Association for Clinical Genetic Science
Part of the British Society for Genetic Medicine

# ACGS best practice guidelines for genetic testing and diagnosis of Lynch syndrome

Prepared and edited by Ian Frayling[1], Ian Berry[2], Andrew Wallace[3], Stewart Payne[4] and Gail Norbury[5]

[1.]All Wales Medical Genetics Service, [2.]Leeds Genetics Laboratory, [3.]Manchester Centre for Genomic Medicine, [4.]North West Thames Regional Genetics Service [5.]Guy's & St Thomas' Genetics Service.

Guidelines reviewed & updated March 2016 following a CMGS workshop held 30th November 2009

## 1. NOMENCLATURE and GENE ID

| Gene name | Symbol | Locus | OMIM gene | Reference | LRG |
|---|---|---|---|---|---|
| mutL homolog 1 | MLH1 | 3p22.3 | 120436 | CCDS2663 NM_000249.3 | LRG_216tl |
| mutS homolog 2 | MSH2 | 2p21 | 609309 | CCDS1834.1 NM_000251.3 | LRG_218tl |
| mutS homolog 6 | MSH6 | 2p16.3 (300 kb from MSH2) | 600678 | CCDS1836 NM_000179.2 | LRG_219tl |
| post meiotic segregation increased 2 | PMS2 | 7p22.1 | 600259 | CCDS5343 NM_000535.5 | LRG_161tl |
| epithelial cell adhesion molecule | EPCAM | 2p21 | 185535 | CCDS1833 NM_002354.2 | LRG_215 |

http://www.genenames.org/cgi-bin/gene_symbol_report?hgnc_id=7127

## 2. DESCRIPTION OF DISEASE

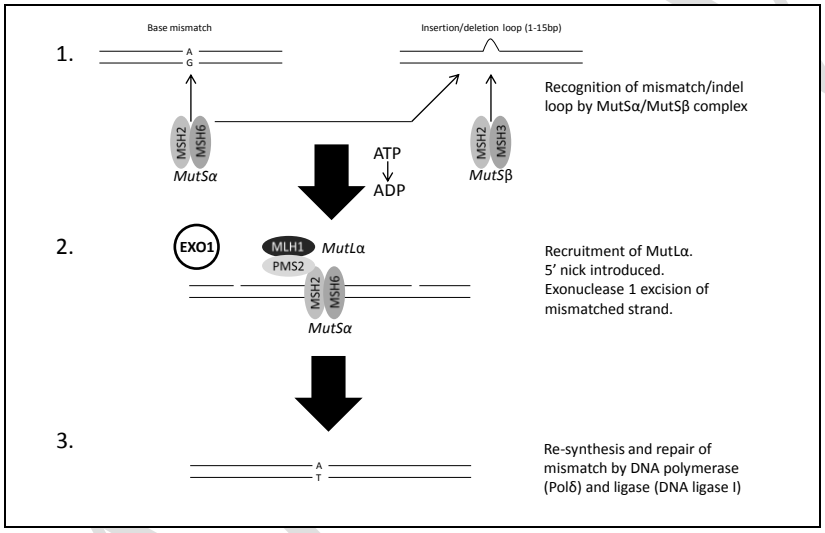
### 2.1 Lynch syndrome

Lynch syndrome (LS), previously known as hereditary non-polyposis colorectal cancer, HNPCC, is an autosomal dominant disorder with a predisposition to colorectal (CRC), and other cancers, in particular endometrial cancer (EC). See list below in section 2.3. Cases identified to date suggest a population frequency of around 1/3000, but studies of incident colorectal cancers indicate a frequency of at least 1/300, implying that about 90% of cases are so far undetected.

## 2.2 Molecular Pathology

**At the cellular level**, Lynch syndrome is caused by defects in DNA mismatch repair (MMR) proteins that act together to detect and repair errors in DNA synthesis that occur during cell division. Without repair, hundreds of random mutations occur which are somewhat more likely in simple repetitive sequences, e.g. microsatellites.

The critical repair elements most relevant to Lynch syndrome are the MutS homologue (MSH) and MutL homologue (MLH) family of proteins. The MSH proteins always work as heterodimers; MSH2 is an obligatory partner and may dimerise with MSH6 or MSH3. The MSH2-MSH6 complex is known as MutSα which preferentially repairs single base mismatch or mononucleotide repeats. The MSH2-MSH3 complex, MutSβ, preferentially recognises larger loop out errors such as at di nucleotide repeats.
MutS heterodimers signal the site for mispairing but additional proteins are required to complete the process. The MutL family consist of one obligatory partner, MLH1 that may dimerise with PMS2, PMS1 or MLH3. DNA between the mismatch and an adjacent nick is excised by exonuclease 1 (EXO1). The excised strand is re-synthesised and thus repaired by DNA polymerase β (POLB).




MMR pathway

**At a genetic level,** loss of MMR in LS is caused in almost all cases by the inheritance of a heterozygous germline mutation in MLH1, MSH2, MSH6 or PMS2, followed by secondary somatic loss of the remaining copy, in the classic Knudson "two-hit" aetiology. LS is therefore inherited in an autosomal dominant manner. Rare biallelic mutations have been reported in each of the 4 MMR genes that leads to a more severe phenotype (constitutional MMR deficiency; CMMR-D).

The multi-component nature and partial redundancy of the mismatch repair process explains in part the geno-phenotype associations. The classic Lynch phenotype being associated with defects in MLH1 & MSH2 critical components. The attenuated phenotype of MSH3/ MSH6 and PMS2/MLH3 reflecting their partially redundant functions.

In addition to functionally null mutations that abolish protein translation (frameshift, nonsense and splicing mutations), a number of missense mutations that appear to cluster within the ATP-binding, DNA-binding and dimerization domains of MLH1, MSH2, MSH6 and PMS2 have been described. Functional relevance of other domains in the MMR proteins is strongly suspected, including the MLH1/EXO1 interaction domain[1], the PCNA binding domain of MSH6[2], and the various structural domains of MSH2/MSH6 involved in the formation of the MutSα complex and 'clamping' of genomic DNA. However, only a limited number of definitively pathogenic missense mutations in these regions have been described, and many of these may affect splicing or cause catastrophic mis-folding of the mature protein.

An important hallmark of tumours with a defect in MMR is **microsatellite instability (MSI).** Repetitive stretches of mono- or dinucleotide repeats are prone to deletion, resulting in a shorter sequence. However microsatellite instability is also detected in around 10-15% of sporadic colon cancers. In this case the molecular defect is commonly loss of expression of *MLH1* as a result of promoter hypermethylation. *MSH2* promoter hypermethylation has also been described. Note that MSI is rare in rectal cancers, but when it does occur it is usually due to Lynch syndrome[3].

Causative mutations in genes other than *MLH1*, *MSH2*, *MSH6* and *PMS2* have not been conclusively proven. A possible causative role in reduced-penetrance LS has been indicated in *MLH3*, however, the tumours described in these cases are microsatellite-stable (indicating an MMR defect is unlikely), and later studies have cast doubt on the functional relevance of previously-reported *MLH3* mutations. Diagnostic genetic testing in genes other than *MLH1*, *MSH2*, *MSH6* and *PMS2* is not recommended.

## 2.3 Cancer Risk

The risks of cancer in individuals heterozygous for a pathogenic mutation have now been determined from an unbiased prospective database[4]. This reveals that mutations in the four main genes have differing penetrance and expression. As a result clinical guidelines are currently being revised.

