COLORECTAL CANCER (CRC) IN YOUNG, BLACK SOUTH AFRICANS: A MORPHOLOGICAL, IMMUNOHISTOCHEMICAL AND MOLECULAR STUDY

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

Johannesburg, 2007

DECLARATION

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

..... [Signature of candidate]

1st day of May, 2007

This thesis is dedicated to everyone fighting this disease.

Acknowledgements

I hereby wish to convey my sincere appreciation to both the people and institutions that made the preparation of this manuscript possible. They include but are not limited to the following....

Professors Paterson and Ramsay whom always showed enthusiasm towards this

Professor Becker from the Biostatistics Unit of the Medical Research Council of South Africa for countless hours spent analyzing the data

Staff of the Department of Anatomical pathology for technical assistance

Erna Kotze and Angela Turner whose knowledge regarding microsatellite instability and genetic sequencing was invaluable

P.A.L.M Microlaser technologies (Bernried, Germany) for gaining experience in laser-assisted microdissection, and their overall hospitality during my visit

AEC Amersham (Mrs Karolina Kuun) and Applied Biosystems (Mrs Christa Swanepoel) for making their PCR instrumentation available for my use

Roche Diagnostics (Anna Kenyon and Leigh Berry) for their assistance in developing the real-time PCR assays

Mrs Carol Crowther from the Department of Haematology and Molecular Medicine for making the Roche LightCycler real-time PCR machine available for my usage

Kevin, who inspires me

My parents for their love, faith and belief in me

All my friends and family for their kindness and support

God, for blessing me in so many ways

Abstract

Background: Amongst black and white patients a disproportionately large number of young blacks present with colorectal cancer (CRC) in South Africa. We proposed that the comprehensive morphological and molecular analysis of a subset of cases might link them to the features associated with either the so-called "serrated pathway" or the hereditary pathway. Such an analysis could direct the diagnosis and treatment of similar patients in an effort to improve prognosis.

Materials and Methods: Pathological review of 1732 cases and molecular analysis on a subset of these cases were retrospectively investigated, without knowledge of family history. Determination of the CpG island methylator phenotype (CIMP) was performed at five loci (methylated in tumour loci MINT1, 2 and 31, and the promoter region of the hMLH1 and MGMT genes) using methylation-specific PCR. Microsatellite status was determined through a multiplex PCR process involving the 5 loci specified by the National Cancer Institute. *BRAF* and *KRAS* gene status was determined by real-time PCR.

Results: Eighty five percent of young patients (<50 years) were black ($p < 0.000$), predominantly male (56%; $p = 0.026$) with proximal tumours (25%; $p = 0.064$). These patients showed significantly more poorly differentiated tumours ($p = 0.001$) and extracellular mucin ($p = 0.006$). Molecular analysis revealed young blacks tended to present with loss of protein expression of the mismatch repair (MMR) proteins hMLH1, hMSH2, hMSH6 and MGMT, which resulted in a low MSI status. Overall methylation phenotype in these patients was low, together with the incidence of mutant *KRAS* genotype. Young white patients less frequently showed loss of mismatch repair protein expression, but MSI-H resulted from hMLH1 promoter hypermethylation in association with KRAS gene mutations and wild-type BRAF although this phenomenon may relate to the small numbers available for study.

Conclusion: Many young black patients may develop CRC through the accumulation of mutations due to MSI that resulted from loss of MMR protein expression. These features are associated with hereditary nonpolyposis colorectal cancer (HNPCC). In contrast, young white patients in this series presented with features reminiscent of sporadic CRC characterized by high levels of CIMP and MSI that results from promoter methylation of the hMLH1 gene, as may be identified in the serrated neoplasia pathway.

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

Research papers submitted for publication or in preparation:

Cronjé L, Paterson AC, Becker PJ. Young Africans with Colorectal Cancer follow the Mutator Pathway. *Submitted to* The Lancet.

