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Molecular profiling of colorectal cancer within South Africa

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Molecular Profiling of Colorectal Cancer within South Africa

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, 2022

Declaration:

I, Michelle McCabe, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other institution.



.....

(31 December 2022)

Publications:

The results of this study have been published in the following journal articles:

1. M. McCabe, Y. Perner, R. Magobo, S. Mirza and C. Penny. Descriptive Epidemiological study of South African Colorectal Cancer patients at a Johannesburg Hospital Academic institution. JGH Open 2019 4(3):360-367 (Chapter 2)

2. M. McCabe, P. Perner, R. Magobo, P. Magangane, S. Mirza and C. Penny. Microsatellite Instability assessment in Black South African Colorectal Cancer patients reveal an increased incidence of suspected Lynch syndrome. Scientific Reports 2019 9(1):15019 (Chapter 3)

3. M. McCabe, C. Penny, P. Magangane, S. Mirza and Y. Perner. Left-sided Colorectal Cancer distinct in Indigenous African patients compared to Other Ethnic Groups in South Africa. BMC Cancer 2022 22:1089 (Chapter 4)

Abstract:

There is a global requirement to characterize colorectal cancer (CRC) by molecular subtyping within subpopulations for the assignment of relevant therapies to improve prediction outcomes. Previous CRC studies conducted within South Africa (SA) have mainly been epidemiological, and subtyping limited to immunohistochemistry (IHC) protein expression analysis. The conclusions from these reports provided limited insight and lacked supportive molecular investigations in the development of CRC specifically within our population.

CRC develops through 3 main molecular pathways; i.e. (1) Chromosomal instability (CIN) (75-85%), (2) Microsatellite Instability (MSI) (~15%), and (3) CpG Island Methylator Phenotype (CIMP) (15-20%) pathway. This study descriptively analyses histopathological and molecular information of CRC patients in a 5-year study cohort (2011-2015), by assessing MSI status to ascertain unique features associated with different population groups, particularly within the African population. This study showed a large proportion (37%) of African CRC patients present with early disease onset (<50 years) in comparison to other ethnic group (OEG) patients (15%). Molecular characterization of CRC revealed MSI CRC within African patients occurred at an increased frequency compared to other ethnic groups (15% vs 8%), lacked a BRAFV600E mutation, and the dominant deficient (d) mismatch repair (MMR) profile was dMSH2 and dMSH6, suggesting hereditary Lynch Syndrome (LS) as the dominant pathway of disease development. These results are unique, as international findings demonstrate, 75% of MSI-CRC are sporadic, associated with dMLH1 and BRAFV00E mutations. OEG SA patients however were mostly associated with MLH1 and BRAFV600E mutations, and therefore follow the more well-established sporadic MSI pathway. Further insight gained through universal MSI screening was the ability to differentiate MSI-H versus MSS and MSI-L CRC. MSS/MSI-L CRC categorized by tumour site (left versus right) and ethnicity revealed unique histopathological features associated with left-sided CRC (LCC) in African patients compared to OEG patients. In addition,

a higher proportion of MSI-L LCC was seen in African patients associated with more advanced disease stage and unique molecular and histopathological features. These findings suggest MSI CRC found in African patients is predominantly of a hereditary form, and further variant screening analysis to determine causative germline pathogenic variants are required. MSS CRC particularly within the left colon, was associated with unique histopathological features in the African population group, suggesting an alternative carcinogenic pathway of development when compared to OEG patients. MSI-L CRC also illustrated unique features at this site in African patients, and suggest this to be a completely separate molecular subtype. Deeper molecular characterization by next generation sequencing, including somatic and germline cancer gene screening is required to provide more insight into the different molecular subtypes and pathways in the development of CRC within the SA. This research will therefore direct better clinical management strategies, improving diagnosis, genetic counselling and testing strategies, prognosis, treatment outcomes, survival, and the overall burden associated with the disease within SA.

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List of Abbreviations:

The abbreviations used in this thesis describe the following unless otherwise indicated.

5-FU	5-fluorouracil
AA	African American
AGO2	Argonaute 2
AIDS	Acquired immunodeficiency syndrome
AJCC	American Joint Committee on Cancer
Ala	Alanine
APC	Adenomatous polyposis coli
BP	Base pair
CD117	Tyrosine-protein kinase KIT
CI	Confidence interval
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
CIR	Crohn's-like inflammatory reaction
CLR	Crohn's-like inflammatory response
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CMS	Consensus Molecular Subtypes
CRC	Colorectal cancer
dMMR	Defective mismatch repair
DNA	Deoxyribonucleic Acid
EMAST	Elevated microsatellite alterations at selected tetranucleotide repeats
EPCAM	Epithelial Cell Adhesion Molecule
FAM	Fluorescein amidites
FDR	False Discovery Rate
FFPE	Formalin fixed paraffin embedded
GSK3b	Glycogen synthase kinase-3 beta
H&E	Haematoxylin and Eosin
HDI	Human development indices
HEX	Hexachord-fluorescein
HG	High grade
HIV	Human immunodeficiency virus
hMLH1	Human MutL homologue-1

hMSH2	Human MutS homologue-2
hMSH3	Human MutS homologue-3
hMSH6	Human MutS homologue-6
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HP	Hyperplastic polyp
HREC	Human Research Ethics Committee
IA	Indigenous African
IHC	Immunohistochemistry
KRAS	Kirsten Rat sarcoma
LCC	Left colon cancer
LG	Low grade
LIS	Laboratory information system
LLS	Lynch-Like Syndrome
LncRNA	Long non-coding RNA
LOF	Loss of function
LOH	Loss of heterozygosity
LS	Lynch Syndrome (LS)
miRNA	Micro-RNA
MMR	Mismatch repair
MSI	Microsatellite Instability
MSI-H	High level of microsatellite instability
MSI-L	Low level of Microsatellite Instability
MSS	Microsatellite stable
MYC	Myelocytomatosis
NCR	National Cancer Registry
NHLS	National Health Laboratory Service
OEG	Other ethnic group
ORG	Other Race group
PCR	Polymerase Chain Reaction
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
pMMR	Proficient mismatch repair
PMS2	Postmeitotic segregation-2
Polε	DNA polymerase epsilon
pri-miRNA	Primary miRNA

RCC	Right colon cancer
RISC	RNA induced silencing complex
RNA	Ribonucleic Acid
SA	South Africa
SCF	Stem cell factor
SCNA	Somatic copy number alteration
SFRP2	Secreted frizzled-related protein 2
SMAD4	Mothers against decapentaplegic homolog 4
SRCC	Signet ring cell carcinoma
SSA	Sessile serrated adenoma
TA	Tubular adenoma
TACST1	Tumour-associated calcium signal transducer 1
TCGA	The Cancer Genome Atlas
TGF-β	Transforming growth factor beta
Thr	Threonine
TIL	Tumour infiltrating lymphocytes
TNM	Tumour node metastases
TP53	Tumour protein 53
TVA	Tubulovillous adenoma
UTRs	Untranslated regions
WNT	Wingless-related integration site

Chapter 1: Introduction

1.1. Global burden of CRC

The burden of colorectal cancer (CRC) remains high globally, ranking 3rd among the most common cancer diagnosed (lung and breast cancer), with an incidence of 1.9 million cases. CRC is the 2nd leading cause of cancer-related mortality (after lung), 935 000 deaths were reported by the WHO in 2020.[1] (Figures 1 and 2) It is also the 2nd most prevalent cancer globally after lung cancer with 5,2 million people living with CRC within 5 years of diagnosis.[1]

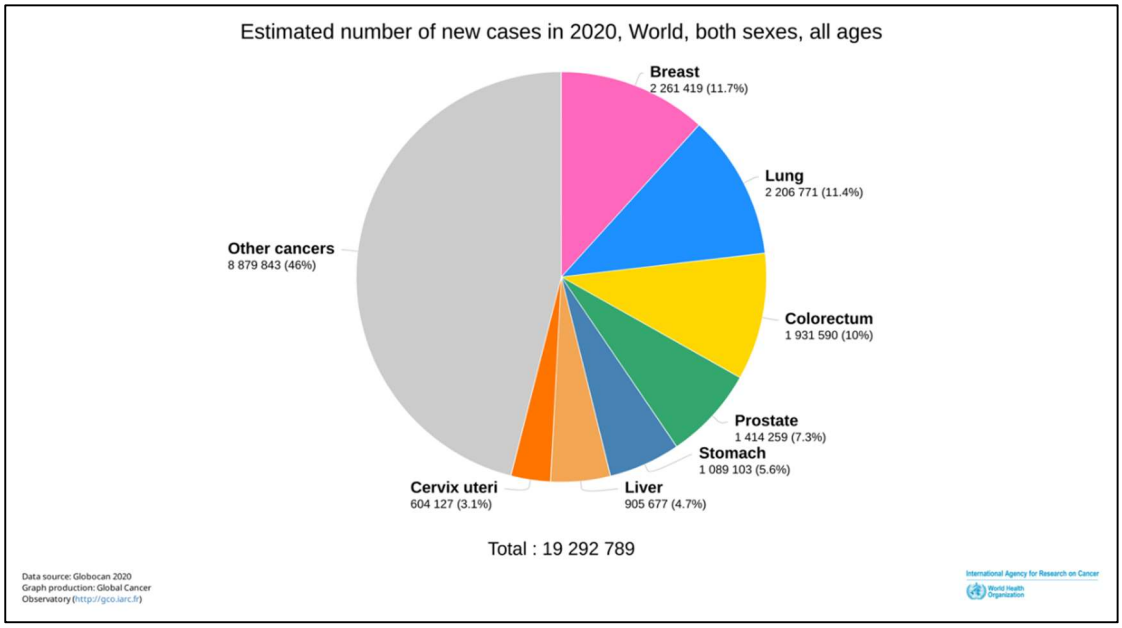


Figure 1: Estimated incidence rate of CRC diagnosed globally. Figure adapted from WHO 2020.[2]

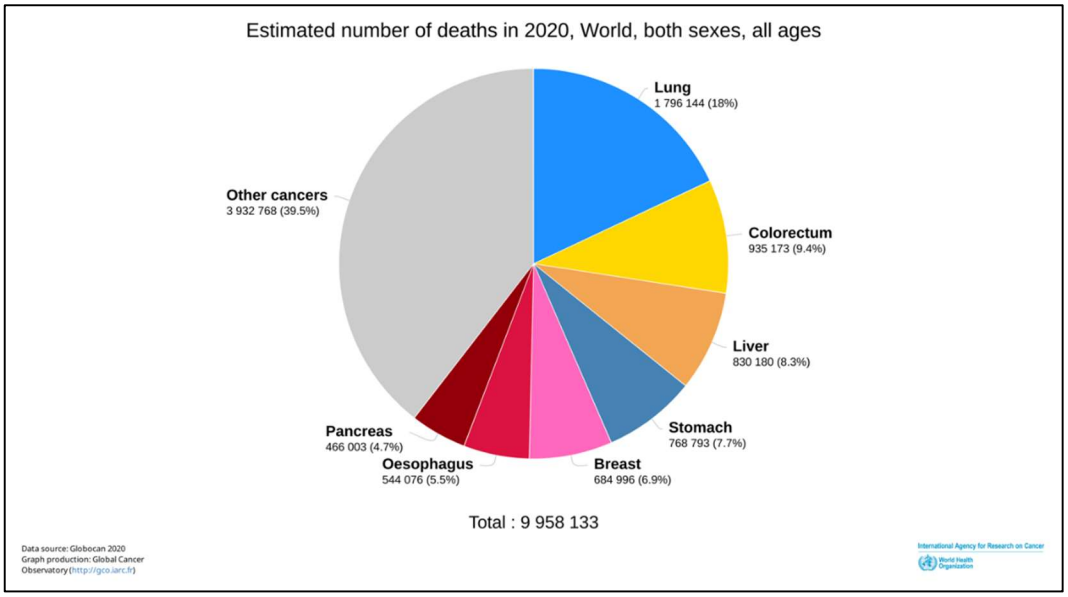


Figure 2: Estimated mortality rate of CRC diagnosed globally. Figure adapted from WHO 2020.[2]

A 10-fold variation in incidence rates exists across the world with very similar patterns in men and women (55% versus 45% respectively) (Figure 3).[1,3–5] The highest rates are reported within the higher human development indices (HDI) or high income countries (i.e. Europe, Australia and New Zealand), whereas the lowest rates were found in the low and medium HDI or low–middle income countries (i.e. South America and Western Asia). [5,6] Even though lower incidence rates are observed in low and medium HDI countries, higher mortality rates of approximately 50-70% are reported reflecting poorer prognosis in these regions.[1,3,5]

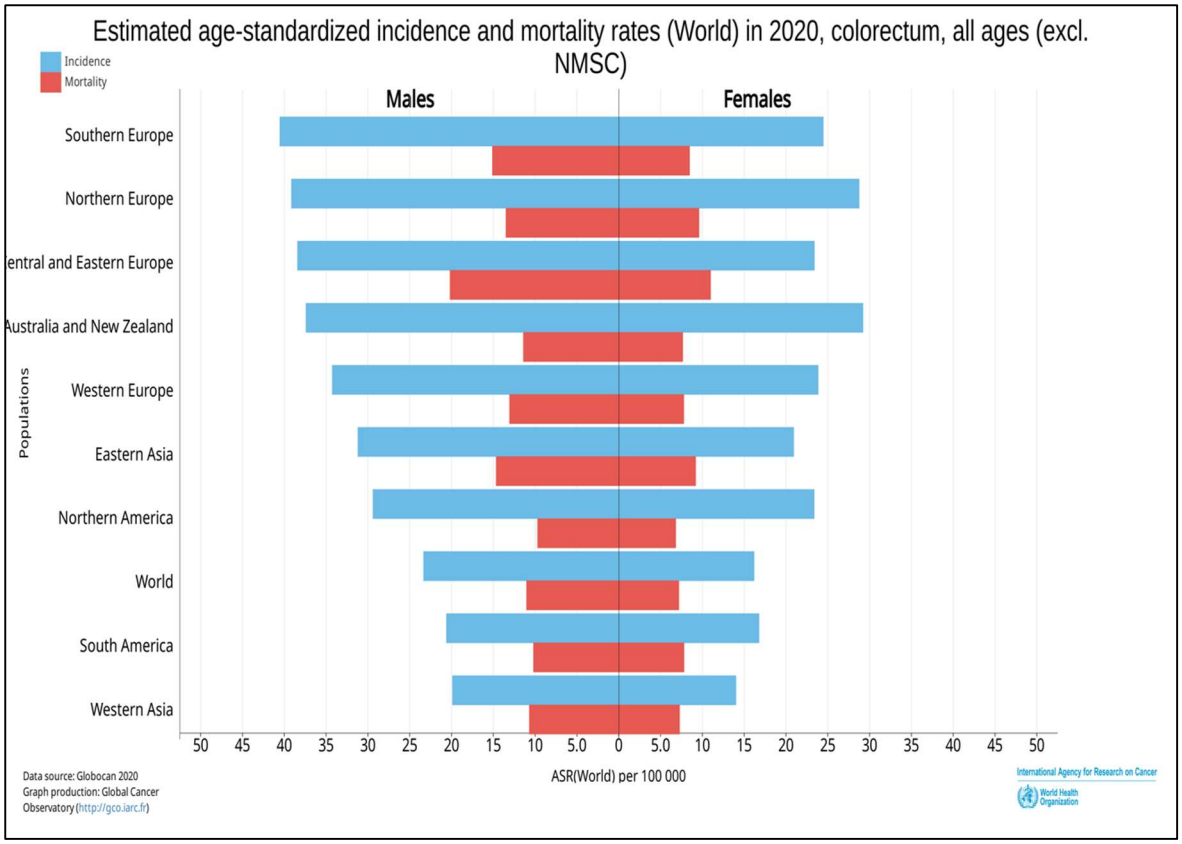


Figure 3: Estimated ASR of incidence and mortality rates of CRC, all ages, worldwide. Figure adapted from WHO 2020.[2]

Incidence rates have however shown an increase in historically low risk countries and a decrease in high risk countries.[3,5,7,8] The shift in epidemiology from low to increased incidence is believed to be linked to changes in dietary and lifestyle factors due to urbanization, such as a more sedentary lifestyle, obesity and smoking.[9] In high risk areas such as the United States of America (USA), a decrease in incidence is believed to be the result of more widespread CRC screening for the early detection and removal of precancerous lesions. [7,10,11]

1.2. CRC in South Africa:

In Sub-Saharan Africa, South Africa (SA) was found to have the highest incidences of CRC in comparison to the other 19 countries studied in the WHO 2018 report.[9,12] Of the most frequently diagnosed cancers, CRC ranked 5th (Figure 4), 3rd among males (after prostate and lung) and 3rd among females (after breast and cervix).[1] CRC was also identified as the 5th most common cause of death after frequently diagnosed cancers, i.e. lung, cervix, breast and prostate (Figure 5).[1]

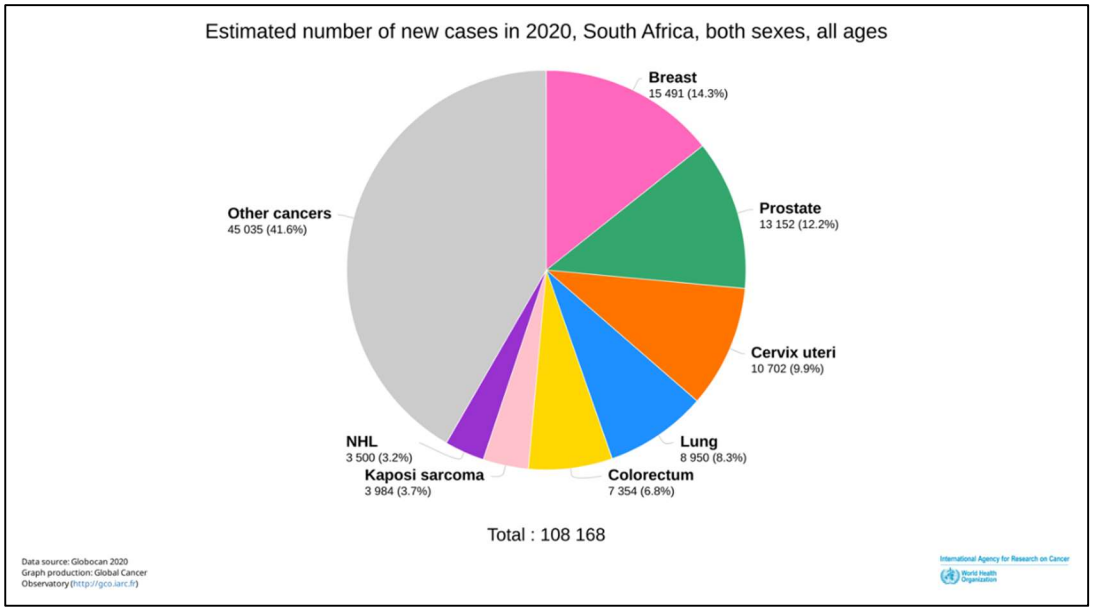


Figure 4: Estimated incidence of CRC diagnosed in South Africa. Figure adapted from WHO 2020[2]

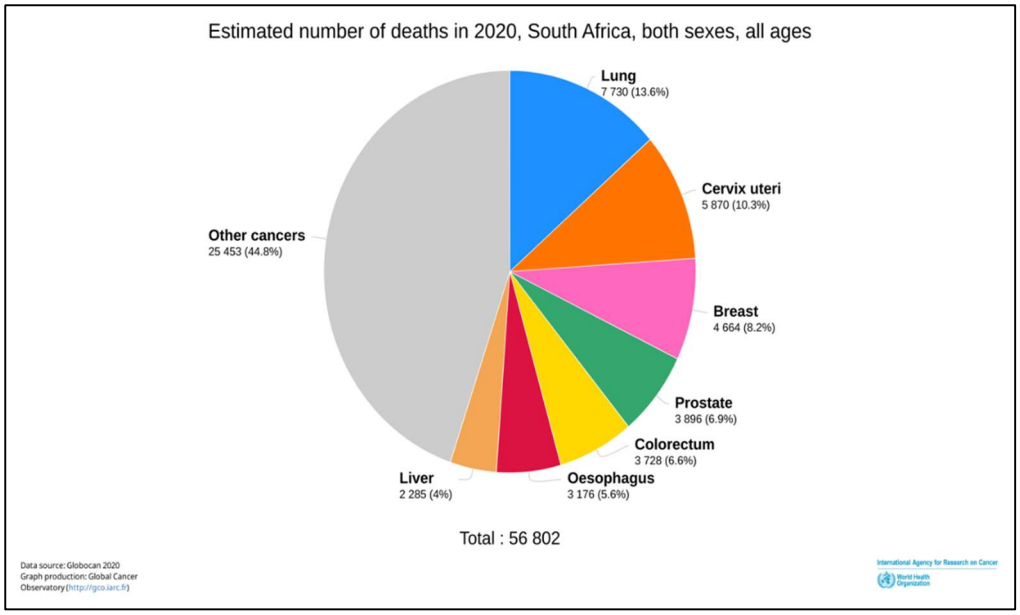


Figure 5: Estimated mortality rate of CRC in South Africa. Figure adapted from WHO 2020 [2]

