Implementation of a Lynch Syndrome Screening Protocol on Colorectal Cancer Patients 70 years of Age and Under: The Initial Manitoba Screening Experience

by

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ABSTRACT

Lynch Syndrome (LS) is responsible for 1-6% of colorectal cancer (CRC). It is associated with mutations in genes involved in deoxyribonucleic acid mismatch repair (MMR), specifically altering the function of proteins MutL homolog 1 (MLH1), MutS protein homolog 2 (MSH2), MutS homolog 6 (MSH6), and Postmeiotic segregation increased 2 (PMS2).

Objective: To assess the ability of a recently instituted Lynch Syndrome (LS) screening protocol in Manitoba to identify potential carriers as compared to literature.

Methods: Three hundred sixteen CRC resection tumour cases, patient age ≤70, were screened for a LS profile. Immunohistochemistry for MMR deficiency (MMRD), v-raf murine sarcoma viral oncogene homolog B1(BRAF) sequencing, and microsatellite instability strategies were used to determine a likelihood of LS. Genetic counselling (GC) was recommended to 'high-likelihood' LS cases.

Results: MMRD was reported in 50 (16%) cases; 3 (0.9%) deficient*(d)* MSH6, 8 *d*MSH2/*d*MSH6 (2.5%), 5 (1.6%) *d*PMS2, and 34 (11%) *d*MLH1/*d*PMS2. Eleven *d*MLH1/*d*PMS2 cases (3.5%) were BRAF-wildtype (WT). GC contacted 26 (96%) of 27 HL LS patients; 17 had germline testing; 9 (2.8%) LS related mutations were identified. MMR intact with high-risk feature(s) was reported in 63 cases; 1 case was MSI-high (MSI-H).

Conclusion: A confirmed LS mutation in 2.8% of cases is a percentage comparable to literature and supports continued use of the protocol.

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LIST OF ABBREVIATIONS

AJCC	American Joint Committee for Cancer
AM	Amsterdam Criteria
AM II	Amsterdam II Criteria
BG	Bethesda Guidelines
BCCA	British Columbia Cancer Agency
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CE	Cannot exclude
CIQC	Canadian Immunohistochemistry Quality Control
CRC	Colorectal Cancer
*d*MLH1	MutL homolog 1 deficient
*d*MSH2	MutS protein homolog 2 deficient
*d*MSH6	MutS homolog 6 deficient
*d*PMS2	Postmeiotic Segregation Increased 2 deficient
DSM	Diagnostic Services of Manitoba
EC	Endometrial Cancer
EGAPP	Evaluation of Genomic Applications in Practice and Prevention
EpCam	Epithelial Cell Adhesion Molecule

GC	Genetic Counselling
GS	Genetic Sequencing
HL	High likelihood
HNPCC	Hereditary Nonpolyposis Colon Cancer
IHC	Immunohistochemistry
IBD	Inflammatory Bowel Disease
LIS	Laboratory Information System
LLS	Lynch-like Syndrome
LS	Lynch Syndrome
MLH1	MutL homolog 1
MSH2	MutS protein homolog 2
MSH3	MutS protein homolog 3
MSH6	MutS homolog 6
MSI	Microsatellite Instability
MSS	Microsatellite Stability
MMR	Mismatched Repair Protein
MMRD	Mismatched Repair Protein Deficiency
PMS2	Postmeiotic Segregation Increased 2

RBG	Revised Bethesda Guidelines
TNM	TNM classification of malignant tumours
UICC	Union for International Cancer Contro

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Colorectal Cancer

Colorectal cancer (CRC) is the 2nd most commonly diagnosed cancer of Canadian men and the 3rd most commonly diagnosed cancer of Canadian women[1]. The Canadian Cancer Society estimates that 1 in 13 males and 1 in 15 females will be diagnosed with CRC in their lifetime[1]. From 1999-2009 it was estimated that 105,000 Canadians had been diagnosed with CRC[1]. It was estimated that 25,100 Canadians will developed colorectal cancer in 2015 and 9,300 will die of the disease[1].The overall five-year survival rate following diagnosis of CRC is 64%[1].

'Western' countries, like the United States, Australia, and Canada have a higher rate of incidence of colon and rectal cancer[2,3]. It has been suggested that the high calorie diet abundant in animal fat common to these countries contribute to the development of CRC[2,3]. The World Health Organization has noted that the introduction of 'western' diet trends into areas with previously low colorectal cancer incidence rates correlates with a marked increase in CRC incidence rates[2]. Although there is evidence to support that a portion of CRCs are hereditary, studies have indicated that obesity, smoking, red meat consumption, alcohol consumption and a sedentary lifestyle are major risk factors[2,3].

1.1.1 Tumour Locations

Colorectal cancer can be found along the entire length of the lower gastrointestinal (GI) tract, including the cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and the rectum[3]. This portion of the GI system functions to absorb excess water, salt, and fat-soluble vitamins from solid undigested food materials passed from the small intestine[4]. The lower portions, including the descending colon, sigmoid colon, and rectum, also serve as storage for and elimination of waste[4].

The cecum, a peritonealised blind-ended pouch-like structure measuring between 3-8 cm in adults, is the first section of the colon[4]. The cecum receives undigested food material from the small intestine. From the cecum, the first portion of the large intestine, the ascending colon, arises.

The ascending colon runs, for approximately 20 cm, superiorly along the right side of the abdominal cavity[4]. The posterior ascending colon remains partially attached to the posterior abdominal wall by loose connective tissue and the anterior and lateral aspects are covered in peritoneum pinning it to the posterior wall[4]. At the site of the liver, the ascending colon makes an abrupt 90 degree turn (hepatic flexure) and begins to run horizontally as the peritonealised transverse colon[4].

The transverse colon is attached to the stomach by a band of fatty tissue called the greater omentum and at the spleen it turns 90 degrees (splenic flexure)[4]. It begins to run along the left side of the abdominal cavity as the descending colon.

The descending colon, approximately 30 cm in length, functions to store faeces that will be emptied into the rectum for future waste expulsion[4]. Like the ascending colon, the descending colon is partially peritonealised, with the posterior aspect remaining attached to the posterior abdominal wall[4]. The descending colon is connected to the rectum by the peritonealised sigmoid colon. The sigmoid colon, along with the rectum, functions to transmit stool toward the anus, to act as collection site and to produce defecation.

The colon and rectum are made up of different tissues that are organized into layers: the mucosa, the submucosa, the muscularis propria, and the serosa (colon only)[3-5]. The mucosa, the innermost lining, is made up of epithelium, lamina propria, and a thin layer of muscle called the muscularis mucosa[3-5]. The submucosa is largely made up of connective tissue, glands, blood and lymphatic vessels, and nerves[3-5]. The muscularis propria layer functions for colonic contraction and consists of an inner circular layer and an outer longitudinal layer[3-5]. The World Health Organization defines colorectal cancer as "a malignant epithelial tumour originating in the large bowel . . . [which] requires invasion through the muscularis mucosae into the submucosa"[2].

1.1.2 Symptoms

Like other diseases, there are symptoms associated with colorectal cancer that can be indicative of the illness; CRC can also by asymptomatic and discovered through screening and surveillance[2,3]. Nonspecific manifestations of CRC can include fever, malaise, weight loss, and abdominal pain[2,3]. More specific symptoms include a change in bowel habits, blood in stool, anaemia, constipation, abdominal distention, bowel obstruction, bowel perforation, and a frequent feeling of or need to evacuate the bowels[2]. These symptoms are used to plan further investigative analysis to determine the cause.

1.1.3 Diagnosis

There are several investigative tools which can be used to guide clinicians to an eventual CRC diagnosis. Investigative procedures, such as colonoscopy and a digital rectal exam are used to visualize and palpate the presence of polyps or masses, respectively. Colonoscopy allows for an internal examination of the mucosal surfaces of the rectum and entire large bowel. A flexible, slender tube attached to a video camera is used to examine the internal surfaces of the large bowel, looking for abnormalities[2]. Colonoscopy allows samples to be taken from suspicious looking areas that can be sent for further pathologic analysis[2,6-9]. If a lesion is small and relatively superficial, meaning it does not appear to extend through the layers of the colon, endoscopic removal of the lesion may be the only required treatment[2,6-9].

Non-invasive techniques such as computer assisted tomography, magnetic resonance imaging, and trans-rectal ultrasonography, can also be used to obtain a visual representation of a

patients' body[2]. These testing strategies, along with positron emission tomography, are also useful in evaluating the stage of a tumour. Magnetic resonance imaging and ultrasonography create cross-sectional images that allow the depth of tumour invasion to be estimated. To evaluates stage of the disease, scintigraphy and positron emission tomography methods use radioactive tracers to target and measure molecules present in certain tissue types within the body[2].

1.1.4 Tumour Staging

Tumour staging is conducted to evaluate the extent of the disease and will determine prognosis and further treatment recommendations[2,9,10]. The TNM classification of malignant tumours (TNM) staging system is used to define the extent of invasion of colorectal cancer[9,10]. This system, developed by the American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC), is widely used and considered to be the most descriptive classification system, uses anatomical markers to evaluate the extent of the disease[10]. The TNM staging system considers: the extent of the primary tumour (T), the absence/presence and extent of regional lymph node metastasis (N), and the absence/presence of distant metastasis (M). The tumour stage is determined by the assignment of a number to each of TNM categories (T0, T1, T2, T3, T4 – N0, N1, N2, N3 – M0, M1)[10]. Tables 1 through 3 display AJCC/UICC TNM staging parameters.

Table 1. Summary of tumour (T) clinical and pathological classifications of colon and rectum tumours based on facts from The International Union Against Cancer TNM Classification of Malignant Tumours 7th ed. 2009[10].

T – Primary Tumour			
TX	Primary tumour cannot be assessed		
T0	No evidence of tumour		
Tis	Carcinoma in situ: intraepithelial or lamina propria invasion		
T1	Tumour invades submucosa		
T2	Tumour invades the muscularis propria		
T3	Tumour invades subserosa *or* Non-peritonealized pericolic or perirectal tissues		
T4	Tumour directly invades other organs or structures *and/or* Perforates visceral peritoneum	T4a	Tumour perforates visceral peritoneum
		T4b	Tumour directly invades other organs and structures

Table 2. Summary of Lymph Node (N) clinical and pathological classifications of colon and rectum tumours based on facts from The International Union Against Cancer TNM Classification of Malignant Tumours 7th ed. 2009[10].

N – Regional Lymph Nodes			
Nx	Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastasis		
N1	Metastasis in 1-3 regional lymph nodes	N1a	Metastasis in 1 regional lymph node
		N1b	Metastasis in 2-3 regional lymph node
		N1c	Tumour deposit(s) in the subserosa, or in non-peritonealized pericolic or perirectal soft tissue without regional lymph node metastasis
N2	Metastasis on 4 or more regional lymph nodes	N2a	Metastasis in 4-6 regional lymph node
		N2b	Metastasis in 7+ regional lymph node

Table 3. Metastasis (M) clinical and pathological classifications of colon and rectum tumours based on facts from The International Union Against Cancer TNM Classification of Malignant Tumours 7th ed. 2009[10].

M – Distant Metastasis	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis is present
M1	Distant metastasis is present

The UICC outlines clinical and pathological classifications of tumours[10]. Clinical classification is a pre-treatment evaluation used to assess stage and determine therapeutic options, specifically local endoscopic therapy or CRC resection for localized disease, and chemotherapy for metastatic disease[10]. Physical examination and imaging are important in determining the clinical stage. Pathological stage refers to the postsurgical histopathological evaluation; it is used to estimate prognosis and determine further treatment[10].

Colorectal cancer can be placed into one of five stages according to the AJCC/UICC TNM: Stage 0, Stage I, Stage II, Stage III, , and Stage IV. Stage 0 references carcinoma in situ (Tis), Stages I tumours are restricted to the colon wall (T1, T2), Stage II tumours penetrate though the wall with negative lymph nodes (IIA (T3), IIB (T4a), IIC (T4b)), Stage III are those with spread into local lymph nodes (IIIA (T1-T2, N1/N1c or T1, N2a), IIIB (T3-T4a, N1/N1c or T2-T3, N2a or T1-T2 N2b), IIIC (T4a, N2a or T3-T4a, N2b or T4b, N1-N2)), and Stage IV are tumours with distant metastasis (IVA (any T, any N, M1a), IVB (any T, any N, M1b))[10].