Cumulative incidences, both sexes combined, to age 70 of any cancer are: MSH2 & MLH1 72%, MSH6 54% and PMS2 18%. In general, *MLH1* mutation carriers have a somewhat greater tendency to GI cancers, whereas *MSH2* mutations are associated with a greater variety of cancers, while *MSH6* and especially *PMS2* mutations are associated with reduced age-related penetrance. Detailed figures for a given patient, of a particular gender, for the risks of particular cancers at a given age, with a mutation in a given gene can be found at: http://www.lscarisk.org/

**Lynch-related cancers**

Colorectal cancer
Endometrial carcinoma
Small intestine carcinoma (MSH2 & MLH1)
Hepato-biliary tract and pancreas cancer (MSH2 & MLH1)
Gastric cancer (MSH2 & MLH1)

Ovarian non-serous cancer (MSH2 and MLH1)
Renal pelvis and ureter carcinoma (MSH2 & MSH6)
Bladder carcinoma (MSH2 & MSH6)
Sebaceous gland carcinoma (and adenoma – see Muir-Torre Syndrome)
Prostate cancer (MSH2)
Breast cancer (MLH1)
Brain cancer

## 2.4 Allelic disorders

### 2.4.1 Muir-Torre syndrome

Muir-Torre syndrome (MTS) is the co-occurrence of a sebaceous skin tumour (either adenoma or carcinoma) with any kind of internal cancer. A large proportion, but not all, of MTS is due to LS. The frequency of an MSI-high phenotype in unselected sebaceous syndrome tumours is approximately 60%[5], however, the vast majority of sebaceous tumours in Muir-Torre patients display an MSI-high phenotype and IHC loss of MMR proteins[6]. Consequently IHC and MSI analysis of sebaceous tumours are recognised as standard investigations in the discrimination of Lynch syndrome. MSI is however significantly less common in sebaceous hyperplasia and is consequently not reliable as a basis for selection of patients at high risk of Lynch syndrome[5].

*BRAF* mutations are rare in sebaceous neoplasms and there are insufficient data to determine whether presence of a *BRAF* mutation is more likely in sporadic lesions; consequently there is no evidence for utility of *BRAF* mutation screening in the identification of sebaceous tumours from Lynch syndrome patients.

### 2.4.3 Endometrial tumours

Approximately 2.5% of all newly diagnosed endometrial cancer patients have Lynch syndrome[7]. Endometrial cancers in Lynch syndrome patients on average are diagnosed 12 years earlier at age 48 than the mean of age 60 for all patients[8]. Of all endometrial carcinomas between 20-30% show MSI, indicating a defect in the mismatch repair pathway[9], a rate higher than most other tumours. *MLH1* promoter hypermethylation is present in approximately 75% of endometrial tumours with MSI[10]; this is closely aligned to the proportion of endometrial tumours with MLH1 loss on IHC, and indicates a high probability that the tumour is sporadic in origin. A 2007 study of 100 women under the age of 50 with a diagnosis of endometrial cancer showed that overall 9% had deleterious mutations detectable by molecular techniques[11]. MSI testing carried out on 95 of the 100 samples identified 25 MSI-high tumours and close to one third (8/25) of these were in patients with a pathogenic *MLH1*, *MSH2* or *MSH6* mutation. Consequently MSI-high status significantly enriches endometrial cancer patients for those at risk of having Lynch syndrome. In the same study the effectiveness of MLH1, MSH2 or MSH6 loss on IHC in identifying Lynch syndrome patients was measured. Of the endometrial cancer patients with IHC loss 38% had Lynch syndrome. Loss of MSH2 had the highest predictive value, with 64% of patients having Lynch syndrome. A high proportion of the patients with MLH1 loss on IHC 12/13 (92%) had *MLH1* promoter hypermethylation. In none of these patients was a clearly pathogenic germline Lynch syndrome mutation identified, indicating that the tumour was highly likely to be sporadic. The remaining

patient with *MLH1* promoter hypermethylation had a pathogenic *MLH1* mutation. Consequently, coupling *MLH1* promoter hypermethylation testing to MMR IHC is the most effective way of identifying endometrial cancer patients at high risk of Lynch syndrome.
*BRAF* mutations are uncommon in sporadic endometrial cancers, so *BRAF* testing is not useful in discriminating between sporadic and Lynch syndrome-associated endometrial tumours[12].

### 2.4.4 Constitutional mismatch repair disorder: CMMR (Biallelic mismatch repair mutations, Turcot's syndrome)

Rare cases have been reported of biallelic mutations in *MLH1*, *MSH2*, *MSH6* and *PMS2*. Affected children typically have multiple colorectal adenomas before age 20. Haematologic cancer, brain tumours and café-au-lait macules also occur, as well as a wide variety of other features, including immune deficiency[13]. The disorder appears to overlap with Turcot's syndrome, characterised by the co-occurrence of colorectal adenomatous polyposis and primary brain cancers. The molecular basis is either a mutation in *APC* or, possibly, one of the MMR genes associated with Lynch syndrome. A majority of cases is due to autosomal recessive inheritance of two mutations in the same mismatch repair gene, i.e. constitutional mismatch repair disorder (CMMR-D). The pathology of the CNS tumour can help distinguish the underlying cause; *APC* mutations are more commonly associated with medulloblastoma; mismatch repair mutations are more commonly associated with glioblastomas and primitive neuroectodermal tumours (PNETs), and such brain tumours exhibit microsatellite instability. In addition, constitutional loss of a particular MMR protein is evident in all cells, both normal and malignant.

## 2.5 Differential Diagnosis

### 2.5.1 Attenuated familial adenomatous polyposis (AFAP)

Polyps and colon cancers associated with AFAP do not usually exhibit MSI.

### 2.5.2 Serrated (SPS) and mixed polyposis

May be due to mutations in *MUTYH*, *PTEN*, *SMAD4*, *BMPR1A*, *STK11* and possibly also *ENG*. Inheritance for MUTYH-associated polyposis (MAP) is autosomal recessive.
Juvenile polyposis syndrome caused by mutations in *SMAD4* and *BMPR1A*.
Peutz-Jeghers syndrome caused by mutations in *STK11*.
PTEN hamartomatous syndromes caused by mutations in *PTEN*.
FAP3 (NTHL1-associated polyposis).

### 2.5.3 Polymerase proof reading polyposis (PPAP)

This is a rare autosomal dominant disorder due to germline mutations in the exonuclease domain of *POLE* (encoding DNA polymerase ε) or *POLD1* (encoding DNA polymerase δ1). Two highly penetrant mutations have been described, POLE p.Leu424Val and POLD1 p.Ser478Asn). Tumours are microsatellite stable. *POLE1* mutations are associated with colonic oligopolyposis (generally 50-70 adenomas) as early as age 20 years, colorectal cancer and duodenal adenomas and carcinomas.

*POLD1* mutation carriers also exhibit increased risk for endometrial cancer and brain tumours.

### 2.5.6 Familial Colorectal cancer including FCCTX

Tumours are microsatellite stable and there is no increased risk for the extra-colonic cancers associated with Lynch syndrome. Familial colorectal type X (FCCTX) is the designation for patients with a family history meeting the Amsterdam Criteria for Lynch syndrome but whose tumours lack microsatellite instability and whose germline DNA lacks MMR mutations. These families are at around 2-fold increased risk for CRC over the general population compared to 6-fold for Lynch patients. FCCTX appears to be a genetically heterogeneous condition.

### 2.5.7 Hereditary diffuse gastric cancer

Gastric cancers caused by mutations in *CDH1* are typically adenocarcinomas.

