MOLECULAR PATHOLOGY OF LYNCH SYNDROME

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Invited review for The Journal of Pathology Annual Review Issue 2020.

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The authors declare no conflicts of interest.

6822 words; 182 references; 1 Table; 2 Figures

Abstract

Lynch syndrome (LS) is characterised by predisposition to colorectal, endometrial and other cancers and is caused by inherited pathogenic variants affecting the DNA mismatch repair (MMR) genes *MLH1, MSH2, MSH6* and *PMS2*. It is probably the most common predisposition to cancer, having an estimated prevalence of between 1/100 and 1/180. Resources such as the International Society for Gastrointestinal Hereditary Cancer's MMR gene variant database, the Prospective Lynch Syndrome Database (PLSD) and the Colon Cancer Family Register (CCFR), as well as pathological and immunological studies are enabling advances in the understanding of LS. These include defined criteria by which to interpret gene variants, the function of DNA mismatch repair in the normal control of apoptosis, definition of the risks of the various cancers, and the mechanisms and pathways by which the colorectal and endometrial tumours develop, including the critical role of the immune system. Colorectal cancers in LS can develop along three pathways, including flat intramucosal lesions, which depend on the underlying affected MMR gene. This gives insights into the limitations of colonoscopic surveillance and highlights the need for other forms of anti-cancer prophylaxis in LS. Finally, it shows that the processes of autoimmunisation and immunoediting fundamentally constrain the development of tumours in LS and explains the efficacy of immune checkpoint blockade therapy in MMR deficient tumours.

Keywords

Lynch syndrome

Colorectal cancer

Endometrial cancer

DNA mismatch repair

Apoptosis

Gene database

Gene variant interpretation

Immunoediting

Immunotherapy

1. Introduction

Lynch syndrome (LS), previously called hereditary non-polyposis colorectal cancer (HNPCC), is probably the most common major cause of inherited susceptibility to cancer, with an estimated prevalence in the general population of between 1/100 and 1/180.[1] It is characterized by predisposition to a range of cancers, involving most frequently the colorectum and endometrium, and also many other organ sites including ovary, stomach, small intestine, hepato-biliary tract, pancreas, urinary tract, prostate, brain and sebaceous skin tumours.[2, 3]

LS is caused by constitutional (germline) pathogenic variants affecting one of four genes encoding the DNA Mismatch Repair (MMR) system components: MLH1, MSH2, MSH6, and PMS2, and hence the current commonly accepted diagnostic definition of LS is carrying such a variant.[1, 4-8] However, while we acknowledge there is an opinion that LS can only be diagnosed in such individuals once cancer has been diagnosed, this is contrary to the hereditary polyposes, which are characterised by the macroscopic syndromic feature of multiple pre-malignant tumours.[9] Allied to this, a major purpose of diagnosis of a cancer predisposing condition is to identify those who would benefit from surveillance and prophylactic surgery to prevent cancer.[5] Hence, our view is that it is inconsistent and to a degree cruel to discriminate against LS by expecting such individuals to develop cancer in order to be diagnosed with the condition, especially as cancer does not always occur.[2, 3, 10] As we discuss later on, the definition of LS could, and perhaps should, therefore, move to one that incorporates recently identified specific microscopic and molecular pre-malignant syndromic features.

DNA MMR recognises and repairs mismatched bases (e.g. C opposite T) and insertions or deletions in repetitive sequences. In LS patients, this MMR gene constitutional variant, when combined with an acquired second pathogenic variant due to somatic mutation in the wild-type allele of the same MMR gene, results in the complete loss of MMR pathway function in the affected cells. Deficiency of MMR (dMMR) leads to hypermutability, resulting in an increase in the mutation rate by 100- to 1000-fold due to uncorrected base mismatches, and to microsatellite instability (MSI) due to variation in the lengths of repetitive sequences (e.g. AAAAAAA… or CACACACA…. or similar, known as microsatellites) due to uncorrected insertion/deletion loops that are prone to occur as DNA replication errors in repetitive sequences.[11] However, crucially, dMMR does not simply fail to repair mismatches, it elicits a reduced susceptibility to apoptosis induced by DNA damage recognised by the MMR pathway.[11-14]

The International Society for Gastrointestinal Hereditary Cancer (InSiGHT) was the first expert group to define pathogenicity of gene variants according to an agreed set of criteria based upon Bayesian probability, using the 5-tier classification system of Plon et al.[4, 15-17] In this system Class 5 variants are pathogenic and Class 4 likely pathogenic, with Class 3 being variants of uncertain significance (VUS), and Classes 2 and 1 being likely benign and benign respectively. InSiGHT maintains the world reference database of variants observed in MMR genes (as Leiden Open Variation Databases (LOVD), which are now linked to ClinVar, as part of the ClinVar-ClinGen partnership), and accordingly, 82% of the Class 4 and 5 variants listed affect *MLH1* and *MSH2*, with 13% affecting *MSH6* and 5% *PMS2*.[16, 18-20] It should be noted that these pathogenic variants are mostly from cases ascertained by family history. As more cases of LS are ascertained from systematic testing of cancer cases and

incidentally from gene panel testing, more patients will be identified with pathogenic variants in *MSH6* and *PMS2* and thus the proportions due to the different MMR genes will alter.[6]

All types of variants are seen as pathogenic: nonsense, frameshift, splice site, missense, insertion-deletion and large deletions/rearrangements, the largest so far being a 10 Mb inversion affecting *MSH2* and which is visible cytogenetically.[21-23] Approximately 60% of all putative pathogenic missense mutations causing LS actually disrupt splicing and are thus, in effect, truncating.[24] Up to 3% of Lynch syndrome cases are due to variants involving the 3′ end of the *EPCAM* gene (immediately adjacent to *MSH2*), that results in the hypermethylation of the *MSH2* promoter or partial deletion of *MSH2.*[25, 26] Another infrequent, but important cause of LS is constitutional methylation of the *MLH1* promoter, which occurs in 1 – 2% of cases.[27-29] This is usually sporadic in nature, so neither inherited nor heritable, and relatives are therefore not at risk. However, in a small number of patients the hypermethylation is secondary to a large deletion typically involving *LRRFIP2*, the gene upstream of *MLH1*, and it is the deletion which is the pathogenic variant: the methylation is secondary.[29] Given the risks to relatives it is therefore important to distinguish such cases, and this is achieved by testing both the tumour and constitutional DNA, and finding *MLH1* promoter methylation in both, perhaps in the context of a LS family history. It has to be borne in mind that approximately 15% of sporadic *colon* cancers are also dMMR due to somatic, so acquired, epimutation of both *MLH1* alleles by promoter hypermethylation.[30] This is a function of such tumours arising from right-sided serrated lesions.[31]. A similar proportion of sporadic endometrial cancers also have biallelic hypermethylation of *MLH1*, and as with colon cancers a small proportion

are due to constitutional methylation plus a somatic mutation in the normal *MLH1* allele.[27, 28, 32, 33]

