2014; Jo et al., 2015), and subsequently led to studies investigating *RNF43* in LS CRCs. Sekine et al. (2017) noticed that *RNF43* mutations seem to be exclusively present in MMR-deficient

adenomas and carcinomas, having not been found in MMR-proficient tissue. However, *RNF43* mutations occur in LS CRCs, although at a lower frequency than in sporadic MSI CRCs (Fennell et al., 2018; Sekine et al., 2017). Interestingly, *RNF43* mutations frequently co-occur with *APC* and *CTNNB1* mutations in LS patients, in contrast to sporadic CRCs (Giannakis et al., 2014; Sekine et al., 2017). Together with the fact that more *RNF43* mutations are found in *MLH1*-associated CRCs (56 %) compared to *MSH2*-associated CRCs (41 %) (Fennell et al., 2018), this could suggest that *RNF43* mutations are synergistic with *CTNNB1* mutations in the MMR-DCF–Carcinoma pathway, helping direct *MLH1*-deficient crypt foci towards this pathway while *MSH2*-deficient foci are less able to do so (Table 1). This, however, is currently only speculation and needs to be tested in further research.

3. Alterations in the RAS-regulated signalling pathways

3.1. KRAS

Along with the WNT/B-CATENIN pathway, several RAS-regulated signalling pathways, such as the RAF/MEK/ERK kinase pathway and the PI3K/PTEN/AKT kinase pathway, are involved in the pathogenesis of various cancers, including CRC (Weinberg, 2014). At the centre of these pathways is a protein named RAS (Fig. 3). RAS proteins are GTPases found in three distinct isoforms: K-RAS, N-RAS and H-RAS (Weinberg, 2014; Yaeger and Saltz, 2014). Activating mutations in *KRAS, NRAS* and *HRAS* lead to overactivity of the downstream RAS pathways, resulting in the upregulation of cell proliferation and survival, and impaired differentiation (Weinberg, 2014; Yaeger and Saltz, 2014; Furtado and Samowitz, 2017). Overall, *KRAS, NRAS* and *HRAS* mutations are found in 45 %, 5% and 0% of all CRCs, respectively (Yaeger and Saltz, 2014; Furtado and Samowitz, 2017). Understandably, most literature focuses on *KRAS*. *KRAS* mutations are found in both MMR-proficient and MMR-deficient CRCs, with a slightly higher percentage in MMR-proficient CRCs (59 % vs. 43 %) (Rajagopalan et al., 2002). Mutations in MMR-deficient CRCs mainly involve codons 12 and 13, which disrupt GTPase activity and lead to the constitutive activation of K-RAS (Weinberg, 2014). Multiple studies have reported that specific hotspot mutations (G12D and G13D) occur more frequently in sporadic MSI and LS CRCs than in MSS sporadic CRCs (Ahadova et al., 2018; Ten Broeke et al., 2018b; Yaeger and Saltz, 2014; Kloth et al., 2016; Young et al., 2001), and in most cases these mutations occur after the onset of MMR deficiency. The latter became clear following the identification of the MMRd-associated unique combination of mutation types, depicted as the Catalogue of Somatic Mutations in Cancer (COSMIC) signature 6 (Ahadova et al., 2018), and by the presence of *KRAS* mutations in LS carcinomas but not in LS adenomas (Giannakis et al., 2014).

KRAS mutations seem to occur frequently in *MLH1-, MSH2-, MSH6-* and *PMS2*-associated CRCs (Table 2) (Oliveira et al., 2004; Ahadova et al., 2018; Ten Broeke et al., 2018b). In a study performed by (Oliveira et al., 2004), the frequency of *KRAS* mutations in *MSH6-* and *MSH2*-associated CRCs was significantly higher than in *MLH1*-associated CRCs. Although this finding has not been directly confirmed by the results of other studies (Ahadova et al., 2018; Ten Broeke et al., 2018b), it supports the idea that different groups of LS carriers develop cancer via different routes. This concept is further strengthened by the finding that the hotspot mutations G12D and G13D accounted for only

Table 2
KRAS, BRAF and *PIK3CA* mutational frequencies in LS CRCs stratified by germline variant.

