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Title: The Impact of Universal Immunohistochemistry on Lynch Syndrome Diagnosis in an Australian Colorectal Cancer Cohort

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Introduction

Lynch Syndrome (LS) is an autosomal dominant condition caused by a germline mutation in one of the DNA mismatch repair (MMR) genes: *MLH1, MSH2, MSH6, PMS2* or *EPCAM*. It is the most common genetic predisposition to colorectal cancer (CRC), underlying 2-4% of all CRC diagnoses[1], and is characterised by earlier onset and right-sided tumours with a microsatellite instability (MSI) phenotype. In addition, LS patients are at increased risk of endometrial, gastric, ovarian and several other cancers.

Screening for LS using immunohistochemistry (IHC) or MSI on the basis of age, family history or tumour histology has been shown to be inadequate[2–4]; studies of universal screening have found that of newly diagnosed LS cases, half are diagnosed after age 50, and almost a quarter do not meet the Amsterdam criteria or Bethesda guidelines[5]. As such, the Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group[6] and the National Comprehensive Cancer Network (NCCN)[7] now recommend screening for LS in all individuals with newly diagnosed CRC to reduce morbidity and mortality in their relatives. A stepwise screening approach is commonly used. Immunohistochemistry for MMR protein expression is a cost-effective, widely accessible initial screening test for LS[3,8], with excellent reported sensitivity. However, up to 85% of MSI CRCs are sporadic[9], most frequently due to acquired hyper-methylation of the *MLH1* promoter. Tumours with loss of

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MLH1 expression on IHC can be further screened for a $BRAF^{V600E}$ mutation or *MLH1* promoter hyper-methylation to exclude sporadic aetiology. Patients still suspected to have LS, where loss of expression of MMR proteins on IHC cannot be explained by a $BRAF^{V600E}$ mutation or *MLH1* promoter hyper-methylation should be referred to a genetics clinic for further evaluation and consideration of germline genetic testing.

In an attempt to identify patients with LS, our pathology service introduced pathologist-initiated MMR IHC during 2010, and subsequent universal screening in 2015. We investigated the impact of pathologist-initiated and universal screening with MMR IHC on screening rates, LS diagnoses, and rates of referral to and utilisation of the genetic counselling service in a large adult Australian cohort at a tertiary institution.

Methods

All cases of CRC that underwent bowel resection between 2010 and 2017 were obtained from the Austin Hospital pathology database in Melbourne, Australia. The Austin pathology department services a large proportion of the North East region of Victoria, including 2 large tertiary hospitals, a private metropolitan hospital and a regional hospital. Only pre-chemotherapy/radiotherapy resection specimens were included for analysis. Biopsies, polyps removed endoscopically, and tumours with a histological type other than adenocarcinoma were not included. The results of MMR IHC were obtained retrospectively from the

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pathology database electronic records. The Family Cancer Centre (FCC) genetics database was then searched for all cases with an abnormal MMR IHC result.

Immunohistochemistry for loss of expression of the MMR proteins was performed on the Ventana BenchMark ULTRA platform, using primary antibodies against MLH1 (clone ES05, diluted 1:25; Dako), MSH2 (clone G219-1129, Cell Marque), MSH6 (clone 44, Ventana) and PMS2 (clone EP51, diluted 1:25, Dako). Tumours were considered to be abnormal if there was complete loss of nuclear expression of one or more MMR proteins. All cases that underwent screening from 2010 to 2012 were tested for all four proteins. In 2013 there was a shift to a two-antibody panel (PMS2 and MSH6 only), based on studies demonstrating equivalent sensitivity to the four-antibody panel[10,11]; MSH2 or MLH1 IHC testing was subsequently preformed if an abnormal result was obtained for PMS2 or MSH6.

BRAF^{V600E} mutant protein detection was performed by IHC using the VE1 clone (Ventana), an anti-BRAF^{V600E} monoclonal antibody, and was scored as positive if there was diffuse positive cytoplasmic staining in the tumour cells, equivalent to positive control staining, with no staining in non-tumour tissues (internal negative control). Testing for BRAF^{V600E} mutant protein was performed for cases with loss of expression of PMS2 and/or MLH1 as part of the screening protocol. Tumours with loss of MMR protein expression and no detectable BRAF^{V600E} mutation were recommended for referral to the familial cancer clinical service in the pathology report.

