Original research

Advances in genetic technologies result in improved diagnosis of mismatch repair deficiency in colorectal and endometrial cancers

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ABSTRACT

Background Testing cancers for mismatch repair deficiency (dMMR) by immunohistochemistry (IHC) is a quick and inexpensive means of triaging individuals for germline Lynch syndrome testing. The aim of this study was to evaluate tumour dMMR and the prevalence of Lynch syndrome in patients referred to the Manchester Centre for Genomic Medicine, which serves a population of 5.6 million.

Methods Tumour testing used IHC for MMR proteins with targeted BRAF and MLH1 promoter methylation testing followed by germline mutation and somatic testing as appropriate.

Results In total, 3694 index tumours were tested by IHC (2204 colorectal cancers (CRCs), 739 endometrial cancers (ECs) and 761 other), of which 672/3694 (18.2%) had protein loss, including 348 (9.4%) with MLH1 loss. MLH1 loss was significantly higher for 739 ECs (15%) vs 2204 CRCs (10%) (p=0.0003) and was explained entirely by higher rates of somatic MLH1 promoter hypermethylation (87% vs 41%, p<0.0001). Overall, 63/134 (48.5%) patients with MLH1 loss and no MLH1 promoter hypermethylation or BRAF c.1799T>A had constitutional MLH1 pathogenic variants. Of 456 patients with tumours showing loss of MSH2/MSH6, 216 (47.3%) had germline pathogenic variants in either gene. Isolated PMS2 loss was most suggestive of a germline MMR variant in 19/26 (73%). Of those with no germline pathogenic variant, somatic testing identified likely causal variants in 34/48 (71%) with MLH1 loss and in MSH2/MSH6 in 40/47 (85%) with MSH2/MSH6 loss.

Conclusions Reflex testing of EC/CRC leads to uncertain diagnoses in many individuals with dMMR following IHC but without germline pathogenic variants or MLH1 hypermethylation. Tumour mutation testing is effective at decreasing this by identifying somatic dMMR in >75% of cases.

INTRODUCTION

Colorectal cancer (CRC) and endometrial cancer (EC) are two of the most common malignancies in humans. They are both characterised by having a relatively high rate of mismatch repair deficiency (dMMR) and similar germline rates (3%) of pathogenic variants in MMR genes. CRC is the third most common cancer in men and women. EC is the most common gynaecological cancer in high-income countries, and its incidence is rising rapidly. Although environmental causes such as diet (CRC) and obesity (particularly EC) and decreased parity (EC) are major contributors to incidence, a significant minority of both cancers (3%) are caused by Lynch syndrome (LS). LS is an inherited susceptibility to malignancies associated with dMMR. Around 1 in 280 of the general population is heterozygous for a pathogenic variant in an MMR gene, MLH1, MSH2 (including deletions of EPCAM), MSH6 or PMS2 (path_MMR), the vast majority of whom are undiagnosed.

Path_MMR heterozygotes have an averaged risk to age 70 years of EC, CRC and ovarian cancer (OC) of 33%, 46% and 11%, respectively, although these vary by gene with lower risks of PMS2. These likelihoods are substantially higher than those of the general population for EC (3%), CRC (4%) and OC (1%). Since the discovery of the MMR genes in 1993–1994, germline testing has been targeted at those most likely to have an inherited pathogenic variant. The Amsterdam criteria were developed in 1991, primarily to select high-risk families, therefore requiring a substantial family history of CRC. While 45%–60% of index cases in families fulfilling criteria are path_MMR variant carriers, the criteria have low sensitivity. The addition of other characteristic tumours of LS, such as EC, OC and urothelial cancers, add little to either the detection rate, or sensitivity. The less restrictive Bethesda guidelines were developed in 1997, which improved sensitivity but resulted in many more samples being tested without detection of all path_MMR variants. More recently, the concept of universal testing of CRC has gained ground and is now recommended national guidance in a number of countries for CRC. This is also gaining traction for EC and is now recommended by the National Institute for Health and Care Excellence in the UK.

We have evaluated our prescreening strategy with immunohistochemistry (IHC) in Lynch-related cancers from 2000 to 2020 and, more latterly, the impact of somatic next-generation sequencing (NGS) of tumours in individuals...
with protein loss on IHC in tumours but without a germline path_MMR.

**METHODS**

**Participants**

Individuals referred to the regional genetics department in Manchester with an LS-related cancer and concerns about the possibility of LS provided consent for tumour and, if necessary, germline analysis. The great majority of evaluated patients had CRC or EC and were selected based on early age at diagnosis or fulfilling Bethesda guidelines or Amsterdam criteria. Occasional cases were tested as deceased first-degree relatives of clinically unaffected index patients. In addition, 500 women from the Proportion of Endometrial Tumours Associated with Lynch Syndrome (PETALS) study with sequential EC were also included (15/NW/9733). Generally, individuals fulfilling Amsterdam criteria did not undergo prescreening and went straight to germline path_MMR analysis.

