

Identifying Children with Constitutional
Mismatch Repair Deficiency (CMMR-D)
Syndrome in the Expanding Lynch
Syndrome population in Cape Town

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Abstract:

INTRODUCTION: Constitutional Mismatch Repair Deficiency (CMMR-D) syndrome is a rare tumour predisposition and polyposis syndrome that presents in childhood. It is caused by mutations in mismatch repair (MMR) genes that result in a tumour spectrum including colorectal cancers, high-grade gliomas, non-Hodgkin T-cell lymphomas and leukaemias. It is characterized by biallelic germline mutation of one of four possible MMR genes resulting in loss of protein expression that can be identified by applying immunohistochemistry to formalin fixed paraffin embedded tissue sections. Use of MMR immunohistochemistry is established in the setting of Lynch syndrome (LS); however, the pattern of loss of staining in the background, non-tumour tissue is unique to CMMR-D syndrome. CMMR-D syndrome is seen in LS families and occurs as a result of consanguinity or founder effect. The South African population has LS families concentrated in the Western Cape and Northern Cape Provinces and the mixed ancestry population shows a unique *MLH1* c1528C>T mutation which may have implications on the incidence, penetrance and severity of CMMR-D syndrome seen in our population. The diagnosis of CMMR-D syndrome includes clinical findings outlined in the European Consortium's Care of CMMRD document and confirmation of the biallelic mutation in one of the MMR genes. MMR immunohistochemistry can be used in the diagnosis of CMMR-D syndrome by identifying cases for targeted molecular genetic tests. However, MMR immunohistochemical staining patterns are not usually described in detail, particularly the loss of staining of the affected gene in the background, non-tumour tissue, the key feature of CMMR-D syndrome.

METHODS: We performed a retrospective analysis of archival formalin fixed paraffin embedded tissue of children attending Red Cross Children's Hospital with tumours that form

part of the CMMR-D spectrum, outlined by the Care for CMMRD criteria. We used the criteria of high-grade gliomas (WHO Grade III or IV) occurring before 25 years of age, cutaneous lesions suggestive of CMMR-D syndrome and patients with a first or second degree relative diagnosed with LS. MMR immunohistochemistry was applied, and the staining pattern was documented in terms of proportion of tumour staining and intensity of staining using a modified Allred Scoring system. Specific attention was given to the characterization of the staining pattern of the background normal tissue.

RESULTS: 21 samples taken from 18 patients were evaluated. 16 samples represented brain tumours, predominantly high-grade gliomas. Three samples were excluded due to suboptimal staining despite positive external controls. 12 samples showed intact staining of all four MMR stains. Two samples showed staining of unknown significance. Four samples from 3 different patients showed staining patterns compatible with MMR deficiency. This included two patients, each with a biopsy showing high-grade glioma and two samples of the same patient taken at a 1-year interval of a Burkitt lymphoma. Of these four samples, three samples showed loss of staining in background non-tumour tissue with positive external control, the unique staining pattern for CMMR-D syndrome. These cases will be referred for confirmatory testing by molecular genetic techniques.

CONCLUSION: MMR immunohistochemistry can be used in the evaluation of CMMR-D syndrome, but care is needed in evaluating adequacy of staining, the pattern and scoring of staining of both the tumour and the background non-tumour tissue. Endothelial cells are easy to identify and evaluate as background tissue which is useful in extra-intestinal tumours. Neurons and choroid plexus can also be evaluated as background tissue in brain tumour samples. Selection bias in this study resulted in the underrepresentation of lymphomas and colorectal carcinomas. Improved characterization and search for Non-Hodgkin T-cell

lymphomas and inclusion of samples of colorectal carcinomas of adolescents and adults would be needed to include these tumours. Use of MMR immunohistochemistry in post-mortem tissue samples is not recommended because of suboptimal staining, even with a short post-mortem interval of 1 day. The diagnosis of CMMR-D syndrome depends on clinical application of Care for CMMRD criteria, MMR immunohistochemistry in conjunction with molecular genetic testing. It is important to identify cases of CMMR-D syndrome and offer cancer screening to prevent development of other cancers in the index patient. It also provides an opportunity for genetic counselling and testing of the parents and at-risk siblings.

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List of Abbreviations:

APC	Adenomatous Polyposis Coli
BMMRD	Biallelic Mismatch Repair Deficiency
CC1	Cell Conditioning solution 1
C4CMMR-D	Care for CMMR-D
CMMR-D / CMMRD	Constitutional Mismatch Repair Deficiency
CNS	Central Nervous System
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
FAP	Familial Adenomatous Polyposis
FFPE	Formalin Fixed Paraffin Embedded
HIV	Human Immunodeficiency Virus
HNPCC	Hereditary Nonpolyposis Colorectal Cancer
IHC	Immunohistochemistry / immunohistochemical
LCS	Liquid Coverslip
LS	Lynch Syndrome
MLH1	MutL Homolog 1
MMR	Mismatch Repair
MRI	Magnetic Resonance Imaging
MSH2	MutS Homolog 2
MSH6	MutS Homolog 6
MSI	Microsatellite Instability
NF	Neurofibromatosis
PCR	Polymerase Chain Reaction
PMS2	PMS1 Homolog 2, mismatch repair system component
POLD	DNA polymerase delta 1, catalytic subunit
POLE	DNA polymerase epsilon, catalytic subunit
UCT	University of Cape Town
WHO	World Health Organization

Introduction:

The DNA Mismatch Repair System:

The Mismatch Repair (MMR) system is responsible for the detection and repair of DNA base pair mismatches during DNA replication in human prokaryotic and eukaryotic cells. The MMR system is also involved in the apoptotic response (Peltomaki, 2003). Four Mismatch Repair (MMR) genes encode four MMR proteins: *MSH2*, *MSH6*, *MLH1* and *PMS2*. These proteins form two pairs of heterodimers which are obligatory partners. *MSH2* is paired with *MSH6* which recognizes and binds to single base mismatched pairs or insertion-deletion loops in newly synthesized DNA. The second pair of MMR proteins, *MLH1* and *PMS2*, joins the complex and recruits an endonuclease to excise the defective daughter strand of DNA. It recruits DNA polymerase to generate a new, correct complementary daughter strand to repair the mismatched DNA (Huang, et al., 2014).

Microsatellites and Microsatellite Instability:

Mutations of MMR genes result in impaired DNA repair of mononucleotide and dinucleotide repeats throughout the genome and these regions are known as microsatellites. These mutations result in microsatellite instability (MSI). There is altered repeat size with shortened length of allele in the tumour DNA. This difference in length can be detected by polymerase chain reaction (PCR). In the setting of Lynch Syndrome, microsatellite instability is established according to the Bethesda guidelines by testing a simplified panel of five microsatellite loci. These include two mononucleotide repeats: BAT25 and BAT26, and 3 dinucleotide repeats: D2S123, D5S346 and D17S250. If ≥ 2 of the 5 markers show microsatellite instability, this is classified as MSI high category. If only one marker shows microsatellite instability, this is

classified as MSI low category. If none of the markers show microsatellite instability, this is classified as MSI stable (Huang, et al., 2014).

Lynch Syndrome and Constitutional Mismatch Repair Deficiency Syndrome:

Lynch syndrome (LS) and Constitutional Mismatch Repair Deficiency (CMMR-D) syndrome are both inherited tumour predisposition syndromes that manifest with gastrointestinal polyps and develop into early onset colorectal carcinomas. What sets them apart from the other tumour polyposis syndromes is that they both result from mutations in the MMR genes. LS is well known to clinicians and pathologists. Routine screening and testing for LS-associated colorectal carcinomas are implemented in most diagnostic laboratories. And there is research and development into screening and testing of LS-associated breast carcinomas, endometrial carcinomas and other tumours. CMMR-D syndrome is a rare and relatively recently recognized entity in comparison to LS. In order to understand CMMR-D syndrome, we need to discuss the concepts around screening and diagnosis of LS. The differences will be discussed and then summarized in Table 3.

Lynch Syndrome:

Lynch syndrome, formerly known as hereditary nonpolyposis colorectal cancer (HNPCC), is a genetic disorder with an autosomal dominant pattern of inheritance. There is an inherited germline mutation of one of the four MMR genes. The remaining wild-type MMR allele undergoes somatic point mutations or loss-of-heterozygosity. This results in frameshift and downstream nonsense mutations that express truncated, non-functional MMR proteins and impairs the MMR DNA Repair system. Replicating DNA is not repaired and there are increased

numbers of spontaneous, single base-pair mismatches with point mutations and insertion-deletions loops. This leads to loss of control of cell growth and cell differentiation, which results in the development of specific types of cancers. LS patients are predisposed to the development of many types of cancers, predominantly colorectal carcinomas and endometrial carcinomas. The Amsterdam and Bethesda criteria are tools used to identify at-risk patients for LS testing (Huang, et al., 2014).

Constitutional Mismatch Repair Deficiency Syndrome:

CMMR-D syndrome occurs when a patient inherits a mutation in both alleles of the same MMR gene, one from each parent. In half of cases, each parent contributes an allele with a different defect in the same MMR gene and this is known as compound heterozygous mutations. In the other half of cases, each parent contributes an allele with an identical mutation in the same MMR gene and this is known as homozygous mutations. Homozygous mutations typically occur in children of LS families as a result of consanguineous unions (Huang, et al., 2014).

Counterintuitively, in spite of the genetic basis of the CMMR-D syndrome, both parents may be unaffected by LS. Family members of the index patient with CMMR-D syndrome do not usually meet the clinical criteria (Amsterdam Criteria) for LS and they are rarely affected by LS-associated cancers (Huang, et al., 2014). Lack of awareness around this feature may result in delays in diagnosis. One reason for the lack of parental expression of LS is that 60% of CMMR-D patients show biallelic germline mutations in *PMS2* and the remaining cases show mutations in *MLH1*, *MSH2* and *MSH6*. The penetrance in monoallelic *PMS2* and monoallelic *MSH6* mutated obligate carriers is less than that for other MMR genes (Tabori, et al., 2017).

The reason for this is still unclear, but it may be because these mutations result in only partial impairment of the PMS2 protein (Huang, et al., 2014). This is in contrast to *MLH1* and *MSH2* mutations which are identified in the majority of patients with LS.

CMMR-D syndrome is clinically distinct from LS and the patients are at higher risk for developing childhood-onset gastrointestinal cancers and extraintestinal cancers when compared to other childhood cancer syndromes. The original constellation of features was colorectal cancers, brain tumours, lymphoma and leukaemia with neurofibromatosis type 1-like cutaneous lesions or café au lait-like cutaneous lesions (Durno, et al., 2017).

CMMR-D syndrome patients can develop metachronous gastrointestinal cancers, commonly colorectal or small intestinal carcinomas with associated adenomatous polyps. The number of adenomatous polyps range from 1 to 50 polyps which can make it difficult to distinguish from other polyposis syndromes such as attenuated Familial Adenomatous Polyposis (FAP) (Huang, et al., 2014). CMMR-D syndrome patients are often initially presumed to have FAP. When *APC* mutations cannot be identified, these patients should be re-evaluated for CMMR-D syndrome (Durno, et al., 2017). Colorectal carcinomas manifest in adolescence or early adulthood and multiple primary synchronous colorectal carcinomas can also occur.

It is important to identify cases of CMMR-D syndrome because it has the highest lifetime risk for gastrointestinal cancers with accelerated progression from adenoma to carcinoma. *POLE* and *POLD1* mutations are acquired early and when paired together with MMR deficiency, it results in tumours with massive numbers of substitution mutations and an accelerated rate of progression. CMMR-D syndrome also predisposes to other gastrointestinal and extra-

gastrointestinal cancers. 40% of patients will develop a secondary metachronous malignancy (Ramchander, et al., 2017).

Small intestinal carcinoma is rare in childhood; however, CMMR-D patients are at increased risk and there have been reported cases of small intestinal carcinoma in children from as young as 11 years of age (Huang, et al., 2014).

The European Consortium's Care for CMMRD (C4CMMRD)

In CMMR-D syndrome, diagnostic difficulties result from the diverse and wide range of neoplasms and the absence of syndrome-specific clinical features. It is a rare entity and the lack of awareness around it contribute to underdiagnosis and underreporting of cases. This results in missed opportunities to screen the index patient and their families for preventable and treatable cancers. A European consortium, 'Care for CMMR-D' (C4CMMR-D), was established and they held a workshop in 2013 to address these issues. Wimmer *et al.* characterized the malignancies of 146 patients with CMMR-D syndrome. They developed the criteria for CMMR-D testing in cancer patients, which is outlined in Table 1 (Wimmer, et al., 2014).

Table 1: Indications for CMMR-D testing in cancer patients, adapted from Wimmer et al. (2014)

Indication for CMMRD testing in a cancer patient	≥3 points
Malignancies/premalignancies: one is mandatory; if more than one is present in the patient, add the points	
Carcinoma from the LS spectrum* at age <25 years	3 points
Multiple bowel adenomas at age <25 years and absence of APC/MUTYH mutation(s) or a single high-grade dysplasia adenoma at age <25 years	3 points
WHO grade III or IV glioma at age <25 years	2 points
NHL of T-cell lineage or sPNET at age <18 years	2 points
Any malignancy at age <18 years	1 point
Additional features: optional; if more than one of the following is present, add the points	
Clinical sign of NF1 and/or ≥2 hyperpigmented and/or hypopigmented skin alterations Ø>1 cm in the patient	2 points
Diagnosis of LS in a first-degree or second-degree relative	2 points
Carcinoma from LS spectrum* before the age of 60 in first-degree, second-degree, and third-degree relative	1 point
A sibling with carcinoma from the LS spectrum*, high-grade glioma, sPNET or NHL	2 points
A sibling with any type of childhood malignancy	1 point
Multiple pilomatricomas in the patient	2 points
One pilomatricoma in the patient	1 point
Agenesis of the corpus callosum or non-therapy-induced cavernoma in the patient	1 point
Consanguineous parents	1 point
Deficiency/reduced levels of IgG2/4 and/or IgA	1 point
*Colorectal, endometrial, small bowel, ureter, renal pelvis, biliary tract, stomach, bladder carcinoma. CMMRD, constitutional mismatch repair deficiency; LS, Lynch syndrome; NHL, non-Hodgkin's lymphomas; sPNET, supratentorial primitive neuroectodermal tumours.	