### 2.5.8 BRCA1/BRCA2 familial breast and ovarian cancer

CRC is common and thus may be seen in other familial cancer syndromes such as hereditary breast and ovarian cancer. The spectrum of cancers seen in BRCA1/2 includes pancreatic and prostate cancer and there is scope for potential phenotype overlap with Lynch, particularly for ovarian cancers. It has been reported that the ovarian tumour subtype in Lynch is of the non-serous subtype[14]. The following table provides a guide as to which types of ovarian cancers may be associated with Lynch syndrome.

| Type of ovarian cancer | BRCA | Lynch |
|---|---|---|
| Mucinous | No | No – unless mixed histology with endometrioid/clear cell/undifferentiated |
| Serous | Yes | No |
| Clear cell | Yes | Yes |
| Endometrioid | Yes | Yes |
| Mixed | Yes | Yes |
| Undifferentiated | Yes | Yes |
| Carcinosarcoma | Yes | No |


| Type of ovarian cancer | BRCA | Lynch |
|---|---|---|
| Mucinous | No | No – unless mixed histology with endometrioid/clear cell/undifferentiated |
| Serous | Yes | No – unless Amsterdam +ve family history |
| Clear cell | Yes | Yes |
| Endometrioid | Yes | Yes |
| Mixed | Yes | Yes |
| Undifferentiated | Yes | Yes |
| Carcinosarcoma | Yes | No – unless Amsterdam +ve family history |

## 3. COMMON REFERRAL CATEGORIES FOR LYNCH SYNDROME

Colorectal cancer is common and multifactorial. There were 33,676 new cases of CRC diagnosed in England in 2013, 18,778 males and 14,898 females. One patient group estimates that there are approximately 160,000 people with Lynch syndrome in England and only 5000 diagnosed (NICE). Clinical selection criteria are used to target genetic testing to those cases most likely to be inherited. Genetic testing is then used to further refine this likelihood, permit predictive testing in at-risk relatives, inform surveillance recommendations in the family and aid surgical and chemopreventation management. The current main referral pathways are through:

- Clinical Genetics due to a cancer family history
- Pathology/Oncology due to the age of occurrence and histopathological features.

At present there is no NICE guidance on the population to be tested or the testing strategy for Lynch syndrome. However, an NIHR Health Technology Assessment has found that systematic testing of colorectal cancers to detect Lynch syndrome would be cost-effective for the NHS[15]. As a result the Royal College of Pathologists Minimum Dataset for Colorectal Cancer was revised in 2014, and now requires all colorectal cancers occurring up to age 50, plus those colorectal cancers determined by pathologists or MDTs as likely due to LS (e.g. on the basis of other cancers and/or pathology), to be tested for MMR deficiency[16]. The RCPath minimum dataset also specifies that while testing of all colorectal cancers is health-economically justified up to age 70, such testing will be optional, until such time as local or national commissioning is established.
The predictive value of MSI in indicating LS is highly age-dependent. About one in four colon cancers at age 35 are due to LS and most will have MSI. The probability that a colon cancer with MSI is due to LS is approximately 90% at age 35-40, reducing to ~20-25% by age 70. Conversely, the probability of a colon cancer having MSI due to sporadic cause (i.e. *MLH1* methylation promoter) rises from 1-3% at age 55 to ~11% by age 70. Hence, the probability that a colon cancer with MSI is due to LS depends on the age at which it occurs:

| Age (y) | P(MSI) | P(LS) | P(Sporadic MSI) | P(MSI tumour is due to LS)* |
|---|---|---|---|---|
| 35 | 23% | 22% | 2% | 92% |
| 40 | 16% | 14% | 2% | 90% |
| 45 | 10% | 9% | 2% | 87% |
| 50 | 7% | 6% | 2% | 79% |
| 55 | 6% | 4% | 2% | 60% |
| 60 | 10% | 3% | 7% | 28% |
| 65 | 11% | 3% | 8% | 32% |
| 70 | 14% | 3% | 11% | 23% |

*Assuming all LS tumours have MSI.

And from this the proportion and probability of LS cases by age is[17]:

| Age (y) | P(MSI) | P(LS) | P(Sporadic MSI) | P(MSI tumour is due to LS)* | Proportion of cases of LS | Proportion of all cases of CRC by age |
|---|---|---|---|---|---|---|

| | | | | | | |
|---|---|---|---|---|---|---|
| <50 | 11% | 10% | 0.7% | 90% | 30% | 13% |
| 51~60 | 8% | 4% | 4% | 50% | 30% | 34% |
| >60 | 12% | 3% | 9% | 25% | 40% | 53% |

## 3.1 Selection criteria used by Clinical Genetics

Note stringency may vary according to whether the selection is for eligibility for mismatch repair testing or cancer surveillance.
The Amsterdam criteria were principally developed to identify Lynch syndrome for research studies. The Bethesda guidelines were developed to identify patients with CRC who should be tested for Lynch syndrome.

### 3.1.1 Amsterdam (Amsterdam II/revised Amsterdam) Criteria

3 or more blood relatives with a Lynch-related cancer (CRC, EC, small intestine, ureter or renal pelvis)
and two or more successive generations affected
and one relative must be a first-degree relative of the other two
and at least one cancer diagnosed <50 y
and FAP excluded in colorectal case(s)
and tumours pathologically verified.

### 3.1.2 Revised Bethesda Guidelines (2004)

These were devised to be more practical than the Amsterdam type criteria. A comparison[18] found them to be more sensitive but less specific.
The Revised Bethesda Guidelines are as follows:
At least one CRC <50
or Synchronous or metachronous CRC or other Lynch-related tumours, any age
or MSI CRC <60
or CRC in one or more first degree relative with a Lynch-related tumour, one to be diagnosed <50
or CRC in two or more first or second-degree relatives with Lynch-related tumours, any age.

Some services will consider testing unaffected individuals where there is an equivalent prior risk of detecting a germline mutation as for familial breast cancer. Examples of such criteria are where there are three or more first- or second-degree relatives with CRC or Lynch-related tumour, all less than 70 years.

### 3.2.3 Polyps

Adenomatous

- 10 or more adenomas <60 (cumulative)
- 20 or more adenomas <70 (cumulative)

Serrated

- 20 or more serrated regardless of location but at least 3 proximal to the sigmoid
- Five or more histologically confirmed serrated polyps proximal to the sigmoid, two of which are at least 1 cm
- Peutz Jegher-type polyp/hamartoma/juvenile polyps
- Two or more histologically confirmed

### 3.2 Selection criteria used by Pathology

CRC <50
Or CRC stage II + III <60,
Or Multiple CRC,
Or Pathological features suggestive for Lynch and <60
Or EC <50
Or Sebaceous adenoma <70 (may be used by Dermatology).

## 4. GENETIC TESTING

Genetic testing may be undertaken on both tumour and / or constitutional DNA.
Various algorithms have been proposed, determined by prior risk, referral pathway, cost effectiveness and sample availability.

### 4.1 Tumour (Biomarker) analysis

Immunohistochemical or DNA analysis may be undertaken. The overall purpose is as a pre-screen for a likely inherited mismatch repair deficiency. There is also reported utility in determining tumour MMR status to guide decision-making for chemotherapy.

#### *4.1.1 Samples*

These are generally formalin-fixed, paraffin-embedded tissue samples that are required to determine the morphology and tumour content as part of the histology analysis. The quantity and quality of DNA that may be extracted from such blocks is variable due to the considerable variation in the fixation process that can itself introduce mutation artefacts that may mimic germline mutations. The Royal College of Pathologists have issued guidance on sample handling by laboratories performing molecular pathology for cancer patients[19].

