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# The role of MLH1, MSH2 and MSH6 in the development of colorectal cancer in Uganda

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## Abstract

**Introduction** In Uganda, colorectal cancer (CRC) is steadily increasing according to the Kampala Cancer Registry. In the West, microsatellite instability is detected in 90% of hereditary nonpolyposis colon cancers (HNPCC) which account for 1–2% of all CRC, and 15% of sporadic CRC. Germline mutations in MLH1 and MSH2 account for 90% of HNPCC in the West, whilst the remainder of cases are due to mutations in MSH6 and PMS2. The aim of this study was to determine the microsatellite instability (MSI) status and determine the proportions of MLH1, MSH2, and MSH6 pathological mutations in Ugandan CRC patients.

**Methodology** This was a cross-sectional study carried out between 1st January 2008 to 15th September 2021. Patients were recruited prospectively from 16th September 2019 to 16th September 2021, from Masaka Regional Referral Hospital, Mulago National Referral Hospital, Uganda Martyrs' Hospital Lubaga and Mengo Hospital. From 1st January 2008 to 15th September 2019, CRC FFPE tissue blocks were obtained from the archives of the Department of Pathology, Makerere University. Data was abstracted from the medical case files for demographics, topography and stage. The histopathological subtype and grade of CRC were obtained by two consultant pathologists from the H&E slides. DNA was extracted from CRC formalin-fixed paraffin-embedded (FFPE) tissue blocks. Library preparation was completed using the Qiagen custom design panel. The custom panel represented 56 genes. The MLH-1, MSH2, MSH6, BRAF and KRAS genes were sequenced using the above library preparation and NGS sequencing. The MSI status was obtained if one of the MSI genes, MLH1, MSH2 or MSH6 was pathologically mutated. If none of the genes was pathologically mutated it was considered MSI negative, microsatellite stable (MSS). Immunohistochemistry was carried out to determine whether MLH1 and PMS2 was MMR proficient or deficient. Categorical data was summarized using frequencies and proportions corresponding to each of the three histopathological subtypes and MSI status subtypes. Continuous and categorical variables were analyzed using the chi-square and Fischer's exact tests. A p -value $\leq 0.05$ was considered statistically significant for all the analyses.

**Results** Out of 127 CRC patients, the mean(SD) age of MSI cases was 55.6(16.9) years and of MSS cases was 55.4(15.5) years. The majority were MSS, 75(59.06%) followed by MSI, 52(40.9%). There were 14(11.02%) MLH-1 mutations, 30(23.62%) MSH2 mutations, and 26(20.47%) MSH6 mutations. BRAF mutational analysis showed only 5(3.9%) having pathologic missense BRAF V600 mutations. KRAS mutations consisted of only 8(6.3%) having pathologic missense KRAS mutations.

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**Conclusions** The high rate of MSI in Ugandan colorectal tumours was mainly associated with a lack of BRAF mutations and a high frequency of MSH2 and MSH6 MMR gene mutations. In CRC patients, identification of the causative mutation is recommended, however in a resource-limited setting, MSI testing and immunohistochemistry is more cost effective. In Ugandan CRC patients who meet at least one of the Bethesda criteria, MSI testing and immunohistochemistry may therefore be offered to obtain the MSI status of the tumour.

**Keywords** Colorectal cancer, Microsatellite instability (MSI), Microsatellite stable (MSS), Immunohistochemistry, Uganda

## Introduction

In terms of incidence colorectal cancer (CRC) is the second in women and the third in men most common malignancy worldwide, and in both sexes is the third leading cause of cancer-related deaths [1].

The Kampala Cancer Registry in Uganda has shown that colorectal carcinoma has a low incidence however there are increases occurring especially among women [2, 3]. The age-standardized incidence rate has increased from 5.2 per 100,000 population for the year period 1991–1995 to 9.0 per 100,000 population for the year period 2006–2010 in females [2, 4]. Male colorectal cancer rates in Uganda increased gradually during the past 20 years, rising from 7.8 to 11 per 100,000 population in the period 2006 to 2015 [4].

Two distinct pathways are responsible for the development of colorectal cancer. The 'suppressor' pathway involves the activation of proto-oncogene K-ras and the loss of tumour suppressor genes APC, DCC and p53. These mutations are responsible for 85% of sporadic colorectal cancers and 1% of cancers associated with familial adenomatous polyposis (FAP) [5, 6]. In the 'mutator' pathway, mutations in MLH1, MSH2, MSH6, PMS1 and PMS2 which are mismatch repair genes (MMR) lead to microsatellite instability (MSI) in genes such as BAX, E2F-4, TGFβRII and ILGF [7–9]. In the West, microsatellite instability is detected in 90% of cancers from hereditary nonpolyposis colon cancer which accounts for 1–2% of all CRC, and 15% of sporadic CRC [10–12].

Microsatellite instability (MSI) is classified according to the level of instability as stable (MSS), low(MSI-L) and high(MSI-H) [13]. Loss of MLH-1 function due to silencing of the gene by methylation of the MLH-1 promotor region is mainly responsible for MSI-H sporadic cases [14, 15].

Hereditary nonpolyposis colon cancer involves germline mutations particularly in the following mismatch repair (MMR) genes: MLH1, MSH2, MSH6, and PMS2 [16–20]. Large deletions in a non-mismatch repair gene called epithelial cellular adhesion molecule (EPCAM), which silences *MSH2* expression, have also been found to cause Lynch syndrome [21]. When treated by standard colorectal resection, patients with hereditary nonpolyposis may develop metachronous colorectal cancer [21]. The Bethesda criteria have been introduced to identify those with hereditary nonpolyposis coli (HNPCC), and advise about analysis of MSI in patients with multiple colorectal carcinomas [22].

In patients with multiple cancers, microsatellite instability has been observed in 33–89% of cancers [23, 24], including any studies that on the basis of the Amsterdam criteria excluded HNPCC clinically [25–27]. The original Amsterdam criteria were not selective and rigorous as the Amsterdam II criteria, and atypical HNPCC associated with MSH6 and other MMR gene mutations were not considered [28]. The underlying genetic pathway in patients who develop synchronous or metachronous cancers is not clear. In the West, 90% of HNPCC cases are due to germline mutations in MLH1 and MSH2, whilst MSH6 and PMS2 account for the remainder of cases [29].

The objective of this study was to investigate the incidence of MSI and determine the proportions of MLH1, MSH2, and MSH6 pathological mutations in Ugandan colorectal cancer patients.