In Wimmer *et al.*'s study, the tumours associated with CMMR-D syndrome were characterized below:

45 patients developed haematolymphoid malignancies, the vast majority were non-Hodgkin lymphomas of T-cell lineage, but also included non-Hodgkin lymphomas of B-cell lineage.

Three patients even showed two different metachronous haematolymphoid malignancies. The study did not specify the histologic type of lymphoma.

78 patients developed brain tumours, the majority were high grade gliomas including glioblastoma (WHO grade IV) and anaplastic astrocytoma (WHO grade III) tumours. Oligodendrogliomas were not as common but were also described by Wimmer *et al.*

59 patients developed LS-associated malignancies. The majority were colorectal carcinomas. 16 patients developed synchronous colorectal carcinomas. Other LS-associated tumours included small bowel carcinomas, endometrial carcinoma, urethral carcinoma and ovarian carcinoma.

Of other tumours, one of each kind was represented including neuroblastoma, nephroblastoma, ovarian neuroectodermal tumour, infantile myofibromatosis, rhabdomyosarcoma, basal cell carcinoma, mucoepidermoid carcinoma of the parotid gland and osteosarcoma.

None of these tumours are unique to CMMR-D syndrome and some may not be related to the underlying mutation. However, suspicion of an underlying tumour syndrome may be raised if multiple tumours occur with an earlier age at presentation (See Figure 1).

Organ	Estimated penetrance, %	Age at diagnosis, median (range), y	References
Small-bowel adenomas ^a	50	12 (10–20)	12,33
Colorectal adenomas ^a	>90	9 (6–15)	12,33
Small-bowel cancer	10	28 (11–42)	12,17,18,25
Colorectal cancer ^b	70	16 (8–48)	12,17,18
Low-grade brain tumors	Unknown	Unknown	
High-grade brain tumors ^c	70	9 (2–40)	17,18,25
Lymphoma	20–40	5 (0.4–30)	17,18,25
Leukemia	10–40	8 (2–21)	17,18,25
Endometrial cancer	<10	(19–44)	17,18,25
Urinary tract cancer	<10	(10–22)	17,18,25
Other sites ^d	<10	(1–35)	17,18,25,33

^aLow- and high-grade adenomas with probable rapid progression.

^bPatients undergo subtotal colectomy and ileal–rectal anastomosis, resulting in a decreased risk of colorectal cancer.

^cHigh-grade glioma, medulloblastoma, and primitive neuroectodermal tumors.

^dFewer than 5 cases of each of the following neoplasms have been reported: neuroblastoma, Wilms tumor, rhabdomyosarcoma, osteosarcoma, breast cancer, melanoma, ovarian neuroectodermal tumor, pilomatricoma, and hepatic adenoma.

Figure 1: Estimated Penetrance and Age of Onset of Neoplasms in BMMRD (CMMR-D syndrome), adapted from Durno *et al.* (2017)

Neurofibromatosis type 1 (NF1) -like features have been observed in most, but not all patients with CMMR-D syndrome. In contrast to CMMR-D syndrome, NF1 is an autosomal dominant disorder which results from mutations in the *Neurofibromin 1* gene. Café-au-lait spots are the main cutaneous manifestation of NF1. Patients with CMMR-D syndrome associated NF1 have no family history of the disease. It has been suggested that the NF1-like manifestations result as a consequence of the failure in the DNA repair mechanism (Lamola, 2018).

Laboratory Diagnosis of CMMR-D Syndrome:

MMR Immunohistochemistry:

Immunohistochemical (IHC) stains may be applied to formalin fixed paraffin embedded (FFPE) tissue sections to identify loss of expression of each of the four MMR proteins. This method is already in use in most diagnostic laboratories because there are established screening protocols for LS in colorectal carcinomas (Huang, et al., 2014). Generally, MMR immunohistochemistry is widely available, more cost effective and has better turnaround times when compared to PCR testing or other molecular techniques.

Use of MMR Immunohistochemistry in Lynch Syndrome:

In LS, the Bethesda criteria and the Amsterdam criteria are used to identify patients for screening by immunohistochemistry. Cases that have intact nuclear staining of all four MMR proteins can be excluded and the pool of suspected LS cases is narrowed. LS patients with missense mutations can show intact staining of all four MMR immunostains, and this is a recognized cause for a false negative result. Diagnosis in these cases depends on strong clinical suspicion and referral for further testing by molecular techniques (Wimmer, et al., 2014).

In the evaluation of MMR immunohistochemistry, both the tumour cells and the background cells are evaluated. In tumour cells, any nuclear staining is considered positive (Bartley, et al., 2014); however, careful evaluation is required because the tumour can show heterogeneous staining with patchy loss of nuclear staining. The background, non-tumour colonic epithelial cells, stromal cells, tumour infiltrating lymphocytes and inflammatory cells are expected to

have intact nuclear positive staining and they are used as inbuilt positive controls (Bartley, et al., 2014).

In the cases that show loss of immunostaining, the pattern of staining allows for targeted molecular genetic testing. Instead of testing all four MMR gene, we can start with the single MMR gene, or the dominant heterodimer that showed loss of immunostaining. Mutations in *PMS2* or *MSH6* result in isolated loss of these proteins, whereas mutations in *MLH1* or *MSH2* leads to concurrent loss of *MLH1/PMS2* or *MSH2/MSH6*, respectively, since *MLH1* and *MSH2* are the obligatory partners (Wimmer, et al., 2014). The pattern of loss of staining in the tumour cells between the four MMR proteins is used to stratify patients into MSI stable tumours and tumours that require further investigations. Diagnostic algorithms are applied to exclude loss of immunostaining caused by other mechanisms such as *MLH1* loss due to promoter hypermethylation (Huang, et al., 2014).

Use of MMR Immunohistochemistry in CMMR-D Syndrome:

In the setting of CMMR-D syndrome, immunohistochemistry is proposed as a diagnostic tool due to its unique staining pattern and was found to be reliable in the evaluation of solid tumours. In contrast to LS, CMMR-D patients show loss of expression of the affected MMR gene in both tumour cells and in the background non-tumour tissue. In general, if this occurs with non-MMR immunohistochemical stains, it prompts checking of the external positive control. If it has failed, the test is considered inadequate and it usually requires review of preanalytical factors with repeat application of staining. In the setting of CMMR-D syndrome, this pattern of staining should not be interpreted as failure of the staining, provided that the external positive control is adequate (Wimmer, et al., 2014). In patients with suspected

CMMR-D syndrome, if there is no tumour material available for testing, a normal skin sample may be submitted for testing to demonstrate the loss of staining in normal tissue (Wimmer, et al., 2014).

In the C4CMMRD and review of the literature of the use of MMR immunohistochemistry in CMMR-D syndrome, there is no explanation or characterization of what is considered loss of staining in tumour cells. It is unclear if the same scoring conventions of MMR immunohistochemistry for Lynch syndrome can be applied in CMMR-D syndrome. It is unclear if reduced staining may be considered a negative staining pattern.

Loss of MMR immunostaining in background non-tumour tissue as a characteristic in CMMR-D syndrome; however, the problem is the variable reporting of what is considered normal tissue or lack of documentation of which normal tissue were or should be evaluated. The tissue types mentioned include lymphocytes within blood vessels, tumour infiltrating lymphocytes, endothelial cells and “normal tissue”. One article even describes loss of staining in endothelial cells, then shows an image of positive staining of endothelial cells (See Figure 2). This is a critical aspect of MMR immunohistochemistry staining, and should be described with more consistency, especially considering the implications of such a result.

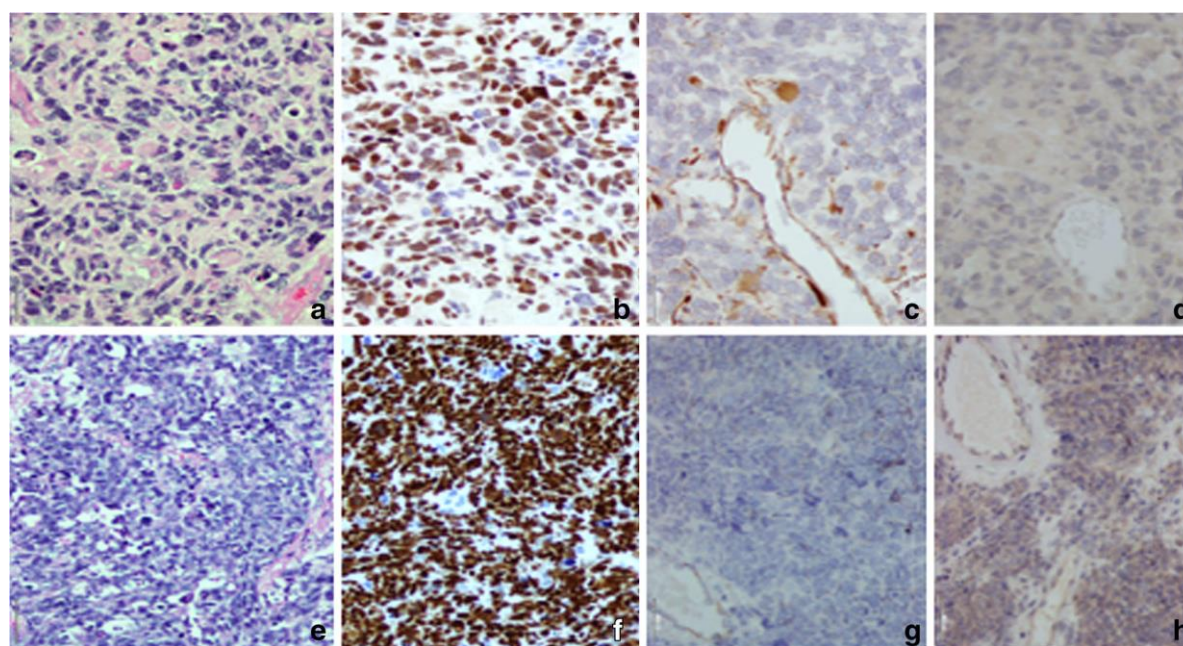


Fig. 2 Glioblastoma multiforme (a–d) and anaplastic medulloblastoma (e–h) tumors. **a** The glioblastoma consisted of pleomorphic cells, some with abundant acidophilic cytoplasm (H&E, ×40); **b** P53 immunostain was positive in the majority of cells (×40); **c**, **d** the mismatch repair proteins MLH1 (**c**) and PMS2 (**d**) were lost in tumor and endothelial

cells (×40). **e** The anaplastic medulloblastoma showed a cellular tumor with many mitotic figures and necrosis (H&E, ×40); **f** P53 immunostain was positive in almost all cells (×40); **g**, **h** the mismatch repair proteins MLH1 (**g**) and PMS2 (**h**) were lost in tumor and endothelial

Figure 2: A figure from Amayiri et al. (2016) describing loss of staining in endothelial cells; however, positive endothelial staining is seen in (c) and (h)

Objective and Reproducible Reporting of Immunohistochemical Stains in General:

In studies that involve the evaluation of staining of immunohistochemical stains, including MMR immunohistochemistry, it is important to evaluate and report the stains in an objective manner. This standardization allows for comparison of the findings between different individual samples, different tumour types and different study cohorts. Fedchencko *et al.* outline semiquantitative scoring systems which are used to standardize the evaluation of immunohistochemical stains and reduce interobserver variability. The parameters, such as intensity of staining and proportion of stained tumour cells, are evaluated and converted into quantitative data, which is then used for statistical analyses. The “gold standard” in the scoring of immunohistochemistry is the evaluation of oestrogen receptor, progesterone receptor and Her2/neu. The Allred scoring system is widely accepted and used by leading

associations and organizations. The percentage of positive cells is scored and the intensity of reaction in most examined fields is scored, which is outlined in Figure 3 . The scores are then added together for a final score that may range from 0 to 8. In this system, scores of 0 and 2 are considered negative, and scores of 3 to 8 are considered positive (Fedchenko & Reifenrath, 2014).

Proportion score A	Positive cells, %	Intensity	Intensity score B
0	0	None	0
1	<1	Weak	1
2	1 to 10	Intermediate	2
3	11 to 33	Strong	3
4	34 to 66	Final score range (A + B): 0-8	
5	≥67		

Figure 3: Allred scoring system outlined in Fedchenko et al. (2014)

Microsatellite Instability Testing in CMMR-D Syndrome:

MSI analysis with current protocols for LS is extremely sensitive and specific for LS-associated tumours, but it may be negative in CMMR-D cancers, especially in extraintestinal cancers (Tabori, et al., 2017). It is not recommended as a screening tool because MSI status may be unreliable in brain tumours and haematological malignancies for reasons that are not yet known (Wimmer, et al., 2014). Different MSI genotypes can be observed in different tumour tissue in patients affected by multiple cancers. MSI status may be tumour specific and not related to the mutation (Huang, et al., 2014). The reason for this is unknown, but there are subtler shifts of microsatellite alleles observed in brain tumours. A different panel of

microsatellite markers could be more sensitive for brain tumours and other tumours in the setting of CMMR-D syndrome.