The standard pathway for non-Amsterdam criteria tumours was an initial test for dMMR using IHC of the MMR proteins. If there was loss of MLH1, the samples were tested for MLH1 promoter hypermethylation and the BRAF c.1799T>A (p.Val600Glu) pathogenic variant. Positive results for either of these is indicative of a somatic mutation of MLH1. All individuals with MLH1 loss, samples and wild type for BRAF and negative for promoter hypermethylation, as well as all sole PMS2 or MSH2/MSH6 loss, underwent germline lymphocyte testing where this was possible. BRAF c.1799T>A (p.Val600Glu) was suspected for EC once it was known this screen was not sensitive (0/23 tested were positive for c.1799T>A (p.Val600Glu)).

**Immunohistochemistry**

IHC for the four MMR proteins was performed in the MFT clinical pathology laboratory using the automated Ventana BenchMark ULTRA IHC/ISH staining module and the OptiView, 3′diaminobenzidine V.S detection system (Ventana Co, USA) according to standard clinical protocols. The proportion of stained tumour epithelial component/intensity of staining was scored by two expert independent observers using tumour stroma as internal control and as described elsewhere. Only tumours with complete loss of protein expression were reported as dMMR (not those with patchy loss).

**Methylation analysis**

Reflex MLH1 methylation testing was performed on tumours showing loss of MLH1 protein on IHC. Purified DNA was amplified with bisulfite-specific primers in triplicate. A region of the MLH1 promoter containing four CpG dinucleotides whose methylation status is strongly correlated with MLH1 expression was sequenced using a pyrosequencer (PSQ 96MA). Two independent scientists interpreted the pyrograms. ‘Hypermethylation’ described >10% mean methylation across the four CpG dinucleotides on two of three replicate analyses. A proportion of MLH1 hypermethylation cases underwent reference standard germline MMR sequencing to exclude coexisting path_MLH1 variants, usually when they had a significant family history. In addition to methylation, analysis testing was carried out for the BRAF c.1799T>A (p.Val600Glu) variant.

**Germline analysis**

DNA was extracted from 2 to 5 mL lymphocyte blood (EDTA anticoagulant) using Chemagic DNA blood chemistry (CMG-1097-D) on an automated PerkinElmer Chemagic 360 Magnetic Separation Module and a JANUS Integrator four-tip Automated Liquid handling platform. DNA was eluted into 400 μl buffer. Extracted DNA samples were measured for DNA yield, concentration and quality using a Nanodrop ND-8000 spectrophotometer. MMR genes MLH1, MSH2 and MSH6 were amplified using long-range PCR followed by NGS using Illumina SBS V2 2×150 bp and Illumina MiSeq to analyse the coding region, flanking sequences to ±15 bp and known splicing variants (minimum 100× coverage depth) of MLH1, MSH2 and MSH6. Variant identification and calling was via an in-house bioinformatic pipeline. Reported sequence changes and regions with <100× coverage were retested via Sanger sequencing using BigDye V3.1. Copy number analysis to detect large genomic rearrangements affecting the MMR genes was performed using MLPA MRC-Holland probe mixes: P003-D1 MLH1/MSH2 and P072-C1 MSH6. Variant nomenclature followed Human Genome Variation Society guidelines (http://www.hgvs.org) using reference sequences: LRG_216, t1(MLH1); LRG_218, t1(MSH2); LRG_219, t1(MLH3). Exons were numbered consecutively starting from exon 1 as the first transcribed exon for each probe mix. Cases with PMS2 protein loss, normal MLH1 methylation and no path MLH1/MSH2/MSH6 variant underwent path_PMS2 analysis at the regional specialist Yorkshire and North East Genomic Laboratory which included MLPA.

![Figure 1](http://jmg.bmj.com/) Study flowchart diagram. Note: germline testing was done for MLH1, MSH2 and MSH6 in cases and PMS2 in select cases. Were a path_MMR was detected in a gene not consistent with the IHC loss, this is shown in brackets below the result. *The majority of these samples were Amsterdam criteria II positive. One sample had constitutional MLH1 hypermethylation. EC, endometrial cancer; CRC, colorectal cancer; IHC, immunohistochemistry; MMR, mismatch repair; path_MMR pathogenic variant.
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Somatic tumour analysis

Tumour specimens were assessed by specialist pathologists. All tissues were formalin-fixed and paraffin-embedded according to local clinical protocols. Tissue blocks with the greatest tumour content (>70%) were chosen for DNA extraction. Tumour was either microdissected from 5×10 µm unstained sections or cored from tissue blocks, depending on tumour content. Non-malignant adjacent tissue was selected for comparative constitutional microsatellite instability (MSI) analysis. MMR genes MLH1, MSH2 and MSH6 were analysed as components of a somatic panel including PTEN, TP53, APC, POLD1 and POLE using a custom NGS approach based on a Qiagen GeneRead amplicon based enrichment. PMS2 was not assessed due to the difficulties with pseudogenes and high copy number variant rate. Formal loss of heterozygosity (LOH) analysis was not part of the initial panel but was introduced with microsatellite repeats after 12 months, but did not include LOH for PMS2.

Statistics

Differences between values were tested by a two-tailed Fisher’s χ² test.