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The Austin Familial Cancer Clinic (FCC) services the North East region of Victoria. Patients referred to the FCC were mailed a family history questionnaire to complete before being offered an appointment with a clinician and/or genetic counsellor. $BRAF^{V600E}$ mutation testing on tumour and/or germline genetic testing of the MMR genes were performed based on IHC results and family history. *MLH1* promoter methylation testing was performed in cases of absent MLH1/PMS2 staining where no $BRAF^{V600E}$ mutation was detected. At a minimum, sequential single gene sequencing and multiplex ligation-dependant probe amplification (MLPA) was performed of the heterodimer pairs (*MLH1*/*PMS2* or *MSH2*/*MSH6*) as directed by IHC testing. MLPA of *EPCAM* was performed for cases with absent MSH2 staining, except for one case which was found by sequencing to have a *MSH2* gene mutation. Due to the complicating pseudogenes, long range PCR of *PMS2* was also performed, as directed by IHC and methylation results. Multigene panel testing, including sequencing and MLPA for all of the Lynch Syndrome genes was introduced as standard in 2015. All genetic testing was performed in Australian NATA accredited laboratories. A diagnosis of Lynch Syndrome was made in cases where a germline MMR gene mutation was identified ,and Lynch-like syndrome was diagnosed in those with dMMR on IHC but no *MLH1* promoter methylation, $BRAF^{V600E}$ mutation, or identifiable germline mutation.

Descriptive statistics were used to analyse pathology and genetic testing results, as well as rates of FCC referral and follow up. The study was approved by the local institutional review board (LNR/18/Austin/308).

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Results

Between January 2010 and December 2017, 1171 consecutive adult patients met the inclusion criteria of having undergone surgical resection of a CRC. The median age at resection was 72 years (range 19-94). Eighty-three patients (7%) were less than 50 years of age; the majority of patients were greater than 60 years (51%). Rates of MMR IHC testing and loss of expression according to age group are presented in Table 1. The rate of MMR testing was lowest in 2011 (19%) and steadily increased to 98% in 2017 (Figure 1a). The increase in testing was greatest in the over 60 age group, while the rate of testing in the under 50 age group remained stable (Figure 1b). The incidence of dMMR also increased (4% in 2011 versus 21% in 2017), as did the rate of $BRAF^{V600E}$ mutation testing in MLH1/PMS2 absent tumours (0% in 2011 vs 80% in 2017).

An abnormal staining pattern on MMR IHC was found in 124 patients (Table 2). One hundred and five (85%) patients with abnormal MMR IHC had loss of PMS2 expression. Simultaneously loss of MLH1 was demonstrated in 86% of cases (90/105), 3% (3/105) retained MLH1 expression and no MLH1 testing was performed in 12/105 cases.. Of the 105 cases with PMS2 and/or MLH1 loss, 48 (46%) underwent $BRAF^{V600E}$ mutation testing at the time of IHC. $BRAF^{V600E}$ mutations were identified in 36 (75%) of the tested tumours. Of the 88 patients with either loss of MSH6, or loss of PMS2 and a negative/unknown $BRAF^{V600E}$ status, 44 (50%) were referred to the FCC; 29 attended an appointment. Germline genetic testing of the MMR genes was performed in 16 (55%) of the patients who attended the FCC;

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BRAF^{V600E} testing was performed in 9 previously untested patients and *MLH1* promoter hyper-methylation testing was performed in the remaining 4 patients. Fifteen patients were referred to the FCC but did not attend an appointment; these patients were contacted several times via phone and post, but either declined an appointment or failed to respond (Figure 3).. There were 44 patients not referred to the FCC; the majority (91%) were over 60 years of age. Thus in total, 59/88 patients with an abnormal IHC result did not undergo further evaluation. Five of these patients were under age 50: four had loss of expression of PMS2 on IHC with no detectable BRAF^{V600E} mutation, and one had loss of MSH6/MSH2 expression on IHC with a strong family history of CRC. Another six patients were between 50-60 years of age. Loss of MSH6/MSH2 expression occurred in 8/59 patients, including one known Familial Adenomatous Polyposis (FAP) patient and one known LS patient.