	MLH1	MSH2	MSH6	PMS2	Study
KRAS	5/9 (56 %)	2/8 (25 %)	2/3 (67 %)	1/1 (100 %)	(Ahadova et al., 2018)
	7/24 (29 %)	7/18 (39 %)	N/A	10/20 (50 %)	(Ten Broeke et al., 2018b)
	29/91 (32 %)	29/61 (48 %)	5/6 (83 %)	N/A	(Oliveira et al., 2004)
BRAF	0/77 (0%)	0/34 (0%)	N/A	N/A	(Domingo et al., 2004)
	0/13 (0%)	0/7 (0%)	N/A	N/A	(Deng et al., 2004)
PIK3CA	8/24 (33 %)	7/18 (39 %)	N/A	5/20 (25 %)	(Ten Broeke et al., 2018b)

NOTE:
N/A. not applicable.

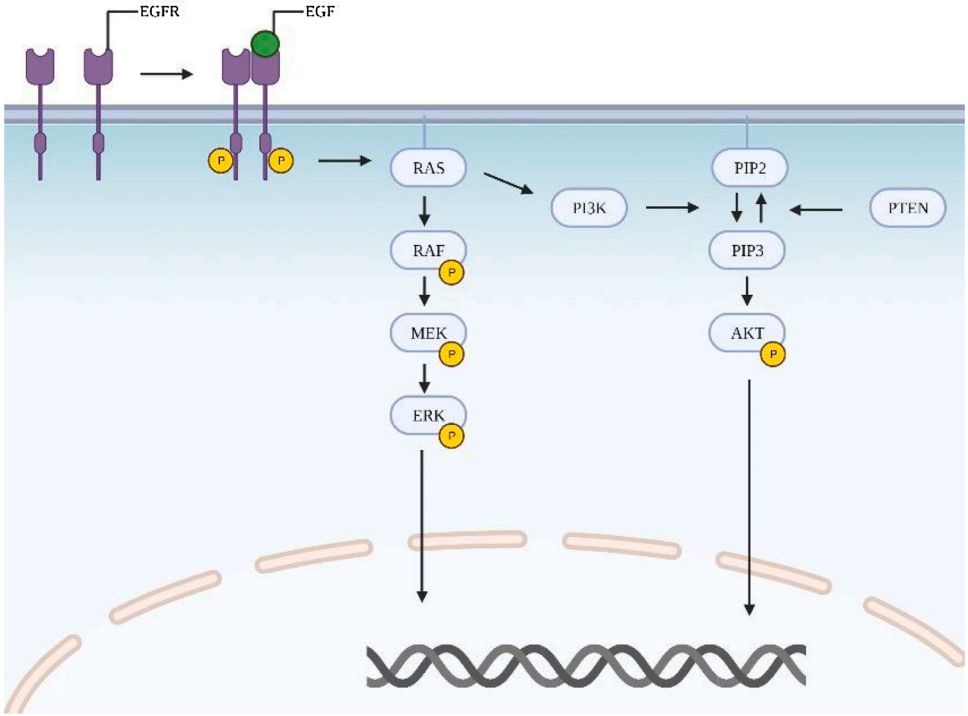

Fig. 3. The RAS-regulated signaling pathways. Upon binding of a growth factor, the growth factor receptor, in this example EGFR, dimerizes and each subunit phosphorylates its counterpart. The resulting phosphotyrosine residues serve as docking sites for a wide variety of proteins. These proteins can form a bridge between the receptor and RAS, located at the plasma membrane, leading to RAS activation. RAS, in turn, may activate several signaling pathways, including the RAF/MEK/ERK and PI3K/PTEN/AKT pathways. In the RAF/MEK/ERK pathway, RAS activates RAF, which then phosphorylates and thereby activates MEK. MEK phosphorylates ERK, which then phosphorylates substrates involved in various cellular processes, including transcription, proliferation and differentiation. In the PI3K/PTEN/AKT pathway, activation of PI3K by RAS leads to the conversion of PIP2 into PIP3. PIP3 activates the kinase AKT, which as in the case of ERK, phosphorylates proteins involved in various cellular processes. PTEN converts PIP3 into PIP2, effectively counteracting the actions of PI3K and serving as a negative regulator of the PI3K/PTEN/AKT pathway.