## Methodology

Retrospective CRC FFPE tissue blocks were obtained from the 1st January 2008 to 15th September 2021, from the archives of the Department of Pathology, School of Biomedical Sciences, College of Health Sciences, Makerere University. We recruited prospectively, consecutive participants attending the surgical Departments of Masaka Regional Referral Hospital, Mulago National Referral Hospital, Uganda Martyrs' Hospital Lubaga and Mengo Hospital from the 16th September 2019 to the 16th September 2021. The histopathologic diagnosis was confirmed as colorectal adenocarcinoma by a consultant pathologist at the Institute of Genetics and Cancer at the University of Edinburgh and another pathologist at the Department of Pathology, School of Biomedical Sciences, College of Health Sciences, Makerere University.

The inclusion criteria included cases with histologically proven diagnosis of colorectal adenocarcinoma. Poor-quality FFPE tissue samples and cases with recurrent colorectal cancer were excluded. Poor quality FFPE tissue samples included those having poor quality DNA, low concentration of extracted DNA, or insufficient tissue for the extraction of DNA. Cases of familial adenomatous polyposis were also excluded. During the study period, all cases meeting the selection criteria were included.

The clinical case files were used to abstract data for demographics, topography of tumour and stage. The radiology reports from the participants' medical case files were retrieved to obtain the stage of CRC.

The three-tier grading system was used to grade CRC cases as follows: G1: well-differentiated CRC with > 95% glandular formation; G2: moderately differentiated CRC with 50–95% glandular formation; and G3: poorly differentiated CRC with < 50% glandular formation [30].

#### DNA extraction

DNA was extracted from formalin-fixed paraffin-embedded (FFPE) tissue blocks. Excluded FFPE tissue blocks included those with DNA degradation. Following the recommendations of the manufacturer, advanced UNG kits (Qiagen GmbH, Hilden, Germany) were used for DNA extraction. The quality of the extracted DNA and concentration were measured using a Nanodrop 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, CO, USA). Qubit was used to check the quality of each sample and make sure that it fell within 100-250ng of DNA required by the DNA protocol. All 127 CRC DNA samples passed the quality check and were stored at -20$^0$C to prevent degradation.

#### Library preparation and NGS sequencing

Library preparation was completed using Qiagen custom design panel (QIAseq DNA panel catalogue identifier: CPHS-43072Z-1294) along with QIAseq targeted DNA Pro kit for Illumina (Qiagen GmbH, Hilden, Germany) [31]. The custom panel consisted of a total of 1,294 primer probes and represented 56 genes. Using 100 to 250 ng FFPE DNA it is designed to enrich selected genes and regions. The fragmentation of the DNA samples, end-repair, and A-tailing was carried out using a single controlled multi-enzyme reaction. Ligation of their 5'-ends was carried out by a sequencing platform-specific adapter containing UMI prepared DNA fragments. A repair step was carried out to generate more FFPE DNA molecules for library construction. In the same tube, the repaired FFPE DNA was placed directly into the fragmentation reaction. The fragmented DNA was ligated using an adaptor containing a 12-base fully random sequence (i.e., UMI). Each DNA molecule in the sample had a unique sequence. Following the UMI assignment, in order to ensure that DNA molecules with UMIs in the sequenced library were sufficiently enriched, target enrichment was performed. Several cycles of targeted PCR using one region-specific primer and one universal primer complementary to the adaptor were subjected to ligated DNA molecules for enrichment. Using universal PCR, amplification of the library and addition of sample indices and platform-specific adaptor sequences were carried out.

After ligation and target enrichment PCRs, an enzymatic reaction was used for cleanup. More consistent library construction was achieved, following enzymatic cleanups, as there were no target enrichment PCRs and no highly variable bead cleanups [31].

In order to overcome errors due to multiplexing sequencing error, image analysis, and oligo synthesis contamination, two unique indices were assigned to each sample to reduce any real misassignment to incorrect samples. Using a dual indexed pared-end sequencing program of 2 × 149-bp reads, the library pool was sequenced on the Illumina MiSeq platform.

#### Bioinformatics analysis

The quality of our raw FASTQ files was initially assessed using FastQC and MultiQC, which provided detailed quality HTML reports. Low-quality bases (Phred score < 25) and adapter sequences were removed using Trim Galore for preprocessing. The cleaned reads were then aligned to the human reference genome (version 38) using the BWA-MEM algorithm to produce alignment files. Variant calling was conducted using the Genome Analysis Toolkit (GATK4) following the Best Practices workflow, with HaplotypeCaller used for variant discovery. Variants were filtered for further analysis based on a minimum read depth of 20X and a variant allele depth of at least 10X. Annotations were performed with ANNOVAR (Wang et al., 2010) to provide functional insights into the identified variants. Variants classified as uncertain significance (VUS) according to ClinVar were analyzed using a suite of nine variant effect prediction tools: SIFT, LRT, MutationTaster, Mutation Assessor, FATHMM, PROVEAN, ClinPred, MutPred, and MetaSVM. A variant was deemed deleterious if at least five of these tools predicted a damaging impact, a threshold chosen to balance sensitivity and specificity based on established consensus approaches. Variants with fewer than five damaging predictions were classified as tolerated. To identify novel mutations, we cross-referenced the variants against major population and mutation databases, including COSMIC, 1000 Genomes, dbSNP, ExAC, GnomAD, ClinVar, Varsome, and Mastermind. This comprehensive screening ensured the reliability of our variant interpretation and its potential translational relevance in clinical contexts.

The MLH-1, MSH2, MSH6, BRAF and KRAS genes were sequenced using the above library preparation and NGS sequencing. The MSI status was obtained if one of the MSI genes, MHL-1, MSH2 or MSH6 was pathologically mutated. If none of the genes was pathologically mutated it was considered MSI negative (MSS).

### Immunohistochemistry for MLH-1 and PMS2 expression

MMR immunohistochemistry was performed on all the 127 colorectal cancer samples, utilizing the Novolink Max polymer detection system Kit and Tris buffered saline (Thermo Scientific) according to the instructions of the manufacturer. The primary monoclonal antibodies used were against MLH-1 (clone ES05; Ref: IR079) in a dilution of 1:100 and PMS-2 (clone EP51; Ref: IR087) in a dilution of 1:100 (DAKO, Agilent, USA) according to the manufacterer kit instructions.