Cronjé L, Ramsay M, Becker PJ, Paterson AC. Hereditary nonpolyposis colorectal cancer is a major contributor to colorectal cancer in young South African blacks. *In preparation.*

Cronjé L, Ramsay M, Paterson AC. What should we look for when diagnosis colorectal cancer in South African patients? Recommendations regarding patient demographics, histological examination and molecular characterization. *In preparation.*

Conference presentations:

Cronjé L, Ramsay M, Paterson AC, Becker PJ. Epigenetics events in Colorectal Cancer: Implication for assessment of toxicity. *Oral presentation at the 4th National Conference on Toxicology: Toxicology in Africa: Emerging technologies, Pretoria, South Africa, June 2006.*

Cronjé L, Ramsay M, Paterson AC, Becker PJ. Colorectal cancer in Africans: On Safari for the etiology. *Oral presentation at the 46th Conference of the Fderation of South African Societies of Pathology: Pathology Revival, Durban, South Africa, July 2006.* Received the prize for the best oral presentation at the conference.

Cronjé L, Ramsay M, Paterson AC, Becker PJ. Colorectal cancer in South Africa: Who is at risk? *Presented at the 11th International Congress of Human Genetics, Brisbane, Australia, August 2006.*

Cronjé L, Ramsay M, Paterson AC, Becker PJ. Colorectal cancer in Africans: On Safari for the etiology. *Oral presentation at the Annual University of the Witwatersrand Health Science Faculty Research day, Johannesburg, South Africa, August 2006.*

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ACF	-	Aberrant crypt foci
APC	-	Adenomatous polyposis coli
APES	-	3-Aminopropyl-triethoxysilane
ASIR	-	Age standardized incidence rate
CA	-	Carcinoma
CIMP	-	CpG island methylator phenotype
CIMP-L	-	Low levels of CpG island methylation
CIMP-H	-	High levels of CpG island methylation
CIN	-	Chromosomal instability
CRC	-	Colorectal cancer
COX2	-	Cyclo-oxygenase 2 enzyme
DAB	-	3,3-diaminobenzidene
dH_2O	-	Distilled water
DNA	-	Deoxyribonucleic acid
FAP	-	Familial adenomatous polyposis
H_2O_2	-	Hydrogen peroxide
H&E	-	Haematoxylin and eosin
HNPCC	-	Hereditary non-polyposis colorectal cancer
JPS	-	Juvenile polyposis syndrome
LOH	-	Loss of heterozygosity
MGMT	-	*O*-6-methylguanine methyltranserase
MHC	-	Major histocompatibility complex
MINT	-	Methylated in tumour
MMR	-	Mismatch repair
MSI	-	Microsatellite instability
MSI-L	-	Microsatellite instability low
MSI-H	-	Microsatellite instability high
MSP	-	Methylation specific PCR
MSS	-	Microsatellite stable
NCI	-	National Cancer Institute

List of abbreviations

PCR	-	Polymerase chain reaction
PJS	-	Peutz-Jeghers syndrome
TGF	-	Transforming growth factor
TGF-β	-	Transforming growth factor beta
TGFβRII	-	Transforming growth factor –beta receptor two

CHAPTER 1

CARCINOMA OF THE COLORECTUM: CLINICOPATHOLOGICAL OVERVIEW

1.1 Introduction

Carcinoma of the colorectum (CRC) is one of the most common causes of morbidity and mortality in many developed countries, being more prevalent in societies that have embraced a typical western lifestyle. In the United States of America, for example, the tumour was identified in 2002 as being the third highest cause of cancer-associated deaths, affecting approximately 148,000 patients, both male and female, and in excess of 55,000 deaths (Jemal *et al*, 2002). By virtue of its frequency in such societies, the disease has significant implications for the medical profession in terms of prevention, surveillance, diagnosis, and therapy.

As will be discussed, dietary and lifestyle factors appear to play a significant role in the development of CRC and the disease may be curbed by addressing these factors. In many cases, there is a well defined precursor lesion, the adenomatous polyp. If identified at time of routine surveillance and removed expeditiously, this will halt progression to invasive tumour. The established cancer is often symptomatic at a relatively early stage and modern surgical technique allows for efficient removal thereof. At the level of anatomical pathology, accurate and meticulous assessment of tumour type and extent of tumour allows for accurate classification and staging of the disease. The information emanating from a high quality histopathological assessment is of inestimable help to the oncologist. Finally, modern chemotherapy is salvaging larger numbers of patients and there is also some preliminary evidence that efficacy of

chemotherapy may be further refined by identifying the molecular characteristics of the tumour when looked at against factors such as age, sex and family history.

