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Original Research

Mismatch repair protein expression defects in endometrioid endometrial adenocarcinoma

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Objectives: Endometrioid endometrial carcinoma (EEC) is the sentinel cancer in over half female patients with heritable mismatch repair (MMR) mutations as part of Lynch syndrome. Immunohistochemical testing for tumoural MMR-protein expression is the primary screening test identifying cases potentially harbouring familial cancer syndrome-related mutations and is also a predictive biomarker for immune-checkpoint blockade response. *Methods*: Following Data Protection and Ethical clearance by the University of Malta, 200 EEC cases were retrospectively identified and categorized into three arms: 151 cases above age 50 at diagnosis, 49 cases at or below age 50 at diagnosis and 30 controls with benign endometrial tissue sampling. H&E case slides were re-examined by an independent pathologist to confirm the diagnosis and identify the block best representing the tumour. Four new slides per case were recut and immunohistochemistry performed for MLH1, PMS2, MSH2, and MSH6 proteins. Protein expression was analysed semiquantitatively using Allred scoring. *Results*: 31% of the overall EEC cases were deficient for one or more MMR-proteins. Dual loss of the MLH1-PMS2 protein heterodimer was the most common deficiency, occurring in 24.5% of cases. Loss of MSH2-MSH6 protein expression represented 3.2% of MMR-deficient cases. Well differentiated tumours had a 76.5% proficiency rate as opposed to grade 2/3 disease with 53.2% and 52.9% proficiency rate respectively. There was no significant difference in MMR status when age 50 was used as a hypothetical testing threshold. After correcting for tumour grade, MLH1 and PMS2 expression was shown to be negatively correlated with age-at-diagnosis while MSH6 expression was positively correlated. *Conclusion*: Reflex MMR proficiency testing of all EEC cases is advisable, as using age 50 as a testing threshold would have missed 82.3% of MMR deficient cases. Prospective evidence is required to clarify the role semi-quantitative scoring plays in MMR status interpretation and patient management in the ever-evolving field of targeted therapeutics.

Keywords

Mismatch repair; MutS homolog 2; MutS homolog 6; MutL homolog 1; PMS-2 protein; Endometrial neoplasms; Neoplastic syndromes

1. Introduction

With an incidence of 29.8 per 100,000 women, endometrial cancer is the fourth leading malignancy in women following breast, lung and colorectal cancer [1]. Endometrial cancer is often diagnosed in early stages of the disease with 66–69% of cases presenting with Stage 1 disease as defined by the International Federation of Gynaecology and Obstetrics (FIGO) [2].

As for several other solid tumour types, familial clustering of endometrial carcinoma is recognised, although research on this topic was slow to materialise. A historic precursor to our modern understanding of genetic susceptibility to endometrial carcinoma occurred in 1913 when Aldred Scott Warthin, a pathologist, described a high propensity for gastrointestinal and uterine cancers in the family of his seamstress, who later also died of endometrial cancer [3]. This pedigree, referred to as family G, was later described as the Lynch syndrome family.

The molecular genetics era for Lynch syndrome or as it is now known, hereditary non-polyposis colorectal cancer (HNPCC), began in 1993 when a genome-wide search and linkage analysis in large families identified cancer susceptibility loci on the p- arms of chromosomes 2 and 3 [4, 5]. During this same period, it was shown that HNPCC tumours had a characteristic genetic change, originally referred to as 'ubiquitous somatic mutations in simple repeated sequences', or a 'replication error' phenotype [6, 7]. These characteristic changes are now called microsatellite instability (MSI) [8, 9] and are a consequence of defective DNA replication-error repair. The repetitive nature of microsatellites makes cells susceptible to mistakes during DNA replication due to slippage of DNA polymerase over these repeats. The identification of germline mutations in the MutL homolog 1 (MLH1) and MutS homolog 2 (MSH2) genes was quickly followed by the discovery of other mismatch repair (MMR) proteins that play a supporting role in the MMR complex.

The detection of MSI in a tumour does not necessarily mean the patient suffers from Lynch syndrome. Hypermutagenic tumour subpopulations that sporadically acquire a defect in the MMR mechanism may survive initial therapeutic modalities and become the major cell line in a heterogeneous tumour mass or subsequent metastases. This process is driven by the genomic instability characteristic of malignant cell populations. It is in fact a continuation of the carcinogenesis process whereby cells accumulate mutations that ultimately result in altered cellular processes such as cell division and transcription.

The MMR mechanism ensures genomic integrity and prevents the deletion or insertion of anomalous DNA segments at microsatellites. This process is reliant on four key proteins: MLH1, MSH2, mutS homologue 6 (MSH6) and post-meiotic segregation increased 2 (PMS2). MSH2-MSH6 is the first heterodimer to bind to mismatched bases in DNA. This is followed by the MLH1-PMS2 heterodimer which removes the abnormal segment and re-synthesizes corrected DNA chains at the mismatch site (Fig. 1).

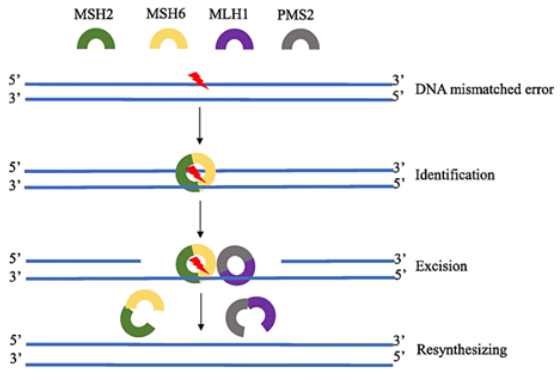

Fig. 1. The MSH2-MSH6 and the MLH1-PMS2 heterodimers play different roles in error recognition, excision and re-synthesis of DNA at mismatched sites [10].

Assessing the general endometrial carcinoma population for HNPCC risk has taken two main approaches. Firstly, patients could be flagged through clinical suspicion at initial presentation and detailed history taking. Patients noted to be at high risk then undergo tumour immunohistochemistry (IHC) and/or MMR gene testing. This approach however misses patients who do not have a suggestive family history, possibly due to a low number of siblings or young siblings.

The second alternative strategy, with higher sensitivity than clinical suspicion, is to identify the subset of MSI hypermutated cases by performing immunohistochemistry for MLH1, MSH2, MSH6 and PMS2 expression on endometrial carcinoma specimens. Patients with abnormal immunohistochemistry are then counselled about germline MMR mutation testing. This technique is consistent with new data on the clinicopathologic characteristics of uterine cancer which has shown that the classical Type 1 (estrogen-dependent and hyperplasia driven) and Type 2 (non-estrogen dependent and non-endometrioid) classification may be too superficial and with limited value with regards to therapeutic choice [11]. The Cancer Genome Atlas identified four molecular subgroups of endometrial cancer: Polymerase E catalytic subunit ultra-mutated, copy number low, copy number high and MSI hyper-mutated which is what this second pathway would be identifying [12].