These monoclonal antibodies were applied to 3-µm deparaffinized formalin-fixed paraffin embedded (FFPE) tissue slide sections. Blockage of the endogenous peroxidase activity was carried out with the Novolink peroxidase-blocking reagent (Novolink maxpolymer) and antigen retrieval performed at 125 degrees centigrade for 36 s at a pH9. The antigen-antibody reaction was visualized with Novolink DAB (diaminobenzidine) solution. Haematoxylin was subsequently used as a counterstain.

Each tissue slide sample was screened by two consultant pathologists for the presence or absence of expression of each of the two MMR proteins. The two deficient MMR proteins assessed were MLH-1 and PMS-2 and complete absence of nuclear staining of the tumour cells was defined as negative protein expression. Deficient MMR (dMMR) was defined if the MMR protein was negatively expressed whilst proficient MMR (pMMR) if the MMR protein was positively expressed in the colorectal cancer tissue slides. Deficient MMR MLH-1 and PMS-2 were subjectively given a staining intensity of 0(-). Proficient MMR MLH-1 and PMS-2 were subjectively given a staining intensity of 1(+). The specificity, dilution, source and clone of antibodies used for IHC analysis of the colorectal adenocarcinomas are shown in Table 1.

### Data analysis

The data consisted of numerical and categorical types and where possible, the numerical data were translated into categorical type using agreeable cutoffs. The data on age was translated to categorical data using the median age as a cutoff. The mean age for the participants corresponding to the MSI status was estimated. Other descriptive analysis was conducted on discrete as well as continuous variables to generate summaries across the samples. Categorical data was summarized using frequencies and proportions corresponding to each of the three histopathological subtypes and MSI status subtypes. Continuous variables were summarized using means (standard deviations) and medians (interquartile range). Continuous and categorical variables were analyzed using the chi-square and Fischer's exact tests.

Case frequencies and the proportions of the histopathologic subtypes and MSI subtypes were measured. A binomial regression (with a log link) analysis to determine the risk factors for MSI CRC was carried out. A log binomial was chosen since the prevalence of MSI positives cases was fairly common, and the use of the binary logistic would yield estimates, that over estimate the relative risk [68]. While running the multivariable models, variables were selected based on biological plausibility, literature review and statistical significance ($P$-value ≤ 0.25 at bi-variable analysis. In the multivariable analysis, a $p$-value < 0.05 was considered statistically significant.

## Results

A total of 127 CRC patients diagnosed with colorectal adenocarcinoma were analyzed to determine the MSI status. Of these cases, the mean (SD) age of the MSI cases was 55.6(16.9) years and of the MSS cases was 55.4(15.5) years. The median (IQR) age for MSI cases was 53(43-68.5) years whilst for MSS was 54 [36, 46–65] years. Left-sided colon tumours were more common at 84(66.1%) whilst right-sided colon tumours constituted 43(33.9%) of all cases. AJCC stage (III + IV) was found in the majority, 109(85.8%) of cases whilst early-stage (I + II) CRC constituted 18(14.2%) cases. AC was the most common histopathological subtype at 114(89.8%) followed by 11(8.66%) invasive mucinous adenocarcinoma and 2(1.57%) SRCC.

### MSI colorectal adenocarcinoma molecular characterization

The majority were MSS, 75(59.06%) followed by MSI, 52(40.9%). MSI gene mutation analysis for MLH-1, MSH-2 and MSH-6 was performed on all the 127 cases. The analysis demonstrated 14/127(11.0%), 30/127(23.6%) and 26/127(20.5%) MLH-1, MSH2 and MSH6 mutations respectively. Furthermore, 4/14 (29%) had MLH1 mutations alone, 14/30 (47%) had MSH6 mutations alone and 18/26 (69%) had MSH2 mutations alone. Of the MSI positive cases, there were: 8/52 (15%) that were a combination of MSH2 + MSH6 mutations, 6/52 (12%) that were a combination of MLH1 + MSH2 mutations and 6/52 (12%) that were a combination of MSH1 + MSH6 mutations. Out of the MSI positive cases 2/52 (3.8%) cases had mutations in MLH1 + MSH2 + MSH6.

**Table 1** MLH-1 and PMS2 antibodies used for IHC analysis

| Primary antibody | Specificity | Dilution | Source | Clone | Reference | Staining time |
|---|---|---|---|---|---|---|
| MLH-1 | Colorectal adenocarcinoma | 1:100 | DAKO Agilent USA | ES05 | IR079 | 3 h |
| PMS2 | Colorectal adenocarcinoma | 1:100 | DAKO Agilent USA | EP51 | IR087 | 3 h |

### MSS and MSI subtypes

MSI positive tumours were found in 52(40.9%) of all CRCs in Ugandans. There was no age difference between MSI cases and MSS ($p = 0.941$). Compared to 46.7% MSS cases which were < 54 years, there were 26(50%) MSI cases which were < 54 years. There was no difference in the two age groups between the MSI and MSS cases ($p = 0.712$). AC was more common in MSS tumours compared to MSI tumours (56.1% versus 43.9%) and this reached statistical significance ($p = 0.039$). The presence of LVI was more common compared to the absence of LVI in MSI positive tumours (42.1% versus 30.8%) however this did not reach statistical significance ($p = 0.431$). Other clinicopathological features in particular topography, stage and grade did not show any significant differences between MSI positive tumours and MSS tumours (Table 2).

Table 3 illustrates a log binomial regression analysis and multivariable analysis for risk factors for MSI CRC. Male patients were 1.36x more likely to have MSI however, this did not reach statistical significance ($p = 0.153$). The risk of SRCC was 1.41x higher in MSI positive cases than MSS cases, however, this did not reach statistical significance ($p = 0.666$). There was also a higher risk of stage II (1.69x), stage III(2.28x) and stage IV(1.35x) having MSI compared to MSS, however, none of these findings were statistically significant (Table 3). The other variables particularly, age, grade and location were not significantly associated with MSI status (Table 3).

### MLH-1 and PMS2 expression defects on immunohistochemistry

Of the 127 participants with CRC, that had MLH-1 immunohistochemistry, 85(66.9%) tumours were MMR protein proficient in MLH1. There were 42(33.1%) tumours that had an MMR protein defect in MLH-1. Among the 42(33.1%) tumours that were MMR MLH-1 deficient, 14(11%) had a mutation in MLH-1 on next generation sequencing, whilst 28(22%) tumours had no MLH-1 mutation on next generation sequencing.