Adenomatous polyps are pre-cancerous lesions and although they can be used to generate useful data where colorectal tumour material is not available, the results are less reliable.
In a study of 109 polyps from patients with genetically confirmed Lynch syndrome, 78/109 (72%) showed MMR loss on IHC. In all cases the expected pattern of IHC loss was observed[20]. In the same study, polyps with high grade dysplasia showed much better concordance with the expected MMR loss on IHC with 12/12 (100%) high grade adenomas showing expected IHC MMR loss compared to 60/79 (76%) low grade adenomas. Similarly villous adenomas from Lynch syndrome patients have a higher rate of the expected MMR loss. Caution should be exercised when analysing adenomatous polyps and where choice is available high grade adenomatous polyps and/or polyps with a villous component should be used.
A study of MSI rates in adenomatous polyps from Lynch syndrome patients showed MSI-high status was only present in 38% of adenomas and 4% of hyperplastic polyps compared with 80-85% of colorectal cancers[21]. Consequently MSI analysis in pre-cancerous polyps is significantly less reliable than that in neoplastic tissue and should be interpreted with caution.

Note that MSI is rare in rectal cancers, but when it does occur it is usually due to Lynch syndrome[3].

Determination of the relative sensitivity and specificity of MSI vs IHC testing to detect LS is complicated by the lack of systematic mutation testing of cases tested, and the variety of techniques, especially IHC. However, a systematic review[15] found:

| | Sensitivity | Specificity |
|---|---|---|
| MSI | 88 – 100% | 68 – 84% |
| IHC | 73 – 100% | 78 – 100% |

- which supports the general statement that MSI is somewhat more sensitive than IHC, but slightly less specific.

There is increasing interest in a move to collection of fresh frozen (vacuum packed) samples to avoid the use of formalin and improve DNA quality.
The identifiers on paraffin blocks are usually limited to reference number and it is important to confirm that the appropriate block has been obtained. The block should be cross-referenced with the histopathology report.

For DNA analysis, it is important that cross-contamination be avoided during preparation of the blocks. A minimum of 30% neoplastic/hyperplastic/dysplastic cell content is recommended (Association of Clinical Genetics Netherlands, 2015). It is recommended that tumours be macro-dissected where feasible, to enrich for neoplastic/hyperplastic/dysplastic cell content.

### *4.1.2 Microsatellite instability analysis*

A consensus panel of mononucleotide markers has been recommended for the determination of microsatellite status, this being BAT25, BAT26, DS123, D17S250 and D5S346. Many laboratories use a commercially available kit that consists of five mononucleotide, quasi-monomorphic markers (BAT-25, BAT-26, NR-21, NR-24, MONO-2). This simplifies interpretation, as most individuals are homozygous for the same common allele for a given marker. In addition the kit includes two pentanucleotide repeat markers (Penta C and Penta D), which are used to detect potential sample mix-ups or contamination.
Stability for BAT26 has been reported in Lynch tumours with large MSH2 deletions. It has also been shown that BAT25 and BAT26 are polymorphic in around 28% of African Americans.
The use of a matched normal control (blood or fixed normal tissue) may aid distinction of potential heterozygosity/instability for some markers and confirmation of sample identity. Some MSH6 mutations have been reported to be microsatellite stable.
Microsatellite instability analyses have generally been validated on colorectal tissue. The Association of Clinical Genetics, Netherlands recommends use of IHC for tumours other than colorectal or endometrial cancer.

Tumours with two or more altered mononucleotide markers should be reported as showing microsatellite instability / high-level microsatellite instability (MSI-H). Tumours which show no altered markers should be reported as showing

microsatellite stability (MSS). In the case where only one of five mononucleotide makers shows instability (not heterozygosity), whilst this is not considered sufficient to be classed as instability associated with Lynch syndrome, it may be of significance and warrant further investigation. This may include testing other tumour types, samples of higher neoplastic/hyperplastic/dysplastic cell content and additional immunohistochemistry.

*MSI testing for adjuvant chemotherapy*
Cytotoxicity of 5-FU is attributed to inhibition of thymidylate synthase and to mis-incorporation of fluoronucleotides into DNA and RNA. Thus a MMR defect is expected to limit its efficacy. Whilst the method of action of 5-FU is not fully understood, it has been reported that patients with stage II colorectal cancer that show microsatellite instability (MSI) have a modestly better prognosis, and this may influence decisions regarding chemotherapy. The European Society for Medical Oncology guidelines suggest that MSI should be evaluated in stage II colorectal cancer patients in order to contribute to treatment decision-making regarding chemotherapy administration[22].

### *4.1.3 Immunohistochemistry (IHC)*
This is a routine technique in histopathology laboratories that can be undertaken rapidly within 24 hours and on any tumour sample. It is recommended that analysis is performed for all four mismatch repair proteins (MLH1, MSH2, MSH6 and PMS2) using monoclonal antibodies. Concerns around IHC testing generally relate to subjectivity of reporting and sensitivity. External quality assessment schemes exist: UK NEQAS ICC and NordiQC. UK NEQAS ICC has issued best practice guidance for MMR IHC[23]. Around 5% of microsatellite unstable tumours have IHC that is not abnormal. Some mutations may not lead to a loss of expression because they do not affect the antigenicity. A mutation in one mismatch repair gene may also lead to loss of additional mismatch repair gene proteins because they exist as dimers.

MLH1 mutation is generally associated with loss of MLH1 and PMS2
MSH2 mutation is generally associated with loss of MSH2 and MSH6
MSH6 mutation is associated with isolated MSH6 loss
PMS2 mutation is associated with isolated PMS2 loss

In addition, at least two mutations have been described which cause loss of expression not of that MMR protein but of another protein because they affect a dimerisation site. Very usefully, data have recently been presented on the underlying causes associated with patterns of MMR IHC in colorectal and endometrial tumours, with MSI found in the setting of families in genetics clinics[24]. This also shows that some mutations are not associated with loss or abnormality of the corresponding protein[25]. Hence, patterns of MMR IHC abnormality are a guide to, but not an absolute indicator of the underlying genetic defect[26].

**Underlying causes of microsatellite instability in colorectal and endometrial cancers in genetics clinic patients, by associated pattern of MMR IHC abnormality.**

| | IHC abnormality | | | | Overall |
|---|---|---|---|---|---|
| | MLH1 (alone, or in combination with PMS2) | MSH2 (alone, or in combination with MSH6) | MSH6 (alone) | PMS2 (alone) | |
| Constitutional *MLH1* mutation | 11.8% | | | 2.0% | 14% |
| Constitutional *MLH1* methylation | 0.4% | | | | 0.4% |
| Constitutional *MSH2* mutation | | 14.2% | 0.4% | | 15% |
| Constitutional *EPCAM* mutation | | 2.0% | | | 2.0% |
| Constitutional *MSH6* mutation | | 0.8% | 10.2% | | 11% |
| Constitutional *PMS2* mutation | | | | 5.9% | 5.9% |
| Acquired *MLH1* methylation | 24.0% | | | | 24% |
| Acquired *MLH1* mutation | 6.7% | | | | 6.7% |
| Acquired *MSH2* mutation | | 2.4% | | | 2.4% |
| Unexplained | 10.2% | 5.9% | 1.6% | 1.6% | 19% |
| Total | 53% | 25% | 12% | 9% | 100% |

(reference [24])

With increasing use of gene panels for DNA analysis there may be less utility associated with pre-screening by IHC to target constitutional mutation analysis, although the IHC may have been done in advance and may guide interpretation of the constitutional analysis required in order to provide a molecular diagnosis of Lynch syndrome and predictive testing for family members.

### *4.1.4 Methylation*

MLH1

Around 15% of sporadic colorectal cancers exhibit microsatellite instability. The molecular basis of this is most commonly hypermethylation of the *MLH1* promoter region, leading to transcriptional inactivation and loss of MLH1 expression. Such tumours commonly acquire the recurrent *BRAF* p.Val600Glu mutation, although the mechanistic relationship between this oncogenic marker and *MLH1* promoter hypermethylation is as yet unclear.