The stage of the tumour is prognostically important, however, it should be recognized that other factors such as microscopic cellular appearance and behaviour contribute to prognosis[2.9,10]. A four-category (G1-G4) grading scale describes the extent to which tumour cells vary from normal colorectal tissue [2]. Cells from G1 are well differentiated, more closely resemble that of normal colorectal tissue and are considered ‘low grade’ [2]. G2 cells are moderately differentiated and G3 cells are poorly differentiated, where G4 cells are

undifferentiated[2]. G1 and G2 categories are often grouped together as 'low grade and G3 and G4 are 'high grade'[2]. Generally, prognosis is better for low-grade cancers than it is for high-grade cancers of the same stage[2].

1.1.5 Types of Colorectal Cancer

More than 90% of colorectal carcinomas are adenocarcinomas that originated from colorectal mucosa epithelial cells[2,9]. Most of these have arisen from non-invasive precursor lesion, so called colonic adenomas[2,9]. An adenoma is a pedunculated or sessile benign tumour of epithelial cells with glandular origin, or which displays glandular characteristics[9]. Adenoma development can be sporadic but the risk of developing an adenoma increases after the age 50[9]. It is estimated that half of adults in Western countries will develop an adenomatous polyp, with 10% becoming malignant[9]. Certain bowel diseases and genetic predispositions can further increase the risk of colorectal cancer development.

Inflammatory bowel disease (IBD) is a group of inflammatory conditions affecting the bowel, and in some cases other segments of the gastrointestinal tract. The term IBD includes ulcerative colitis and Crohn disease[2,3,9]. Chronic inflammation of the mucosa and submucosa of the colon and rectum can result in dysplastic changes[2,9]. These changes sometimes result in the formation of flat, raised, polypoid, or subtle velvety appearing lesions within the inflamed areas [2,9]. It is these areas where CRC will develop; those patients with ulcerative colitis have a 5-30% chance of developing CRC and those with Crohn disease are three times more likely to develop CRC over those without a IBD[2]. While the exact cause of IBD is unknown, there is strong

evidence that environmental factors combine with hereditary genetic predisposition to produce the disease.

There are several known hereditary genetic disorders that greatly increase risk of colorectal carcinoma development within the patients' lifetime; these disorders include Familial Adenomatous Polyposis (FAP), Peutz-Jeghers Syndrome, Juvenile Polyposis Syndrome, and Lynch Syndrome (LS)[2,3,9]. LS is the focus of this project it is described in length in the literature review to follow.

1.2 Lynch Syndrome

Lynch syndrome is an autosomal dominant genetic condition characterized by early onset (<50 years of age) and development of colorectal cancer, endometrial cancer (EC), and cancers of the stomach, ovary, ureter, renal pelvis, brain, small bowel, pancreas, and biliary tract[7,11-16]. It is primarily the result of germline mutations in genes which code for specific mismatch repair proteins (MMR) and is associated with microsatellite instability (MSI)[15]. The term LS has previously been synonymous with hereditary non-polyposis colorectal cancer (HNPCC), however, a more recent line of thought separates the two based on identification methods[16]. LS is defined genetically through confirmation of MMR germline mutations, whereas HNPCC is defined through recorded and reported ancestry, a pedigree, of a patient[16]. It should be noted that some familial disorders strongly mimic LS clinical features, but without a germline mutation they cannot be identified as LS. Instead these disorders are referred to as Lynch-like syndrome (LLS) and may be due to somatic mutations[16].

LS is a type of cancer syndrome suspected to be the cause of an estimated 1-6% of all colorectal cancers[11,12,15,16]. While individuals with LS may never develop a colorectal carcinoma, they have up to an 82% lifetime risk of CRC development at a mean age of 44-61 years at diagnosis[7,11,15,17].

Carcinogenesis of LS associated CRC, adenoma to carcinoma, occurs at a rapid pace, 1-3 years, compared to MMR-intact tumours,1-2 decades[7,16,18]. These tumours can appear mucinous, are of signet ring cell or medullary histologic type, are poorly differentiated, have a lymphocytic

infiltrate or are rimmed by a Crohn-like germinal centre-producing lymphoid reaction[9,15,17,19]. Clinical data, such as suspicious histology, in combination with a heavy reliance on family history has been the primary method of LS detection and diagnosis in the past[17]. A greater understanding of the genetics of LS syndrome and its relationship with MMR proteins has allowed the development of cost effective strategies for Lynch screening[20, 21, 22].

The MMR genes highly associated with LS are the MutL homolog 1 (MLH1), MutS protein homolog 2(MSH2), MutS homolog 6 (MSH6), and Postmeiotic segregation increased 2 (PMS2). These genes are responsible for identifying and repairing single nucleotide mismatches which occur during cell growth and division [7,11,12,15].

MLH1 and MSH2 pathogenic variants account for approximately 90% of LS cancers[7,11,16,17]. Patients with a MLH1 or MSH2 mutation present with cancers at a younger age, 40-50 years[16]. MSH6 and PMS2 pathogenic variants are observed in <10%[16,17]. These patients are diagnosed with CRC between ages 50-65 and the variants are associated with a higher rate of endometrial cancer[16]. MMR proteins form functional heterodimer complexes, a protein complex composed of two different polypeptide chains, with one another which recognize and repair base-base mismatches and insertion/deletion loops ([23], [12], [16]). MSH2 can bind with MSH6 or MSH3 and MLH1 protein complexes with PMS2 or PMS1. Recognition of base-base mismatches and insertion/deletion loops is performed by the heterodimer of MSH2-MSH6, called the MutSα heterodimer[12,16,17,23]. Repair of the base-base mismatches and insertion/deletion loops, is primarily managed by MLH1-PMS2, the MutLα heterodimer[12,16,17,23]. MLH1-PMS2

and MSH2-MSH6 are obligatory complexes, where MLH1 and MSH2 stabilize PMS2 and MSH6, respectively[12,16,17,23]. While MLH1 and MSH2 can form complexes with other MMR proteins, called secondary heterodimer pairings, loss of MLH1 results in the degradation of PMS2 and, similarly, loss of MSH2 leads to MSH6 degradation[12,16,17,23]. The heterodimeric nature of these key MMR provides a clinical utility which can be harnessed to determine the probability of LS.

When MMR genes are mutated, as in LS, mistakes in nucleotide sequences, such as insertion and deletion exon loops, are not removed and corrected. Failure of MMR proteins to correct errors that occur during DNA replication inflicts genomic instability, resulting in short tandem repeats of DNA, known as microsatellites. Accumulation of microsatellites results in microsatellite instability (MSI)[16]. Fifteen percent of CRC demonstrate MSI, of only approximately 3% total are due to LS[14,16]. This is because most MSI relates to somatic hypermethylation of MMR genes not germline mutation[14,16].

Identifying MLH1 promoter hypermethylation as the basis for tumour formation can eliminate a LS diagnosis. Most hypermethylation occurs when a methyl group is added to a DNA nucleotide of the promoter region of MLH1. This process, which silences gene expression in the tumour tissue, yields MSI[24-27]. Some authors have suggested that a LS cannot be eliminated solely by the presence of MLH1 promoter hypermethylation[14,28]. Niessen et al (2009) suggest MLH1 promoter hypermethylation can be a "second hit" event in an individual with a pre-existing germline MLH1 single allele pathogenic variant[14], if this appears it is a rare

phenomenon. Methylation of the remaining functional allele results in the homozygous inactivation of the gene resulting in tumour development[14]. Somatic methylation of non-MLH1 functional alleles has been observed, however, they are not known causes of sporadic CRC[26]. Therefore, one strategy used to eliminate LS in MLH1 patients is hypermethylation screening[14,26].

A second strategy for ruling out sporadic MHL1 deficient tumours is BRAF analysis. A specific BRAF mutation, BRAFV600E, is strongly associated with MSI, MLH1 methylation in sporadic MSI tumours [25,26,29]. Considering this, a BRAF mutation is strong evidence against a MLH1 mutation, and presence of a mutation can eliminate the need for further genetic testing. A BRAF-WT results in an MLH1d tumour means the patient remains at risk for LS and that genetic testing is required. BRAF-WT status is very specific for LS in this scenario, but is not particularly sensitive, as many patients who are MLH1 and BRAF-WT do not have LS[24,30]. However, like patients with 'high-risk' MMR, deficient patients of this type are considered to be Lynch-like in the absence of a genetic mutation[16]. BRAF mutation specific immunohistochemistry antibodies have been developed which have good concordance with traditionally used molecular/stain based techniques[24,26].

BRAF is a gene which helps to regulate cell growth and division. Up to 79% of BRAF mutations are associated with sporadic tumours demonstrating a MLH1 promoter hypermethylation[24]. The most common BRAF mutation, V600E, is also present in 10-20% of CRCs and is highly associated with MSI-H tumours[26,31]. A BRAF V600E mutation is considered

to be rare in LS cancers; its presence decreases the likelihood of LS [26,30]. Sinicrope et al (2013) determined IHC can be used to verify the presence of or absence of a BRAF V600E mutation by demonstrating the ability of a BRAF mutation-specific (V600E) antibody to detect tumour with BRAF mutations to have complete concordance with a DNA-based method[31].

Hypermethylation of MSH2 as the result of epigenetic silencing due to deletions in the 3' end of epithelial cell adhesion molecule (EpCAM) is a novel cause of LS[14,27,28]. A germline deletion in EpCam, a transmembrane glycoprotein which lies next to MSH2 on chromosome 2, accounts for approximately 2-3% of LS cases[14,27,28]. While the gene itself is not involved in the processes involved in impaired DNA repair, deletions in the 3' end of EpCAM gene and consequent inactivation of MSH2 interrupts DNA repair processes of MSH2; this results in an observed *d*MSH2 on IHC testing, indicating LS[14,15,17,28].

Understanding the hereditary nature of LS and mechanisms by which MMRD occurs allows for development of LS identification methods and strategies. Considering the increased lifetime risk of cancer development associated with LS and the genetic implications, identification of persons with the syndrome is advantageous for that person and their relatives.

1.2.1 Methods of Identifying and Diagnosing Lynch Syndrome

1.2.1.1 *Amsterdam II Criteria and Bethesda Guidelines*

The diagnosis of LS has previously relied heavily on self-reported family medical histories, as outlined by the Amsterdam Criteria (AM) and Bethesda Guidelines (BG)[17]. These guidelines have been criticized for their lack of ability to correctly diagnose the syndrome[31].

The AM and the BG documents primarily rely on family medical history to determine the likelihood of LS[32-37]. Low sensitivity has called into question the reliability of these guidelines to identify LS[32]. According to the most current publication of the AM II, a revised version published in 1999, a diagnosis of LS requires evidence of the following: (1) 3 or more relative with an associated cancer, (2) 2 or more successive generations much be affected by said cancer, (3) 1 or more relatives must have been diagnosed with this cancer before 50 years of age, (4) 1 should be a first degree relative of the other two, (5) familial adenomatous polyposis (FAP) should be excluded, and (6) tumours should be verified by pathologic examination[34]. A list of these criteria can be viewed in Table 4.

Table 4. Clinical indications of Lynch Syndrome as outline by the Amsterdam II and revised Bethesda guidelines[34].