Out of the 85(66.9%) tumours that were MLH-1 protein proficient, 84(66.1%) had no MLH-1 mutation on next generation sequencing, whilst 2(1.6%) tumours had a mutation in MLH-1 on next generation sequencing.

Of the 127 participants with CRC that had MLH-1 and PMS2 immunohistochemistry, 37(29.1%) had at least one MMR protein defect in either MLH1 or PMS2. Among them, 21(16.5%) participants were identified with isolated loss of PMS2 expression; 26(20.5%) had a defect in the expression of both MLH-1 and PMS2. The 21(16.5%) participants with isolated PMS2 MMR defect expression had no pathologic mutations in MLH-1, MSH-2 and MSH-6. Representative images of MLH-1 and PMS2 staining at different magnifications are shown in Figs. 1 and 2.

### KRAS and BRAF mutation analysis

BRAF mutational analysis was performed on all the 127 cases with only 5(3.9%) having pathologic missense BRAF V600 mutations and 1(0.8%) was a benign synonymous variant. Only 1(0.9%) case had a BRAF mutation

**Table 2** Clincopathological characteristics of MSI and MSS CRC subtypes

| Variable | | MSI ($n = 52$) | MSS ($n = 75$) | *p*-value* |
|---|---|---|---|---|
| Age(median) | | 53.0(43-68.5) | 54.0(46–67) | 0.941* |
| | ≥ 54 years | 26(50) | 40(53.3) | 0.712* |
| | < 54 years | 26(50) | 35(46.7) | |
| Sex | Female | 19(32.8) | 39(67.2) | 0.085* |
| | Male | 33(47.8) | 36(52.2) | |
| Topography | Left-sided colon | 34(40.5) | 50(59.5) | 0.881* |
| | Right-sided colon | 18(41.9) | 25(58.1) | |
| Stage | I | 4(22.2) | 14(77.8) | 0.116* |
| | II | 12(36.4) | 21(63.6) | |
| | III | 29(51.8) | 27(48.2) | |
| | IV | 7(35.0) | 13(65.0) | |
| Grade | I | 6(35.3) | 11(64.7) | 0.776* |
| | II | 41(42.7) | 55(57.3) | |
| | III | 5(35.7) | 9(64.3) | |
| Histological subtype | AC | 50(43.9) | 64(56.1) | 0.039** |
| | MAC | 1(9.1) | 10(90.9) | |
| | SRCC | 1(50.0) | 1(50.0) | |
| LVI | Present | 48(42.1) | 66(57.9) | 0.431* |
| | Absent | 4(30.8) | 9(69.2) | |

*$p$-values derived using the Pearson Chi-Square test

** $P$-values derived using the Fisher's Exact Chi-Square test

**Table 3** Log binomial regression analysis for risk factors for MSI CRC

| Variable | category | Crude estimates | | | Adjusted estimates | | |
|---|---|---|---|---|---|---|---|
| | | Risk ratio | 95% CI | *p*-value | Risk ratio | 95% CI | *p*-value |
| Sex | Female | 1 | | | | | |
| | Male | 1.46 | 0.94–2.27 | 0.094 | 1.36 | 0.89–2.07 | 0.153 |
| Age | < 54 years | 1 | | | | | |
| | ≥ 54 years | 0.93 | 0.61–1.42 | 0.727 | | | |
| Location | Left-sided colon | 1 | | | | | |
| | Right-sided colon | 1.03 | 0.67–1.60 | 0.880 | 0.93 | 0.61–1.41 | 0.727 |
| LVI | Negative | 1 | | | | | |
| | Positive | 1.37 | 0.59–3.18 | 0.466 | | | |
| Grade | I | 1 | | | | | |
| | II | 1.21 | 0.61–2.40 | 0.585 | | | |
| | III | 1.01 | 0.39–2.62 | 0.981 | | | |
| Stage | I | 1 | | | | | |
| | II | 1.64 | 0.62–4.34 | 0.322 | 1.69 | 0.65–4.39 | 0.285 |
| | III | 2.33 | 0.95–5.73 | 0.066 | 2.28 | 0.94–5.55 | 0.070 |
| | IV | 1.57 | 0.55–4.50 | 0.397 | 1.35 | 0.46–3.98 | 0.581 |
| Histological subtype | AC | 1 | | | | | |
| | MAC | 0.21 | 0.03–1.36 | 0.101 | 0.23 | 0.03–1.47 | 0.120 |
| | SRCC | 1.14 | 0.28–4.63 | 0.855 | 1.41 | 0.30–6.67 | 0.666 |

in all MSI positive cases. The other 3(2.4%) BRAF mutations were in MSS (MSI negative) patients. The two pathologic variants included Glu269Gly and Gly506Glu. The commonest pathologic BRAF mutation variant was Glu269Gly (Table 4). The BRAF variant which was clinically benign, was a synonymous variant (Gln501Gln) (Table 4).

KRAS mutational analysis was performed on all 127 cases with only 8(6.3%) having pathologic missense KRAS mutations and 1(0.8%) case was a benign synonymous variant. The pathologic variants included Gly13Asp, Gly12Asp, Gly12Ser, Gln61His and Asn116Ser (Table 5). These included two Gly12Asp pathogenic variants, 2 Gly12Ser pathogenic variants and 2 Asn116Ser pathogenic variants. There was one Gln43Gln benign KRAS synonymous variant (Table 5).

## Discussion

This study was the first attempt to analyze the molecular characteristics of colorectal cancer in Uganda and in East Africa. The steady increase in the number of treated colorectal cancer patients and increasing life expectancy in Uganda may pose a challenge in the management of this non-communicable disease in future. Therefore, successful management of colorectal cancer in the Ugandan population requires a better understanding of the tumour biology and genetic biomarkers in this population. Hence determining the MSI status of CRC was crucial for CRC management. Some authors have argued that the advanced-stage presentation in Uganda and generally in Sub-Saharan Africa may be due to the rapid progression of an aggressive CRC type of tumour rather than to a late presentation with CRC [32–35].

