# Deficient mismatch repair: Read all about it (Review)

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**Abstract.** Defects in the DNA mismatch repair (MMR) proteins, result in a phenotype called microsatellite instability (MSI), occurring in up to 15% of sporadic colorectal cancers. Approximately one quarter of colon cancers with deficient MMR (dMMR) develop as a result of an inherited predisposition syndrome, Lynch syndrome (formerly known as HNPCC). It is essential to identify patients who potentially have Lynch syndrome, as not only they, but also family members, may require screening and monitoring. Diagnostic criteria have been developed, based primarily on Western populations, and several methodologies are available to identify dMMR tumours, including immunohistochemistry and microsatellite testing. These criteria have provided evidence supporting the introduction of reflex testing. Yet, it is becoming increasingly clear that tests have a limited sensitivity and specificity and may yet be superseded by next generation sequencing. In this review, the limitations of diagnostic criteria are discussed, and current and emerging screening technologies explained. There is now useful evidence supporting the prognostic and predictive value of dMMR status in colorectal tumours, but much less is known about their value in extracolonic tumours, that may also feature in Lynch syndrome. This review assesses current literature relating to dMMR in endometrial, ovarian, gastric and melanoma cancers, which it would seem, may benefit from large-scale clinical trials in order to further close the gap in knowledge between colorectal and extracolonic tumours.

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*Abbreviations:* BRAF, v-Raf murine sarcoma viral oncogene homolog B; CRC, colorectal cancer; DFS, disease-free survival; dMMR, deficient mismatch repair; EPCAM, epithelial cell adhesion molecule; EXO1, exonuclease-1; HNPCC, hereditary non-polyposis colorectal cancer; IHC, immunohistochemistry; mCRC, metastatic colorectal cancer; MLH1, mutL homologue 1; MMR, mismatch repair; MSH2, mutS homologue 2; MSH6, mutS homologue 6; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSI-l, microsatellite instability-low; MSS, microsatellite stable; NGS, next generation sequencing; OS, overall survival; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PFS, progression-free survival; pMMR, proficient mismatch repair; PMS2, post-meiotic segregation increased 2; Pol δ, DNA polymerase δ; RFA, replication factor A; RFC, replication factor C; VUS, variants of unknown significance

*Key words:* Lynch syndrome, deficient mismatch repair, microsatellite instability, prognostic, predictive

**Contents**



## 1. Introduction

DNA mismatch repair (MMR) is a very highly conserved cellular process, involving many proteins, resulting in the identification, and subsequent repair of mismatched bases, likely to have arisen during DNA replication, genetic recombination or chemical or physical damage (Fig. 1). The MMR genes play additional roles in double-strand break repair, apoptosis and recombination. The four key genes identified to date are mutL homologue 1 (*MLH1*), mutS homologue 2 (*MSH2*), mutS homologue 6 (*MSH6*) and postmeiotic segregation increased 2 (*PMS2*), so named because of their homology to the *E. coli* MMR genes. The MSH2 and MSH6 proteins form a heterodimeric complex (mutSα) which is involved in the initial identification of mismatched bases, and initiates DNA repair. Binding to the mismatch results in an ATP-dependent conformational change, which subsequently recruits mutLα, a heterodimer comprising of MLH1 and PMS2. Other proteins are recruited to complete the DNA repair, but are not discussed further in this review. The repair complexes ensure that it is the