With respect to family history, although the vast majority of CRCs are typically identified as a lifestyle phenomenon in older individuals peaking at 60 to 79 years, a significant subset have an identifiable genetic predisposition. It is estimated that 20 to 30% of CRC have such a tendency (Grady, 2003). These patients will often escape the surveillance net as they are frequently younger than those with a putative environmental aetiology. As such, it would be of importance to identify those genetic and molecular factors that may be in play in younger patients with CRC.

South Africa is a country of extremes in terms of racial composition, cultural practices and socio-economic development. As will be discussed, the white population is historically a more affluent minority migrating mainly from mainland Europe and the United Kingdom at any time up to 300 years before present. They are identified as having a high incidence of CRC, thought to have a typical dietary and lifestyle origin. Conversely, the black population, also migrant from more central and northern part of the continent, are historically identified as having a low incidence of CRC. However, there are numbers of younger black patients who develop CRC, a phenomenon that has passed virtually unnoticed for at least the past fifty years. It is against this background of extremes that it would appear pertinent to identify the characteristics of CRC amongst the two diverse populations.

1.2. Colorectal cancer: clinicopathological perspectives

In its more common form, CRC occurs in an ageing population as a result of dietary factors (high saturated fat and calorie diet accompanied by a sedentary lifestyle) (Hamilton *et al.*, 2000). Other risk factors may include smoking, alcohol and chronic inflammatory bowel disease. Putative protective factors include a diet high in vegetables, the use of aspirin and other non-steroidal anti-inflammatory agents, and physical activity. The role of fibre is somewhat contentious. As discussed later (Chapter 2), although Burkitt originally postulated a high fibre diet as being protective for CRC in Africa (Burkitt, 1971), later studies have noted a declining fibre content in black without an increase in prevalence of CRC in this population (Oettlé and Segal, 1985; Segal and Walker, 1986; O'Keefe *et al.*, 1999). Conversely, a more recent multi-ethnic study by Peters *et al.* (2003) indicated that a high intake of dietary fibre, specifically from grains, cereals and fruits, was associated with a decreased risk of developing distal colonic adenoma and may partially explain why there is a low incidence of CRC in black patients.

Clinically, patients with the more common CRC originating in the left side of the bowel (rectum and sigmoid colon) commonly present with fresh rectal bleeding, changing bowel habits and tenesmus, or pain during defaecation. Furthermore, altered bowel habits or a general feeling of insufficient bowel emptying and weight loss are frequently associated with the diagnosis. In contrast, less prominent disturbances are noted for right-sided lesions. These include fatigue, weakness, iron deficiency anaemia and unexplained weight loss. They may be discovered radiologically or

during colonoscopy at an early stage. A poorer prognosis is associated with rectal and sigmoid lesions as these tend to be more infiltrative at the time of diagnosis. A well defined adenoma-carcinoma sequence is identified in many of these patients and surveillance programs for detection and management of adenomas are in place in many developed countries, including faecal blood detection and routine colonoscopy at defined intervals of up to 10 years.

With onset of the overt tumour, spread of disease mainly occurs through direct extension through the wall of the bowel and into adjacent structures including peritoneum, metastasis to regional lymph nodes and vascular spread to liver. Later, metastatic sites may include lungs and bones (Crawford, 1999).

The aetiology of CRC relating to environmental factors may be explained through different mechanisms, including the production of bile acids, reactive oxygen species or volatile fatty acids. Similarly, the decreased faecal transit time may also contribute to the incidence of CRC (Hamilton *et al.*, 2000; Rosai, 2004). Although the molecular mechanisms underlying these above-mentioned associations are only poorly understood, their respective roles may prove to be important.

1.3. Colorectal cancer: morphology

The distribution of cancer throughout the colorectum is not uniform and occurs predominantly in the rectosigmoid region with a significant but lesser percentage in the caecum. Smaller numbers of CRC are identified in transverse and descending colon

(Figure 1.1) (Crawford, 1999). The majority of cases occur singly, but in cases where multiple carcinomas are present, they often occur in disparate sites.