20 % of all *KRAS* mutations in *PMS2*-associated CRCs, whereas this figure was considerably higher in *MLH1-, MSH2-* and *MSH6*-associated CRCs (Ten Broeke et al., 2018b). The latter finding suggests that adenomas and/or CRCs in *PMS2* variant carriers acquire *KRAS* mutations before they develop PMS2 deficiency, since types of mutations other than those related to MMR deficiency predominate (Ten Broeke et al., 2018b). Again, this is in line with the idea that *PMS2* carriers develop cancer only via the Adenoma–Carcinoma pathway, as discussed in previous sections, since PMS2 deficiency apparently occurs as a late event and not as an initiating step. It also supports the hypothesis that MSH2 deficiency can accelerate the adenoma-carcinoma pathway, explaining the observed interval carcinomas. Unfortunately, no data were available on the specific KRAS mutations found in *MSH6*-associated cancers (Ten Broeke et al., 2018b), as it would have been interesting to see if the G12D and G13D mutations were also less common in the MSH6 subgroup, in line with the lower rate of interval cancers in MSH6.

3.2. BRAF

RAF is found directly downstream of RAS in the RAF/MEK/ERK pathway (Fig. 3) (Weinberg, 2014; Yaeger and Saltz, 2014). B-RAF is the isoform most commonly involved in cancer, and similarly to *KRAS* mutations, activating mutations in *BRAF* lead to continuous proliferation and disordered differentiation (Weinberg, 2014; Yaeger and Saltz, 2014; Thiel and Ristimaki, 2013). B-RAF is a serine/threonine kinase which phosphorylates and thereby activates downstream proteins, particularly MEK (Weinberg, 2014). V599E and V600E are two common mutations found in *BRAF*, and both result in a constitutively active protein kinase domain (Rajagopalan et al., 2002; Thiel and Ristimaki, 2013). In total, 5–10 % of CRCs have a *BRAF* mutation (Furtado and Samowitz, 2017). Although these mutations are relatively common in sporadic MMR deficiency and MSI (Rajagopalan et al., 2002; Nowak and Hornick, 2016), they are only rarely found in LS CRCs (Table 2) (Sekine et al., 2017; Rajagopalan et al., 2002; Domingo et al., 2004; Deng et al., 2004; Thiel and Ristimaki, 2013; Cohen et al., 2016). Moreover, *BRAF* and *KRAS* mutations are thought to be mutually exclusive (Yaeger and Saltz, 2014; Rajagopalan et al., 2002). Taken together, these data suggest that *BRAF* mutations have a minimal role in LS. As the *BRAF* V600E variant is strongly associated with somatic *MLH1* hypermethylation, many laboratories conduct *BRAF* analysis as a surrogate marker to assess *MLH1* hypermethylation (Parsons et al., 2012; Chen et al., 2014)

3.3. PI3K and PTEN

Another important pathway downstream of RAS is the PI3K/PTEN/AKT pathway (Fig. 3). In this pathway, RAS activates a phosphatidylinositol 3-kinase (PI3K), which then phosphorylates phosphatidylinositol-4,5-diphosphate (PIP2) to form phosphatidylinositol-3,4,5-triphosphate (PIP3) (Weinberg, 2014; Zhao and Vogt, 2008). PIP3, located at the plasma membrane, is mainly associated with the activation of AKT/PKB, which in turn activates a series of proteins that lead to cell proliferation, cell growth and resistance to apoptosis (Weinberg, 2014; Zhao and Vogt, 2008). One particular PI3K, PIK3CA, is involved in several types of cancer, including 15–25 % of all CRCs (Fearon, 2011). Activating mutations in *PIK3CA* leads to overproduction of PIP3 and therefore unchecked cellular proliferation, growth and survival. Of note, these mutations are usually concurrent with either *KRAS* or *BRAF* mutations (Fearon, 2011; Yaeger and Saltz, 2014). This suggests that mutations in *KRAS* mainly effect the RAF/MEK/ERK pathway but have little or no impact on the PI3K/PTEN/AKT pathway, since additional *PIK3CA* mutations are required in order impede the latter pathway (Fearon, 2011). Mutations in *PIK3CA* occur more frequently in MSI CRCs than in MSS CRCs (Kloth et al., 2016). When comparing the different subgroups of MSI CRCs, *PIK3CA* mutations appear more common in MMRd CRCs, in which both alleles of a single MMR gene are somatically mutated, than in LS CRCs or *MLH1* hypermethylated CRCs (Cohen et al., 2016). When stratifying LS CRCs based on germline variants, *MLH1-, MSH2-* and *PSM2*-associated CRCs show no significant differences in *PIK3CA* status (Ten Broeke et al., 2018b), albeit additional literature to corroborate this observation is lacking and no data at all are available on *MSH6*-associated CRCs (Table 2).