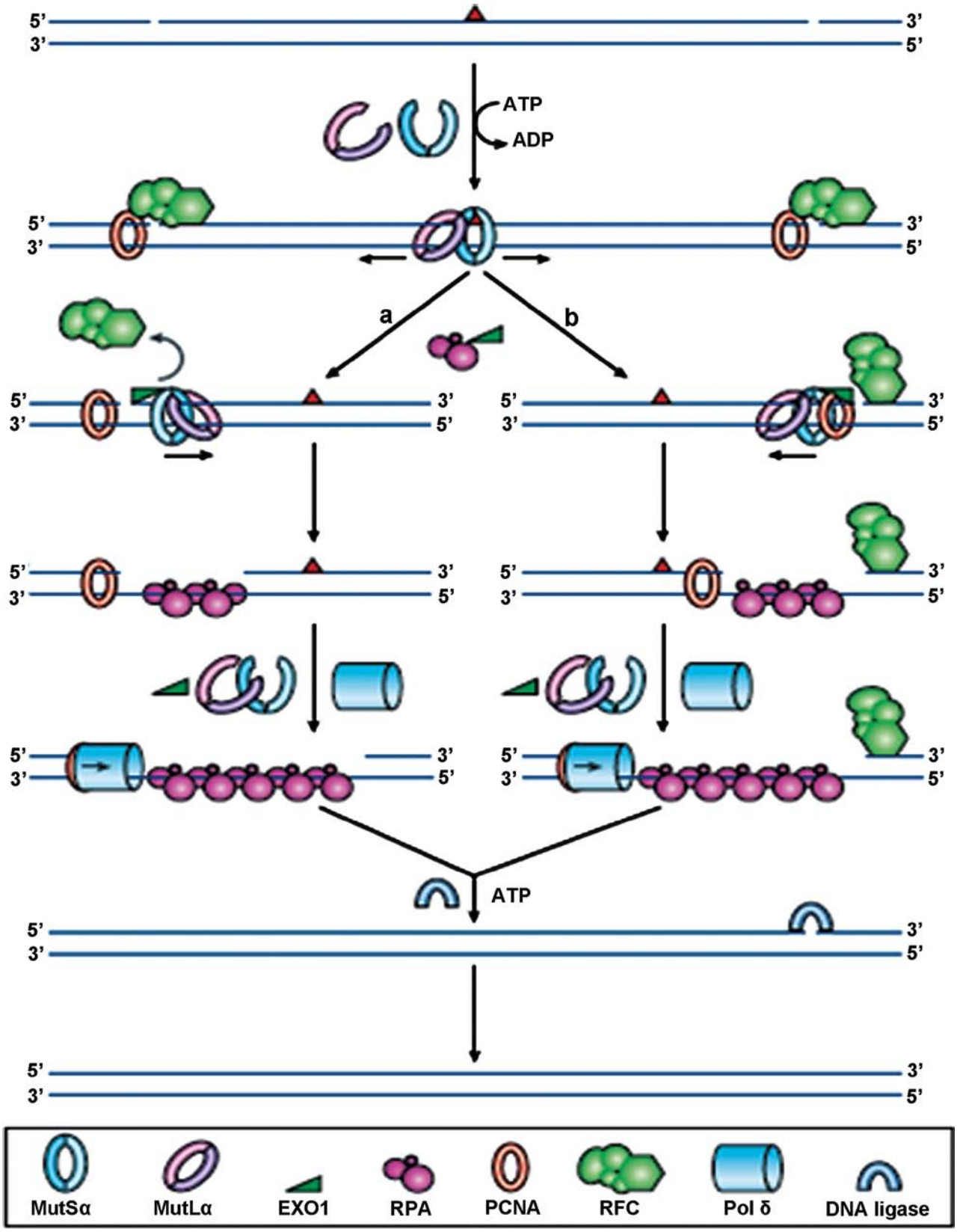


Figure 1. Reprinted by permission from Macmillan Publishers Ltd., Nature Reviews Molecular Cell Biology, 7 (5): 335-346, copyright 2006. DNA mismatch repair. In normal cells, any mismatched base pairs (or incorrect insertion or deletion loops) are repaired by the complex machinery which forms the DNA mismatch repair process. MSH2 and MSH6 form a heterodimeric complex, called mutSα, which identifies and binds to the error, resulting in an ATP-dependent conformational change, which recruits mutLα, a heterodimer consisting of MLH1 and PMS2. The resultant complex undergoes an ATP-driven conformational alteration, releasing it from the error site. If it diffuses upstream, it displaces replication factor C (RFC) and loads exonuclease-1 (EXO1). This degrades the strand in the 5'→3' direction. Replication factor A (RPA) then stabilises the single-stranded DNA, while a complex of DNA polymerase Pol δ (Pol δ) and proliferating cell nuclear antigen (PCNA) fills the gap and finally DNA ligase seals the remaining nick to finalise the repair. If the mutSα/mutLα complex diffuses downstream, EXO1 is recruited and degrades the region of the DNA strand, up to the RFC complex. As stated before, the single-strand is stabilised by bound RPA, which also inhibits EXO1 activity. Pol δ fills the gap and finally DNA ligase I seals the remaining nick to finalise the repair.

newly synthesised strand of DNA which is targeted for repair, not the parental strand.

When the MMR system develops a functional error or defect, this results in a particular phenotype called microsatellite instability (MSI). This is characterised by the insertion or deletion of short, repetitive sequences of DNA, resulting in mutations in cancer-related genes. The increase in the rate of mutations in cells exhibiting deficient mismatch repair (dMMR), may confer a Darwinian survival advantage. The cause of the dMMR system is different depending upon whether the tumour is sporadic in origin, or as a result of the autosomal dominant inherited predisposition condition, Lynch syndrome.

## 2. dMMR in sporadic colorectal cancer (CRC)

Many people wrongly group colon and rectal tumours together as 'colorectal', when referring to rates of dMMR. It is in fact very rarely seen in rectal cancers (1) but accounts for between 10 and 15% of sporadic colon cancers. This MSI phenotype is associated with several clinicopathological features such as a proximal primary tumour location, high grade, mucinous pathology, early stage, diploid and the presence of the BRAF p. (V600E) mutation (2). In addition, they tend to also be associated with being female, smoking and older age at onset. Furthermore, most of these sporadic MSI tumours are thought to arise from sessile serrated adenomas or polyps (3). This pathway of colorectal cancer development is different to the Fearon and Vogelstein adenoma-carcinoma pathway (4). In the majority of tumours, the defect in the MMR system is the inactivation of *MLH1*, through methylation of CpG islands in the promoter, causing transcriptional silencing of the gene. Limited data also suggest that inactivation in a small subset of tumours is caused my mutation of the *MLH1* gene itself (5-9).

## 3. dMMR in Lynch syndrome

Lynch syndrome (10) (formerly known as hereditary non-polyposis colorectal cancer; HNPCC) is the most common hereditary cancer predisposition syndrome, and is associated with a high risk of colorectal cancer and also extra-colonic tumours, particularly endometrial. In fact, the risk of endometrial cancer in women within some affected families may actually be greater than the risk of CRC (11). The average age at onset, of <45, is significantly lower than that for sporadic tumours and the cause of the defect in the MMR system in Lynch syndrome is constitutional mutations of the *MLH1* or *MSH2* genes, rather than methylation-induced inactivation of *MLH1*. The InSiGHT database (12) has been developed to record all mutations observed in patients with Lynch syndrome, and data from this suggest that mutations in *MLH1* account for 42% of Lynch syndrome, mutations in *MSH2* account for 33% and the remainder are found in *MSH6* (18%) and *PMS2* (7%).

A very small subset of Lynch syndrome patients is characterised by the presence of 'constitutional epimutations' of *MLH1*. These are characterised by promoter methylation and transcriptional silencing of a single allele of a gene in normal tissues, in an otherwise intact gene. Since they appear to confer a similar phenotype to that caused by sequence mutations, they are considered to be an alternative aetiological mechanism for Lynch syndrome (13). This phenomenon was first recognised in 2002 by Gazzoli *et al* (14). Several more recent studies (15-17) screened constitutive DNA samples for *MLH1* methylation, in CRC patients who had lost MLH1 expression in their tumours, without deleterious germline mutations in *MLH1*. Each study found low levels of constitutional MLH1 epimutations, but Ward *et al* suggest expanding screening programmes to include such patients, since testing of relatives identified paternal transmission (16).

In 2009, Ligtenberg *et al* proposed an alternative mechanism causing a defect in the MMR system in a subset of Lynch syndrome families (18). The study of patients from Dutch and Chinese families identified tumours which were deficient in *MSH2* as a result of the presence of heterozygous germline deletions of the 3' exons of the epithelial cell adhesion molecule (*EPCAM*; also known as *TACSTD1*) gene. Such deletions in *EPCAM* cause transcriptional read-through, which silences *MSH2*, and has been termed *MSH2* 'epimutation'. In 2011, Kloor *et al* suggested that loss of EPCAM protein expression, as assessed by immunohistochemistry (IHC) may be a suitable method of identifying Lynch syndrome patients with *EPCAM* germline deletions, as the majority of tumours with *EPCAM* germline deletions also showed loss of protein expression (19). Further to this study, Huth *et al* hypothesised that, as loss of expression did not always correlate with the presence of *EPCAM* germline deletions, that it was potentially the actual type of second somatic hit that determined EPCAM protein expression. Using multiplex ligation-dependent probe amplification (MLPA) to assess allelic deletion status, tumours with loss of EPCAM expression showed biallelic deletions, whereas tumours retaining EPCAM expression demonstrated monoallelic retention of the *EPCAM* gene. The group therefore concluded that EPCAM protein expression is dependent upon the actual localisation of the second somatic hit that inactivates *MSH2* (20). More recently, a study by Musulen *et al*, showed a high specificity between the presence of *EPCAM* germline mutations and loss of EPCAM expression, and recommended the addition of EPCAM IHC into diagnostic Lynch syndrome testing, in patients with *MSH2*-negative tumours (21).