Five main deficiency patterns have emerged from studies investigating MMR deficiency by immunohistochemistry: complete loss of all four proteins, loss of the MSH1-PMS2 heterodimer, loss of the MSH2-MSH6 heterodimer, and isolated loss of either MSH6 or PMS2. Loss of the MLH1-PMS2 heterodimer ranges from 5.78–26.92% of cases while loss of the MSH2-MSH6 heterodimer ranges from 4.1–9.72%. Isolated MSH6 has been reported in 1.98–6.48% of cases while isolated PMS2 loss is less common at 0.14–1.45% of cases [13–21]. Complete loss of all four proteins is rare, with only a handful of cases reported in published literature.

MMR-dependent response to DNA damage is important as it also impacts response to chemotherapy. The European Society for Medical Oncology (ESMO) clinical practice guidelines advise the use of Platinum-based alkylating chemotherapy for Stage II–III endometrial cancer cases and for FIGO Stage 1 Grade 3 disease when other risk factors are present (Age, lymphovascular space invasion and high tumour volume) [22]. For this reason, the impact of MMR deficiency on platinum agents is crucial. Preclinical studies have shown that MMR defective tumour cells have an inherent resistance to Carboplatin and Cisplatin [23–27].

Platinum resistance in MMR-deficient cell lines can be explained using the 'futile cycling' model. The MMR pathway primarily recognises Guanine-Guanine intrastrand crosslinks that are generated by platinum-containing agents [27]. However, normal MMR processing of alkylator damage persistently regenerates methylguanine-Cytosine and methylguanine-Thymine mispairs because the newly incorporated Cytosine or Thymine residues on the daughter strand are targeted once again for excision. These unregulated attempts at repair result in replication fork arrest, cell-cycle arrest and eventually trigger senescence and/or cell death. In MMR-deficient cells these mispairs persist and cells survive with increasing mutagenicity [28].

The second proposed mechanism is the 'direct signalling' model whereby MMR proteins serve as a scaffold for the ATR-ATRIP (ataxia telangiectasia-mutated and Rad3-related protein kinase and ATR-interacting protein) complex which transduces genomic stress signals and halts cell cycle progression [29]. Lack of this direct signal contributes towards MMR-deficient cell survival during Platinum therapy.

MMR status has also made its mark on immunotherapeutic treatment. It is now established that MMR-deficient tumours are susceptible to inhibitors of programmed death-ligand 1 (PD-L1) and programmed cell death protein 1 (PD-1) [30]. One such ICB drug is Pembrolizumab which tar-

gets PD-1, a protein on T-cells. It is FDA-approved as a single agent in MMR-deficient endometrial cancer cases that have progressed following first-line treatment and who have no satisfactory alternative treatment options [31]. PD-1 activation normally inhibits T-cell mediated attack on malignant cells. By blocking PD-1, Pembrolizumab boosts the immune response against cancer cells. For advanced endometrial cancers that are not MMR deficient, Pembrolizumab has only been approved as combination treatment with the kinase inhibitor Lenvatinib [32]. This followed findings of the Keynote-146 Phase II trial which showed an overall response rate(ORR) of 40.7% (95% CI 31.4–50.6%) in the study population [33]. In MMR-proficient cases the combination demonstrated an ORR of 38.3% (95% CI: 28.5%–48.9%) while in the 11 patients with tumours that were MMR-deficient it demonstrated an ORR of 63.6% (95% CI: 30.8%–89.1%).

It is hypothesized that the increased efficacy of immunotherapy on MMR-deficient tumours is linked to the proportion of cellular somatic mutations that serve as triggers or targets for a T-cell mediated immune response. Using exome sequencing, Le DT *et al.* reported a mean 1782 somatic mutations and 578 potential neoantigens in MMR-deficient tumours, as opposed to 73 mutations and 21 neoantigens in MMR-proficient tumours (P = 0.007) [30]. A greater proportion of neoantigens and somatic mutations were associated with better treatment response and higher progression-free survival (PFS). Furthermore, MMR-deficient tumours have a dense infiltration of cluster of differentiation 8 (CD8+) tumour-infiltrating lymphocytes, which induce a better and more robust response [34, 35].

The study presented in this article investigates the prevalence of tumoral mismatch repair protein expression defects in Maltese endometrial cancer cases and the role age at presentation plays in MMR IHC testing. With a mean 71 local patients per year diagnosed with endometrial carcinoma, data on the prevalence of MMR deficiency would improve the quality of counselling based on reliable local data, guide targeted adjuvant treatment and help formulate immunohistochemical and/or genetic screening protocols for patients potentially susceptible to familial cancer syndromes [36, 37]. In an era where focus is shifting to personalised treatment, targeted testing will pave the way towards personalised screening and management while empowering patients in a setting of comprehensive cancer care.

2. Methodology

2.1 Ethical issues and permissions

Data protection clearance for access to personal data was acquired from the Data Protection Officer at Mater Dei Hospital as required by Article 16 of the Maltese Data Protection Act. Patient and specimen identification was performed by an intermediary. Identification numbers were converted into encrypted identifiers which were used during immunohistochemistry, scoring and result analysis. Ethical clearance was acquired through the University of Malta Research Ethics Committee (UREC).

2.2 Case selection

200 patients suffering from endometrioid endometrial cancer were retrospectively identified from the Mater Dei Hospital histopathological laboratory information system using IBM Cognos analytics. Cases were subcategorised into three arms: those above the age of 50 years at diagnosis, those at or under the age of 50 years at diagnosis, and a control group. 151 patients over the age of 50 years were randomly identified in the period 2013–2020. All patients diagnosed with endometrioid endometrial carcinoma at or below the age of 50 years between 2000–2020 were included in the second arm. A control group was compiled using endometrial tissue samples from patients with benign pathologies such as leiomyomas and uterine prolapse between 2000–2020.

Cases with endometrioid endometrial carcinoma on curettage, Pipelle endometrial sampling and/or hysterectomy specimen were originally included in the study. Initial pilot immunohistochemistry runs identified false-negative IHC defects in hysterectomy specimens due to fixation-related issues. For this reason, only cases with an endometrial sampling specimen were included in the final dataset. These endometrial samples were acquired via dilatation and curettage or outpatient clinic Pipelle endometrial sampling. Patients with non-endometrioid Type 2 endometrial cancers were excluded from the study due to low absolute case numbers and the limited literature available on the role of mismatch repair testing in these subtypes. Cases with an unclear tumour origin, such as suspected primary ovarian endometrioid tumours were also excluded.