A total of 9 patients (1.3% of IHC tested, 0.8% of cohort) received a new diagnosis of Lynch or Lynch-like Syndrome. The median age of resection in this group was 72 years (range 35-90). A germline mutation was identified in 7 patients (4 *MSH6*, 2 *MSH2*, 1 *PMS2*); the other two patients had no identifiable germline mutation and were diagnosed with Lynch-like Syndrome (Figure 2). The first was a 71-year-old patient with loss of PMS2 staining, no BRAF^{V600E} mutation, and no detectable *PMS2* germline mutations. The other was a 62-year-old male with loss of MSH6 and MSH2 on IHC, but no detectable germline mutation in either gene. The number of new diagnoses per year are displayed in Figure 2. Families of the 7 patients newly diagnosed with Lynch Syndrome were offered genetic testing resulting in 2 subsequent diagnoses of Lynch Syndrome to date.

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The rate of testing, MMR IHC abnormalities and LS diagnoses in the two years pre and post implementation of universal testing were compared (Table 3). Rates of MMR testing, dMMR (17% vs 10%, p=0.02), BRAF^{V600E} testing (79% vs 25%, p<0.0001), and genetics referral (61% vs 33%, p<0.0001) were significantly greater post implementation, however FCC attendance rates (65% vs 67%, p =0.59) and LS incidence (5/321; 1.6% vs 0/320; 0%, p=0.06) were similar.

A subset of 206 cases from 2010 to 2014, enriched for cases with pathological features associated with LS (high grade or mucinous differentiation), underwent retrospective analysis for MMR IHC and BRAF^{V600E} mutation by a single gastrointestinal pathologist. Eighty-six (42%) had undergone MMR IHC testing during initial histopathological analysis. Of the remaining 120 cases, 25 (21%) had abnormal staining on IHC, of which 24/25 (96%) were over 60 years of age. All 25 cases had loss of PSM2 staining, and 21/25 were BRAF^{V600E} mutation positive. The four BRAF^{V600E} mutation negative cases were over 80 years of age; only one was subsequently referred to the FCC, but failed to attend any appointments. These results were not included in the results analysis of the overall cohort.

Discussion

In this large retrospective study of 1171 consecutive CRCs, the implementation of a universal screening policy resulted in greater detection of MMR deficient tumours, but did not increase the rate of Lynch Syndrome diagnosis. Despite higher rates of referral to the FCC and greater

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use of BRAF^{V600E} mutation analysis to refine the cohort of patients referred for genetic counselling, uptake of genetic testing remained low. Several changes to our institutions' screening policy have contributed to the pattern of IHC testing rates observed in Figure 1. Recommendations from the Cancer Council Australia in 2005 included screening of all CRC cases under 50 years of age. With the initial shift from clinician-initiated to pathologist-initiated testing, this guideline was applied, which unexpectedly led to a fall in testing in 2011. Soon after, screening was expanded to patients under 70 years old, in line with the Jerusalem Guidelines[12], and then to all CRC in 2015.

Previous studies have reported the advantages of universal screening[3,8], including identification of up to 50% more LS-affected patients and incremental cost-effectiveness[13]. In keeping with previous studies[14], our retrospective subset analysis suggests that screening based on histopathological features is unlikely to detect many more germline mutations than clinical criteria. We found that reflex IHC for MMR was feasible, with 98% of resected tumours tested in the 2nd year of implementation. However, the clinical benefit of screening is dependent upon the downstream management of abnormal results. Rates of referral to the FCC were worryingly low, an issue not unique to our cohort[15,16]. While reasons for this were not analysed in detail, in most cases it seems that there was either a low clinical suspicion of LS or the patient declined further investigation. Barriers to referral and uptake of genetic counselling have previously been explored; possible contributions include lack a of understanding of genetic syndromes and the benefits of testing[17–19], unfamiliarity with genetics services, and the burden of concurrent cancer treatment or other comorbidities.