## 4. Who (and how) to test for mismatch repair deficiencies?

*Diagnostic criteria and guidelines*

*Amsterdam criteria*. The identification of a patient with a colorectal or endometrial tumour raises the question of whether to screen for the presence of Lynch syndrome. Various criteria have been in place for the past 35 years, to help guide this decision. In 1991, the Amsterdam criteria arose from a meeting of the International Collaborative Group on Hereditary Non-Polyposis Colon Cancer (ICG-HNPCC) where an attempt was made to standardise international criteria for identifying HNPCC patients for research purposes (22). These criteria were known as the '3-2-1 rule': a) at least three relatives should have histologically confirmed CRC, with one being a first degree relative of the other two; b) there must be two successive generations affected; and c) one or more relatives must be diagnosed by the age of 50.

The Amsterdam criteria was later renamed Amsterdam criteria I, following the subsequent identification of the genes involved, which lead to the expansion of the criteria and its renaming Amsterdam Criteria II.

*Amsterdam Criteria II*. Based upon further research identifying the fact that Lynch syndrome tumours were not confined to the colon or rectum, the criteria were further expanded and updated in 1998, and renamed the Amsterdam II criteria (23). These new criteria added in the fact that at least three relatives should have a histologically confirmed HNPCC-associated cancer (colorectal, endometrial, small bowel, ureter or renal pelvis), rather than just a colorectal tumour.

*Bethesda Guidelines*. At around the same time, the National Cancer Institute of the USA published its own set of guidelines (24). These included the following criteria: a) individuals with cancer in families that meet the Amsterdam criteria; b) individuals with two HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extra-colonic cancers (endometrial, ovarian, gastric, hepatobiliary, or small bowel cancer or transitional cell carcinoma of the renal pelvis or ureter); c) individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extra-colonic cancer and/or a colorectal adenoma: one of the cancers diagnosed by age 45, and the adenoma diagnosed by age 40; d) individuals with colorectal cancer or endometrial cancer diagnosed by age 45; e) individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed by age 45; f) individuals with signet-ring-cell-type colorectal cancer diagnosed by age 45; and g) individuals with adenomas diagnosed by age 40.

*Revised Bethesda Guidelines*. In 2004, the NCI revised these guidelines, and went on to publish the Revised Bethesda Guidelines (25). These remain the most recent clinical diagnostic criteria upon which a patient is identified as likely having Lynch syndrome; a) individuals with CRC diagnosed by age 50; b) individuals with synchronous or metachronous CRC, or other HNPCC-associated tumours regardless of age; c) individuals with CRC and MSI-H histology diagnosed by age 60; d) individuals with CRC and more than 1 first degree relative with an HNPCC-associated tumour, with one cancer diagnosed by age 50; and e) individuals with CRC and more than 2 first degree relatives or second degree relatives with an HNPCC-associated tumour, regardless of age.

*Jerusalem criteria*. In 2009, the 'Jerusalem criteria' were published, recommending that either dMMR IHC or MSI testing be carried out on every colorectal tumour, where the patient is under the age of 70 at diagnosis (26). The idea behind this broader screening programme was to identify potential Lynch syndrome patients with an *MSH6* or *PMS2* mutation, who tend to present at a later age, and would not be included for screening, under the revised Bethesda guidelines.

## 5. Does 'one size' really fit all?

All of the above criteria for selecting patients for screenings have been based upon North American and European populations. In order for these criteria to be used worldwide, this makes the assumption that there are no population-specific differences. A study by Yan *et al*, has questioned this very point in relation to a Chinese population, where there is a strict one child policy (27). The resultant large number of small families makes it almost impossible to meet all the specified criteria regarding the number of affected relatives. As a result this increases the likelihood of overlooking and not screening a high proportion of potential or actual Lynch syndrome patients.

A second factor bringing the relevance of using the Amsterdam or Bethesda criteria in Asian populations into question, is the fact that gastric and hepatocellular cancers are the most common extracolonic tumours seen in Chinese patients with Lynch syndrome, rather than endometrial tumours as seen in the West. Furthermore, it becomes difficult to gauge how specific this is for Lynch syndrome, when the rates of gastric and hepatocellular carcinoma (HCC) are so high in Asia due to *Helicobacter pylori* (*H. pylori*) and chronic hepatitis B virus (HBV) infections respectively. An *H. pylori* infection induces an inflammatory response, in addition to causing genetic changes which result in genetic instability (28). The oncogenic effects of HBV such as genomic instability result from its integration into the host genome (29).

A third factor is that several studies have reported a predominance of left-sided CRC in Asian populations, which is different to what is seen in Western patients, where there is a predominance of right-sided tumours. Wang *et al* (30) noted that 60.6% of 60 Lynch syndrome patients under study had distal colorectal tumours. Chew *et al* (31) undertook a study of 6,736 CRC patients, who underwent surgery for their disease at Singapore General Hospital between 1989 and 2005; 52 (0.8%) fulfilled the Amsterdam I or Amsterdam II criteria, so were included for analysis and 69% of these patients had left-sided tumours, the majority of which were located in the sigmoid colon (31). In a very recent study of 116 Chinese families with suspected Lynch syndrome, 32 of whom had confirmed *MLH1* or *MSH2* germline mutations, 56.5% of the colorectal tumours were left-sided (32). These observations could be as a result of the fact that rectal cancers are more prevalent in Asian populations, or simply the fact that this is a feature of Asian Lynch syndrome.

In Western populations, we know that 10-15% of sporadic CRC tumours are dMMR. This figure may be much higher in Asian populations, based upon a study carried out in Singapore on 240 CRC patients, under the age of 50 at presentation. MMR IHC was performed and 21% of patients showed loss of expression of at least one of the MMR proteins. The authors identified the fact that, had selection for screening been based solely on the Amsterdam criteria, a staggering 86% of patients would have not been identified as high risk of Lynch syndrome, and would thus not have been screened (33). This provides further evidence for the introduction of population-specific diagnostic screening criteria.

## 6. Reflex testing

In essence, reflex testing is the routine screening of all newly diagnosed colorectal tumours for dMMR, to increase the likelihood of identifying Lynch syndrome patients. Obviously early diagnosis will result in increased surveillance, thus hopefully reducing morbidity and mortality, not only for the affected individual, but also family members.

Several studies have proven the cost-effectiveness of such a screening approach (34-38). A Dutch study by Sie *et al* (39)

recommended increasing the cut-off age for testing all CRC from 50 to 70 years old, and still found this strategy cost-effective. However, in spite of the potential financial savings, reflex testing is proving difficult to implement, with areas requiring attention being highlighted at a multidisciplinary working group meeting of the Centers for Disease Control and Prevention in the US (40). The group identified the lack of primary care provider knowledge of Lynch syndrome and testing issues, as the main barrier to implementation. Furthermore, it was recognised that there is a requirement for a strategy to ensure that at-risk relatives are identified and counselling offered. There is also very limited data available on the feasibility of carrying out such testing, so one recommendation is for additional 'real-world' studies to be carried out to generate such data.