Clinicopathological characteristics for these cases were acquired from histological reports and included age, tumour subtype, grade, FIGO stage, surgical management and oncological treatment administered.

Haematoxylin and Eosin (H&E) stained sections mounted on glass slides representing malignant endometrioid endometrial tissue for these cases were retrieved from the histopathology lab archives. Slides were re-examined by an independent pathologist to confirm the diagnosis as well as to identify the best block representing the tumour. In the control group, slides representing benign endometrial tissue were identified. The corresponding formalin-fixed and paraffin-embedded (FFPE) block for each case was retrieved and four sections from each case were cut at 3 micrometers (μm) for immunohistochemical analysis. Leica Biosystems ready-to-use (RTU) stains were used (LBSPA0988, LBSPA0989, LBSPA0990, LBSPA0991). After a rigorous optimisation process, all four antibodies were validated using five specimens with a known MMR proficiency status. The optimal nuclear staining with minimal background staining for all four antibodies was achieved at H2 40 (high pH antigen retrieval incubated for 40 minutes).

Table 1. The Allred scoring system used for MMR IHC status evaluation.

Proportion of positive staining score (PS)		Staining intensity score (IS)	
PS	Range (%)	IS	Type
0	0	0	No staining
1	< 1	1+	Weak positive staining
2	1–10	2+	Moderate positive staining
3	11–33	3+	Strong positive staining
4	34–66	Allred score = PS + IS	
5	67–100		

Table 2. Clinicopathological characteristics and overall MMR status in the three study arms.

Arm	n	Mean age (years)	Grade at presentation	Stage at presentation	Proportion receiving radiotherapy	Proportion receiving chemotherapy
1	151	66	1- 64.9% (n = 98) 2- 25.8% (n = 39) 3- 9.3% (n = 14)	IA- 56.3% (n = 85) IB- 29.1% (n = 44) II- 5.3% (n = 8) IIIA- 4.0% (n = 6) IIIB- 2.6% (n = 4) IIIC- 0.7% (n = 1) IV- 0.7% (n = 1) Unknown- 1.32% (n = 2)	46.3% (n = 70)	12.6% (n = 19)
2	49	44	1- 77.6% (n = 38) 2- 16.3% (n = 8) 3- 6.1% (n = 3)	IA- 59.2% (n = 29) IB- 10.2% (n = 5) II- 10.2% (n = 5) IIIA- 4.1% (n = 2) IIIB- 2.0% (n = 1) IIIC- 2.0% (n = 1) IV- 4.1% (n = 2) Unknown- 8.2% (n = 4)	10.2% (n = 5)	14.3% (n = 7)
3	30	46.3	/	/	/	/

2.3 Immunostain scoring

The immunostained sections were evaluated for retention or loss of nuclear MMR protein expression by a histopathologist, an IHC lab scientist and the main investigator. Scoring was performed using the Allred scoring system whereby visual quantification of positive cells and stain intensity was done on areas of endometrial carcinoma cells that were deemed representative of the overall tumour. The percentage positivity and intensity score were combined to create the final Allred score as shown in Table 1.

Normal expression was defined as nuclear staining within tumour cells, using the nuclei of normal endometrial cells and stromal cells as positive internal controls. Negative expression was defined as the complete absence of nuclear staining within tumour cells (Allred score of 0), but with the presence of positive staining in normal endometrial and stromal cells. MMR deficiency was defined as the complete loss of expression of at least one of the four MMR proteins. To facilitate further sub-group analyses, cases with weak nuclear IHC staining (Allred score 1–4) were denoted as 'Weak expression' while cases with a stronger nuclear IHC staining (Allred score 5–8) were denoted as 'Positive'. Cases with one or more 'Weak expression' IHC stains were still considered to be overall MMR proficient.

3. Results

3.1 Clinicopathologic observations

151 endometrioid endometrial carcinoma patients over the age of 50 were identified in the period 2013–2020. These had a mean age of 66 years at EEC diagnosis. 64.9% of these cases involved well-differentiated EEC, with 56.3% of patients in this arm presenting with FIGO Stage IA disease. All 49 EEC cases identified in patients $\leq$ 50 years old within the extended 2000–2020 period were included in the study. This extended period of inclusion increased representation of the younger age-groups, making statistical correlation testing feasible but not allowing any temporal-based analyses to be made. Cases in this second arm had a mean age of 44 years at EEC diagnosis. 77.6% of these younger patients presented with well-differentiated EEC, with 59.2% of patients in this arm presenting with FIGO Stage IA disease. The baseline clinical and pathologic characteristics of the study population are represented in Table 2.

The majority of cases (68%) in the overall study population presented with well differentiated EEC, while 23.5% and 8.5% of patients presented with Grade 2 and Grade 3 disease respectively. 57% of patients presented with FIGO IA disease and underwent definite curative-intent surgical treatment. 24.5% presented with FIGO IB disease, while 15.5% of cases were diagnosed at more advanced stages due to locoregional invasion or metastasis.

Table 3. MMR expression patterns in the overall study population.

MMR expression pattern	Number of patients	Proportion of MMR-deficient cases (%)	Proportion of overall EEC cohort (%)
Normal expression of all four proteins (Appendix 1)	131	/	65.5
MLH1-PMS2 loss (Appendix 2)	49	79.0	24.5
Isolated weak expression of PMS2	4	/	2
Isolated MSH6 loss (Appendix 3)	3	4.8	1.5
Weak expression of MLH1 and PMS2	3	/	1.5
PMS2 loss with weak expression of MLH1	2	3.2	1.0
MSH2-MSH6 loss	2	3.2	1.0
Isolated weak expression of MSH6	2	3.2	1.0
Isolated MLH1 loss	2	3.2	1.0
MSH6 loss with weak expression of MSH2	1	1.6	0.5
MSH2 loss with weak expression of MSH6 (Appendix 4)	1	1.6	0.5

A large proportion of patients (59%) did not require any further treatment following surgery for low-grade, early-stage EEC. Radiotherapy was the sole adjuvant treatment in 27% of cases while chemotherapy (or a combination of chemotherapy and radiotherapy) was indicated in 24 patients, representing 12% of cases. Two patients did not receive any oncological treatment after diagnosis due to severe comorbidities or a secondary, terminal malignant condition. A combination of Carboplatin AUC5-6 (Area under the curve) and Paclitaxel was the most common chemotherapeutic regimen utilized in these cases. The most common approach to radiotherapy was external-beam radiotherapy, delivering 45 Gray(Gy) of ionising radiation in 25 fractions.

3.2 Immunohistochemical mismatch repair protein expression

In the overall study cohort 69% of cases were MMR proficient with positive immunostaining to all four MMR proteins, while 31% of cases were deficient for one or more MMR proteins. Table 3 details the immunohistochemical MMR protein expression patterns in the study population. Dual loss of the MLH1 and PMS2 heterodimer protein expression was the most common deficiency and occurred in 24.5% of the EEC population. Loss of MSH2-MSH6 heterodimer protein expression was less common and represented 3.2% of MMR-deficient cases.