Interestingly, *PIK3CA* mutations might be of importance when predicting the efficacy of aspirin as a preventive therapy in LS carriers. Aspirin has been shown to reduce the risk of CRC in the general population (Cook et al., 2013) and does this by inhibiting cyclooxygenase 2 (COX-2), which produces prostaglandin E2 (PGE_2) (Nguyen et al., 2020). PGE_2 is involved in the regulation of cell proliferation and aids in tumorigenesis. A significant reduction in 10-year risk of developing CRC has now also been shown in LS patients (HR 0.65). It is not yet known whether cancers that develop during aspirin therapy have a specific molecular signature or whether the reduction in risk differs between *MLH1-, MSH2- MSH6- or PSM2*-LS patients (Burn et al., 2020).

Analysing the contribution of PIK3CA to this process might possibly generate new insights. The exact effects of *PIK3CA* variants on aspirin therapy in general are still controversial. Wang et al. (2015) demonstrated that PGE_2 exerts its tumorigenic effects partly by activating the PI3K/PTEN/AKT pathway, which suggests that activating mutations in *PIK3CA* confer resistance to preventive aspirin therapy, as PIK3CA acts downstream of PGE_2. On the other hand, since PIK3CA signalling can result in the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) Wang et al. (2015), and because NF-κB induces transcription of the *COX2* gene (Dixon et al., 2013), increased PIK3CA signalling leads to increased synthesis of PGE_2, suggesting that pre-malignant cells with activating *PIK3CA* variants are actually susceptible to preventive aspirin therapy.

Acting in the opposite direction to PIK3CA, PTEN dephosphorylates PIP3 to PIP2 and thereby blocks the signalling pathway (Fig. 3) (Weinberg, 2014; Zhao and Vogt, 2008). *PTEN* is a TSG and inactivating mutations in both *PTEN* alleles provide a second mechanism by which the PI3K/PTEN/AKT pathway can become deregulated. Between 10 % and 20 % of CRCs carry *PTEN* mutations (Cohen et al., 2016; Laurent-Puig et al., 2009), with a higher prevalence in MSI CRCs compared to MSS CRCs (Cohen et al., 2016). As with *PIK3CA* mutations, *PTEN* mutations co-occur with *KRAS* and *BRAF* mutations (Fearon, 2011), providing further support for the idea that *KRAS* mutations alone are not able to activate the PI3K/PTEN/AKT pathway. Although *PTEN* mutations in LS EC are a hot topic, the exact role of *PTEN* mutations in LS CRCs remains to be investigated. In one study, 24 % (4 out of 18) of LS CRCs harboured a *PTEN* mutation (Cohen et al., 2016), indicating that *PTEN* mutations may play a role in a subset of CRCs associated with LS, though more research concerning the effect of these mutations is needed. No literature could be found regarding *PTEN* mutations in LS CRCs stratified for germline variants.

4. Other genes and their role in LS CRCs

4.1. TP53

TP53 is one of the best studied TSGs and is the most commonly mutated gene in all human cancers (Weinberg, 2014). The protein it encodes, P53, acts as a transcription factor and can impose cell cycle arrest and apoptosis in response to a wide variety of signals, including DNA damage (Weinberg, 2014). Mutations in *TP53* are found in 60 % of all CRCs (Nakayama and Oshima, 2019), with a higher prevalence in MSS CRCs compared to MSI CRCs (Kloth et al., 2016). The presence or absence of such mutations in LS CRCs could be very important because they have been associated with nonpolypous carcinogenesis and might therefore be involved in the MMR-DCF–Carcinoma pathway (Ahadova et al., 2018). If this is indeed the case, a higher frequency of *TP53* mutations would be expected in *MLH1*-associated and *MSH2*-associated LS

CRCs, since these carcinomas show the highest frequency of interval growth (Moller et al., 2018). However, this hypothesis is not directly supported by currently available literature, as significant numbers of *TP53* mutations have been found in LS CRCs associated with all four germline MMR mutations (Ahadova et al., 2018; Ten Broeke et al., 2018b). This topic therefore remains an important subject of investigation (Table 3). Using larger cohorts, it would be interesting to determine whether *TP53* mutations, like *CTNNB1* mutations, occur more frequently in *MLH1*-associated CRCs compared to *MSH2*-associated CRCs, as this would potentially shed more light on the preferential pathways of CRC development in MLH1 versus MSH2 carriers.