The SA national cancer registry (NCR) largely provides SA's cancer incidence rates, based on annual surveillance reports from histology, cytology and bone marrow aspirate samples analysed from newly diagnosed cancer patients nationally. NCR 2000-2016 reports indicated a steep increase in incidence of CRC over recent years.[13] (Figure 6)

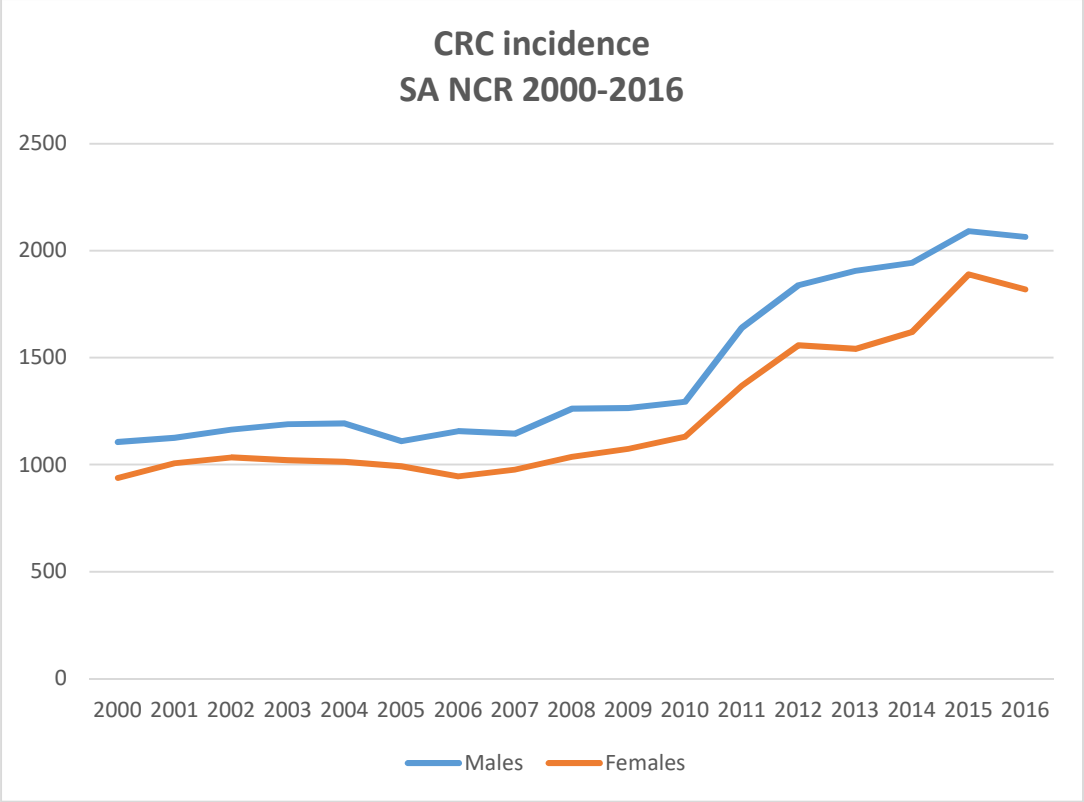


Figure 6: Incidence of SA CRC among males and females. Figure adapted from NCR report 2016.[14,15]

There are four main population groups within SA; the dominant group diagnosed with CRC is the white (Caucasian) population group with a frequency of 48-54%. The second is the black (indigenous African) ethnic group with a proportion of 27-31%, followed by the coloured population (mixed ancestry) (13-15%) and the lowest percentages occurring in the Asian (Indian) population group (5-7%). [13–18]

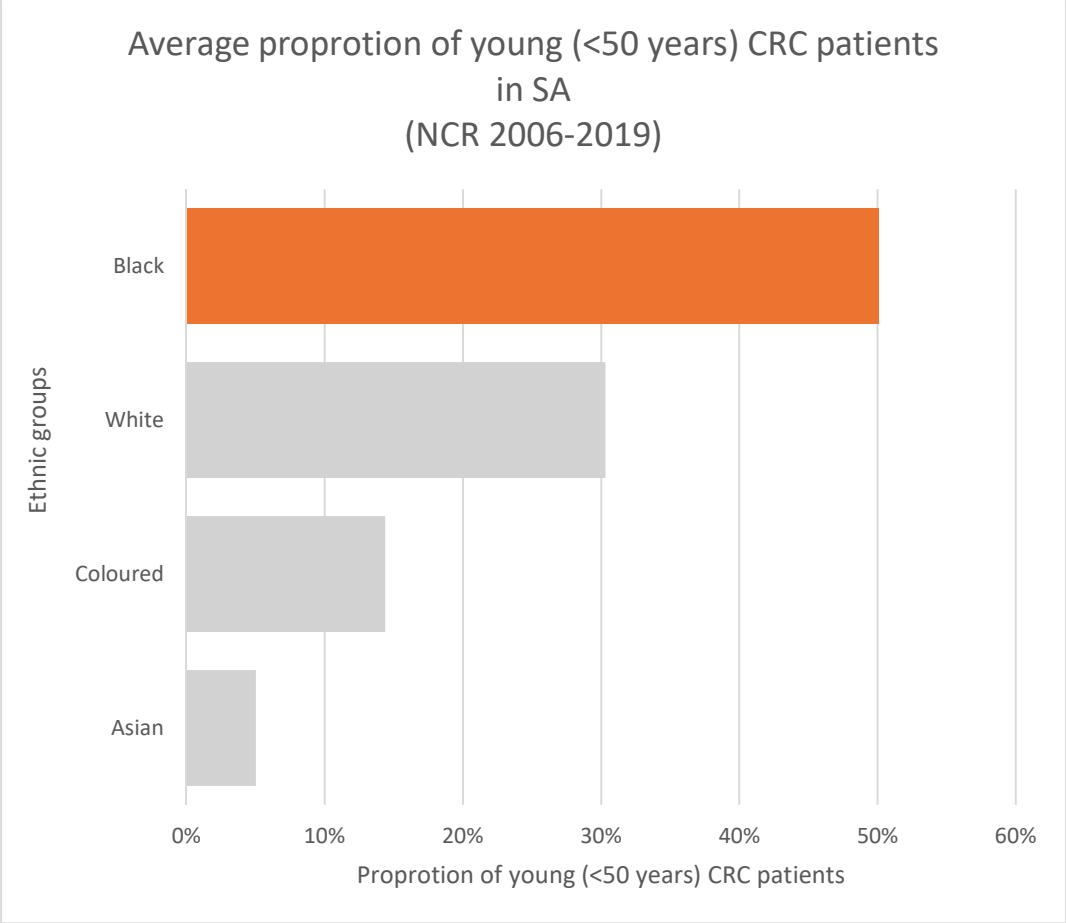


Figure 7: Young CRC patients showing population group proportions in SA. Data from NCR 2006-2019.[13,14,24–27,16–23]

Of great concern is the considerably high frequency of young CRC patients (20%) in SA, particularly within the black population group, contributing on average half of all young CRC cases in SA (Figure 7). [13,15,28] This high incidence of young onset CRC particularly in the young black population raises the concern of a possible hereditary contribution. A previous SA CRC study demonstrated a possible familial cancer trend, as approximately 50% of patients had a relative previously diagnosed with cancer, and CRC shown to be the most common.[29]

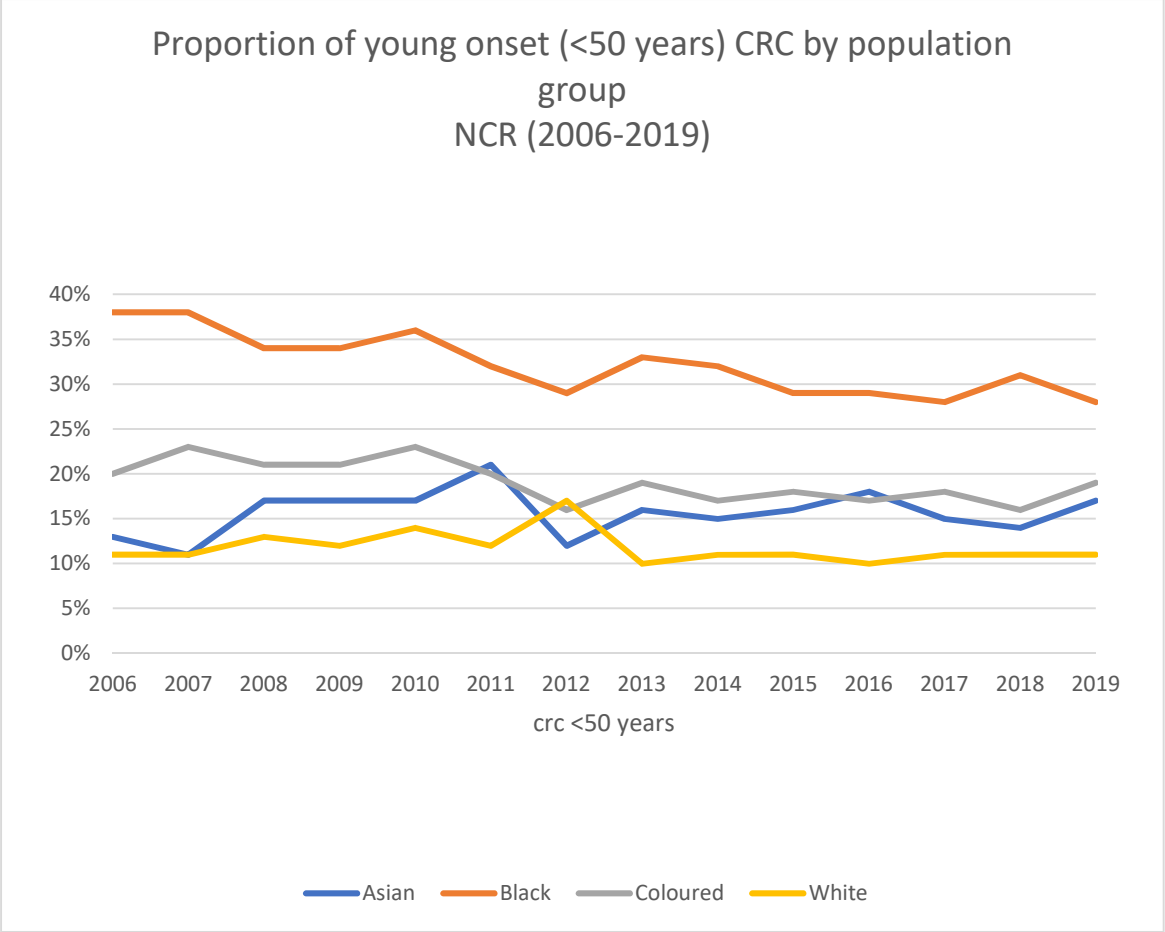


Figure 8: Proportions of young CRC patients per population group in SA. Data from NCR 2006-2019.[13,14,23,25–27,15–22]

The trend of young onset (<50 years) CRC within the African population group illustrates a downward trend from 40% to 30% according to NCR 2006-2019 data (Figure 8). The incidence of CRC has increased; this suggests a shifting epidemiology with later age onset (50-65 years) within the black population group. SSA studies consider the shift from rare to rising incidence due to western diet and lifestyle adoption, however no definite aetiological factors have been identified and associated with this trend to date.[9]

1.3. CRC Pathogenesis:

CRC is a heterogeneous and multifactorial disease, with the majority (50-60%) of cases due to sporadic changes, such as epigenetic alterations in critical genes, due to unhealthy diet and lifestyle choices.[30] The remainder are a result of familial cancer (20-30%) or genetic (5-10%) predispositions.[30,31] Other studies have also shown bacterial and viral aetiological factors, such as gut microbiota cross-talk in the tumour microenvironment playing a role in the development of CRC. [32–35] These factors lead to a specific sequence of genetic or epigenetic events, transforming healthy epithelial cells to a preneoplastic form and progressing towards more malignant stages as theorized by Fearon and Vogelstein.[36,37] CRC has been shown to arise mainly through 3 different molecular pathways. The majority of CRC (70-80%) develop through the chromosomal instability (CIN) pathway, while the remainder to a much lesser degree follow the microsatellite instability (MSI) (~15%) and CpG island methylator phenotype (CIMP) (15-20%) pathways. [38–42] In addition, regulatory molecules such as non-coding RNAs i.e. microRNA (miRNA), circular (circRNA), long non-coding RNA (lncRNA), packaging RNA (pRNA) and transfer RNA (tRNA), have over recent years illustrated its crucial role in mechanisms of tumorigenesis.[43,44] miRNAs are of particular research interest as they mainly function as inhibitory molecules blocking targeted RNA transcripts from being translated into proteins through transcript degradation.[45]

1.3.1. CRC Molecular Pathways:

In 2013, CRC classification by The Cancer Genome Atlas (TCGA) was based on two major molecular subgroups based on genetic instability; (1) MSI accounting for ~16% of cases and the CIN group ~84% of cases. [46] MSI cancers were further characterized into hypermutated (13%) and ultramutated (3%) cancers, with hypermutated cancers occurring as a result of a defective mismatch repair (dMMR) system (~13%) and ultramutated cancer due to pathogenic variants

in the DNA polymerase epsilon (*Polε*) gene. [47] CIN cancer was characterized with aneuploidy and high copy number variation (CNV), however with a microsatellite stable (MSS) genome. Frequently affected genes in this pathway include oncogene Kirsten Rat sarcoma (*KRAS*), and tumour suppressors, Adenomatous polyposis coli (*APC*), Tumour protein 53 (TP53), Mothers against decapentaplegic homolog 4 (*SMAD4*) and Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha (*PIK3CA*).[41,47,48] However in 2015, the Consensus Molecular Subtypes (CMS) Consortium categorized the majority of CRC into four main CMS groups based on meta-analysis CRC expression profiling data. [48] Nearly all hypermutated MSI cancers with prominent immune activity grouped into CMS1 (~14 %), the remainder MSS/CIN cancer subcategorised into CMS2 which follow the canonical *WNT* and *MYC* signalling epithelial pathways constituting the majority (37 %), CMS3 which represent epithelial tumours but with considerable metabolic dysregulation (13%), CMS4 showcase mesenchymal tumours with increased Transforming growth factor beta (TGF-β) signalling (23%), and the residual unclassified group defined by tumours with a heterogeneous phenotype with a combination of features (13%) .[41,48]

1.3.1.1 Chromosomal instability (CIN) pathway

Most CRC develop via the CIN pathway (70-80%), portrayed by gross chromosomal abnormalities (loss of heterozygosity (LOH), insertions, deletions, etc.) A sequence of key molecular events has been well described within this pathway and associated with various stages of tumour development in CRC.[49] Key driver genes include the tumour suppressor genes *APC* and *TP53,* and oncogene *KRAS*, that when altered dysregulate critical molecular signalling pathways leading to cellular transformation [41,50] (Figure 9). The 1st image in Figure 9 illustrates normal mucosa. The first genetic change often detected when cells transition to the next stage of aberrant crypt focus, is due to a loss of function (LOF) mutation in the *APC* gene. This leads

to genomic instability. Additional alterations in the oncogene *KRAS*, allow the cell to proliferate and progress to an early adenoma stage, progressively increasing in size to a late adenoma stage. The final stage of progression includes advanced or high grade (HG) adenoma transitioning to malignant carcinoma and are usually associated with *TP53.* A subset of advanced adenomas may progress due to mutations in *PIK3CA* and chromosome18 LOH.

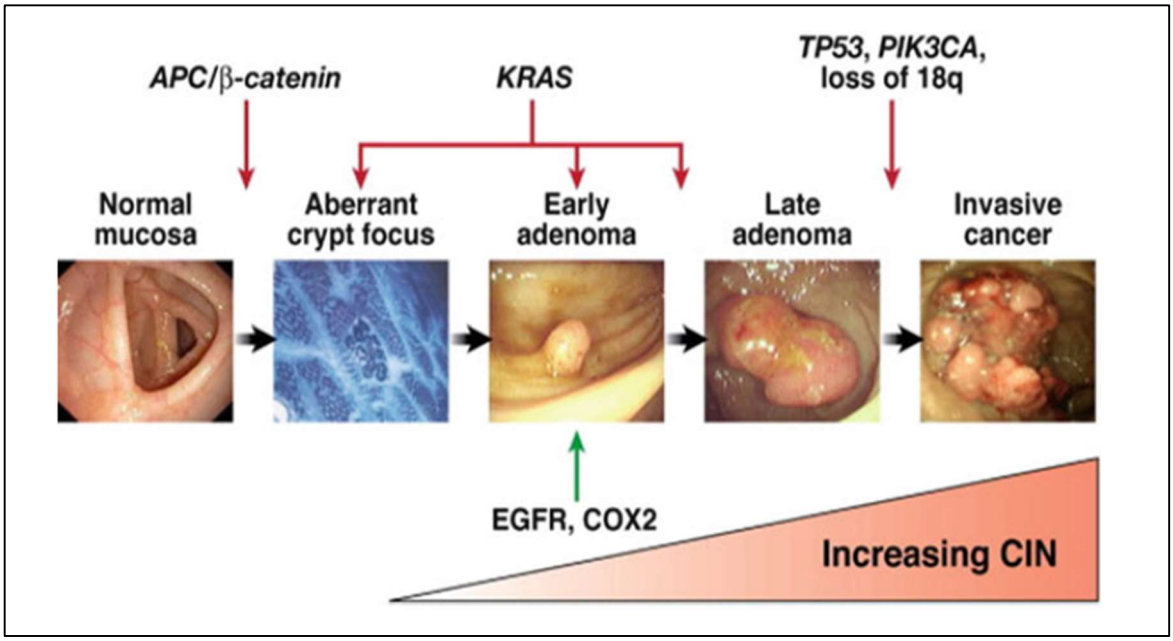


Figure 9: Photographic images of the adenoma to carcinoma tumour progression model. Figure adapted from Pino MS, et al (2010)[51]

Patients with CIN type tumours most often have poor prognostic outcome and possess distinct clinical and pathological features in comparison to those with MSI tumours. Determining the molecular and phenotypical features of CRC is essential in ascertaining the appropriate and successful therapeutical regime for successful treatment outcomes. [52]

1.3.1.1.2 APC (WNT/β-catenin) signalling pathway

CIN is mainly linked to the canonical WNT signalling pathway, due to pathogenic variants within the *APC* gene.[41] APC is an important component of the Axin-APC complex, and its main function

is to aid destruction of β-catenin involved in WNT signalling, limiting proliferation in epithelial cells. When *APC* alterations occur and this complex is ineffective, β-catenin is implicated in continuous WNT signalling and the activation and upregulation of important genes such as *MYC* leads to aberrant cell processes and proliferation.[38] In normal colon cells, *APC* is found at increased levels in epithelial cells and decreased levels in base crypt stem cells . The 1st stage of CRC development involves the transition of normal tissue to preneoplastic lesions in aberrant crypt foci cells due to APC LOF, continuous WNT signalling and cell proliferation extending outside crypt baseline cell boundaries (See figure 9).[38] Genomic instability follows this event permitting cells to rapidly progress to an early adenoma stage. Inactive APC are found in sporadic CIN CRC as well as germline mutated *APC* associated Familial Adenomatous Polyposis (FAP).[50]

1.3.1.1.3 RAS (MAPK signalling pathway)

RAS genes are proto-oncogenes that encode for GTPase enzymes involved in the mitogen-activated protein kinase (MAPK) signalling pathway. RAS oncogenes result in the loss of inherent GTPase cleaving activity, leaving GTP in a continuously active form, causing constitutive signalling through the downstream MAPK pathway for continuous cell growth and tumorigenesis. Subsequent events that facilitate tumour progression from a benign to malignant state are initiated by *KRAS* gene alterations. Colorectal tumours harbour up to 50% of *RAS* mutations, with KRAS being the most common mutation (~40%) and *NRAS* least common (2-5%).[53,54] The frequency of *RAS* gene mutations has been found to vary across cancers originating from different tissue types, with the assumption that specific *RAS* isoforms are favoured solely based on the degree of expression. A study by Russo et. al. in 2014 demonstrated increased expression due to altered *KRAS* was mainly associated with colon cancer, whereas altered *NRAS* was seen mostly in rectal cancer. These findings support the theory that *RAS* isoforms having distinct roles in driving cancer from different tissues.[55] *RAS*

mutation status serves as both a prognostic and predictive indicator. Studies have also shown *KRAS* alterations in codon 12 correlated with an enhanced oncogenic phenotype when compared to CRC with mutations in alternative codons such as codon 13. Researchers suggest the latter could therefore only be involved in the initial adenoma-carcinoma transition stage; in contrast to codon 12 variants involved in the advancement of tumour cells to invade and metastasize in the process of carcinogenesis.[56] Codon 12 mutations are therefore associated with poorer prognosis when compared to codon 13 mutations. Screening for *KRAS* and *NRAS* mutations also serve as important prediction markers, as patients with a wildtype genotype for these genes could potentially benefit from anti-EGFR targeted therapy,.[57] Inherited pathogenic variants in *KRAS* and *NRAS* are rarely found and are therefore mostly screened in sporadic CRC.