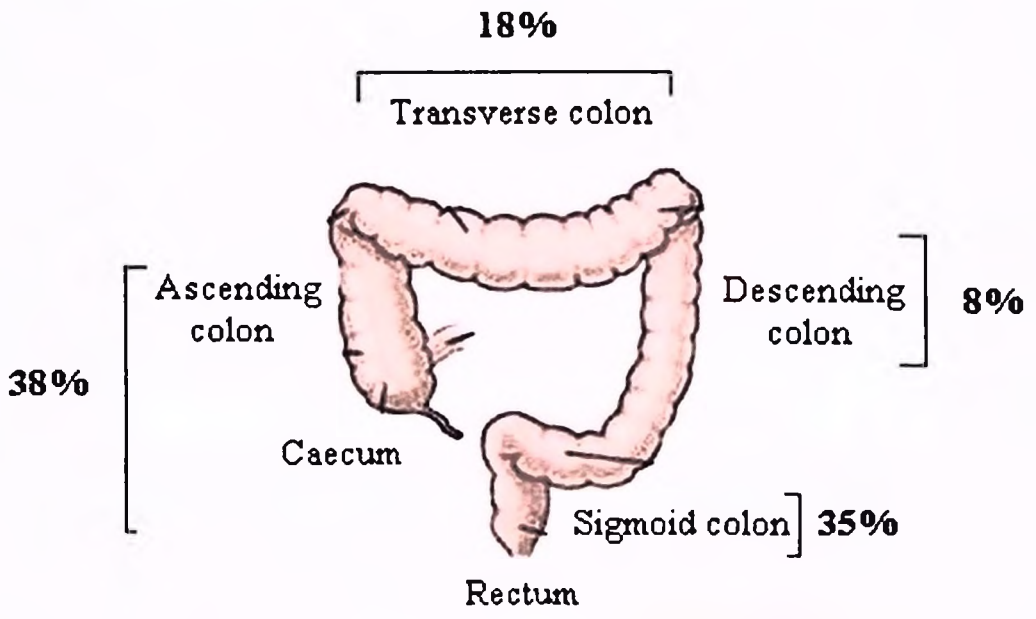


Figure 1.1: **The distribution of the cancers in the colorectum in a western population.** Adapted from Lui and Crawford, 2005

The evolution of colorectal adenoma to carcinoma occurs through different morphologic patterns, their pathways being described in section 1.4. Tumours arising in the proximal colon tend to have an exophytic, polypoid growth pattern, with a less frequent obstructive tendency arising from an adenoma (Figure 1.2) (Crawford, 1999). This is in contrast to tumours in the distal colon that tend to be annular, encircling

lesions that cause constriction of the bowel (Figure 1.3). These distal lesions are classically ulcerating, white masses.