Other genetic conditions relating to Lynch syndrome

Muir-Torre syndrome (MTS) is a descriptive diagnosis of an individual in which both skin sebaceous neoplasms (and keratoacanthoma-like lesions) and a "visceral" carcinoma of any sort has occurred.[34, 35] It is a frequently seen combination in LS, indeed on close inspection many, perhaps most patients with LS who have suffered a carcinoma probably have a sebaceous skin lesion and thus MTS.[36, 37] However, MTS is also seen in other heritable predispositions, e.g. *MUTYH*-associated adenomatous polyposis, or it may be sporadic, so while a marker of possible LS, MTS is not diagnostic.[38, 39] Notably, although sebaceous lesions and hyperplasia are often rightly a cause for concern regarding LS and a patient with sebaceous lesions and a visceral cancer warrants testing, it should be noted that in the absence of a family or personal history of LS-associated cancer they are at low risk and do not warrant testing.[40, 41]

Individuals may inherit pathogenic variants in both copies of a DNA MMR gene. When both are in the same gene it causes Constitutional Mismatch Repair Deficiency (CMMR-D) syndrome. CMMR-D is a classic recessive DNA repair disorder typically characterised by childhood onset leukaemia, lymphoma, colorectal and brain cancers, but also, in contrast to LS, some patients may have multiple colorectal adenomas.[42-44] Signs suggestive of neurofibromatosis type 1 also often occur, such as café-au-lait macules and cutaneous neurofibromata, plus other features such as immune deficiency.[45] In CMMR-D patients abnormal MMR IHC is seen in both normal and

tumour cells (hence the necessity always to test all four markers) and it is possible to detect constitutional MSI in e.g. blood.[46, 47] Patients with CMMR-D due to *MSH6* or *PMS2* variants may show milder disease and later onset.[48] Comprehensive diagnostic criteria and care pathways have been published.[7, 45]

Patients may also be found who have pathogenic variants in more than one MMR gene, so-called digenic LS. It is not clear if this is more severe than LS due to a pathogenic variant in one gene, but has clear implications for clinical genetic counselling. However, LS patients can and do co-inherit other forms of predisposition to cancer (or indeed any other genetic condition), and if this is suspected should be pursued.[49]

Finally, Turcot syndrome, a combination of colorectal cancer or adenomas and central nervous system tumours, with dominant or recessive inheritance, is another historical descriptive diagnosis that has been related to LS. However, it is now known that it can be due to CMMR-D, Familial Adenomatous Polyposis and probably other conditions, and so as an ambiguous term it should be abandoned.[42, 44, 50]

2. DNA Mismatch Repair Mechanisms

The MMR pathway is a highly evolutionarily-conserved mechanism responsible for the correction of base mismatches (e.g. C or G opposite T or A) and insertion/deletion loops (occurring in repetitive sequences such as AAAAAA … or CACACACA … due to insertion or deletion of an extra repeat unit during stalled DNA replication of these repetitive sequences). Such stalling is probably mostly due to replication associated

errors, but is also caused by DNA damage due to oxidative stress, lipid peroxidation, base deamination, methylation, and alkylation.[51]

A base mismatch or single nucleotide insertion/deletion error is recognized by the MutSα complex which is composed of MSH2 and MSH6 proteins. Insertion/deletion loops of 2-8 nucleotides are recognized by the alternative complex MutSβ, composed of MSH2 and MSH3 proteins. MutSα complex activation is characterized by ATPase activity, which is important for the interaction with the mismatched DNA and initiation of repair. The binding of MutSα stimulates ATP-hydrolysis leading to a conformational change which subsequently triggers the recruitment of a second complex MutLα, composed of MLH1 and PMS2 proteins. The tetrameric complex, by sliding on the DNA, searches for the single-strand DNA mismatch on the new strand (daughter strand). This, in turn, activates proliferating cell nuclear antigen (PCNA) and Replication factor C (RFC). MutLα possesses an intrinsic ATP-stimulated endonuclease activity that requires activation by PCNA in order to create an incision in the recently synthesised daughter strand (containing the error). The incision step is followed by the recruitment of exonuclease 1 (EXO1) that removes the newly-synthesized DNA strand up to and beyond the mismatch or loop. DNA polymerase δ re-synthesizes the DNA, whilst Ligase 1 seals the remaining nick.[52]

The MMR pathway is involved in a signalling cascade that leads to cell cycle arrest and / or apoptosis, if DNA damage has previously occurred.[53] It has been observed that MMR-deficient cells fail to recruit ataxia-telangiectasia mutated (ATM) and ATM and Rad3-related (ATR); and this prevents p53 phosphorylation in response to DNA damage.[12, 54] The underlying mechanisms by which MMR proteins promote DNA damage-induced cell cycle arrest and apoptosis have not been fully elucidated. Two

models have been hypothesised: the futile cycling model and the direct damage signalling model. In the futile cycling model, MMR recognizes the mismatches triggering the excision of the newly synthesized strand, although the persistent offending damage on the template strand cannot be excised. MMR initiates futile repair cycles, eventually resulting in the formation of DNA double-strand breaks and thus, activating the ATM/ATR/p53 signalling pathway to activate cell cycle arrest and / or apoptosis.[55] In the direct damage signalling model, MutSα and MutLα directly recruit ATM/ATR and cause cell cycle arrest and / or apoptosis.[56]