Taking a whistle-stop tour of current practice worldwide, it would appear that much still needs to be done in terms of implementation. In the UK, despite reflex testing being mandated by the Royal College of Pathologists and recommended by the British Society of Gastroenterologists, less than 50% of National Health Service Hospital Trusts currently carry out screening on patients presenting with the disease under the age of 50. This is the case in England, Wales and Scotland, however, all social care trusts in Northern Ireland have successfully implemented screening. The National Services Division Scotland and the Molecular Pathology Consortium are currently trying to implement national screening throughout Scotland, with the rest of the UK hopefully following suit, once this model is in place (data from a Bowel Cancer UK freedom of information request sent out across the UK to establish the level of implementation) (41). The main reason for not screening was put down to the additional financial burden. A further reason given is a current lack of National Institute for Health and Care Excellence (NICE) guidance. NICE is an executive non-departmental public body within the Department of Health in the UK, and publishes guidelines in, amongst other areas, clinical practice. Another rather interesting reason is the potential impact on patients and their families. The fact remains, and must not be overlooked, that patients simply may not wish to undergo genetic testing. There are many negative perceptions of this type of screening, and unless patients are educated appropriately as to the potential benefits, this could remain a barrier to implementation.

A study in Canada by Tomiak *et al* determined that in order to increase the uptake of genetic services by patients with suspected Lynch syndrome, several areas needed addressing, such as improving health literacy for the general population, newly diagnosed patients, and perhaps a little surprising, healthcare professionals (42). The study highlighted a general lack of awareness of hereditary cancers and a lack of understanding of the need for, and potential benefits of, genetic screening and what is done with, and who has access to, the results. The requirement for psychosocial support was also highlighted as an area to be addressed. Tomiak *et al* concluded that these gaps need to be filled for the successful implementation of universal screening, planned by the US Office of Public Health Genomics, by 2020.

In 2012, Beamer *et al* carried out a questionnaire-based review of reflex testing practise across the United States of America, similar in design to that undertaken by Bowel Cancer UK, in the United Kingdom (43). They found that the level of reflex testing implementation was dependent primarily upon the level of cancer program [ranging from Community Hospital Cancer Programs (CHCP), to Community Hospital Comprehensive Cancer Programs (COMP), and finally up to the most complex level of National Cancer Institute-designated Comprehensive Cancer programs (NCI-CCC)]. Seventy-one percent of NCI-CCCs, 36% of COMPS yet only 15% CHCPs had already implemented reflex testing. Another point arising from this study is whether written patient consent is required. Currently this is not the case, presumably because screening a tumour provides phenotypic, rather than genotypic information, but it will be interesting to see whether this aspect becomes a barrier to worldwide reflex testing.

Back in 2008, in the state of Western Australia, routine screening for Lynch syndrome was implemented. All patients under the age of 60 at the time of diagnosis are screened and figures published recently estimate that the majority of Lynch syndrome cases are being identified as a result of this programme (44).

Table I. Loss of MMR protein expression.

| Protein expression lost (determined by IHC) | Interpretation (defective protein) |
|---|---|
| PMS2 | PMS2 |
| MLH1 and PMS2 | MLH1 |
| MSH6 | MSH6 |
| MSH2 and MSH6 | MSH2 |

Due to the heterodimeric nature of the MMR proteins, loss of expression of a particular protein may in fact be due to the loss of expression of its paired protein. For example, loss of PMS2 alone indicates a defect in PMS2, whereas, when expression of both MLH1 and PMS2 are lost, this is likely due to loss of MLH1, as this results in unstable PMS2. The same is true for MSH6 and MSH2, respectively.

## 7. MMR Immunohistochemistry (IHC)

MMR IHC is a quick and relatively simple assay to determine protein expression of MLH1, MSH2, MSH6 and PMS2 (Fig. 2). Tumours with dMMR will usually show complete loss of expression of one or more protein. Assessing all four proteins provides further information to determine the actual defective protein. We know that MLH1 forms a heterodimer complex with PMS2. Loss of expression of PMS2 alone is indicative of a defect in the *PMS2* gene. However, combined loss of PMS2 and MLH1 suggests the defect lies in MLH1, as MLH1 is responsible for the stability of PMS2. A similar situation is seen with MSH6 and MSH2, with loss of MSH6 only indicating defective MSH6, whereas loss of expression of both proteins would indicate the defect is within MSH2 (Table I). Based on a recent publication by Mensenkamp *et al* (45) this may in fact be a real oversimplification of the actual situation. The group sequenced dMMR CRC tumours and endometrial tumours which appeared to have neither a germline mutation in any MMR gene, or hypermethylation of the *MLH1* promoter.