The present study found a high incidence of MSI positive tumours (41%) and a low incidence of BRAF mutations (3.9%). The findings are in keeping with studies in West Africa, particularly in Ghana and Nigeria which found a high rate of MSI positive tumours. In Ghana, Raskin L et al., reported a high MSI positive rate of 40% and in Nigeria, Irabor DO et al., reported an MSI positive rate of 43% [33, 34]. Similar to the present study, two MSI positive tumours among five randomly selected CRCs have been found to be MSI positive in a pilot study conducted in Nigeria [36]. The study from Ghana on 90 CRCs, showed similar findings to the present study with a high MSI positive rate and no BRAF mutations [34]. The findings in the present study and those in West Africa are also similar to the findings from African-American studies of MSI in CRC [37–40] (Table 6).

These findings are in contrast to the 10–15% MSI positive CRC tumours in white populations [41]. A study from South Africa showed that only 9.7% of MSI positive cases had a BRAF V600 mutation. This South African study suggested that a large proportion of MSI positive CRC patients possibly develop through a hereditary pathway [42]. This finding is in contrast to that found in the West where BRAF mutations constitute 40–60% of MSI-positive CRC [43]. In Uganda only one patient was BRAF mutated from all the MSI positive CRC patients and therefore the prevalence of sporadic MSI is low in keeping with findings from South Africa.

The present study showed that only one (1.9%) BRAF mutation was found in MSI positive patients in a patient

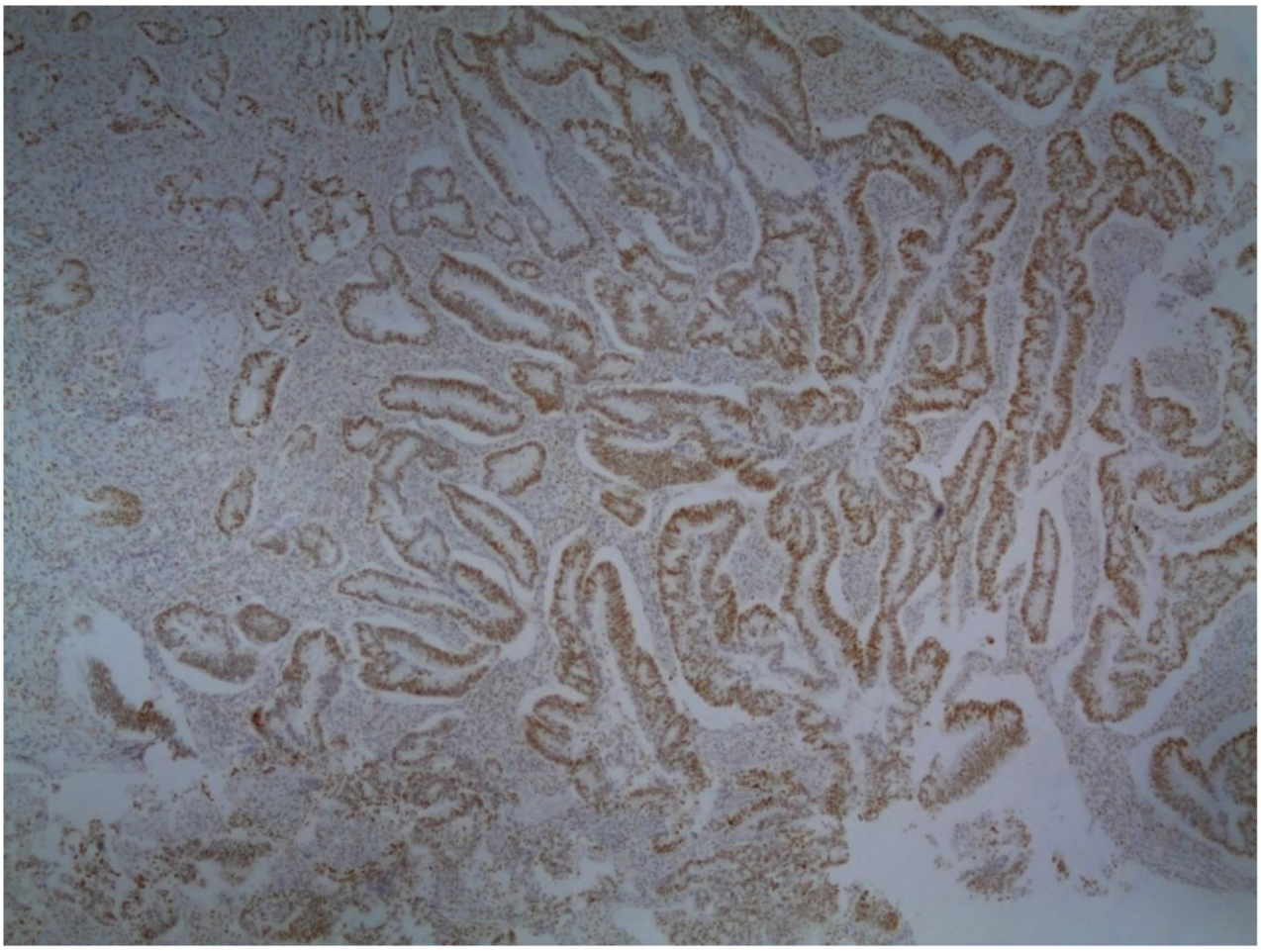

**Fig. 1** MLH1 immunohistochemistry showing the presence of MLH1 staining in the colon tumour epithelium. Those cells that have stained positive nuclei are considered MLH1 proficient whilst those cells that stained negative nuclei are considered MLH1 deficient. Magnification x40

with mutations in MLH1 and MSH6. The other three (2.4%) BRAF mutations were in MSS (MSI negative) tumour patients. In white populations a meta-analysis showed that BRAF-activating mutations have a high prevalence of MSS tumours (5%) and in tumours which have MLH1 promotor hypermethylation there is a high prevalence (63.5%) [44]. In the present study as in West African studies, the high numbers of MSI positive tumours had a low number of BRAF mutations and the majority were left-sided colon tumours [33, 34]. These West African studies and a study from South Africa therefore have suspected that these high numbers of MSI cases could possibly represent Lynch syndrome cases [33, 34, 42].

In the present study nearly all patients that were mutated for MLH1 were dMLH1 deficient on immunohistochemistry. However, there were 21(16.5%) CRC cases that were PMS2 deficient with no mutation in any other MMR gene. In the West, mutations in MLH1 and MSH2 account for 90% of Lynch-syndrome causing mutations [45]. The remaining 10% are caused by MSH6 and PMS2 genes [45]. Studies have shown that isolated PMS2 gene mutations represent 2% of the pathogenic mutations in Lynch syndrome families [46]. PMS2 mutations are associated with an older age, weaker family history for Lynch syndrome and an attenuated phenotype [47]. A limitation in the present study was that PMS2 was not genetically sequenced in 16.5% of dMMR PMS2 cases, however, since PMS2 mutations are only responsible for 2% of Lynch syndrome then it is unlikely to have had a significant affect to the MSI status and proportion in Lynch syndrome in this study.