According to Wimmer *et al.* a recent, simpler assay analysing “stutter” peaks was presented for the detection of MSI in CMMR-D syndrome patients. Stutter peaks are associated with microsatellite PCR products. A new, publically available software application is used to quantify the peak height of the “stutter” peaks in selected dinucleotide microsatellites. In CMMR-D syndrome patients, this peak significantly increases compared to normal controls, but is limited to *PMS2*, *MLH1* and *MSH2* mutations. The peak height in patients with biallelic *MSH6* mutations is not altered (Wimmer, et al., 2014).

Molecular Genetic Testing:

Genetic testing is recommended to help confirm the diagnosis of CMMR-D syndrome. It is used to detect biallelic mutations in one of four MMR genes. These genes have many variants of unknown significance. The *PMS2* gene also has multiple pseudogenes which makes it difficult to sequence (Tabori, et al., 2017).

Tabori *et al.* suggest that MSI should not be used as a diagnostic tool for CMMR-D syndrome because of the risk of false negative results and that immunohistochemical stains should be used as a screening tool on any index patients and family members (Tabori, et al., 2017).

In the C4CMMRD guidelines, it is acknowledged that both immunohistochemistry and MSI testing have potential pitfalls. They recommend a combination of both methods if needed.

Final confirmation comes from the identification of the causative biallelic mutation (Wimmer, et al., 2014).

Relevance to the Lynch Syndrome Families of Cape Town:

There is a well-known population of LS affected families in the Western Cape and Northern Cape Provinces of South Africa. They are regularly followed up by clinicians and clinical geneticists as part of the University of Cape Town (UCT) Human Genetics registry. LS populations across the world can show varied predominance of the affected MMR gene and specific mutations. In the South African population, the *MLH1* c1528C>T mutation is the most common mutation. It is unique to the mixed ancestry population and is recognized as a founder mutation. The mutation aging analysis traced this mutation to be an estimated 225 years old (1789 – 1814); however, the “founder” of this founder effect in this population has not yet been identified. The founder effect may explain the higher frequency of LS in the Cape mixed ancestry populations of the Western and Northern Cape provinces compared to the rest of South Africa (Lamola, 2018).

Consanguinity and founder effect are the two mechanisms that contribute to the development of CMMR-D syndrome (Ramchander, et al., 2017). Mutations in both *MLH1* and *MSH2* contribute to the entire cohort of LS cases in the University of Cape Town (UCT) Human Genetics registry. This is a concern because CMMR-D syndrome arising from mutations in *MLH1/MSH2* result in a more severe phenotype. The percentage of biallelic mutation carriers with more than one malignancy is lowest in *MLH1/MSH2* carriers (22%) and highest in *PMS2* biallelics (42%) (Wimmer, et al., 2014). This may indicate that patients with *PMS2* biallelic

patients are more likely to survive to develop a second metachronous tumour, where as those with *MLH1/PMS2* are less likely to survive to develop a second metachronous tumour. The incidence of CMMR-D syndrome may be different in our population because the majority of cases of CMMR-D syndrome are attributed to mutations in *PMS2*, but there is a predominance of *MLH1/MSH2* mutations in our population. Table 2 shows the average age of onset of different tumours in biallelic *MLH1* carrier cases.

Table 2: Average age of malignancy presentation / detection among reported biallelic MLH1 mutation carrier cases, adapted from Bruwer et al. (2014)

Site of malignancy	Average age of onset for specific malignancy
Haematological	2.9 years
Brain	4.5 years
Colorectal	17.8 years
Mean age of onset of any malignancy	8.4 years
Range	1 year to 35 years

In contrast to the founder effect seen in the South African population, a study in Jordan looked at a region with high rates of consanguinity. They found that there is a higher prevalence of CMMR-D syndrome compared to a North American cohort. They found that up to 51% of childhood gliomas in Jordan may harbour germline mutations in MMR genes (Amayiri, et al., 2016).

The first published report of predictive genetic testing for CMMR-D syndrome came from our institution and the University of Cape Town (UCT) Human Genetics registry. Both the mother and father showed heterozygous mutation in *MLH1* c1528C>T, the most common mutation in our population. The parents were of mixed ancestry and they were not related to each other. They had two daughters and the parents were offered predictive testing and genetic

counselling due to the concern from CMMR-D syndrome. The older daughter had café au lait spots and was tested and confirmed to have biallelic mutations in *MLH1* c1528C>T, CMMR-D syndrome. She later died from complications of a high-grade diffuse astrocytoma of the brain stem, cerebellum and thalamus despite normal screening tests. The brain MRI and blood work were normal six months prior to the development of symptoms. The younger daughter was also tested and showed heterozygous mutation in *MLH1* c1528C>T (Bruwer, et al., 2014).

Wimmer *et al.* characterized the expression of tumours in patients with an established diagnosis of CMMR-D syndrome. They suggested that there is an under-recognition and under-diagnosis of CMMR-D syndrome because there is a varied presentation, overlapping features with other tumour syndromes such as NF1 and FAP and lack of specific features of this syndrome. The diagnosis depends on increased awareness and a higher index of suspicion to avoid a missed or a delayed diagnosis. This study aimed to address this issue. When paediatricians identify a patient with clinical suspicion for CMMR-D syndrome, by applying MMR immunohistochemistry to tumours that fit part of the CMMR-D syndrome spectrum, can we identify cases that are CMMR deficient? Is there a role of the pathologist to flag these cases for the attention of the clinicians and trigger genetic testing and genetic counselling of affected families? This may be different in the South African population because we have established LS families with a unique *MLH1* mutation and potentially a more aggressive phenotype.

Table 3: Comparison of Lynch Syndrome and CMMR-D Syndrome

Lynch Syndrome		CMMR-D Syndrome
GENETICS		
Autosomal dominant	Inheritance pattern	Autosomal recessive
Founder effect	Mechanism of inheritance	Founder effect and/or consanguineous unions
Germline mutation in an MMR gene with second acquired somatic mutation	Type of mutation	Biallelic germline mutation in the same MMR gene
<i>MLH1</i> and <i>MSH2</i> genes Unique <i>MLH1</i> c1528C>T mutation in mixed ancestry population in South Africa	Predominantly mutated MMR genes	<i>PMS2</i> and <i>MSH6</i> genes
CLINICAL FINDINGS		
Expected autosomal dominant pedigrees	Family history of Lynch Syndrome	May not be present. <i>PMS2</i> mutations have partial penetrance, due to possible partial inactivation
± 50 years	Average age of onset of MMR-deficient colorectal carcinomas	17.8 years
Not classically described	Cutaneous manifestations	Café-au-lait-like macules and multiple pilomatrixomas
Breast carcinoma and endometrial carcinoma	Commonly associated extraintestinal tumours	High grade gliomas, Non-Hodgkin T-cell lymphoma, leukaemia
Amsterdam and Bethesda criteria	Tools for evaluation of at-risk patients	Care for CMMR-D (C4CMMR-D) criteria
MMR IMMUNOHISTOCHEMISTRY		
Established protocols for routine use in colorectal carcinomas	Use as a screening tool	Some conflicting recommendations. Most recommend MMR IHC as the first investigation, to direct targeted molecular testing
Mutated MMR gene ±heterodimer: Loss of nuclear staining in tumour cells & Non-mutated genes Intact nuclear staining in tumour cells	Expected staining in tumour cells (patients <u>with</u> MMR deficiency)	Mutated MMR gene ±heterodimer: Loss of nuclear staining in tumour cells & Non-mutated genes: Intact nuclear staining in tumour cells
In <u>both</u> mutated and non-mutated genes:	Expected staining in background tissue	Mutated MMR genes ±heterodimer:

Intact nuclear staining in stromal cells, inflammatory cells and non-tumour epithelial cells (established for colorectal carcinomas) Used as an inbuilt positive control	(patients <u>with</u> MMR deficiency)	Loss of nuclear staining in background tissue At risk of being interpreted as failed staining & Non-mutated genes: Intact nuclear staining in background tissue
Intact nuclear staining in tumour cells of all 4 MMR IHC	Expected staining in tumour cells (patients <u>without</u> MMR deficiency)	Intact nuclear staining in tumour cells of all 4 MMR IHC
Intact nuclear staining in background tissue of all 4 MMR IHC	Expected staining in background tissue (patients <u>without</u> MMR deficiency)	Intact nuclear staining in background tissue of all 4 MMR IHC
Colorectal carcinomas: MLH1 loss can also be seen in tumours with promotor hypermethylation (false positive) Lynch syndrome with missense mutations can show falsely intact staining (false negative)	Recognized causes for false results	Missense mutations can show falsely intact nuclear staining (false negative)
MSI LOCUS TESTING		
MMR immunohistochemistry determines if MSI testing is recommended Extremely sensitive and specific for Lynch syndrome associated tumours	MSI locus testing	Not recommended as a screening tool. May be unreliable in brain tumours and haematological malignancies. Different MSI genotypes can be observed in different tumour tissue in patients affected by multiple cancers
MOLECULAR GENETIC TESTING		
Gold standard. Immunohistochemistry can identify which gene to test. Recommended if there are conflicting results from immunohistochemistry and MSI testing	Molecular genetic testing	Immunohistochemistry can identify which gene to test. Recommended to use IHC in conjunction with molecular genetic testing

Methods:

Design and Setting:

This study was a retrospective study using archival formalin fixed, paraffin embedded (FFPE) tissue samples of paediatric patients attending Red Cross Children's Hospital. The C4CMMR-D criteria, outlined by Wimmer *et al.*, was used to identify at-risk patients. MMR immunohistochemical stains were applied to tissue sections of each case: *MLH1*, *PMS2*, *MSH2* and *MSH6*. Decreased or absent staining of one or more of these stains indicated abnormalities in mismatch repair protein expression and the possible diagnosis of CMMR-D syndrome. These cases will be referred to the Department of Human Genetics for genetic counselling and proposed confirmatory testing.

Sampling:

A clinical search was performed to identify paediatric oncology patients with a family history of LS, patients with cutaneous lesions suggestive of CMMR-D syndrome and patients diagnosed with tumours in the spectrum of CMMR-D syndrome. A computerised search of the entire Red Cross Hospital histopathology database was performed to retrieve relevant cases from 1988 to 2014.

A modified version of Wimmer *et al.*'s "*Indication for CMMRD testing in a cancer patient*" criteria (See Table 1) to determine the types of CMMR-D associated tumours to search. The search included patients with one or more of the following conditions or neoplasms:

- Endometrial, colorectal, small bowel, ureter, renal pelvis, biliary tract, stomach and bladder carcinoma diagnosed <25 years of age (LS spectrum carcinomas)
- Multiple bowel adenomas / single high-grade adenoma, < 25 years of age
- WHO grade III or IV gliomas, <25 years of age

- Non-Hodgkin T-cell lymphoma or supratentorial primitive neuroectodermal tumour <18 years of age
- Any malignancy at age <18 years with café au lait spots (≥ 2 hyperpigmented and/or hypopigmented skin macules)
- Multiple pilomatrixomas
- Agenesis of corpus callosum or non-treatment related cavernoma
- Deficiency/reduced levels of IgG2/4 and/or IgA

Surgical biopsies, frozen section samples and post-mortem samples were included. Cytological specimen, samples with insufficient viable tumour material and adult samples were excluded.

Study Procedures:

Each sample was assigned a unique study number to protect patients' identifying details. The electronic documents used to capture this information were password protected and access was limited to the study staff only. The patients' age, diagnosis, date and site of sample were documented. A selected folder review of cases with features compatible with CMMR-D syndrome was performed.

The Haematoxylin and Eosin (H&E) slides of each sample were evaluated and the best slide was selected to minimize factors that would interfere with the staining process, such as excessive tumour necrosis and poor tissue preservation. The corresponding tissue blocks were retrieved from archives. Additional sections were made, and each sample was stained with the four immunohistochemical stains: *PMS2*, *MLH1*, *MSH2* and *MSH6*.

Methodology

Immunohistochemistry was performed using the commercially-available, automated detection system – Roche Optiview DAB detection system and automated supplier antibodies. The preparation of all buffers and reagents used in the staining protocol are listed in Appendix A.

Three-micron FFPE tissue sections were cut on a Leica Rotary Microtome RM2125 RTS. The sections were floated on a water bath and picked up on coated Histobond slides. The sections were then heat fixed on a hotplate at 60°C for 10-15 minutes.

Thereafter, the various MMR antibody protocols were selected on the auto-stainer, and a barcoded slide label with the study number and date was generated.

The system generated label was fixed to the slide and then loaded onto the auto-stainer. The required bulk reagents were re-constituted with water (Appendix A), loaded, and registered on the auto-stainer. The detection system was also loaded and registered on the auto stainer. The run was then started, and a brief dewaxing took place using EZPREP. Antigen retrieval, using CC1 was then performed for 64 minutes. After antigen retrieval, each of the primary antibodies was applied automatically, and incubation took place for 36 minutes. The Optiview DAB detection kit reagents were then applied to the slides to visualise the antigen/antibody complex. The slides were then automatically counterstained with haematoxylin and blued using bluing reagent. When the staining protocol had run to completion, the slides were removed from the auto-stainer and washed briefly in diluted washing up detergent and water in order to remove the LCS.