A crucial consequence of this is that the low background level of DNA damage in normal cells may stimulate MMR and thus inhibit the cell cycle, or if severe even stimulate apoptosis, and so net cell turnover does not reach its theoretical maximum. However, if MMR deficiency should occur in such cells there is then no such limitation by stalling of the cell cycle or activation of apoptosis and net cell division increases in an uncontrollable fashion, allied to which as a secondary phenomenon the mutation rate increases, which is manifest as MSI and/or abnormal MMR IHC. This is very useful diagnostically, but it is important to appreciate that it is not the increased mutation rate *per se* that is driving the carcinogenic process, and neither does it make adenomas progress any quicker than usual.[14] However, because mutations are strongly biased towards repetitive DNA sequences in dMMR cells this has profound consequences for the biology of such tumours and patients with LS due to the strong immunological responses this elicits.[57, 58] The critical consequences of this are manifest in how LS tumours develop and potentially evade the immune system.[59]

Notably, some MMR gene variants are associated with abnormal MMR IHC in tumours, but not MSI, and vice versa.[4, 15] In addition, only one of 149 *PMS2*

pathogenic variants causative of LS is a missense mutation.[18] However, many other *PMS2* missense variants are seen in CMMR-D (a recessive DNA repair disorder), which is consistent with common, but not complete, overlap between loss of DNA (MMR-recognised) damage repair and apoptotic functions of MMR.[18, 60, 61]

3. Lynch Syndrome Databases

In addition to the MMR gene variant database maintained by InSiGHT, there are other phenotypic databases aimed at understanding the precise risks LS patients face. Initial risk estimates were liable to ascertainment bias and thus tended to overestimate by being necessarily retrospective. The penetrance and expressivity of MMR pathogenic variants differs in LS patients according to the MMR gene, age, sex, and environmental / lifestyle factors.[62] Several lifestyle factors, such as smoking, alcohol, obesity, are associated with an increased risk of sporadic cancer and have been suggested to have similar effects in LS patients. Therefore, as it is fundamental to quantify accurately the risks of developing cancer for LS patients, then in order to provide adequate data for surveillance and care, as well as understand the underlying biology, the Prospective Lynch Syndrome Database (PLSD) was established in 2012 by the Mallorca Group of InSiGHT.[2, 63] The PLSD collects data on LS patients from Lynch Syndrome expert centres and registries worldwide, who are thus undergoing colonoscopic surveillance with polypectomy, and may also be having therapeutic or prophylactic surgery. It therefore provides information on the natural history of the disease course and the effects of interventions and lifestyle factors. The PLSD is linked to the InSiGHT MMR LOVD: all patients on the PLSD must have a Class 4 or 5 MMR variant, so pathogenic or likely pathogenic according to the InSiGHT

classification.[4, 15] The PLSD includes basic information on pathogenic genetic variants, sex, and age, plus information such as cancers or pre-cancers diagnosed, age at diagnoses, age at prophylactic surgical removal of organs and information on pre-cancers. Every patient is followed as an individual, as the family history is ignored so as not to introduce bias, and the database now has >50,000 patient-years of observations.[10] The PLSD website is public and allows anyone to determine the risks to an individual of an LS-associated cancer in an interactive graphical form according to their affected gene, age, gender and whether previously affected by cancer.[63] Risks of LS-associated cancers to age 75 are summarised in Table 1; those for *PMS2* affecting both sexes are combined due to the smaller numbers available and numbers have been rounded throughout for clarity.[10]

Table 1.

Important parallel efforts have been made in defining risks in Lynch syndrome by the Colon Cancer Family Registry (CCFR) and the International Mismatch Repair Consortium (IMRC).[64, 65] The CCFR is an international consortium of six institutes in the United States, Canada and Australasia formed as a resource to support studies on the aetiology, prevention, and clinical management of colorectal cancer, and utilises a form of modified segregation analysis to minimise retrospective ascertainment bias.[65, 66] It currently has data on >42,500 individuals from over 15,000 families on its records and has made significant advances in demonstrating how environmental and lifestyle factors affect cancer risks in LS, such as smoking, raised body mass index and alcohol consumption.[67-69] By contrast, reduced cancer risk is seen with, e.g. hormone replacement therapy, vitamin and mineral supplements, NSAID use, and parity, but there is no change in risk associated with oral contraceptive

use.[70, 71] The IMRC is a worldwide collaboration of more than 115 investigators from 59 centres, with 20,000 individuals with LS from 8,800 families, facilitated by InSiGHT and the Collaborative Group of the Americas on Inherited Gastrointestinal Cancer (CGA-IGC).[64, 72]

Lastly, is the initiative to determine the effects of aspirin prophylaxis on LS patients, although this is a series of clinical trials rather than a database. Remarkably, the CAPP2 trial has shown that only two-four years of treatment with 600 mg/d of aspirin very significantly reduces the risk of colorectal cancer up to more than 10 years post treatment, and likely also reduces the risks of other LS cancers, although this is less certain. Notably, side-effects possibly attributable to aspirin were at a low rate and actually slightly more common, though non-significantly, in the placebo compared to treatment arm.[73-75] A follow on randomised double blind dose non-inferiority trial, CaPP3, is now in progress to determine the optimum dose of aspirin for long term prophylaxis.[76]

4. Pathology of Lynch Syndrome Cancers

Typical histological features of Lynch Syndrome tumours are best exemplified by colorectal cancer (CRC), which often shows a combination of the presence of prominent tumour-infiltrating lymphocytes, Crohn-like peritumoural lymphoid aggregates, poor differentiation, frequently with areas of mucinous and / or signet-ring cell patterns, sometimes with a medullary growth pattern.[77, 78] These characteristics can be seen in both Lynch Syndrome CRC and sporadic dMMR bowel cancers, but are not sufficiently specific to distinguish them from pMMR cancers.

Less data have been published about non-colorectal LS-associated cancers. LS-associated endometrial cancers can be seen more frequently in the lower uterine segment than sporadic cancers, are mostly of endometrioid type, often with poor differentiation, solid or dyscohesive, with prominent tumour-infiltrating lymphocytes, and Crohn-like peritumoural lymphoid aggregates.[79-84] LS-associated ovarian cancers are typically of endometrioid or clear cell type, with some tumour-infiltrating lymphocytes.[79, 80, 85, 86] LS-associated gastric carcinomas are mostly of intestinal-type with fewer of diffuse-type, and rarely of mucinous type, and an associated immune gastritis is reported.[87-90] LS-associated small intestinal adenocarcinomas often display mucinous, signet-ring cell or medullary differentiation, with tumour-infiltrating lymphocytes and Crohn-like reactions, as do ampullary adenocarcinomas.[91] LS-associated pancreatic cancers are mostly acinar cell carcinomas and medullary carcinomas.[92]