RESULTS

A total of 3694 index cases aged 8–91 years at diagnosis had a tumour (figure 1 and table 1) prescreened with tumour IHC of MMR proteins with 672 (18%) showing loss of at least one protein (table 2). CRC (n=2204 mean age 50.8 years) and EC (n=739 mean age=61 years) were by far the most frequently tested, and further analysis was largely confined to those two tumour types (online supplemental figure 1A,B). However, we also tested 761 other cancers and benign tumours (mean age=50, table 1).

A total of 211 patients with CRC underwent germline MMR testing without an IHC prescreen with 123 (58%) demonstrating

| Table 1 | Tumour samples tested, age at diagnosis, IHC loss and path_MMR rate |
|---------|---------------------------------------------------------------|
| IHC (n) | Age range (median) | IHC loss | % IHC loss | Path_MMR with loss |
|---------|-------------------|----------|------------|-------------------|
| Colorectal cancer | 2204 | 14.5–91 (50.8) | 422 | 19.15 | 155 | 7.03 |
| Colorectal polyps | 244 | 8.4–82 (54) | 8 | 3.28 | 3 | 1.23 |
| Endometrial cancer | 739 | 183 | 24.76 | 44 | 5.95 |
| Genetics service | 239 | 16–79 (51) | 28 | 11.7 |
| PETALS | 500 | (65) | 16 | 3.2 |
| Gastric cancer | 58 | 17–79 (48) | 5 | 8.62 | 0 | 0.00 |
| Ovarian cancer | 261 | 16–89 (49) | 27 | 10.34 | 8 | 3.07 |
| TCC/kidney | 13 | 32–61 (45) | 3 | 23.08 | 1 | 7.69 |
| Non-melanoma skin cancer | 29 | 37–75 (57) | 12 | 41.38 | 3 | 10.34 |
| Cholangiocarcinoma | 20 | 32–76 (50) | 5 | 25.00 | 0 | 0.00 |
| Pancreas | 13 | 37–71 (53) | 1 | 7.69 | 0 | 0.00 |
| Brain | 12 | 9–84 (48) | 4 | 33.33 | 1 | 8.33 |
| Breast | 15 | 33–74 (49) | 0 | 0.00 | 0 | 0.00 |
| Small bowel including ampulla | 21 | 29–72 (48) | 1 | 4.76 | 0 | 0.00 |
| Unknown primary | 19 | 26–71 (40) | 0 | 0.00 | 0 | 0.00 |
| Oesophagus | 16 | 21–61 (51) | 1 | 6.25 | 0 | 0.00 |
| Other | 30 | 0 | 0.00 | 0 | 0.00 |
| Total | 3694 | | 672 | 215 |

TCC urinary tract; others include cervix (n=4), prostate (n=4), sarcoma (n=3), melanoma (n=3), thyroid (n=2) and lung (n=2).

IHC, immunohistochemistry; MMR, mismatch repair; path_, pathogenic variant; PETALS, Proportion of Endometrial Tumours Associated with Lynch Syndrome; TCC, transitional cell carcinoma.

| Table 2 | IHC loss and germline path_MMR detection rates in all index samples tested |
|---------|---------------------------------------------------------------|
| All | Tested (n) | IHC loss | % | Tested germline (n) | Germline PV | % Germline |
| MLH1 loss | 3694 | 348 | 9.42 | 191 | 66* | 34.55 |
| PMS2 loss alone | 3694 | 33 | 0.89 | 26 | 19 | 73.08 |
| MSH2 loss | 3694 | 198 | 5.36 | 166 | 90 | 54.22 |
| MSH6 loss | 3694 | 215 | 5.82 | 176 | 102 | 57.95 |
| Either MSH2 or MSH6 | 3694 | 291 | 7.88 | 239 | 130 | 54.30 |
| Any loss | 3694 | 672 | 18.19 | 456 | 215 | 47.15 |
| MSH6 loss alone | 3694 | 53 | 1.43 | 73 | 38 | 52.05 |

| Tested (n) | | | |
| Tested (n) | | | |
| MLH1 loss hypermethylation | 268 | 1671 | 62.3 | 57 | 1 | 1.75 |
| MLH1 loss Braf c.1799T>A | 181 | 45 | 24.86 | 11 | 3 | 27.3 |
| No loss | 3022 | 0 | 0 | 329 | 19 | 5.78 |