The *MLH1* promoter contains numerous CpG dinucleotide motifs that are potential substrates for conversion by DNA methyltransferases to 5-methyl-CpG.
Studies have shown that of the 5’ promoter sub-regions, designated 'A' (most distal) to 'D' (most proximal), the region designated 'C' (containing 16 CpG sites between

nucleotides -401 to -198 relative to the ATG methionine translational start codon) is most strongly associated with *MLH1* expression. Methylation of CpG sites at the distal A and B regions (encompassing nucleotides -754 to -392) was not associated with *MLH1* silencing in the absence of methylation in the more proximal C region[27].

Numerous assays to directly test for CpG methylation have been described. Many entail pre-treatment of test DNA with sodium bisulphite, which specifically converts unmethylated cytosine to uracil whilst leaving methylated cytosine unmodified. Resulting modified (unmethylated) template can be distinguished from unmodified (methylated) template by a number of established techniques including methylation-specific PCR, sequencing or pyrosequencing and Combined Bisulphite Restriction Analysis (COBRA). Other assays exploit the fact that certain restriction enzymes (e.g. *Hha*I) are specific to sites containing unmethylated CpG dinucleotides but will not digest at 5-methyl-CpG sites, an example being methylation-specific MLPA (MS-MLPA). Direct methylation assays have the potential disadvantage of requiring robust optimisation in the case of bisulphite-based tests or being relatively resource-intensive in the case of MS-MLPA.

Constitutional (germline) *MLH1* promoter methylation has been reported as the underlying cause of a Lynch phenotype in a small number of cases. For this reason, it is recommended that a matched constitutive sample (blood or normal tissue) be analysed either in parallel or following identification of promoter methylation in the tumour. The precise mechanism for this is yet to be fully established and several patterns have been reported including a genomic rearrangement in the adjacent region. Methylation may be reversed during gametogenesis, such that it represents an epimutation that is not transmissible from one generation to the next, or may be fixed and associated with a sequence alteration that is inherited in a Mendelian fashion. Additionally, constitutional methylation may vary significantly in a tissue-specific manner, such that methylation levels in leucocyte DNA may not reflect accurately methylation levels in colorectal mucosal cells. For these reasons, detection of constitutional *MLH1* promoter methylation may be useful to identify the cause of disease in an index case but should not be used as the basis for predictive testing for at-risk relatives without further investigation. Constitutional *MLH1* promoter hypermethylation is not associated with *BRAF* p.Val600Glu mutation. This means that *BRAF* mutation status will not detect cases with constitutional *MLH1* epimutation.

Analysis of MLH1 promoter methylation is recommended over tumour testing of BRAF activating mutations (e.g. BRAF p.Val600Glu) as such mutations are detected in Lynch syndrome mutation carriers (i.e. the predictive value of BRAF testing is lower). Combined testing of *MLH1* promoter and BRAF p.Val600Glu alongside consideration of tumour content may improve reliability, though at additional cost.

MSH2

Somatic methylation of the *MSH2* promoter has rarely been reported and anecdotally has not reliably been seen in a large number of tumours tested in a clinical diagnostic setting.

Constitutional *MSH2* methylation is a relatively rare but recognised event that is associated with deletion of upstream sequences including the 3' region of the *EPCAM* gene. Deletions may extend into the *MSH2* promoter or further into 5' regions of the *MSH2* coding and intronic sequence. Such deletions result in loss of the *EPCAM* termination codon and 3'UTR and cause transcriptional read-though from *EPCAM* into *MSH2*. Deletions that extend into *MSH2* are assumed to be pathogenic by virtue of loss of the *MSH2* methionine start codon and downstream coding sequence. Smaller deletions cause methylation of the *MSH2* promoter and transcriptional silencing. MLPA kits that include probes to *EPCAM* 3' exons and UTR/*MSH2* promoter are commercially available. Deletions that do not extend into *MSH2* coding sequence can be assumed to be pathogenic by causing *MSH2* promoter methylation, and this can be confirmed by MS-MLPA.

Deletions that extend from *EPCAM* into *MSH2* behave phenotypically as *MSH2* mutations, whereas deletions of the 3' end of *EPCAM* which indirectly silence *MSH2* via methylation are associated with a cancer phenotype restricted to the GI tract, i.e. untypical of *MSH2* mutations. So, it is important to distinguish this when reporting.

### 4.1.5 Somatic mismatch repair gene analysis

Biallelic somatic mutations in mismatch repair genes may account for a considerable number of tumours with microsatellite instability of no known aetiology. Commercial assays using next generation sequence analysis[28] are now available to test both single and a panel of genes associated with Lynch-related and associated disorders. MSI and the exon 1-7 *MSH2* inversion may be captured in the same test. One recent study identified biallelic mutations in *MLH1* and *MSH2* in 13 out of 25 such tumours[24] and another study analysing *MLH1*, *MSH2*, *MSH6*, *PMS2*, *EPCAM*, *POLE* and *POLD1* found somatic mutations in 23 of 32 patients with MMR deficiency but no germline mutation[29]. Further information is needed on likely false positives, somatic mosaicism and phase of mutations. However in the near future, tumour analysis for mutations in MMR genes may prove cost-effective in helping to distinguish sporadic and familial cases of patients with colon or endometrial cancer with MMR deficiency but no germline mutation and thus guiding surveillance.

## 4.2 Constitutional Testing

Mutation spectrum

| Gene | Relative freq of mutations | Coding exons | Different mutations (HGMD) | Large deletions | Coding exons |
|---|---|---|---|---|---|
| *MLH1* | 40% | 19 | *1015* | 5-10% | 19 |
| *MSH2* | 40% | 16 | *1196* | 17-50% | 16 |
| *MSH6* | 13% | 10 | *546* | Rare | 10 |
| *PMS2* | <5% | 15 | *249* | Rare | 15 |
| *EPCAM* | 1-3% | 15 | 60 | ~25% | 15 |

Over 3000 different mutations have been identified (The Human Gene Mutation Database).
There are many recurring mutations. For example, c.350T>C p.Thr117Met (*MLH1*, exon 4) and c.942+3A>T (*MSH2* exon 5). A deletion of exon 16 in *MLH1* is one of three founder mutations in Finland and other founder mutations are found elsewhere, e.g. Portugal, Poland, and Newfoundland. The c.1906G>C p.Ala636Pro mutation in *MSH2* is present in around 0.6% of the Ashkenazi Jewish population with CRC.

### 4.2.1 Constitutional mutation screening

Generally a gene panel approach is used for sequence and dosage analysis of *MLH1*, *MSH2*, *MSH6* and *PMS2*. *PMS2* is challenging because of the number of highly homologous pseudogenes.
Sequence analysis may be by Sanger or Next Generation Analysis (NGS) using either hybridisation- or amplification-based target capture and enrichment methods. Some regions may not be amenable to NGS analysis and require a composite approach. Sequence and bioinformatics analysis should be performed in accordance with the ACGS best practice guidelines (referenced below).

Large rearrangements and dosage analysis may be performed by different methods including NGS copy number, MLPA, long-range PCR or targeted array comparative genome hybridisation (aCGH)
Primer sequences should be checked for polymorphisms that may cause allele drop-out and interfere with interpretation.

The mutation nomenclature used should be in accordance with the Genbank accession number quoted in section 1 and HGVS nomenclature guidelines should be followed, with codon one coinciding with nucleotide one.