5. Testing for Lynch Syndrome Cancers

Testing of (usually selected) patients with CRC, EC and/or other types of LS-associated cancer is recommended by many guidelines and organizations, generally starting with testing the tumours for either the presence of MSI or the absence (or abnormal expression) of mismatch repair proteins. There is no consensus regarding whether MMR immunohistochemistry or MSI testing is the better first test in *colorectal cancers* as they have similar test performance characteristics in detecting LS: sensitivity of MSI is 88 ~ 100% and IHC 73 ~ 100%, with specificity of MSI 68 ~ 84% vs IHC 78 ~ 98%.[93, 94] They may be used serially, or in combination.[93-97] However, evidence is now emerging that IHC may be the preferred option when testing

endometrial cancers (systematic testing of which is now under consideration by NICE) as a recent UK study has shown that while MSI and IHC have similar specificity (83.7 vs. 81%), MSI has only 56.3% sensitivity compared to 100% for IHC.[82, 83]

MMR immunohistochemistry is the better option for small biopsies, cancers with a low tumour cell proportion or intense inflammatory reaction. Subsequent testing for *MLH1* promoter hypermethylation and somatic (rather than constitutional / germline) mutations can be used to clarify the risk of inherited pathogenic variants in suspected Lynch Syndrome patients. *MLH1* promoter hypermethylation testing may be used as an alternative to *BRAF* V600E mutation analysis in colonic cancers.[98-100] The use of larger targeted gene mutation panels (or whole exome / genome sequencing) that includes MMR tumour testing with mutation analyses is becoming more widespread.[101]

Immunohistochemical staining for the four major DNA mismatch repair proteins (MLH1, MSH2, MSH6 and PMS2) is probably the most common test used to screen CRCs and other tumours for dMMR.[93, 95, 97] The nuclear expression of all four proteins suggests mismatch repair proficiency with microsatellite stability.[102-104] Loss or abnormality of nuclear staining for any of the proteins indicates dMMR and suggests the most likely MMR gene involved.[103, 104] Loss of MSH2 alone or loss of both MSH2 and MSH6 suggests a mutation or abnormality in MSH2 is most likely. Similarly, loss of MLH1 alone or loss of both MLH1 and PMS2 suggests an underlying mutation, abnormality or promoter methylation in MLH1. Combined loss of both MSH2 and MSH6 (or of both MLH1 and PMS2) reflects the heterodimeric binding of MSH2 with MSH6 (or of MLH1 with PMS2) in the mismatch repair complex MutSα (or of MutLα), such that loss of the first protein partner generates instability and loss of the

second [37]. Usually, there is nuclear staining in the nuclei of both tumour cells and adjacent normal epithelial cells, stromal cells and lymphocytes.

In a dMMR tumour due to *MSH2* mutation there is loss of nuclear expression of MSH2 and MSH6 and intact staining for MLH1 and PMS2. In a dMMR tumour due to *MLH1* mutation there is loss of nuclear expression of MLH1 and PMS2 and intact staining for MSH2 and MSH6. This pattern of combined MLH1 and PMS2 loss could be seen either in a sporadic tumour (most commonly due to *MLH1* promoter methylation) or in LS due to constitutional *MLH1* mutation. Correct MMR IHC interpretation requires adequate internal control staining of the adjacent stromal and lymphoid cells to confirm good fixation of the tissue region.[102, 103] Patchy intact nuclear staining may occur due to variable fixation, or tissue hypoxia, or unequal antibody diffusion.[105, 106] Cytoplasmic staining may occur, but if nuclear staining is lost this is considered abnormal indicating dMMR.[107] Weak, patchy, nucleolar staining, or sometimes absence of MSH6 expression has been described in rectal tumours following neoadjuvant treatment without MSI or a mutation confirmed by molecular testing.[108, 109] Notably, heterogeneous staining or loss of MSH6 expression can be due to a secondary (non-germline) acquired somatic mutation in the *MSH6* coding mononucleotide tract.[110, 111] Approximately 3–10% of LS-associated dMMR tumours show no abnormality on immunohistochemical testing (presumably because of variants that disrupt normal MMR protein function, but nonetheless enable protein detection by IHC).[112]

Testing DNA extracted from tumours for microsatellite instability (MSI), involves investigating for the presence of extra alleles (longer or shorter) at a microsatellite locus compared with normal microsatellite length (as determined from normal tissue

or blood) from the same individual.[113] Microsatellites vary in their propensity to show instability, and thus the frequency with which the same microsatellite is altered varies in different tumour types. Instability is more likely to be observed at mononucleotide repeats (e.g. AAAAA …), than at dinucleotide repeats (e.g. CACACA …). Microsatellite loci or markers used in colonic cancer MSI testing are known to have reduced sensitivity at detecting MSI in non-colonic cancers, including endometrial, small intestinal, or gastric cancers, in tumours from LS patients with *MSH6* or *PMS2* mutations and in colonic adenomas.[114] Therefore, a proportion of LS-associated tumours may not appear to have MSI using the standard MSI test (but might be identified by abnormal MMR immunohistochemistry).

The efficacy of MMR IHC and MSI may be significantly enhanced by testing more than one tumour from the same individual or family, particularly if there are tumours that are multiple or rarely seen in LS (e.g. colorectal adenomas, small intestinal cancers, hepato-biliary, upper urinary tract and cutaneous sebaceous tumours).[15, 115] Consistent immunohistochemical abnormality of one mismatch repair protein in more than one tumour from an individual or family represents very good evidence for variant pathogenicity.[15, 116] Some CRCs due to *MUTYH*-associated polyposis (MAP) or proof-reading polymerase-associated polyposis (PPAP) may exhibit MSI and/or abnormal immunohistochemistry due to somatically acquired MMR gene mutations.