A small study by Adebamowo CA et al., in Nigeria found two MSI positive tumours for every five randomly selected colorectal cancer patients (40%) [36]. These results are consistent with the MSI positive rate in the present study which is 41%. In the West, MSI positive tumours have been associated with a younger age, female gender and a right-sided colon tumour location. However, the present study in Uganda and other studies

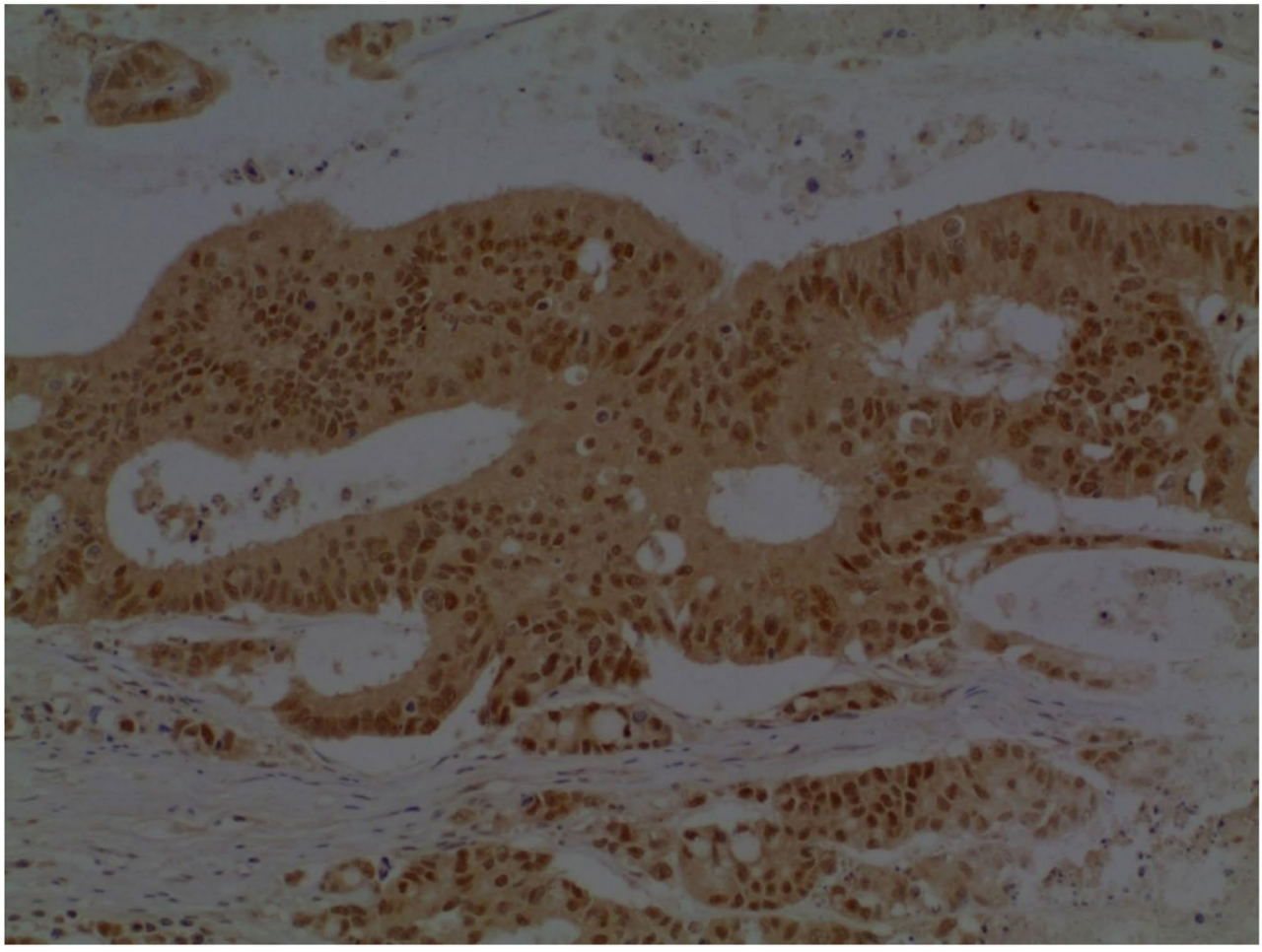

**Fig. 2** PMS2 immunohistochemistry showing the presence of PMS2 staining in the colon tumour epithelium. Those cells that have stained positive nuclei are considered PMS2 proficient whilst those cells that stained negative nuclei are considered PMS2 deficient. Magnification x200

**Table 4** Details of BRAF variants in Ugandan patients with colorectal cancer

| No. | dbSNP.ID | HGVS.c | HGVS.*p* | Clinical significance | No. of cases with mutations |
|---|---|---|---|---|---|
| 1 | - | c.806 A > G | p.Glu269Gly | Pathogenic | 4 |
| 2 | rs121913351 | c.1517G > A | p.Gly506Glu | Pathogenic | 1 |
| 3 | rs759843625 | c.1503 A > G | p.Gln501Gln | Benign | 1 |

**Table 5** Details of KRAS variants in Ugandan patients with colorectal cancer

| No. | dbSNP.ID | HGVS.c | HGVS.*p* | Clinical significance | No. of cases with mutations |
|---|---|---|---|---|---|
| 1 | rs112445441 | c.38G > A | p.Gly13Asp | Pathogenic | 1 |
| 2 | rs121913529 | c.35G > A | p.Gly12Asp | Pathogenic | 2 |
| 3 | rs121913530 | c.34G > A | p.Gly12Ser | Pathogenic | 2 |
| 4 | rs17851045 | c.183 A > C | p.Gln61His | Pathogenic | 1 |
| 5 | - | c.129 A > G | p.Gln43Gln | Benign | 1 |
| 6 | rs202247812 | c.347 A > G | p.Asn116Ser | Pathogenic | 2 |

from West Africa did not find any of these associations in MSI positive CRC patients. In these studies, a high number of young patients with left-sided colon MSI positive tumours which were moderately and poorly differentiated with the presence of LVI were reported. A higher proportion of mucinous adenocarcinoma and signet ring colorectal carcinoma have also been found in the present study in Uganda compared to white populations which may partially explain the high prevalence of MSI positive tumours in Uganda.