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As IHC testing for MMR and $BRAF^{V600E}$ mutations expanded, the proportion of appropriate referrals to the FCC also increased. However, a similar proportion of patients declined or failed to attend a FCC appointment each year, suggesting that even more cases of LS may have been missed in the later years. For example, in 2016 to 2017 there were four cases under age 50 who declined or failed to attend the FCC: one with MSH6 loss on IHC and two with loss of MSH6/MSH2. We observed a much lower overall incidence of germline mutations in our cohort (0.6%), compared to the reported incidence (2.2%) in similar cohorts[5]. Although this was improved after introduction of universal testing (1.6%), it is still likely to be an underestimate of the true incidence.

$BRAF^{V600E}$ mutation testing was performed with increasing frequency during our study period, however one fifth of PMS2-negative tumours still did not have this tested reflexively during the last two years. Reflex $BRAF^{V600E}$ testing has been demonstrated to provide substantial time and cost savings[20,21], and would have spared nine patients in our study from requiring genetics input. Testing for the $BRAF^{V600E}$ mutation has been established as an important component in a cost-effective screening algorithm[13], and spares up to 40% of patients from unnecessary genetic testing[20]. Commercially-available monoclonal antibodies such as VE1 offer a rapid, convenient, and lower cost alternative IHC screening compared to the traditional PCR-based testing. While initial studies concluded that the sensitivity and specificity of $BRAF^{V600E}$ IHC was too low to be useful[22], subsequent studies have reported high sensitivity (96-100%) and specificity (98-100%) [23–27] using automated Ventana stainers,

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which were used in this study. Furthermore, BRAF testing has additional predictive and prognostic utility [28–30], playing a role in predicting response to both anti-EGFR therapies[31,32] and potentially immune checkpoint inhibitors[33].

The role of *MLH1* promoter methylation analysis is less clear, having not been well studied until recent years. During the study period, it was used in six cases to confirm sporadic aetiology where MMR IHC was abnormal but no $BRAF^{V600E}$ mutation was identified and the clinical suspicion of LS was low. Recent studies propose that *MLH1* methylation should be used instead of $BRAF^{V600E}$ due to its higher specificity (78% vs 40%)[34], and two studies have reported superior performance in terms of cost effectiveness and efficiency compared to $BRAF^{V600E}$ analysis[34,35].

The strength of this study is in its large population-based cohort across a number of different practice settings over a period of 8 years, during which several changes to recommended LS screening protocols occurred. The testing and diagnosis rates are therefore reflective of real-world practice. The limitations of this study include its retrospective nature and inclusion of only a single pathology service from a single state. It is entirely possible that patients and family members may have attended other Clinical Genetics services and were therefore not captured in the FCC database. Additionally, as the true incidence of IHC abnormalities and germline mutations remains unknown, we are unable to make definitive conclusions about the effect of these policy changes.

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In conclusion, our study suggests that universal screening using MMR IHC followed by analysis for $BRAF^{V600E}$ mutation and/or *MLH1* hyper-methylation has the potential to increase the identification of patients at risk of Lynch Syndrome, and should be implemented where possible. However, the full benefit of universal screening was limited by low uptake of genetic testing, and further strategies are needed to overcome these barriers.

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Figure legends

Figure 1.a) Overall rate of mismatch repair testing by immunohistochemistry, and b) by age group

Figure 2. Number of new diagnoses of Lynch Syndrome and Lynch-like Syndrome each year

Figure 3. Management of patients referred to the Familial Cancer Clinic

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Table 1. Rates of mismatch repair testing and loss of expression

Age group	Number of patients	MMR tested	MMR loss (if tested)	Lynch/Lynch-like Syndrome
All ages	1171	680 (58%)	124 (18%)	9 (1.3%)
<50	83	81 (98%)	8 (10%)	3 (3.7%)
50-60	196	148 (76%)	14 (9.5%)	2 (1.4%)
>60	892	451 (51%)	102 (23%)	4 (0.9%)

MMR = mismatch repair

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Table 2. Patients with abnormal mismatch repair immunohistochemistry on initial testing