4.2. FBXW7

FBXW7, a member of the F-box protein family, is classified as a TSG and contributes to various types of cancer (Sailo et al., 2019; Yeh and Bellon, 2018). *FBXW7* encodes a protein that forms part of the Skp1-Cdc53/Cullin-F-box protein (SCF) complex, which functions as a ubiquitin ligase and controls the degradation of various oncoproteins including cyclin E, c-MYC and mTOR. Mutations in *FBXW7* that lead to a defective SCF complex cause the accumulation of oncoproteins and therefore promote tumorigenesis (Sailo et al., 2019; Yeh and Bellon, 2018). *FBXW7* is commonly mutated in CRC, at frequencies of 6–20 % (Malapelle et al., 2016; Miyaki et al., 2009). Miyaki et (Miyaki et al., 2009) al. detected *FBXW7* mutations in 9% of LS CRCs and in 10 % of sporadic CRCs, suggesting that *FBXW7* mutation frequencies are roughly similar between these groups. This contrasts with a study performed by ten Broeke et al. (Ten Broeke et al., 2018b), which found a significantly higher prevalence of *FBXW7* mutations in LS CRCs compared to sporadic CRCs (Table 3). In the latter study, 20 % of *PMS2*-associated CRCs harboured *FBXW7* mutations, compared to 17 % in *MLH1*- and *MSH2*-associated CRCs and 0% in sporadic CRCs. Since no significant differences were found between the LS subgroups, *FBXW7* most likely plays a similar role in each group, except possibly for *MSH6*-associated CRCs for which data are not available. Since alterations in *FBXW7* have been associated with both a poor prognosis (Korphaisarn et al., 2017; Iwatsuki et al., 2010) and an earlier onset of sporadic CRC (Kothari et al., 2016), it would be interesting to see if similar correlations can also be found between *FBXW7*-positive and *FBXW7*-negative LS CRCs.

4.3. ARID1A

ARID1A forms part of the highly conserved SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin-remodelling complex, which is a master regulator of transcription and acts as a tumour suppressor (Reisman et al., 2009). Mutations in *ARID1A* have been found in a range of cancer types and lead to a dysfunctional SWI/SNF complex (Reisman et al., 2009; Jones et al., 2012). The exact prevalence of *ARID1A* mutations in CRC remains to be validated, but is predicted to be around 10 % (Jones et al., 2012). These mutations are strongly related to MMR deficiency and MSI (Jones et al., 2012; Ye et al., 2014; Cajuso et al., 2014; Chou et al., 2014), but the mechanism underlying this relationship remains a topic of discussion. *ARID1A* mutations could either be a result of MMR deficiency or the cause. Evidence is currently lacking regarding the former option, as, for example, mutations in *ARID1A* are distributed along the entire gene rather than clustered at microsatellite regions (Cajuso et al., 2014). The latter option has already been considered for ECs (Bosse et al., 2013). In these cancers *ARID1A* mutations are associated not with LS but with *MLH1* hypermethylation (Bosse et al., 2013), based on the underlying idea that mutations in *ARID1A* cause epigenetic alterations that may include *MLH1* hypermethylation, indirectly causing MSI (Bosse et al., 2013). In the case of CRC, an association between *MLH1* hypermethylation and ARID1A loss has been demonstrated using immunohistochemistry (IHC) (Ye et al., 2014), suggesting that this mechanism might indeed also hold for CRC as well. Based on IHC, it has also been shown that CRCs lacking expression of PMS2 only (n = 2), or both MSH2 and MSH6 (n = 9), lacked expression of ARID1A. In addition, 1 out of 4 carcinomas lacking expression of MSH6 only, stained negative for ARID1A (Ye et al., 2014). Nevertheless, these results do not yet clarify the role of *ARID1A* in LS-associated CRC, and additional NGS analysis of large cohorts of LS CRCs will be needed to resolve this issue. In the course of these analyses it would be interesting to consider a possible association between *ARID1A* mutations and alterations in the PI3K/PTEN/AKT pathway. This idea arose because alterations in the PI3K/PTEN/AKT pathway are more commonly found in ARID1A-negative ECs, which may imply that ARID1A is associated with this pathway (Bosse et al., 2013). On the other hand, an inverse relationship between *TP53* and *ARID1A* mutations has been noted in endometrial (Bosse et al., 2013), ovarian (Xiao et al., 2012) and gastric (Cajuso et al., 2014) cancers, suggesting that P53 and ARID1A mediate their tumour suppressor functions via roughly similar mechanisms.