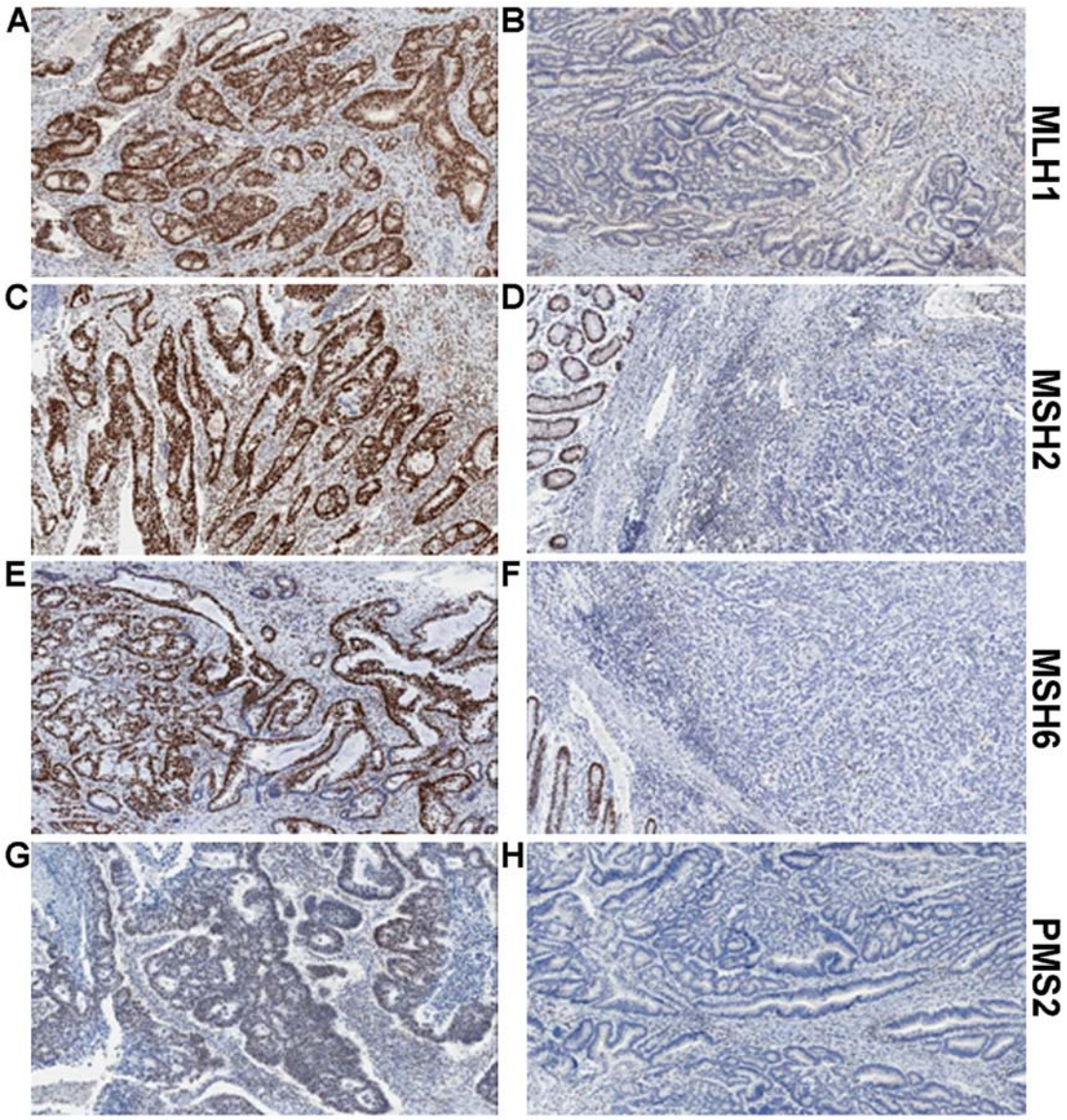


Figure 2. Examples of MLH1, MSH2, MSH6 and PMS2 immunohistochemistry. (A) Positive MLH1 staining and (B) absence of MLH1 staining in tumour epithelium yet showing the positive internal control staining of lymphocytes in the stroma. (C) Positive MLH2 staining and (B) absence of MLH2 staining in tumour epithelium, yet showing positive staining in the adjacent normal colonic epithelium. (E) Positive MSH6 staining and (F) absence of MSH6 staining in tumour epithelium yet with positive staining in the adjacent normal colonic epithelium. (G) Positive PMS2 staining and (H) absence of PMS2 staining in tumour epithelium yet with positive internal control staining of lymphocytes in the stroma.

In more than half of these tumours, somatic mutations were identified as the underlying cause of deficient mismatch repair.

Unfortunately, as is often the case with a seemingly straightforward assay, there are exceptions to the rules. Sometimes expression is reduced in intensity, or patchy, rather than completely lost. This may be a result of the expression of a truncated protein with limited stability, and is likely to be accompanied by the predicted normal strong nuclear staining within adjacent stromal cells or lymphocytes. It is often the case that the abnormal staining is seen in both binding partners, i.e., MLH1 and PMS2, or MSH2 and MSH6. Another unusual situation is where staining is seen localised to the cytoplasm, rather than within tumour cell nuclei. This may be caused by a defect in the nuclear localisation signal, and would most likely be reported as dMMR, although PCR-based MSI testing may be requested for confirmation. The single biggest problem in the assessment of MMR IHC is the variability in fixation of the tumour tissue. The actual fixative used, the time in formalin prior to embedding and the uniformity of fixation are all factors which can affect the quality of staining seen. Fadhil and Ilyas compared staining of the four MMR proteins in 30 matched pre-surgical diagnostic biopsy samples and the matched resection tissue, and concluded that not only was the staining in the biopsies identical to that in the resection, but the interpretation was made easier by the staining being more intense and thus easier to interpret (46). This difference was deemed to be a result of more uniform and complete fixation in the biopsy samples, compared to the resection specimens.

A further complication in terms of the interpretation of the MMR IHC was reported by Bao *et al* in a study of 51 colorectal cancer patients undergoing neoadjuvant chemoradiation (47). Nine of these tumours showed reduced, but not complete loss

of MSH6 staining, yet upon MSI analysis, all were microsatellite stable, suggesting that the reduced expression was a result of the chemoradiation treatment.

A slightly contentious issue, worthy of a mention, is whether missense mutations in the MMR genes are associated with reduced or patchy immunohistochemical staining. Missense mutations result in a protein with a single amino acid change, which could lead to no defect at all, or a dysfunctional or 'pathogenic' mutation. Difficulty arises in the assessment of the pathogenicity of a missense mutation. Criteria which would have to be met would include: a) the mutation not being present in control subjects; b) the mutation co-segregating with a phenotype in a family; c) the mutation resulting in a non-conservative amino acid alteration; and d) the codon in which the mutation arose being evolutionarily conserved (48,49). PubMed searches for this review failed to identify any studies reporting reduced levels of MMR protein expression, which were attributed to missense mutations. At the present time, this phenomenon may have to remain an 'urban myth'.

Once an abnormal expression pattern of the MMR proteins has been established, it is vitally important it determine whether the tumour is from a patient with Lynch syndrome. The MMR protein expression profile most commonly associated with Lynch syndrome is loss of both MLH1 and PMS2; however, this would also be seen in a sporadic tumour, if caused by *MLH1* methylation. The *BRAF* p. (V600E) mutation is observed in up to 70% of tumours which have loss of expression of MLH1 and PMS2 or exhibit *MLH1* methylation (50,51), but the mutation is almost never seen in Lynch syndrome-associated tumours (52,53). Thus the presence of the *BRAF* mutation strongly indicates a dMMR tumour of sporadic origin. *BRAF* mutation testing is currently carried out routinely by traditional sequencing methodologies, such as Sanger sequencing, but in 2011, the first report was published by Capper *et al*, that used an antibody specific for the V600E mutant protein (VE1), allowing direct immunohistochemical testing of a tumour section (54). Several groups have published data showing very favourable results with the antibody (including refs. 55,56) however, concerns have been voiced regarding the usefulness, and sensitivity of this antibody, particularly when assessing colorectal tumours. Adackapara *et al* noted a high level of weak staining in wild-type and *KRAS* mutant tumours, in addition to non-specific nuclear staining. They determined the sensitivity and specificity to be 71 and 74%, respectively, and deemed the antibody not to be a surrogate for standard genotyping (57). A study by Loes *et al* in 2015 assessed three methods of *BRAF* mutation detection [IHC, Sanger sequencing and a single probe-based high-resolution melting assay (LightMix) which has clamped wild-type allele amplification] in both melanoma and colorectal tumour samples. Data were available for all three assays in 99 colorectal tumours, of which 63 were wild-type by all methods, 12 were *BRAF* mutant by all methods, and yet 22 gave discordant results. Using the IHC data alone would have misinterpreted 10 patients as being *BRAF* mutant, and also failed to detect mutations in a further two patients. The authors conclude that the high level of unexplained, non-specific staining seen in colorectal tumours, much more so than for melanoma tumours, would support that the antibody be used solely as a screening tool, rather than a diagnostic test (58). It is worth noting that the antibody will only identify the specific V600E mutation, so there is always the risk of missing other BRAF mutations, but these are extremely rare, particularly in colorectal tumours.