Interpretation of mutations should be in accordance with ACGS best practice guidelines for the evaluation of pathogenicity and the reporting of sequence variants in Clinical Molecular Genetics.

The International Society for Gastrointestinal Hereditary Tumours (InSiGHT) maintains a helpful resource on variants and the InSiGHT LOVD, as reflected on ClinVar, Ensembl and Decipher, is now recognised by the HVP/GA4GH as the sole global repository for MMR gene mutations and their interpretation. [http://chromium.lovd.nl/LOVD2/colon_cancer/home.php]. For reasons of quality and to minimise disparity in interpretation between centres this should be regarded as the primary source of interpretations of MMR gene mutations.

The current criteria used for the interpretation of MMR mutations[30] are given on the InSiGHT website [http://insight-group.org/criteria/]. The evidence supporting each classification is given on the LOVD listings and can include segregation and tumour analyses (incorporated in a multifactorial Bayesian model), as well as mRNA and other analyses. The prior probability of pathogenicity of all possible MMR gene missense mutations has been calculated using a customised version of PolyPhen v2.1 in conjunction with MAFF (a LS-specific in vitro model)[31]. Using conditional probabilities of pathogenicity from e.g. tumour tests, posterior probabilities of pathogenicity can be established.

In the case of variants not listed on the LOVD, or not classified, or where extra evidence is sought to establish a classification, they should be submitted to the InSiGHT LOVD curator for inclusion on the database. The InSiGHT Variant Interpretation Committee will then be happy to give a classification, or the reasons it is unable to do this. Those submitting as yet unlisted mutations, or wishing to follow an unclassified mutation, can choose to be notified of a classification or change of classification via the online 'Follow Variant' facility.

The value of such a resource is dependent on the submission of all occurrences of all and any variants and mutations, whether suspected pathogenic, non-pathogenic, or unclassified, and so this is to be encouraged. It may not be possible for one laboratory to establish pathogenicity, but often with the combined efforts of more than one laboratory this is possible.

### 4.2.2 Targeted constitutional testing

4.4.2.1 3' *EPCAM* deletion leading to *MSH2* promoter hypermethylation

*MSH2* can be inactivated by an upstream deletion of the 3' *EPCAM* gene including its polyadenylation signal. This causes read-through of *EPCAM* and hypermethylation of the *MSH2* promoter 15kb downstream. As 3' *EPCAM* deletions are heritable, so too is the *MSH2* promoter methylation epimutation. The risk of colorectal cancer in carriers of such *EPCAM* deletions is comparable to that of *MSH2* mutation carriers.

*EPCAM* deletions may be detected by MLPA for the Lynch gene (SALSA MLPA P003). No further confirmation of methylation status is deemed necessary.

4.2.2.2 *MSH2* inversion

Inversions of exons 1-7 of *MSH2* has now been reported as a frequent cause of unexplained Lynch syndrome. This may be detected by allelic drop out on long range PCR[32].

The most recent MLPA kit now includes probes for the 10 Mb inversion of *MSH2* exons 1-7, which is prevalent in the UK population[33]. Breakpoint PCR is an acceptable alternative method for detecting this mutation. However, while the inversion is detectable by conventional cytogenetic analysis, the sensitivity is approximately 50% when the analyst is unaware of the possibility, and aCGH is generally unable to detect the inversion.

4.2.2.3 Predictive testing

Once a pathogenic mutation has been identified in a family member, predictive testing may be offered to at-risk relatives.

4.2.2.4 Linked marker testing

This is not considered appropriate for diagnostic use because of genetic heterogeneity and variable penetrance.

### 4.3 Tumour samples

*Essential*

- The report should state the tissue type, collection date and tumour content if the sample has not been macro-dissected.
- Colorectal adenocarcinoma tissue is considered to be the preferable tissue to test if multiple neoplasms are available; sensitivity of MSI and IHC has been shown to be sub-optimal in extra-colonic samples.

*Guidance*

- Analysis of pre-cancerous polyps is significantly less reliable than that of neoplastic tissue.
- Consideration should also be given to potential fixation artefacts and selection of the most appropriate tumour type and age to test.
- Low neoplastic/hyperplastic/dysplastic cell content may result in a false negative. Association of Clinical Genetics, Netherlands recommend a neoplastic cell content of at least 30%.
- If the neoplastic cell content is not provided or a suitable normal control sample is not available, then this should be noted on the report.

### 5.2 Microsatellite analysis

*Essential*

- Analysis should include a minimum of 5 markers of which at least 3 should be mononucleotide repeat.
- MSI analysis has mainly been validated for use in CRC and EC. Where two tumour types are available from a family/individual, CRC should therefore be used is used in preference
- Tumours with more than one of five mononucleotide markers should be reported as having microsatellite instability (MSI / MSI-H).
- Tumours with none of five mononucleotide markers should be reported as having microsatellite stability (MSS).
- Tumours with one of five mononucleotide markers showing instability (not heterozygosity) should be reported as insufficient to be classed as instability associated with Lynch syndrome. Further testing may be indicated depending on the context (family history, tumour type, sample status).

*Guidance*

- MSI may be used as a pre-screen for Lynch syndrome. Selection over IHC depends on a number of factors including sensitivity, sample size, cost and reporting time.
- MSI is generally considered more sensitive but less specific than IHC.
- MSI status may be used to inform decision around adjunct chemotherapy in stage II CRC.
- Matched constitutive (normal) control is helpful to detect marker heterozygosity and sample identity

### 5.3 Immunohistochemistry

*Essential*

- Analysis should follow guidance that has been issued by the UK NEQAS ICC[23].

*Guidance*

- IHC may be used as a pre-screen for Lynch syndrome.
- Preference over MSI depends on a number of factors including specimen size, specificity, costs and reporting time.
- IHC may have a specific role in testing of tumours other than CRC and EC and in the interpretation of VUCS.

### 5.4 Methylation analysis

#### 5.4.1 MLH1

*Essential*

- BRAF p.Val600Glu alone is not recommended as a surrogate for *MLH1* promoter methylation due both to false positives in Lynch mutation carriers and to false negatives in constitutional *MLH1* epimutation.
- *MLH1* promoter methylation analysis should include sub-region C of the *MLH1* promoter.
- Constitutional *MLH1* epimutations are usually not inherited, and predictive testing should not be offered without further investigation. In those cases in which inheritance is seen, this can be due to large-scale rearrangements involving *MLH1* and nearby genes e.g. *LRRFIP2*. The rearrangements are the primary defect of *MLH1* and the methylation is thus a secondary effect which can be used as a surrogate marker of the underlying mutation (not unlike the situation of *EPCAM* deletions causing methylation of *MSH2*)[34].

*Guidance*

- *MLH1* promoter methylation analysis may be used to help distinguish common sporadic tumours from Lynch syndrome-related cancers.

#### 5.4.2 MSH2

*Essential*

- *MSH2* promoter methylation is associated with 3' *EPCAM* deletions, which are generally assumed to be pathogenic and may be used as the basis for predictive testing in at-risk relatives.

### 5.5 Somatic gene analysis

*Guidance*

The utility of this in term of assay performance and clinical value has yet to be demonstrated.

### 5.6 Constitutional gene analysis

*Essential*

- Full coding sequence and dosage analysis is recommended, including for *MSH2* the ex1-7 inversion.

- Diagnostic genetic testing in genes other than *MLH1*, *MSH2*, *MSH6* and *PMS2* is not currently recommended. There is no evidence that constitutional mutations in other MMR genes are associated with LS or Lynch-like syndrome or FCCX. In particular, *PMS1* c.1376_1377insA, p.(Ile459fs) is not pathogenic.