**Table 6** Frequency of the MSI status of CRC tumours comparing African populations and African-Americans in different studies

| Ethnicity | Author | MSI positive | MSS |
|---|---|---|---|
| Uganda | Wismayer R et al., 2023 | 52(41%) | 75(59%) |
| Nigeria | Irabor DO et al., 2017 | 15(43%) | 20(57%) |
| Ghana | Raskin L et al., 2013 | 29(41%) | 41(59%) |
| African-Americans | Ashktorab H et al., 2005 | 22(43%) | 29(57%) |
| African-Americans | Ashktorab H et al., 2003 | 10(45%) | 12(55%) |
| African-Americans | Brim H et al., 2012) | 29(31%) | 66(69%) |
| African-Americans | Sylvester BE et al., 2012) | 29(15%) | 170(85%) |

Studies from Asia have shown that mucinous colorectal cancer constitutes 6–25% of cases and is associated with a younger age, MSI positive tumours and a poor prognosis [48]. Whilst a study in Nigeria also found a high frequency of mucinous tumours (27%) and signet ring colorectal carcinoma (40–50%) presenting at a young age which may explain the high prevalence of MSI positive tumours [49].

While MSI-high colorectal cancer is typically associated with better prognosis, its higher prevalence in Uganda compared to Europe and the United States might be explained by delayed access to healthcare, limited screening and other regional factors leading to late-stage diagnosis. In Uganda, access to quality healthcare, especially in rural areas, can be limited due to factors like infrastructure, distance and cost. Therefore, patients present with late-stage CRC, and MSI-high tumours, even with their generally better prognosis, may have already progressed [3, 4]. As MSI-high tumours are generally considered to have a better prognosis, the ability to accurately stage and diagnose these tumours early on is crucial for effective treatment. Hereditary factors like Lynch syndrome may play a role and therefore further research is needed to determine if there are any differences in the prevalence of Lynch syndrome or other genetic predispositions in Uganda compared to other regions.

Our study in Uganda found that the MSH2 mutation was the commonest MSI gene mutation at 34.6% followed by the MSH6 mutation at 26.9% and the MSH2/MSH6 mutations at 11.5%. These findings are consistent with a study from South Africa, by Cronje et al., which found that a high number of black patients having MSH2 loss of MMR protein expression in comparison with white CRC patients suggesting a possible heritable aetiology [50]. A recent study from South Africa also confirmed a higher frequency of dMSH2, particularly dMSH2/MSH6 loss of expression which is associated with young black South African CRC patients respectively [42]. The same study found that black patients more commonly had dMSH2/6 loss of expression whilst other ethnic groups had more commonly dMLH1/PMS2 loss of expression [42]. These findings are confirmed by previous MSI studies in South Africa which have shown that MLH1 mutations are mainly responsible for sporadic and hereditary CRC in coloured and white populations [51–53].

Studies have shown that PMS2 mutations are associated with a lower risk for Lynch-syndrome related cancer compared to other MSI gene mutations and are only responsible for 1–6% of all Lynch syndrome mutations. In Uganda, this study showed that 16.5% of participants had an isolated defect in the expression of the PMS2 MMR. However, since sequencing of the PMS2 gene was not carried out, it is expected that the PMS2 gene is pathogenically mutated in an even lower number of participants.

Many of our CRC patients in Uganda presented at a young age but at an advanced stage which is associated with a poor prognosis. Studies in the West have shown that the prevalence of dMMR in patients with CRC is higher in early stages than in advanced disease [69, 70]. Therefore, we do not believe that the difference in MSI rate between Uganda and the West is due to differences in clinical stage of the CRC patients at the time of presentation.

Several studies indicate that dMMR/MSI-H prognostic value may differ by clinical stage of disease [Zhao F et al., 2019]. MSI-H or dMMR is associated with a better prognosis and plays a negative predictive role for adjuvant fluorouracil-based chemotherapy in patients with early stage resected CRC. On the contrary, dMMR/MSI-H is associated with a poorer outcome in patients with advanced disease and an increased response to immune checkpoint inhibitors [71]. The response to 5-fluorouracil and immunotherapy needs to be investigated in future studies in Ugandan CRC patients.

In the present study KRAS mutations were all found in left-sided colon tumours. This is in contrast to the findings of Buchanan DD et al., and Samara M et al., which showed a higher frequency of BRAF and KRAS mutations in right-sided colon tumours [54, 55].

Low KRAS mutation rates below 30% have been observed in African studies. In Nigeria, Tunisia and Morocco KRAS gene mutation rates of 21%, 23.9% and 15.4% have been reported [56–58]. In Uganda the rate of KRAS mutations in our CRC patient population in the present study has been reported to be 6.3%. This is in contrast to the findings in the West from Buchannan DD et al., where KRAS mutations have been found to comprise 35–40% of all colorectal carcinomas [54]. Compared to other regions of the world, other countries in Sub-Saharan Africa have reported lower KRAS mutation rates and the reasons are not fully understood [72]. This low prevalence of KRAS mutations, may be due to differences linked to environmental factors, dietary factors, and genetic backgrounds, all contributing to the lower incidence of CRC in the region.

In the West BRAF V600 mutations represent 7–10% of CRC and define a subtype with a poor prognosis [55, 59]. BRAF mutations constitute 40–60% of MSI positive CRC in the West [43]. However, in Uganda only one patient was BRAF mutated in an MSI positive patient and therefore the prevalence of sporadic MSI is low. Colorectal medullary carcinomas may be diagnosed based on their unique characteristics of harbouring dMLH1 expression and the BRAFV600E mutation [73]. Considering the difficulties in distinguishing between medullary carcinoma with poorly differentiated adenocarcinoma and neuroendocrine tumours, universal screening for both the BRAFV600E mutation and dMLH1 biomarkers may be useful in diagnosing medullary carcinoma and determining the course of treatment for the CRC patient.

The results from this study have shown important CRC tumour peculiarities in Ugandan patients particularly with the high rate of MSI positive tumours. The high rate of MSI in Ugandan colorectal tumours is mainly associated with a high frequency of MSH2 and MSH6 MMR gene mutations and a lack of BRAF V600 mutations. Whereas identification of the causative mutation is recommended in CRC patients, MSI testing, and immunohistochemistry is more cost effective than genetic sequencing especially in a resource limited setting. MSI testing and immunohistochemistry may therefore be offered to CRC patients who meet at least one of the Bethesda criteria in Uganda.