1.3.1.1.4 *SMAD* (TGF-β signalling pathway)

TGF-β signalling pathway, is a tumour suppressing pathway involving regulation of normal colonic epithelial cell homeostasis in control proliferation, differentiation and apoptosis.[58] In the more advanced tumour stages however, TGF-β signalling has been shown to inversely promote tumour progression.[59] *SMAD4* is a transcription factor in the TGF-β pathway, and it's sequence located on chromosome 18. CRC is often linked to LOH of chromosome 18, which includes the loss of *SMAD4,* and has been found in up to 20% of CRC.[60] Studies have shown disruption of TGF-β signalling leads to more progressive disease within the CIN pathway, and SMAD4 mutation CRC associated with poorer patient survival. The hamartomatous juvenile polyposis syndrome, (JPS), is affected by germline mutations in *SMAD4* or BMPR1A genes and these occur at a frequency of <1% of CRC. [61]

1.3.1.1.5 *TP53 (TP53* signalling pathway)

TP53 gene alterations are associated with adenoma progression to an invasive malignancy stage.[51] *TP53* is a tumour suppressor gene that is usually only activated in response to DNA damage, stress and oncogene activation.[62] *TP53* has many direct and indirect gene targets (~500 genes) involved in the process of apoptosis and cell cycle arrest.[62] A recent study conducted by Schell et al. 2016, showed pathogenic variants in *APC* , *KRAS* and *TP53* had significantly poorer outcomes when compared to only APC or KRAS mutations, and this could be an important prognostic marker in a routine clinical setting, in predicting outcomes and appropriate therapy.[63] *TP53* germline mutations are associated with Li-Fraumeni syndrome cancers, however CRC are rarer cancers associated with this syndrome.[64]

1.3.1.2 CpG island methylation phenotype (CIMP) pathway

The 3rd mechanism (CIMP) is an epigenomic pathway, as a consequence of global methylation of CpG dinucleotide regions in promoter sequences of critical genes throughout the genome.[42] This results in silencing of crucial tumour suppressor genes involved in important cell processes such as the regulation of the cell cycle, DNA repair and apoptosis..[39] CIMP features have been indicated in a fraction of CRC (20-30%) and the associated clinicopathological features found to be similar and overlapping with the MSI phenotype, which include late age onset, female sex, proximal tumours, *BRAF* and *PIK3CA* mutations, and Fusobacterium nucleatum.[65,66] CIMP tumours were also found to be associated with wild-type *APC*, *KRAS* and *TP53* genes, indicating CIMP to be independent of the canonical *WNT* signalling pathway.[65]

1.3.1.3 Microsatellite Instability (MSI) pathway:

Fifteen to sixteen percent of CRC is attributed to the MSI pathway caused by disruption of the DNA Mismatch Repair (MMR) system. The functional MMR system is composed of 4 subunits, i.e. human MutS homologue-2 (h*MSH2*), -6 (h*MSH6)*, human MutL homologue-1 (h*MLH1*) and Post-meiotic segregation-2 (*PMS2*). Approximately 12-13% of MSI CRC are the result of somatic alterations (sporadic form), due to methylation silencing of the promoter sequence of *MLH1*, often a feature in older patients.[67] Sporadic MSI CRC also harbour the *BRAF V600E* oncogenic variant as well as clinical and histopathological features similar to CRC following the CIMP pathway. The remaining subset (~3%) are triggered by germline pathogenic variants in either of the 4 MMR genes or the Epithelial Cell Adhesion Molecule (*EpCAM)* gene causing Lynch syndrome (LS), previously referred to as Hereditary Non-Polyposis Colorectal Cancer (HNPCC).[68] LS usually occurs in younger patients.[69] Heritable epimutations although rare, have been described in defective *MLH1* and *MSH2* genes in LS families.[70–72] Methylation of *MSH2* has been reported to occur as a second hit in defective *MSH2* LS patients, and germline mutations in the *EPCAM* gene (formerly called Tumour-associated calcium signal transducer 1 [*TACSTD1]*), located adjacent to *MSH2*, has also been associated with MSH2 heritable somatic epimutations.[73,74]

MSI screening also serves as a useful genetic marker when predicting CRC patient prognosis and survival and assisting clinicians in determining the best patient treatment strategies. MSI is a well-established marker of favourable prognosis and has been promoted to be utilized as a routine test to assist in establishing more accurate prognosis and pathologic staging. [5] MSI is a negative predictor in therapy decision making as MSI tumours have been shown to be resistant to 5-fluorouracil (5-FU) chemotherapy.[2] *POLE* germline mutations have been related to increased risk for inherited CRC and adenomatous polyposis syndromes, particularly when MMR variants have not been identified.[9]

A small subset of MSI tumours have been found with a lack of pathogenic variants in MMR genes, however numerous microRNAs (miRNAs) that potentially inhibit MMR genes transcript from being translated have been found overexpressed in MSI-H CRC, e.g. miRNA-155 and miRNA21.[11,12] The miRNA-155 has been found to downregulate MMR transcripts *MLH1*, *MSH2* and *MSH6*, and miRNA-21 targets the *MSH2* and *MSH6* transcripts.

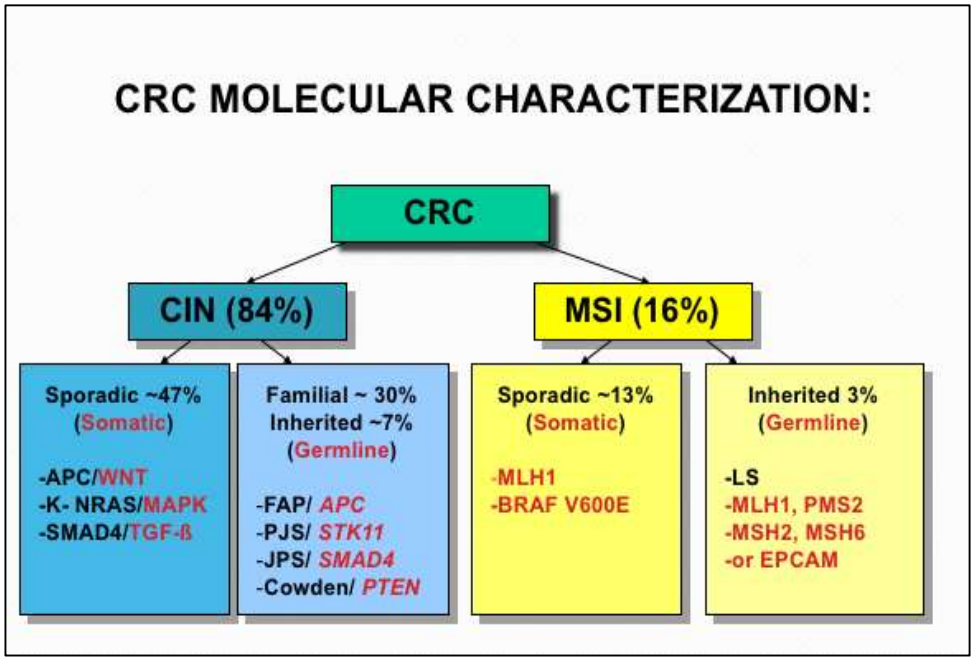


Figure 10: Summary or CRC molecular pathways/subtypes. Majority of CRC develop through the CIN pathway. Approximately 30% of CRC is due to a familial component and ~10% inherited. LS contributes to the majority of hereditary CRC cases (3%). FAP-Familial adenomatous polyposis (FAP), Peutz-Jeghers syndrome (PJS) and Juvenile polyposis syndrome (JPS) are the main CIN pathway hereditary syndromes.

1.3.1.4. Role of miRNAs in CRC

Approximately 3-4% of the human genome comprises of MicroRNA (miRNA) sequences. MiRNAs are non-coding RNA molecules ranging between 18 and 23 nucleotides in length. They are located within intergenic, intronic or untranslated regions of the genome and are regulated

by protein coding genes.[75] These molecules are critical key players in developmental and biological processes, including cellular differentiation, proliferation, signal transduction, apoptosis, etc.[76] These molecules bind to 3' untranslated regions (UTRs) of target mRNA in the majority of cases, leading to downregulation of gene expression.[77] Literature has also shown miRNAs bind to the 5' UTRs suppressing gene expression, however reports of miRNAs binding to gene promoter regions resulting in transcript upregulation have also been shown.[77–80] A single miRNA has been shown to regulate multiple transcripts, and over 2000 miRNAs have been discovered to date, regulating approximately a third of protein coding genes.[81,82]

miRNAs are also found in clusters, where miRNAs (~2-3) located within close proximity of each other are transcribed together and each have their own specific target.[83,84] miRNAs have been reported to be secreted into extracellular fluid i.e. plasma, serum, saliva, urine, tears etc. and therefore serve as potential cell free biomarkers in disease diagnosis and prognosis.[85–90]

MiRNA synthesis occur via canonical and non-canonical pathways in the nucleus (See figure 11).[75] The canonical pathway occurs through transcription of the host gene, producing a primary miRNA (pri-MiRNA) transcript. These molecules are spliced, capped, and polyadenylated, similar to mRNAs. Cleavage occurs by the Drosha enzyme producing a precursor miRNA, which subsequently moves into the cytoplasm. An endonuclease Dicer, then cleaves the top or loop region of this precursor molecule generating the mature miRNA strand. The mature miRNA strand is then added to the RNA induced silencing complex (RISC), which silence and prevents mRNA targets being translated into protein products. The alternative strand released after Dicer cleavage usually is degraded, however in some cases both strands of the miRNAs may be loaded into RISC, and in these instances strands at the 5′ end of the loop are denoted as "5p" and the 3′ end as "3p". [75] There are alternating non-canonical biogenesis pathways which includes bypassing the Drosha cleavage step, where instead a splicing process generates the precursor. The second is where a miRNA precursor is directly bound and cleaved

by Argonaute 2 (AGO2) instead of Dicer, also resulting in a mature miRNA. The subsequent steps remain the same for each alternating pathway. [75,82]

KRAS has been shown to be inactivated by miR-143, preventing CRC progression, while another study showed miR224 to increase WNT/ß-catenin signalling by deregulation of Glycogen synthase kinase-3 beta (GSK3b) and Secreted frizzled-related protein 2 (*SFRP2*) to translocate ß-catenin in CRC.[91,92] Many studies in CRC illustrating potential roles of miRNA molecules in the downregulation and upregulation of oncogenes, therefore serving as promising tools for future treatment interventions.[84,93,94]

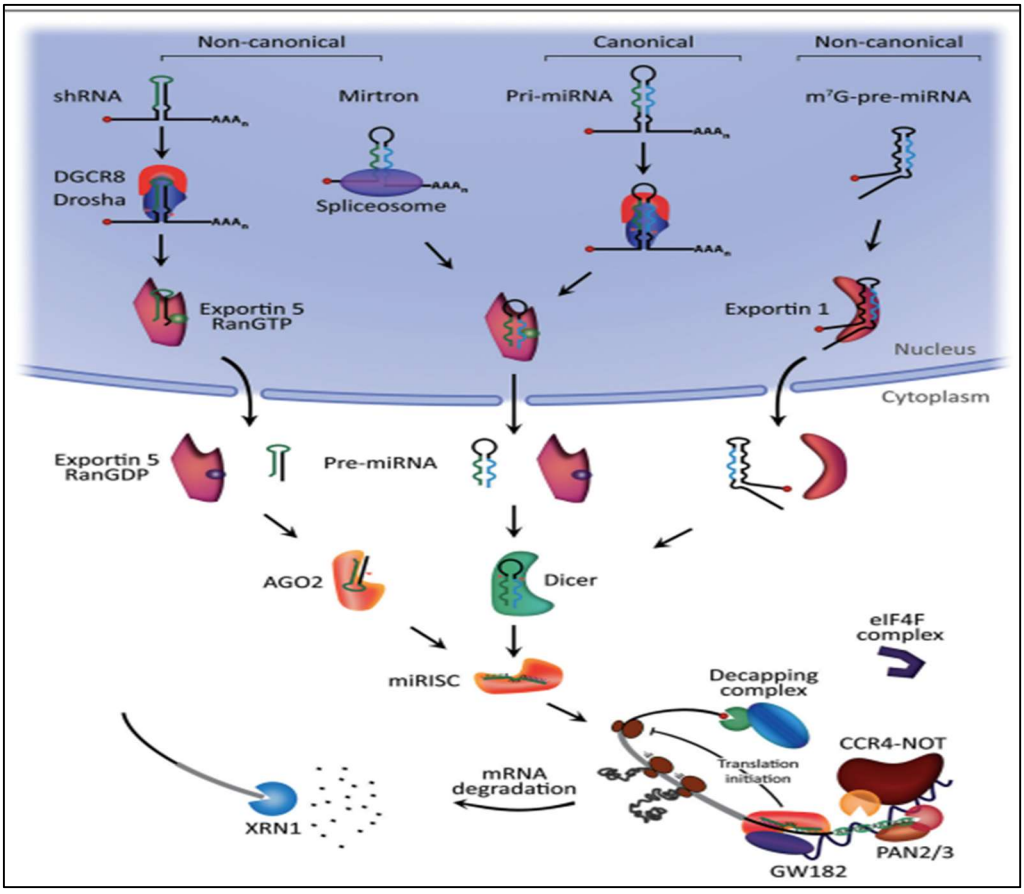


Figure 11: MicroRNA biogenesis. The canonical pathway involves the initial step of pri-miRNA transcript generation, followed by the Drosher enzyme complex cleaving it into a precursor-miRNA(pri-miRNA) molecule. It is then exported to the cytoplasm (through Exportin5/RanGTP) and cleaved by Dicer to produce the mature miRNA molecule. Subsequently it is inserted into AGO2 to form miRISC. Figure adapted from O Brien J, et al (2018)[75]

1.3.1 Thesis outline and main objectives

Presented in this thesis are the main research findings generated by this study, which includes a deeper understanding of CRC subtypes in SA patients characterized by histopathological and microsatellite instability assessment. These are shown in the form of three original research published journal articles. Chapter 2 includes the published descriptive epidemiological study of CRC patients diagnosed at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) over a 5-year period (2011-2015). The results from this study provided insight into the number of patients diagnosed, dominant sex, age, site, ethnicity and histopathological associations seen in the SA CRC population. The findings confirmed the alarming rate of approximately 40% of CRC patients within the African population group are < 50 years of age, compared to ~10% of patients of other ethnic groups. While results from this study identified clinicopathological features associated with site and young age, unique histological features associated specifically with African CRC patients was not seen. This led to our second objective to further characterize by molecular subtyping through MSI screening of all patients diagnosed within a 2-year study period (2011-2012). This was included in our second publication (Chapter 3), where our research showed 12% of CRC patients had a MSI subtype. With additional categorization by ethnicity, interestingly African patients revealed an increased frequency of MSI CRC (15%) when compared to other ethnic groups (8%). In addition, unique associated features included increased MSH2/MSH6 protein expression loss, and the absence of BRAF mutations, suggesting the hereditary form of disease (LS) could be the main cause of MSI CRC in the African population.

The final published journal article characterized the non-MSI CRC group (MSS/MSI-low) to determine whether distinct associations could be identified subsequent to omitting MSI-H patient samples. These findings are presented as an original research article in Chapter 4. MSS and MSI-low samples were descriptively analysed by categorization of ethnicity (African versus

other ethnic groups (OEG) and site (right-sided colon cancer (RCC) versus left-sided colon cancer (LCC). Additional investigations of African CRC categorized by site and MSI subtype revealed associations with gender, site, histological and molecular subtype. These results support the concept that MSI-L CRC possibly is a standalone molecular subtype with a worse prognosis, compared to counterparts MSS and MSI-H CRC.

In conclusion, Chapter 5 summarizes the major findings of our study and suggest prospective research in the much needed understanding of the molecular mechanisms involved in CRC development in SA.

Chapter 2:

Descriptive epidemiological study of South African colorectal cancer patients at a Johannesburg Hospital Academic institution

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ORIGINAL ARTICLE

Descriptive epidemiological study of South African colorectal cancer patients at a Johannesburg Hospital Academic institution

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Key words
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Abstract

Background and Aim: Epidemiological studies of colorectal cancer (CRC) in South Africa (SA) have been poorly characterized. Black and white SA population groups have demonstrated distinct CRC clinical presentations, suggesting that black SA patients follow a different carcinogenic pathway than their white counterparts. Thus, the aim of this study was to identify unique demographic and histopathological features associated with black SA patients to facilitate earlier diagnosis and to improve disease management.

Methods: This preliminary descriptive epidemiological study included 665 retrospective CRC cases diagnosed between the period 2011 and 2015 at the Charlotte Maxeke Johannesburg Academic Hospital. Demographic and histopathological features in black *versus* other race groups (ORG) were compared, and Student's *t*-test, Chi-square, and Fischer's exact tests were used for statistical analysis.

Results: Statistical analysis demonstrated that patients with left-sided tumors of invasive adenocarcinoma were predominantly black and male. These patients were considerably younger when compared to ORG (median 56 *vs* 62 years, respectively), $P < 0.0001$. However, no significant propensity for other histological features was illustrated. Polyps were mostly tubular adenomas (51%) and tubulovillous adenomas (TVAs) (44%). TVAs were mostly high-grade lesions ($P < 0.0001$) and associated with left-sided CRC ($P = 0.0325$).

Conclusion: These findings verify that black SA CRC patients have an earlier disease onset in comparison to ORG; however, no increased tendency for tumor site, precursor lesion, stage of disease, or gender was evident. Thus, a deeper molecular characterization of CRC is required to understand the underlying causes associated with earlier disease onset in black SA CRC patients.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide and the fourth most common cause of cancer-related mortalities.[1] Within South Africa (SA), it is the fourth most commonly diagnosed cancer, being the second most common among males and the fourth most common among females and the sixth leading cause of cancer mortalities in SA.[1,2] Male and female ratios have remained constant (53–55% *vs* 45–47%, respectively).[2–6] Male patients have a 1.27 estimated lifetime risk of developing CRC, and females have a lower risk of 0.75.[6] CRC incidence rates within SA have progressively increased over the years, with the annual crude incidence in SA CRC male and female patients in 2014 reported as 7.34 and 5.86 per 100 000, respectively.[6] CRC occurs mostly within the white (Caucasian) population group in SA patients (52–54%), followed by black (African) (26–28%), colored (mixed ancestry) (14–15%), and Asian (Indian) patients (4–7%).[2–6] The pathogenesis of CRC is complex and diverse and is influenced by multiple factors, including diet, lifestyle, and genetic predispositions. It is reported that the white patient population group in SA appears to follow the classic Western trend, which presents at a later age, having an association with diet and lifestyle factors, although the molecular pathology has not been extensively investigated.[7,8] Comparatively, the black SA population has a higher frequency of young (<50 years) CRC patients, possibly because of diet and lifestyle changes either due to urbanization or a familial contribution.[7–9] A study by Prodehl *et al.* showed that 49.0% of SA CRC patients reported a family history of cancer, with CRC the most frequently diagnosed, where maternal (35%) and fraternal (33%) members were the most affected, followed by paternal members (25%).[10]

High prevalence rates for young CRC patients, <40 years of age, have been reported globally, with the lowest rates found

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in the United States, Europe, and New Zealand (1–6%) and the highest in Asian and Middle Eastern countries (10–39%).[11–13] The current SA National Cancer Registry (NCR) statistics, spanning a 5-year period (2010–2014), show that young CRC patients, under 50 years of age, contribute an average of 18% of all CRC cases, of which approximately 7% were younger than 40 years of age.[2–6] The NCR 2014 report also showed an exponential growth in CRC incidence in male patients from 2010 to 2014. A correlation between race and age was also demonstrated as the majority of young patients younger than 50 years old were black (32%) *versus* white patients (11%) ($P < 0.0001$) (NCR 2013–2014).[5,6] The burden of disease in SA has also been a challenge to assess due to underreporting of CRC cases as registries are mainly limited to small urban areas.[14]

Thus, this descriptive epidemiological study will serve as a preliminary study to obtain a greater understanding of the development of CRC, particularly in black SA CRC patients. This will include screening demographic and histopathological data in the hope of identifying key features associated with the disease to assist with earlier detection and improvement of prognosis, particularly within this population group.

Methods

A retrospective laboratory information system (LIS) search was conducted on all patients who had biopsy samples or colorectal resections reported by the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) branch of the National Health Laboratory Service (NHLS)/Anatomical Pathology Division, Faculty of Health Sciences, University of the Witwatersrand. Histological reports of all patients diagnosed with colorectal adenocarcinoma between 2011 and 2015 were reviewed, and data were described with respect to age, race, gender, tumor site ([distal to the splenic flexure [left-sided] and proximal to the splenic flexure [right-sided]), histological subtype and grade, presence and grade of precursor lesion, invasive tumor including the presence of tumor infiltrating lymphocytes (TIL), Crohn's-like inflammatory reaction (CIR), and the tumor stage (TNM). The Ethics Committee of the University of the Witwatersrand (clearance number M120994) approved this study.

Statistical analysis. Statistical associations between race groups were investigated using Student's *t*-test, Fisher's exact test, and Chi Square test analysis. Non-black race patients were included in a single group, termed the "Other Race" group (White, Colored/mixed ancestry, Indian) due to small case numbers in the colored and Indian groups in comparison to the black patient group. Race was compared with age, tumor site, and morphological features as stated above. Statistical data analysis was completed using Stata Intercooled 7.0 (Stata, College Station, TX, USA) and Graphpad Prism version 7 (GraphPad Software, La Jolla, CA, USA). The results were considered statistically significant when $P < 0.05$* and highly significant when $P < 0.001$***.