*This rose to 65/134 (48.5%) unmethylated samples.
†One patient with colorectal cancer had germline MLH1 methylation.
EC, endometrial cancer; IHC, immunohistochemistry; PV, pathogenic variant.
Table 3  IHC loss and germline path_MMR detection rates in CRC and EC index samples tested

| Colorectal | Number tested | IHC loss | % | Number tested germline | Germline path_MMR | % Germline path_MMR |
|------------|--------------|----------|---|------------------------|-------------------|-------------------|
| MLH1 and PMS2 loss | 2204 | 171 | 7.8 | 104 | 40† | 38.5% | 37 MLH1, 3 PMS2 |
| MLH1 loss alone | 2204 | 51 | 2.3 | 33 | 24† | 72.7% | 24 MLH1 |
| PMS2 loss alone | 2204 | 25 | 1.1 | 21 | 14 | 66.7% | 14 PMS2 |
| MSH2 and MSH6 loss | 2204 | 81 | 3.7 | 76 | 54 | 71.0% | 48 MSH2, 6 MSH6 |
| MSH2 loss alone | 2204 | 45 | 2.0 | 32 | 8 | 25.0% | 8 MSH2 |
| MSH6 loss alone | 2204 | 41 | 1.9 | 30 | 15 | 50.0% | 15 MSH6 |
| Either MSH2 or MSH6 | 2204 | 175 | 7.9 | 140 | 77 | 55.0% | 56 MSH2, 21 MSH6 |
| Any loss | 2204 | 422 | 19.1 | 298 | 155 | 52.0% | |

| EndometrialC | Tested (n) | Positive (n) |
|--------------|------------|--------------|
| MLH1 loss hypermethylation | 163 | 67* | 41.1 | 23 | 1 | 4.3% | MLH1 |
| MLH1 loss BRAF c.1799T>A | 170 | 45 | 26.5 | 11 | 3 | 27.3% | |
| No loss | 1782 | 0 | 0.0 | 176 | 18† | 10.2% | 4 MLH1, 7 MSH2, 3 MSH6, 4 PMS2 |

| Tested (n) | Positive (n) |
|------------|--------------|
| MLH1 loss hypermethylation | 109 | 95 | 87.2 | 32 | 0 | 0.0% | |
| MLH1 loss BRAF c.1799T>A | Not systematically tested | |
| No loss | 556 | – | 0.0 | 120 | 1 | 0.8% | 1 MSH6 |

* One patient with CRC had germline MLH1 methylation, #13/74 (17.6%) Amsterdam criteria, 5/102 (4.9%) non-Amsterdam.
† This rose to 2/15 unmethylated samples.
‡ This rose to 63/114 (55.3%) of unmethylated samples.

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a pathogenic variant (56 MLH1, 59 MSH2 and 8 MSH6). Similarly, 24 women with EC fulfilling Amsterdam criteria went direct to germline testing, of whom 19 (79%) had a path_MMR (4 MLH1, 10 MSH2, 4 MSH6 and 1 PMS2).

Of the 672 tumours with dMMR IHC loss in prescreened samples (Table 2), loss of MLH1 was most common (9.4%) with 7.9% having loss of either MSH2 or MSH6 or both. There were 215 path_MMR present in 456 lymphocyte samples tested (47.4%–63 MLH1, 79 MSH2, 51 MSH6 and 22 PMS2). The relatively low detection rate of only 34.5% for those with MLH1 loss is partially explained by screening of 57 samples showing MLH1 methylation of which only one had a path_MMR variant (26 samples from PETALS and 31 clinical samples were tested). The patient with a germline MLH1 had a caecal tumour aged 45 and met Amsterdam criteria. Thus, the true rate of MLH1 promoter methylation-negative samples in this group was 65/134 (48.5%). The highest detection rates of path_MMR were for those with PMS2 loss alone (73%) and MSH6 loss alone (58%). Overall 12/22 (54.5%) with a PMS2 germline path_MMR had a large rearrangement.

Table 3 shows the dMMR tumours and the pathogenic variants detected for CRC and EC, respectively. Overall, 2204 index CRCs underwent IHC and 422 (19.1%) showed dMMR with the highest proportion demonstrating MLH1 loss (10%). For EC, 183/739 (24.8%) samples were dMMR with 15% demonstrating MLH1 loss. Both overall dMMR rates (p=0.001) and MLH1 loss rates (p=0.0003) were significantly higher in EC, although the difference is entirely driven by MLH1 loss. MLH1 promoter hypermethylation rates were much higher in EC at 87% (95/109) compared with only 41% (67/163) in CRC (p<0.0001). BRAF c.1799T>A was identified in only 26.5% (45/170) dMMR CRC samples, compared with 41% (67/163) with MLH1 promoter hypermethylation (p=0.005). BRAF testing was of no value in EC and was abandoned for the PETALS study. The difference in path_MMR variant rates between CRC and EC was most striking for MSH6 with pathogenic variants identified in 24/44 (54.5%) cases of EC compared with 21/155 (13.5%) for CRC (p<0.0001). Equally, MLH1 variants were more common in CRC with 61/155 (39%) pathogenic variants compared with 2/44 (0.5%) in EC (p<0.0001).

A subset of samples with EC and CRC still underwent full germline testing despite no IHC loss. This was in general because of a strong family history, and it remains routine practice to test all Amsterdam criteria cases regardless of IHC loss.27 For CRC, 13/74 (17.6%) Amsterdam criteria and 5/102 (4.9%) non-Amsterdam criteria cases tested positive for a path_MMR despite normal IHC. However, for EC, only 1/120 (0.8%) tested positive for a path_MSH6 and nil in other MMR genes. The higher figures for CRC may be because these are more common, and a sporadic tumour without IHC loss could explain at least some of these false negative results. Sensitivity for IHC loss in CRC was 153/173 (89.6%) and that for EC was 44/45 (97.8%), although these figures might drop further if all samples with retained IHC staining were tested.