### 5.6.1 Reports where no mutation detected

*Essential*

- Reports should state which genes were tested, the extent of the analysis, including coverage for next generation sequencing, and the sensitivity of these methods.
- If a variant of uncertain clinical significance is detected it is helpful to indicate what further evidence or information would allow further evaluation. This may include IHC, MSI and further family history.

### 5.6.2 Reports where pathogenic mutation detected

*Essential*

- Reports must comply with current relevant ACGS guidelines on reporting and targeted next generation sequencing where this technology has been used [http://www.acgs.uk.com/committees/quality-committee/best-practice-guidelines/].This includes stating which genes were tested, the extent of the analysis, including coverage for next generation sequencing, and the sensitivity of these methods.
- Details of novel mutations should be submitted to the InSiGHT database.
- The report wording should state that the result confirms a diagnosis of Lynch syndrome and that predictive testing may be offered to at-risk family members.

### 5.6.3 Predictive test – family mutation not detected

*Essential*

Although the high family (prior) risk is removed, there is still a risk of sporadic disease. Therefore an appropriate phrase is that the risk of Lynch-related tumours is reduced to that of the general population.

### 5.6.4 Linked marker testing

Essential

- This is not appropriate.

## 6. REFERENCES


1 Andersen, S. D., Liberti, S. E., Lutzen, A., Drost, M., Bernstein, I., Nilbert, M., Dominguez, M., Nystrom, M., Hansen, T. V., Christoffersen, J. W., Jager, A. C., de Wind, N., Nielsen, F. C., Torring, P. M. & Rasmussen, L. J. Functional characterization of MLH1 missense variants identified in Lynch syndrome patients. *Hum Mutat* **33**, 1647-1655, (2012).

2 Lau, P. J. & Kolodner, R. D. Transfer of the MSH2.MSH6 complex from proliferating cell nuclear antigen to mispaired bases in DNA. *J Biol Chem* **278**, 14-17, (2003).

3 Nilbert, M., Planck, M., Fernebro, E., Borg, A. & Johnson, A. Microsatellite instability is rare in rectal carcinomas and signifies hereditary cancer. *Eur J Cancer* **35**, 942-945, (1999).

4 Moller, P., Seppala, T., Bernstein, I., Holinski-Feder, E., Sala, P., Evans, D. G., Lindblom, A., Macrae, F., Blanco, I., Sijmons, R., Jeffries, J., Vasen, H., Burn, J., Nakken, S., Hovig, E., Rodland, E. A., Tharmaratnam, K., de Vos Tot Nederveen Cappel, W. H., Hill, J., Wijnen, J., Green, K., Lalloo, F., Sunde, L., Mints, M., Bertario, L., Pineda, M., Navarro, M., Morak, M., Renkonen-Sinisalo, L., Frayling, I. M., Plazzer, J. P., Pylvanainen, K., Sampson, J. R., Capella, G., Mecklin, J. P. & Moslein, G. Cancer incidence and survival in Lynch syndrome patients receiving colonoscopic and gynaecological surveillance: first report from the prospective Lynch syndrome database. *Gut*, (2015).

5 Kruse, R., Rutten, A., Schweiger, N., Jakob, E., Mathiak, M., Propping, P., Mangold, E., Bisceglia, M. & Ruzicka, T. Frequency of microsatellite instability in unselected sebaceous gland neoplasias and hyperplasias. *J Invest Dermatol* **120**, 858-864, (2003).

6 Arshad, M. D. & D'Angelis, C. Sebaceous Tumors of the Skin and Muir Torre Syndrome – A Mini-Review. *N A J Med Sci* **7**, 87-92, (2014).

7 Hampel, H., Frankel, W., Panescu, J., Lockman, J., Sotamaa, K., Fix, D., Comeras, I., La Jeunesse, J., Nakagawa, H., Westman, J. A., Prior, T. W., Clendenning, M., Penzone, P., Lombardi, J., Dunn, P., Cohn, D. E., Copeland, L., Eaton, L., Fowler, J., Lewandowski, G., Vaccarello, L., Bell, J., Reid, G. & de la Chapelle, A. Screening for Lynch syndrome (hereditary nonpolyposis colorectal cancer) among endometrial cancer patients. *Cancer Res* **66**, 7810-7817, (2006).

8 Vasen, H. F., Watson, P., Mecklin, J. P., Jass, J. R., Green, J. S., Nomizu, T., Muller, H. & Lynch, H. T. The epidemiology of endometrial cancer in hereditary nonpolyposis colorectal cancer. *Anticancer Res* **14**, 1675-1678, (1994).

9 Zighelboim, I., Goodfellow, P. J., Gao, F., Gibb, R. K., Powell, M. A., Rader, J. S. & Mutch, D. G. Microsatellite instability and epigenetic inactivation of MLH1 and outcome of patients with endometrial carcinomas of the endometrioid type. *J Clin Oncol* **25**, 2042-2048, (2007).

10 Simpkins, S. B., Bocker, T., Swisher, E. M., Mutch, D. G., Gersell, D. J., Kovatich, A. J., Palazzo, J. P., Fishel, R. & Goodfellow, P. J. MLH1 promoter methylation and gene silencing is the primary cause of microsatellite instability in sporadic endometrial cancers. *Hum Mol Genet* **8**, 661-666, (1999).

11 Lu, K. H., Schorge, J. O., Rodabaugh, K. J., Daniels, M. S., Sun, C. C., Soliman, P. T., White, K. G., Luthra, R., Gershenson, D. M. & Broaddus, R. R. Prospective determination of prevalence of lynch syndrome in young women with endometrial cancer. *J Clin Oncol* **25**, 5158-5164, (2007).

12 Kawaguchi, M., Yanokura, M., Banno, K., Kobayashi, Y., Kuwabara, Y., Kobayashi, M., Nomura, H., Hirasawa, A., Susumu, N. & Aoki, D. Analysis of a correlation between the BRAF V600E mutation and abnormal DNA mismatch repair in patients with sporadic endometrial cancer. *Int J Oncol* **34**, 1541-1547, (2009).

13 Wimmer, K., Kratz, C. P., Vasen, H. F., Caron, O., Colas, C., Entz-Werle, N., Gerdes, A. M., Goldberg, Y., Ilencikova, D., Muleris, M., Duval, A., Lavoine, N., Ruiz-Ponte, C., Slavc, I., Burkhardt, B. & Brugieres, L. Diagnostic criteria for constitutional mismatch repair deficiency syndrome: suggestions of the European consortium 'care for CMMRD' (C4CMMRD). *J Med Genet* **51**, 355-365, (2014).

14 Chui, M. H., Gilks, C. B., Cooper, K. & Clarke, B. A. Identifying Lynch syndrome in patients with ovarian carcinoma: the significance of tumor subtype. *Adv Anat Pathol* **20**, 378-386, (2013).

15 Snowsill, T., Huxley, N., Hoyle, M., Jones-Hughes, T., Coelho, H., Cooper, C., Frayling, I. & Hyde, C. A systematic review and economic evaluation of diagnostic strategies for Lynch syndrome. *Health Technol Assess* **18**, 1-406, (2014).

16 Loughrey, M. B., Quirke, P. & Shepherd, N. A. Standards and datasets for reporting cancers: dataset for colorectal cancer histopathology reports. *Royal College of Pathologists*, (2014).

17 van Lier, M. G., Leenen, C. H., Wagner, A., Ramsoekh, D., Dubbink, H. J., van den Ouweland, A. M., Westenend, P. J., de Graaf, E. J., Wolters, L. M., Vrijland, W. W., Kuipers, E. J., van Leerdam, M. E., Steyerberg, E. W. & Dinjens, W. N. Yield of routine molecular analyses in colorectal cancer patients </=70 years to detect underlying Lynch syndrome. *J Pathol* **226**, 764-774, (2012).