MLH1 and PMS2 antibodies performed in an analogous manner. MLH1 deficient cases were 96.1% as likely to be PMS2 deficient and vice-versa. On the other hand, MSH2 and MSH6 performed in an analogous fashion when proficient (same IHC outcome in 98.5–100% of cases) but less so when deficient (same outcome in 33.3%–66.6% of cases).

3.3 Correlation analysis with MMR immunohistochemical status

A Chi-squared test of association demonstrated a statistically significant relationship between tumour grade at presentation and mismatch repair proficiency on IHC (P = 0.004). Well differentiated tumours were more likely to be MMR proficient. A discrepancy was most noticeable between grade 1 tumours, with a 76.5% proficiency rate, and grade 2/3 disease with 53.2% and 52.9% proficiency rates respectively.

Further statistical analysis was performed using ANOVA to assess the correlation between tumour grade and mean IHC Allred score for each MMR protein. Lack of tumour differentiation was associated with a higher risk of MLH1 (P = 0.02), MSH6 (P = 0.393) and PMS2 (P = 0.011) expression deficiency. This was once again most pronounced between well-differentiated tumours and grade 2/3 disease (Fig. 2). Overall MMR status or MMR protein expression patterns were not significantly correlated with FIGO stage at presentation.

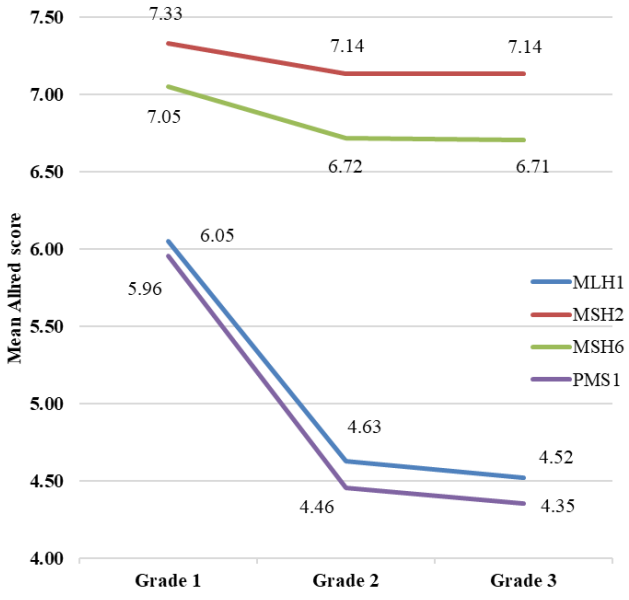

Fig. 2. Tumour grade at presentation and MMR protein Allred score means plot.

Chi-squared testing identified no statistically significant difference ($P = 0.136$) in overall MMR status between Arms 1 and 2 ie. when age 50 was used as a hypothetical testing threshold (Table 4). All control endometrial tissue samples positively expressed all four MMR proteins.

3.4 Correlation analysis with MMR Allred scores

Age at diagnosis (as a discrete variable) showed a statistically significant Pearson correlation with MLH1, MSH6 and PMS2 Allred scores. MLH1 and PMS2 were negatively correlated with increasing age ($P = 0.01$, 0.017 respectively) while MSH6 was found to be positively correlated with increasing age ($P = 0.016$) (Table 5). These findings remained statistically significant when correcting for the effect of tumour grade as a confounding variable using regression analysis. MSH2 Allred scores were not correlated with age at EEC diagnosis.

Table 4. MMR status in the three study arms.

Arm	MMR proficient	MMR deficient
1: > 50 years	66.2% (n = 100)	33.8% (n = 51)
2: ≤ 50 years	77.5% (n = 38)	22.4% (n = 11)
3: Control	100% (n = 30)	0%

MMR deficient cases were more likely to require radiotherapy ($P = 0.014$) but this association was no longer significant after correcting for tumour grade. There was also no association between MMR status and adjuvant chemotherapy administration.

4. Discussion

The significance of the mismatch repair mechanism in carcinogenesis and its links to other intracellular pathways is a prominent research topic in endometrial cancer studies, especially in the ever-developing world of targeted diagnostics and therapeutics. Due to the historically clearer association between Lynch syndrome (HNPCC) and colorectal cancer, protocols to determine MMR status in endometrial carcinoma have only recently been developed but have several important clinical implications. Primarily, loss of MMR protein expression may be due to heritable pathogenic mutations of HNPCC. In these cases, immunohistochemical MMR defects serve as the initial marker when selecting patients who would most benefit from germline testing. Secondly, tumoral MMR defects serve as biomarkers to predict the response to checkpoint inhibitor agents and immunotherapy as they represent cells with a higher phenotypic neoantigen load [30]. Clinical outcomes in MMR-deficient cases can be improved both directly by guiding patient treatment and indirectly by guiding genetic testing, family counselling and surveillance. This study sought to identify the local MMR protein expression trends in Maltese EEC patients and to assess the extent to which age at EEC diagnosis affects MMR status as assessed by IHC.

4.1 Mismatch repair protein expression patterns

This study is the first one investigating the MMR status in Maltese gynaecological cancer patients and has shown that tumoral MMR IHC deficiency is present in 31% of EEC cases. Overall deficiency of IHC MMR protein defects in other published studies lies at 22.5–35.9% (13–21) but one should point out that almost all these investigators included both endometrioid and non-endometrioid histologies in their cohort or made no reference to subtype. This study only included Type 1 endometrioid malignancies to avoid tumour subtype from acting as a confounding variable. A study by Kim *et al.* [18] in 2018 was one of the few that performed MMR IHC testing solely on Type 1 EEC cases. Kim et al reported MMR IHC loss in 26% of cases, in a study that was also done retrospectively and in a very comparable study population of 173 cases.

Mismatch repair proteins act as heterodimer pairs. The MSH2-MSH6 heterodimer is the first to bind to mismatched bases in the DNA strand and is then followed by the MLH1-PMS2 heterodimer which excises the anomalous segment and re-synthesizes DNA at the mismatch site. Knowledge of this MMR protein pairing mechanism is essential for the correct interpretation of IHC testing results. Although MLH1 forms a heterodimer with PMS2, it does occasionally pair up with other mismatch proteins, unlike PMS2 that can only bind to MLH1. As a result, MLH1 loss automatically leads to loss of PMS2 expression since it would have lost its binding partner. When there is loss of the PMS2 protein, MLH1 may still be expressed and detected immunohistochemically as it not fully dependent on the presence of PMS2. In the presented study, IHC loss of both MLH1 and PMS2 occurred in 24.5% of cases while isolated loss of PMS2 was noted in 1% of cases. Isolated MLH1 loss occurred in a further 1%. The reported loss of the MLH1-PMS2 heterodimer in literature is once again comparable and ranges from 5.78–26.92% of cases while isolated loss of PMS2 is also less frequent at 0.14-1.45% of cases [13–21].