Approximately 13-15% of sporadic colonic cancers have dMMR, usually due to epigenetic silencing of both alleles of *MLH1* by promoter hypermethylation. Hence, while overall unselected dMMR colonic cancers have a relatively poor positive predictive value for LS, as the proportion of colonic cancers with MSI due to LS varies with age then this can be exploited clinically. Under the age of 57 y, more than half of

all dMMR colonic cancers will be due to LS, whereas over this age less than half will be, although even at age 70 approximately 25% dMMR colonic cancers will be due to LS (Figure 1).[8, 104, 117] In contrast, pMMR has a good negative predictive value for LS. Further tests (*BRAF* mutation and *MLH1* methylation tests) are required for MLH1-negative cancers to distinguish between LS and sporadic origin.[93, 94, 96] Another important practice point is that *rectal* cancers are distinct from *colonic* cancers in the diagnosis of LS. Because sporadic colonic cancers with dMMR largely arise from right-sided serrated lesions, sporadic rectal cancers with dMMR are correspondingly rare, if they occur at all, and hence a rectal cancer with dMMR at any age must be considered to be due to LS until proven otherwise.[118, 119]

(Figure 1)

The activating missense variant *BRAF* p.V600E occurs in sporadic colonic cancers with dMMR, but not in those due to LS; therefore, *BRAF* p.V600E is highly predictive of the tumour being of sporadic origin rather than LS.[31, 93, 94, 96, 120] However, sporadic tumours may occasionally occur in patients with LS, so the absence of *BRAF* p.V600E does not definitively diagnose LS, but it does indicate that LS is more likely. Alternatively, detection of *MLH1* gene promoter hypermethylation in a colonic cancer provides good, although not unequivocal, evidence that the tumour is sporadic in origin, as occasional sporadic tumours do occur in LS and constitutional *MLH1* promoter methylation can be found in a small proportion of patients with LS.[28, 29] *BRAF* p.V600E testing is only of use in distinguishing colorectal cancers; it has no utility in e.g. endometrial cancer.

LS is definitively diagnosed following tumour testing by constitutional/germline MMR gene sequencing to identify a pathogenic constitutional variant.[4-6, 121] Patients with

digenic LS, who have inherited pathogenic variants in more than one MMR gene, are occasionally seen (and more will be as gene panel testing becomes prevalent), but it is unclear if their risks are increased over those patients with a pathogenic variant in one MMR gene. It is often useful to have samples from more than one individual in the family, because case segregation studies may be required in order to determine pathogenicity or whether an individual is a phenocopy.[4, 15] If the family shows evidence of hereditary transmission of LS, but no point mutation is found, then tests for large-scale mutations, such as deletion of a whole exon (or more), can be performed; some 12 – 40% of pathogenic variants are of this type.[21, 23, 122-127] LS-related tumour types that are rare in the general population, and thus have a high predictive value for LS, such as small intestinal and hepatobiliary cancer, upper urinary tract and bladder (under age 60y) transitional cell carcinoma, or skin sebaceous adenoma/carcinomas are therefore worth testing.[40] Synchronous or metachronous bowel cancers are also significant, as is the development of any two LS-related tumours (e.g. CRC and endometrial cancer), and all such cases warrant testing for LS.

6. Neoplastic Precursors in Lynch Syndrome

When LS was being defined in the early 1990s the only known pathway to CRC was based on the work of Dukes, Bussey and Morson on Familial Adenomatous Polyposis: firstly Dukes' concept of "simple tumours and cancer" in 1925 as part of his system for the staging of rectal cancer in FAP, followed by the adenoma to carcinoma pathway in 1958.[128-130] Naturally, the reasonable assumption was made that the same pathway applied in LS and thus colonoscopic surveillance to remove premalignant

adenomas would be beneficial in LS. When early data started to come in on the efficacy of surveillance in LS it became obvious that there was a large number of interval cancers, and moreover these occurred despite the interval between colonoscopies being steadily reduced to less than three years and sometimes even less than one year, because it appeared that LS cancers developed much more rapidly than sporadic ones, assuming they all arose from adenomas.[6, 131] Moreover, because of the increased mutation rate, the MSI, observed in LS cancers allied to the prevailing concept that genomic instability characterised all cancer, it was further assumed that this must be what was driving a faster adenoma-carcinoma progression, although not all parties were convinced.[11, 14, 132] In addition, although dMMR adenomas could be found in LS patients, further doubts were raised when aspirin treatment failed to reduce the incidence of adenomas (although it later reduced that of CRCs), and results from the PLSD became available.[2, 73, 116, 133, 134] LS patients on colonoscopic surveillance at various intervals could finally be compared.[133] Remarkably, within the limits measurable, colonoscopy did not appear to reduce the rate at which colorectal cancers were arising in LS patients, despite it being associated with a significant reduction in mortality and, in addition, stage was not related to the interval since last colonoscopy – completely the opposite of population screening programmes which are based primarily on adenoma removal.[2, 133-135] What was going on? Although LS patient survival is certainly improved by colonoscopic surveillance enabling earlier diagnosis and a degree of downstaging, together this mass of evidence was leading to the conclusion that a pathway independent of adenomas must be occurring and moreover a pathway in which precursors were less obvious on colonoscopy.[94]

At about the same time, it was discovered that LS patients harbour an enormous number of dMMR crypts in the colorectum (~1/cm^2 mucosa, so ~10,000 crypts/patient), which are not dysplastic, and yet LS patients only go on to eventually develop between zero and perhaps one, two or three cancers.[136, 137] Could these be leading to cancers, perhaps by an occult route? This was answered shortly afterwards by the finding of flat intra-mucosal cancers in which the wnt pathway was activated by mutations not in *APC*, as in classical adenomas, but in beta-catenin, indeed mutations in repetitive coding sequences, exactly as predicted would result from dMMR.[138] Intriguingly, subsequent work to sequence LS cancers has shown that some 61% of *APC* mutations are predicted to occur after MMR deficiency occurs, as they are found in repetitive sequences, exactly as would be expected in dMMR tumours. Hence, a proportion of these beta-catenin-mutant flat lesions acquire secondary *APC* mutations, thence to become polypoid adenomas and subsequently cancers.[139, 140] Thus, it is now understood that there are at least *three* pathways to colorectal cancers in LS, not including the occasional sporadic colonic cancer due to a right-sided sessile lesion, which are occasionally observed (Figure 2). The first pathway is via sporadic adenomas which acquire secondary dMMR. The second pathway is via flat cancers within the mucosa that arise directly from dMMR crypts, and the third pathway is LS-specific adenomas that arise from these flat lesions due to secondary *APC* mutations.[139] Hence, a proportion of LS colorectal cancers arise from flat lesions which are inherently more difficult to detect, let alone remove, on colonoscopy, explaining at least in part the apparent high rate of interval cancers, but also removing the need to invoke a faster progression rate in LS. Building on this, it has been shown that the cancers in patients with LS due to *PMS2* mutations arise largely along pathway 1, from sporadic adenomas, going further to explain why

patients with pathogenic *PMS2* variants have only a small increased risk of CRC.[141] Intriguingly, very recently it has been found that pathway 2 predominates over pathway 3 in patients with pathogenic *MLH1* variants, but pathway 3 predominates over pathway 2 in those with *MSH2* pathogenic variants.[142]. These fascinating findings have clear implications for future surveillance strategies, which of necessity must now also include modalities such as aspirin prophylaxis and vaccines to address the inherently limited efficacy of colonoscopy and increased risks of cancers in LS at sites other than the large bowel, which are becoming the predominant cause of mortality in LS patients under surveillance.[2, 3]