Amsterdam II Criteria	Revised Bethesda Guidelines
1. At least 3 relatives with a LS associated cancer[a]	1. CRC diagnosed is a patient who is less than 50 years of age
2. 1 relative is a first degree relative[b] of the other two	2. CRC with a synchronous, metachronous or other Lynch-associated malignancy[a] regardless of age
3. 2 successive generations affected (minimum)	3. CRC with microsatellite instability-high in a patient who is less than 60 years of age
4. 1 individual (minimum) was diagnosed before 50 years	4. CRC in more than 1 first degree relative[b] with a LS-related tumour with one cancer diagnosed at less than 50 years of age
5. FAP is excluded in the individuals with CRC	5. 5. CRC in more than 2 first or second degree relatives, regardless of age
6. Tumours should be verified by pathologic examination	

a. Lynch-associated malignancies include CRC, endometrial, ovarian epithelial, stomach, pancreas, ureter and renal pelvis, biliary tract, brain, sebaceous gland adenomas and keratocanthomas and carcinoma of the small bowel.

b. First degree relatives include parents, siblings, and children. Second degree relatives are aunts/uncles, nieces/nephews, grandparents, and grandchildren

The National Cancer Institute published a set of recommendations, the Bethesda guidelines, in 1997[19,34]. These Guidelines, most recently updated in 2004 titled revised Bethesda Guidelines (RBG), were developed based on a superior understanding of clinical and histologic presentation of LS to provide a more sensitive set of guidelines[19,34.37]. These guidelines are used to assess those patients which do not meet AM II to determine which patients would benefit from molecular testing[34]. The criteria include the following elements: (1) diagnosis of CRC in a patient less than 50 years of age, (2) presence of synchronous or metachronous CRC or other LS associated tumours (regardless of age), (3) a CRC demonstrating histology of high microsatellite instability in a patient less than 60 years old, (4) 1 or more first-degree relative less than 60 years old diagnoses with CRC LS associated tumours, and (5) CRC diagnosed in two or more first-degree relatives (regardless of age) with LS associated tumours[19,34]. These criteria are listed in Table 5.

When all elements of the AMII or RBG criteria are fulfilled, they can act as significant predictor of a germline pathogenic variant in an MMR gene. However, frequently not all elements are met by or known to a patient reporting a family history. This lowers the sensitivity and specificity of the guidelines, especially when compared to other screening methods. Alternative methods are discussed later. They both fail to identify a large portion of individuals with an MMR mutation, low sensitivity, and include many individuals without LS, low specificity[33-36]. The criteria have been critiqued over their lack of sensitivity, meaning their ability to correctly identify individuals with the syndrome. Sjursen et al (2010) reports sensitivity of the AM II to be 87%, 62%, 38%, and 48% when identifying an individual with a MLH1, MSH2, PMS2, or MSH6 MMR germline variants, respectively[32]The RBG are reported to have a

higher sensitivity than the AM II[35], which is sited to range from 50% to 94%[33,35,36]. A study conducted by Tranø et al (2010) compared the molecular tumour testing results to evaluate the performance and accuracy of the RBG and AM II in 336 CRC patient tumour tissues. Eighty-seven patients fulfilled the RBG, 8 patients met AM II, and molecular testing identified 12 tumours as possible LS[33]. The RBG were revealed to have a 50% sensitivity when only 6 of the 12 molecular tested tumours met the guidelines[33]. The AM II falsely identified 5 of the 8 patients who fulfilled the criteria as probable LS[33]. Similar reviews revealed a 22%[35] and 14% [37] failure rate to identify a LS profile by the AM II and the RBG, respectively. These results favour the use routine screening to diagnosis LS. To overcome the diagnostic limitations associated with these criteria, more technical methods of diagnosis have been introduced to assess tumour tissues for MMR pathogenic variants and associated MSI. MSI testing and immunohistochemistry are two tissue based screening methods which can be used to assess tumours for MMR defects indicative of LS.

1.2.1.2 *IHC Strategy*

Immunohistochemistry (IHC) testing, a staining process which utilizes binding of applied antibodies to antigens in tumour tissues, can be used to detect absent MMR proteins on microscopic slides. It allows the presence or absence of a protein to be visualized by exploiting an antibody-antigen interaction. Attaching a colour-producing enzyme to the antibody results in colour formation when an antibody and antigen interact. If the protein is present (an antibody-antigen interaction), the tissue is positive, and a brown stain is present within tumour cell nuclei; a positive MMR-IHC result, see Figure 1. Protein expression is considered absent (no antibody-

antigen interaction) when there is a complete lack of staining in tumour cell nuclei; a negative MMR-IHC result, see Figure 2.

Figure 1. A positive IHC-MMR microscopic slide prepared for MSH2 analysis demonstrating stained nuclei. *A.* Low microscopic power, 50X. *B.* High microscopic power, 200X.

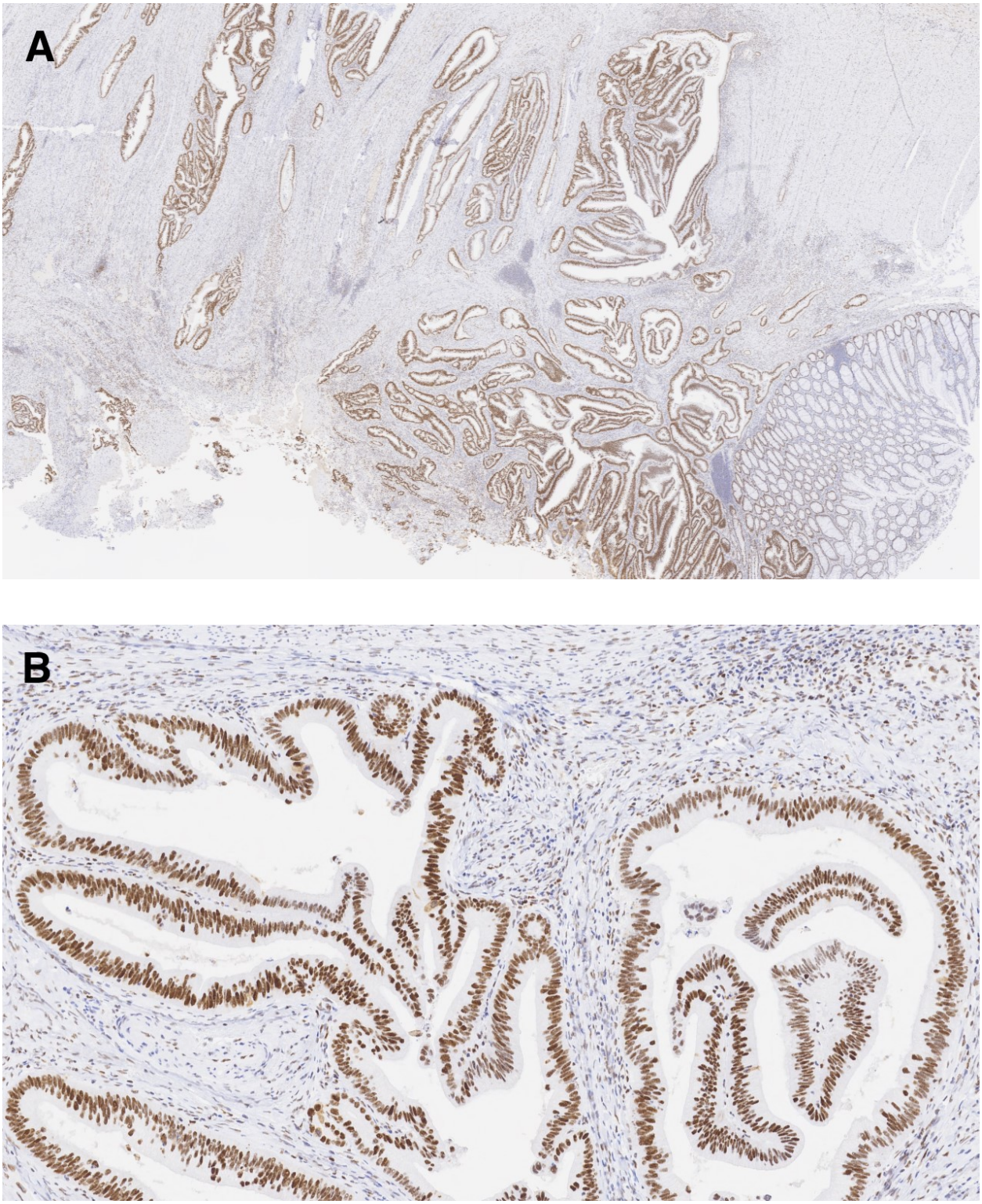
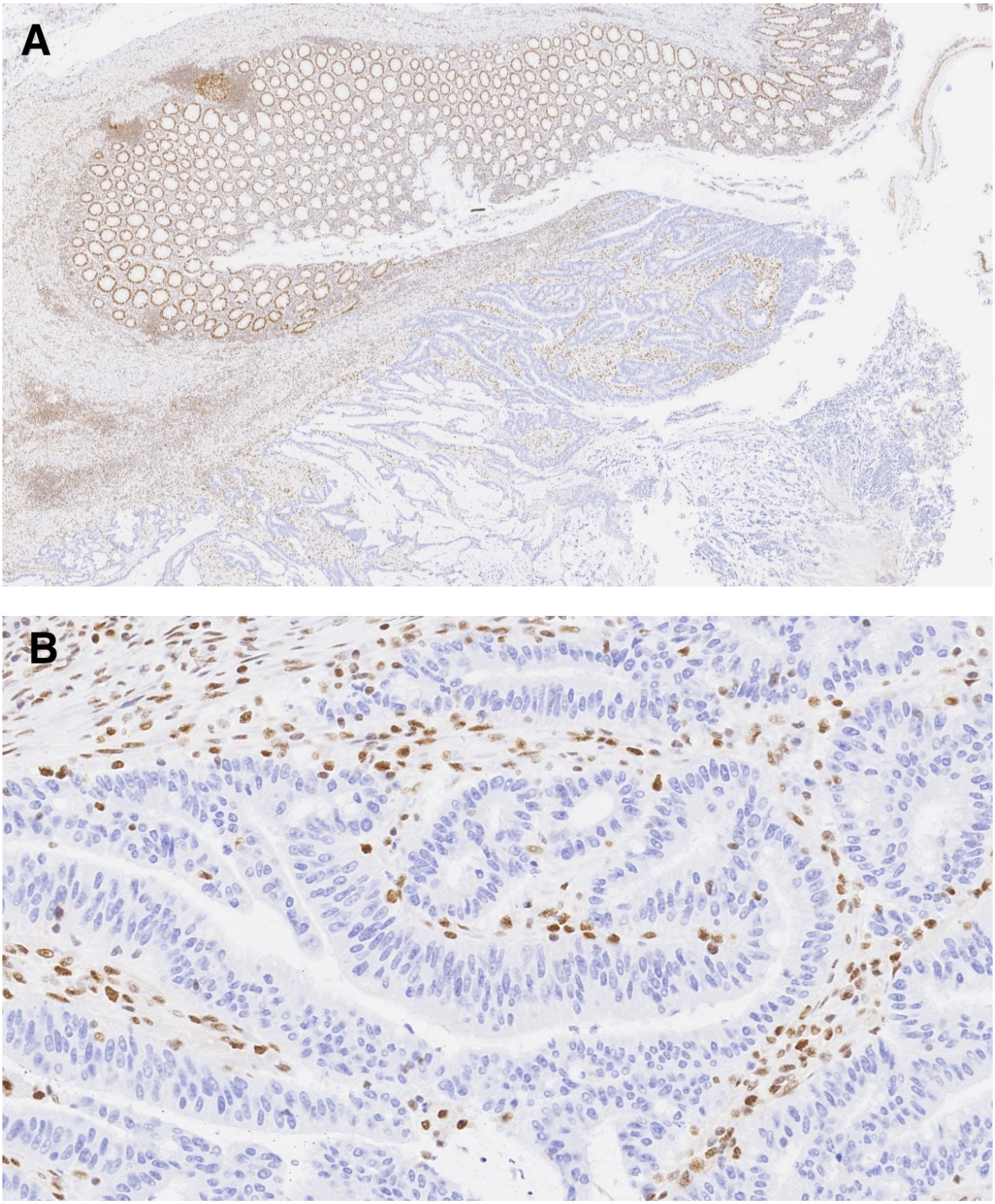


Figure 2. A negative IHC-MMR microscopic slide prepared for MLH1 analysis demonstrating a lack of stained nuclei. *A.* Low microscopic power, 50X. *B.* High microscopic power, 200X.



It is highly unlikely a patient has LS when immunostains for all MMR genes products demonstrate positivity[38]. A patient is classified at a high-risk for LS when MSH2, PMS2 or MSH6 proteins stain negative. When MLH1 is negative, LS cannot be excluded (CE) and further testing is required to rule out MLH1 hypermethylation. These changes are identified through microscopic identification of routine tumour sample. A 4-antibody panel for mismatch repair deficiency screening, using monoclonal antibodies for MLH1, MSH2, MSH6 and PMS2, has become the general standard when using IHC to determine the likelihood of a LS mutation[15,17,38,39].