Results

A total of 665 CRC patients were diagnosed at CMJAH between 2011 and 2015, with the majority of patients being black (391/665; 59%) (Table 1). Overall, the patients were predominantly male (366/665; 55%), and no variation of gender was seen among race groups. The median age of CRC was 59 years (interquartile range: 49–68). A significant association was seen between patient age and race (black *vs* other race group [ORG]) (median age: 56 *vs* 62 respectively), ($P < 0.0001$). Most tumors occurred in the left colon (427/621; 69%), had an invasive adenocarcinoma subtype (596/646; 92%), were low-grade (LG) (568/624; 91%), and presented as TNM stage III (180/350; 52%). The majority of CRC cases did not contain TILs (224/345; 65%) or a CIR (263/345; 76%), nor did they show lymphatic invasion (296/422; 70%). A minority of polyps was found (140/408; 34%), with tubular adenomas (TAs) being reported as the predominant subtype (72/140; 51%), followed by tubulovillous adenomas (TVAs) (61/140; 44%). TAs mostly displayed LG dysplasia (40/67; 60%); however, TVAs were mostly associated with high-grade (HG) dysplasia (49/60; 82%) ($P < 0.0001$). All the aforementioned pathological characteristics lacked any correlation with race. BRAF V600E mutations, however, exclusively occurred in ORG patients (5/21; 24%) ($P = 0.0011$).

Correlation between age groups, young *versus* old (≤50 *vs* >50 years) and CRC subtype, tumor grade, TIL, and lymphatic invasion was identified (Table 2). Signet ring cell carcinoma occurred at a higher frequency in the young patient group (16/183; 9%) *versus* the older patient group (4/463; 1%) ($P = 0.0030$), while a mild to moderate TIL response was more commonly found in the older patient group (95/247; 38%) than in the younger group (26/98; 27%) ($P = 0.0450$). Lymphatic invasion was associated more with younger patients (45/120; 37%) than with the older group (81/302; 27%) ($P = 0.0341$), and BRAF V600E mutations were exclusively found in older patients (5/36; 24%) *versus* younger patients (0/40; 0%) ($P = 0.0204$). No significant association was found between gender and histopathological data (Table 3).

The majority of polyps were found in the left colon (81/131; 62%) and in male patients (89/140; 64%) (Tables 3 and 4). A particular association between the side of colon and grade of TVAs was noted here ($P = 0.0325$) as left-sided CRCs were mostly associated with HG TVAs (31/34; 91%). BRAF V600E mutations occurred exclusively in the right colon (5/34; 15%), and none were associated with left CRC tumors (0/42; 0%) ($P = 0.0151$).

Discussion

The focus of this study was to ascertain common features, particularly demographic and histopathological characteristics, associated with black patients to assist in the earlier detection and better management of the disease. Although this study demonstrated no significant differences between male and female patients, black *versus* ORG and younger *versus* older patient groups illustrated significant differences in a number of parameters.

A total of 665 CRC patients were diagnosed at CMJAH over a 5-year period, from 2011 to 2015. Patients were predominantly male and black, with a considerable proportion being young. The median age of black patients compared to ORG patients (56 *vs* 62 years, respectively) was significantly younger. The data confirmed local and international reports

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Table 1 Descriptive histopathological analysis of colorectal cancer cases diagnosed at Charlotte Maxeke Johannesburg Academic Hospital between 2011 and 2015: Black *versus* other race groups

Demographic data	Number of cases (%)	Stratified by race groups no. of cases (%)		Statistical analysis:
		Black (B)	Other race groups (O)	
Gender	665	391	274	$P = 0.9369$
Male	366 (55)	216 (55)	150 (55)	
Female	299 (45)	175 (45)	124 (45)	
Age	660	389	271	***P* < 0.0001***
Median	59	56***	62	
Min–Max	15–94	15–94	25–92	
Mean ± SD	57 ± 14	55 ± 15	62 ± 13	
P25-P75	49–68	45–65	55–71	
95% CI	[56–59]	[53–56]	[60–64]	
≤50 years	184 (28)	142 (37)***	42 (15)	***P* < 0.0001***
>50 years	476 (72)	247 (63)	229 (85)	
Tumor site	621	368	253	$P = 0.7634$
Left	427 (69)	250 (68)	177 (70)	
Right	190 (30)	115 (31)	75 (30)	
Left and right	4 (1)	3 (1)	1 (0)	
Tumor subtype	646	380	266	$P = 0.2878$
Invasive adenocarcinoma	596 (92)	349 (92)	247 (93)	
Mucinous adenocarcinoma	29 (5)	16 (4)	13 (5)	
Signet ring cell adenocarcinoma	20 (3)	15 (4)	5 (2)	
Neuroendocrine carcinoma	1 (0)	0 (0)	1 (0)	
Tumor grade	624	372	252	$P = 0.4793$
LG	568 (91)	336 (90)	232 (92)	
HG	56 (9)	36 (10)	20 (8)	
AJCC TNM staging	350	208	142	$P = 0.4625$
I	46 (13)	26 (13)	20 (15)	
II	109 (31)	59 (28)	50 (35)	
III	180 (52)	114 (55)	66 (46)	
IV	15 (4)	9 (4)	6 (4)	
Tumor-infiltrating lymphocytes	345	202	143	$P = 0.4231$
None	224 (65)	135 (67)	89 (62)	
Mild–moderate	121 (35)	67 (33)	54 (38)	
Crohn's-like inflamatory response	345	202	143	$P = 0.3077$
None	263 (76)	158 (78)	105 (73)	
Mild–moderate	82 (24)	44 (22)	38 (27)	
Lymphatic invasion	422	254	168	$P = 0.1935$
Absent	296 (70)	172 (68)	124 (74)	
Present	126 (30)	82 (32)	44 (26)	
Polyps	408	234	174	$P = 0.2934$
Absent	268 (66)	160 (68)	108 (62)	
Present	140 (34)	74 (32)	66 (38)	
Polyp subtype	140	75	65	$P = 0.1856$
Hyperplastic polyp	4 (3)	1 (1)	3 (5)	
Pseudopolyp	2 (1)	2 (3)	0 (0)	
Sessile serrated adenoma	1 (1)	0 (0)	1 (1)	
TA	72 (51)	43 (57)	29 (45)	
TVA	61 (44)	29 (39)	32 (49)	
TA grade	67	39	28	$P = 0.2161$
LG	40 (60)	25 (65)	15 (54)	
HG	27 (40)	14 (35)	13 (46)	
TVA grade	60	28	32	
LG	11 (18)	2 (7)	8 (25)	
HG	49 (82)	25 (93)***	24 (75)	
BRAF V600E	76	55	21	***P* = 0.0011***
Wild-type	71	55 (100)	16 (76)	
Mutation	5	0 (0)	5 (24)***	

*Statistically significant.

***Statistically highly significant.

AJCC, American Joint Committee on Cancer; CI, confidence interval; HG, high grade; LG, low grade; TA, tubular adenoma; TNM, tumor node metastases; TVA, tubulovillous adenoma.

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Table 2 Descriptive histopathological analysis of colorectal cancer cases diagnosed at Charlotte Maxeke Johannesburg Academic Hospital between 2011 and 2015: Younger (≤50 years) *versus* older (>50 years) patients

Demographic data	Number of cases (%)	Stratified by age groups no. of cases (%)		Statistical analysis:
		≤50 years	>50 years	
Gender	665	189	476	$P = 0.1005$
Male	366 (55)	114 (60)	252 (53)	
Female	299 (45)	75 (40)	224 (47)	
Age	660	389	271	***P* < 0.0001***
Median	59	41	64	
Min–Max	15–94	15–50	51–94	
Mean ± SD	57 ± 14	40 ± 8	65 ± 9	
P25-P75	49–68	34–46	57–71	
95% CI	[56–59]	[39–41]	[64–66]	
Tumor site	621	179	442	$P = 0.2999$
Left	426 (68)	119 (66)	307 (69)	
Right	191 (31)	60 (34)	131 (30)	
Left and right	4 (1)	0 (0)	4 (1)	
Tumor subtype	646	183	463	***P* < 0.0001***
Invasive adenocarcinoma	596 (92)	159 (87)	437 (94)	
Mucinous adenocarcinoma	29 (5)	8 (4)	21 (5)	
Signet ring cell adenocarcinoma	20 (3)	16 (9)***	4 (1)	
Neuroendocrine carcinoma	1 (0)	0 (0)	1 (0)	
Tumor grade	624	178	446	***P* = 0.0030****
LG	568 (91)	152 (85)	416 (93)	
HG	56 (9)	26 (15)**	30 (7)	
AJCC TNM staging	350	100	250	$P = 0.2282$
I	46 (13)	9 (9)	37 (15)	
II	109 (31)	27 (27)	82 (33)	
III	180 (52)	59 (59)	121 (48)	
IV	15 (4)	5 (5)	10 (4)	
Tumor-infiltrating lymphocytes	345	98	247	***P* = 0.0450***
None	224 (65)	72 (73)	152 (62)	
Mild–moderate	121 (35)	26 (27)	95 (38)*	
Crohn's-like inflamatory response	345	98	247	$P = 0.1613$
None	263 (76)	80 (82)	183 (74)	
Mild–moderate	82 (24)	18 (18)	64 (26)	
Lymphatic invasion	422	120	302	***P* = 0.0341***
Absent	296 (70)	75 (63)	221 (73)	
Present	126 (30)	45 (37)*	81 (27)	
Polyps	408	111	297	$P = 0.1610$
Absent	268 (66)	79 (71)	189 (64)	
Present	140 (34)	32 (29)	108 (36)	
Polyp subtype	140	32	108	$P = 0.6291$
Hyperplastic polyp	4 (3)	0 (0)	4 (4)	
Pseudopolyp	2 (1)	1 (3)	1 (1)	
Sessile serrated adenoma	1 (1)	0 (0)	1 (1)	
TA	72 (51)	18 (56)	54 (50)	
TVA	61 (44)	13 (41)	48 (44)	
Polyp grade				$P = 0.6828$
TA	67	18	49	
LG	40 (60)	10 (56)	30 (60)	
HG	27 (40)	8 (44)	19 (40)	
TVA	60	12	48	
LG	11 (18)	3 (25)	8 (17)	
HG	49 (82)	9 (75)	40 (83)	
BRAF V600E	76	40	36	***P* = 0.0204***
Wildtype	71	40 (100)	31 (76)	
Mutation	5	0 (0)	5 (24)*	

*Statistically significant.

***Statistically highly significant.

AJCC, American Joint Committee on Cancer; CI, confidence interval; HG, high grade; LG, low grade; TA, tubular adenoma; TNM, tumor node metastases; TVA, tubulovillous adenoma.

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Table 3 Descriptive histopathological analysis of colorectal cancer cases diagnosed at Charlotte Maxeke Johannesburg Academic Hospital between 2011 and 2015: Male *versus* female

Demographic data	Number of cases (%)	Stratified by gender no. of cases (%)		Statistical analysis:
		Male	Female	
Gender	665	366 (55%)	299 (45%)	
Age	660	364	296	$P = 0.2560$
Median	59	58	60	
Min–Max	15–94	15–94	20–92	
Mean ± SD	57 ± 14	56 ± 14	59 ± 14	
P25-P75	49–68	47–67	51–69	
95% CI	[56–59]	[55–58]	[58–61]	
Tumor site	621	344	277	$P = 0.9653$
Left	426 (68)	237 (69)	189 (68)	
Right	191 (31)	105 (31)	86 (31)	
Left and right	4 (1)	2 (0)	2 (1)	
Tumor subtype	646	183	463	$P = 01663$
Invasive adenocarcinoma	596 (92)	437 (87)	267 (94)	
Mucinous adenocarcinoma	29 (5)	21 (4)	14 (5)	
Signet ring cell adenocarcinoma	20 (3)	4 (9)	9 (1)	
Neuroendocrine carcinoma	1 (0)	1 (0)	0 (0)	
Tumor grade	624	344	280	$P = 01611$
LG	568 (91)	308 (85)	260 (93)	
HG	56 (9)	36 (15)	20 (7)	
AJCC TNM staging	350	100	250	$P = 0.2282$
I	46 (13)	9 (9)	37 (15)	
II	109 (31)	27 (27)	82 (33)	
III	180 (52)	59 (59)	121 (48)	
IV	15 (4)	5 (5)	10 (4)	
Tumor-infiltrating lymphocytes	345	192	153	$P = 0.1119$
None	224 (65)	132 (69)	92 (60)	
Mild–moderate	121 (35)	60 (31)	61 (40)	
Crohn's-like inflamatory response	345	192	153	$P = 0.0569$
None	263 (76)	154 (80)	109 (71)	
Mild–moderate	82 (24)	38 (20)	44 (29)	
Lymphatic invasion	422	234	188	$P = 0.6698$
Absent	296 (70)	162 (69)	134 (71)	
Present	126 (30)	72 (31)	54 (29)	
Polyps	408	236	174	$P = 0.0917$
Absent	268 (66)	147 (62)	123 (71)	
Present	140 (34)	89 (38)	51 (29)	
Polyp subtype	140	89	51	$P = 0.4160$
Hyperplastic polyp	4 (3)	3 (4)	1 (2)	
Pseudopolyp	2 (1)	2 (2)	0 (0)	
Sessile serrated adenoma	1 (1)	0 (0)	1 (2)	
TA	72 (51)	43 (48)	29 (57)	
TVA	61 (44)	41 (46)	20 (39)	
Polyp grade				$P = 0.4732$
TA	67	36	28	
LG	40 (60)	21 (58)	17 (54)	
HG	27 (40)	15 (42)	11 (46)	
TVA	60	41	19	
LG	11 (18)	6 (15)	5 (28)	
HG	49 (82)	35 (85)	14 (72)	
BRAF V600E	76	44	32	$P = 0.6444$
Wildtype	71	42 (95)	29 (91)	
Mutation	5	2 (5)	3 (9)	

*Statistically significant.

***Statistically highly significant.

AJCC, American Joint Committee on Cancer; CI, confidence interval; HG, high grade; LG, low grade; TA, tubular adenoma; TNM, tumor node metastases; TVA, tubulovillous adenoma.

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Table 4 Descriptive histopathological analysis of CRC cases diagnosed at Charlotte Maxeke Johannesburg Academic Hospital between 2011–2015: Left *versus* right-sided colon cancer

Demographic data	Number of cases (%)	Stratified by tumor site no. of cases (%)		Statistical analysis:
		Left-sided	Right-sided	
Tumor site	617	426 (69%)	191 (31%)	
Tumor subtype	607	419	188	***P* < 0.0001***
Invasive adenocarcinoma	596 (92)	398 (95)	162 (86)	
Mucinous adenocarcinoma	29 (5)	9 (2)	18 (10)***	
Signet ring cell adenocarcinoma	20 (3)	12 (3)	7 (4)	
Neuroendocrine carcinoma	1 (0)	0 (0)	1 (0)	
Tumor grade	587	399	188	*P* = 02845
LG	533 (91)	366 (92)	167 (89)	
HG	54 (9)	33 (8)	21 (11)	
AJCC TNM staging	350	192	153	*P* = 0.6086
I	46 (13)	28 (15)	17 (11)	
II	109 (31)	60 (31)	46 (30)	
III	180 (52)	96 (50)	80 (52)	
IV	15 (4)	8 (4)	10 (7)	
Tumor-infiltrating lymphocytes	338	193	145	***P* = 0.0210***
None	220 (65)	136 (70)	84 (58)	
Mild–moderate	118 (35)	57 (30)	61 (42)*	
Crohn's like inflamatory response	338	193	145	*P* = 1.0000
None	258 (76)	147 (76)	111 (77)	
Mild–moderate	80 (24)	46 (24)	34 (23)	
Lymphatic invasion	417	254	163	***P* = 0.0009****
Absent	293 (70)	194 (76)	99 (61)	
Present	123 (30)	60 (24)	64 (39)***	
Polyps	392	231	161	*P* = 0.4468
Absent	261 (67)	150 (65)	111 (69)	
Present	131 (33)	81 (35)	50 (31)	
Polyp subtype	131	81	50	*P* = 0.1484
Hyperplastic polyp	3 (2)	3 (4)	0 (0)	
Pseudopolyp	2 (1)	0 (0)	2 (4)	
Sessile serrated adenoma	1 (1)	0 (0)	1 (2)	
TA	68 (52)	43 (53)	25 (50)	
TVA	57 (44)	35 (43)	22 (44)	
Polyp grade	119			*P* = 0.0615
TA	64	40	24	
LG	38 (59)	21 (52)	17 (71)	
HG	26 (41)	19 (48)	7 (29)	
TVA	55	34	21	
LG	10 (18)	3 (9)	7 (33)	
HG	45 (82)	31 (91)*	14 (67)	
BRAF V600E	76	42	34	***P* = 0.0154***
Wildtype	71	42 (100)	29 (85)	
Mutation	5	0 (0)	5 (15)*	

*Statistically significant.

***Statistically highly significant.

AJCC, American Joint Committee on Cancer; CI, confidence interval; CRC, colorectal cancer; HG, high grade; LG, low grade; TA, tubular adenoma; TNM, tumor node metastases; TVA, tubulovillous adenoma.

regarding male *versus* female ratios (55 *vs* 45%, respectively),[1,5] with black patients showing an earlier age of onset in comparison to the white group.[7,15] In comparison, in African Americans (AA), the median age of onset in males and females were reported to be 66 and 70 years, respectively, compared to 72 and 77 in white men and women, respectively.[16] In this SA cohort, the median age for male and female black patients was 55 and 56 years, respectively, *versus* 62 and 65 years for males and females of ORG, respectively. The median age for white male and female patients (separated from Indian and colored or mixed race) remained 62 and 65 years, respectively. These data showed that SA black and white patients are at least 10 years younger in comparison to U.S. AA and U.S. white patients.

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The frequency of young black patients younger than 50 years of age in this cohort (37%) was similar to that of young black SA CRC patients in the NCR 2014 report (32%). The majority of tumors occurred in the left colon, had an invasive adenocarcinoma subtype, LG tumors, and presented at an advanced TNM stage (III and IV) These findings were in concordance with international published data that reported an approximate 70% occurrence of left-sided CRC, with about 90% being of an invasive adenocarcinoma subtype, that was moderately to well differentiated, with approximately 50–60% of CRC cases presenting at a more advanced stage.[17,18] Male and female patients had equal frequencies for tumor site (69% left-sided *vs* 31% right-sided). This finding is contrary to other studies showing right-sided colon cancer to be increased in female patients and left-sided CRC to be increased in males.[19]

In the present analysis, with regard to polyps, conventional adenomas (TA, TVA, and villous adenomas [VAs]) are mostly found in the left colon,[20] with TAs described as the predominant subtype (80%), TVAs accounting for 10–20%, and VAs less than 5%.[21–23] In this study, polyps were found in 34% (140/408) of cases, with only 1% being sessile serrated adenoma (SSA), 4% hyperplastic polyps (HPs), 51% TAs, and 44% TVAs. TAs were mostly associated with LG dysplasia (60%), and TVAs that also occurred at a considerably high frequency than that stated in literature were mostly associated with HG dysplasia (83%) ($P < 0.0001$). The size of the adenomatous polyp (AP) was not recorded in the majority of reports, only the quantity of APs and grade of dysplasia. This study showed no association of APs with race, gender, or patient age; however, HG TVAs were more commonly found in the left colon ($P = 0.0325$). The transition from LG APs to more advanced APs or CRC takes approximately 3 years, suggesting that frequent surveillance colonoscopies for the earlier detection of CRC would benefit patients. Guidelines indicate repeat surveillance every 5–10 years for 1–2 small TAs (<1 cm) with LG dysplasia, and where 3–10 large APs are found (≥1 cm) or in those with HG dysplasia or with villous features, colonoscopy should be performed every 3 years.[20,23]

The literature indicates that AA patients are more likely to have right-sided tumors than left.[15,24,25] This SA study, however, showed no relationship between race and tumor site. BRAF V600E mutational analysis was carried out in 76 patients, and an association was only found in older white patients with right-sided colon cancers, possibly indicating development via the microsatellite instability CRC pathway.

CRC research in AA patients has demonstrated higher incidence and mortality rates, earlier age of diagnosis, more advanced stage of the disease, and a greater proportion of right-sided colon cancers in comparison to other ethnic groups. This has led American professional societies to change guidelines, recommending colonoscopy screening by 45 instead of 50 years of age, which has led to a great reduction in CRC incidence in this population group.[26] Although statistically significant differences concerning stage and tumor site were not seen when comparing black *versus* ORG patients, later stage of disease and earlier age of onset in black patients remained significant factors, and a policy recommending earlier screening in this population group should be considered to reduce incidence rates.