(Figure 2)

It is of note that a number of observers have now found dMMR glands in morphologically normal endometrium from LS patients, which in turn has implications for the understanding of LS carcinogenesis in that organ.[143]

7. Immune Escape of Lynch Syndrome Neoplasms

The early general observations of large local tumours and a lower rate of metastasis, together with a strong immune reaction to LS cancers, such as increased tumour infiltrating lymphocytes (TIL) and tertiary lymphoid structures (TLS; also termed Crohn-like peritumoural lymphoid aggregates or follicles), later backed up by gene expression signatures characteristic of immune cell activation, all indicate an important role for the immune system in LS.[57, 144, 145] However, this needs to be put in the context of the normal immune architecture in the bowel. Gut-associated lymphoid tissue (GALT) comprises both isolated and aggregated lymphoid follicles in both the small and large

intestines. Humans have approximately 30,000 isolated lymphoid follicles (ILF), scattered throughout the large and small intestine, but especially in the colon.[146] ILFs vary in their distribution within the large and small intestines, may be mucosal or sub-mucosal, and at their simplest may consist of a single follicle, with or without some T-cells.[147, 148] They are considered to be the main source of immune priming in the colon and from where Crohn's disease originates, and they have specialised follicle-associated epithelium (FAE), which overlies a subepithelial dome containing numerous macrophages, dendritic cells, T, B lymphocytes, and special antigen sampling microfold/M/cells.[149, 150] The FAE has a crucial role in the initiation of the mucosal and systemic immune responses.[151] However, the relationship between Crohn-like follicles in LS and ILF in normal colon is not completely clear. In LS, the follicles are generally peritumoural and not located inside cancers, and, although they do not have FAE, they do have T cells, B cells and germinal centres.[58, 152] Whether they are induced *de novo*, as in chronic inflammation or develop from submucosal ILFs remains to be determined.[153] Crohn-like follicles/TLS are found in CRCs apart from LS, however, the frequency/number is highest in LS patients compared to sporadic dMMR and pMMR CRCs which is not simply related to age.[58]

In recent years, there has been a growing interest in TLSs in a variety of cancers including CRC, in particular as prognostic indicators of cancer progression and responses to immunotherapy.[150, 154] In hepatocellular carcinoma, intratumoral TLSs correlated with a lower risk of early relapse after surgery.[155] In sarcoma, melanoma and renal cell carcinoma, both intratumoural location and the presence of B cells (but not T cells) and particularly germinal centres correlate with improved outcomes to checkpoint blockade immunotherapy.[156-158] Further studies of the

location, cellular composition and presence of germinal centres in TLS in hereditary dMMR may therefore shed light on their role in Lynch Syndrome.

In dMMR cells predictable mutations can and do occur in repetitive protein coding sequences and result in frameshift peptides (FSP).[159-161] Such FSPs are novel antigens and elicit both humoral and cellular immune responses, which are seen as TILs around the dMMR crypts in LS patients as well as in dMMR cancers, both sporadic and due to LS.[136, 160, 162, 163] In the face of such responses how is it possible for tumours, let alone cancers, to develop in LS? The answer lies in the 3-step process of elimination, equilibrium and escape.[59, 164] Cells generating FSPs, presented on their surface by MHC-I are subjected to attack by cytotoxic T-lymphocytes (CTL), resulting in *elimination*. However, such cells that randomly manage to acquire activating mutations in PD-L1 before they are eliminated can hold the immune system to a local standstill (activating the PD-1 – PD-L1 immune checkpoint) – the process of *equilibrium*. Subsequently, if before being eliminated these cells manage to acquire inactivating mutations in MHC-I or MHC-II (HLA Classes I & II) that abrogate presentation of FSPs on their surface they are then able to *escape* the immune system, at least locally. Given the huge number of dMMR crypts in an LS patient, but that the average LS patient manifests between zero and one, two or three CRCs, it is clear that the process of elimination must be highly efficient, giving a different perspective on cancer biology.[2, 139]

A number of different escape mechanisms have been observed. The commonest, seen in approximately 30% of dMMR CRCs, is mutation of beta-2-microglobulin (*B2M*) which prevents MHC-I presentation of FSPs. This was an early observation, the full significance of which is only now apparent.[165-169] The outgrowth of such *B2M*

mutant clones is a prime example of cancer immunoediting, which has been further related to variation in host immune function, e.g. mucosal density of FOXP3-positive T-cells, indicating that such factors may be additional modifiers of LS.[170] Importantly, *B2M* mutations in dMMR cancers are significantly associated with an almost zero rate of metastasis, and thus indicate highly favourable prognosis.[171, 172] In addition to mutations in *B2M*, mutations of *CIITA* or *RFX5* are seen in approximately 20% of dMMR CRCs, and prevent MHC-II antigen presentation, while approximately 10% of dMMR CRCs have mutations of *TAP1* or *TAP2*, which are antigenic peptide transporters responsible for antigen presentation, thus also preventing antigen presentation on the cell surface.[173, 174] In such ways tumour cells escape the attentions of the host's immune system, both locally and in the circulation, but are in turn liable to attack with help from immunotherapy, such as anti-PD-1 or PD-L1 immune checkpoint blockade.[175, 176]

The full variety of mechanisms by which tumours, and dMMR tumours in particular, manage to evade the immune system has yet to be determined. Undoubtedly, the colorectal microbiome plays an important part in the process of CRC development in LS as well as sporadically.[177] Intriguingly, the immune response to dMMR CRCs in the form of development of high endothelial venules (HEV) responsible for trafficking lymphocytes into lymphoid follicles/TLS is stronger in LS patients, than in sporadic dMMR colonic cancers, and especially high HEV densities in *B2M*-mutant tumours support the concept of immunoediting during tumour evolution.[58] Such higher HEV densities in *B2M*-mutant tumours imply that under strong immunoselective pressure created by immune cells recruited via HEVs, tumour cells which have lost MHC class I antigens gain growth advantage due to immunoediting, thus revealing a major role of HEVs in enhancing the immunoselective pressure on highly immunogenic cancers.