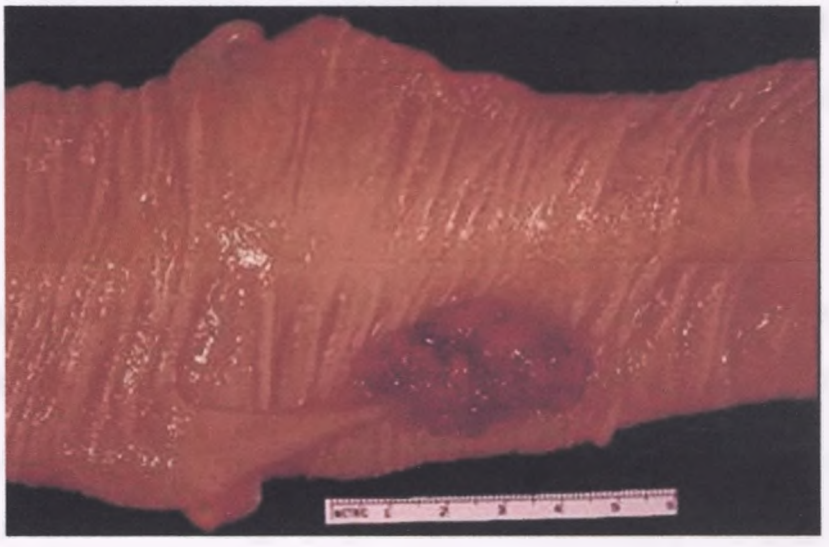


Figure 1.2: **Polypoid growth pattern of a solitary colorectal adenoma.**

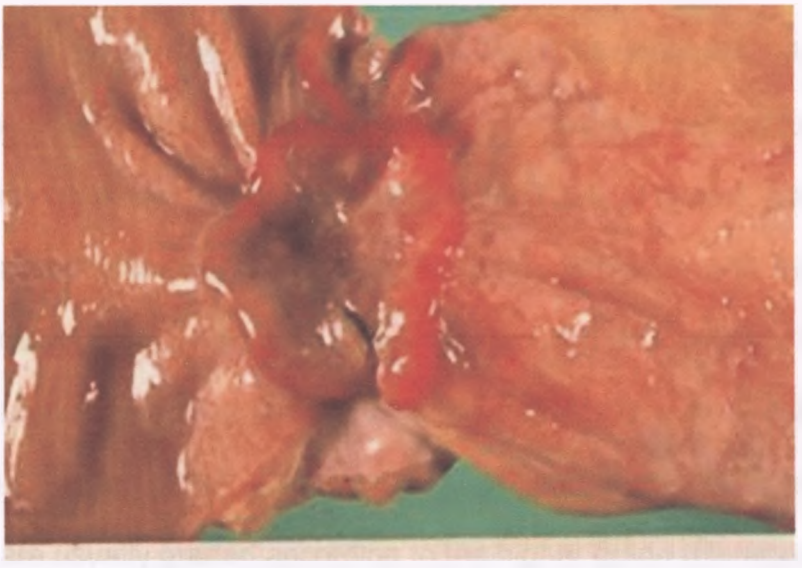


Figure 1.3: **A constricting, ulcerated carcinoma of the colon.**

Microscopically, these lesions are very similar. Differentiation may range from well-differentiated gland-forming structures, to undifferentiated carcinomas. Mucin secretion is also frequent, and may aid in metastasis and worsen the prognosis (Hamilton *et al.*, 2000). Malignant cells may take on a signet-ring cell or small cell appearance and, rarely, focal endocrine differentiation may also be evident.

1.3.1 Tumour grading and staging systems

Following diagnosis with CRC, tumours are classified according to the presence or absence of features that may have a profound effect on prognosis. These include overall tumour differentiation, tumour type, lymphocytic infiltration (e.g. peritumoural, Crohn's-like or tumour infiltrating), lymph node involvement, spread (e.g. local, distant, venous), margin and peritoneal involvement (Jass and Morson, 1987).

Adenocarcinomas are graded in terms of the level of differentiation. As illustrated in Figure 1.4, well differentiated adenocarcinomas have a gland architecture that is well-defined with uniform nuclei that has a basal location (20% of cases), while moderately differentiated adenocarcinomas show irregular gland formation with large nuclei and nuclear stratification (60%). Together these are identified as low-grade CRCs. Poorly differentiated adenocarcinomas present as diffuse sheets of tumour cells with total or near-total gland loss (20%) (high-grade tumours). Tumours showing variable differentiation are usually graded according to the higher grade (Hamilton *et al.*, 2000).