In conclusion, this study shows that black SA CRC patients most likely follow a different carcinogenic pathway in comparison to other SA ethnic groups due to earlier age of onset. Black SA patients also seem to present at least a decade earlier when comparing Africans found in the Western world, with more left-sided CRC occurring in SA patients compared to a right-sided CRC association seen in AAs. Moreover, a lack of BRAF V600E mutation in the black SA CRC population in this study suggests that there is a different carcinogenic pathway in black SA CRC patients compared to other ethnic groups, which still needs to be evaluated in a larger cohort of patients. TVAs found at a frequent rate across different ethnic groups in SA patients could possibly suggest a dominant adenocarcinoma sequence in the development of the disease. The initiation of earlier screening and more frequent surveillance policies in black SA patients could possibly result in better management of the disease. Further epidemiological research linked to molecular characterization in SA population groups is still needed to assist in determining the underlying cause and designing more personalized treatment strategies for better management of the disease.

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Chapter 3:

Microsatellite Instability assessment in Black South African Colorectal Cancer patients reveal an increased incidence of suspected Lynch syndrome.

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Microsatellite Instability assessment in Black South African Colorectal Cancer patients reveal an increased incidence of suspected Lynch syndrome

M. McCabe[1*], Y. Perner[1], R. Magobo[1], P. Magangane[1], S. Mirza[2] & C. Penny[2]

Microsatellite Instability (MSI) is a hallmark of colorectal cancer (CRC) and occurs in 15–16% of CRC. Molecular biological information of CRC in South Africa (SA) is largely unrecorded. This study was undertaken to determine the frequency of MSI, with particular reference to Lynch syndrome (LS) with a view to improve surveillance and prevention strategies. This was a retrospective study on CRC samples diagnosed between 2011–2015 at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). Samples diagnosed between 2011–2012 were screened for MSI by PCR and mismatch repair (MMR) immunohistochemistry (IHC), and additional BRAFV600E mutational analysis performed. T-tests, Fischer's exact and Chi square statistical tests were applied. Twelve percent of patients displayed MSI, with increased frequency in black (15%) versus other ethnic group (OEG) (8%) patients. MSI patients were significantly younger than microsatellite stable (MSS) patients, however when stratified by ethnicity, black patients were predominantly younger (median age: 47), with increased MSH2/6 loss, and no BRAF mutations. These findings suggest a large proportion of young black SA CRC patients develop via the LS pathway due to earlier age onset and predominant MSH2/6 protein loss. SA patients of other ethnicities appear to follow the more well established sporadic MSI pathway.

Colorectal cancer (CRC) develops through three major molecular pathways namely: Chromosomal Instability (CIN), Microsatellite Instability (MSI), and the epigenetic instability or CpG island methylator phenotype (CIMP) pathway[1–3]. CIN or microsatellite stable (MSS) tumours account for 65–75% of CRC and mainly develop through mutant adenomatous polyposis coli (APC) gene with subsequent KRAS mutational activation, TP53 inactivation and somatic copy number alterations (SCNAs)[4,5]. MSI CRC occurs in approximately 15–16% of CRC due to inactivation of the DNA Mismatch Repair (MMR) system (MLH1, MSH2, MSH6, PMS2), with the majority (12%) presenting as sporadic MSI CRC as a result of epigenetic mutation (methylation silencing) of the promoter sequence of MLH1, and BRAF V600E oncogenic mutations[2,6–10]. The remaining subset (2–3%) is caused by germline mutations in either one of 4 MMR genes or the EpCAM gene and is a feature of Lynch syndrome (LS)[8,9,11–14]. CIMP CRC (10–20%) demonstrate hypermethylation of several promoter CpG island loci throughout the genome, leading to tumour suppressor and tumour-related gene inactivation. The CIMP and the MSI pathway overlaps in sporadic MSI CRCs, as these tumours display high levels of CIMP and MSI and are characterized by a different type of precursor lesion in comparison to LS[15,16]. LS follows the classic adenoma-carcinoma sequence pathway as they present mainly with tubular adenomas (TAs) or tubulovillous adenomas (TVAs), whereas sporadic MSI CRCpremalignant lesions are sessile serrated adenomas (SSAs) developing through the serrated neoplasia pathway[15–17]. Clinicopathological features of sporadic MSI CRC include predominant occurrence in female patients, within the right colon and associated morphology includes signet ring cell and mucinous features with

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tumour infiltrating lymphocytes (TIL)[15,18–20]. LS patients are mainly associated with younger patients, with no gender preference and similar morphology as sporadic MSI tumours[12].

Previous CRC studies conducted in SA showed a higher frequency of MSI CRC in young black patients through MMR deficiency than white patients[21–23]. These studies suggested a high frequency of LS, however additional validation studies and further molecular characterization of MSI CRC in black SA patients was recommended[24].

MSI assessment has also shown important prognostic and predictive roles in CRC patient care, as MSI tumours are associated with a better prognosis than MSS tumours in early stage CRC, and treatment of MSI stage II tumours are not well responsive to 5-fluorouracil (5-FU) (standard treatment)[25,26]. MSI/BRAF wild-type tumours are also more suggestive of LS and is crucial for improving cancer surveillance and prevention screening for patient family members, due to their increased risk of developing cancer. Identifying LS patients and treating with aspirin (600 mg per day) have also shown to reduce the risk of CRC[27].

This study assesses the frequency and features associated with MSI CRC over a 5-year period in a cohort of newly diagnosed CRC patients at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) within SA. This data will provide insight into the CRC histopathological and molecular features associated with MSI CRC in black SA patients, with particular reference to MSI CRC frequency and the occurrence of suspected LS, a heretofore largely unassessed aspect of the disease.

Methodology

Patient demographics and tumour pathological characterisation. This was a retrospective study, comprising a 5-year cohort of 675 patients who had biopsy samples or colorectal resections fulfilling the histological criteria for adenocarcinoma of the colon or rectum from January 2011–December 2015, reported by the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) branch of the National Health Laboratory Service (NHLS)/Anatomical Pathology Division, Faculty of Health Sciences, University of the Witwatersrand. A total of 439 CRC cases with an MSI status by MMR immunohistochemistry (IHC) or MSI PCR result were included in this study. All cases were stratified by age, gender, ethnicity, tumour site, histological subtype, grade, stage (TNM classification by AJCC staging), presence and grade of precursor lesion, presence of tumour infiltrating lymphocytes (TIL) and Crohn's-like inflammatory reaction (CIR). Tumour site was considered left-sided if distal from the splenic flexure and right-sided if proximal to the splenic flexure. This information was obtained from histology reports. A semi-quantitive H&E assessment using the Klintrup-Mäkinen score was used to determine the presence of TILs[28]. Immunophenotypic profiling of TILs was not performed. Family histories were not available from the histological reports. This research was conducted under the institution's blanket ethics clearance (M10744) which allows for research to be carried out on all archived pathology specimens without informed consent from study participants. Additional study-specific ethics clearance was also obtained by the Ethics Committee of the University of the Witwatersrand (clearance number M120994), and all tests were performed according to the relevant guidelines and regulations.

Molecular characterisation. Molecular characterization of patient samples for frequency assessment was limited to the first 2 years (2011–2012) mainly due to the availability of funds (Figure 1). A total of 275 CRC cases were diagnosed during this period and a cohort of 267 archival CRC tumour specimens was retrieved, examined and subjected to molecular analysis. MSI PCR was conducted and samples with a MSI-High profile had additional BRAF V600E mutational analysis performed.

Cases without MMR IHC results as ascertained from the histology reports were screened for MMR protein loss by IHC.

DNA was extracted from 4 × 10μm sections of tumour tissue from formalin fixed paraffin embedded (FFPE) blocks using the QIAamp DNA FFPE tissue kit (Qiagen, Valencia, CA USA). The extracted DNA was subjected to a pentaplex PCR panel of 5 markers comprising mononucleotide repeats amplified by 5 sets of primers, i.e. (BAT-25, BAT-26, NR-21, NR-24, and NR-27) as described in Haghighi *et al.*[29]. Each antisense primer was labeled with 1 of the fluorescent markers FAM, HEX, or NED. The Applied Biosystems® True Allele® PCR master mix was used according to manufacturer's instructions with slight modifications for successful amplification of the multiplex PCR. Fluorescent PCR products were subjected to capillary electrophoresis on the Applied Biosystems PRISM 3500 automated genetic analyzer, and allelic size was determined using Genemapper 4.0 software (Applied Biosystems). Samples with no allelic size variations in the 5 microsatellite markers were classified as microsatellite stable (MSS), allelic size variations found in 1 of the 5 microsatellites markers were classified as microsatellite instability-low (MSI-L) and variation in 2 or more microsatellite markers was classified as microsatellite instability-high (MSI-H)[29]. Previous studies have indicated black people show polymorphic variation frequencies within alleles NR21, BAT25 and BAT26 of approximately 10%[30–32]. For tumour samples that demonstrated instability in only 2 of these 3 markers, matched normal tissue was assessed to determine the instability status. In cases where no normal tissue could be assessed, samples were regarded as MSS.

MMR IHC was performed on MSI-H samples that were not previously assessed, utilizing the Dako EnVision™ FLEX detection system Kit and Autostainer Link 48 equipment (Dako, Glostrup, Denmark) according to manufacturer's instructions. The primary monoclonal antibodies used were FLEX monoclonal mouse antibodies against MLH1 (clone ES05, ref ISO79, ready-to-use), MSH2 (clone FE11, ref IRO85, ready-to-use), MSH6 (clone EP49, ref IRO86, ready-to-use) and PMS2 (clone EP51, IRO87, ready-to-use) (Dako, Glostrup, Denmark). These were applied to 4-μm deparaffinized formalin fixed paraffin-embedded (FFPE) tissue sections. Blockage of endogenous peroxide activity was carried out with the EnVision™ FLEX Peroxidase-Blocking Reagent (Dako, Glostrup, Denmark) and Antigen retrieval performed at 97 °C for 20 minutes (pH 9.0). Antigen-antibody reaction was visualized with the DAB (Diaminobenzidine) solution, and subsequently counter-stained with hematoxylin. Each sample was screened by an expert pathologist for the absence or presence of expression of each of the

4 MMR proteins. Negative protein expression was defined as complete absence of nuclear staining within tumor cells. Expression of MMR proteins in normal epithelium, lymphocytes and stromal cells was used as positive internal controls for each case. The four deficient (d) MMR patterns assessed were 1: dMLH1/PMS2; 2: dMSH2/MSH6; 3: dMSH6 and 4: dPMS2.

All MSI-H samples were assessed for BRAF V600E mutations using the real-time Therascreen RGQ BRAF V600E mutation kit (Qiagen, Whitehead Scientific WHS), according to the manufacturer's instructions. The kit uses ARMS® and Scorpions® technology, and includes a control assay to evaluate the adequacy of quality and quantity of DNA in a sample, before assessing the presence or absence of mutated DNA. Results were only deemed valid if the control run passed.

Data obtained from the molecular characterization study (2011–2012) led to a more comprehensive study which included cases with an MSI status from histological reports for the total 5-year study period (2011–2015).

Statistical analysis. All clinical data was collected in an excel sheet and statistical analysis was performed using Stata Intercooled 7.0 (Stata, College Station, TX, USA) and Graphpad Prism version 7 (GraphPad Software, La Jolla, CA, USA). MSS and MSI-L cases were grouped together into one group (MSS), to establish unique associations with the counterpart MSI-H group. Patients were also analysed on the basis of ethnicity: black (African) versus other ethnic groups i.e., white (Caucasian), coloured (Mixed Ancestry), Indian (Asian); age and gender. The ability to draw statistically significant results on all ethnic groups was not possible due to small case numbers of certain groups. For this reason, White, Indian and Coloured patients were grouped into one "Other Ethnic Group" (OEG) to allow comparison with their black counterpart. Histopathological features were assessed as stated in the methodology section, and associations were evaluated using Fischer's exact and Chi-square tests. A result with a P value less than 0.05 was considered statistically significant.

Results

Patient demographics. A total 439 of 672 CRC patients diagnosed between January 2011-December 2015 had MSI CRC status assessment. Of these cases, 247 (56%) were male and 192 (44%) female, with an overall median age of 58 years. Black patients contributed to 60% (262/439) of cases. (Column1 of Table 1).

Pathological characteristics. Further descriptive analysis was done with respect to histological characteristics, with left sided tumours accounting for the majority of cases (63%; 273/430) (Column1 of Table 1). The most common subtype was invasive adenocarcinoma (384/430; 89%), followed by invasive mucinous adenocarcinoma (30/430; 7%). AJCC tumour stage II and III accounted for the majority of cases (259/287; 90%) (Table 1). Further stratification by MSI and MSS subtype is illustrated in the 2nd and 3rd column in Table 1.

MSI CRC molecular characterization (2011–2012). MSI PCR performed on 267 cases (2011–2012) illustrated the majority were MSS (191/267; 71%), followed by MSI-L (45/267; 17%) and MSI-H (31/267; 12%). MMR IHC results demonstrated dMLH1 in most MSI CRC cases (13/31; 42%), followed by dMSH2/6 (10/31; 32%), dPMS2 (2/31; 6%) and dMSH6 (1/31; 3%). BRAF mutational analysis was performed on all MSI-H cases with only 10% (3/31) harbouring a mutation, and all 3 (3/13; 23%) occurred in dMLH1 tumours (See Fig. 1).

MSI versus MSS CRC subtyping (2011–2015). Male patients comprised 56% of the entire CRC cohort. Correlation between subtype and age was found ($P = 0.0033$) as median age was significantly lower in MSI CRC compared to the MSS subtype (51 vs 59 years respectively) (Table 1). A higher frequency of MSI tumours was seen in blacks ([22/148; 15%] versus OEG patients [9/119; 8%]), for the period 2011–2012. A significant relationship was seen between CRC subtype and tumour site ($P < 0.0001$), as MSS was associated with left sided tumours (261/370; 70%) and MSI with the right sided tumours [(47/60; 78%). A link between CRC molecular subtype and tumour subtype was also found ($P < 0.0001$), as mucinous adenocarcinoma occurred at a more frequent rate in MSI CRC; (13/60; 22%) than MSS CRC (17/370; 5%). Tumour infiltrating lymphocytes (TIL) response and subtype was found to have a correlation ($P = 0.0045$) as this occurred more often in the MSI group (26/47; 55%) than the MSS (77/236; 33%). Polyp subtypes tubular adenoma (TA) and tubulovillous adenoma (TVA) were associated with molecular subtypes ($P = 0.0216$) as TAs occurred more frequently in MSS CRC (49/84; 58%) and TVAs in MSI CRC (11/17; 65%).

Within the MSI CRC subgroup, additional association evaluations between ethnicity, gender, tumour site, defective MMR proteins and BRAFV600E mutation status were performed (Table 2). A correlation with age was shown ($P = 0.0006$) as black patients were significantly younger compared to OEG patients (median age: 47 versus 62, respectively). Tumour site and ethnicity showed an association for 2011–2012 data ($P = 0.0105$), as the majority of tumours were right sided within OEG patients (8/9; 89%), whereas black patients had left (8/22; 36%) and right-sided tumours (13/22; 59%) (Supplementary Data Table 2). Data for 2011–2015 however showed no such association ($P = 0.1817$). An association between ethnic groups and dMMR protein expression was found ($P = 0.0098$), where dMLH1/PMS2 expression was more commonly seen in OEG patients (15/22; 68%), and dMSH2/6 in black patients (19/39; 49%). BRAF V600E mutation status and ethnicity were related in the 2011–2012 data ($P = 0.0187$) (Supplementary Data Table 2), as all black patients were wild-type for BRAF gene status, however within the OEG, BRAF mutations were only found in dMLH1 tumours (3/5; 60%). For the 2011–2015 BRAFV600E data analysis, 2011–2012 results were dominantly represented with an additional 3 cases from 2013–2015 reports with BRAFV600E status. An increased association between BRAFV600E and OEG patients was shown ($P = 0.0045$) for this period.

Table 3 illustrates a multivariate analysis including variables considered clinically significant for MSI status. Total number of observations were 277. Age, tumour site, mucinous adenocarcinomas and TILS associated after controlling for confounding factors. Patients >50 years were 67% less likely to have MSI and right sided tumours were 8X more likely to have MSI. Invasive adenocarcinomas were 73% less likely to show MSI than mucinous

	Number of cases (%)	CRC subtype		Statistical analysis: Significance indicated by*
Frequency:	**All CRC cases**	**MSI**	**MSS**	
2011–2015	**439**	**61 (14)**	**378 (86)**	
Demographical data (2011–2015)				
GENDER	**439**	**61**	**378**	
Male	247 (56)	34 (56)	213 (56)	P = 1.0000
Female	192 (44)	27 (44)	165 (44)	
AGE	**436**	**61**	**375**	
Min-Max	15–92	27–77	15–92	
Mean ± SD	56 ± 14	52 ± 13	57 ± 14	**P = 0.0033****
Median	58	51**	59	
P25-P75 (Interquartile Range)	47–67	40–62	48–68	
95% CI	[55–58]	[48–55]	[56–59]	
Categorical Age	**436**	**61**	**375**	
≤50 years	137 (31)	29 (48)**	108 (29)	
>50 years	299 (69)	32 (52)	267 (71)	**P = 0.0046****
Ethnic groups	**439**	**61**	**378**	
Black	262 (60)	39 (64)	223 (59)	P = 0.4858
Other Ethnic Groups (OEG)	177 (40)	22 (36)	155 (41)	
Histological characteristics (2011–2015)				
TUMOUR SITE	**430**	**60**	**370**	
Left (distal from splenic flexure)	273 (63)	12 (20)	261 (70)	**P < 0.0001*****
Right (proximal to splenic flexure)	154 (36)	47 (78)***	107 (29)	
Left and Right	3 (1)	1 (2)	2 (1)	
TUMOUR SUBTYPE	**430**	**60**	**370**	
Invasive Adenocarcinoma	384 (89)	45 (75)	339 (91)	**P < 0.0001*****
Mucinous Adenocarcinoma	30 (7)	13 (22)***	17 (5)	
Signet Ring Cell Adenocarcinoma	16 (4)	2 (3)	14 (4)	
TUMOUR GRADE	**371**	**45**	**326**	
Low Grade	346 (93)	40 (89)	306 (94)	P = 0.1022
Medium/High Grade	25 (7)	5 (11)	20 (6)	
AJCC TNM STAGING	**287**	**45**	**242**	
I	11 (4)	2 (4)	9 (4)	
II	101 (35)	17 (38)	84 (35)	P = 0.6226
III	158 (55)	22 (49)	136 (56)	
IV	17 (6)	4 (9)	13 (5)	
TUMOUR INFILTRATING LYMPHOCYTES (TIL)	**283**	**47**	**236**	
None	180 (64)	21 (45)	159 (67)	**P = 0.0045****
Mild-moderate	103 (36)	26 (55)**	77 (33)	
CHROHN'S LIKE INFLAMMATORY RESPONSE	**283**	**47**	**236**	
None	206 (73)	34 (72)	172 (73)	P = 1.0000
Mild-moderate	77 (27)	13 (28)	64 (27)	
LYMPHATIC INVASION	**346**	**54**	**292**	
Absent	242 (70)	34 (63)	208 (71)	P = 0.2582
Present	104 (30)	20 (37)	84 (29)	
POLYPS	**313**	**49**	**264**	
Absent	210 (67)	31 (67)	179 (68)	P = 0.6197
Present	103 (33)	18 (33)	85 (32)	
POLYP SUBTYPE	**101**	**17**	**84**	**P = 0.0216* TA vs TVA**
Hyperplastic Polyp	3 (3)	0 (0)	3 (4)	
Pseudopolyp	2 (2)	1 (6)	1 (1)	
Sessile serrated Adenoma (SSA)	1 (1)	1 (6)	0 (0)	
Tubular Adenoma (TA)	53 (52)	4 (23)	49 (58)*	
Tubulovillous Adenoma (TVA)	42 (42)	11 (65)*	31 (37)	

Table 1. Descriptive analysis of CRC cases diagnosed at CMJAH between (2011–2015) with Microsatellite Instability status, stratified by CRC subtypes: MSI versus MSS.