The second heterodimer is composed of MSH2 and MSH6. As with the MLH1-PMS2 heterodimer, the MSH2 protein can pair up with other MMR proteins while MSH6 solely binds to MSH2. Loss of MSH2 protein expression therefore leads to loss of MSH6 staining, but not vice versa. Literature reports loss of the MSH2-MSH6 heterodimer in 4.1–9.72% of endometrial cancer cases while isolated MSH6 has been reported in 1.98–6.48% of cases [13–21]. In this study we report MSH2-MSH6 IHC loss in 1% of cases, isolated MSH6 loss in 2% of cases and isolated MSH2 loss in 0.5% of cases. This slight disparity in the incidence of MSH2-MSH6 deficiency is likely to be secondary to the relatively low study population size in which each case mathematically represents a significant percentage of the overall cohort.

The heterodimer mechanism facilitates the interpretation of MMR IHC results (Table 6). Dual loss of MLH1 and PMS2 immunohistochemical expression often indicates somatic MLH1 promoter methylation or germline MLH1 mu-

Table 5. Pearson correlation analysis between age and MLH1, MSH2, MSH6 and PMS2 Allred scores.

MMR protein	Pearson Correlation coefficient (Allred score vs age)	Statistical significance	Statistical significance when correcting for tumour grade
MLH1	-0.184	S ($P = 0.01$)	S ($P = 0.004$)
MSH2	0.097	NS ($P = 0.175$)	NS
MSH6	0.17	S ($P = 0.016$)	S ($P = 0.037$)
PMS2	-0.17	S ($P = 0.017$)	S ($P = 0.004$)

Table 6. Interpretation of the likely defective protein responsible for IHC expression defects.

IHC expression loss	Interpretation (defective protein)
PMS2	PMS2
MLH1 + PMS2	MLH1
MSH6	MSH6
MSH2 + MSH6	MSH2

tation. Further testing such as MLH1 promoter hypermethylation analysis or DNA sequencing for the BRAF V600E mutation can help distinguish between these two possibilities and is thus an essential step in laboratory IHC testing protocols. Unlike the case of MLH1-PMS2 heterodimer expression loss, dual loss of MSH2 and MSH6 expression often indicates a germline MSH2 mutation. Isolated loss of PMS2 or MSH6 expression typically indicates a germline mutation in the respective gene [38].

A proportion of MMR IHC defects are secondary to sporadic non heritable tumour mutations. Mesenkamp *et al.* sequenced MMR IHC-deficient colorectal tumours and endometrial tumours from patients with normal germline sequencing and which appeared to have no tumoral hypermethylation of the MLH1 promoter [39]. In more than half (52%) these tumours, the underlying source of deficient MLH1 and MSH2 expression were non-hereditary somatic mutations. This emphasizes the important role that sporadic somatic mutations and gene sequencing will play in the management of endometrial cancer. Sanger gene sequencing has been the predominant technique employed in this field, but next generation sequencing (NGS) also offers great potential as it enables parallel sequencing of a large number of fragments per run in a simultaneous manner.

4.2 Fixation quality and MMR immunostaining

One of the major challenges encountered during the pilot phase of this project was the lack of reliable findings from uterine specimen section immunostaining. A large proportion of slides only showed weak or patchy epithelial surface staining with negative internal controls, while stained slides from the respective diagnostic curettings or Pipelle endometrial samples performed optimally (Fig. 3).

Resection specimens with positive MMR expression also exhibited a noticeably clear gradual loss of IHC staining that was directly dependent on the distance from the section surface. The central areas of sectioned endometrial tissue thus remained invariably negative; a problem that was attributed to suboptimal fixation in the uterine specimen. These effects were most prominent with MSH6 and PMS2 antibody stains.

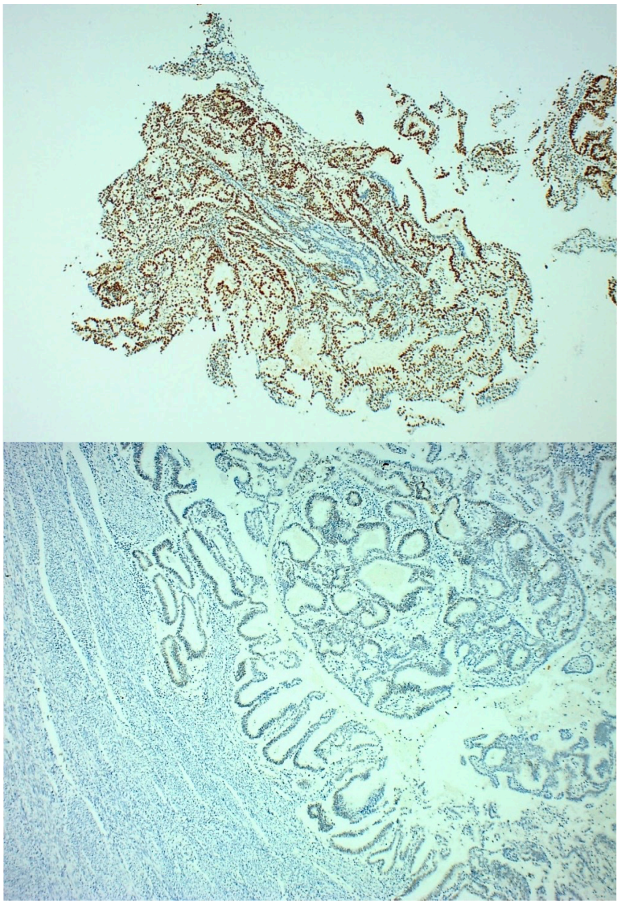

Fig. 3. Comparative MLH1 stain of endometrial diagnostic biopsy (above) and resection specimen (below) showing the fixation-dependent discrepancy in IHC intensity.

The suboptimal fixation of uterine resection specimens can be attributed to several factors. It is local practice at Mater Dei Hospital to place diagnostic endometrial biopsies directly into a formalin filled container. Biopsies are small and easily

penetrated by formalin during the fixation process. Resection specimens, on the other hand, are larger and with a lower surface area-to-volume ratio. Following hysterectomy, it is standard practice by local gynaecologists not to open the uterine body as this could interfere with macroscopic assessment and margin testing by histopathologists. Following hysterectomies for both benign and malignant indications, the specimens are opened upon arrival to the lab (transfer of specimen may take up to 24 hours) and left to fix for at least 24 hours depending on the size of the specimen and friability of the tumour.