IHC is considered a sensitive screen to ID patients with potential LS. Several studies comment on the specificity and sensitivity of IHC to detect MMR mutations[38,40,41]. After reviewing several studies, in 2008 Shia reports an overall 94% sensitivity of IHC to detect MMR mutations[38]. Shia suggests that use of a four-anitbody panel can increase previously reported low sensitivities of MLH1, thereby increasing overall IHC sensitivity to be comparable to that of MSI[38]. Similarly, a review conducted by Kheiresleid et al (2013) presents a sensitivity range of 27%-100% and a specificity range of 43%-100% with a suggested average sensitivity and specificity of 74% and 77%, respectively[12]. Aside from competitive levels of sensitivity and specificity, IHC is readily available at most centers requiring no additional equipment or personal to implement it as a screening method. Testing is easy to perform from a technical standpoint and is associated with faster turn-around times and lower costs[17,24,38,42-44].

To further decrease the cost of MMR IHC some authors suggest using a 2-antibody panel over the standard 4-antibody panel. Based on the biomedical properties and the heterodimeric nature of these MMR proteins, it is suggested that a 2-antibody panel using monoclonal antibodies for PMS2 and MSH6 alone, may be as predictive as a 4-antibody panel[23,45]. A study conducted by Shia et al (2009) revealed that, in cases which demonstrated a deficient MMR proteins, a concordant loss of MLH1-PMS2 and MSH2-MSH6 were the most prominent[45]. In the same study, isolated MMR loss was observed only in PMS2 and MSH6 proteins[45]. Similarly, after conducting an audit of cohort slides for concurrent loss of MLH1-PMS2 and MSH2-MSH6, Hall et al (2010) could conclude that a PMS2 and MSH6 2-antibody panel had a 100% sensitivity and specificity that matched that of a 4-antibody panel[23]. Potentially, costs of an IHC screening program can be reduced by replacing the four antibody panels with a two-antibody panel, where PMS2 and MSH6 alone are used, without decreasing sensitivity or specificity of testing[23].

Despite these advantages, IHC screening has limitations. Technical issues, such as tissue fixation, amount of tissue used, and staining parameters can play a role in interpretation of MMRD. While the preferred tissue for IHC testing is that from a colorectal carcinoma tumour, testing can be performed adenomatous polyp biopsies or tumour tissue biopsies[24,46-48]. Both Shia et al (2011) and Kumarasinghe et al (2010) concluded that biopsied tumour tissue can be a useful alternative to resected tumour tissue for IHC analysis[47,48], where Kumarasinghe produced completely interpretable testing results in 100% of biopsied tissues[48]. Paraffin embedded biopsied tumour tissues may be the only tissue remaining in patients who have undergone a CRC resection who underwent neo-adjuvant therapy. Unlike the previously described studies, Walsh

et al reported test results on biopsied adenomatous polyps. In this study, only 79% of 109 adenomatous polyps from 69 individuals with a known pathogenic variant expressed MMRD on IHC[46]. Each author concluded use of biopsy tissue to be suitable alternative for IHC testing when tumour resection tissue is not available[46-48].

IHC has been criticized for its lack of reproducibility and variation between different laboratories, with issues of both technical concordance and inter-observability interpretation variation[38,41]. Weak or ambiguous focal staining patterns can lead to an incorrect interpretation[38]. As noted by Kheirelseid, IHC is unable to detect functional loss of a protein in germline variants which do not result in significant structural changes to the protein[12]. It also cannot account for secondary heterodimer pairings, such as MSH2/MSH3; each of which could result in equivocal staining patterns[12,24,41,42,44]. Despite hesitation by some authors to use IHC as the sole method of LS detection[24,29,30,44,46], the affordable, accessible, and quick qualities of IHC screening make it a useful and desirable method of LS screening.

1.2.1.3 MSI Strategy

Microsatellite instability (MSI) testing, a polymerase chain reaction-based technique detects MSI and loss of functional MMR proteins in tumour tissues. This method can be used to identify patients at risk for LS. MSI has a well-established clinical utility. Exposing tumour tissue to a routine panel of 5 microsatellite markers and comparing the number of nucleotide repeats in the tumour tissue to those in normal tissue creates a readable profile[12,42]. A tissue is microsatellite stable (MSS) when the same number of nucleotide repeats is present in all 5

markers in tumour and normal tissue[12,42]. MSI occurs when the number of repeats in the tumour and normal tissue differs. MSI-high (MSI-H) tissues occur where 2 or more markers in the panel show instability and is commonly observed in LS associated tumour tissues[12,42]. MSI-low (MSI-L) occur when 1 of the five markers from the panel show instability; MSI-L is frequently observed in sporadic colon cancer[12,42].

The Evaluation of Genomic Applications in Practice and Prevention (EGAPP) Working Group reports a sensitivity of up to 91% and a specificity of 90% for MSI among individuals with MLH1 and MSH2 mutations[43]. Although the sensitivity of MSI testing decreases slightly when MSH6 and PMS2 cases are included, 55-77%, the specificity remains the same[43]. Similar rates of MSI sensitivity and specificity are described by several other authors[17,38,40,42].

MSI testing can decipher MSI status of tumours suggestive of an MMRD, however, it cannot identify the gene which is most likely to be mutated or recognize the small portion of LS tumour which do not demonstrate MMRD[17,38,43].

Interpretation of MSI results does not usually require great technical skill, but technical issues can arise. Hampel et al (2005) describe difficulty in detecting MSI in mucinous tumour tissues as the lack of DNA in these samples doesn't allow for proper conduction of the test[40]. Furthermore, unlike most LS related tumours which are associated with MSI-H, MSH6 deficient tumours could demonstrate MSI-L due to the ability of the MSH2/MSH3 heterodimer to partially

compensate functionally for a MSH6 loss[41,49]. MSI-L is reported in this scenario because MSI is limited to mononucleotide tandem repeats, explaining lower MSI sensitivity among those with a reported dMSH6[41]. As MSI-L tumours are not considered suspicious for LS, the individual would thus not receive further genetic testing, genetic counselling, or enter a screening program. However, if there is a high clinical suspicion of LS in a MSI-L individual, Zhang et al (2008) suggest that addition of a mononucleotide marker, such as BAT-40, can provide clarity to reclassify some MSI-L tumours to MSI-H[41]. MSI is also associated with higher costs, is not readily available across centers, and despite results being easy to interpret, is technically more difficult to perform requiring special skill sets[17,38,43].

Despite these limitations, Bartley et al (2012) encourage use of MSI testing[44]. MSI testing is highly reproducible, decreasing inter-observer interpretation variation, and is regulated by the College of American Pathologists (CAP) administered proficiency testing[17,41,43]. MSI testing may not be able to detect a specific protein deficiency, however, it is able to indicate mutations outside of those used on a IHC screening antibody panel[17,41,43]. MSI testing can also correctly identify a MMRD tumour where IHC falsely indicated an intact MMR profile when protein translation remained intact despite a tumour-causing mutation[17,41,43].

Each type of analysis has advantages and limitations which must be considered when developing a LS testing strategy. There is debate over the most useful methods of MMRD detection concerning CRC but most authors[13,20-22,36,50] agree that increasing the number of LS

individuals identified is feasible and imperative for disease management in patients and their relatives.

1.2.2 Development of a Lynch Syndrome Screening Algorithm

It is estimated that less than 5% of individuals with Lynch Syndrome have been identified[51], illustrating the need for a comprehensive systemic approach for LS diagnosis. LS diagnosis based solely on clinical and some pathologic parameters is ineffective. Several screening algorithms have been developed to circumvent limitations of these diagnostic techniques. The Cleveland Clinic study[36] and the Dutch Study[21] each laid groundwork which provided insight into the various elements required to develop a successful screening protocol.

Heald et al (2013) describe the implementation of a progressively comprehensive universal screening program conducted by one academic center, the Cleveland Clinic, between January 2004 and January 2012[36].The study describes three separate approaches where each subsequent strategy builds on the previous. The study demonstrates the significance of a universal screening method and highlights the impact of interdepartmental communication and shared responsibility on patient follow-up. In approach 1 MSI testing was applied only to CRC tumours of patients ≤50 years of age, to tumours located on the right side, and to tumours demonstrating MSI-H histology; surgeons alone were responsible referral to genetic counselling. This approach failed to identify 17 patients eligible for genetic counselling[36]. A universal MSI-IHC testing strategy was used in the second approach. It also allowed genetic counsellors to receive testing results and an increased number of referrals to genetic counselling was noted[36]. The final approach

allowed genetic counsellors to directly contact patients about results and schedule genetic counseling. As a result, genetic counselling referrals further increased and shorter time intervals between referral and the genetic counselling appointment were observed[36]. Overall, from a cohort of 1108 patients, the study identified 21 mutations due to LS. Eighty-eighty percent of patients who were referred to GC proceeded with genetic testing[36]. Furthermore, Heald et al (2013) were also able to determine, in the subset of those patients who underwent GC, that 20.3% and 66.1% satisfied the AM and RBG, respectively[36]. The Cleveland Clinic study provides a template for practical implementation of universal MSI/IHC testing. A similar study, conducted by van Lier et al (2012)[21], reports on the implementation of a routine molecular analysis on CRC when applied to a wide catchment area.

Referred to as the Dutch Study, van Lier et al (2012) describe the yield of a universal screening program in a cohort of 1117 CRC patients ≤ 70 years of age; data was collected from 11 different hospitals and 5 pathology laboratories[21]. By examining the results of MSI, IHC-MMR and MLH1-promoter methylation, the study successfully identified 4.5% of patients to have a molecular profile consistent with LS. Emphasizing the limitations of the age guidelines of the Amsterdam and Bethesda Criteria, 70% of these patients demonstrating a LS consistent profile were older than 50 years of age[21]. At the time of publication of the study, over 80% of these patients had been referred to and underwent genetic counselling, where 26 MMR mutations had been identified[21]. Eighty-three percent of patients referred for counselling did not meet the Amsterdam II Criteria and only 57% satisfied the RBG[21].

Heald et al (2013) and van Lier et al (2012) demonstrated successful and effective implementation of combined MSI, IHC and hypermethylation analysis to identify LS. Other studies have examined the cost-effectiveness of this combination[20,22,50,52]. A recent study by Barzi et al (2015) reported universal testing using screening methods such as those described by Heald et al (2013) and van Lier et al (2012) to not be cost-effective[52]. Barzi also suggested that a universal screening method when combined with retrieval of accurate familial history to be cost effective[52]. However, as previously described, there are limitations to patient reported family history. The dependability of pedigrees is weakened by lack of familial knowledge and decreasing family sizes. This was demonstrated in the Cleveland and Dutch studies when large percentages of LS patients did not meet the AM, AM II, or RBG.

Despite the Cleveland study's LS identification success, like the AM II and RBC it used a cut off age of ≤50 years. This strict age cut off accounts for some of the lack of sensitivity to of the AM II and RBC. A predictive model produced by Gudgeon et al (2013) concluded that over 50% of LS cases would be missed when using a cut off age of ≤50 years of age[53], suggesting the age cut off should be increased. Leenen et al (2016) reported small cost increases were associated with screening all CRC ≤ 70 years of age compared to ≤ 50 years when considering life years gained[20]. A balance between screening effectiveness and cost can be achieved.

1.2.3 Surveillance of Lynch Syndrome Individuals

It is important to identify individuals with a mutated MMR allele to offer them surveillance for prevention of CRC and other malignancies associated with LS[54-58]. Follow up with colonoscopy is effective for early detection of CRC and adenomatous polyps[6-8,54,55]. Currently, literature suggests that follow up colonoscopies for LS individuals should be completed between a 1-2-year time frame[7]. While some studies have shown a CRC rate reduction by up to 62% in LS families using a three-year time interval[54,55]. Furthermore, CRC discovered on colonoscopy after a lapsed screening interval have a less favourable prognosis and increased stage compared to those discovered when suggested screening intervals are followed[8].