				PMS2 loss only†			
	Number of patients	**PMS2 loss**	**MSH6 loss**	**BRAF mutant**	**BRAF wildtype**	**BRAF not tested**	**Referral to genetics**‡
All	124	105 (85%)	19 (15%)	36	12	57	44/88 (50%)
<50	9	3 (33%)	6 (67%)	0	2	1	7/9 (78%)
50-60	13	7 (54%)	6 (46%)	0	2	5	11/13 (85%)
>60	102	95 (93%)	7 (7%)	36	8	51	26/66 (39%)

† $BRAF^{V600E}$ immunohistochemistry was performed in cases with PMS2 loss
‡ $BRAF^{V600E}$ mutant patients excluded from denominator

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Table 3. Comparison of outcomes in the two year periods before and after implementation of universal screening in 2015

	2013-2014	2016-2017	p value
Total patients	320	321	
MMR tested	170 (53%)	295 (92%)	**<0.0001***
Number of dMMR	33	54	
Incidence of dMMR	33/320 (10%)	54/321 (17%)	**0.016***
BRAF testing *in PMS2 deficient*	8/32 (25%)	34/43 (79%)	**<0.0001***
BRAF mutation *in PMS2 deficient*	6/32 (19%)	26/43 (60%)	**<0.0001***
Referral to FCC†	9/27 (33%)	17/28 (61%)	**<0.0001***
Attendance at FCC	6/9 (67%)	11/17 (65%)	0.59
New LS cases	0	5	
LS incidence	0%	1.6%	0.06

† BRAF mutant excluded
dMMR = mismatch repair deficiency; FCC = Family Cancer Clinic; IHC = immunohistochemistry; LS = Lynch Syndrome

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Abstract

Background: Current guidelines recommend a step-wise screening algorithm for all colorectal carcinomas (CRC) to identify patients with Lynch Syndrome (LS).

Aim: We describe the frequencies of mismatch repair deficiency (dMMR), BRAF^{V600E} mutations and *MLH1* methylation in resected CRCs, and evaluate the impact of universal screening on LS detection.

Methods: Retrospectively, 1171 consecutive cases of resected CRC were identified between 2010 and 2017 from a large multi-centre pathology service. Testing for dMMR by immunohistochemistry (IHC) was initiated by the reporting pathologist from 2010, until universal testing was introduced in 2015. Patients with dMMR were referred to the Family Cancer Clinic (FCC) for consideration of germline mutation analysis.

Results: IHC was performed on 680 tumours, with abnormal expression in 124 (18%). Referral to FCC was made for 44 of the 88 patients with abnormal IHC (excluding those with BRAF^{V600E} mutations). Of the 29 who attended, 16 underwent germline genetic testing, and LS was diagnosed in 7 with a germline mutation. After implementation of universal testing, there was a greater incidence of dMMR (17% vs 10%, p=0.02), rate of BRAF^{V600E} testing (79% vs 25%, p<0.0001), and referral to FCC (61% vs 33%, p<0.0001), but no difference in FCC attendance rate (65% vs 67%, p =0.59) or new LS diagnoses (1.6% vs 0%, p=0.06).

Conclusion: Universal IHC testing may increase the detection of LS, and should be implemented where possible. However, the full benefit was limited by low referral to and uptake of genetic testing, and further strategies are needed to overcome these barriers.

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Key words: Lynch Syndrome, immunohistochemistry, mismatch repair, universal screening, genetic counselling