The results of somatic analysis are shown in Table 4 for CRC and EC, respectively. In seven cases with IHC loss, the
individuals were deceased and a germline path_MMR was found on somatic testing and confirmed in normal tissue material. Initial somatic MMR testing in the first 40 CRC samples did not include an analysis of LOH, and thus many variants were only monoallelic. Overall, 15/20 monoallelic CRC samples did not have a formal LOH analysis. However, in EC, 17/18 tumours showed at least one somatic variant with 13/18 (72%) biallelic. Four tumours had a single variant at low allele frequency (MSH2 c.2458+1G>A (5.04%), MSH2 c.1003dupA (5.99%), MSH6 c.3261delC (10.1%) and MSH6 c.718C>T (13.2%)), which precluded a sensitive LOH analysis. Of the 183/739 (24.7%) EC samples showing IHC loss, 95 (52%) were explained by MLH1 promoter methylation; 157 had a germline path_MMR; and 17 (9.3%) showed evidence of somatic involvement of the relevant gene. This leaves only 27/183 (14.7%), but six samples did not undergo germline testing and three did not have promoter methylation with MLH1 loss. Most of the remainder, bar 1, did not have tumour somatic testing. Assuming the same detection rates for somatic testing as in the 18 EC samples, this would leave no more than 3 of 183 (1.6%) unexplained and only 3/739 (0.4%) of the whole IHC prescreen cohort. Of the 422/2204 (19.1%) CRC samples showing IHC loss, 67 (15.9%) were explained by MLH1 promoter methylation; 155 (36.7%) had a germline path_MMR; and 65 (15.4%) showed evidence of somatic involvement of the relevant gene. This leaves 135/422 (32%), but 73 samples did not undergo germline testing as patients were deceased and 17 did not have promoter methylation with MLH1 loss.

**DISCUSSION**

We have reported IHC tumour prescreening in 3694 tumour samples, which, to our knowledge, is the largest such series in the literature. Although IHC does not have 100% sensitivity, it has the advantage of identifying the relevant likely genes involved and allows targeted MLH1 promoter methylation in a lower number of samples than MSI testing. We have previously shown that for EC, in particular with the higher rates of involvement of MSH6 (55% in the present study), MSI is significantly less sensitive (58%) with IHC detecting 100% of 16 path_MMR. The high proportion of MSH6 in EC is confirmed in other studies with 5/9 (55.5%) in a US universal testing study. An Australian study limiting testing to ECs<60 found 10/22 (45.4%) of those with path_MMR had a path_MSH6. The study confirms the utility of MLH1 promoter methylation particularly for EC with 87% of MLH1 loss being explained. This is similar to the 86.3% in a meta-analysis of 29 studies with 1159 showing loss of MLH1. Although MLH1 promoter methylation is less useful in CRC, it is still superior to BRAF testing. The significantly higher rates of promoter methylation in EC seem to account entirely for the higher rates of MLH1 loss. As methylation is a mechanism that is used to coordinate menstruation in the endometrium, we propose that there is increased opportunity for regions in the DNA to be erroneously methylated, which may explain increased promoter methylation of MLH1 in EC.

The current study has confirmed the high predictive value of isolated loss of PMS2 and, to a lesser extent, MSH6, although isolated MLH1 loss was also quite specific with 73% being caused by a germline path_MMR. We have also shown the importance of somatic MMR testing in cases with IHC loss unexplained by either MLH1 promoter methylation or a germline path_MMR. Somatic biallelic path_MMRs are found in a high proportion of these cases. Of the 284 patients with non-methylated MMR loss in a joint Ohio and Icelandic cohort, 157 had a germline path_MMR, (55%) and 92 (32.4%) had probable biallelic (double) somatic variants. They concluded that 19 (6.7%) were unexplained and 17 had incorrect IHC. While we demonstrated this well in EC, it was less well shown in CRC. This may be due to low neoplastic cell counts that preclude a sensitive assessment of LOH. Recutting tumour FFPE sections for higher neoplastic content may well overcome this issue. Furthermore, some IHC loss may be spurious (an overcall) and reanalysis or assessment of MSI in those that still remains with unexplained IHC loss may resolve the issue. For EC we have shown that <1% of cases undergoing IHC are left with an unresolved diagnosis. In reality, ‘Lynch’ like syndrome, which was thought to be due primarily to missed path_MMR or another inherited mechanism, appears to be a relatively uncommon situation once somatic testing has been performed especially in EC.

Although there was a low rate of path_MMR in patients with tumours with MLH1 loss on IHC and promoter methylation as a prescreen, we have previously demonstrated that 4/71 (5.6%) individuals with CRC and germline pathogenic variants in MLH1 had evidence of promoter methylation. Three of these four fulfilled Amsterdam criteria did not have an IHC prescreen (they were tested after path_MMR was found); therefore, overall MLH1 promoter methylation still left a >10% chance of a germline path_MMR. Similarly, those with Amsterdam criteria who had proficient MMR tumour on IHC also had a path_MMR rate above 10%. As such, we would still recommend that those with CRC fulfilling Amsterdam criteria undergo germline
path_MMR testing irrespective of the IHC or MLH1 promoter methylation result. The same may not be true for EC with the much higher rates of MLH1 promoter methylation and low rate of pathogenic germline variants in those tested with proficient MMR on IHC.