The sections were dehydrated in alcohol, cleared in xylene, and then the slides were cover slipped using glass coverslips and Entellan.

Outcome Measures:

Evaluation of Immunohistochemical Stains:

The MMR staining was evaluated by Dr S Tu on an Olympus BX43 microscope. A modified Allred method of scoring immunohistochemistry was used to quantify proportion of viable tumour cells that showed positive nuclear staining and the intensity of the staining. Any nuclear staining of tumour cells is considered positive staining for MMR immunohistochemistry in the setting of Lynch Syndrome (Bartley, et al., 2014).

In this study, the Allred method (See Table 5) is used to record the intensity and proportion of each sample in an objective manner. The system is modified to exclude the final score because we do not know yet which scores are significant. The samples included in this study are from patients with suspected CMMR-D syndrome and have not been genetically confirmed.

Comments on staining of the background tissue were also included.

These results were documented on a password protected electronic spread sheet.

Results:

A total of 21 samples were selected from 18 patients. Two patients had two samples that were taken at the same instance with concordant diagnoses. One patient had two samples taken at an interval of 6 months with concordant diagnoses.

A total of four post-mortem samples were evaluated, with an average post-mortem interval of 1.75 days and a range of 1 to 4 days. One sample was from an intraoperative frozen section and the remaining 16 samples were routine surgical biopsies.

20 samples were submitted to the laboratory at Red Cross Hospital. One sample was submitted to the laboratory at Frere Hospital, a referral hospital located in East London, Eastern Cape Province and was subsequently sent to Red Cross Children's Hospital as a referral case.

The ages of the children were converted into months, to allow for fair comparison from infants to older children. Red Cross Children's Hospital has a cut-off age for admissions of 14 years. The referral case from East London did not include the age or date of birth of the patient. This sample was excluded in the calculation of the average age of patients. The average age of the patients was 81.588 months (6 years 9 months).

Of the 21 samples taken from 18 patients, the diagnoses were as follows:

Table 4: Histologic diagnosis and anatomic site of tissue samples

Organ system	Histologic diagnosis	Site of tumour	Number of cases
CNS malignancies (16)	Glioblastoma (WHO Grade IV)	Brainstem	3 (2 cases had 2 samples each)
		Frontal lobe	2
		Thalamus	3
		Parietal lobe	1
		Site not specific	3
	Anaplastic astrocytoma (WHO Grade III)		2
	Low grade oligodendroglioma		1
	Choroid plexus carcinoma		1
Haematolymphoid malignancies (1)	Burkitt lymphoma	Left cervical region	1 (1 case with 2 samples taken at a 1-year interval)
Other (1)	Anaplastic embryonal rhabdomyosarcoma	Testicular	1

Evaluation of MMR Immunohistochemical Stains:

Each of the four MMR stains had a confirmed positive external control which excludes failed immunohistochemistry as a cause for negative staining tumour cells. A normal lymph node was used as the control tissue.

Three samples showed suboptimal staining. Samples 2,3 and 4 showed absent to weak nuclear staining in all four MMR immunostains in the tumour cells.

12 samples showed strong, diffuse, intact nuclear staining in all four antibodies in the tumour cells.

Two samples showed staining patterns that are not recognized in CMMR-D syndrome: Sample 19 showed absent or reduced nuclear staining in PMS2 and MSH2. Sample 21 showed absent or reduced nuclear staining in MSH2, PMS2 and MSH6.

Four samples showed staining patterns compatible with CMMR-D syndrome. Sample 16 showed isolated reduced nuclear staining of PMS2. Sample 1 showed reduced staining of MLH1 and PMS2. Sample 8 and 18 were taken from the same patient at a 6-month interval. Both samples showed loss of MSH2 and reduced staining of MSH6.

The scoring of the immunohistochemical staining was summarized in Table 5. Areas of necrosis, cautery and poorly preserved tumour cells were avoided as these areas are known to show unreliable staining.

Table 5: Modified Allred scoring for MMR immunohistochemistry in each tissue sample

[proportion (P) and intensity (I)] (white cells indicate cases of interest with MMR loss in tumour cells; grey cells indicate intact MMR staining; dark grey cells indicate suboptimal staining)

	MSH2		MLH1		PMS2		MSH6	
Case	P	I	P	I	P	I	P	I
1	5	2	2	1	1	1	4	2
2	1	1	2	1	1	1	0	0
3	2	1	0	0	1	1	0	0
4	2	2	0	0	0	0	0	0
5	5	3	5	3	5	3	5	3
6	5	3	5	3	5	3	5	3
7	4	3	5	3	5	3	5	3
8	0	0	5	3	5	2	2	1
9	5	3	5	3	5	3	5	3
10	5	3	5	3	5	3	5	3
11	5	3	5	3	4	3	5	3
12	5	3	5	3	4	3	4	3
13	5	3	5	3	5	3	5	3
14	5	3	5	3	5	3	5	3
15	4	3	5	3	5	3	4	2
16	5	3	5	2	1	1	5	3
17	5	3	5	3	5	3	5	3
18	0	0	5	3	5	3	4	1
19	1	1	5	3	2	2	4	3
20	5	3	5	2	5	2	5	3
21	2	2	5	3	2	3	3	3

Comments on the background tissue staining characteristics were included (see Table 6). The majority of samples had representation of vessels and endothelial cells. In samples of high-grade glioma, the neurons, vessels and choroid plexus showed consistent and strong positive nuclear staining. In lymphomas, endothelial cells showed consistent and strong positive nuclear staining. Tumour infiltrating lymphocytes were difficult to evaluate. In the control tissue of normal lymph node, the germinal centre lymphocytes showed nuclear positivity. The lymphocytes in the interfollicular zone shows variable, but predominantly negative nuclear staining. Intravascular, circulating lymphocytes were difficult to identify.

Table 6: Characterization of MMR staining in the background, non-tumour tissue

(white cells indicate cases of interest identified in table 4 and comments on staining pattern of background non-tumour tissue; grey cells indicate remaining cases with intact MMR staining; dark grey cells indicate suboptimal staining in tumour cells)

	MSH2	MLH1	PMS2	MSH6
	comment	comment	comment	comment
1	endothelial cells, lymphocytes and blood are negative	background tissue negative, generally faded slide	background tissue negative, generally faded slide	endothelial cells positive, neural tissue positive
2	endothelial cells, lymphocytes and blood are negative	generally faded slide	endothelial cells neg	Endothelial cells neg
3	some endothelial cells positive, lymphocytes negative, Edge artefact noted	focal staining, not accepted, looks mostly cytoplasmic	weak endothelial cell staining	Endothelial cells neg
4	choroid plexus and cortical cells positive, variable endothelial cell staining	choroid plexus, vessels and neural tissue positive	choroid plexus, vessels and neural tissue positive	choroid plexus positive, endothelial cells positive, neural tissue neg?
5	cortical tissue positive too, endothelial cells positive,	neural tissue positive, vessels variably staining	endothelial cells positive, neural tissue positive	endothelial cells positive, neural tissue positive
6	cortex also positive	edge loss, no other tissue types, small fragment	edge loss, small fragment	edge loss, small fragment
7	variable staining in vessels, smooth muscle positivity, focal glial tissue negative	neural tissue positive, vessels variable	endothelial cells positive, neural tissue positive	Endothelial cells positive,
8	endothelial cells negative	endothelial cells positive	endothelial cells positive	endothelial cells negative
9	small frag, vessels variable, edge minimally lost	edge loss, no other tissue types, small frags	edge loss, small frag	edge loss, small frag
10	haematoma negative	positivity in the background neural tissue	endothelial cells positive, neural tissue positive	endothelial cells positive, neural tissue positive
11	poor staining? areas show loss in tumour and background	endothelial cells positive	endothelial cells positive, neural tissue positive	endothelial cells positive, neural tissue positive
12	small fragment, large negative blood clot	endothelial cells positive, are of haemorrhage and necrosis	Endothelial cells positive	Endothelial cells positive
13	clot negative, neurons positive, astrocytes negative	neurons positive, endothelial cells variable	endothelial cells positive, neural tissue positive	endothelial cells positive, neural tissue positive
14	vessels variable, necrosis negative, scanty neural tissue negative	neurons positive, endothelial cells variable	endothelial cells positive, neural tissue positive	endothelial cells positive, neural tissue positive
15	small frag, excluded edge artefact	edge loss, no other tissue types, small frags	edge loss, small frag	edge loss, small frag
16	necrosis and blood negative, neural tissue positive, endothelial cells positive	necrosis and blood negative, neural tissue positive, endothelial cells positive	endothelial cells, neurones and intratumoural lymphocytes positive	endothelial cells positive, neural tissue positive
17	edge loss, variable endothelial positivity	neural tissue positive, vessels variable	endothelial cells positive	endothelial cells positive, neural tissue positive
18	endothelial cells negative	endothelial cells positive	endothelial cells positive	endothelial cells only positive in a small focus
19	cautery present 10%, , endothelial cells negative	cautery and necrosis negative, endothelial cells positive	faded slide, neg endothelial cells	endothelial cells positive
20	uncertain which are the tumour cells, large cells in follicles positive, smaller interfollicular cells show scattered positivity	follicular cells and interfollicular cells	follicular cells and large interfollicular cells	positive in the follicular cells and scattered large interfollicular cells
21	endothelial cells negative	endothelial cells positive	endothelial cells neg	endothelial cells focal positive

Figure 4 shows the process of elimination of samples that are unlikely to confirm CMMR-D syndrome. The pattern of loss of MMR staining is described in the remaining four samples with comments on the background tissue and the histologic tumour type.



Figure 4: Outcomes of MMR immunohistochemistry staining with positive external controls

Selected Folder Review:

A selected folder review was performed on cases with staining patterns compatible with CMMR-D syndrome.

Sample 1:

The patient was a 7-year-old girl who was brought to Red Cross Children's Hospital by her mother and aunt. She presented with a one week history of difficulty in speech, dysphagia and weakness in the left arm and leg. She was assessed with a left-sided hemiparesis, a right-sided upper motor neuron 7th nerve palsy, a left-sided 6th nerve palsy and a pseudobulbar 10th nerve palsy. There were no skin rashes identified and café au lait-like lesions were not described. Screening tests for HIV and tuberculosis were negative. A brain MRI was performed and showed a poorly enhancing lesion in the brainstem. The largest component was present in the medulla with extension into the pons. The rhomboid fossa showed upward displacement. It was assessed as a diffuse pontine glioma without features of hydrocephalus. In view of the diffuse nature and extent of the lesion, the tumour was not resectable. Her parents were counselled regarding the prognosis and palliative care was provided. She died in hospital, 8 days after admission. Her parents were counselled and consented for a post-mortem examination to be performed. Histology confirmed a high grade pontine glioma. There was no documentation of a genogram or siblings in the hospital notes.

Sample 8 and 18:

The patient was a 2-year-old boy who was referred from private paediatrician with one and a half week history of a left cervical mass. It was noted that he had cutaneous neurofibromatosis at birth without a family history of neurofibromatosis. There was no documentation of confirmatory genetic testing. At Red Cross Oncology, he was assessed with

Burkitt Lymphoma on 27/10/1988 (sample 18) and commenced on cyclophosphamide, vincristine, methotrexate, prednisone and allopurinol. He had local and bone marrow recurrence in February 1989 (sample 8). He was started on chemotherapy (French protocol) on 06/03/1989. It was documented that there was an uncomplicated induction course. He developed a pseudomonas septicaemia with thrombocytopenia and neutrophilia and a slow resolution on antibiotics. The cervical mass showed initial reduction in size, but started progressively enlarging again. In view of this, it was decided to stop further chemotherapy with continuation of palliative care only. He was discharged on 05/04/1989. There was no documentation of a genogram or siblings in the hospital notes. The oncology folder was reviewed. The medical folder was not found and was documented as destroyed.

Sample 16:

The oncology folder only contained a note that she presented on 16/11/2004 at 3 years of age with neck stiffness and that a debulking craniotomy was performed 2 days after admission. The medical hospital folder was documented as destroyed. In electronic medical records, the date of death was documented as 16/04/2005, 5 months after the date of biopsy confirming a glioblastoma (WHO Grade IV).

Discussion:

Suboptimal Staining:

Three samples, sample 2, 3 and 4 showed absent to weak staining in all four MMR stains despite adequate positive external controls for all four MMR immunostains (See Figure 5). All three samples were post-mortem samples and the post-mortem interval may have resulted in autolysis, reduced antigen retrieval and reduced staining across all four antibodies. Sample 2 and 3 were from the same patient with a post-mortem interval of 1 day. Sample 4 had a post-mortem interval of 4 days.

Sample 1 is the fourth and final post-mortem sample but was evaluated as an adequate sample, to be discussed below under staining patterns compatible with CMMR-D syndrome. Sample 1 had a post-mortem interval of 1 day. Even though it was evaluated as adequate, it is important to note that the MMR proteins that showed intact or retained nuclear staining were patchier with reduced intensity of staining.

Overall, MMR testing of post-mortem specimens by immunohistochemistry is not recommended due to the probability of failed testing, especially in cases with a prolonged post-mortem interval and marked autolysis. The H&E slides may be used to evaluate the degree of autolysis and selected cases with better preserved tissue may proceed to testing. Further testing by MSI analysis or molecular genetic analysis would not be advised because of the potential poor preservation of tumour tissue, even in samples with a short post-mortem interval of 1 day. In suspected cases of CMMR-D syndrome, the alternative would be to clinically screen and counsel the parents for genetic testing to ensure good quality samples

and reliable results. It may reveal more pertinent information on the risk and need for testing in any surviving siblings.