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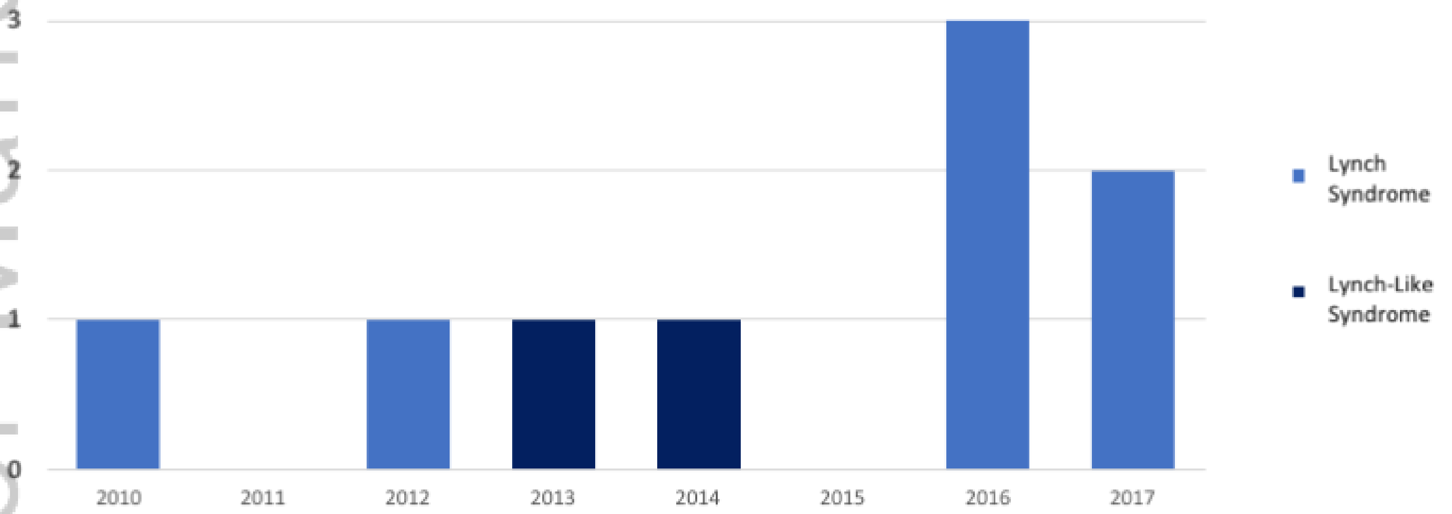

IMJ_14230_Fig2 IMJ LS.png

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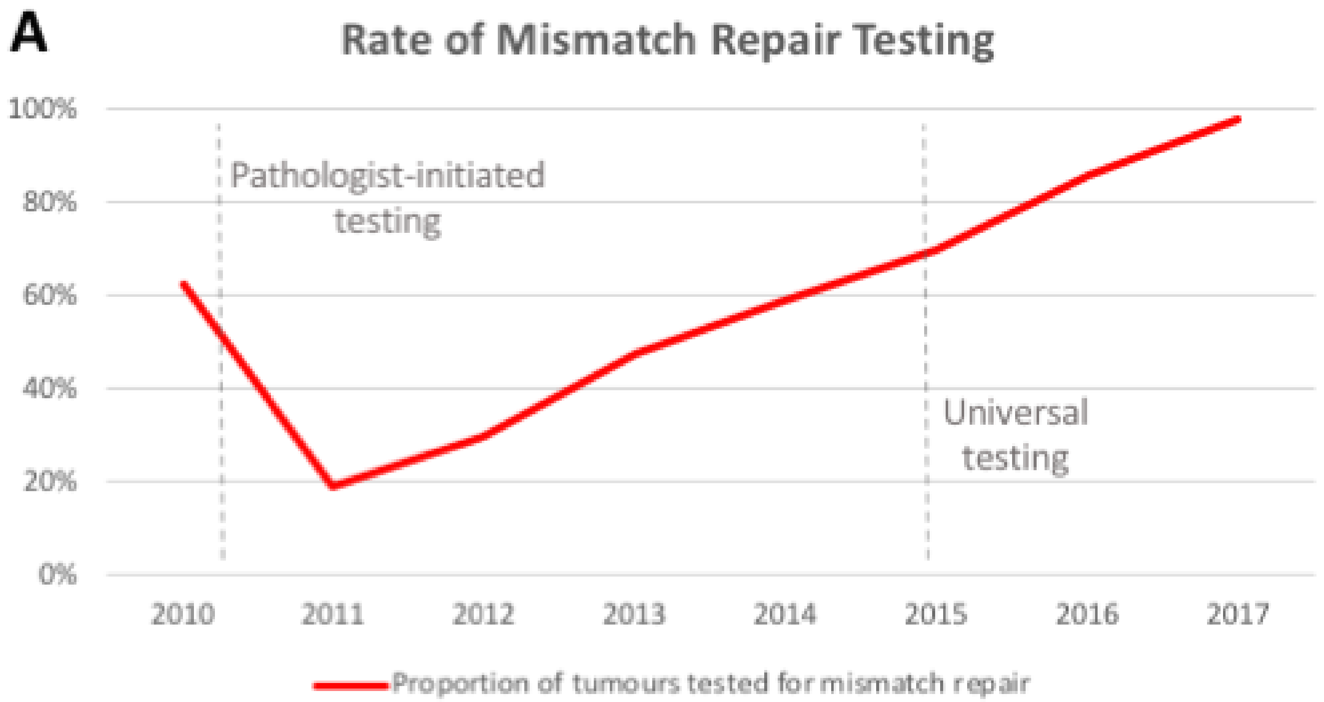