Our results show the value of a combined tumour somatic and germline test after IHC loss. This can especially be seen in the population-based PETALS study where the 3.2% detection rate for germline path_MMR in EC is similar to that seen in unselected CRC. By combining a tumour somatic approach with germline testing in 500 ECs, this comprehensive testing left just 1.9% (2/106) MMR deficient tumours unexplained by a path_MMR variant/epigenetic silencing.25 As such, only 2/500 (0.4%) were left still in the Lynch-like category after testing. A similar mainstreaming approach for CRC as well as EC would leave far fewer with an uncertain diagnosis, and only those with a path_MMR or unexplained IHC would need referral to genetics. Unfortunately, we cannot be certain the results would be as good in CRC based on our analysis as this did not involve LOH analysis for many samples, but others have found a high rate of double somatic events in CRC.33 While families can be reassured when double somatic events account for IHC loss this will still leave some where the age of the patient or family history requires ongoing management as Lynch-like. Testing of benign colorectal polyps is quite specific but does not have a high yield, although it can detect germline path_MMR in individuals with strong family histories suggestive of LS.

There are some limitations to the present study. We did not perform MSI testing on all the samples with IHC and cannot therefore make a direct comparison, although for EC, we have previously shown reduced sensitivity of MSI.23 The selection criteria for testing for CRC was stronger than for EC, and therefore comparisons are likely to overestimate the contribution of IHC loss in CRC compared with the EC tested in this study. Nonetheless, this is likely to strengthen further some of the differences identified between CRC and EC. We also did not typically prescreen individuals meeting Amsterdam criteria, meaning that the detection rates for IHC loss and path_MMR may be underestimated compared with studies that included individuals meeting Amsterdam criteria. We would still test patients meeting Amsterdam criteria even if they had hypermethylation of MLH1 as evidenced by the case presented here. Some authors now advocate starting analysis with a tumour somatic approach.31 It is certainly plausible that this will become more mainstream and may reduce the requirement for a prescreen for LS testing. However, given the high rate of copy number variants in LS (11%-46%)44 and especially in this study for PMS2 (54.5%), the sensitivity to detect these in tumour samples needs to be fully validated first. PMS2 is known to be difficult to screen in lymphocyte DNA, and therefore testing in stored non-frozen tissue samples requires a bespoke approach.

In conclusion, we have undertaken prescreening of a very large series of tumour specimens with IHC for dMMR. Detection rates for germline path_MMR are similar to previous estimates. We have shown the superiority of MLH1 promoter hypermethylation over BRAF testing and the higher utility in EC compared with CRC. Furthermore, we have shown that somatic MMR testing with NGS removes most patients from the ‘Lynch’-like category with previously unexplained IHC loss.

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Contributors DGE was principal investigator for the study and is its guarantor. DGE and EJC designed the study and supervised its execution. DGE and EJC wrote the manuscript. All authors contributed to the data collection, analysis and interpretation; provided critical comment; edited the manuscript; and approved its final version.

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REFERENCES
1. Ryan NAJ, Glaire MA, Blake D, Cabrera-Dandy M, Evans DG, Crosbie EJ. The proportion of endometrial cancers associated with Lynch syndrome: a systematic review of the literature and meta-analysis. Genet Med 2019;21:1267–80.
2. Ferlay J, Soejomotaram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359–86.
3. Crosbie E, Morrison J. The emerging epidemic of endometrial cancer: time to take action. Cochrane Database Syst Rev 2014;10:CD000095.
4. Crosbie EJ, Zwahlen M, Kitzchen HC, Egger M, Rehenhan AG. Body mass index, hormone replacement therapy, and endometrial cancer risk: a meta-analysis. Cancer Epidemiol Biomarkers Prev 2010;19:3139–30.
5. Jiang W, Cai M-Y, Li S-Y, Bei J-X, Wang F, Hampel H, Ling Y-H, Frayling IM, Sinicrope FA, Rodriguez-Bigas MA, Dignam JJ, Kerr DJ, Rosell R, Mao M, Li J-B, Guo Y-M, Wu Y-Y, Kong L-H, Tang J-H, Wu X-D, Li C-F, Chen J-R, Ou Q-J, Ye M-Z, Gu F-M, Han P, Wang Q-W, Wan D-S, Li L, Xu R-H, Pan Z-Z, Ding P-R, XD W, CF L, QJ O, RH X, Written on behalf of AME Colorectal Cancer Cooperative Group. Universal screening for Lynch syndrome in a large consecutive cohort of Chinese colorectal cancer patients: high prevalence and unique molecular features. Int J Cancer 2019;144:2161–8.
Evans DG, et al. J Med Genet 2021;58:1–7. doi:10.1136/jmedgenet-2020-107542

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6 Win AK, Jenkins MA, Dowty JG, Antoniou AC, Lee A, Giles GG, Buchanan DD, Clendenning M, Rosy C, Ahnen DJ, Tribouleeau SN, Casey G, Gallinger S, Le Marchand L, Haile RW, Potter JD, Zheng Y, Lindor NM, Newcomb PA, Hopper JL, Madan RJS. Prevalence and penetrance of major genes and polygenes for colorectal cancer. *Cancer Epidemiol Biomarkers Prev* 2017;26:404–12.