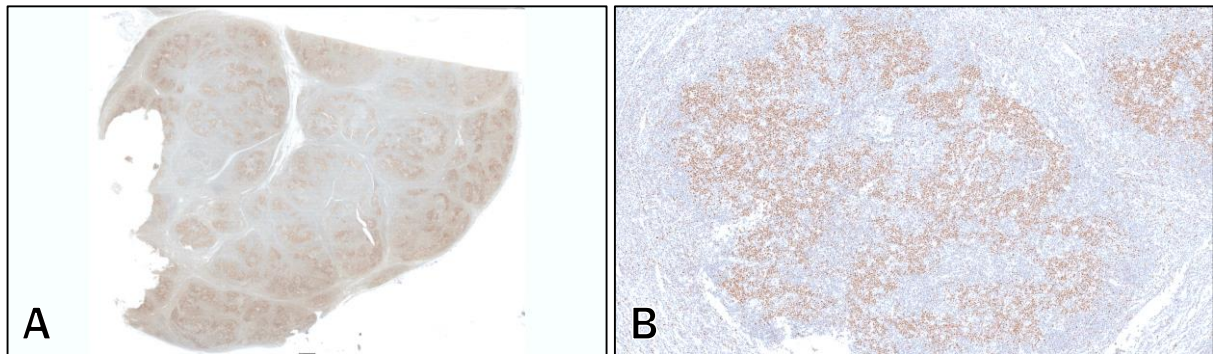


Figure 5: MSH2 control – A normal lymph node used as positive external control tissue

A: Low power view shows a normal lymph node with preserved follicular architecture.

B: Medium power view shows positive staining of central germinal centre lymphocytes and variable staining of peripheral interfollicular lymphocytes

Staining Pattern Unlikely to be Associated with CMMR-D Syndrome:

A total of 12 samples showed strong, diffuse, intact nuclear staining in all four antibodies. These cases are unlikely to represent CMMR-D syndrome. A potential for a false negative result occurs in the setting of CMMR-D syndrome with underlying missense mutations which may show intact immunohistochemical stains. The frozen section sample (sample 15) formed part of this group and the staining was adequate. This gives an indication that frozen section samples may be suitable for testing by MMR immunohistochemistry; however, a bigger sample size would be needed to make a definitive recommendation.

Staining Pattern of Unknown Significance:

A total of two samples showed loss of staining in a pattern that was not compatible with CMMR-D syndrome. One sample showed absent or reduced staining in PMS2 and MSH2

which are not heterodimer pairs. This sample was noted to have cautery artefact which may have impaired the staining process. One sample showed absent or reduced staining in MSH2, PMS2 and MSH6 which is also not described. The reduced staining in more than expected MMR stains may indicate a poorly preserved specimen.

Staining Pattern Compatible with CMMR-D syndrome:

A total of four samples showed loss of staining in a pattern that is compatible with CMMR-D syndrome. Intermediate to strong nuclear staining was interpreted as intact nuclear staining. These cases will be referred to the Department of Human Genetics, for further confirmatory testing. These cases are outlined below.

Sample 1 (See Figure 6) showed loss of PMS2 and MLH1, a heterodimer pair. This sample was a post-mortem sample of a brainstem glioma. MSH2 and MSH6 were expected to show strong intact nuclear staining; however weaker and patchy staining of the tumour cells was present. This inconsistency in staining is possibly as a result to a prolonged post-mortem interval.

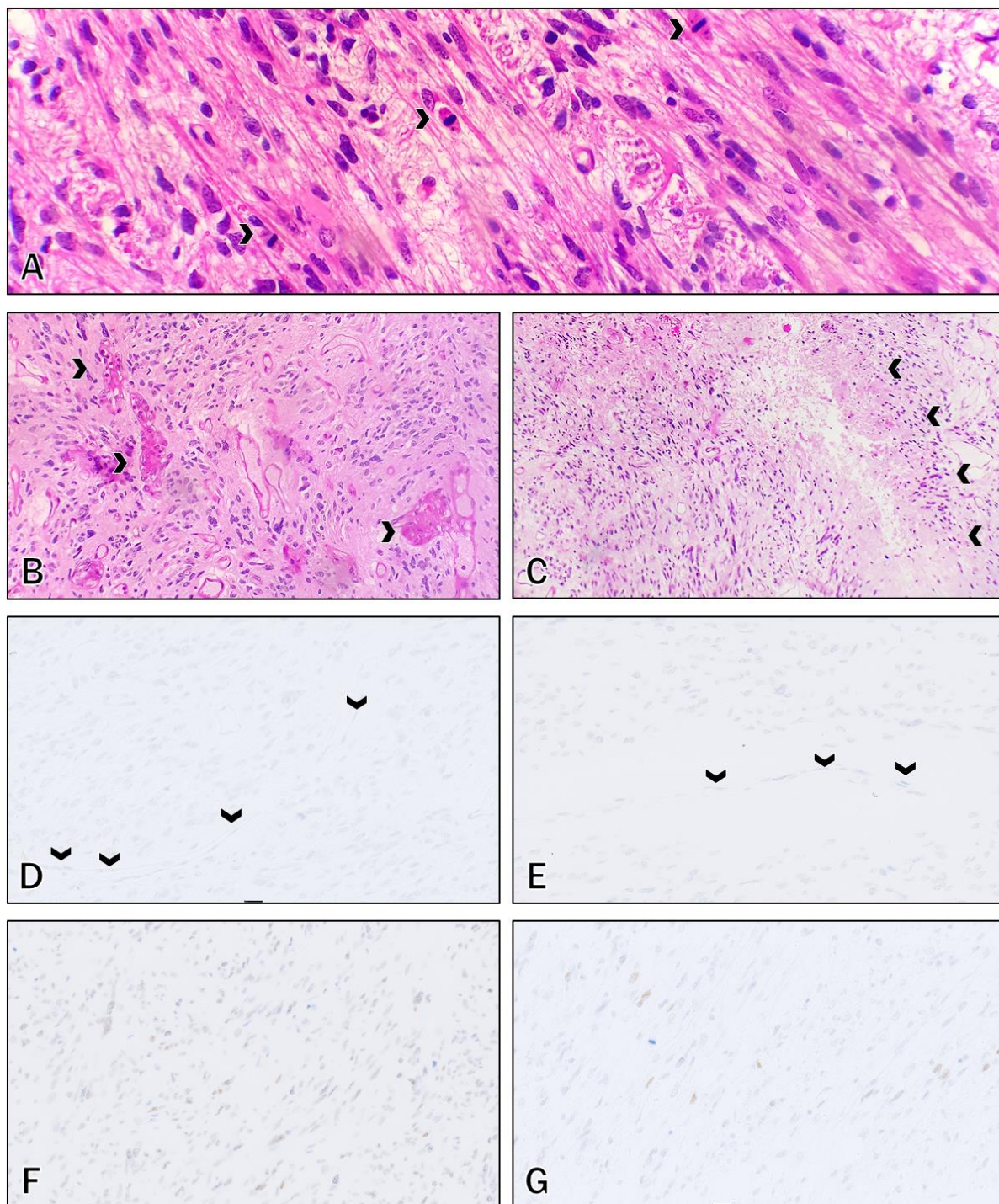


Figure 6 (A-D): MMR immunostains of Sample 1 (Brainstem glioblastoma)

A: H&E high power view – Malignant glial cells with increased nuclear to cytoplasmic ratios, nuclear pleomorphism and increased mitotic activity (arrows)

B: H&E low power view – Areas of microvascular proliferation and glomeruloid structures (arrows)

C: H&E low power view – A focus of palisaded necrosis (arrow)

D: MLH1 – Intensity = 2; Proportion = 1; absent staining in endothelial cells (arrows)

E: PMS2 – Intensity = 1; Proportion = 1; absent staining in endothelial cells (arrows)

F: MSH2 – Intensity = 2; Proportion = 5

G: MSH6 – Intensity = 2; Proportion = 4

Sample 18 (See Figure 7) and sample 8 (See Figure 8) were taken from the same patient with the same diagnosis of Burkitt lymphoma. Sample 18 was the first biopsy and sample 8 was a second biopsy taken after a 6-month interval. Both samples showed absent staining of MSH2 in the tumour cells with absent staining in the background endothelial cells. MSH6, the heterodimer of MSH2, shows markedly reduced staining in the tumour cells and absent staining in the background endothelial cells.

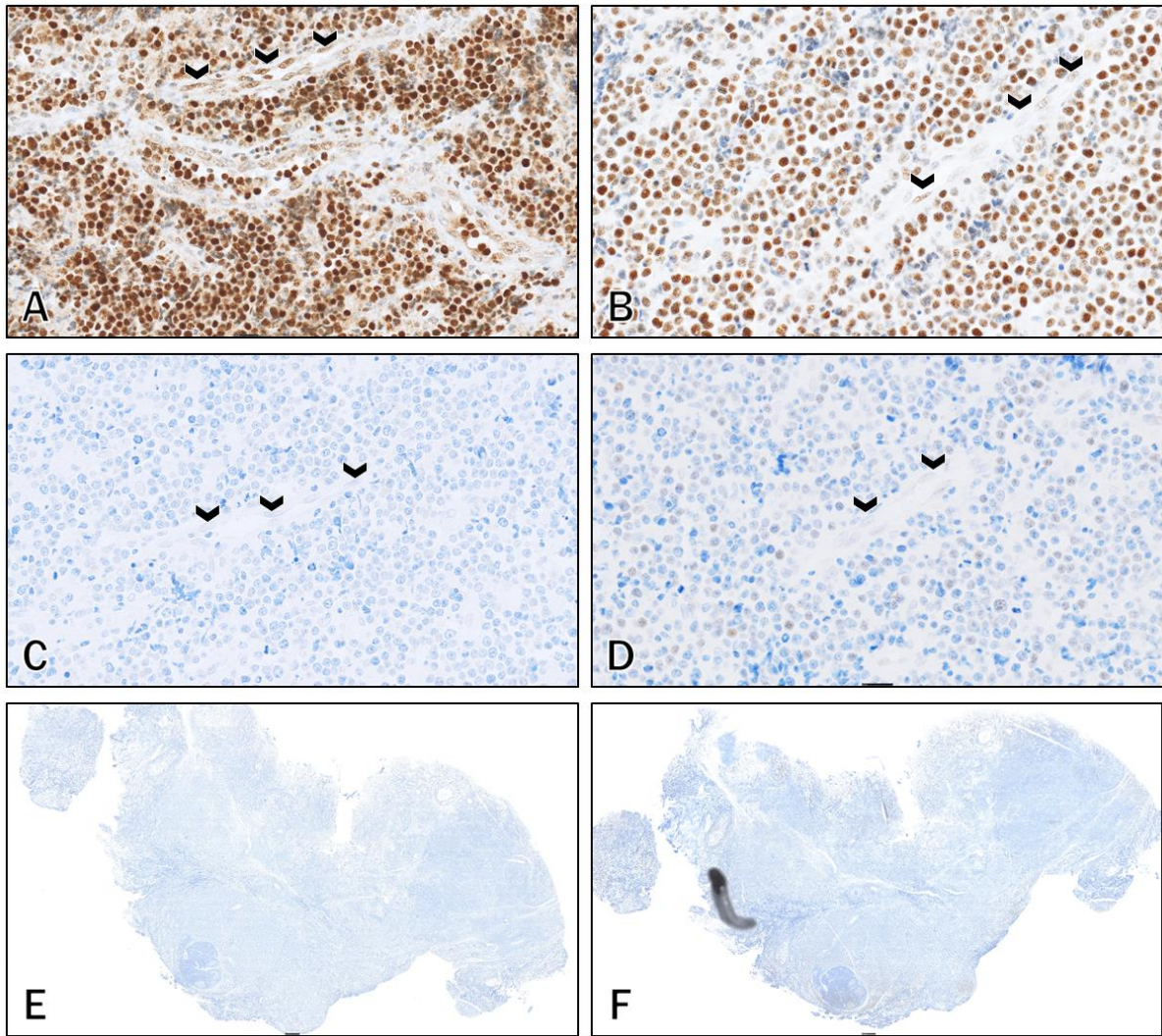


Figure 7 (A-F): MMR immunostains of Sample 18 (First biopsy; Burkitt Lymphoma)

A: MLH1 – Intensity = 3; Proportion = 5; positive staining in endothelial cells (arrows)

B: PMS2 – Intensity = 3; Proportion = 5; positive staining in endothelial cells (arrows)

C: MSH2 – Intensity = 0; Proportion = 0; absent staining in endothelial cells (arrows)

D: MSH6 – Intensity = 1; Proportion = 4; absent staining in endothelial cells (arrows)