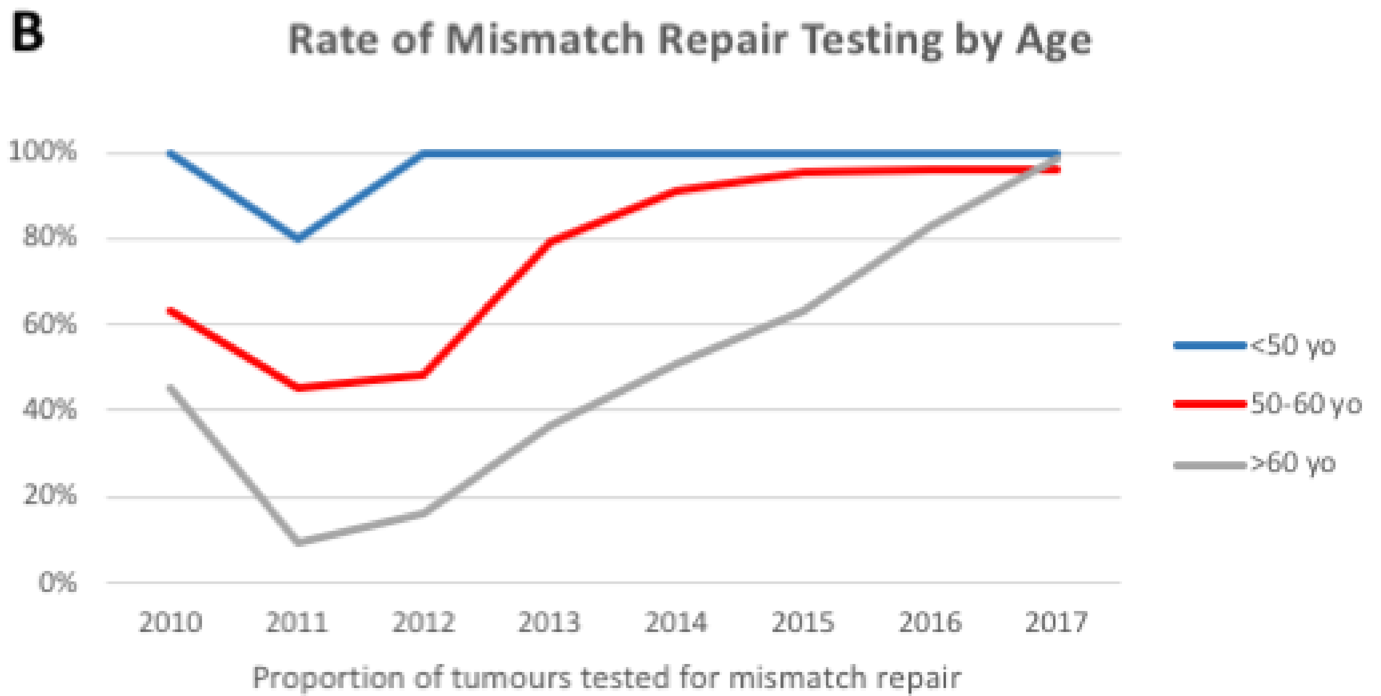

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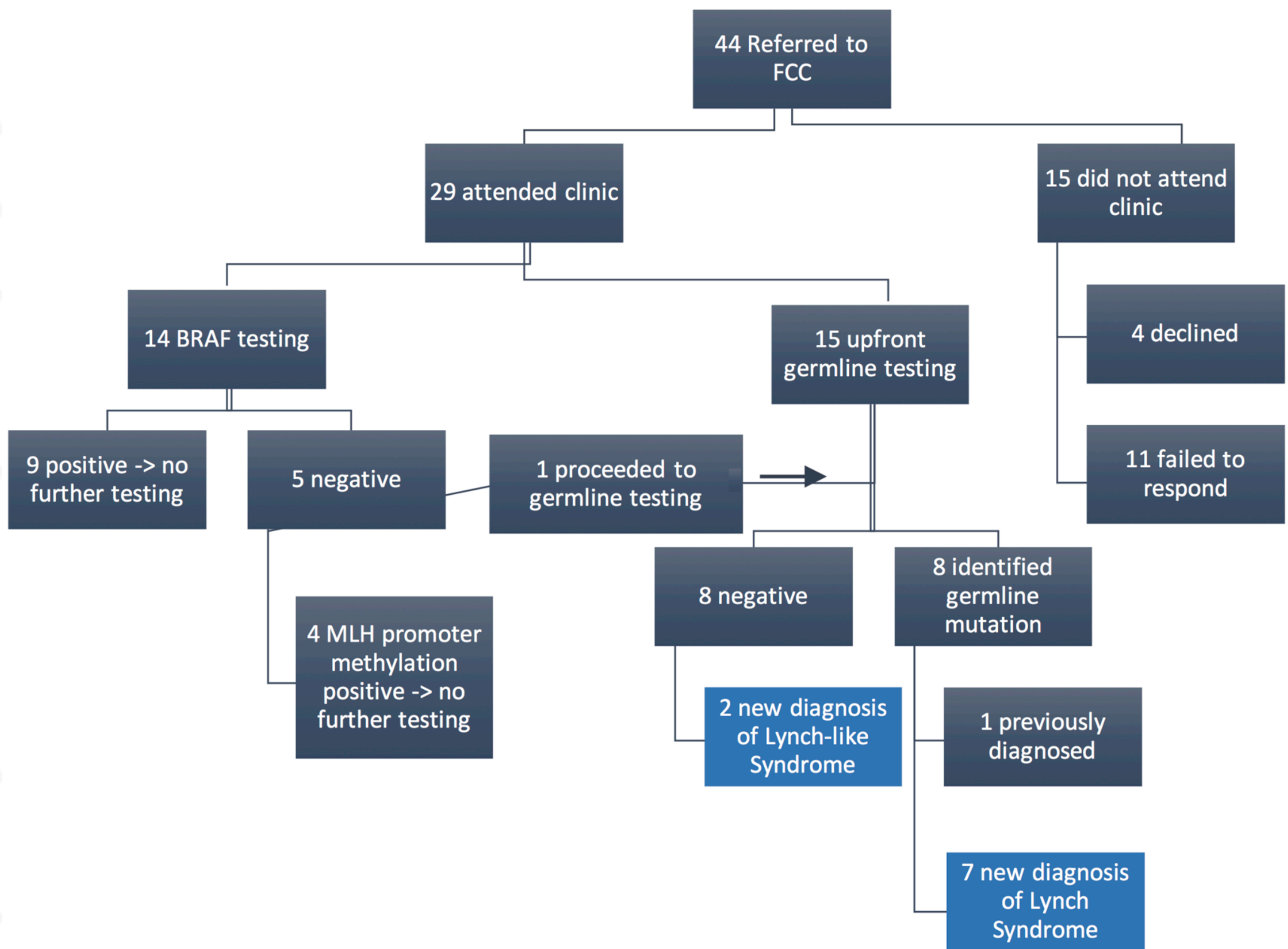

IMJ_14230_Figure3 IMJ LS.png

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Title: The Impact of Universal Immunohistochemistry on Lynch Syndrome Diagnosis in an Australian Colorectal Cancer Cohort

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