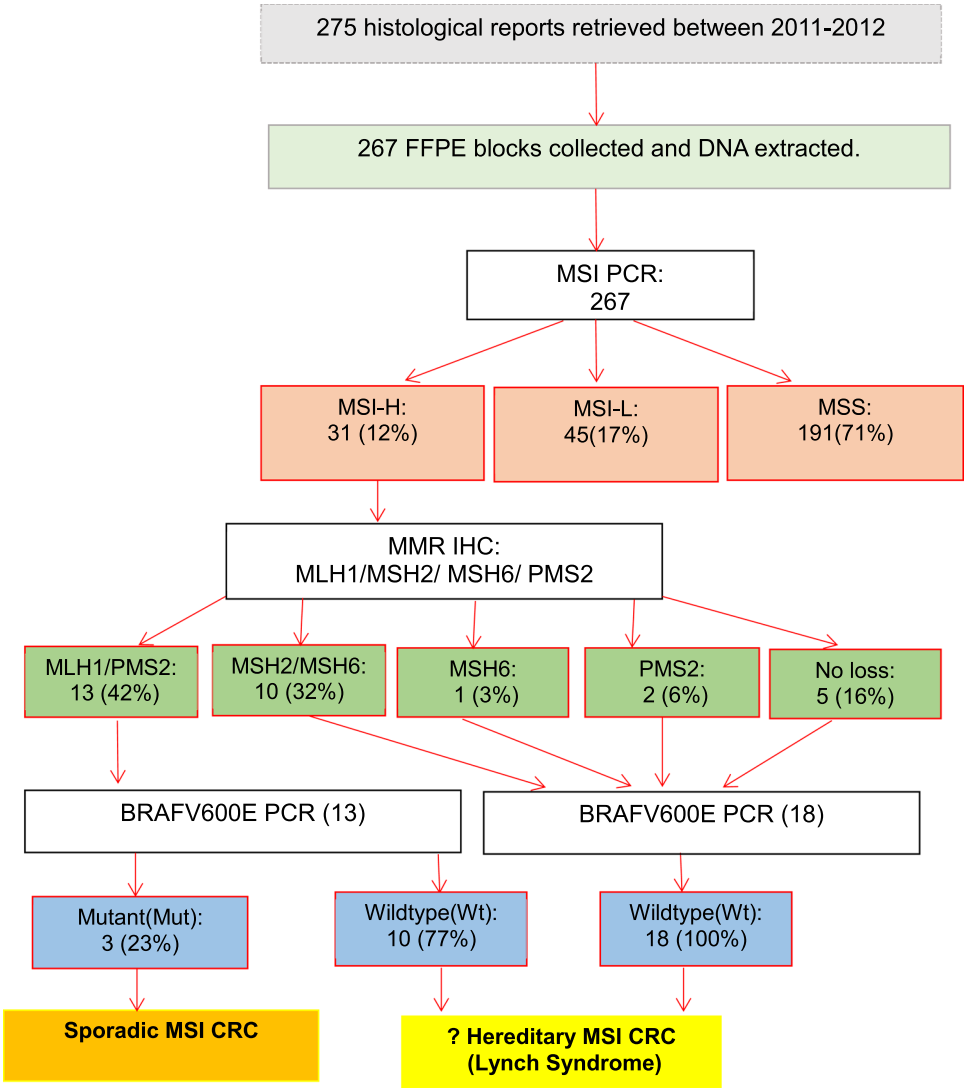


Figure 1. Flow diagram illustrating the molecular methodology and frequency results of CRC cases diagnosed between 2011–2012. MSI CRC was found in 12% of CRC cases, with predominant MLH1 loss, however only 23% of deficient MLH1 cases harboured BRAFV600E mutations. These findings suggest a large proportion of SA MSI CRC patients could develop through a hereditary pathway.

adenocarcinomas. Absence of a TIL response was 60% most likely not to have MSI in comparison to those with TILs. TVAs were not included due to the small sample size, reducing the power and value of the analysis.

Discussion

MSI CRC was detected in 12% of CRC patients diagnosed during the period 2011–2012, however when stratified by ethnic groups, a higher frequency was found in black versus OEG patients although only of borderline significance (15% versus 8% respectively; $P = 0.0831$). Black patients with MSI tumours occurred in younger patients with left- (36%) and right-sided (59%) tumours, associated with dMSH2/6 (45%), and dMLH1/PMS2 (36%) tumours with a lack of BRAF mutations, indicating a possible LS cause[10,33–35]. MSI CRC in Western population groups are shown to dominantly occur in female patients with right-sided tumours, a serrated morphology and BRAFV600E mutations, MLH1 protein loss due to promoter methylation and a high CIMP molecular phenotype[2,20].

In the SA cohort, MSI CRC was associated with younger age, right sided tumours, mucinous adenocarcinomas, TIL response, and TVA precursor lesions. This correlates well with findings in the literature[36,37]. Histopathological features were further stratified by ethnic groups (black versus OEG) to assess if any associations occurred with regard to gender, age, tumour site, MMR protein expression profile and BRAF mutation status. Within the 2011–2012 cohort, patients within the OEG were predominantly older (median age: 62), female (67%), possessed right sided tumours (89%), with deficient MLH1 (56%) expression, of which 60% harboured BRAF mutations. The 2011–2015 cohort however demonstrated an equal ratio of male to female patients, which could signify an under-diagnosis of female patients with MSI within the OEG. All other variables illustrated

		MSI CRC stratified by ethnic groups (2011–2015)		
		No. of cases (%)		
Demographic Data	**Number of cases (%)**	**Black**	**OEGs**	**Statistical Significance**
GENDER	**61**	**39**	**22**	
Male	34 (56)	23 (59)	11 (50)	P = 0.5946
Female	27 (44)	16 (41)	11 (50)	
AGE	**61**	**39**	**22**	
Median	51	47***	62	
Min-Max	27–77	28–74	27–77	
Mean ± SD	52 ± 13	47 ± 11	59 ± 14	**P = 0.0006*** **
P25-P75	40–62	38–55	48–70	
95% CI	[48–55]	[44–51]	[53–65]	
Categorical Age	**61**	**39**	**22**	
≤50 years	29 (48)	23 (59)*	6 (27)	**P = 0.0316***
>50 years	32 (52)	16 (41)	16 (73)*	
TUMOUR SITE	**60**	**39**	**21**	
Left	12 (20)	10 (26)	2 (10)	
Right	47 (78)	28 (72)	19 (90)	P = 0.1817 Left vs Right
Left and Right	1 (2)	1 (2)	0 (0)	
MMR PROTEIN IHC	**61**	**39**	**22**	
dMLH1/PMS2	31 (51)	16 (41)	15 (68)**	
dMSH2/MSH6	22 (36)	19 (49)**	3 (14)	**P = 0.0098**** MLH1/ PMS2 vs MSH2/MSH6
dMSH6	1 (2)	1 (2.5)	0 (0)	
dPMS2	2 (3)	1 (2.5)	1 (4)	
pMMR	5 (8)	2 (5)	3 (14)	
***BRAF V600E**	**34**	**24**	**10**	
Wildtype	30 (88)	24 (100)**	6 (60)	**P = 0.0045****
Mutation	4 (12)	0 (0)	4 (40)**	

Table 2. Descriptive analysis of MSI CRC diagnosed at CMJAH (2011–2015), stratified by ethnic groups: Black versus Other Ethnic Groups (OEGs). Left (Colon distal from splenic flexure); Right (Colon proximal to splenic flexure); Microsatellite Instability (MSI); Mismatch repair (MMR); Deficient (d); Proficient (p). Significance indicated by an asterix. *Majority of the samples screened for BRAFV600E mutations (31/34; 91%) were from the 2011–2012 cohort. The remaining 3 were retrieved from histological reports for the remaining period (2013–2015).

increased statistical associations within the 5-year cohort study. Collectively these features suggest the majority of OEG patients follow the more well established sporadic MSI CRC pathway[19,20]. Within the black patient group, all 2011–2012 data analysed with statistical significant associations revealed increased significance within the 5 year cohort, with the exception of tumour site where 2011–2012 data illustrated significance with an increased incidence of left-sided MSI tumours. This could possibly be due to pathologists generally screening more right than left-sided tumours for MSI CRC.

MSI CRC also showed a high percentage of proficient (p) MMR expression (16%) for the universal 2-year screen, despite high concordance rates (95–96%) for MSI PCR versus IHC reported[38]. This could possibly be indicative of aberrant non-functional proteins expressed or an alternative MMR protein involved in the development of MSI in this group of patients. This would suggest the 5 mononucleotide panel for MSI testing is the more sensitive tool for detecting instability in the South African setting. Perhaps this could be explored further for alternative causes of dMMR in these patients. For example, MSH3 loss of expression has recently been shown to be associated with MSI in African American patients[39,40].

Multiple logistic regression analysis was also conducted to control for the effect of confounding factors, young age (≤50 years), right-sided tumours, mucinous adenocarcinomas and TILS were associated with MSI CRC in SA patients.

Limitations of the study included patient selection bias as patients from the 2013–2015 cohort were only selected if an MSI status was available. In addition, only a few patient samples with available data in certain subgroup analysis, such as TNM stage of disease, TILs, polyp subtype could be analysed, and this is mainly due to biopsy samples also included in the cohort. Only a subset of MSI samples (34/61; 56%) for the period 2011–2015 were included for BRAFV600E analysis, these largely from cases between period 2011–2012. This also represents a limitation of the study. The majority of patients diagnosed at CMJAH are of the black population group, therefore selection bias could play a role in the selection effect due to smaller sample numbers in the comparator OEG patient group. Previous MSI studies in SA however, have largely shown MLH1 mutations as the cause of hereditary and sporadic CRC in OEG patients (specifically the coloured and white population groups in the Western and Northern Cape regions of SA)[23,41,42]. A study carried out by Cronje *et al*. in 2009 at CMJAH in Johannesburg

Variable	Multivariate	
	Odds ratio with CI	P-Value
Gender		
Male	0.834 (0.394, 1.762)	0.634
Age Category		
>50 years	0.330 (0.146, 0.745)	**0.008****
Ethnic Group		
Black	0.902 (0.411, 1.983)	0.799
Tumour Site		
Right	7.661 (3.150, 18.630)	**0.000*****
Subtype		
Invasive Signet ring cell	0.971 (0.007, 1.249)	0.074
Invasive Adenocarcinoma	0.270 (0.099, 0.731)	**0.010***
Grade		
LG	0.535 (0.142, 2.007)	0.354
Tumour Infiltrating Lymphocytes		
None	0.392 (0.182, 0.845)	**0.017***
Lymphatic Invasion		
Present	0.829 (0.376, 1.825)	0.642

Table 3. Multiple logistic regression factors at risk for MSI CRC. Number of observations were 277. Young Patients under 50 years, with right-sided tumours, displaying mucinous features with TILs were more likely to have MSI. Significance indicated by an asterix.

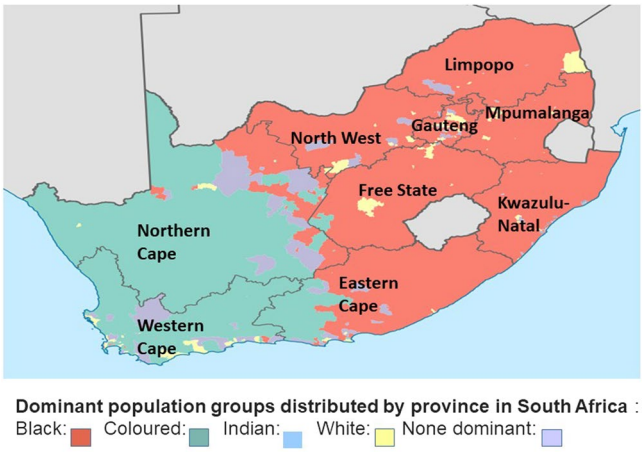


Figure 2. Map of South Africa illustrating the distribution of population groups by the different provinces, (adapted from the Wikipedia map of South Africa 2011)[54].

demonstrated a large number of young black patients had increased loss of MMR protein expression, particularly MSH2, in comparison to their white counterpart, also suggesting a heritable cause[21]. Findings from this study confirm dMLH1 and dMSH2 associations within young OEG and black SA patients respectively.

South Africa is comprised of 9 provinces, with an estimated mid-year population of 58.78 million. The Northern Cape comprises 2.2% of the population, Western Cape 11.5%, and Gauteng 25.8%[43,44]. The Western and Northern Cape comprises mainly of the coloured population within SA, and LS have largely been identified in these 2 provinces (See Fig. 2)[23,41,45]. Genetic services are offered to newly diagnosed LS patients and family members in these regions mainly through academic centres based in the Western Cape, and to smaller surrounding towns and rural areas through outreach programmes[23,41,45,46].

The Gauteng province, although the smallest, is the most densely populated and urbanized with nearly 700 people per square kilometre[43,44]. This is largely due to the discovery of gold in Johannesburg during the 1800's,

with a large influx of migrants from Africa and Europe[46]. The black population is the predominant group (77.4%) followed by white (15.6%) in this region[43,44]. LS genetic screening in Gauteng and remaining 6 provinces have been poor to non-existent, even though preliminary findings have suggested a possible hereditary cause, particularly in black patients from the 1990's[21,47–49].

The healthcare financial system in SA is based on private and public funding, with the majority (57%) of the population utilizing public healthcare services, however 80% of healthcare expenditure in SA occurs in the private sector[46,50]. Genetic services such as testing and counselling are mostly offered in academic centres, and subsidies for these services are limited primarily due to the high medical costs associated with the HIV/AIDS epidemic[46]. Pathologists within the National Health Laboratory Services include a possible hereditary cause in histopathological reports of these patients with suspected LS, but genetic services are limited to counselling, particularly within the public sector of the Gauteng province. Colonoscopic surveillance of family members at risk for LS is difficult and expensive in a resource-poor country, however previous LS studies carried out within the Western Cape region of SA have shown the identification of the causative-mutation simplifies and reduces costs of surveillance considerably[22,45,51].

If LS is the more prevalent MSI subtype in black SA patients, identifying the causative mutation together with regular colonoscopy screening is recommended for all affected individuals and families by the 2nd decade of life, to be repeated every 1–2 years[52]. LS patients treated with aspirin have a considerably reduced risk and reduced mortality rates associated with CRC[27,53].

Conclusion

This study revealed MSI in black SA CRC patients is associated with earlier age onset, higher frequency of MSH2/6 loss and a lack of BRAFV600E mutations, suggesting Lynch or "Lynch-Like Syndrome" (LLS) as the main hereditary driver. Implementation of universal MSI screening is recommended as a routine diagnostic test within the SA setting to increase the detection rate of LS or LLS. This data warrants further molecular investigations in defining the underlying germline or somatic mutations. Appropriate early detection, surveillance and prevention strategies will reduce the risk and improve overall management of the disease in suspected LS patients and affected family members, particularly in the black population group in SA.

Compliance with ethical standards. This was a retrospective study on already available biological material and ethics clearance was obtained from the Human Ethics Research Committee, Faculty of Health Sciences: Ethics clearance reference number: M120994.

Data availability

The dataset generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

M.M., Y.P. and C.P. were involved in the design and research methodology of the study. M.M. performed the analysis and wrote the manuscript. R.M. and P.M. contributed to data analysis. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Chapter 4:

Left-sided Colorectal Cancer distinct in Indigenous African patients compared to Other Ethnic Groups in South Africa.

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RESEARCH

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Left-sided colorectal cancer distinct in indigenous African patients compared to other ethnic groups in South Africa

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Abstract

Introduction: A large proportion of indigenous African (IA) colorectal cancer (CRC) patients in South Africa are young (< 50 years), with no unique histopathological or molecular characteristics. Anatomical site as well as microsatellite instability (MSI) status have shown to be associated with different clinicopathological and molecular features. This study aimed to ascertain key histopathological features in microsatellite stable (MSS) and low-frequency MSI (MSI-L) patients, to provide insight into the mechanism of the disease.

Methods: A retrospective cohort (2011–2015) of MSS/MSI-L CRC patient samples diagnosed at Charlotte Maxeke Johannesburg Academic Hospital was analyzed. Samples were categorized by site [right colon cancer (RCC) versus left (LCC)], ethnicity [IA versus other ethnic groups (OEG)] and MSI status (MSI-L vs MSS). T-test, Fischer's exact and Chi-square tests were conducted.

Results: IA patients with LCC demonstrated an increased prevalence in males, sigmoid colon, signet-ring-cell morphology, MSI-L with BAT25/26 marker instability and advanced disease association.

Conclusion: This study revealed distinct histopathological features for LCC, and suggests BAT25 and BAT26 as negative prognostic markers in African CRC patients. Larger confirmatory studies are recommended.

Keywords: Colorectal cancer, African, Microsatellite stable, Low level microsatellite instability, BAT25, BAT26

Introduction

Right-sided colon cancer (RCC) and left-sided colon cancer (LCC) show distinct mechanisms of development and are associated with different clinicopathological features [1–3]. During embryological development, the RC develops from the midgut and the LC from the hindgut, supporting the view that RCC and LCC develop through different developmental/embryological pathways genetic mechanisms [1, 4]. The incidence of RCC (~30%) is lower than LCC (~70%). RCC presents with larger tumours, a higher rate of tumour node metastases (TNM stage), mucinous features, and comprises of an overall poorer survival than LCC [1, 2, 4–6]. Older female patients are at higher risk of developing RCC compared to younger male patients associated with increased risk of developing LCC [1]. The literature shows population groups with a lower risk of developing CRC moving to high-risk areas acquire the risks associated with the new area, and this could be linked to dietary, environmental, cultural and genetic factors [1, 7]. This speaks to a possible role for the increasing incidence of CRC in indigenous African (IA) patients moving from rural to urbanized areas [8–10].

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There are 3 main pathways involved in the development of CRC: 1) Microsatellite instability (MSI) caused by a defective mismatch repair (MMR) system, most often (~70–95%) caused by an alteration of MLH1,2) Chromosome instability (CIN) pathway which develops due to gross chromosomal changes and 3) the CpG island methylator phenotype (CIMP) pathway, arising through methylation of CpG islands in promoter sequences, leading to inactivation of tumour suppressor genes throughout the genome [11, 12]. MSI and CIMP tumours mostly occur in the right colon, whereas CIN CRC is associated with LCC [11, 13, 14]. Four main consensus molecular subtypes (CMS) were established in 2015. These are differentiated by unique molecular features: CMS1 (14%, MSI pathway, immune activation); CMS2 (37%, Canonical WNT/MYC pathway, epithelial signature); CMS3 (13%, epithelial and metabolic dysregulation), and CMS4 (23%; Mesenchymal TGF- β pathway; stromal invasions and angiogenesis) [11, 15].

To date, fewer research outputs on low frequency microsatellite instability (MSI-L) CRC have been published compared to MSI-H and MSS CRC. MSI-L is usually grouped with MSS CRC, as literature states all CRCs display some level of MSI [16, 17]. Some researchers interpret MSI-L tumours as precursors of MSI-H CRC, whereas others believe it to be a completely separate entity [18, 19]. MSI-L tumours have illustrated different clinicopathological features and have been considered to be a worse prognostic group in a few CRC studies [20–23]. Jass et al. reported that MSI-L LCC showed distinctive clinicopathological features, with a male predilection, a moderately differentiated histopathological grade, KRAS mutations, CIMP-Low status and DNA aneuploidy. In contrast, MSI-L RCC was found to occur more frequently in females, being associated with a serrated adenoma precursor lesion, mucinous adenocarcinoma histological subtype and poorly differentiated grade. BRAF mutations, CIMP-High status and diploid DNA content, features associated with a worse prognosis, were also associated with the MSI-L RCC group [24].

A disproportionate number of indigenous African (IA) patients display a younger age of onset (< 50 years of age) with no distinct histopathological features to assist with early diagnosis and management [25–28]. Previous work by McCabe et al. described MSI-H CRC in detail according to ethnicity groups and found an increased association of MSH2/MSH6 MMR protein expression loss in right sided CRC in young IA patients [29]. This study aims to characterize proficient MMR (MSS/MSI-L) CRC, by ethnicity (IA versus OEG) and anatomical site (LCC versus RCC), to potentially identify a unique subtype associated with young IA CRC patients.

Methodology

Patient demographics and tumour histopathological characterization

This retrospective study was conducted on a 5-year cohort (2011–2015) of 428CRC patient samples with known anatomical site and MMR status, diagnosed at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) branch of the National Health Laboratory Service (NHLS). Informed consent was waived by the Human Research Ethics Committee (HREC) (Medical) of the University of the Witwatersrand for this study, as research was conducted under the institutional blanket ethics clearance (M10744) obtained from the HREC (Medical), which allows for research to be carried out on all archived pathology specimens without informed consent from study participants. Additional project-specific ethical clearance was also obtained from the HREC (Medical) (M120994), and all tests were performed according to the relevant guidelines and regulations. A total of 59 (14%) patient samples had a deficient MMR (dMMR) profile (MSI-H) and the remaining 369 (86%) a proficient MMR (pMMR) status. All pMMR CRC cases were categorized into 4 groups (see Table 1). Tumour site the main group: RCC (tumour primary site proximal to splenic flexure) versus LCC (tumour primary site distal from splenic flexure), further sub-grouped by ethnicity: Indigenous African (IA) versus Other Ethnic Groups (OEG) [Caucasian, Mixed Ancestry, Asian]. Demographic and histopathological information were analyzed within these categories i.e., gender, age, tumour subtype, grade, site, TNM stage (American Joint Committee on Cancer [AJCC] TNM stage), presence of tumour infiltrating lymphocytes (TIL) using the Klintrup-Mäkinen scoring assessment, Crohn's-like inflammatory reaction (CLR), polyp subtype, venous, perineural and lymphatic invasion, all of which were obtained from histology reports. Immunophenotypic profiling of TIL was not conducted. No family histories were available from these reports.