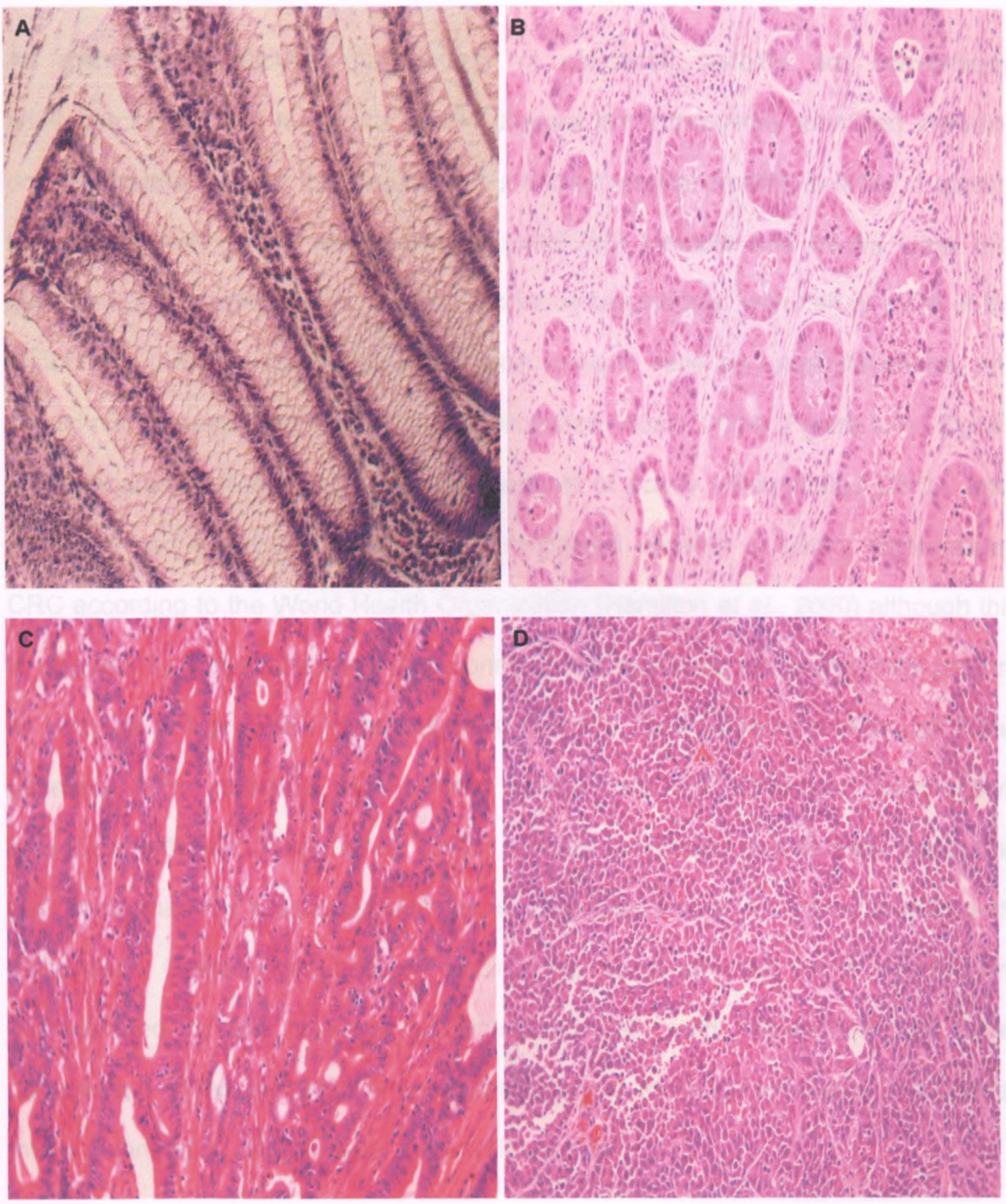


Figure 1.4: Features of colonic adenocarcinoma. *A* represents normal colonic epithelium, *B* represents well differentiated adenocarcinoma with ordered gland formation and uniform nuclei, *C* shows moderately differentiated adenocarcinoma with slightly irregular gland formation, and *D* represents a poorly differentiated adenocarcinoma with no gland formation.

As mentioned previously the majority of colorectal cancers are conventional adenocarcinomas but numerous other subtypes are infrequently identified including undifferentiated tumours that require immunohistochemical profiling in order to confirm their origin from colon, neuroendocrine (carcinoid) tumours and others (Jass and Morson, 1987; Hamilton *et al.*, 2000). Mucinous carcinomas, with half or more of the tumour volume showing extracellular mucin, usually have an exophytic shape, and should not be equated to the signet-ring carcinoma (Rosai, 2004) (Figure 1.5). Signet-ring tumours are frequently considered variants of poorly differentiated CRC. Both mucinous and signet-ring tumours are considered variants of poorly differentiated CRC according to the World Health Organization (Hamilton *et al.*, 2000) although this is disputed by some authorities who consider that mucinous carcinoma may present as both low or high grade forms (JR Jass, personal communication). Although it has generally been accepted that tumour typing does not confer an independent prognostic indicator, Wang *et al.*, (2003) recently mentioned that at least signet-ring cell carcinomas are associated with poorer prognosis and an increased tendency for metastasis. Overall signet-ring cell carcinomas, mainly affecting young patients, are characterised by the presence of signet-ring cells with crescent-shaped nuclei compressed to the periphery of cells, secondary to the accumulation of intracellular mucin (Wang *et al.*, 2003; Rosai, 2004) (Figure 1.6).