Table 3
TP53 and *FBXW7* mutational frequencies in LS CRCs stratified for germline variants.

	MLH1	MSH2	MSH6	PMS2	Study
TP53	4/9 (44 %)	1/8 (13 %)	1/3 (33 %)	1/1 (100 %)	(Ahadova et al., 2018)
	6/24 (25 %)	5/18 (28 %)	N/A	5/20 (25 %)	(Ten Broeke et al., 2018b)
FBXW7	4/24 (17 %)	3/18 (17 %)	N/A	4/20 (20 %)	(Ten Broeke et al., 2018b)

NOTE:
N/A. not applicable.

5. Clinical implications and future recommendations

Patients with LS suffer from a hereditary predisposition to CRC. Before the introduction of the EHTG and ESGE guidelines in September 2020, in most countries all LS patients were screened and treated according to the same guidelines. As previously discussed, LS is considered to be a highly heterogeneous disease, with significant differences in, for example, the penetrance of cancer and the age at which cancer develops. A growing number of NGS analyses have also shown that most of this heterogeneity is attributable to a variety of different molecular pathways of tumour development, and only partly depends on which MMR gene is mutated. The varying distribution of mutations in important onco- and tumour suppressor genes supports the idea that LS can be divided into distinct subgroups, and furthermore suggests that in the future the diagnosis, surveillance and treatment of LS should be specifically optimized for each subgroup.

It is now recognized that LS CRCs develop via one of three pathways and that the relative occurrence of these pathways depends on the underlying germline MMR mutation. Combining available evidence, *CTNNB1* mutations appear to be associated with the MMR-DCF–Carcinoma pathway, while *APC* mutations are more closely related to the development of adenomas. In future experiments, it would be interesting to explore the mechanisms underlying these associations. Many questions regarding this topic still remain to be answered: Can functional redundancy account for the differences in *CTNNB1/APC* mutational frequencies between subgroups or is some other mechanism at play? Do *CTNNB1* mutations actually initiate the MMR-DCF–Carcinoma pathway? And what is the role of genes such as *RNF43* and *TP53* in this process? To answer these questions more research using larger cohorts is needed. Clarifying the mechanisms behind each pathway will not only lead to a better understanding of disease, but will also aid in the provision of optimal care to each patient. For example, changes in surveillance procedures might be beneficial, with shorter surveillance intervals for *MLH1* and possibly *MSH2* carriers, and longer intervals for *MSH6* and *PMS2* carriers. In addition, new insights could form a basis for subgroup-dependent treatments. Although MMRd CRCs, including LS CRCs, in general are known to be especially sensitive to

immunotherapies, considering the fact that the presence of MSI leads to a high mutational load with an increased expression of neoantigens, these therapies are not applicable to all LS patients, for example due to the high risk of immune-mediated adverse reactions (Chang et al., 2018). For this reason, the development of other therapies, such as molecular targeted therapies, is of vital importance as well. A recent study in mice for example showed that β-catenin short hairpin RNA therapy suppresses *APC*-mutated tumour growth but not *CTNNB1*-mutated tumour growth (Mologni et al., 2010). Extrapolating this outcome to humans, comparable results might be beneficial in patients with *PMS2* variants but not in patients with *MLH1* variants, highlighting the importance of specific care for each subgroup.