There is ongoing debate about when to open the uterine body in uterine specimens. The most commonly practiced technique is to open the uterine body as soon as possible to facilitate the formalin penetration through the myometrial wall and endometrium [40]. This approach prevents autolysis and fixation artifacts that could cause difficulties with interpretation, possibly to the detriment of the patient. The main drawback to this method is uterine wall distortion secondary to myometrial contraction. This distortion may affect the assessment of tumoral invasion within the myometrium, which may alter the reported FIGO stage of endometrial carcinoma.

The locally used method that delays cut-up until day one post-surgery preserves the uterine wall without distortion but appears to result in delayed fixation of the endometrium. This delay may result in autolytic changes such as altered nuclear features including chromatin pattern, shape, and nucleolar features. Alturkustani *et al.* investigated the delayed protocol by analysed H&E sections of uterine specimen that were only opened after the uterus was fixed in formalin for a minimum of 16 hours [41]. The investigators confirmed that delayed formalin fixation of the endometrium was associated with autolytic changes and affected the nuclear features of the glandular cells. This could further complicate the interpretation of endometrial changes, some of which already mimic nuclear atypia regardless of the fixation protocol used. Although these investigators did not specifically test the effect of the fixation protocol on immunostain quality, we hypothesise this was a major player in the problems faced in the pilot phase of our project. Some authors have prevented these autolytic changes by injecting formalin through the cervical os to improve fixation while preventing uterine wall distortion [42].

4.3 The use of endometrial biopsies for IHC studies

It is well documented that immunostaining with MMR antibodies is fixation-dependent and that poor fixation may cause difficulties with immunostaining and interpretation. The fixative agent being used, the duration in formalin prior to the embedding process and the uniformity of fixation are all factors which can influence the final immunostain quality. In 2012, Fadhil *et al.* assessed tumour sections from 30 colorectal cancer specimens and their corresponding presurgical diagnostic biopsies [43]. The authors concluded that staining is typically more intense and reliable in biopsies and that these faithfully replicate the diagnosis in the resection specimen. This was deemed to be due to more uniform and complete fixation in the colorectal biopsy samples when compared to the final resection specimens. As observed in our study, this phenomenon was most stark in MSH6 and PMS2 in which corroboration through loss of the heterodimer partner may not be possible.

Two challenges that arise from using biopsies are the limited amount of tissue available which may restrict the number of immunostains performed, as well as the risk that the biopsied material may not fully represent the final resection histopathological outcome. The second problem was preempted in our study as a histopathologist analysed and compared H&E slides from both the diagnostic endometrial tissue and the final resection specimen to ensure that the FFPE block being considered for slide re-cuts was truly representative of the patient's disease grade and subtype.

4.4 Semiquantitative analysis of MMR expression

Most centres and research groups have until now interpreted MMR IHC stains in a binary manner whereby tissue is declared deficient when malignant cell nuclei do not take up the stain and proficient if nuclei taken up the stain, regardless of the proportion of stained cells and strength of the stain. Aberrant function of the MMR mechanism typically results in complete loss of nuclear immunostaining especially when this is secondary to MLH1 promoter hypermethylation, as it causes complete gene silencing [44]. However, weak MMR IHC expression was noted in some cases in our study (Table 7). Weak expression was defined as an Allred score of between 1 and 4. For the sake of overall MMR status interpretation such cases were still considered MMR proficient. It is worth noting that weak immunostaining did not occur randomly, as would be expected with suboptimal reagents, epitope retrieval or staining technique. Weakly expressed proteins followed the previously discussed heterodimer-related patterns. In some cases, both components of a heterodimeric pair would stain weak while in others, a tissue that is deficient in one MMR protein would stain very weakly to the other heterodimeric partner. As in the classic 'binary' proficient/deficient system, isolated MSH6 or PMS2 weak expression cases were also identified.

These patterns show that this finding was not the result of an artefact but a true representation of relative MMR protein concentrations in cancer cells. The issue of weak MMR protein expression has been tackled in some publications, but the prevalence of this staining pattern and possible implications on tumoral or germline DNA mutation findings have not been well elucidated.

In HNPCC, the large number of potential DNA mutations may have varying downstream effects on epitope expression, from complete silencing to low or full expression of one or both heterodimer proteins [45, 46]. Weak immunohistochemical staining may be a result of the transcription and translation of a truncated protein with reduced stability and is likely to be accompanied by normal strong nuclear staining of internal controls such as neighbouring stro-

Table 7. Summary of the cases that exhibited weak MMR protein expression (Allred score 1–4).

MMR expression pattern	Number of patients	Proportion of all MMR-deficient cases (%)	Proportion of overall EEC cohort (%)
Isolated weak expression of PMS2	4	/	2
Weak expression of MLH1 and PMS2	3	/	1.5
PMS2 loss with weak expression of MLH1	2	3.2	1.0
Isolated weak expression of MSH6	2	3.2	1.0
MSH6 loss with weak expression of MSH2	1	1.6	0.5
MSH2 loss with weak expression of MSH6 (Appendix 4)	1	1.6	0.5