It is suggested that suspected LS individuals be screened ten years prior to the age of familial relation diagnosis or even beginning at 20-25 years old[8,17]. Some authors suggest that colonoscopy screening methods for LS individuals could vary depending on the type of MMR mutation responsible for the disease[8]. Later onset of CRC is seen in LS patients who possess a PMS2 and MSH6 mutation compared to CRC in carriers of MLH1 mutations[8]. This suggests that PMS2 and MSH6 carriers could enter surveillance screening programs at a later age[17].

A LS diagnosis, combine with proper follow-up and surveillance leads to improved management and reduction in morbidity and mortality for both, that individual and their relatives [6,8,54-58]. Knowledge of a hereditary condition predisposing an individual to various cancers allows them to be participate in pre-symptomatic screening and surveillance programs. Informed

patients and their relatives can make decisions on screening, surveillance, and begin consideration of prophylactic surgery to decrease the risk of cancer development.

1.2.4 The Development of a Lynch Syndrome Screening Protocol in Manitoba

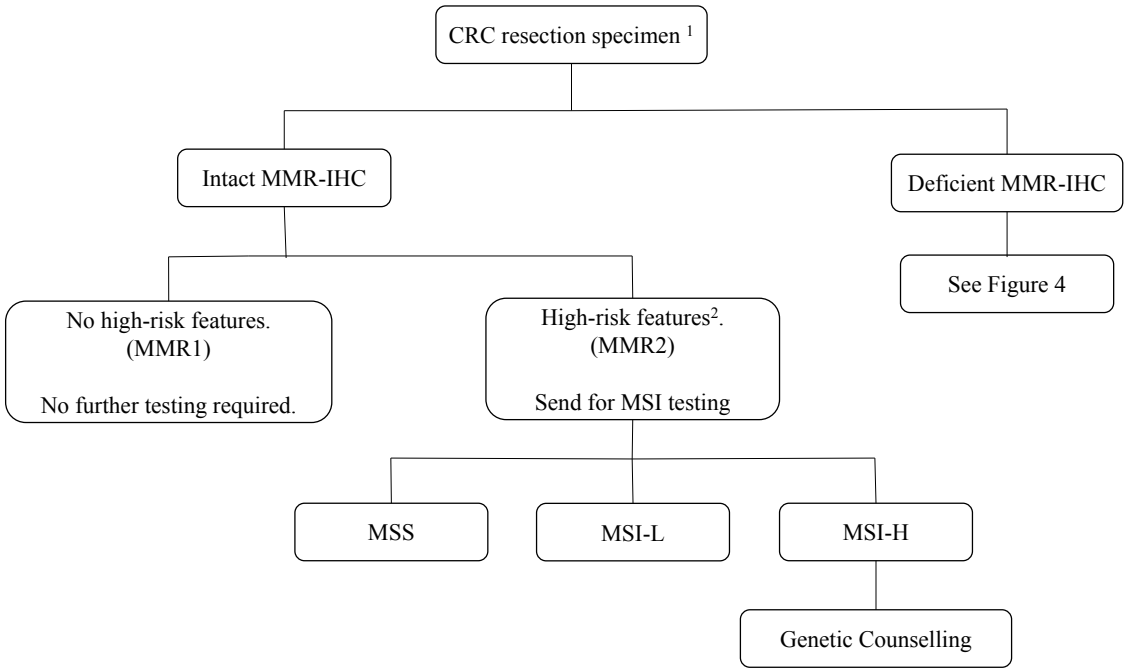
Before 2013, Manitoba had no designated policies for detection of LS in patients[59]59. It was unclear which care provider was responsible for identification of a cases suspicious for LS, requesting tumour tissue testing, and ensuring appropriate use of test results. Patients clinically suspicious of LS, who met AM II and the RBG, were referred to medical genetics for counselling. Approximately 40-50 patient tissues were sent annually to external agencies, such as the British Columbia Cancer Agency (BCCA) or Mount Sinai Hospital, to determine LS status[59]. Each sample was screened using the preferred testing method of that site; for example, MMR-IHC with or without MSI testing or MSI alone.

Inconsistencies with initial LS recognition, tissues tested, testing methods, and use of test results put Manitoba in a suboptimal position, as only a fraction of LS cases were being identified. While some patients were appropriately tested, and received valuable results, others were tested without clear logic and received unclear results; some patients were lost in the system and to follow up[59]. There was a strong desire amongst the pathology and genetics communities to standardize testing and to promote the benefits a provincial screening program could provide to patients and their families[59].

In 2012 a core group of interested parties, mainly pathologists, technical directors, IHC technologists and laboratory management representatives alike, collaborated with colleagues from medical genetics, gastroenterology, colorectal surgery and oncology to push forward with implementation of an in-house LS testing strategy[59].

This group developed a set of inclusion criteria, a screening algorithm, and post-screening clinical testing pathways based on techniques previously used to determine LS on local patient samples by external third parties (BCCA, Mount Sinai Hospital)[59]. The testing algorithm can be view in Figures 3 and 4 below.

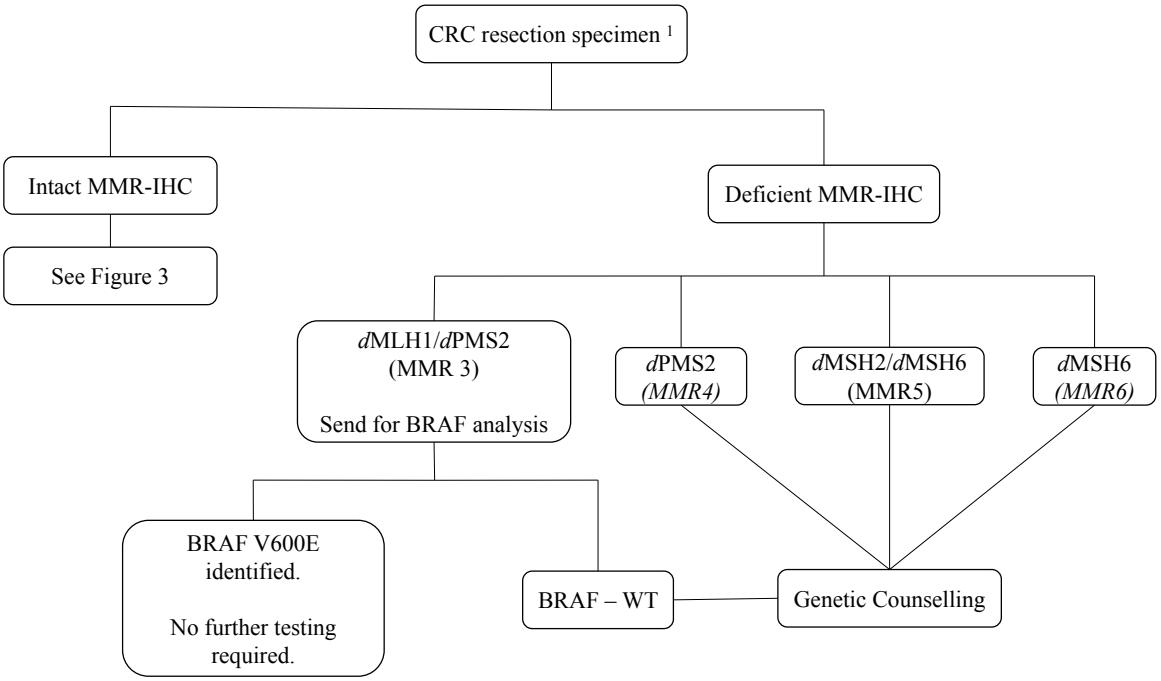
Figure 3. Testing strategy algorithm for CRC tissues demonstrating intact MMR-IHC after reflex MMR-IHC screening using a four-antibody panel. LIS insertion codes in parenthesis where applicable.



[1] Where the patient ≤70 years, reflex MMR-IHC conducted (MLH1, MSH2, MSH6, PMS2).

[2] High-risk features include clinical suspicion, MSI-H histology, patient less than 50 years.

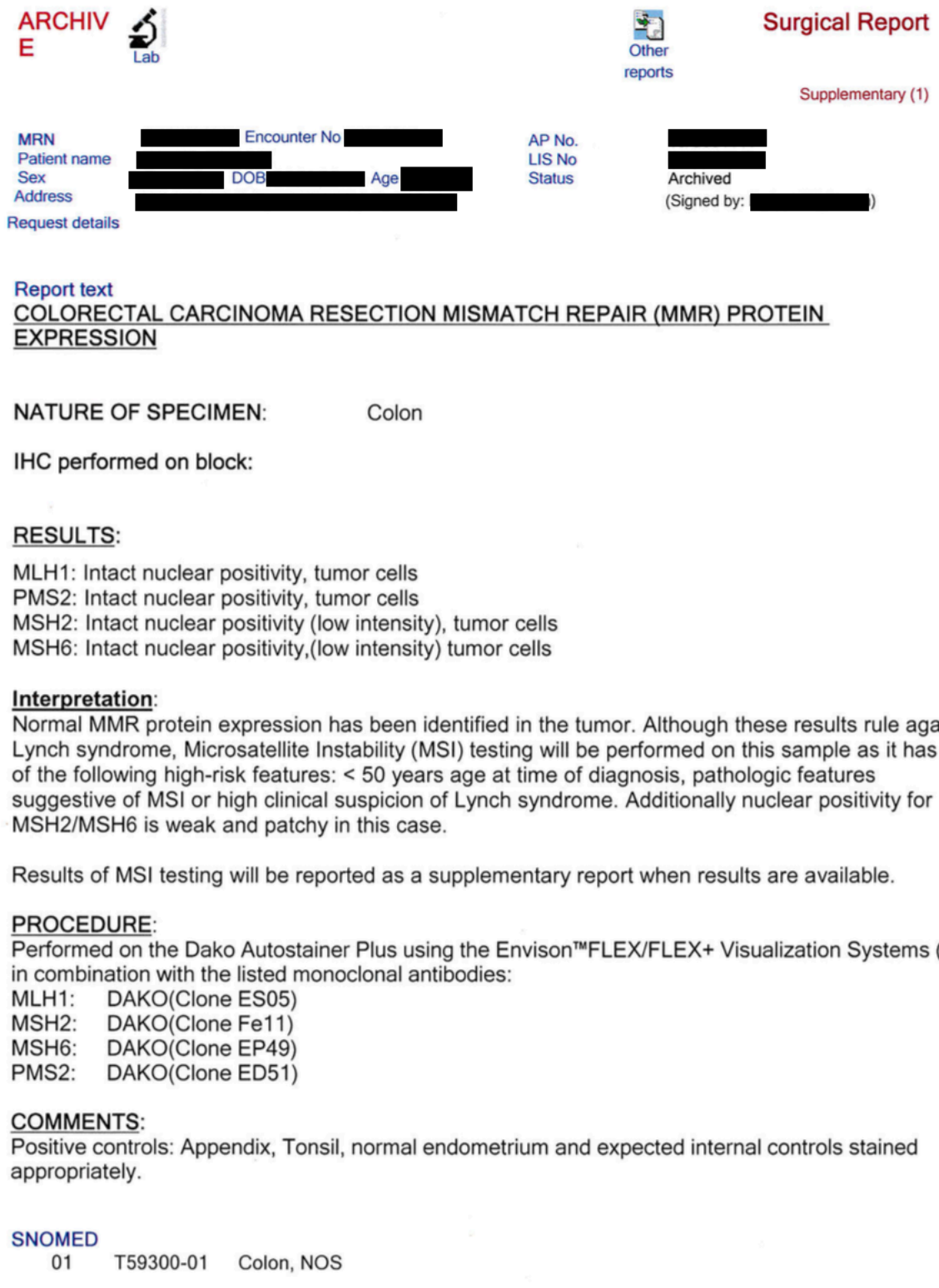
Figure 4. Testing strategy algorithm for CRC tissues demonstrating abnormal MMR-IHC after reflex MMR-IHC screening using a four-antibody panel. LIS insertion codes in parenthesis where applicable.



[1] Where the patient ≤70 years, reflex MMR-IHC conducted (MLH1, MSH2, MSH6, PMS2).