Taken together with the high numbers of dMMR crypts in LS and the low numbers of CRCs that actualy manifest, these findings all point toward a longer process of immunoediting in Lynch syndrome CRCs, possibly due to the pre-existing dMMR crypts immunising LS patients against their own propensity to cancer, and explaining the higher proportion of *B2M* mutations in LS compared to sporadic CRCs.[58, 136, 137] However, these HEV generally recruit naïve lymphocytes from the blood in to tissues and HEV-containing Crohn-like aggregates are generally seen in a peritumoural location in both pMMR and dMMR cancers, so our understanding of HEV and TLS in LS is as yet incomplete.[58, 152, 178]

8. Conclusions

The major carcinogenic effect of dMMR is to reduce apoptosis and increase the net cell turnover rate. The raised mutation rate *per se* is of little or no consequence, but the bias to frameshift mutations in repetitive sequences caused by dMMR is of considerable fundamental consequence, firstly in auto-immunising LS patients to FSPs and secondly in thus modifying the evolution of dMMR cancers by immunoediting.

That some MMR gene variants are associated with abnormal MMR IHC in tumours, but not MSI, and vice versa, plus the observation of, for example, certain *PMS2* variants in cases of CMMR-D , but not LS, challenges the concept of *in vitro* functional tests in assessing variant pathogenicity.[4, 15, 18, 60, 61] Which function should be tested: DNA repair, or apoptosis, or both?[4, 179-181] We would therefore advise caution in the interpretation of such assays until this is clarified.

It is now understood that there are three main pathways to CRC in LS, including via sporadic adenomas, but also via occult intramucosal dMMR crypts and then Beta-Catenin-mutant flat cancers which are directly invasive, some of which acquire secondary *APC* mutations to become polypoid.[138, 139] Moreover, the likelihood that tumours follow particular pathways is related to the underlying gene affected by a pathogenic variant, and hence the variation in expression of LS starts to be explained and may lead to surveillance protocols becoming more tailored to genotype.[3, 10, 141, 142]

Currently, LS is defined by an individual carrying a pathogenic variant in an MMR gene, but of necessity to interpret variants as pathogenic often requires tests on tumours for dMMR.[4, 6] As part of the interpretational process tests of MMR function may be performed *in vitro*, but this too is problematic and so interpretation cannot be based solely on such analyses.[4, 179-181] However, the recent advances in understanding the pathways and responses to cancer in LS brings forward the possibility of alternative means to diagnose LS independent of cancer itself, indeed perhaps even an alternative definition involving the immune system. The nature of the intense immune response to cancers in LS, manifest as the stronger HEV response seen in individuals with LS from an early age (16y), allied to specific T and B-cell responses to FSPs can be considered syndromic features characteristic of LS.[58, 160, 163] In addition, the dMMR crypts seen in the normal large bowel mucosa, and the dMMR glands seen in normal endometrium can also be considered syndromic features characteristic of LS.[136] So, all taken together we may be approaching a definition of LS in molecular terms, one in which the development of cancer, while of great importance clinically, is not necessary to achieve diagnosis. This in turn may enable

the classification as pathogenic of many variants in MMR genes, and *MSH6* and *PMS2* in particular, which is currently problematic due to their reduced penetrance.

Acknowledgements

We wish to thank William Agace, Allan Mowat, Emma Crosbie, Aysel Ahadova and Matthias Kloor for their advice and helpful discussions during preparation of this work.

Author contributions

All authors contributed to preparation and review of the manuscript.

Table 1

Average Risks of Lynch syndrome-associated Cancers to age 75y

Data from the Prospective Lynch Syndrome Database (PLSD).[10, 63] Note that the individuals studied are under colonoscopic surveillance, and may have had prophylactic or therapeutic surgery, which is allowed for in the estimates.

Males				
Cancer type	***MLH1***	***MSH2***	***MSH6***	***PMS2***
Any cancer	71% [63 - 81%]	75% [66 - 86%]	42% [25 - 67%]	See below
Colorectal (bowel)	57% [49 – 68%]	51% [41 – 65%]	18% [8 – 43%]	See below
Stomach, small bowel, bile duct, gallbladder and pancreas	22% [16 – 30%]	20% [14 – 28%]	8% [3 – 30%]	See below
Ureter and kidney	5% [3 – 10%]	18% [13 – 25%]	2% [<1 – 24%]	See below
Urinary bladder	7% [4 – 13%]	13% [8 – 21%]	8% [3 – 30%]	See below

Prostate	14% [9 – 22%]	24% [17 – 33%]	9% [3 – 31%]	5% [<1 – 68%]
Brain	0.7% [<1 – 5%]	8% [4 – 15%]	2% [<1 – 24%]	See below

Females				
Cancer type	***MLH1***	***MSH2***	***MSH6***	***PMS2***
Any cancer	81% [74 – 88%]	84% [77 – 91%]	62% [47 – 78%]	See below
Colorectal (bowel)	48% [41 – 57%]	47% [39 – 55%]	20% [12 – 41%]	See below
Endometrium	37% [30 – 47%]	49% [40 – 61%]	41% [29 – 62%]	3% [5 – 50%]
Ovaries	11% [7 – 20%]	17% [12 – 31%]	11% [4 – 39%]	3% [<1 – 43%]
Stomach, small bowel, bile duct, gallbladder and pancreas	11% [7 – 17%]	13% [9 – 19%]	4% [3 – 30%]	See below

Ureter and kidney	4% [2 – 8%]	19% [14 – 27%]	6% [2 – 27%]	See below
Urinary bladder	5% [3 – 11%]	8% [5 – 14%]	1% [<1 – 23%]	See below
Brain	2% [<1 – 5%]	3% [1 – 8%]	1% [<1 – 23%]	See below

Both Sexes combined	
Cancer type	***PMS2***
Any cancer	34% [19 – 60%]
Colorectal (bowel)	10% [3 – 41%]
Stomach, small bowel, bile duct, gallbladder and pancreas	4% [1 – 34%]
Ureter and kidney	4% [<1 – 34%]

Urinary bladder	<1% [0 – 31%]
Brain	<1% [0 – 31%]