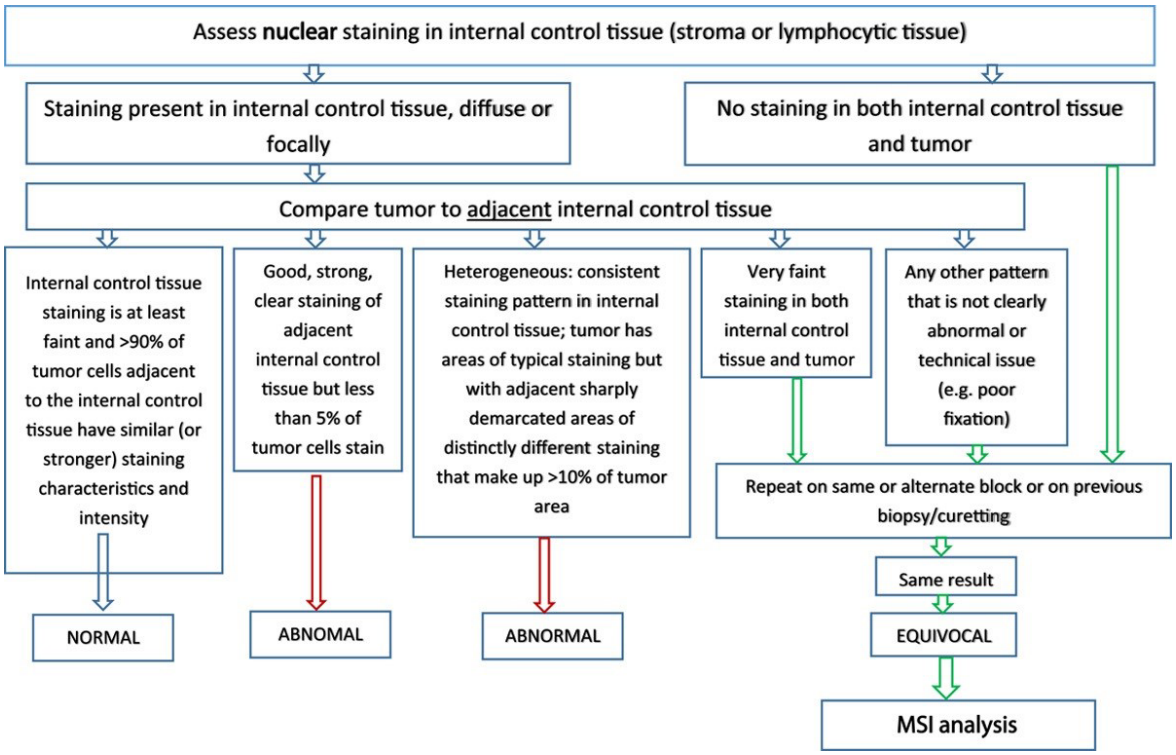

Fig. 4. The decision tool developed by Sari *et al.* [48] with a high degree of interobserver agreement for immunohistochemical MMR expression analysis.

mal cells, normal endometrial lining or lymphocytes. Barrow et al confirmed that protein expression may still occur in the context of known pathogenic germline mutations after performing IHC expression analysis for all four MMR proteins on tumour sections from 51 known heritable mutation carriers and 17 controls. This is a potential pitfall in triaging algorithms that are based on a primary immunohistochemical screen [47].

Semiquantitative IHC analysis of MMR proteins may become clinically relevant in the future. Although a laborious and time-consuming process, Allred scoring of all endometrial tissue sections in our study provided us with the opportunity to perform correlation analysis between clinicopathologic variables and MMR expression as assessed by validated tool that combines both the intensity and proportion of stained cells. Decision tools have been developed to facilitate MMR status analysis and decrease the impact of fixation-related issues and preanalytical conditions on MMR status outcomes. Sari *et al.* proposed one such tool (Fig. 4) which resulted in an interobserver agreement rate of 92% [48].

Similarly, Stormorken and colleagues investigated this semiquantitative technique for MMR expression evaluation [49]. They quantified MMR protein expression as a percentage of nuclear immunopositivity with a 0–3 scale, zero equating to MMR protein deficiency. This study was performed on a relatively small number of subjects, immunostaining tumour sections from eleven MSH2, four MSH6 and four MLH1 germline mutation carriers. The authors showed that only 21% (8 of 38) gene mutation carriers scored zero on the respective antibody stain. This confirms that contrary to previous understanding, staining for MMR proteins does not give a binary response to the presence of a corresponding genetic mutation. In the future, the more widespread use of digital image analysis will facilitate the objective quantification of protein expression in malignant tissue sections, thus decreasing interobserver and intraobserver variability.

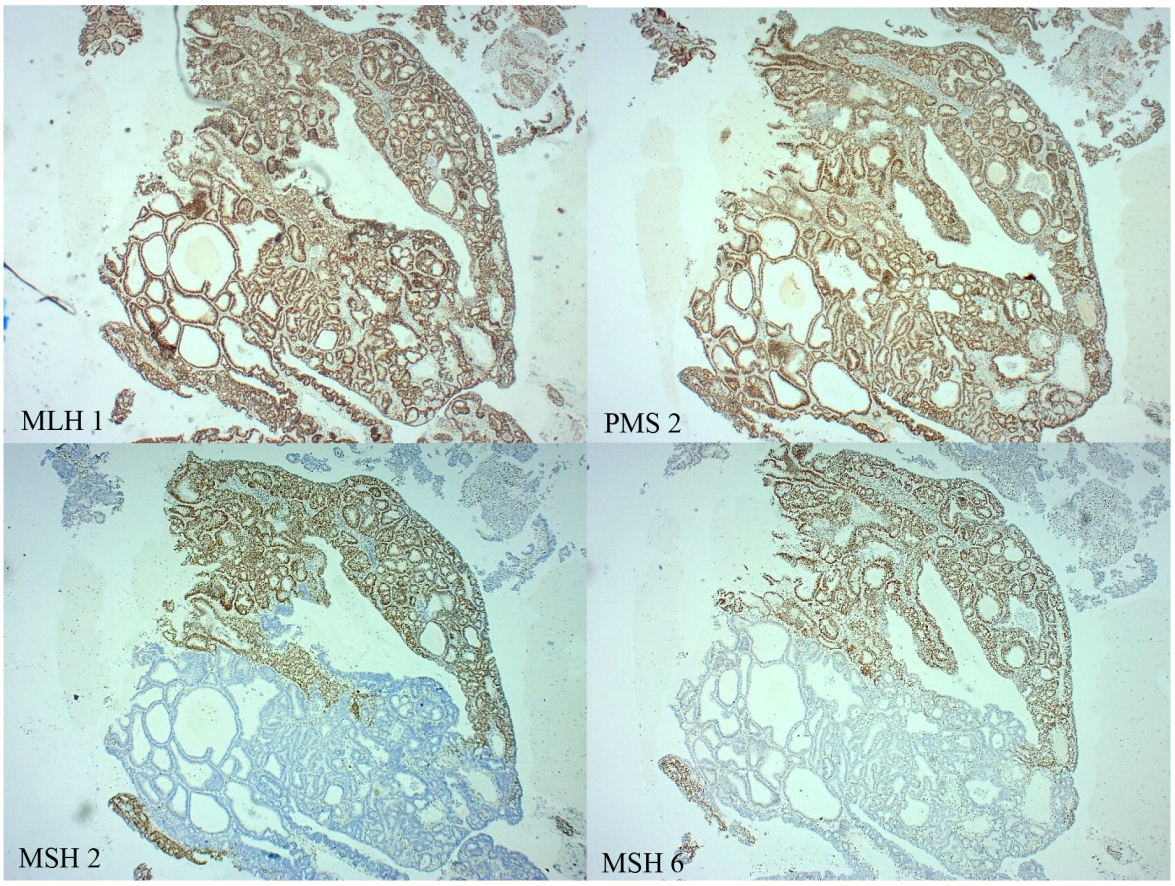

Fig. 5. MMR IHC set showing heterogenous staining for MSH2 and MSH6.

Table 8. Summary of the local EEC cases with heterogenous MMR protein staining patterns.