A series of reporting strategies were developed to ensure consistency of reports for all cases. A standardized template for reporting of MMR-IHC screening results was developed to report the nature of the specimen, reference block, detailed IHC results, interpretive comments, and technical details[59]. Figure 5 shows a sample supplementary report.

Figure 5. Sample MMR-IHC supplementary report. Page 1 of 2.



ARCHIV E Lab

Other reports

Surgical Report

Supplementary (1)

MRN [redacted] Encounter No [redacted]
Patient name [redacted]
Sex [redacted] DOB [redacted] Age [redacted]
Address [redacted]
Request details

AP No. [redacted]
LIS No [redacted]
Status Archived
(Signed by: [redacted])

Report text

COLORECTAL CARCINOMA RESECTION MISMATCH REPAIR (MMR) PROTEIN EXPRESSION

NATURE OF SPECIMEN: Colon

IHC performed on block:

RESULTS:

MLH1: Intact nuclear positivity, tumor cells
PMS2: Intact nuclear positivity, tumor cells
MSH2: Intact nuclear positivity (low intensity), tumor cells
MSH6: Intact nuclear positivity,(low intensity) tumor cells

Interpretation:
Normal MMR protein expression has been identified in the tumor. Although these results rule against Lynch syndrome, Microsatellite Instability (MSI) testing will be performed on this sample as it has one of the following high-risk features: < 50 years age at time of diagnosis, pathologic features suggestive of MSI or high clinical suspicion of Lynch syndrome. Additionally nuclear positivity for MSH2/MSH6 is weak and patchy in this case.

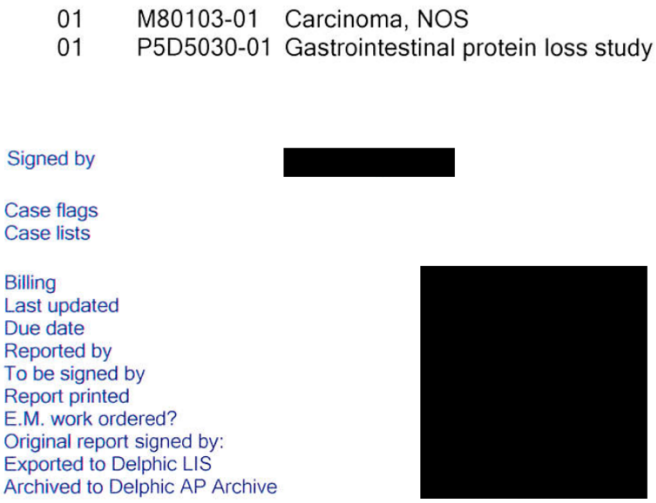
Results of MSI testing will be reported as a supplementary report when results are available.

PROCEDURE:
Performed on the Dako Autostainer Plus using the Envison™FLEX/FLEX+ Visualization Systems (link) in combination with the listed monoclonal antibodies:
MLH1: DAKO(Clone ES05)
MSH2: DAKO(Clone Fe11)
MSH6: DAKO(Clone EP49)
PMS2: DAKO(Clone ED51)

COMMENTS:
Positive controls: Appendix, Tonsil, normal endometrium and expected internal controls stained appropriately.

SNOMED
01 T59300-01 Colon, NOS

Figure 5. Sample MMR-IHC supplementary report. Page 2 of 2.



A group of selected pathologists underwent MMR-IHC interpretation training to ensure high quality interpretation of test results. These pathologists were issued a MMR-IHC visual user guide (a step by step user manual including screening shots of the reporting process for MMR-IHC cases)[59].

The dedication of this group, combined with the efforts a patient advocacy group (public awareness, letter writing, fund raising, letter writing)[59], lead to the approval of development and implementation of an in-house Manitoba LS screening system in 2013.

2 HYPOTHESIS AND RATIONALE

Implementation of an in-house LS screening protocol in Manitoba, Canada is expected to result in the identification of LS carriers by a number which corresponds to the 1-6% of all CRC cases LS is responsible for, as documented in current literature. This will result in a decrease of the number of unidentified and missed LS carriers post treatment for colorectal resection of CRC. A screening protocol that incorporates and promotes communication between multidisciplinary aspects of patient care is expected to reduce the number of patient cases lost within the system and ensure proper follow up care is available to patients and their relatives.

3 STUDY OBJECTIVES

The objective of this study was to assess the effectiveness of a recently instituted Lynch Syndrome screening protocol in Manitoba, Canada against those of similar screening methods described in current literature. The ability of the protocol to detect a Lynch Syndrome profile in patients who underwent colorectal resection for CRC treatment and genetic counselling follow-up of those patients will be assessed. The province of Manitoba, Diagnostic Services of Manitoba (DSM) and its partners hoped to establish a convention for screening that would better service those patients with LS related CRC.

4 MATERIALS AND METHODS

4.1 Materials

Equipment

Dako Autostainer Plus

Envision™FLEX/FLEX+ Visualization Systems

Monoclonal Antibodies

MLH1: DAKO(Clone ES05)

MSH2: DAKO(Clone Fe11)

MSH6: DAKO(Clone EP49)

PMS2: DAKO(Clone ED51)

4.2 Methods

4.2.1 Inclusion criteria

Cases included were limited to patients, male or female, who underwent curative colorectal surgery in Manitoba, at the age of 70 years old or younger, between October 1, 2013 and March 31, 2015, (an 18-month period). Cases were examined by pathologists stationed at the Health Science Centre (Winnipeg), St. Boniface General Hospital (Winnipeg), Grace General Hospital (Winnipeg), and Brandon Regional Health Centre (Brandon).

4.2.2 Screening Algorithm

After initial IHC-MMR testing on all cases, further screening followed the design formulated agreed upon by the working group of pathologists, technical directors, IHC technologists and laboratory management representatives as show in Figure 3 and Figure 4.

Intact MMR-IHC with clinical or histopathological high-risk features were sent for MSI testing; those reported as MSI-H were referred to GC. No further testing was required for intact MMR-IHC which demonstrated no high-risk features. Refer to Figure 3.

Cases reported as *d*PMS2*,* dMSH2/*d*MSH6, or *d*MSH6 were sent to GC. Deficient *d*MLH1*d*PMS2 cases were sent for BRAF V600E molecular analysis. Cases which were identified as BRAF-WT were referred to GC. No further testing was required for cases demonstrating a BRAF V600E mutation. Refer to Figure 4.

4.2.3 Case Reporting Methods

4.2.3.1 *MMR-IHC*

MMR immunochemistry was ordered in Delphic on CRC resection as defined above to determine the expression of the proteins MLH1, MSH2, MSH6, and PMS2. Results were reported on separately generated supplementary reports, see Figure 5.

The procedure was performed on the Dako Autostainer Plus using Envision™FLEX/FLEX+ Visualization Systems (link) in combination with monoclonal antibodies (MLH1: DAKO(Clone ES05); MSH2: DAKO(Clone Fe11); MSH6: DAKO(Clone EP49); PMS2: DAKO(Clone ED51)). The following positive controls were used: appendix, tonsil, endometrium.

Codes for MMR interpretation based on protein expression (MMR1, MMR2, MMR3, MMR4, MMR5, and MMR6) were created and inserted into the "Results" section of the supplementary report. Based on immunohistochemical expression of MLH1, MSH2, MSH6, and PMS2 in tumour cells and presence/absence of high-risk features, additional testing was performed as per the defined algorithm and additional supplementary reports issued with MSI or BRAF V600E mutation results. See Table 5.

Table 5. Laboratory information system (LIS) insert code interpretation of MMR protein expression (intact or loss) of a four-monoclonal antibody panel (MLH1, PMS2, MSH2, and MSH6).

LIS Code		MMR1	MMR2	MMR3	MMR4	MMR5	MMR6
Protein Expression	MLH1	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	***Loss*** of nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells
	PMS2	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	***Loss*** of nuclear positivity, tumour cells	***Loss*** of nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells
	MSH2	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	***Loss*** of nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells
	MSH6	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	*Intact* nuclear positivity, tumour cells	***Loss*** of nuclear positivity, tumour cells	***Loss*** of nuclear positivity, tumour cells
Interpretation		*Unlikely LS*	*LS cannot be excluded, demonstrates high-risk feature(s)*	*LS cannot be excluded, patient remains at risk for hereditary CRC*	*High probability LS*	*High probability LS*	*High probability LS*

4.2.3.2 *MSI Analysis*

Microsatellite instability analysis was performed at the DMS Molecular Diagnostic Laboratory. DNA was extracted from a portion of tumour, genotyped and compared against amplified normal tissue DNA. Elizabeth L. Spriggs, PhD, FCCMG interpreted the results as microsatellite instable (MSSI), where, one of mononucletotide microsatellite markers exhibited instability (MSI-Low) or two or more of the five mononuecleotide microsatellite markers exhibited instability (MSI-High) or were reported as microsatellite stable (MSS), where, all five mononucleotides were scored as stable.

3.2.3.3 *BRAF V600E Mutation Analysis*

BRAF V600E mutation detection testing was performed at the DSM Molecular Diagnostic Laboratory. Results were interpreted by Elizabeth L. Spriggs, PhD, FCCMG to "rule against Lynch syndrome" (detection of BRAF V600E mutation) or as "not diagnostic of Lynch syndrome . . . patient remains at risk for lynch syndrome, genetic consultation is recommended" (wildtype BRAF; no mutation identified at codon 600 of the BRAF gene).

Supplementary reports where results suggested a high likelihood of LS (loss of PMS2, MSH2, MSH6, MLH1/PMS2 with BRAF WT, or MSI-H) were copied to Dr. Bernie Chodirker for genetic follow up of patient.

4.2.4 Data Acquisition

4.2.4.1 *Winnipeg Cases*

To obtain the desired cases from the Winnipeg catchment, Dr. H.R. Wightman requested a search for all colorectal resections with an MRR request from the Diagnostic Services of Manitoba LIS within the desired time frame. Cases that fell within the criteria were sorted out and all others were discarded. Each case was individually examined by Laura Sexsmith to acquire clinical and patient information.

4.2.4.2 *Brandon Cases*

To obtain the desired cases from the Brandon catchment, Dr. M. Dupré requested all colorectal reports from Westman Regional Laboratory. Dr. M. Dupré sorted the cases into those that fit the inclusion criteria and all others were discarded. The cases were examined for clinical and patient information by Dr. M. Dupré. It should be noted that as per availability, Brandon data was collected and included from October 1, 2013 to December 31, 2014, a 15-month period. This data was eventually removed from the study because a complete set of data, from case acquisition to genetic counselling information, could not be obtained. This was in part due to lack of accessibility to the laboratory information system used by Westman Regional Laboratory. It was therefore agreed, between Laura Sexsmith and her thesis supervisor, Dr. Wightman, that removal of the Brandon data would result in more sound and valid study results.

4.2.4.3 *Clinical Information Collected from Each Case Report*

Clinical information collected from each case included pathology number, year, and sex and age of patient. Pathological data collected included the results of all supplementary reports, including MMR immunohistochemistry and BRAF and MSI molecular testing. This information was manually collected from each individual case report.

4.2.4.4 *Genetic counselling*

The Winnipeg Regional Health Authority's Genetics and Metabolism program offered complete genetic counselling opportunities to patients. All high-likelihood LS patients (dPMS2, dMSH2, dMSH6 and dMLH1 BRAF-WT) were copied to genetics, see Figure 4.

Initially, the office of the clinician or the surgeon would contact each patient through a mailed letter offering genetic counselling. After this point, a genetic counsellor would contact the patient and do a preliminary telephone conversation to determine if a further appointment was required. This conversation would further explain the role of genetic counselling, allow the patient to accept or decline said counselling, and/or include a preliminary screening through pedigree chart creation. If genetic counselling was accepted, each patient was assigned a genetics counsellor.

However, within the last few months of the first year of protocol genetic counsellors began to directly contact patients by telephone after receiving confirmation that a referral was issued. This was done to decrease the time between patient surgery and initial genetic

counselling interaction. These conversations were used to inform the patient of their genetic counselling options and to communicate a follow up letter would be received in the mail.

Once consented genetic counselling was completed, those patients with a strong likelihood of hereditary cancer were sent for genetic sequencing to determine if and which genetic variation they carried. It should be noted, initial contact with one patient was not possible and 6 others simply declined counselling or sequencing for various to unknown reasons.