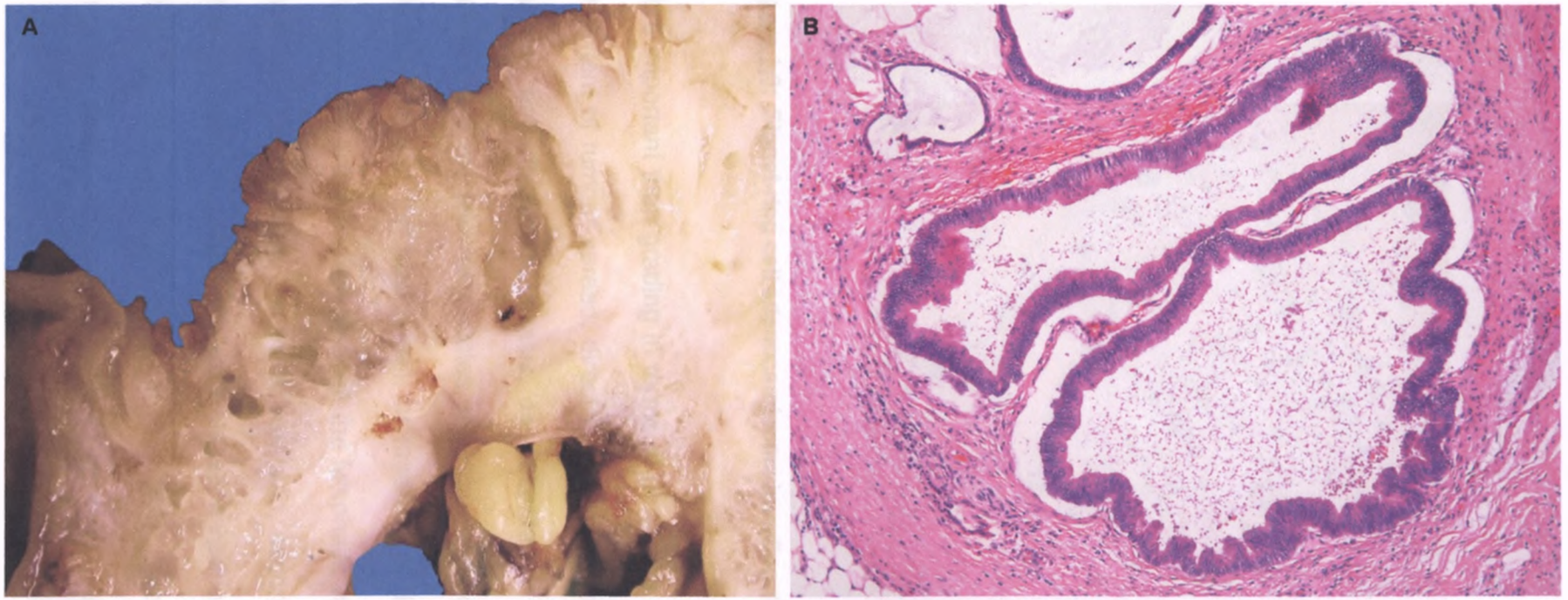


Figure 1.5: Adenocarcinoma of the colon showing the abundant excretion of mucin. *A* represents the macroscopic view of a mucinous carcinoma, while *B* is the representative microscopic view. When more than 50% of the adenocarcinoma shows mucin, the tumour is classified as mucinous (Hamilton *et al.*, 2000).