Although *BRAF* mutations do not seem to contribute to carcinogenesis in LS syndrome patients, the opposite is the case for *KRAS, PIK3CA* and *PTEN* mutations and might thus affect distinct LS subgroups in different ways. Knowing the status of *KRAS, BRAF, PIK3CA* and *PTEN* would also help in choosing the proper treatment. For example, *PIK3CA* mutations might influence the effectiveness of aspirin in preventing CRC, as mentioned earlier. Moreover, patients with metastatic CRC harbouring *KRAS* or *BRAF* variants show a poorer response to anti-EGFR therapies such as cetuximab and panitumumab (Raponi et al., 2008; Lievre et al., 2006). These monoclonal antibody therapies work by blocking epidermal growth factor receptors (EGFRs), which normally activate the RAS signalling pathways after the binding of a growth factor. Since *KRAS* and *BRAF* are downstream of the EGFR, blocking the EGFR will not prevent mutated *KRAS* or *BRAF* from signalling and therefore *KRAS* and *BRAF* status can be used to predict treatment response (Yaeger and Saltz, 2014; Furtado and Samowitz, 2017; Kloth et al., 2016; Seruca et al., 2009). A similar relationship is expected in the case of *PIK3CA* and *PTEN*, however, to date the use of *PIK3CA* and *PTEN* mutations to predict anti-EGFR therapy responses has produced conflicting results and thus requires further research (Furtado and Samowitz, 2017).

Besides genes involved in the WNT/B-CATENIN, RAF/MEK/ERK and PI3K/PTEN/AKT pathways, additional genes involved in other processes and signalling pathways are associated with CRC and LS. Amongst these are *TP53, ARID1A* and *FBXW7,* together with other genes that were not discussed in this review such as *ERBB2, SMAD4, NF1, ATM, BRCA1/2* and *CREBBP*. For most of these genes, however, little research has been undertaken with regard to LS. A better understanding of the status of these genes in LS CRCs, with stratification for germline variant, will provide new insights in the development of CRC in LS patients and might also aid our understanding of the three pathways of carcinogenesis in LS patients.

One emerging development that could be especially helpful in future studies investigating the LS molecular profile is the extraction of mutational signatures from sequencing data (Alexandrov et al., 2013; Ma et al., 2018). Seven mutational signatures are associated with MMRd, including signatures 6 (already mentioned with regards to *KRAS* variants), 14, 15, 20, 21, 26 and 44 (Alexandrov et al., 2013; Helleday et al., 2014; Van Hoeck et al., 2019; Viel et al., 2017; Haradhvala et al., 2018; COSMIC, 2020). When analysing sporadic variants, looking at mutational signatures would make it easier to determine the timing of mutational events and to identify mutations attributable to a MMRd. Of note, these signatures are not only useful during research but also exhibit considerable potential in the diagnosis of LS. For example, signatures 6, 10, 15 and 26 indicate to be suitable for detecting MSI and as such can distinguish MMRd CRCs from MMR-proficient CRCs (Viel et al., 2017; Drost et al., 2017; Georgeson et al., 2020). Mutational signatures might also be used to detect hereditary predispositions by discriminating LS CRCs from sporadic MMRd CRCs. Recent studies focused on another DNA repair mechanism, base excision repair, demonstrated that deficiencies of Nth like DNA glycosylase 1 (NTHL1) correspond with the presence of signature 30 (Drost et al., 2017; Grolleman et al., 2019), while deficiencies of MUTYH result in signatures 18 and 36 (Viel et al., 2017; Pilati et al., 2017). This suggests that deficiencies within a single repair mechanism can result in more than one signature.

In conclusion, the first steps in the molecular profiling of *MLH1*-, *MSH2*-, *MSH6*- and *PMS2*-associated CRCs have been taken, but further comprehensive genetic analysis of mutations in these cancers is vital in order to confirm the findings of previous studies and to provide a more complete picture of carcinogenesis in LS patients. Ultimately, our goal should be to use molecular profiles as guidance for surveillance, treatment, preventive strategies and other aspects of LS patient care.

Author contributions

Noah Cornelis Helderman: conceptualization, writing, visualization, investigation. **Sanne W. Bajwa - ten Broeke:** reviewing and editing. **Anne-Sophie van der Werf – 't Lam:** reviewing and editing. **Manon Suerink:** reviewing and editing. **Diantha Terlouw:** reviewing and editing. **Hans Morreau:** reviewing and editing. **Tom van Wezel:** reviewing and editing. **Maartje Nielsen:** conceptualization, supervision, reviewing and editing.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.critrevonc.2021.103338.

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