These findings also have direct clinical importance in the treatment of patients as MSI positive tumours have a low response to adjuvant 5-fluorouracil-based chemotherapy which is a main adjuvant chemotherapeutic agent in CRC [60–63]. A differential response to 5-FU based chemotherapy has been found with MSI but also with CpG island methylator phenotype (CIMP) positive colorectal cancer with no increase in survival reported [64]. A differential response to the anti-EGFR therapies (egs: cetuximab) on the basis of BRAF and KRAS mutations is also known [65–67].

## Conclusions

This study in Uganda revealed a high prevalence of MSI positive patients with a lack of BRAF V600E mutations and a higher frequency of MSH2/MSH6 mutations than the Western world. Immunohistochemistry and MSI testing is more cost effective at identifying the MSI gene mutation than genetic sequencing in a resource-limited setting. In Ugandan CRC patients who meet at least one of the Bethesda criteria, MSI testing and immunohistochemistry may therefore be offered to obtain the MSI status of the tumour. Our study showed important tumour biology peculiarities in CRC patients from Uganda. This knowledge may have important clinical implications for all East African colorectal cancer patients. This research study went on to investigate the molecular subtypes of CRC and determine the prevalence of Lynch syndrome in Ugandan patients.

### Limitations

The entire population may not be represented with hospital-based studies. However, there was a high level of confidence about the generalizability of the findings in this study, as there was a fair representation of study participants from all four major regions of the country. Participants were excluded when there was missing data in the hospital clinical case files especially regarding whether they have had neoadjuvant chemotherapy or radiotherapy prior to their operative procedure for colorectal cancer. There were a few of these cases which did not adversely affect the study. Since there were a significant number of FFPE tissue blocks which were excluded due to poor quality or inadequate quantity of DNA then high standard laboratory testing which included those samples with a phred score of $\geq 20$, were included on the remaining samples to mitigate this limitation. Although the fixation time was controlled for 24 h in 10% formalin for biopsies and 72 h in 10% formalin for resected colorectal specimens there was no control over the quality of fixation.

Left-sided tumours involved rectal tumours however, they have a distinct biological behaviour from right-sided and left-sided colon tumours. Compared to right- and left-sided colon tumours, rectal tumours have higher rates of T0P01 expression and Her2/neu amplification. Stage II and III rectal tumours receive neoadjuvant radiotherapy whilst similar stage colon tumours do not receive this modality of treatment. Therefore, colon tumours should be considered a different disease entity from rectal tumours.

Antigen degradation of archival material was another limitation. In order to overcome this influence in the prospective arm of the study a high standard of laboratory testing was followed together with maintenance of a short period of specimen storage. Although labour intensive, whole tissue sections were used instead of tissue microarray for MLH1 and PMS2 immunohistochemical analysis. However, this laboratory technique prevented false-negative rates by avoiding tumour heterogeneity. The DNA samples analyzed passed quality control with a phred score of 20. The majority of samples passed this score. In the present study the PMS2 gene was not genetically sequenced in 16.5% of dMMR PMS2 cases. However, since PMS2 mutations are only responsible for 2% of Lynch syndrome then it is unlikely that the proportions of MSI positive cases and Lynch syndrome would be significantly higher in this study. These limitations had no significant effect on the results.

There were 28(22%) tumours that were MLH-1 MMR deficient however they were found to have no MLH-1 mutation on next-generation sequencing. We did not perform MSI array testing, on these MLH-1 MMR deficient tumours, hence there may possibly have been more MLH-1 MMR deficient tumours that were MSI positive, which may result in a slightly higher MSI status for colorectal tumours in Uganda.

**Abbreviations**

| | |
|---|---|
| AC | Classical adenocarcinoma |
| APC | Adenomatous Polyposis Coli |
| AJCC | American Joint Committee on Cancer |
| CRC | Colorectal cancer |
| CIN | Chromosomal instability |
| CSS | Cancer specific survival |
| DNA | Deoxyribonucleic acid |
| FAP | Familial Adenomatous Polyposis |
| FFPE | Formalin fixed paraffin embedded |
| HNPCC | Hereditary Non-Polyposis Colorectal Cancer |
| HD-REC | Higher Degrees Research and Ethics Committee |
| IHC | Immunohistochemistry |
| LS | Lynch syndrome |
| LVI | Lymphovascular invasion |
| MAC | Mucinous adenocarcinoma |
| MSI | Microsatellite Instability |
| MMR | Mismatch repair |
| MSS | Microsatellite stability |
| PCR | Polymerase chain reaction |
| SRCC | Signet ring colorectal cancer |
| TNM | Tumor Node Metastasis |

## Supplementary Information

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Supplementary Material 1

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**Author contributions**

RW conceived the concept and proposal, collected data, performed data analysis and wrote the paper. RM extracted DNA from colorectal cancer tissue samples. CW designed and performed mutation analysis and DNA sequencing. FEK and ST carried out bioinformatics analysis of the variant data. MO and HW interpreted all the immunhistochemical slides. JK performed data analysis and provided statistical support. HW, MO and IT carried out critical reviews of the manuscript for intellectual content. All authors approved the final manuscript.

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**Data availability**

The data supporting the findings of this study are available within the paper and its supplementary information.

## Declarations

**Ethical approval and consent to participate**

This study was part of the PhD work, which was approved by the Doctoral Committee and Higher Degrees Research and Ethics Committee of the School of Biomedical Sciences, College of Health Sciences, Makerere University for the corresponding author (SBS-HDREC-630). Final approval of this research study was obtained from the Uganda National Council for Science and Technology (HS-2574). Written informed consent was obtained from prospective participants included in the study before completing the Data Extraction Form. All the data and specimens pertaining to the research were kept confidential. For the retrospective arm of the study, data were abstracted from the case files in the respective hospitals. Therefore, a waiver of consent was obtained from the Higher Degrees Research and Ethics Committee, School of Biomedical Sciences, College of Health Sciences, Makerere University, to access the data and perform the experiments on the tissue block samples. The patient data, which were accessed from the medical case files in the respective hospitals, were anonymized and maintained with confidentiality. The conduct of the study was in accordance with the Declaration of Helsinki.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no competing interests.

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