## 8. Microsatellite (MSI) testing

As an alternative, or indeed in combination with MMR IHC testing, PCR-based MSI screening may be undertaken. The recommended NCI-reference panel comprises two mononucleotide repeats (BAT-25 and BAT-26) and three dinucleotide repeats (D5S346, D2S123 and D17S250). There is also a commercially available kit, consisting of five mononucleotide markers (BAT-25, BAT-26, MONO-27, NR-21 and NR-24), as data are emerging to suggest that there is a higher level of both sensitivity and specificity in the detection of the MSI-H phenotype when only mononucleotides are used (59). Where available, DNA from normal mucosa is compared to that extracted from the tumour. However, the nature of the mononucleotide markers means that it is not essential to have normal DNA for testing. The tumour is classed into one of three phenotypes; if none of the markers show instability, the tumour is classed as microsatellite stable (MSS). If one of the markers show instability, the tumour is classed as microsatellite-low (MSI-L), and if two or more of the markers show instability, the tumour is classed as microsatellite-high (MSI-H). Often MSS and MSI-L tumours are classified as a single subset, as very few tumours of either phenotype will exhibit loss of expression of any of the MMR proteins. Data surrounding clinical differences between the two tumour phenotypes is still inconclusive (60-63).

*IHC or MSI?* There have been several studies carried out to assess the correlation between IHC and MSI-testing, and the overall results seem to suggest that firstly neither test is 100% accurate in the detection of MSI-H tumours and secondly, there is actually a high level of concordance between both technologies. The largest study to date was performed by Cicek *et al* in 2011, when almost 6,000 tumours from patients in the Colorectal Cancer Family Registry were analysed. The group showed a 90-95% concordance between those cases identified as dMMR by MSI and those detected by IHC. Furthermore, only 2.7% of the 3964 tumours with IHC data available, would have been miscalled, had only these data been used in the initial assessment (64).

## 9. Next generation sequencing

There is no doubt that sequencing methodologies have been transformed over the past few years, with the advent of next generation sequencing platforms. Several companies are now producing panels and kits, allowing the massive parallel sequencing of MMR genes. This additional depth of sequencing may cause the problem with the identification of variants of unknown significance (VUS). Furthermore, there will undoubtedly be mutations detected at lower levels than previous technologies have allowed. The issue with these is that the clinical significance has not yet been determined, thus with the technology being still in its infancy, there remains the need to validate such panels. Pritchard *et al* carried out one

such validation study of the ColoSeq panel, which correctly identified all 28 previously characterised mutations in *MLH1, MSH2 MSH6, PMS2, EPCAM, APC* and *MUTYH*. Two VUS were also detected in 19 samples from patients without cancer (65). The significance of such variants should become apparent once more data are available and they can be related to pathogenicity.

**10. Deficient MMR and clinical outcomes**

*Prognostic value in sporadic colorectal cancer.* The majority of the data published recently on the prognostic and predictive value of MMR has been gathered on CRC patients. There is definitely a distinction between the prognostic benefit of dMMR in early (stage II/III) and late (stage IV) disease. Several studies and meta-analyses have shown that dMMR in stage II +/or III tumours is a positive prognostic factor. Back in 2003, a study of 570 stage II or II CRC patients showed that those patients whose tumours were MSI-H had an improved 5-year OS, compared to MSI-L or MSS tumours (HR for death was 0.31 (95% CI, 0.14-0.72, p=0.004) (66). In 2010, a large meta-analysis pooled data from 12,782 CRC patients, including 1,972 MSI-H patients. The odds ratio (OR) for disease-free survival (DFS) was 0.58, 95% CI 0.47-0.72, p<0.0001 and a similar value obtained for OS (OR=0.6, 95% CI 0.53-0.69, p<0.0001) (67). This was confirmed by Sargent *et al*, in a further meta-analysis of 457 patients, where it was reported that dMMR status was associated with improved DFS (HR, 0.46; 95% CI, 0.22-0.95; p=0.03) and a trend was seen towards improved OS (HR, 0.51; 95% CI, 0.24-1.10; p=0.06) (68). The QUASAR (QUick And Simple And Reliable trial provided a more recent dataset on which to confirm the positive prognostic significance of dMMR. The recurrence rate in the dMMR cohort was 11% (25/218), compared to 26% (438/1695) in the pMMR cohort [risk ratio (RR), 0.53; 95% CI, 0.40-0.70] (69).

Because of the fact that dMMR appears to be a good prognostic marker in early CRC, it stands to reason that prevalence of dMMR would be lower in advanced CRC (aCRC), since these patients should be less likely to develop metastatic disease (70). This has been reported in several studies (71-73). The question remains as to why these tumours appear to metastasise less frequently. This may be as a result of the increased immune response seen in dMMR tumours. Tikidzhieva *et al*, have suggested a possible mechanism, involving β2-microglobulin (*B2M*) (74). Mutations in *B2M*, within microsatellite coding regions, are reported frequently in MSI-H tumours, and result in the inability to present antigens at the cell surface, through HLA-class I molecules. This in turn, may stimulate natural killer (NK) cell-mediated tumour cell death.

In terms of the prognostic value of dMMR in aCRC, a recent large meta-analysis by Venderbosch *et al* (75) of patients in four randomised clinical trials (CAIRO, CAIRO2, FOCUS and COIN) provides convincing evidence of the negative prognostic effect of dMMR in the metastatic CRC (mCRC) setting. Data on dMMR was gathered on 3,063 patients, recruited into the four clinical trials. PFS and OS were significantly reduced in the dMMR cohort, in comparison to the pMMR cohort (PFS, 6.2 versus 7.6 months respectively; HR, 1.33; 95% CI, 1.12-1.57; p=0.001; and OS, 13.6 versus 16.8 months respectively; HR, 1.35; 95% CI, 1.13-1.61; p=0.001). The analysis also demonstrated the negative prognostic effect of the presence of the *BRAF* p. (V600E) mutation, but ruled out any interaction between the two poor prognosis markers. The group suggest that the negative value of dMMR is as a result of the mutant BRAF status, since significantly more dMMR tumours also contained the mutation.