Figure Legends

Figure 1: Probability by Age that a Colonic Cancer with dMMR is due to Lynch Syndrome

This graph shows the probability by age that a colonic cancer with dMMR is due to Lynch syndrome.[8, 104, 117] Note that this does not apply to rectal cancers, because right-sided serrated lesions that give rise to sporadic dMMR colonic cancers do not occur in the rectum, and hence a rectal cancer with dMMR is due to Lynch syndrome until proven otherwise.[118, 119]

Figure 2: Pathways to Colorectal Cancer in Lynch Syndrome

There are three pathways to dMMR colorectal cancers in Lynch syndrome.[138, 139] Pathway 1: Classic sporadic adenomas, initiated by WNT pathway activation due to mutations in *APC* (*APC*^{m}), acquire dMMR through somatic mutation of the remaining normal MMR allele (*MLH1*^{m}, *MSH2*^{m}, *MSH6*^{m} or *PMS2*^{m}). This can occur at any stage of the adenoma, from early through to adenoma-carcinoma transition. Pathway 3: crypts which have acquired dMMR due to somatic mutation of the normal MMR allele

are not dysplastic, but if they undergo somatic mutation of Beta-Catenin (*CTNNB1*^{m}) which activates the WNT pathway they become flat carcinomas which later acquire mutations in *TP53* (*TP53*^{m}), an otherwise rare event in dMMR CRCs.[138, 182] Pathway 2: a proportion of Pathway 3 lesions acquire secondary *APC* mutations and thus become polypoid adenomas. Because of this unique combination of somatic events these Pathway 2 adenomas are, as far as is known, specific to LS (*hence* "Lynch-specific dMMR Adenomas"). Regarding Pathways 2 & 3 in their original proposal, Ahadova and colleagues remark: "*For better visibility, pre-malignant lesions that do not develop into cancer are not included in the diagram, because their number greatly exceeds the number of carcinomas.*"[139] Pathway 1 predominates in patients with LS due to *PMS2* pathogenic variants, while *CTNNB1*-mutant tumours are more likely in *MLH1* patients and *APC*-mutant tumours are more likely in *MSH2* and *MSH6* patients.[141, 142] Note that sporadic colonic cancers that arise from serrated lesions with MLH1 deficiency due to somatic biallelic hypermethylation of the *MLH1* promoter can and do occur in LS, albeit perhaps less often due to the enhanced immunity in LS patients against dMMR cells, because of chronic autoimmunisation from the novel frameshift peptides generated from dMMR crypts.[31, 57, 59, 136, 139, 162, 169]

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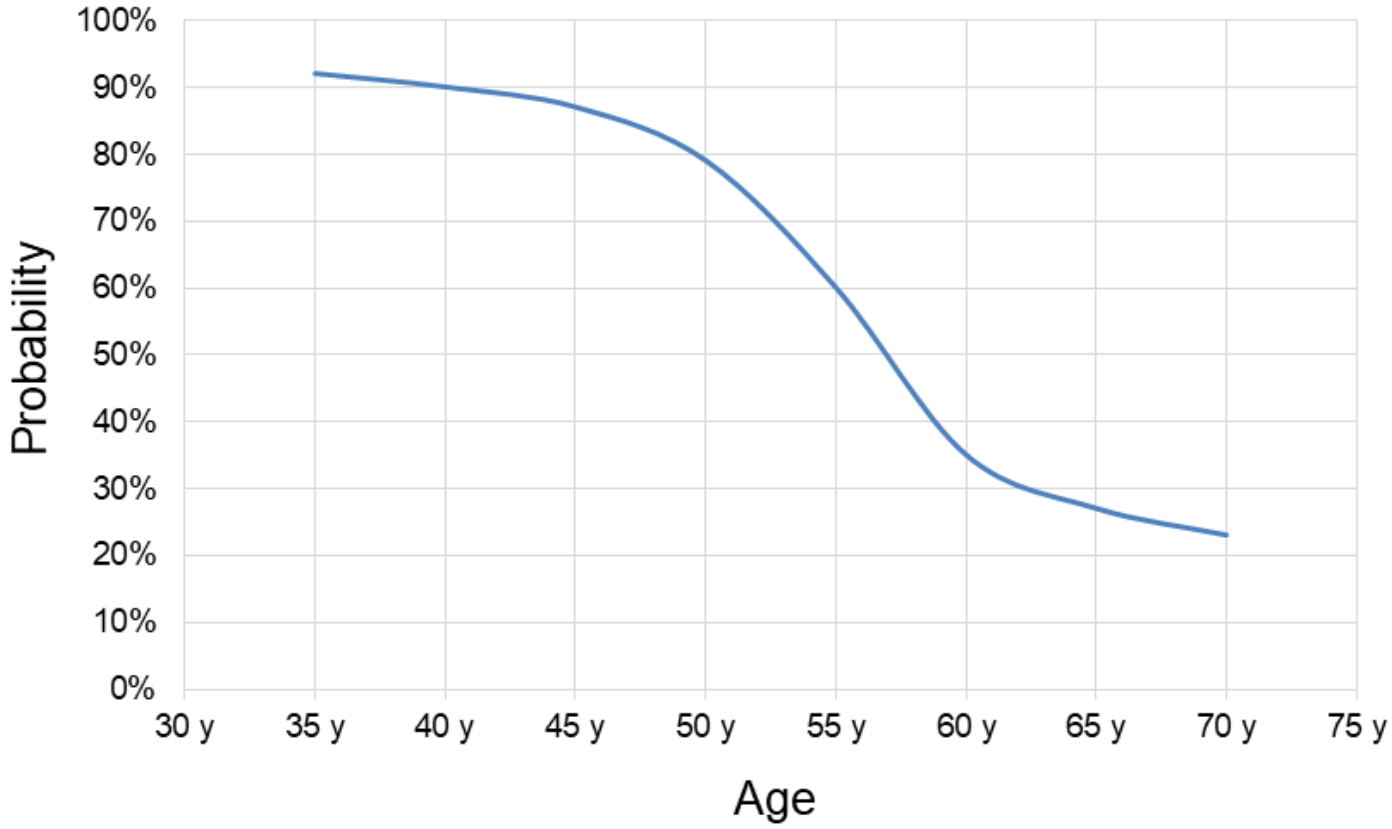
Probability by Age that a Colonic Cancer with dMMR is due to Lynch Syndrome



Pathways to Colorectal Cancer in Lynch Syndrome