4.2.4.5 *Genetic Sequencing*

Results of genetic sequencing testing performed on patients identified from October 1, 2013 and March 31, 2015 were requested from Elizabeth L. Spriggs, PhD, FCCMG. A list of patients who had received genetic counselling and subsequent germline sequencing was obtained up to and including September 2015. Patient date of birth and personal health identification numbers, sequencing test conducted and the subsequent result were obtained. The patient date of birth and personal health identification number were used to cross reference patients with pathology case number to ensure patient eligibility and study inclusion.

The results of the sequencing were cross referenced with the findings of the original pathology case and supplementary reports to determine efficacy of the tumour screening protocol to determine LS.

4.3 Statistical Analysis

A comparative data analysis was conducted. The Manitoba LS screening protocol algorithm was compared to similar screening algorithms in the literature by examining its' ability to detect a high-likelihood LS profile in a study population. To assess this, the number of MLH1, MSH2, MSH6 and PMS2 deficiencies in the Manitoba Study cohort were quantified and displayed in tabulation format (Table 6). The observed number for each protein was then compared to those in the literature. Similar comparative analysis was used to evaluate the uptake of genetic follow-up and genetic sequencing mutation results (Table 7). Sums, averages, and percentages in this study were created using Microsoft® Excel® for Mac 2011 version 14.0.0 (100825).

4.4 Quality Assurance

Participation in externally conducted quality assurance through the Canadian Immunohistochemistry Quality Control (CIQC) MMR IHC challenge and use of in house tissue microarrays (TMA) established adequate (>90%) concordance with the gold standard (CIQC or BCCA/Mount Sinai results). A >95% interpretive accuracy was required of all reporting pathologists.

4.4.1 Canadian Immunohistochemistry Quality Control (CIQC)

Each laboratory used to administer and interpret IHC MMR test, were participants in the Canadian Immunohistochemistry Quality Control board MMR immunohistochemistry challenge. CIQC assessors blindly reviewed slides from 36 participating laboratories to analyze and rank IHC status, or quality of the stain, for MLH1, PMS2, MSH2, and MSH6 antibodies. For each antibody, every laboratory was given a sensitivity, specificity, positive predictive value, negative predictive value and Cohen's kappa score, a percent score-able and percent concordance with reference, and a pairwise complete observations number. The three laboratories that participated received nothing lower than an "Adequate" scoring in IHC status for all antibodies with the majority receiving an "Optimal" score.

4.4.2 *Validation Tissue Microarray*

Tissue samples from multiple previous known cases of CRC LS individuals were used to create tissue microarrays for reference for LS MMRD IHC. The use of tissue microarrays

allowed for simultaneous analysis of MMR protein levels under identical, standardized conditions on a single glass slide. It was an effective and practical method for analysis of different tumour type with varying MMRD in LS individuals exposed by IHC. It was, as well, a valuable learning, reference, and verification tool.

5 RESULTS

5.1 Patient Characteristics

The study population was comprised of 316 cases. The average patient was 58 years (57.98) with a range between 13-70 years.

It should be noted that several hundred additional cases were retrieved for review based on the MMR markers used to flag the cases. Those outside of study parameters were removed to obtain the final study case cohort of 316 cases.

5.2 MMR-IHC with Intact Proteins

Of the 316 cases included in the study population, 203 cases (64%) demonstrated intact MMR-IHC expression with no high-risk clinical or pathological features. See Table 6.

Table 6. Results from initial 18 months of LS screening protocol. *A:* MMR-IHC findings. *B:* MSS status of sequenced intact MMR-IHC with a high-risk feature from part A. *C:* MMRD protein identified by IHC from part A. *D:* Deficient MLH1/PMS2 from part C sequenced to determine BRAF status.

A. MMR-IHC results

Intact MMR-IHC	Intact MMR-IHC with a high-risk feature	Deficient MMR-IHC	Total Cases
203 (64%)	63 (20%)	50 (16%)	316

B. Microsatellite stability (MSS) status of sequenced intact MMR-IHC with a high-risk feature

MSS Status	
MSS	59 (93%)
MSI-L	3 (4.8%)
MSI-H	1 (1.6%)

C. MMR protein identified as abnormal using IHC

*d*MMR Protein	
*d*MSH6	3 (0.9%)
*d*PMS2	5 (1.6%)
*d*MSH2/*d*MSH6	8 (2.5%)
*d*MLH1/*d*PMS2	34 (11%)

D. Deficient MLH1/PMS2 sequenced to determine BRAF status (V600E mutation or Wild Type (WT))

BRAF Status	
BRAF V600E	23 (7.3%)
BRAF-WT	11 (3.5%)

5.3 MMR-IHC with Intact Proteins Demonstrating High-Risk Features

Sixty-three cases (20%) had intact MMR-IHC but were identified as having at least one high-risk feature. These cases were sent for downstream MSI testing, where 59 (93%) were MSS, 3 (4.8%) were MSI-L, and 1 (1.6%) was MSI-H. See Table 6.

5.4 MMR-IHC with Deficient Proteins

Fifty (16%) cases demonstrated abnormal MMR-IHC expression. Of these cases, 16 (5.1%) demonstrated abnormal MMR-IHC expression patterns that were indicative of high-likelihood of LS. A PMS2 deficiency was observed in 5 (1.6%) cases, 8 (2.5%) showed a MSH2/MSH6 deficiency, and 3 (0.9%) showed a MSH6 deficiency. See Table 6.

The remaining 34 (11%) cases, which expressed a MLH1/PMS2 deficiency, were sent for BRAF mutational analysis. BRAF V600E (a mutation) was identified in 23 (73%) cases; these were interpreted as 'highly unlikely to be LS'. BRAF-WT (no mutation) was identified in 11 (3.5%) cases; these cases were interpreted as a moderate to high-likelihood of LS. See Table 6.

5.5 Follow-up of High Risk Cases

Twenty-seven (8.5%) of 316 cases demonstrated high-risk findings and were referred to GC. This study population subset consisted of 18 males and 9 females. The average age was 56 years with a range from 43-70 years. Refer to Table 7 for patient characteristics.

Table 7. Genetic mutations identified in 27 LS suspected cases, including patient age, patient sex, IHC-MMRD, and the likelihood of LS based on IHC-MMRD. Where applicable, the mutated protein and germline status are included. *A.* PMS2 suspected mutation. *B.* MSH2 suspected mutation. *C.* MSH6 suspected mutation. *D.*MLH1 suspected mutation. LS causing pathogenic germline mutations are in bold. Patients with no LS causing mutations are in standard font. Italic font indicates a declined appointment, declined germline testing or GC unable to contact patient.

		Tumour MMR-IHC			Germline Status	
Age	Sex	IHC-MMRD	LS Likelihood	Mutated Protein	Nucleotide change	Protein Mutation
A.	PMS2 suspected mutation					
60	**M**	**PMS2**	**HL**	**PMS2**	**c.736_74delCCCCCTinsTGTGT GTGAAG**	-
70	**M**	**PMS2**	**HL**	**PMS2**	**c.2536G>T**	-
43	**M**	**PMS2**	**HL**	**PMS2**	**c.2500_2501delinsG**	-
70	F	PMS2	HL	-	-	-
48*	M	PMS2	HL		Unknown	
B.	MSH2 suspected mutation					
62	**M**	**MSH2**	**HL**	**MSH2**	**Deletion exon 16 c. 2635-?_2805+?del**	**-**
49	**F**	**MSH2**	**HL**	**MSH2**	**Deletion exon 14 c.2211-1A>G**	**-**
59	**M**	**MSH2**	**HL**	**EpCAM**	**EpCAM deletion exon 3-9**	-
56	M	MSH2	HL	-	-	-
69	F	MSH2	HL	-	-	-
58	*M*	*MSH2*	*HL*	-	-	-
53	*M*	*MSH2*	*HL*	-	-	-
46*	M	MSH2	HL		Unknown	
C.	MSH6 suspected mutation					
70	**F**	**MSH6**	**HL**	**MSH6**	**Deletion exon 4 c.[1571dupA]**	**p.Tyr542Ter**

59	**M**	**MSH6**	**HL**	**MSH6**	**Not Available**	**-**
67**	M	MSH6	HL	-	-	-
D.	MLH1 suspected mutation					
45	**M**	**MLH1**	**HL**	**MLH1**	**c.298C>T**	
58	M	MLH1	HL/CE	MLH1	Exon9, c.788A>T, an unknown variant	
55	F	MLH1	HL/CE	-	-	-
44	M	MLH1	HL/CE	-	-	-
69	F	MLH1	HL/CE	-	-	-
69	F	MLH1	HL/CE	-	-	-
60	*F*	*MLH1*	HL/CE	-	-	-
50	*M*	*MLH1*	HL/CE	-	-	-
68	*M*	*MLH1*	HL/CE	-	-	-
62	*M*	*MLH1*	HL/CE	-	-	-
65	*F*	*MLH1*	HL/CE	-	-	-

*Patient underwent germline testing, result was not available at time of data collection
**Patient underwent neoadjuvant therapy
IHC - immunohistochemistry; MMR,- mismatch repair protein, MMRD- mismatch repair protein deficiency, HL – High likelihood; CE – cannot exclude

5.6 Genetic Counselling Follow-up

Genetic counsellors were able to contact 26 (96%) of 27 patients, of which 6 declined appointments or genetic testing for various reasons. A LS causing mutation was confirmed through germline sequencing in 9 (2.8%) of 316 patients; there were 3 PMS2 mutations, 3 MSH2 mutations, 2 MSH6 mutations, and 1 MLH1 mutation. See Table 7 for the specific mutation identified in each case.

No LS associated mutation was observed in 9 cases. This included 1 PMS2, 2 MSH2, 1 MSH6, and 5 MLH1 cases, see Table 7. However, one of the five MLH1 cases did express a

mutation of an unknown variant, unrelated to LS. It should also be noted that patient associated with the one MSH6 case underwent neoadjuvant therapy.

Two cases, one PMS2 and one MSH2, received germline testing but results, mutation or no mutation, were unavailable at the time of data collections. The exact nucleotide change of one MSH6 mutated case was not available to report.

6 DISCUSSION

This study demonstrated the ability of a routine screening protocol for LS, in CRC patients ≤ 70 years, to identify a high-risk immunohistochemical profile for LS in 8.5% of the study cohort. LS germline mutations represent 2.8%, nine of 316, of the CRC population cohort screened. This percentage falls comfortably within the reported 1-6% of all colorectal cancers[11,12,15,16,21,58,57] that LS is responsible for.

The average age of the patient at time of colorectal resection, who demonstrated a high likelihood of LS, was 56 years. The average of patients with a confirmed LS mutation was 57; this number exceeds the age criterion used for LS assessment used in routine clinical assessment (Amsterdam II and Revised Bethesda Criteria)[34]. Use of a CRC surveillance program has been shown to reduce CRC morbidity and mortality by 65-70%[21,57], highlighting the importance of identification of these potential LS patients outside of the routine criterion.

IHC indicated a possible *d*MSH2/*d*MSH6 involvement in 8 (2.5%) cases of the entire study cohort; of all CRC patients IHC identified as HL LS (27 cases), *d*MSH2/*d*MSH6 was indicated in 30%. This number is higher than those reported by van Lier et al (2012), 20%[21], and by Heald et al (2013), 10%[36]. However, it is lower than that reported by Steinke et al (2014)[60].

IHC indicated a possible *d*MLH1/*d*PMS2 involvement in 34 (11%) cases of the entire study cohort results; of all CRC patients IHC identified as HL LS (27 cases), *d*MLH1/*d*PMS2, after BRAF-WT identification was indicated in 41% of cases. This percentage is like those described by several authors[21,60] but, is half of that reported by others[36].

According to the literature, MLH1 and MSH2 deficiencies should account for a combined 90% of LS cases[17,61-65]. There was a relatively high uptake of germline sequencing by HL LS identified patients. However, comparing patient up-take of germline sequencing between subgroups shows that fewer patients which demonstrated *d*MLH1 and *d*MS2 accepted sequencing. This could have contributed to a lower than expected 71% MLH1 and MSH2 representation. Of this subgroup, higher than estimated levels of MSH6 and PMS2 were reported. This is discussed in a later section.