E: MSH2 – Low power view showing absent staining of the tumour cells

F: MSH6 – Low power view showing markedly reduced staining of the tumour cells

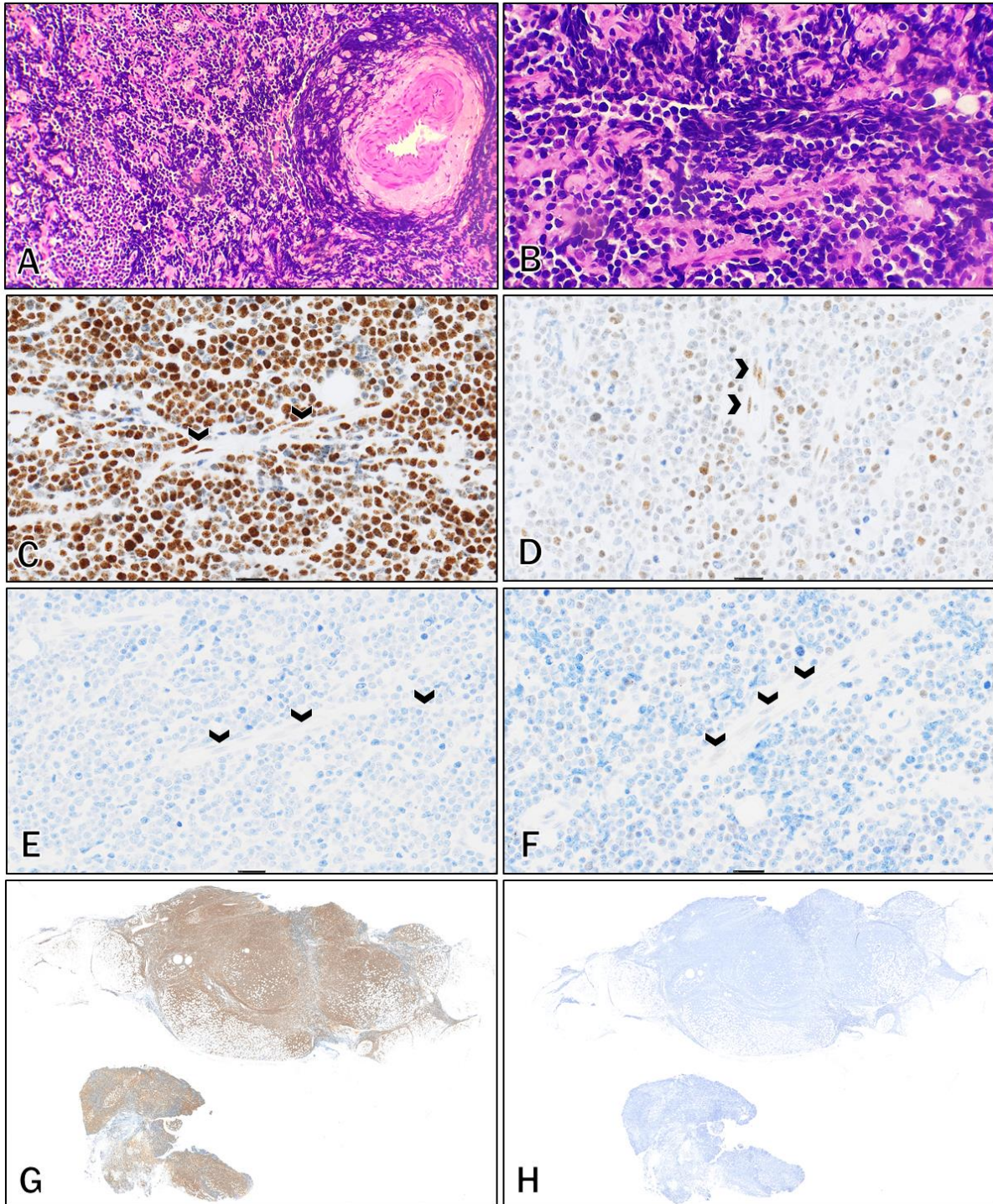


Figure 8 (A-F): MMR immunostains of Sample 8 (Second biopsy after a 6-month interval; Burkitt Lymphoma)

A: H&E low power view – Biopsy taken from the cervical region showing infiltration of discohesive malignant cells

B: H&E high power view – The discohesive malignant cells show increased nuclear-to-cytoplasmic ratios, nuclear hyperchromasia, features consistent with a high-grade lymphoma

C: MLH1 – Intensity = 3; Proportion = 5; positive staining in endothelial cells (arrows)

D: PMS2 – Intensity = 2; Proportion = 5; positive staining in endothelial cells (arrows)

E: MSH2 – Intensity = 0; Proportion = 0; absent staining in endothelial cells (arrows)

F: MSH6 – Intensity = 1; Proportion = 2; absent staining in endothelial cells (arrows)

G: MLH1 – Low power view showing positive staining, infiltrative tumour cells

H: MSH2 – Low power view showing absent staining of the tumour cells

Sample 16 (See Figure 9) showed loss of expression of PMS2, which is the most common mutation seen in CMMR-D syndrome. This tumour was a glioblastoma of the frontal lobe. The background neurons, intratumoural lymphocytes and endothelial cells showed positive nuclear staining, which is contrary to the described loss of staining in the normal tissue seen in CMMR-D syndrome. This tumour was initially scored with a high proportion of 3, but when the case was reviewed, the nuclear positive cells were identified as tumour infiltrating lymphocytes and endothelial cells. This is a potential pitfall in scoring of MMR immunohistochemistry, an overestimation of the proportion of tumour cells by including background non-tumour cells. In this sample, this misinterpretation was easy to make because the tumour cell nuclei were similarly sized to lymphocytes and endothelial cells. It is important to take care in evaluating a DAPI stained slide because clues to cell type, such as morphology and character of cytoplasm, are not as easily assessable as it is on a Haematoxylin and Eosin stained slide and the cells can be misidentified.

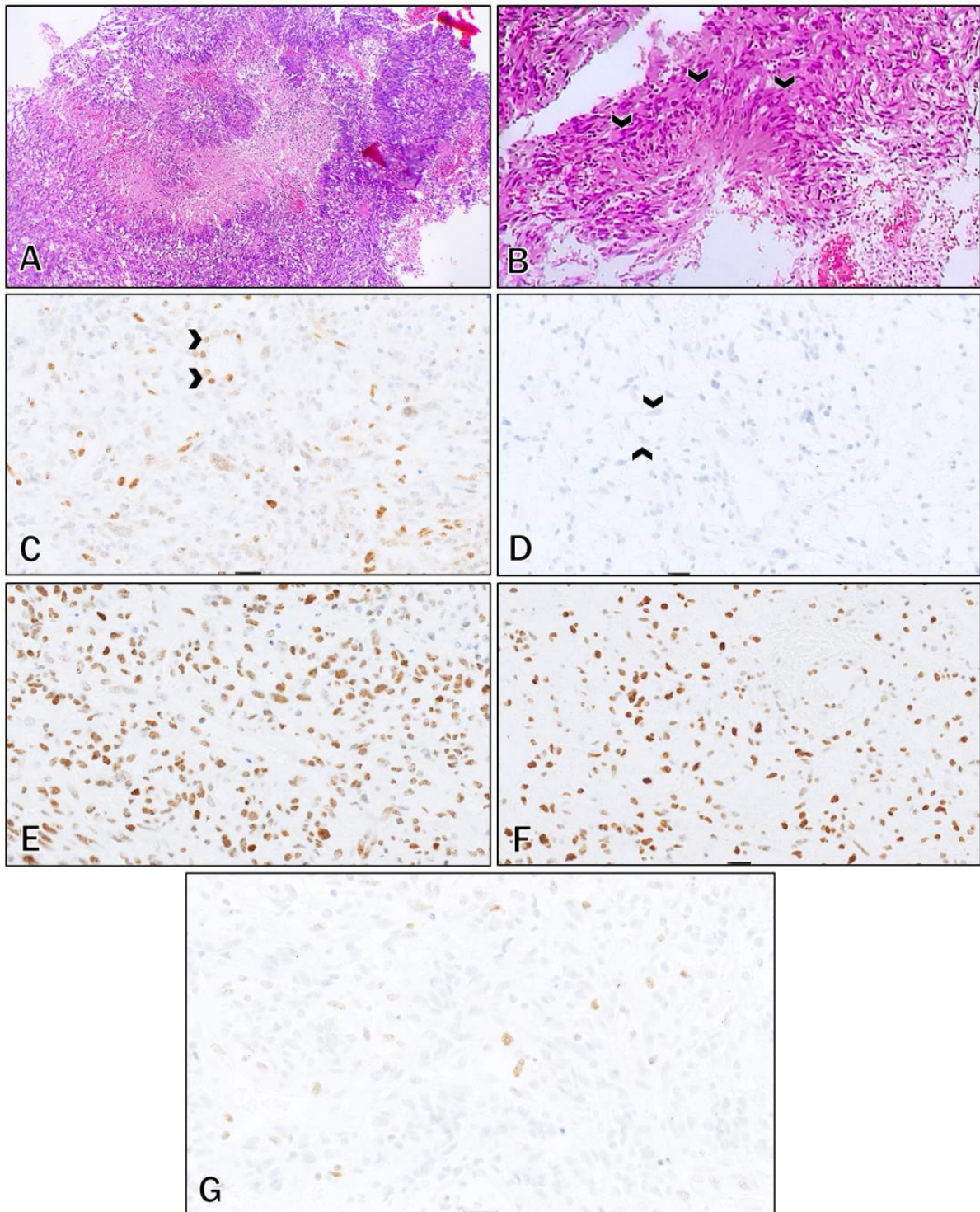


Figure 9 (A-E): MMR immunostains of Sample 16 (Frontal lobe glioblastoma)

A: H&E low power view: A fragment of tissue showing increased cellularity and a central area of palisaded necrosis

B: H&E high power view: The malignant cells show increased nuclear-to-cytoplasmic ratio, nuclear pleomorphism and an area of palisaded necrosis (arrows)

C: MLH1 – Intensity = 2; Proportion = 5; positive staining in endothelial cells (arrows)

D: PMS2 – Intensity = 1; Proportion = 3; absent staining in endothelial cells (arrows)

E: MSH2 – Intensity = 3; Proportion = 5

F: MSH6 – Intensity = 3; Proportion = 5

G: PMS2 – Higher power view showing focal weakly positive tumour cells and positive intratumoural lymphocytes

Only sample 16 of the four samples showed isolated loss of PMS2 staining, a pattern associated with *PMS2* gene mutations which is the classically described mutation seen in CMMR-D syndrome. This may reflect a reduced frequency of *PMS2* mutation in the South African LS population.

Staining Characteristics of the Background Non-tumour Tissue:

It is described that loss of MMR staining in the background tissue is a classical finding in CMMR-D syndrome; however, this has been poorly characterized in studies. This gives pathologists little guidance when evaluating a specimen for potential CMMR-D syndrome. Some biopsies may be small, cauterized, necrotic and contain minimal normal tissue which hampers this assessment. We are also evaluating slides stained with DAPI which also makes it difficult to identify cellular details and normal tissue types, for example lymphocytes look very similar to glial cells. This is not seen in the setting of LS because normal colonic mucosa is typically easy to identify. This problem could be overcome by comparison with the H&E slides or double staining techniques, but this is cumbersome and may still be unreliable.

In this study, the easiest tissue landmark to identify and evaluate were the blood vessels. One of the hallmarks of cancer is induced angiogenesis and even biopsies of solid tumours contain dispersed blood vessels. In samples with intact staining of MMR proteins, the endothelial cells showed positive nuclear staining which was easy to identify. Even in the samples with patchy staining, at least some of the endothelial cells showed some positive staining. This may be

attributed to endothelial cells being more resistant to ischaemic damage or autolysis because they are in direct contact with oxygenated blood. This is an important finding because in brain biopsies, lymphocytes are difficult to distinguish from glial cells. Lymphocytes are not native to the central nervous system and may not be represented. Intravascular circulating lymphocytes may be sparse. In the samples with intact MMR staining, glial cells seem to stain negative or variably. In the samples with intact MMR staining, neurons (See Figure 10) and choroid plexus and choroid plexus carcinoma (See Figure 11) also stained consistently and strongly positive. Our sample size was small, and a larger sample size will be needed to ensure that this finding is consistent.