MSS versus MSI-L molecular subtyping

Samples were screened for proficient MMR status through MMR immunohistochemistry (IHC) and/or MSI polymerase chain reaction (PCR). IHC images and MSI electropherograms are illustrated in Figs. 1 and 2 respectively. The MMR IHC panel included antibodies targeting MutL Homolog 1(MLH1), MutS Homolog 2 and 6 (MSH2/ MSH6) and Post Meiotic Segregation Homolog-2 (PMS2) protein expression. Only samples with a MMR proficient profile detected via IHC and a MSS or MSI-L profile determined via PCR were included in this cohort [29]. MSI PCR included the 5-mononucleotide PCR panel (NR27, NR21, NR24, BAT25 and

Table 1 Descriptive analysis of MSS CRC cases diagnosed at CMJAH between (2011–2015). Categorized by site: LCC *vs* RCC and Ethnicity: Indigenous African (IA) *vs* Other Ethnic Group (OEG)

		Total number cases (%)	Left-sided Colon Cancer (LCC)		Statistical analysis:	Right-sided Colon Cancer (RCC)		Statistical analysis:
			IA	OEG		IA	OEG	
Frequency		**369**	**154 (42)**	**108(29)**		**63(17)**	**44(12)**	
Demographical data								
GENDER		**369**	**154**	**108**	***P*=0.0111***	**63**	**44**	***P*=0.6932**
Male		**209(57)**	**98(64)***	**51(47)**		**34(54)**	**26(59)**	
Female		**160(43)**	**56(36)**	**57(53)**		**29(46)**	**18(41)**	
AGE		**365**	**152**	**107**	***P*<0.0001*****	**62**	**44**	***P*=0.0103***
Min–Max		**15–92**	**20–90**	**28–92**		**15–79**	**25–86**	
Mean ± SD		**57 ± 14**	**53 ± 15**	**62 ± 13**		**54 ± 13**	**60 ± 11**	
Median		**59**	**54*****	**62*****		**55***	**61***	
P25-P75 (Interquartile Range)		**47–67**	**41–65**	**55–72**		**47–64**	**54–68**	
95% CI		**[55-58]**	**[51-56]**	**[60-65]**		**[51-57]**	**[57-65]**	
Categorical Age		**365**	**152**	**107**	***P*<0.0001*****	**62**	**44**	***P*<0.0001*****
≤ 50 years		**107 (29)**	**62(41)*****	**15(14)**		**24(39)*****	**6(14)**	
> 50 years		**258(71)**	**90(59)**	**92(86)**		**38(61)**	**38(86)**	
Histological characteristics								
TUMOUR SUBTYPE		**360**	**152**	**104**	***P*=0.0257***	**61**	**43**	***P*=0.2608**
Invasive Adenocarcinoma		**329(91)**	**138(91)**	**100(96)**		**54(89)**	**35(81)**	
Mucinous Adenocarcinoma		**17(5)**	**4(3)**	**4(4)**		**3(5)**	**6(14)**	
Signet Ring Cell Adenocarcinoma		**14(4)**	**10(6)**	**0(0)**		**4(6)**	**2(5)**	
TUMOUR SITE:		**359**	**151**	**105**	***P*=0.0221***	**61**	**42**	***P*=0.2431**
Left	**Right**							
Splenic Flexure	**Hepatic Flexure**	**14(4)**	**5(3)**	**0(0)**		**6(10)**	**3(7)**	
Descending colon	**Ascending Colon**	**58(16)**	**21(14)**	**11(10)**		**11(18)**	**15(36)**	
Sigmoid	**Transverse Colon**	**83(23)**	**46(31)***	**21(20)**		**10(16)**	**6(14)**	
Rectum	**Caecum**	**204(57)**	**79(52)**	**73(70)**		**34(56)**	**18(43)**	
TUMOUR GRADE		**348**	**148**	**95**	***P*=0.0930**	**61**	**44**	***P*=1.0000**
Low Grade (LG)		**316(91)**	**132(89)**	**91(96)**		**54(89)**	**39(89)**	
High Grade (HG)		**32(9)**	**16(11)**	**4(4)**		**7(11)**	**5(11)**	
AJCC TNM STAGING		**240**	**85**	**61**	***P*=0.0922**	**57**	**37**	***P*=0.4995**
I-II		**92(38)**	**31(38)**	**31(51)**		**20(36)**	**10(27)**	
III-IV		**148(62)**	**54(62)**	**30(49)**		**37(64)**	**27(73)**	
TUMOUR INFILTRATING LYMPHOCYTES (TIL)		**230**	**81**	**60**	***P*=0.8512**	**54**	**35**	***P*=0.6591**
None		**156(68)**	**58(72)**	**44(73)**		**34(63)**	**20(57)**	
Mild-moderate		**74(32)**	**23(28)**	**16(27)**		**20(37)**	**15(43)**	
CROHN'S LIKE INFLAMMATORY RESPONSE		**230**	**81**	**60**	***P*=0.4406**	**54**	**35**	***P*=0.3392**
None		**168(73)**	**62(77)**	**42(70)**		**41(76)**	**23(66)**	
Mild-moderate		**62(27)**	**19(23)**	**18(30)**		**13(24)**	**12(34)**	
LYMPHATIC INVASION		**285**	**115**	**76**	***P*=0.2921**	**56**	**38**	***P*=0.2896**
Absent		**203(71)**	**85(74)**	**62(82)**		**36(64)**	**20(53)**	
Present		**82(29)**	**30(26)**	**14(18)**		**20(36)**	**18(47)**	
VENOUS INVASION		**232**	**82**	**59**	***P*=0.3937**	**55**	**36**	***P*=1.0000**
Absent		**181(78)**	**68(83)**	**45(76)**		**41(75)**	**27(75)**	
Present		**51(22)**	**14(17)**	**14(24)**		**14(25)**	**9(25)**	
PERINEURAL INVASION		**241**	**91**	**60**	***P*=0.1110**	**54**	**36**	***P*=1.0000**
Absent		**187(78)**	**67(74)**	**51(85)**		**41(76)**	**28(78)**	
Present		**54(22)**	**24(26)**	**9(15)**		**13(24)**	**8(22)**	
POLYPS		**80**	**31**	**19**	***P*=0.5516**	**16**	**14**	***P*=0.7131**
Tubular Adenoma (TA)		**49(58)**	**21(68)**	**11(58)**		**10(63)**	**7(50)**	
Tubulovillous Adenoma (TVA)		**31(37)**	**10(32)**	**8(42)**		**6(37)**	**7(50)**	

Table 1 (continued)

	Total number cases (%)	Left-sided Colon Cancer (LCC)		Statistical analysis:	Right-sided Colon Cancer (RCC)		Statistical analysis:
		IA	OEG		IA	OEG	
MSS/MSI-L PCR CONFIRMED CASES	**233**	**94**	**85**	***P*=0.0157***	**28**	**26**	***P*=0.3582**
MSS	**189(81)**	**72(77)**	**77(91)**		**19(70)**	**21(81)**	
MSI-L	**44(19)**	**22(23)**	**8(9)**		**9(30)**	**5(19)**	
MSI PCR MARKERS (MSI-L)	**42**	**22**	**8**	***P*=0.0138***	**7**	**5**	***P*=0.2222**
1 single unstable marker				**BAT25/26**			**BAT25/26**
BAT25	**20(56)**	**11(50)***	**5(62.5)**	**Vs**	**3((43)**	**1(20)**	***Vs***
BAT26	**15(22)**	**11(50)***	**0(0)**	**NR21/24/27**	**3(43)**	**1(20)**	**NR21/24/27**
NR21	**4(12)**	**0(4)**	**2(25)**		**0(0)**	**2(40)**	
NR24	**3(10)**	**0(0)**	**1(12.5)**		**1(14)**	**1(20)**	
NR27	**0(0)**	**0(0)**	**0(0)**		**0(0)**	**0(0)**	

Levels of statistical significance indicated by an asterix *. *P*-values <0.05, is indicated with (*), *P*-values <0.01, (**), and *p*-values < than 0.001 (***)

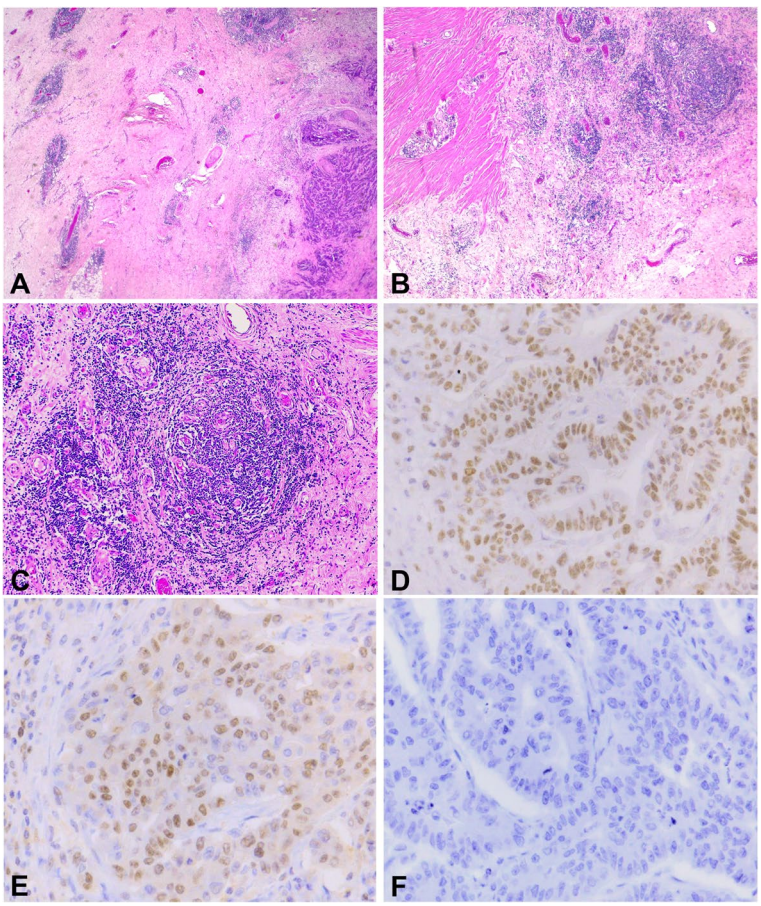


Fig. 1 Haematoxylin and Eosin (**A-C**) images and Immunohistochemistry (**D-F**) images of colorectal (CRC) tissue sections. **A** Low power (40X) of serosa showing CRC and mild Crohn's-like response (CLR). **B** Low (100X) power of serosa with moderate CLR. **C** Similar CLR at higher magnification (200X). **D** MSH2 retained nuclear expression (400X). **E** MLH6 retained nuclear expression (400X). **F** MLH1 loss of nuclear expression, showing MSIphenotype (400X)

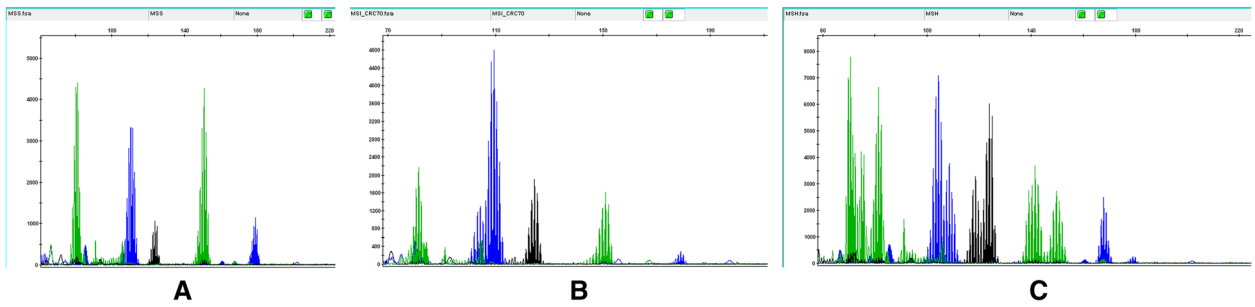


Fig. 2 Electropherograms illustrating instability patterns using the 5-mononucleotide MSI PCR panel. **A** MSS: 5 markers stable **B** MSI-Low: 1 marker unstable **C** MSI-H: 5 markers unstable

BAT26); and a MSS or MSI-L result was ascribed when an allelic size varied in none or only one of the 5 markers respectively [29, 30]. The literature indicates that people of African descent exhibit normal variation within loci BAT25 and BAT26 [31–34]. A multipopulation study by Buhard et al. 2006, revealed approximately 10% show normal variation in one of five markers and 2% in 2 markers [33]. In silico analysis of BAT 25 and BAT 26 PCR primer sets were performed against the global 1000 Genomes dataset (www.internationalgenome.org/data) and local AWI-GEN dataset (https://www.wits.ac.za/research/sbimb/research/awi-gen). Data analysis from the 1000 Genomes dataset revealed 2 single nucleotide polymorphisms (SNPs) in each primer set and occurred at a rare frequency of 0.02%. AWI-GEN data analysis showed one SNP for BAT26 primers at position Chr2: 47,641,434, and occurred at a frequency of 0.5%. No SNPs were detected in BAT25 primer set. The 5-mononucleotide panel remained the assay of choice due to the additional 3 quasimonomorphic mononucleotide repeat markers found in Caucasian and African germ-line DNA, ensuring the panel is extremely sensitive in detecting somatic alterations in MSI-H tumours and distinguishing between MSS/MSI-L tumours. As recommended by the authors Suraweera et al. 2002, in tumour samples with instability in BAT25 and/or 26 markers with a proficient MMR profile via IHC, matched normal samples were assessed to establish the true instability status. In cases where these markers matched instability in normal colon tissue, the status was regarded as normal or germline variation and reported as MSS. In biopsies this was not possible due to the limited size and mixture of normal and neoplastic tissue, increasing the chance of contamination when assessing normal tissue.

Statistical analysis

All data was collected in an excel sheet and statistical analysis was performed using Stata Intercooled 7.0 (Stata, College Station, TX, USA) and Graphpad Prism version 9.0 (Graphpad Software, La Jolla, CA, USA). Unpaired t-tests were used to assess differences between groups on continuous normally distributed variables while Fischer's exact and Chi-square tests were used to assess associations between categorical variables; and a result with a P value less than 0.05 was considered statistically significant. Additionally, analysis of MSI status (MSI-L versus MSS and MSI-H CRC) stratified by tumour site (LCC versus RCC) was conducted to determine if any association occurred with demographic variables (gender, age and ethnic groups) as well as TNM stage, CLR and TIL (Table 2). R/Rstudio was used to perform multiple comparisons on MSI status stratified by tumour site with other categorical variables eg gender to ascertain pairwise associations and the False Discovery Rate (FDR) adjusted p-value (q-value) was reported and considered statistically significant if less than 0.05.

Results

Patient demographics

LCC revealed a higher frequency in males in IA than in OEG patients (64% vs 47%, respectively; $P=0.0111$) (Table 1). The IA patients were younger in comparison to the OEG patients (median age: 54 vs 62 years, respectively; $P<0.0001$).

Pathological characterization

Signet ring cell carcinomas (SRCC) were more frequently found in IA (14/213; 7%) versus OEG patients (2/147; 1%) ($P=0.0221$). When further stratified by site, SRCC was only associated with LCC in IA patients, as compared to OEG patients ($P=0.0257$).

MSI-L, MSS and MSI-H molecular subtypes

Ethnicity was linked to mononucleotide instability markers, with BAT25 and BAT26 markers being more

Table 2 Multiple comparison analysis of CRC cases diagnosed at CMJAH (2011–2015). Categorized by MSI status: MSI-L *vs* MSS *vs* MSI-H CRC and site: LCC *vs* RCC

	Number of cases (%)	MSI-L CRC		MSS CRC		MSI-H CRC		Statistical analysis: Multiple comparison analysis: *(Associations between 2 groups italicized)*
	CRC 2011–2015	A: LCC	B: RCC	C: LCC	D: RCC	E: LCC	F: RCC	
Frequency/ Prevalence	**303**	**30(10)**	**12(4)**	**160(53)**	**42(14)**	**12(4)**	**47(15)**	
GENDER	303	30	12	160	42	12	47	$X^2 = 2.6877$, df = 5
Male	160(53)	16(53)	6(50)	88(55)	24(57)	6(50)	20(43)	$P = 0.748$
Female	143(47)	14(47)	6(50)	72(45)	18(43)	6(50)	27(57)	*No association of sex*
AGE	303	29	12	160	42	12	47	FDR (q value = 0.8238) Q = 0.05
Min–Max	15–92	24–91	37–84	20–92	15–80	33–67	27–77	
Mean ± SD	57 ± 14	56 ± 17	59 ± 13	58 ± 15	58 ± 13	50 ± 12	53 ± 14	*E vs C: P = 0.0415**
Median	59	56	60	59	58	52	51	*F vs C: P = 0.0274**
P25-P75 (IQR)	48–69	40–72	47–67	48–69	53–67	38–61	43–62	
95% CI	[56-59]	[49-62]	[50–67]	[55-60]	[53-62]	[42-57]	[49-57]	
ETHNIC GROUPS	303	30	12	160	42	12	47	$X^2 = 9.2824$, df = 5, $P = 0.09832$
Indigenous African	171(56)	22(76)	7(58)	83(47)	21(50)	10(83)	28(60)	*A vs C: P = 0.0442**
Other Ethnic groups	132 (44)	8(24)	5(42)	77(53)	21(50)	2(17)	19(40)	*E vs C: P = 0.0393**
AJCC TNM STAGING	178	9	8	76	37	7	41	$X^2 = 7.6681$, df = 5, $P = 0.1755$
I-II	77(43)	1(11)	4(50)	38(50)	12(32)	4(57)	18(44)	
III-IV	101(57)	8(89)	4(50)	38(50)	25(68)	3(43)	23(56)	*A vs C: P = 0.0348**
CROHN'S LIKE INFLAMMATORY RESPONSE	172	9	8	73	35	7	40	$X^2 = 11.185$, df = 5, $P = 0.04782$*
Mild-moderate	49(28)	6(67)	1(12)	19(26)	10(29)	4(57)	9(23)	*A vs C: P = 0.0205**
Absent	123(72)	3(33)	7(88)	54(74)	25(71)	3(43)	31(77)	
TUMOR INFILTRATING LYMPHOCYTES	178	15	8	73	35	7	40	$X^2 = 12.898$, df = 5, $P = 0.02435$* *D vs C: P = 0.0497**
Mild-moderate	68(38)	5(33)	2(25)	19(26)	16(46)	5(71)	21(52)	*E vs C: P = 0.0231**
Absent	110(62)	10(67)	6(75)	54(74)	19(54)	2(29)	19(48)	*F vs C: P = 0.0072***

Levels of statistical significance indicated by an asterix *. *P*-values <0.05 indicated with (*), and *P*-values <0.01 with (**)

frequently unstable in IA patients (28/29; 97%) within the MSI-L subgroup. NR21, NR24 and N27 instability was commonly demonstrated in OEG patients (7/13; 54%) ($P = 0.0053$). An increased rate of MSI-L vs MSS CRC (21% vs 9%; $P = 0.0442$) and MSI-H vs MSS CRC (11% vs 2.5%; $P = 0.0393$) was found in the left colon, particularly in IA compared to OEG patients (Table 2). In addition, the MSI-L subtype was associated with more advanced disease stage (III-IV) (8/9; 89%) when compared to MSS CRC (38/76; 50%) ($P = 0.0348$). CLR was associated with MSI-L LCC (6/9; 67%), when compared to subtypes MSI-L RCC (1/8; 12%) ($P = 0.0498$), and MSS LCC (19/73; 26%) ($P = 0.0205$). TIL was associated with subtypes MSI-H LCC ($P = 0.0231$), MSI-H RCC ($P = 0.0072$) and MSS-RCC ($P = 0.0497$).

Discussion

CRC has been shown to have unique clinicopathological features associated with tumour site and different molecular subtypes (CMS 1–4) [11]. CRC molecular subtypes and age of onset have also been described to vary considerably among geographically distinct ethnic groups. Within this cohort, (40%) of IA patients was shown to be younger (< 50 years) compared to OEGs with pMMR CRC. Patients of OEGs in this cohort displayed RCC with poor prognostic factors compared to LCC. Increased frequencies of HG tumours (11% vs 4%) advanced staged tumours (73% vs 49%), perineural invasion (22% vs 15%), mucinous and signet ring morphology (19% vs 4%) were seen in RCC. While the median age of onset was similar for LCC vs RCC (61 vs 62), more males however were diagnosed with RCC (59%) than LCC (47%).

Within the IA patient group, both left and right colon cancers showed similar frequencies for poor prognostic factors. Higher frequencies for HG tumours (11%), advanced tumour stage (62–64%), perineural invasion (24–26%), SRCC (6%), younger age onset (median age 54–55), with more males presenting with LCC compared to RCC (64% vs 54%).

When comparing ethnic groups and right versus left-sided CRC, significant differences were observed for IA

patients with LCC. The IA population group showed a propensity to occur in males, within the sigmoid colon, to present with a SRCC histological pattern, and an MSI-L status. Notably, SRCC is recognized as a rare histological subtype (1%) of CRC and is associated with young adults in other geographical locations [35]. Previous studies have shown SRCC to have a RCC dominance. However, more recently, SRCC has been reported to have an even site distribution within the colon, with a slight male predominance [36]. Moreover, the SRCC histological subtype is known to have an adverse prognostic significance independent of tumour stage and molecular subtype [37, 38]. Poor tumour grade and advanced TNM staging are usually associated with worse survival outcomes [39, 40]. In this study, these features were found to have borderline significance in IA patients with LCC compared to OEGs, with slight increases in frequencies of HG (11% vs 4%, respectively; $P=0.0930$) and advanced disease stage (62% vs 49%, respectively; $P=0.0922$).