*Predictive value in colorectal cancer.* Since its introduction into clinical practice almost 40 years ago, 5-fluorouracil (5-FU) has, until recently, been the 'gold standard' chemotherapy agent in the treatment of CRC. As a result of this, there is much, and it has to be said, conflicting data regarding the predictive value of MMR status and response to 5-FU-based therapy, with some studies reporting benefit from 5-FU (76,77) whilst most reporting no benefit or indeed a dis-benefit (66,68,78,79).

The final results from the MOSAIC trial where 2,246 stage II or II CRC patients were randomised between 5-FU plus leucovorin (LV5FU2) and FOLFOX (LV5FU2 + oxaliplatin), provided convincing evidence that the addition of oxaliplatin resulted in improved 5-year DFS and 6-year OS, and in particular, ought to be given to stage III patients after surgery (80). Following this, studies were performed to assess whether microsatellite status was predictive of response to oxaliplatin; Zaanan *et al* (81) analysed 233 MSI-H stage III patients, receiving either 5-FU/LV or FOLFOX, and finding that those on FOLFOX had an improved 3-year DFS compared to those on 5-FU/LV. However, in the same year, a study of 135 patients receiving FOLFOX following surgery, found no difference in DFS or OS when patients were stratified for MMR status (82). In the metastatic setting, Muller *et al* in a 108-patient study, comparing two oxaliplatin and 5-FU-containing regimens, demonstrated a lower rate of disease control in MSI-H patients compared to non-MSI-H patients (p=0.02) (73). Kim *et al* however, showed that MMR status did not predict response to oxaliplatin-based treatment, when 171 recurrent or mCRC patients were analysed (83).

There is also conflicting data as to the predictive value of MMR status and response to irinotecan. Bertagnolli *et al* showed that patients with dMMR/MSI-H had improved DFS, compared to MSS patients, when irinotecan was added to standard 5-FU/LV treatment, with this benefit not being seen in patients treated with 5-FU/LV alone (84). However, this was not confirmed by the PETACC-3 study (85) or by a Korean study of almost 300 patients (86), or by the UK MRC FOCUS study (71).

It would be difficult to summarise the prognostic and predictive value of MMR status in both the adjuvant and metastatic CRC settings, based on the data presented above. It is apparent that dMMR/MSI-H in the adjuvant setting is a good prognostic marker, but in the metastatic setting, the evidence suggests the complete opposite effect. As for the predictive value, there are conflicting data regarding each treatment regimen. One can speculate as to why this is the case; perhaps we are seeing population differences, perhaps the method of determining MMR status had differing sensitivities. The small numbers of patients in some of the studies should also be taken into account. It is without doubt safe to say, that one cannot use only MMR status for the prediction of response to therapy.

*Prognostic and predictive value in extra-colonic tumours*. The majority of published data regarding the role of the mismatch repair system in carcinogenesis, and the resultant prognostic and predictive value, is within colorectal cancer. There are, however, several extra-colonic cancers where there are high percentages of dMMR have been reported, yet little is known of the prognostic or predictive value.

*Endometrial cancer*. dMMR has been reported in 20-30% of endometrial cancers (87), yet there are scarce data available regarding the prognostic and predictive impact of mismatch repair deficiencies. In a study reported earlier this year, Kato *et al* analysed 191 endometrial tumours, and found that 40% of them were deficient in at least one of the MMR proteins, as assessed by IHC (88). This cohort displayed differences in tumour grade histology and International Federation of Gynecology and Obstetrics (FIGO) stage, when compared to the proficient MMR tumours. Furthermore, dMMR cases had improved PFS and OS, with MMR status being an independent prognostic factor for OS in endometrial cancers. A further study, admittedly smaller, of 66 patients with endometrial cancer and lymphatic invasion, also reported improved disease specific survival (DSS) (p=0.04) and OS (p=0.03) in dMMR patients, compared to those with pMMR. The authors also reported increased OS particularly in FIGO stage 3C and stage 4 dMMR patients, which may suggest that despite the lymphatic invasion and lymph node metastases, this subgroup has a better prognosis than patients with an intact MMR system. The other factor that cannot be ignored is the effect that adjuvant chemotherapy has contributed to this improved survival (89). A third study, of 477 patients, investigated whether MMR status impacted upon response to chemotherapy or pelvic teletherapy [also known as external beam radiotherapy (EBRT)]. There was no significant difference in PFS or OS between dMMR and pMMR subgroups, when stratified by treatment. However, when patients were stratified between endometrioid and non-endometrioid tumours, significantly improved OS (p=0.003) and PFS (p=0.004) was seen for dMMR/non-endometrioid tumours, receiving teletherapy. The opposite was seen for patients receiving adjuvant chemotherapy, where those with intact MMR showed improved PFS and OS (90). Taking these data together, it would possibly appear that dMMR in endometrial cancers, or at least within subgroups, is a positive biomarker. However, Ruiz *et al* reported no association between MMR status and survival, in a study of 212 endometrioid tumours (91), and a further study actually reported an increased risk of disease-specific death in dMMR high-grade endometrioid carcinomas (HGEC). Interestingly in this study, dMMR was only seen in these HGEC tumours, and not serous or clear cell tumours, suggesting the use of MMR testing to aid in tumour-type diagnosis (92). Cohn *et al* reported improved DFS in a cohort of endometrial cancer patients who had retained expression of both MLH1 and MSH2, in comparison to patients who displayed abnormal expression (p=0.035) (93). A large meta-analysis carried out in 2013 summarised very eloquently the lack of concrete evidence of an association between MMR status and clinical outcome, where in a pooled analysis of 23 studies (published between 1980 and 2011), the group failed to show a significant association between MSI and a worse OS (p=0.11) or DFS (p=0.66) (94). The heterogeneous nature of the method of determining MSI status, combined with variability in the study populations, still make it very difficult to determine the usefulness of MMR status in relation to outcome in this disease.