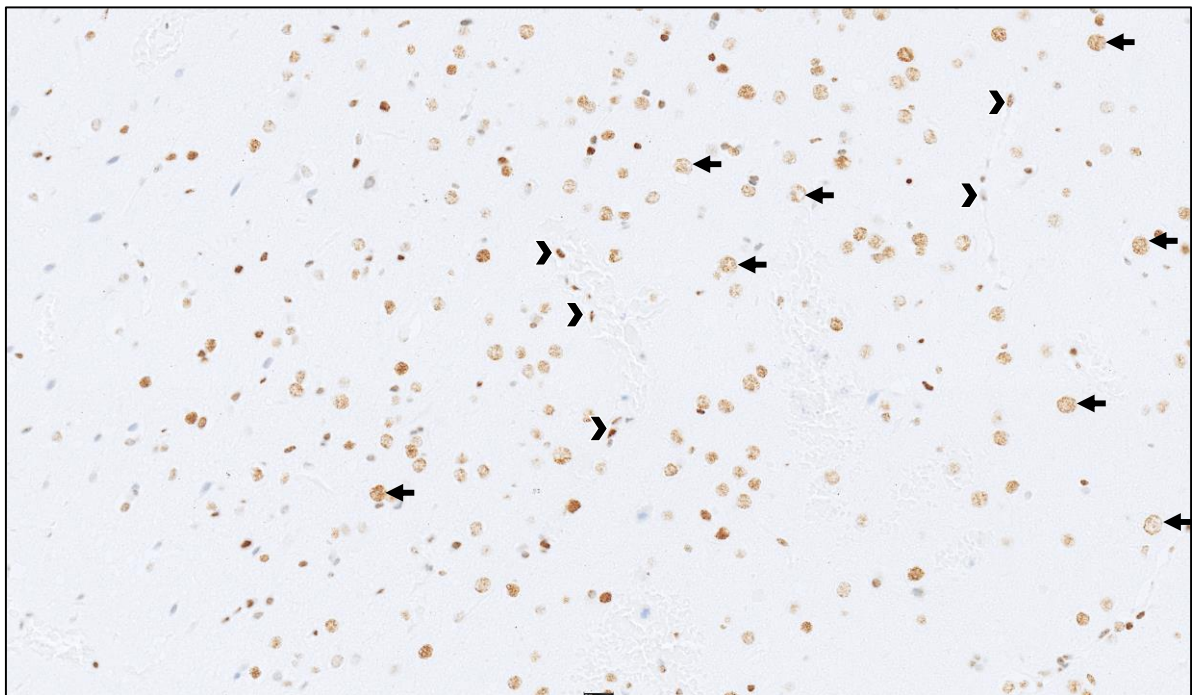


Figure 10: Positive nuclear staining in normal neurons and endothelial cells

MHL1 immunostain of Sample 13 – Nuclear positivity in normal background neurons (arrowheads) and endothelial cells (“>” - shaped arrows)

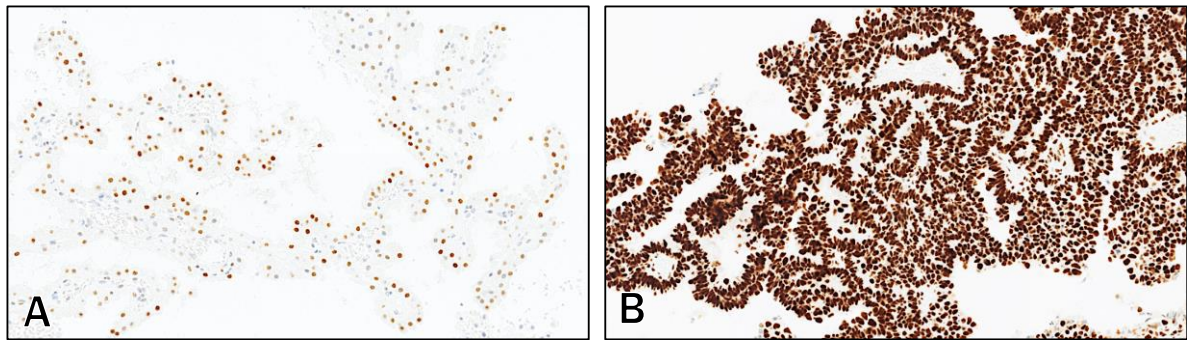


Figure 11: Positive nuclear staining in both normal choroid plexus and choroid plexus carcinoma

A: MLH1 immunostain of Sample 4 – Background normal choroid plexus showing nuclear positivity

B: MSH2 immunostain of Sample 5 – Choroid plexus carcinoma showing strong nuclear positivity

In the lymphomas, the staining pattern was more difficult to evaluate because normal lymph nodes show positive staining in follicles and reduced staining in the interfollicular zones. High-grade lymphoma tumour cells are easy to identify; however, this may be more difficult in indolent, small cell lymphomas. The tumour cells are small, and the distribution of neoplastic cells might be difficult to evaluate, particularly in small biopsies or core biopsies. This may result in the underrepresentation of small cell lymphomas in the CMMR-D syndrome tumour spectrum. Endothelial cells were still more easily identifiable landmarks. Until there is better characterization of MMR staining in the different kinds of lymphocytes, endothelial cells are a more reliable and consistent tissue type to evaluate for background staining.

Interpretation of the Results:

Only four samples showed staining patterns consistent with CMMR-D syndrome and only three samples showed the additional loss of staining of the background tissue. The recommendation would be to confirm a biallelic mutation in one of the MMR genes by molecular genetic methods and these cases have been referred to the Department of Human

Genetics for further evaluation. Once the cases of CMMR-D syndrome are confirmed, the Allred scores for these samples can be reviewed, and the cut off points for negative and positive cases can be proposed. We expected a predominance in MLH1 loss in our population or even predominantly PMS2 loss which is classically described. However, we only had three patients with staining compatible with CMMR-D syndrome: one with MLH1/PMS2 loss, one with MSH2/MHS6 loss and one with isolated PMS2 loss.

16 of the 21 samples represent high-grade gliomas which is similar to the Wimmer *et al.* study where a large proportion of tumours were high grade gliomas. Of the 16 high-grade gliomas, only two showed a pattern of loss or reduced MMR staining that was compatible with CMMR-D syndrome, far fewer than the study in Jordan that showed 51% of paediatric gliomas associated with germline mutations in MMR genes. This highlights a difference in frequency of MMR deficient high-grade gliomas in a country with predominantly founder effect compared to a country with high rates of consanguinity. The high-grade gliomas in our population are predominantly MMR stable and it may be interesting to test the tumours for other mutations common in the paediatric age group such as the diffuse midline glioma, H3 K27M mutant.

Even though CMMR-D syndrome is a polyposis syndrome, there is no representation of colorectal polyps or colorectal carcinomas. This was attributed to selection bias. The patients attending Red Cross Children's hospital are younger because the hospital admissions policy has a cut-off age of 14 years. The colorectal manifestations of CMMR-D syndrome occur in adolescence or early childhood. This could be addressed by including samples from the adult referral laboratories.

Lymphomas were severely underrepresented in this study. In Wimmer *et al.*'s study, lymphomas were the second most represented tumours. The majority were Non-Hodgkin T-cell lymphomas; however Non-Hodgkin B-cell lymphomas were also described. Non-Hodgkin T-cell lymphomas are generally less common compared to Non-Hodgkin B-cell lymphomas. In the articles reviewed, some did not classify the lymphomas into specific histological diagnoses, such as Burkitt lymphoma or diffuse large B cell lymphoma. This may have hampered our computerised search for cases because there may not be specific codes for Non-Hodgkin T- cell lymphoma. The classification of lymphomas has also expanded and many of the cases may require reclassification by present day criteria. Immunohistochemistry may have been available internationally at the time but may not have been readily available in our institution, particularly in the 1980s. Even with available immunohistochemistry, there may still be interobserver variability and confirmation by molecular techniques may still be required. In future studies, the classification and spectrum of lymphomas associated with CMMR-D syndrome should be defined. A reason for selection bias is that in our hospital, bone marrow aspirates and trephines are evaluated by the Department of Haematology. This may exclude potential cases that were diagnosed on bone marrow samples. Another variable is that the phenotype of CMMR-D syndrome arising from the unique *MLH1* c1528C>T mutation is not well characterized. There may be less expression of lymphomas in this phenotype and that may explain why despite clinical screening of patients and the computerized search of cases, only a single lymphoma was represented. Our study only analysed archival formalin fixed and paraffin embedded tissue and this would exclude any presentation of leukaemia which are typically diagnosed on peripheral blood samples.

Multiple metachronous tumours are described in CMMR-D syndrome, but only one patient had a second interval biopsy during the study interval. Both samples were taken from the left neck region 6 months apart and were reported as Burkitt lymphoma. The clinical interpretation was that the second biopsy represented a recurrence of the tumour. We also expected a more aggressive phenotype in our population due to the predominance of *MLH1* mutations. Even though there was no outright predominance of *MLH1* loss, all three of the cases with staining compatible with CMMR-D syndrome died shortly after diagnosis or discharged home for palliation. The patient from the South African case report of *MLH1* mutated CMMR-D syndrome described in the introduction also did not survive very long after diagnosis. In our population, multiple metachronous tumours may not be a feature of CMMR-D syndrome because of an aggressive phenotype and patients not surviving long enough to develop a second tumour. There may be another unidentified reason for this.

During our selection process, we decided to exclude the non-malignant entities associated with CMMR-D syndrome. Future studies may be used to characterize the staining characteristics of these lesions, but due to their benign or indolent course, there is less urgency to do so.

Strengths and Limitations of the Study:

This is the first study in our country to use the C4CMMRD criteria to identify at-risk patients for testing by MMR deficient tumours by immunohistochemistry. There was a focus on the evaluation, scoring and interpretation of the immunohistochemical staining patterns because it is key in distinguishing CMMR-D syndrome from LS. Some articles had inconsistencies and variable description of the normal tissue evaluated for loss of staining. Some articles report

reduced staining and some articles report absent staining as indicative of MMR gene mutation. By applying a modified Allred scoring system, the staining could be described in more precise and comparable measurements. We were able to differentiate cases that have complete absence of staining from cases that show reduced staining and describe, by proportion and intensity, the reduction in staining. Fortunately, the MMR proteins show nuclear staining which is easier to evaluate than cytoplasmic staining which could be confounded by aberrant staining.

This is also one of the only studies that focused on the characterization of the background staining in CMMR-D syndrome. And identified specific structures such as endothelial cells, neurons and choroid plexus that can be used to evaluate the presence of loss of background staining. This is critical information when the defining feature of CMMR-D syndrome is the loss of background staining.

This study also re-emphasizes the importance of establishing the adequacy of the tissue sample and staining technique in the assessment of MMR immunohistochemistry. Avoiding samples with poor preservation, the areas with cautery and necrosis and ensuring an adequate external positive control. The pitfall is to interpret the loss of staining in tumour and background tissue and a failed stain despite a positive external control. We also identified that post-mortem samples are generally not suitable for assessment by MMR IHC which was not recognized in other studies.

There was also identification of the gap in characterization of lymphomas associated with CMMR-D syndrome. Selection bias resulted in the underrepresentation of lymphomas in this

study. Inclusion of bone marrow samples and peripheral blood samples may have improved the yield of haematolymphoid malignancies.

CMMR-D syndrome is a rare entity and our overall sample size was small which has an impact on the significance of the findings. Paediatric tissue samples submitted for tumour diagnostics in the Western Cape Province are split by drainage areas between Red Cross Children's Hospital and Tygerberg Hospital, the major histopathology laboratories in the province. Other cases may be sent to the private laboratories in the region. A multi-centre study may be proposed to increase the number of cases and give a true reflection of the population in the Western Cape Province and also the Northern Cape Province where the known LS families reside.

Implication of the Results and Recommendations:

Pathologists may be familiar with the evaluation of MMR immunohistochemistry for screening of LS in colorectal carcinomas, but they also need to be aware of the different pattern and interpretation of staining in paediatric cases suspected of CMMR-D syndrome. They may even be able to detect cases of CMMR-D syndrome when performing routine screening for LS.

Causes for false negative and false positive results are recognized and molecular genetic testing is still recommended if there is a strong clinical suspicion. Molecular genetic testing should also be considered in cases with reduced but not absent staining and cases where the background cells do not show the classic loss of staining seen in CMMR-D syndrome. In these cases, molecular genetic testing could detect either LS or CMMR-D syndrome. We agree with

the *Care for CMMRD* document that both immunohistochemistry and molecular genetic testing should be used to confirm the diagnosis of CMMR-D syndrome and immunohistochemistry should be performed first to identify cases for molecular genetic testing.

In future studies of CMMR-D syndrome patients that involve MMR immunohistochemistry, it is important to characterize the staining pattern in both the tumour tissue and normal background tissue. This characterization should involve a scoring system, such as the Allred scoring system to document the intensity and proportion of staining to control for interobserver variability. One of the potential pitfalls recognized in sample 16 of this study is that the tumour-infiltrating, non-tumour lymphocytes may show nuclear positivity and falsely raise the score of proportion of positive tumour cells. This can easily occur when the tumour cell nuclei are small and are of similar size to normal lymphocyte nuclei on DAPI stained cells. The background tissue assessed should include endothelial cell staining because they are present in intestinal and extraintestinal tumours and are readily identified. Other background cells in the central nervous system could include neurons and choroid plexus. Testing of post-mortem samples is not recommended. Testing of frozen section tissue was adequate, but we only had one frozen section sample and testing of a larger sample size is required before a recommendation can be made. Studies of confirmed CMMR-D syndrome cases will be needed to determine the sensitivity and specificity of MMR immunohistochemistry, but in the meantime, immunohistochemistry should be used in conjunction with molecular genetic testing.

Studies involving CMMR-D syndrome-associated lymphomas should report the histological classification of lymphomas. To study the scope of CMMR-D spectrum of tumours, and a prolonged study interval or older cohort is needed to include the gastrointestinal carcinomas. Collaboration with haematologists and the inclusion of haematological specimens is needed to include leukaemias.

It may be useful to review the University of Cape Town (UCT) Human Genetics LS registry for family members where both parents carry mutations in the same MMR gene. Their risk of having a child with CMMR-D syndrome is 25%. If enough cases can be recruited, this could be used to determine if the incidence, phenotype and severity is different due to the unique *MLH1* gene mutation that is present in our LS population. This may also require more intense cancer screening protocols.

Discussion with our paediatric oncology colleagues will determine if routine MMR immunohistochemical screening of these tumours should be performed or if it should be performed on request. In the Red Cross Children's hospital population, high-grade gliomas, non-Hodgkin T-cell lymphomas and supratentorial PNETs do not occur frequently. In addition to screening children with carcinoma and multiple adenomas, it would be better to screen the abovementioned tumours in our paediatric population, even if they occur without the additional features in *Indications for CMMR-D screening in cancer patients* outlined by Wimmer *et al.* (See Table 1). The motivation for this is that there are serious implications for the index patient and their families and we should use every opportunity to screen for CMMR-D syndrome. Clinicians need to be aware the parents typically do not meet the criteria for LS and multiple metachronous tumours may not be a feature of CMMR-D syndrome in our

population. This emphasizes the importance of using a genogram or pedigree in children with suspected CMMR-D syndrome and suspected tumour syndromes in general.