IHC analysis indicated a possible *d*MSH6 involvement in 3 (0.9%) cases of the entire study cohort; of all CRC patients IHC identified as HL LS (27 cases), *d*MSH6 was indicated in 11%. This number is slightly higher than those in literature, where most sources report a *d*MSH6 range between 7-10%[17,61-64]. However, higher involvement has been described by van Lier et al (2012), 24%[21] or Ramsoekh et al (2008), 52%[65].

IHC indicated a possible *d*PMS2 involvement in 5 (1.6%) cases of the entire study cohort; of all CRC patients IHC identified as HL LS (27 cases), *d*PMS2 was indicated in 19%. This percentage is higher than expected[17,61-64]. However, higher rates of involvement have been

described by van Lier et al (2012) who suggest underrepresentation of PMS2 incidence, along with MSH6, due to atypical presentation of these proteins in LS[21]. PMS2 deficiencies mutations have also been associated with later ages of cancer development and diagnosis[13,62]. Studies by Talseth-Palmer et al (2010) and Heald et al (2013) each reported *d*PMS2 involvement in the <5% range[36,62]. However, unlike this study, Talseth-Palmer et al 2010) studied a population solely of LS individuals[62], regardless of age, and Heald et al (2013) focused on a CRC population ≤50 years[36]. In this study three of five suspected *d*PMS2 cases fell above the age cut-off (≤50 years) used in routine screening methods. In two of these three cases where a LS germline mutation was reported the age at colorectal resection was 10 and 20 years above the cut off age. A higher age cut off for screening parameters could have allowed sampling of a previously unobserved *d*PMS2 population to be evaluated, thereby pushing the number above what was estimated.

Interpretation of cases can become complicated when protein expression is focally strong and weak, patchy or faint; retained protein expression of a stabilizing heterodimer, such as MSH2 in an MSH2-MSH6 complex, could compound the issue. The College of American Pathologists (CAP) recommends interpretation of a tissue with faint nuclear expression in tumour cell nuclei where stronger nuclear expression in controls to be reported as intact[59]. However, considering such cases as absent would maximize screening sensitivity and patient opportunity to receive genetic counselling.

Neo-adjuvant therapy can contribute to unclear interpretation of MMR-IHC results. Bao et al (2010) and Vilkin et al (2014) each described the ability of neo-adjuvant therapy to alter

MMR protein expression in cancer cells; reporting a loss or reduced expression in up to 20% of cases[66,67]. Neo-adjuvant therapy could be the cause of unusual MSH6 expression patterns. One patient, MMR-IHC *d*MSH6, from our study cohort sent for germline testing, is known to have undergone neoadjuvant therapy. Germline sequencing detected no mutation and intact MSH6 IHC was reported on pre-therapy biopsy tissue of the patient. This phenomenon was also reported by Vilkin et al (2014) after repeat IHC staining of pre-therapy biopsy materials showed intact MSH6 where post-therapy tissues indicated *d*MSH2 and *d*MSH6[67]. It should be noted that unlike MMR-IHC, MSI status is not influenced by neoadjuvant chemotherapy[21,66,67]. Therefore, MSI can be used to asses post treatment resection tissues and could be considered when there is an unresolved MMR-IHC interpretation discrepancy.

Sixty-three (20%) MMR intact cases were defined as 'cannot exclude (CE)' LS because of high-risk clinical (predominantly age) and histopathological features. The results in this subgroup exceeded that of the average literature[36]. While most were MSS, three MSI-L cases and one MSI-H case were reported. Of interest are the three reported MSI-L cases, as none were anticipated.

The biological significance of the MSI-L phenotype is up for debate[41,49, 68, 69]. There is little evidence suggesting that all MSI-L tumours are the direct result of undetected MSH6 mutations[49,69]. However, the heterodimeric nature of MMR proteins could explain cases of LS associated MSI-L which may be expressed as IHC positive. For example, MSH6 and MSH3 have some overlapping functions and each can form a heterodimer complex with MSH2, the

stabilizing protein. If MSH6 is deficient, MSH3 will bind to MSH2 and this pairing will partially compensate for a *d*MSH6[49,69]. A MSH2-MSH3 pairing can result in weakened or focal expression at IHC when tested for *d*MSH6[49,69]. As a result, if MSI-L tumours are not further screening potential LS carriers will go undiagnosed.

MSI-L cases can be reported due to a lack of sensitivity of dinucleotide MSI panel markers to detect MSI. It is known that mononucleotide markers are more sensitive to MSI changes [41,49,69]. Inclusion of additional mononucleotide markers to a MSI screening panel, as suggested by Zhang, can result in recognition of increased MSI tandem repeats[41]. Repeat MSI testing of MSI-L with a panel including extra mononucleotide could therefore reclassify MSI-L tumours to MSS[41,49,69].

Only one case was determined to be MSI-H in this subgroup. Assuming the MSI-L can be reclassified as MSS, this represents a 98% concordance between MMR-IHC and MSI testing in the study population suspicious for LS. This data is being further tracked in ongoing analysis. Despite having a high-risk feature, MMR-IHC was able correctly identify positive MMR-IHC results, intact MMR. This is a result that exceeds usual sensitivity of MMR-IHC versus MSI in literature[12,43.] It supports the utilization of MMR as a stand-alone screening method. Considering this, routine MSI of this high-risk feature population testing could be eliminated in the future. However, this also demonstrates the usefulness of MSI testing as a screening quality assurance method. It is unlikely MSI testing will be eliminated completely other quality assurance measures are put into place.

Although MSI is associated with LS, most MSI relates to somatic hypermethylation of MLH1 MMR and does not reflect a germline mutation[14,16]. Because hypermethylation leads to silencing of MLH1, it would report as MMR-IHC *d*MLH1. Identification of 23 BRAF V600E mutations, strongly correlated with sporadic CRC MLH1 promoter hypermethylation[24], increased the overall sensitivity of the screening protocol; the ability of the of the protocol so correctly identify those patients with LS. BRAF mutation analysis was successfully utilized in this study to reduce the number of cases sent for germline testing.

While this study used BRAF molecular sequencing to distinguish between BRAF V600E, a mutation almost uniquely associated with sporadic MSI-H tumours and BRAF-WT, Capper et al (2013) demonstrated the ability of a BRAF V600E-specific IHC (clone VE1) to do the same. Capper et al (2013) reported a 100% sensitivity and 98.9% specificity of BRAF IHC when compared to BRAF molecular sequencing[29]. In contrast, Adackapara et al (2013) found the sensitivity and specificity implementation of a VE1 antibody to be much lower, 71% and 74%, respectively[30]. The authors describe weak cytoplasmic and non-specific nuclear staining where only 71% of known BRAF V600E mutant tumours stained properly[30]. Despite this finding, incorporation of a VE1 monoclonal antibody may be a useful addition to the LS IHC diagnostic panel. It could potentially reduce BRAF sequencing costs and decrease result turn-around-time.

The use of hypermethylation specific testing could allow for initial exclusion of a larger percentage of *d*MLH1 from germline testing; it would exclude a portion of the BRAF-WT cases screened in this study. However, hypermethylation of MLH1 has been observed in up to 15% of LS individuals[70] but authors suggest it is most likely due to a false positive or to methylation as a second hit event in a LS individual[24, 70]. This calls into question the sensitivity of hypermethylation testing with concern about the potentially high false positive rate. This concern is shared by Moreira et al (2015) who detected a somatic MLH1 hypermethylation through hypermethylation testing in three patients with known LS related *d*MLH1[70]. These patients would not have been identified as LS carriers if BRAF testing was not also conducted; the study reported a 100% sensitivity for BRAF analysis and concluded that hypermethylation analysis alone could not provide the sensitivity required to identify LS[70]. Higher costs are associated with molecular testing, for both BRAF and hypermethylation analysis. A shift toward BRAF IHC combined with low sensitivity of hypermethylation analysis would suggest use of hypermethylation analysis would not be beneficial or cost-effective.

6.1 Study Limitations

Limitations to this study do exist. Despite intensive communication efforts from the project lead, including a thorough description of testing parameters, a full understanding of who should be tested for LS was not established. Protocol initiation memos outlining protocol parameters, MMR-IHC order entry procedures, LIS MMR code inserts, and tissue sample parameters were distributed amongst the various stakeholders. However, cases were assigned to pathologists outside of the screening working group who were unfamiliar with the protocol. This resulted in parameter discordant cases being tested and failure to test some concordant cases.

A common misunderstanding was that tumour tissues with histologic high-risk features were to be screened regardless of age. This was observed as most discordant cases were those where patient age exceeded 70 years but had a high-risk histological feature. Within the MMR reporting group, the provisional visual guide and standardized MMR insert codes resulted in essentially full compliance. The one exception was incomplete timely copying of reports to genetics.

Despite MMR insertion code compliance by the working group, data collection and tracking using MMR marker insertion codes was not efficient or consistent. Many cases were collected which fell outside of protocol parameters, largely age >70, those before October 2013, and non-CRC tissues. However, this did not reflect a communication failure. Rather, these were almost certainly all cases marked by various separate authorities (for example genetics) handling backlog cases requested through a different tracking method. Inclusion of these cases in initial data collection concluded in many hours human revision to ensure a proper data set.

Data collection was further complicated by the use of two different LIS systems in Manitoba (Winnipeg and Brandon sites). Access to the Brandon LIS system was not readily available from Winnipeg, where most of the data collection and analysis took place. Data received from Brandon did not include certain patient and tumour characteristics (age, stage, site, histologic features, etc) that were more easily accessed in Winnipeg. Unfamiliarity with some of the details of this data subset, due to lack of access by Laura Sexsmith, ultimately led to removal

of the Brandon data. A strong, well rounded, user friendly LIS system would accelerate rate of data retrieval and increase search accuracy thereby decreasing time intervals between results and GC referral.

Data collection did not include family history for the study cohort and would have been uneasy to access based on the collection methods used. Because of this, the yield of the Manitoba protocol in term of LS detection cannot be compared to other strategies such as the revised Bethesda Criteria and Amsterdam II Criteria.

Frustrations over inclusion and timely communication of results to clinical teams, lead to a change in approach where genetic counsellors were directly contacted patients via telephone once a HL of LS was report. Heald et al (2013) observed a statistically significant decrease in the number of days between referral and GC when a similar method was used to contact patients[36]. Despite these initial frustrations, this shift most likely contributed to the impressive 96% of patients contacted for GC and subsequent 76% follow-up with germline sequencing. These numbers exceed those observed by Heald et a (2013)l[36].

6.2 Future Considerations

With an established LS screening protocol and standardized algorithm in place, Manitoba might consider expanding the protocol criteria to include endometrial cancer (EC) tumour tissues. While LS is most commonly related to colorectal cancer, women with LS have a 40-60% chance of developing EC as their first LS malignancy. Women also have a greater overall risk of

developing EC than CRC[11,71-73]. Despite an uncertainty surrounding best LS EC detection methods, largely because few studies choose to focus on LS related EC, reflex testing of all newly diagnosed EC for LS should be considered.

7 CONCLUSION

Implementation of an in-house LS in Manitoba screening program led to the identification of a "high likelihood" LS profile in CRC patients ≤70 years in 8.5% of patients, and identification in 2.8% of cohort, meeting those criteria described in literature. These numbers can be in part be contributed to a strong uptake of genetic counselling and efforts made by GC to contact patients.

Most of these patients did not meet the age criterion cut off (≤50 years) used in routine clinical assessment guidelines (AM II and RBG). This highlights the advantages of a screening protocol with a high age cut off, such as this one.

A unified cross province LIS system or a more inclusive collection process would allow for more efficient and accurate data collection. Formalized and strong communication strategies between various facets would decrease the confusion and wait times between IHC results, supplementary testing, and genetic counselling.

Efficiency and effectiveness of the screening protocol will increase as inconsistencies and weaknesses are corrected. An increased number of potential LS carriers will be identified allowing affected families and carriers to consider prophylactic surgeries and enrollment in

surveillance and screening programs. A successful LS program has been established in the province of MB.

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