When assessing PCR confirmed cases only to accurately categorize MSI-L versus MSS and MSI-H CRC in right versus left CRC, significant associations were seen for MSI-L LCC with advanced disease stage and the IA ethnic group (Table 2). This data was perceived to be similar to the findings of Devaraj 2010, linking elevated microsatellite alterations at selected tetranucleotide repeats (EMAST) with advanced disease stage, rectal cancers and peri-tumoral infiltration in patients of African American (AA) descent [41, 42]. Even though MSI-L status was not available for the data in the study of Devaraj et al. 2010, previously published data closely linked MSI-L tumours to EMAST and has been associated with a poorer prognosis [20, 22, 43]. The most frequent unstable markers in tumours from IA patients in our cohort were BAT25 (15/29; 52%) and BAT26 (13/29; 45%). MSI-L LCC tumours showed a tendency to be of a more advanced disease stage (AJCC TNM stage: III-IV) compared to MSS LCC (89% vs 50%, respectively; $P=0.0348$).

Previous studies have shown these markers to be polymorphic within the African population, linked to the theory that older population groups show increased genetic variation [31–33]. Within this cohort, 17 CRC patients had shown instability in one or both markers (BAT25 and/or 26) in tumour and matched normal tissue, with a proficient MMR protein expression profile. These samples were assumed to be due to increased genetic polymorphisms. What was interesting and noteworthy to mention, was all these patients were exclusively of IA descent, with no differences observed in allele deletion sizes (5-15 bp deletion) between germline and somatic instability, and the majority were LCC (15/17; 88%) with advanced disease stage at diagnosis (7/10; 70%). Based on these findings, limiting as it is in size, BAT25/26 instability (whether of polymorphic/germline or somatic variation) was associated with advanced disease stage in proficient MMR LCC patients of IA descent. Even though survival data was not available for this study, literature has shown poor clinical prognosis and overall survival associated with MSI-L CRC, particularly for advanced disease stage CRC [21–23, 44]. The somatic MSI-L LCC group was associated with CLR when compared to MSI-L RCC (67% vs 12%; $P=0.0498$) and MSS LCC (67% vs 26%; $P=0.0205$). Polymorphic/germline MSI-L tumours within 2 markers (11/17; 65%) however displayed no CLR. Literature has indicated tumours with CLR to have a better prognosis compared to stage-matched tumours without [45–47]. This raises a plausible argument that germline MSI-L tumours could have a worse prognosis compared to somatic MSI-L tumours, due to the lack of the host's immune response to the cancer.

BAT26 marker (26(A) repeats) is located in intron 5 of the MSH2 gene on chromosome 2p21. This marker is situated immediately downstream of exon 5, which is susceptible to large intragenic deletions and accounts for nearly a third of dMSH2 mutations [48–50]. Studies by Pastrello et al. 2006 and Jaskowski et al. 2007 indicated that instability in Bat26 was associated with overall instability of dMSH2 tumours. Confirmatory IHC to determine dMMR protein expression is therefore important, however exceptions of cases with mutations in intronic nucleotides close to splice sites could result in expressed non-functional proteins, such as (*MSH2* c.913G>A p.Ala305Thr) which has been reported with proficient MMR activity and a MSI-L genotype [50]. This variant however had no aberrant splicing and normal subcellular localization and interaction with MSH6 was shown [50, 51].

BAT25 (25(T) repeats) is situated within intron 16 of the c-kit proto-oncogene on chromosome 4q12. cKit (CD117) the receptor for Stem cell factor (SCF) involved in haemopoiesis has more recently shown to be involved in lymphopoiesis. The CD117 receptor has shown to be expressed on mature $CD8^+$ T cells following initial activation, suppressing differentiation and increasing its response to apoptosis. CD117 expressed $CD8^+$ T cells could therefore play a role in CD117-blockade, an important mechanism in tumour immune evasion. BAT25 instability in the CD117 gene could potentially play a role in immune evasion in MSI-L CRC, and additional studies are required to determine CLR and its association with MSI-L LCC.

A study by Carethers et al. 2014 showed an increased incidence of MSI-H CRC in AA patients, however had poorer prognosis and higher mortality rates compared to their Caucasian counterparts [52]. This was thought to be

due to AA patients showing a lower infiltration of $CD8^+$ T cells compared to Caucasian patients, suggesting an altered immune function in AA patients. It has been well established that the increased tumour-infiltrating $CD4^+$ and $CD8^+$ T cells in patients with MSI-H CRC (due to the increased mutator phenotype of the tumour stimulating the host immune response) significantly improved patient outcomes when treated with immunotherapy when compared to MSS CRC with decreased immune response [53, 54]. The study by Carethers et al. 2014 illustrate despite having the same disease subtype and stage, ethnicity can be a negative prognostic factor in CRC disease.

A frequency of 17% MSI-L CRC in SA CRC patients has been reported in our previous study [29]. Further evaluation in this study has demonstrated MSI-L LCC to occur predominantly in IA patients, and associated with advanced disease stage, with considerable number of germline/polymorphic MSI-L LCC also presenting at an advance stage compared to MSI-L RCC and MSS LCC.

Based on these findings, universal MSI PCR screening is recommended as a first-line screening method for all newly diagnosed CRC patients, to not only identify MSI-H CRC, but also increase the detection rate of MSI-L CRC. Local ethnic polymorphisms however have to be taken into account when implementing diagnostic marker panels in certain geographical settings. If instability is required in 30% of markers used in a panel for a diagnosis of MSI, it is important to confirm markers included are non-polymorphic in the local population.

CRC is a heterogeneous disease and more studies are required to unravel the complexity associated with it by investigating different ethnic groups in the context of site. This study has shown that ethnicity and tumour site play an important role in the prognostication of tumours and should be taken into consideration for effective treatment planning, especially in geographical regions with diverse population groups such as South Africa. Limitations of this study include selection bias, as only samples with an MSI status were included. This resulted in smaller sample sizes for the analysis of certain features such as polyps, TILs, TNM staging, MSI-L and BAT25/26 instability status. The lack of universal screening for MSI within the study institution, as well as the inclusion of biopsy samples in addition to resections, have contributed to providing limited information. Confirmation of MSI-L status in biopsies was not possible, increasing the likelihood of a small percentage of false positive MSI-L samples with normal variation.

A study by Ozaki et al. found that MSI-L colon tissue occurred in a few but not all intestinal crypts, and both in malignant and normal tissues. The presence of MSI-L in non-neoplastic mucosa could indicate a primary step in tumorigenesis and could potentially be used as an early diagnostic and prognostic marker in CRC [55].

Additional AA patient studies have illustrated increased frequencies of MSI-L/EMAST markers in rectal cancers most likely due to somatic inactivation of an alternative MMR gene (MSH3) [21, 44, 56, 57]. Dysfunctional MSH3 has shown to lead to MSI, appearing to be inflammation-related within the tumour microenvironment [58]. Regular intake of anti-inflammatory drugs such as aspirin and non-steroidal anti-inflammatory drugs (NSAID) has been reported to prevent the development of colorectal adenomas, tumour growth and progression, as well recurrence and metastasis after curative surgery, prolonging colorectal cancer patient survival [59–61]. Anti-inflammatories could therefore possibly have a positive effect not only in MSI-H CRC, but also in this subgroup of MSI-L LCC patients. In addition, due to the presence of CLR, MSI-L LCC could potentially be an eligible subgroup for immunotherapeutic strategies in metastatic disease and further studies are recommended.

Conclusion

This SA CRC study indicated that in considering categorization of CRC according to anatomical site, microsatellite instability status and ethnicity, unique clinicopathological features were identified. In particular, IA CRC patients with LCC are more likely to be male, have an MSI-L subtype, show BAT25/BAT26 marker instability and have advanced disease stage. This study suggests that BAT25 and BAT26 instability are negative prognostic markers in African CRC patients, and larger confirmatory studies are recommended. Further exploratory studies of MSH3, EMAST, KRAS and immune cell infiltration in the tumour microenvironment are indicated in SA CRC patients. This will assist in establishing molecular profiles to accurately improve diagnostic, prognostic and personalized predictive markers for the effective management of early onset CRC.

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Authors' contributions

MM, YP and CP were involved in the design and research methodology of the study. MM conducted the tests, performed the analysis and wrote the manuscript. All authors reviewed the manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials
The dataset generated and analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
This was a retrospective study on already available biological material and ethical clearance was obtained from the Human Ethics Research Committee (Medical), University of the Witwatersrand Ethics clearance reference number: M120994. The need for informed consent was waived by the HREC (Medical), University of the Witwatersrand for this study, as research was conducted under the institutional blanket ethics clearance (M10744) obtained from the HREC (Medical), which allows for research to be carried out on all archived pathology specimens without informed consent from study participants. All experimental protocols were approved by the Postgraduate Research and Protocol committee within the Faculty of Health Sciences, University of the Witwatersrand, and all experiments were performed according to the relevant guidelines and regulations.

Consent for publication
Not applicable.

Competing interests
The authors have no conflicts of interest to declare.

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Chapter 5: Concluding comments

CRC remains a complex disease with multiple environmental and genetic factors involved in its aetiology. The main contributing factors are environmental, with the adoption of a westernized diet and a less active lifestyle. Disease onset predominantly presents from the 7th decade of life, and has been linked to high income countries globally, as elevated incidence rates occur within these regions compared to much lower incidence reported in the under-developed regions, such as Africa.[1,95] To date the incidence of CRC is reported to be decreasing in the developed countries due to effective surveillance and awareness programs in place, however in lower income or developing countries incidences continue to rise.[1] On a global scale however, young onset CRC incidence is increasing, with no clear explanation to the cause in the shift of epidemiology. [96]

Early CRC studies in Africa reported the disease predominantly occurring in the Caucasian population group, and a rare occurrence among Africans.[12,95,97] NCR reports suggest this to be a major underestimate due to inefficient or non-existent cancer registries within rural areas and less developed parts of the country at the time.[24] Alternatively the introduction of an environmental factor such as lifestyle changes (urbanization) or a major genetic founder effect occurring at certain point in time could have contributed to the rise in incidence over the years within the African population.[98–100]

To date the incidence rates of CRC is on the rise in all population groups, and drastic interventions need to be implemented to alleviate the burden of the disease.[9,27] A major concern is the high number of young African patients diagnosed with the disease.[27,28,101] Minimal studies have been published on the prognosis, survival and burden of disease in this patient group.[29,97] The elevated proportion of young onset cancer has been shown in a few studies over the past 2 decades, however no significant strategies or interventions to

determine the causative and underlying genetic factors to assist in the eradication of the disease has been implemented.[101–103]

This study was undertaken to provide further clarity into the molecular subgroups and clinicopathological characteristics of the disease. Overall these findings revealed African CRC patients display unique molecular pathological features compared to other ethnic groups within SA. Universal microsatellite instability screening demonstrated an increase in the detection of MSI CRC patients compared to only using the Bethesda guidelines when selecting patients for MSI testing. The Bethesda criteria for predicting MSI CRC (based on the MSI phenotype, tumour site (RCC), young age of onset and family cancer history), was more restrictive demonstrating a lower pick up rate when comparing to universal MSI PCR screening of SA CRC patients.[104,105] This was due to a proportion of MSI CRC in our population also detected in the left colon and over the age of 50 years. The IHC technique also showed to be less sensitive in comparison to PCR when detecting MSI CRC, this is mainly due to some cases showing expression of non-functional proteins and being detected by IHC. An additional benefit of universal MSI PCR screening is the ability to differentiate MSI-L CRC from the MSS CRC, a subtype demonstrating unique histopathological and molecular features and perceived to be a completely separate molecular entity. MSI genetic polymorphism in 2 markers (BAT25/26) was also associated with advanced disease stage in our cohort. These preliminary results indicated a possibility of BAT25/26 to be utilized as a negative prognostic marker in African CRC patients. This also raises the question whether this genetic polymorphism could be a possible predisposing genetic factor in African patients. Larger confirmatory as well as functional studies are recommended to determine if this 2 marker instability pattern results in an expressed but non-functional protein in these patients. CLR has been associated with this subtype, and literatue has shown improved stage specific prognosis with an immune host response infiltration, and further research into treatment strategies for tumours with increased CLR need to be explored, as illustrated with MSI-H tumours,TIL and immune checkpoint inhibitor therapy.

Futher characterization of all CRC genes, including those providing predictive and prognostic information, such as KRAS variatns and- anti-EGFR therapy should be explored in SA, which has been long overdue and lacking in the effective treatment of the disease, particularly in the public sector.[57,106]

There is a global need to gain greater understanding into the various subtypes of CRC.[41] This includes unravelling molecular signatures of all subtypes to gain insight into the best appropriate targeted treatment and management strategies, to improve patient survival outcomes, quality of life and the overall burden associated with the disease. Considering all research outputs from this study, the implementation of a routine universal MSI PCR screening tool in diagnostic histopathology laboratories is recommended in SA. Subsequent to confirming MSI status, IHC analysis should be performed on all MSI-H CRC to determine the causative defective protein. Additional BRAFV600E testing can be conducted on all *MLH1* deficient tumours to differentiate between sporadic and hereditary forms of the disease. This study highlighted *MSH2* as the predominant causative gene of young onset MSI CRC in the African population, and recommend additional investigation by next generation sequencing to determine the causative pathological variants in somatic and germline cells of deficient MSH2 CRC patients. This will assist in ascertaining whether the dominant MSI molecular subtype in African patients is due to a genetic predisposition or sporadic cause. As there are multiple possible genetic causes of the disease, including single nucleotide variation (SNV), copy number variation (CNV), epigenetic variation etc., next generation sequencing (NGS) screening of intronic and exonic regions of associated CRC genes is recommended as a first line screening method (Figure 1). Larger deletions, such as those seen in the *EPCAM* gene will not be detected by technologies such as NGS, and therefore CNV by MLPA (multiple ligation probe analysis) or larger deletions by microarray analysis should be implemented as a second line screening tool. If there are no pathological variants identified through this method, additional epigenetic analysis of promoter regions in CRC-related genes need to be pursued.

LS CRC studies within the Western Cape identified a founder mutation (*MLH1*:c.1528C>T) in the mixed ancestry (coloured) population group mainly in the Northern Cape of South Africa.[70,107,108109] Identifying this variant allowed a rapid and cost-effective screening tool to be developed to screen and diagnose patients presenting with symptoms of the disease on a large scale. This allowed for appropriate interventions to be implement in this part of the country within the mixed ancestry population.[108,110–112]

In determining the causative pathological variants in the African population, will allow the same appropriate and effective diagnostic, predictive and management strategies to be implemented.

Policy that all suspected hereditary CRC patients be referred by clinicians to the genetics clinic for follow up should be implemented. Greater effort and priority should given in determining causative factors and underlying germline or somatic pathogenic variants in the aetiology of the disease. Once the common or founder variants have been identified in the causative genes, cost effective genetic screening tools can be implemented in routine diagnostic laboratories to aid in the accurate diagnosis of patients.[113] This information will assist geneticists in the early diagnosis and management of patients, as well the implementation of effective prevention strategies by cascase testing of high risk family members of patients. This information will also guide clinicians in stratifying the best treatment and surveillance strategies in CRC patients.

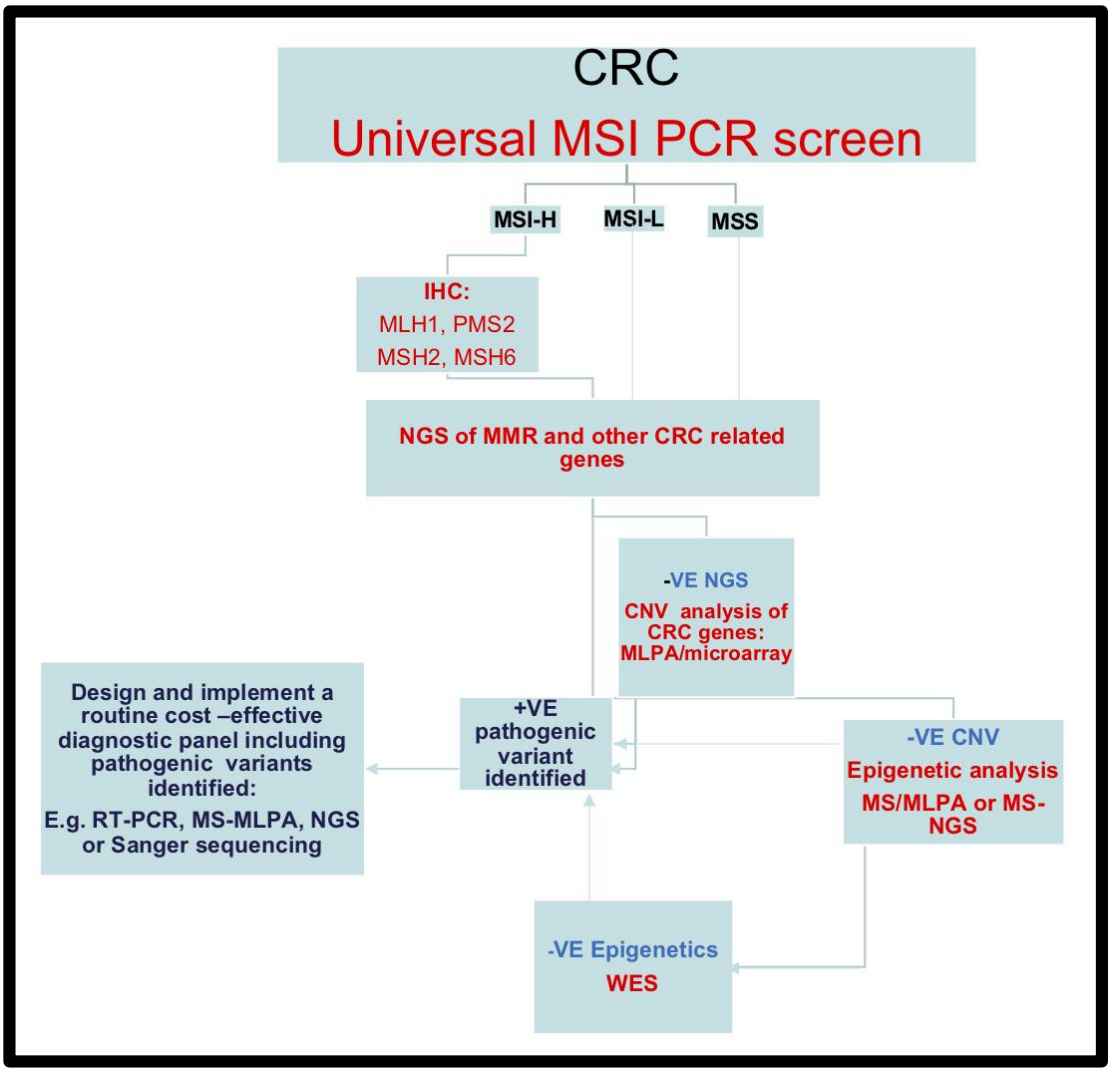


Figure 1: Algorithmic approach recommended for the molecular characterization of CRC in SA

Universal microsatellite instability (MSI) assessment of all CRC tissue samples followed by immunohistochemistry (IHC) of MSI tumours would assist pathologists in effectively determining the molecular subtype of CRC for appropriate treatment and management. The next approach should include next generation sequencing (NGS) utilising targeted CRC-related gene panels for assessment of High-level-MSI (MSI-H), low-level-MSI (MSI-L) and microsatellite stable (MSS) tumours. Both tumour and matched blood specimen types should be screened to establish the underlying genetic cause whether germline or somatic. Samples negative for pathogenic variants by NGS, should be assessed for copy number variation (CNV) through technologies such as multiplex ligation probe analysis (MLPA) and/or microarrays screening larger deletions or duplications. Samples negative for CNV, additional methylation profiling of CRC-related genes should be assessed through methylation specific MLPA or NGS technologies. The final recommended assessment would be whole exome sequencing (WES) of samples with no identifiable genetic cause of disease. Identification of clinically significant variants through this algorithmic approach would assist in the design and implemention of a cost-effective routine diagnostic screening tool for the effective management of CRC patients in SA.

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APPENDIX A:

R14/49 Professor Martin Hale

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150884

NAME: (Principal Investigator)	Professor Martin Hale
DEPARTMENT:	Division of Anatomical Pathology NHLS
PROJECT TITLE:	Use of Pathology Specimens Archived in the Department of Anatomical Pathology (Previously M00/08/29/Prof A Paterson)(M10744)
DATE CONSIDERED:	Adhoc
DECISION:	Approved unconditionally
CONDITIONS:	Renewal (2015 - 2020)
SUPERVISOR:	
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	09/09/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. I agree to submit a yearly progress report.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Ms Michelle McCabe

CLEARANCE CERTIFICATE	**M120994**
PROJECT	Microsatellite Instability Assessment in Colorectal carcinoma Tumour Biopsies: Mismarch Repair Immunohistochemistry versus Microsatellite PCR
INVESTIGATORS	Ms Michelle McCabe,
DEPARTMENT	School of Anatomical pathology
DATE CONSIDERED	Ad hoc
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 28/09/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor :

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

APPENDIX B:

Plagiarism declaration for written work

I Michelle McCabe, as a postgraduate student registered for a PhD at the University of the Witwatersrand declare that this thesis is my own written work, except where I have stated otherwise and indicated by referencing the work, thoughts and ideas of others



Signature ……..