7 Hampil H. Genetic counseling and cascade genetic testing in Lynch syndrome. *Fam Cancer* 2016;15:423–7.

8 Hampil H, de la Chapelle A. The search for unaffected individuals with Lynch syndrome: do the ends justify the means? *Cancer Prev Res* 2011;4:1–5.

9 Møller P, Seppälä T, Bernstein I, Holinski-Feder E, Sala P, Evans DG, Lindblom A, Macaee F, Blanco I, Sijmons R, Jeffries J, Vasen H, Burn J, Nakken H, Hovig E, Redland EA, Thammaratnam K, de Vos Tol Nederveen Cappel WH, Hijl W, Jijnen G, Green K, Laloo F, Sunde L, Mints M, Bertario L, Pineda M, Navarro M, Morak M, Rikkenen-Sinisalo L, Fraying IM, Plazzer J-P, Pylvainen K, Sampson JR, Capella G, Mecklin J-P, Möslin G, Mallorca Group. Cancer incidence and survival in Lynch syndrome patients receiving colonoscopic and gynaecological surveillance: first report from the prospective Lynch syndrome database. *Gut* 2017;66:464–72.

10 Siegel RL, Miller KD, Jemal A. Cancer statistics, 2017. *CA Cancer J Clin* 2017;67:7–30.

11 Vasen HF, Mecklin JP, Khan PM, Lynch HT. The International Collaborative group on hereditary non-polyposis colorectal cancer (HGG-HNPCC). *Col Rectum* 1991;34:424–5.

12 Park J-G, Vasen HFA, Park YJ, Park KL, Petolmaki P, de Leon MP, Rodriguez-Bigas MA, Lubinski J, Beck NE, Bisgaard M-L, Miyaki W, Jijnen BT, Baba S, Lindblom A, Madlensky L, Lynch HT. Suspended HNPCC and Amsterdam criteria II: evaluation of mutation detection rate, an international collaborative study. *Int J Colorectal Dis* 2002;17:109–14.

13 Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group. Recommendations from the EGAPP Working Group: genetic testing strategies in newly diagnosed individuals with colorectal cancer aimed at reducing morbidity and mortality from Lynch syndrome in relatives. *Genet Med* 2009;11:35–41.

14 Shia J. Evolving approach and clinical significance of detecting DNA mismatch repair deficiency in colorectal carcinoma. *Semin Diagn Pathol* 2015;32:552–61.

15 Vasen HF, Watson P, Mecklin JP, Lynch HT. New clinical criteria for hereditary nonpolyposis colorectal cancer (HNPCC, Lynch syndrome) proposed by the International Collaborative group on HNPCC. *Gastroenterology* 1999;116:1453–6.

16 Rodriguez-Bigas MA, Boland CR, Hamilton SR, Henson DE, Jass JR, Khan PM, Lynch H, Perou M, Smyrk T, Sobin L, Srivastava S. A national cancer institute workshop on hereditary nonpolyposis colorectal cancer syndrome: meeting highlights and Bethesda guidelines. *J Natl Cancer Inst* 1997;89:1578–62.

17 Giulia C, Tékollat C, Broquet O, Vallot C, Zimmermann U, Mitty E, Radivanj F, Rouleau E, Lidéreau R, Crouzet F, Otshwagw P, Roubou G, Bessinger P, Fischler J, Beaudoin P, Géhenet-Gabriel H, Identification in daily practice of patients with Lynch syndrome (hereditary nonpolyposis colorectal cancer): revised Bethesda guidelines-based approach versus molecular screening. *Am J Gastroenterol* 2008;103:2825–35.

18 Balmain A, Balaguer F, Castelvi-Bel S, Steyerberg EW, Andraw M, Llor X, Jover R, Castells A, Snyal S, Gastrointestinal Oncology Group of the Spanish Gastroenterological Association. Comparison of predictive models, clinical criteria and molecular tumour screening for the identification of patients with Lynch syndrome in a population-based cohort of colorectal cancer patients. *J Med Genet* 2008;45:557–63.

19 Byfield SAD, Syngal S. Clinical guidelines versus universal molecular testing: are we ready to choose an optimal strategy for Lynch syndrome identification? *Am J Gastroenterol* 2008;103:2837–40.

20 Adar T, Rodgers LH, Shannon KM, Yoshida M, Ma T, Mattia A, Lauwers GY, Ifatse AJ, Hartford NM, Oliya E, Chung DC. Universal screening of both endometrial and colon cancers increases the detection of Lynch syndrome. *Cancer* 2018;124:3145–53.