18 Syngal, S., Fox, E. A., Eng, C., Kolodner, R. D. & Garber, J. E. Sensitivity and specificity of clinical criteria for hereditary non-polyposis colorectal cancer associated mutations in MSH2 and MLH1. *J Med Genet* **37**, 641-645, (2000).

19 Cree, I. A., Deans, Z., Ligtenberg, M. J., Normanno, N., Edsjo, A., Rouleau, E., Sole, F., Thunnissen, E., Timens, W., Schuuring, E., Dequeker, E., Murray, S., Dietel, M., Groenen, P. & Van Krieken, J. H. Guidance for laboratories performing molecular pathology for cancer patients. *J Clin Pathol* **67**, 923-931, (2014).

20 Walsh, M. D., Buchanan, D. D., Pearson, S. A., Clendenning, M., Jenkins, M. A., Win, A. K., Walters, R. J., Spring, K. J., Nagler, B., Pavluk, E., Arnold, S. T., Goldblatt, J., George, J., Suthers, G. K., Phillips, K., Hopper, J. L., Jass, J. R., Baron, J. A., Ahnen, D. J., Thibodeau, S. N., Lindor, N., Parry, S., Walker,

N. I., Rosty, C. & Young, J. P. Immunohistochemical testing of conventional adenomas for loss of expression of mismatch repair proteins in Lynch syndrome mutation carriers: a case series from the Australasian site of the colon cancer family registry. *Mod Pathol* **25**, 722-730, (2012).

21 Yurgelun, M. B., Goel, A., Hornick, J. L., Sen, A., Turgeon, D. K., Ruffin, M. T. t., Marcon, N. E., Baron, J. A., Bresalier, R. S., Syngal, S., Brenner, D. E., Boland, C. R. & Stoffel, E. M. Microsatellite instability and DNA mismatch repair protein deficiency in Lynch syndrome colorectal polyps. *Cancer Prev Res (Phila)* **5**, 574-582, (2012).

22 Saridaki, Z., Souglakos, J. & Georgoulias, V. Prognostic and predictive significance of MSI in stages II/III colon cancer. *World J Gastroenterol* **20**, 6809-6814, (2014).

23 Arends, M., Ibrahim, M., Happerfield, L., Frayling, I. & Miller, K. Interpretation of Immunohistochemical Analysis of MisMatch Repair (MMR) Protein Expression in Tissue Sections for Investigation of Suspected Lynch / Hereditary Non-Polyposis Colorectal Cancer (HNPCC) Syndrome. *UK NEQAS* **1**, (2008).

24 Mensenkamp, A. R., Vogelaar, I. P., van Zelst-Stams, W. A., Goossens, M., Ouchene, H., Hendriks-Cornelissen, S. J., Kwint, M. P., Hoogerbrugge, N., Nagtegaal, I. D. & Ligtenberg, M. J. Somatic mutations in MLH1 and MSH2 are a frequent cause of mismatch-repair deficiency in Lynch syndrome-like tumors. *Gastroenterology* **146**, 643-646 e648, (2014).

25 Grindedal, E. M., Aarset, H., Bjornevoll, I., Royset, E., Maehle, L., Stormorken, A., Heramb, C., Medvik, H., Moller, P. & Sjursen, W. The Norwegian PMS2 founder mutation c.989-1G > T shows high penetrance of microsatellite instable cancers with normal immunohistochemistry. *Hered Cancer Clin Pract* **12**, 12, (2014).

26 Frayling, I. M. & Arends, M. J. How can histopathologists help clinical genetics in the investigation of suspected hereditary gastrointestinal cancer? *Diagnostic Histopathology* **21**, 137-146, (2015).

27 Deng, G., Chen, A., Hong, J., Chae, H. S. & Kim, Y. S. Methylation of CpG in a small region of the hMLH1 promoter invariably correlates with the absence of gene expression. *Cancer Res* **59**, 2029-2033, (1999).

28 Pritchard, C. C., Smith, C., Salipante, S. J., Lee, M. K., Thornton, A. M., Nord, A. S., Gulden, C., Kupfer, S. S., Swisher, E. M., Bennett, R. L., Novetsky, A. P., Jarvik, G. P., Olopade, O. I., Goodfellow, P. J., King, M. C., Tait, J. F. & Walsh, T. ColoSeq provides comprehensive lynch and polyposis syndrome mutational analysis using massively parallel sequencing. *J Mol Diagn* **14**, 357-366, (2012).

29 Haraldsdottir, S., Hampel, H., Tomsic, J., Frankel, W. L., Pearlman, R., de la Chapelle, A. & Pritchard, C. C. Colon and endometrial cancers with mismatch repair deficiency can arise from somatic, rather than germline, mutations. *Gastroenterology* **147**, 1308-1316 e1301, (2014).

30 Thompson, B. A., Spurdle, A. B., Plazzer, J. P., Greenblatt, M. S., Akagi, K., Al-Mulla, F., Bapat, B., Bernstein, I., Capella, G., den Dunnen, J. T., du Sart, D., Fabre, A., Farrell, M. P., Farrington, S. M., Frayling, I. M., Frebourg, T., Goldgar, D. E., Heinen, C. D., Holinski-Feder, E., Kohonen-Corish, M., Robinson, K. L., Leung, S. Y., Martins, A., Moller, P., Morak, M., Nystrom, M., Peltomaki, P., Pineda, M., Qi, M., Ramesar, R., Rasmussen, L. J., Royer-Pokora, B., Scott, R. J., Sijmons, R., Tavtigian, S. V., Tops, C. M., Weber, T., Wijnen, J., Woods, M. O., Macrae, F. & Genuardi, M. Application of a 5-tiered scheme for standardized classification of 2,360 unique mismatch repair gene variants in the InSiGHT locus-specific database. *Nat Genet* **46**, 107-115, (2014).

31 Thompson, B. A., Greenblatt, M. S., Vallee, M. P., Herkert, J. C., Tessereau, C., Young, E. L., Adzhubey, I. A., Li, B., Bell, R., Feng, B., Mooney, S. D., Radivojac, P., Sunyaev, S. R., Frebourg, T., Hofstra, R. M., Sijmons, R. H., Boucher, K., Thomas, A., Goldgar, D. E., Spurdle, A. B. & Tavtigian, S. V. Calibration of multiple in silico tools for predicting pathogenicity of mismatch repair gene missense substitutions. *Hum Mutat* **34**, 255-265, (2013).

32 Rhees, J., Arnold, M. & Boland, C. R. Inversion of exons 1-7 of the MSH2 gene is a frequent cause of unexplained Lynch syndrome in one local population. *Fam Cancer* **13**, 219-225, (2014).

33 Wagner, A., van der Klift, H., Franken, P., Wijnen, J., Breukel, C., Bezrookove, V., Smits, R., Kinarsky, Y., Barrows, A., Franklin, B., Lynch, J., Lynch, H. & Fodde, R. A 10-Mb paracentric inversion of chromosome arm 2p inactivates MSH2 and is responsible for hereditary nonpolyposis colorectal cancer in a North-American kindred. *Genes Chromosomes Cancer* **35**, 49-57, (2002).

34 Morak, M., Koehler, U., Schackert, H. K., Steinke, V., Royer-Pokora, B., Schulmann, K., Kloor, M., Hochter, W., Weingart, J., Keiling, C., Massdorf, T. & Holinski-Feder, E. Biallelic MLH1 SNP cDNA expression or constitutional promoter methylation can hide genomic rearrangements causing Lynch syndrome. *J Med Genet* **48**, 513-519, (2011).