Conclusion:

CMMR-D syndrome is a rare tumour predisposition syndrome that results in lethal, but potentially preventable or treatable childhood cancers. CMMR-D syndrome and LS are linked because they arise from mutations in MMR genes; but it is important to know the differences to accurately diagnose cases of CMMR-D syndrome.

In our study, we used selected criteria from the *Care for CMMRD's indications for testing* to identify patients with CMMR-D syndrome spectrum of tumours. Our samples were predominantly of high-grade gliomas with underrepresentation of gastrointestinal carcinoma, lymphoma and leukaemia. We performed a retrospective analysis on archival formalin fixed paraffin embedded tissue by applying MMR immunohistochemistry. A modified Allred scoring technique was used to define the staining patterns of the tumours and special note of background staining was documented. Loss of nuclear staining is expected in MMR deficient tumour cells. The unique and concomitant loss of staining in the background non-neoplastic tissue, despite a positive external control, is a critical feature in the diagnosis of CMMR-D syndrome. Only four of 21 samples showed staining compatible with CMMR-D syndrome. MMR deficient gliomas were not as frequent as in areas of high rates of consanguinity. We identified endothelial cells as a consistent, reliable and easily identifiable background tissue as well as choroid plexus and neurons. Lymphocytes were not reliable because of variable staining. Post-mortem samples were not suitable for MMR immunohistochemistry, even in cases with a 1-day post-mortem interval. It is important for pathologists to be aware of the different staining patterns and potential pitfalls in interpretation. If there are inconsistent results and a strong clinical suspicion, or non-classic

staining patterns, referral for molecular genetic confirmation is recommended. MMR immunohistochemistry should be the first investigation because it allows for identification of MMR deficient cases for further molecular genetic testing.

The South African LS population has predominantly MLH1 mutations with a unique *MLH1* c1528C>T mutation attributed to the founder effect. This may have impact on the incidence, penetrance and severity of CMMR-D in South Africa which is yet to be fully defined. Critically, CMMR-D syndrome patients may not have LS affected parents. In the four cases with staining patterns compatible with CMMR-D syndrome, the patterns were mixed and MLH1 deficient tumours were not overrepresented. The clinical findings of the four samples suggest that our phenotype is aggressive, and patients do not survive long enough to develop a second metachronous tumour for reasons not yet identified. Further studies are required to characterize gastrointestinal carcinomas and lymphomas which are the two other main manifestations of CMMR-D syndrome.

CMMR-D syndrome is a rare and potentially lethal childhood tumour predisposition syndrome. It is probably underrecognized and this can be improved by increased clinician awareness around the entity with application of the criteria for indication of CMMR-D testing to identify potential cases. It also requires increased awareness by pathologists of the unique staining pattern of the MMR immunohistochemistry and confirmation by molecular genetic techniques. Diagnosis is critical to provide genetic counselling, testing and applying tumour screening protocols for affected patients and their families.

Appendix A

Equipment

Ventana Autostainer
Leica Rotary Microtome RM2125 RTS
Waterbath
Hotplate

Consumables and reagents

Glass slides – Histobond charged coated
Glass coverslips 22x40
Microtome Blades Low profile
Gloves – Latex free
Entellan
Xylene
Absolute Alcohol
Dishwashing liquid

Kits and automated reagents

Optiview DAB detection system
Haematoxylin II
Bluing reagent
EZ Prep
Liquid Coverslip (LCS)
CC1
Reaction Buffer

Antibodies

VENTANA anti-MSH2 (G219-1129) Mouse Monoclonal Primary Antibody
Clone Name: G219-1129
Species: Mouse
Localization: Nuclear
Dilution: Used as is

VENTANA anti-MLH1 (M1) Mouse Monoclonal Primary Antibody
Clone Name: M1
Species: Mouse
Localization: Nuclear
Dilution: Used as is

VENTANA anti-PMS2 (A16-4) Mouse Monoclonal Primary Antibody
Clone Name: A16-4
Species: Mouse
Localization: Nuclear
Dilution: Used as is

VENTANA anti-MSH6 (SP93) Rabbit Monoclonal Primary Antibody
Clone Name: SP93
Species: Rabbit
Localization: Nuclear
Dilution: Used as is

Reagents and Buffers

Immunohistochemistry – semi-automated method on Ventana XT auto-stainer reagents and buffers.

10X EZ Prep solution

EZ prep solution was used for paraffin removal in both IHC and ISH automated staining procedures. Two litres of EZ Prep reagent concentrate were made up to 20 litres using distilled water in the manufacture supplied plastic container. This was stored on the shelf and decanted into the machine bulk container as required.

Reaction buffer concentrate

Reaction buffer is a Tris based buffer solution which was used to rinse between IHC and ISH staining procedures. 2L of Reaction buffer reagent concentrate was made up to 20 litres using distilled water in the manufacture supplied plastic container. This was stored on the shelf and decanted into the machine bulk container as required.

LCS

Also known as liquid coverslip, this solution is used as is, and is available in a 2L plastic bottle, prediluted. LCS is a high temperature coverslip solution which is used to prevent the aqueous

solution from evaporating and the section drying out. This allows for a stable IHC environment.

CC1

Also known as cell conditioning solution 1, this solution is used as is and is available in a 2L plastic bottle. CC1 is a tris-based pre-treatment or antigen retrieval buffer used for automated immunohistochemical reactions.

OptiView DAB IHC Detection Kit

This kit was used for the semi-automated IHC staining. It contained propriety reagents in six 25ml dispensers, which were automatically applied by the analyser. These reagents were peroxidase, universal linker, HRP multimer, hydrogen peroxide (H₂O₂), 3,3'-Diaminobenzidine (DAB) and copper (copper sulphate).

Hematoxylin II

Hematoxylin II counterstain reagent is a modified Mayer's hematoxylin and was available in a 25ml dispenser. This reagent was used as is.

Bluing reagent

Bluing Reagent is an aqueous solution of buffered lithium carbonate and was available in a 25ml dispenser. This reagent was used as is.

Dishwashing detergent (LCS wash)

Three drops of Sunlight® dishwashing soap was added to 500ml of water

Absolute Alcohol

This reagent was used as is

Xylene

This reagent was used as is

Entellan – mounting media

This reagent was used as is

Appendix B

Data Extraction Form

MSH2			MLH1			PMS2			MSH6			age in month	final diagnosis
P	I	comm	P	I	comm	P	I	comm	P	I	comm	age	
1	5	2 endoth	2	1 genera	1	1	1	1 endoth	4	0	2 endoth 7y	85	brainstem glioblastoma (anaplastic astrocytoma, focal transition to glioblastoma)
2	1	1 endoth	2	1 genera	1	1	1	1 endoth	0	0	0 endoth 7y	87	Pontine glioblastoma (arising in a diffuse astrocytoma)
3	2	1 some e	0	0 focal st	1	1	1 weak e	0	0	0 endoth "	0		
4	2	2 choroid	0	0 choroid	0	0	0 choroid	0	0	0 choroid 2y8m	0	31	HISU: Low grade oligodendroglioma
5	5	2 in neocortical	5	3 neural	5	3	3 endoth	5	3	3 endoth 1y8m	5	20	Choroid plexus carcinoma
6	5	3 cortex	5	3 edge la	5	3	3 edge la	5	3	3 edge la 10y	5	125	Glioblastoma (possible small cell variant)
7	4 patchy	variabl	5	3 neural	5	3	3 endoth	5	3	3 endoth 3y2m	5	38	
8	0	0 endoth	5	3 endoth	5	2	2 endoth	5	2	1 endothelial cells negative	2		burkitt lymphoma
9	5	3 small fr	5	3 edge la	5	3	3 edge la	5	3	3 edge la 14y	5	169	Anaplastic astrocytoma
10	5	3 haemat	5	3 positiv	5	3	3 endoth	5	3	3 endoth 1y7m	5	19	Testicular anaplastic embryonal rhabdomyosarcoma
11	5	3 poor st	5	3 endoth	4	3	3 endoth	5	3	3 endoth 11y	5	137	Brainstem glioblastoma
12	5	3 small fr	5	3 endoth	4	3	3 endoth	4	3	3 endoth "	4		
13	5	3 clot neg	5	3 neuron	5	3	3 endoth	5	3	3 endoth 7y	5	94	Glioblastoma (parietal lobe)
14	5	3 vessels	5	3 neuron	5	3	3 endoth	5	3	3 endoth 6y	5		Glioblastoma (frontal lobe) Frere referral***
15	4	3 small fr	5	3 edge la	5	3	3 edge la	4	2	2 edge la 9y	4	119	Anaplastic astrocytoma (thalamus)
16	5	3 necrosi	5	2 necrosi	1	1	1 endoth	5	3	3 endoth 2y11m	5	35	Glioblastoma (frontal lobe)
17	5	3 edge la	5	3 neural	5	3	3 endoth	5	3	3 endoth 7y	5	92	Glioblastoma arising in a lower grade astrocytoma (Left hemisphere)
18	0	0 endoth	5	3 endoth	5	3	3 endoth	4	1	1 endoth 2y2m	4	26	burkitt lymphoma, same patient as 223/89
19	1	1 strange	5	3 cautery	2	2	2 faded s	4	3	3 endoth 7y	4	91	Glioblastoma (thalamus)
20	5	3 uncerte	5	2 follicul	5	2	2 follicul	5	3	3 positive 10y	5	128	Glioblastoma (cerebral)
21	2	2 endoth	5	3 endoth	2	3	3 endoth	3	3	3 endoth 7y	3	91	Glioblastoma (thalamus)

Appendix C

First Ethics Approval Letter 2015



UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee



Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6338 • Facsimile [021] 406 6411
Email: sumayah.arietdien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

03 June 2015

HREC REF: 236/2015

Dr K Pillay
Anatomical Pathology
Room 116
ICH Building
Red Cross Children's Hospital

Dear Dr Pillay

PROJECT TITLE: IDENTIFYING CHILDREN WITH CONSTITUTIONAL MISMATCH REPAIR DEFICIENCY (CMMR-D) SYNDROME IN THE EXPANDING LYNCH SYNDROME POPULATION IN CAPE TOWN (MMed candidate - Dr A Wessels) - Sub-study linked to 333/2014

Thank you for your response letter dated 18 May 2015, addressing the issues raised by the Human Research Ethics Committee (HREC).

It is a pleasure to inform you that the HREC has formally approved the above-mentioned study.

Approval is granted for one year until the 30th June 2016.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

We acknowledge that the following student:- Dr Annesu Wessels is also involved in this project.

Please quote the HREC reference no in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.

Hrec/ref: 236/2015

Updated Ethics Approval Letter 2020

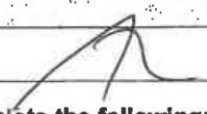


UNIVERSITY OF CAPE TOWN
TYUNISIJEN, TATAAPA - UNIVERZITEIT VAN KAAPSTAD

HUMAN RESEARCH ETHICS COMMITTEE
FACULTY OF HEALTH SCIENCES
 Human Research Ethics Committee
13 FEB 2020
HEALTH SCIENCES FACULTY
UNIVERSITY OF CAPE TOWN



Form FHS007: Amendment – study staff

HREC office use only (FWA00001637; IRB00001938)			
☒ Approved			
This serves as notification that all changes to the study staff and documentation described below are approved.			
Chairperson of the HREC signature		Date	14/2/20

Principal Investigator to complete the following:

1. Protocol information

Date (when submitting this form)	20/1/2020
HREC REF Number	236/2015
Protocol title	IDENTIFYING CHILDREN WITH CONSTITUTIONAL MISMATCH REPAIR DEFICIENCY (CMMR-D) SYNDROME IN THE EXPANDING LYNCH SYNDROME POPULATION IN CAPE TOWN
Protocol number (if applicable)	
Principal Investigator	DR KOMALA PILLAY
Department / Office Internal Mail Address	DEPARTMENT OF CLINICAL LABORATORY SERVICES DIVISION OF ANATOMICAL PATHOLOGY
1.1 Does this protocol receive US Federal funding?	
	☐ Yes ☒ No

2.1 Staff changes (tick ✓)

Are new personnel being added to this research?	☒ Yes	☐ No
Are current personnel being removed from this research?	☒ Yes	☐ No
Is the principal investigator for this research being changed?	☐ Yes	☒ No
If yes, please attach revised conflict of interest and PI declaration statements. (Refer: sections 7 and 8.3 in the New Protocol Application Form - FHS013)		
Do the consent and assent forms need modification to reflect these staff changes?	☐ Yes	☒ No
If yes, please attach copies of the revised forms, with all changes highlighted or tracked and listed in the documents for approval.		



2.2 Amended study staff details

Title, first name, surname	Department/Division	E-mail	Role of new staff member
DR SINDY TU	ANATOMICAL PATHOLOGY	SINDYTU@GMAIL.COM	CO-INVESTIGATOR

3. List of documentation for approval

Please list below all staff documentation such as CVs, declarations, GCP certificates and revised consent forms which need approval. This information must correspond to all 'yes' answers in 2.1 above. This form will be signed and returned to the PI as notification of approval. Please add extra pages if necessary.

--

4. Signature

My signature certifies that I will maintain the anonymity and/ or confidentiality of information collected in this research. If at any time I want to share or re-use the information for purposes other than those disclosed in the original approval, I will seek further approval from the HREC.

Signature of PI		Date	23/01/2020
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