21 National Institute for Health and Care Excellence. Molecular testing strategies for Lynch syndrome in people with colorectal cancer (DG27), 2017. Available: https://www.nice.org.uk/guidance/dg27

22 Yozu M, Kumaarasinghe MP, Brown IS, Gill AJ, Rosti C. Australasian gastrointestinal pathology Society (AGPS) consensus guidelines for universal defective mismatch repair testing in colorectal carcinoma. *Pathology* 2019;51:233–9.

23 Croibia EJ, Ryan NAJ, Arends MJ, Bosse T, Burn J, Cornes JM, Crawford R, Eccles D, Fraying IM, Ghaem-Maghadi S, Hampil H, Kauff ND, Kitchin SJ, Manchanda R, McMahon RFT, Monahan KJ, Menon U, Müller P, Möslin G, Rosenthal A, Sasiain P, Seif MW, Singh N, Skarpt R, Snowsill TM, Steele R, Tischkowitz M, Evans DG, Manchester International Consensus Group. The Manchester international consensus group recommendations for the management of gynaecological cancers in Lynch syndrome. *Genet Med* 2019;21:2390–400.

24 National Institute for Health and Care Excellence. Testing strategies for Lynch syndrome in people with endometrial cancer (DG42), 2020. Available: https://www.nice.org.uk/guidance/dg42.

25 Ryan NAJ, McMahon R, Tobi S, Snowsill T, Esquibel S, Wallace AJ, Bunstone S, Bowers N, Mosneag JE, Kitson SJ, O’Flynn H, Ramchander NC, Sivalingham VN, Fraying IM, Bolton J, McKay RJ, Evans DG, Croibia EJ. The proportion of endometrial tumours associated with Lynch syndrome (petals): a prospective cross-sectional study. *PLoS Med* 2020;17:e1003263.

26 Metcalf AM, Spurle DB. Endometrial tumour BRAF mutations and MLH1 promoter methylation as predictors of germline mismatch repair gene mutation status: a literature review. *Fam Cancer* 2014;13:1–12.

27 Newton K, Jorgensen NM, Wallace AJ, Buchanan DD, Laloo F, McMahon RFT, Hill J, Evans DG. Tumour MLH1 promoter region methylation testing is an effective precursor for Lynch syndrome (HNPCC). *J Med Genet* 2014;51:789–96.

28 Barrow E, Evans DG, McMahon R, Hill J, Byers R. A comparative study of quantitative immunohistochemistry and quantum dot immunochemistry for mutation carrier identification in Lynch syndrome. *J Clin Pathol* 2011;64:208–14.

29 Hampil H, Frankel W, Panescu J, Lockman J, Sotamaa K, Fix O, Comeras I, La Jeunesse J, Nakagawa H, Westman JA, Prior TW, Clendenning M, Penzoe P, Lombardi J, Dunn P, Cohn DE, 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* 2006;66:7810–7.

30 Buchanan DD, Tan YW, Walsh MD, Clendenning M, Metcalf AM, Ferguson K, Arnold ST, Thompson BA, Lass EA, Parsons MT, Walters RJ, Pearson S-A, Cummings M, Dehler MK, Blomfield PB, Quinn MA, Kirk JA, Stewart CJ, Obermair A, Young JP, Web PM, Spurle DB. Tumor mismatch repair immunohistochemistry and DNA MLH1 methylation testing of patients with endometrial cancer diagnosed at age younger than 60 years optimizes triage for population-level germline mismatch repair gene mutation testing. *J Clin Oncol* 2011;29:300–100.

31 Kahn RM, Gordinhasas S, Maddy BP, Balthch Nelson B, Askig G, Christos PJ, Caputo TA, Chapman-Davis E, Holomb K, Frey MK. Universal endometrial cancer tumor typing: how much has immunohistochemistry, microsatellite instability, and MLH1 methylation improved the diagnosis of Lynch syndrome across the population? *Cancer* 2019;125:3172–83.

32 Mortock S, Restudi R, Levien R, Girling JE, Holdsworth-Carson SJ, Healey M, Zhu Z, Qiu T, Wu Y, Lukowski SW, Rogers PAV, Yang J, McRae AF, Fung JN, Montgomery GW. Genetic regulation of methylation in human endometrium and blood and gene targets for reproductive diseases. *Clin Epigenetics* 2019;11:49.

33 Pearlman R, Haraldsdottir S, de la Chapelle A, Jonasson JG, Lykanarachi S, Frankel WL, Rahafar T, Stefansson K, Pritchard CC, Hampil H. Clinical characteristics of patients with colorectal cancer with double somatic mismatch repair mutations compared with Lynch syndrome. *J Med Genet* 2019;56:462–70.

34 Smith MJ, Urecht HE, Harkness EF, Miles EK, Bowers NL, Byers HJ, Bulman M, Gokhale C, Wallace AJ, Newman WG, Evans DG. The contribution of whole gene deletions and large rearrangements to the mutation spectrum in inherited tumour predisposing disorders. *Hum Mutat* 2016;37:250–6.