Age at diagnosis	Tumour Grade	FIGO surgical staging	MLH1 (Allred score)	PMS2 (Allred score)	MSH2 (Allred score)	MSH6 (Allred score)
53	2	FIGO IA	Heterogenous 8/0	Heterogenous 8/0	0	3
54	2	FIGO IA	8	8	Heterogenous 8/0	Heterogenous 7/0
74	1	FIGO IB	Heterogenous 8/0	Heterogenous 8/0	7	8
77	1	FIGO IB	Heterogenous 8/0	7	8	7

4.5 Focal and heterogenous MMR immunostaining

Another phenomenon that was noticed in this study is focal or heterogenous MMR immunostaining, with sharply contrasting areas scoring 7/8 and 0 on the Allred system (Fig. 5).

The heterogenous staining pattern observed in these cases was well delineated, with sharp contrast between the MMR proficient and deficient regions. This should be differentiated from the patchy staining secondary to poor fixation or staining as the latter often shows a gradient of diminishing stain intensity without clear delineation between the different areas. Once again this phenomenon was observed to follow the heterodimeric staining patterns. Studies have found several potential causes for these heterogenous patterns. These include variable epitope expression, areas of dedifferentiation, and second-hit mutations or methylation in tumour subclones.

MMR protein expression could also potentially be under the influence of microenvironmental tumour factors such as oxidative stress and hypoxia [50]. Table 8 exhibits the four heterogeneous MMR staining cases encountered in our study.

Watkins *et al.* published a study investigating these uncommon heterogenous MMR expression patterns in endometrial cancer cases [51]. The authors reported discrete subclonal loss of MMR protein expression in 7.2% of tumours (9 of 125 cases), and such observations were limited to endometrioid portions of the tumour. Discordant subclones were separately analysed for MLH1 promoter methylation and microsatellite instability and this confirmed that these heterogenous tumours consisted of contrasting areas that were either MMR proficient and microsatellite stable or MMR deficient due to MSI or MLH1 promoter hypermethylation. Of note, the investigators emphasised that subclonal

Table 9. IHC-protocol performance according to hypothetical age thresholds.

Age threshold for IHC testing (years)	MMR deficient cases detected (n)	Hypothetically missed MMR deficient cases (n)	Percentage of all MMR deficient cases missed (%)
50	11	51	82.3
60	27	35	56.5
70	51	11	17.7
80	58	4	6.5

MLH1 defects in such cases appeared to be secondary to somatic or methylation events, without evidence of underlying germline mutations. It is essential to recognise these IHC patterns to avoid false-positive and false-negative MMR reports.

4.6 The role of age in MMR testing

The role patient age plays in MMR testing protocols can be examined from two different standpoints. Primarily, age has been shown to be an important predictor of the likelihood of HNPCC in patients with endometrial or colorectal carcinoma, a phenomenon on which both the Amsterdam and revised Bethesda guidelines are based. Both the Amsterdam guidelines and the revised Bethesda guidelines use 50 years as the threshold under which familial cancer germline defects would be deemed more likely. The Jerusalem criteria, on the other hand recommend that IHC or MSI testing be carried out for patients under the age of 70 years at presentation [52]. The reasoning behind this broader inclusion criteria is to identify HNPCC patients with an MSH6 or PMS2 mutation as patients with one of these two gene mutations tend to present later in life and would thus not be picked up using the Amsterdam or Revised Bethesda criteria [53].

The second standpoint from which to analyse the role of age in MMR testing is in its role to guide therapeutic choice in patients with an active colorectal or endometrial malignancy. For this sole purpose, the correlation can be tested using age at diagnosis and immunohistochemical tumour phenotype, rather than patient genotype. Our study findings have shown that there is no statistically significant difference in overall MMR IHC status when comparing patients under or over the age of 50. This shows that when using IHC to guide targeted therapy such an age threshold is not well suited at triaging cases for MMR expression status assessment.

This study has also shown that when the four MMR proteins were investigated separately, patients in Arm 2 ($\leq$ 50 years) were more likely to be MLH1 and PMS2 proficient. This younger cohort was 1.29 times more likely to be MLH1 proficient ($P = 0.004$) and 1.25 times more likely to be PMS2 proficient ($P = 0.011$). Our study also assessed the correlation between age at diagnosis and the semiquantitative assessment of MMR protein expression using Allred scores. MLH1, MSH6 and PMS2 expression showed a statistically significant Pearson correlation with age. MLH1 and PMS2 were negatively correlated with increasing age ($P = 0.01, 0.017$ respectively) while MSH6 was found to be positively correlated with increasing age ($P = 0.016$). These findings remained statistically significant when correcting for the effect of tumour grade as a confounding variable using regression analysis. We hypothesise that the observation of decreased MLH1 and PMS2 proficiency with increasing age is due to MLH1 hypermethylation which has been shown to be more frequent with advancing age [54, 55]. On the other hand, MSH2 and MSH6 deficiency has been shown to reflect a germline defect in a large proportion of cases [56]. This may be the reason behind our observation that younger EEC patients are more likely to be MSH6 deficient. Definite confirmation of these two hypotheses will be confirmed in prospective studies that include tumoral and germline DNA sequencing studies.

In our study, a 50-year age-at-diagnosis threshold for IHC testing would have missed 51 cases with MMR expression defects, representing 82.3% of all MMR defective cases in the overall study cohort. This would have given the protocol an unacceptable sensitivity of 17.7% for MMR expression defects. A relatively high sensitivity of 93.5% was only achieved when using 80 years as a hypothetical age-at-diagnosis threshold. This is unlikely to be practical and sensible in clinical practice (Table 9).

5. Conclusions

There is no statistically significant difference in overall qualitative immunohistochemical MMR status when using the age of 50 as a threshold for tumour analysis. Such a threshold should not be included in lab algorithms for EEC IHC analysis unless its sole purpose is the identification of potential HNPCC patients. Using such a threshold in this cohort would have missed 82.3% of cases with immunohistochemical tumour MMR deficiency. There is a significant correlation between MLH1, MSH6, PMS2 and age at EEC diagnosis when IHC is analysed semiquantitatively using Allred scoring. MLH1 and PMS2 expression is negatively correlated with increasing age while MSH6 expression is positively correlated, even after correcting for the confounding effect of tumour grade. More prospective evidence is required to clarify the role IHC scoring and semi-quantitative analysis should play in MMR status interpretation and patient management especially in the ever-evolving field of targeted therapeutics.

Author contributions

MRB designed the research study, collected data and wrote the manuscript under the supervision of YMB. TB, SF, SA, SB and ISH assisted with immunohistopathological lab work and interpretation of slides. JD provided input from the medical oncology point of view. NC assisted with statistical data analysis.

Ethics approval and consent to participate

Ethical clearance was acquired from the University of Malta Research Ethics Committee (UREC) on 12th July 2018. Pseudoanonymised data identifiers were used via a professional intermediary and patients had no direct participation in the project.

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Conflict of interest

The authors declare no conflict of interest.

Appendix

Appendices associated with this article can be found, in the online version, at https://ejgo.imrpress.com/EN/10.31083/j.ejgo.2021.02.2331.

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