*Ovarian cancer*. Ovarian cancer is the 7th most common cancer worldwide for females, with over 239,000 new cases diagnosed in 2012, and has the highest mortality rate of all the gynaecological cancers (95). Early detection is difficult, and as a result, only 15% of women present with localised disease (96). Women with Lynch syndrome, have a lifetime risk of ovarian cancer of approximately 8% (97-99). As we find in common with other extracolonic cancers, data on MMR is sparse. Several authors have attempted to clarify dMMR or MSI rates through meta-analyses; Xiao *et al* (100) found disparities between reported rates of MSI frequency, ranging from 5 to 13% (101-103). Murphy and Wentzensen combined results from 22 studies, arriving at a figure for MSI of 10% for unselected ovarian cancer patients (104). This figure was further refined to 9%, when only patients who had been tested for MSI using the five Bethesda markers were analysed. Pal *et al* also suggest that 10% of ovarian cancers show MSI, analysing data from 18 studies (105). In terms of dMMR as assessed by IHC, larger differences were observed; ranging from 2 to 29% across the 12 studies analysed by Xiao *et al* (100). One feature common to most studies was the fact that there was an overrepresentation of the non-serous tumours within the MSI cohorts, which parallels the overrepresentation of mucinous and endometrioid histologies in CRC and endometrial cancers respectively. In terms of data relating to the effect of dMMR or MSI on prognosis or response to chemotherapy, very little has been published, and the results are varied. Scartozzi *et al* found that loss of expression of MLH1 correlated with increased survival in patients with stage III/IV disease, although the study size was only 34 patients (106). Zhia *et al* assessed 322 tumours for MSH6 expression, and found no correlation with survival. The group did find a correlation between loss of expression and clear cell, mucinous and endometrioid histologies (p<0.007) (107). Another study finding no association between MSI and survival was carried out on a series of Danish patients by Begum *et al*, who used a panel of 16 dinucleotide markers to assess status (108). In terms of response to therapy, there have been two reports of a correlation between a lack of MSH2 and response to platinum-based chemotherapy; Ercoli *et al* showed that patients who did not respond to treatment had lower levels of MSH2 than patients who had at least a partial response (109). A report by Marcelis *et al* described two Lynch syndrome patients, both carrying a deletion in exon 6 of *MSH2*, who developed a rapid resistance to cisplatin-based therapy (110). Based upon current literature, very little can be reasonably or reliably concluded regarding the role of the MMR proteins in ovarian cancer survival or response. There is clearly a need for large, randomised studies in this disease field, where one can control for factors such as MMR assessment criteria, tumour histology, treatment regimen and sample size.

*Melanoma*. Malignant melanoma is the 19th most common cancer worldwide, with around 232,000 new cases diagnosed in 2012 (111). MSI has been reported to be present in anywhere

between 2 and 30% of primary tumours (112-116) and 20-77% of metastatic lesions (117-123). Castiglia *et al* suggest that the inactivation of the MMR system, in combination with the deregulation of the Wnt/beta-catenin pathway may act cooperatively to promote the development of melanoma (124). It may be that in melanoma, it is a downregulation of the MMR proteins, rather than a complete loss of expression, or gene inactivation that is important, as seen in a study by Korabiowska *et al*, who confirmed the downregulation by both IHC and *in situ* hybridisation in 59 malignant melanomas (125). Alvino *et al* also reported a reduction in expression of MLH1, MSH2 and PMS2 in primary melanomas compared to benign nevi. Interestingly they also noted the opposite for MSH6, and this increased expression was also associated with increased risk of melanoma mortality (R, 3.76; 95% CI, 1.12-12.70) (126). With such little data available on the MMR proteins in melanoma, the only conclusion that can be reliably drawn is that as the cancer progresses from benign nevus, through primary melanoma to metastatic melanoma, the level of MSI increases. This may, however, only be at an MSI-L level, rather than MSI-H. The significance of this is yet to be determined.

*Gastric cancer.* Gastric cancer is the 5th most common cancer worldwide, with more than 951,000 new cases diagnosed in 2012 (127). In gastric cancer, MSI exists in approximately 10-20% tumours (128-130). Such tumours are associated with older patients, distal location, lower pTNM stage and intestinal subtype and reduced lymph node involvement. Several large studies have assessed the prognostic effect of the MSI phenotype, all showing that MSI correlates with improved survival; back in 2000, Schneider *et al* showed that in MSI-H and MSI-L patients, there was an increased median survival time, compared to MSS patients (p=0.027) (131). In 2002, Lee *et al* analysed 327 consecutive gastric cancers, assessing MSI status with the BAT-26 marker. Patients with MSI had improved overall survival compared to those with MSS tumours (p=0.046) (130). Beghelli *et al*, determined the MSI status of 510 sporadic gastric cancers, also concluding that MSI correlated with improved survival, but only in stage II disease (p<0.011) (128). In a study of 159 patients, Falchetti *et al* demonstrated an association between MSI-H phenotype and improved survival at 15 years (p=0.01) (132). Finally Fang *et al* showed that there was an improved 5-year OS benefit in the MSI-H cohort (p=0.03) and also a trend towards an improved 3-year disease-free survival (p=0.076), when analysing 214 gastric cancer patients (129). However, as one has come to expect in this field, there is conflicting data to suggest that MSI status has no influence on survival; Perez *et al* found no survival benefit in the MSI patients, compared to the MSS patients, however, it must be noted that there were only 24 patients in this study (133). In a slightly larger study of 83 patients, An *et al* also did not find an association between MSI status and survival (134). Given the disparity between sample sizes, the evidence is pointing to the direction that gastric cancer patients with an MSI-H tumour are likely to have improved survival compared to patients whose tumours are MSS. Looking at MSI status and its predictive value in terms of response to 5-FU-based chemotherapy, there is yet again conflicting data; a large study by An *et al*, of 1990 patients, identified an MSI-H rate of 8.5%. The group determined that MSI status was not prognostic, as DFS between MSI-H and the MSI-L/MSS groups was not significantly different, even taking each disease stage separately. However, DFS was improved in the MSI-L/MSS group treated with 5-FU-based chemotherapy (p=0.008) (135). Oki *et al*, determined that there was no correlation between MSI status and survival following 5-FU-therapy, in their study of 240 patients, collected over a 9-year period (136). Clearly the gastric cancers with MSI form a distinct subset, and as such, are likely to be driven by slightly different signalling pathways. It still remains to be determined, how to identify and best and treat these patients.

## 11. Conclusion

Deficiencies in the DNA mismatch repair system have been identified in many unrelated cancer types. These deficiencies may be the result of either the inactivation of *MLH1*, through methylation, as seen in sporadic cancers, or through germline mutations of *MLH1* or *MSH2*, as seen in inherited cancers. Despite it being almost 50 years since the initial observations by Henry Lynch, which subsequently lead to the term 'Lynch syndrome', there are still gaps in our knowledge of the role of dMMR in cancer. Progress is being made, however, particularly in the field of colorectal cancer. We now have evidence that the prognostic role of dMMR is stage-dependent, and steps are beginning to be implemented, to ensure that every patient who may require screening actually has access to this service. In terms of identifying dMMR or MSI patients, there is now some standardisation of IHC and adoption of the use of the Bethesda marker panel, but with the recent introduction of next generation screening, the additional depth of sequence data, may complicate the situation as more VUS are identified. Furthermore, the clinical significance of low-level variants is yet to be elucidated, adding a further layer to complexity to the use of this emerging technology. Extracolonic cancers trail far behind in terms of what is known of the prognostic and predictive value of MMR, and, our understanding will remain limited unless large controlled trials are performed.

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