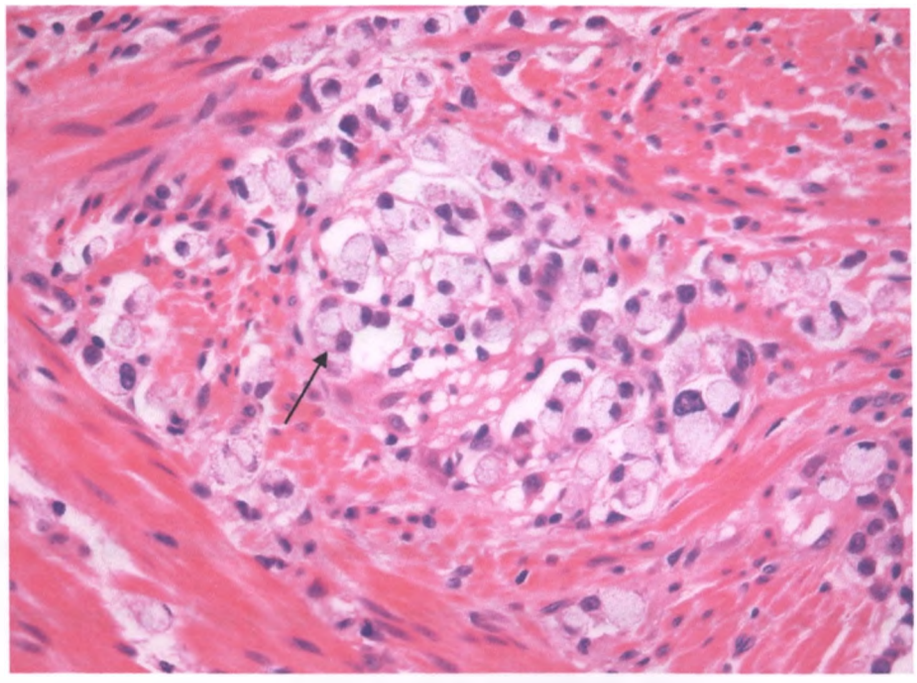


Figure 1.6: Signet-ring cell adenocarcinoma invading the muscularis externa.

More important than grading in terms of prognosis and survival is tumour stage, *viz.*, the extent of tumour spread, directly through the bowel wall, as well as lymph node invasion and distant metastasis, as illustrated in Figure 1.7 (Ouchi *et al.*, 1996). Overall there are two main routes of tumour spread, involving either direct extension into and through the bowel wall, or invasion of the vascular structures. Regional lymph nodes are accessed through the lymphatic vessels, while other organs may be invaded through, firstly, the portal vein and, secondarily, by traversing the systemic circulatory system. Although lymph vessel invasion is of slightly less importance than blood vessel invasion, Shirouzu *et al.* (1995) illustrated that it does adversely affect prognosis when present extensively in tumours of an advanced stage. In his original

paper on the spread of rectal cancer and its effect on prognosis, Dukes showed that when more than 10 lymph nodes were involved, the five year survival statistics dropped to only 2%, compared to the 64% survival in patients where only one lymph node was involved (Dukes, 1932). Perineural invasion is also a sign of advanced disease and Rosai (2004) noted that it is usually accompanied by other unfavourable pathologic findings. The establishment of metastases would conversely adversely affect the successful local treatment.

There are several clinical and pathological factors that influence the prognosis of colorectal carcinoma. The individual worth of these determinants is, however, difficult to ascertain as the majority of the studies examining them are not conclusive, and often contradict each other (Rosai, 2004). Age seems to adversely affect the prognosis in very young and very old patients (Griffin *et al.*, 1987), although it seems to play a more important role in rectal carcinomas than in colonic tumours (Tominaga *et al.*, 1996). Male patients have been reported to have a significantly worse prognosis than females (Griffin *et al.*, 1987). Furthermore, tumour location, size and advancing edge are important, although clear correlations are still to be established (Rosai, 2004). Obstruction and perforation seem to be linked to a poor prognosis (Steinberg *et al.*, 1986; Wolmark *et al.*, 1987), while a pushing margin and lymphocytes at the advancing edge of the tumour, is of great prognostic value (Jass and Morson, 1987) (Figure 1.8a). The lymphocytes, as well as eosinophils, neutrophils and plasma cells, follow the contour of the invasive margin and the resulting inflammatory layer has been said to resemble the contour of the normal

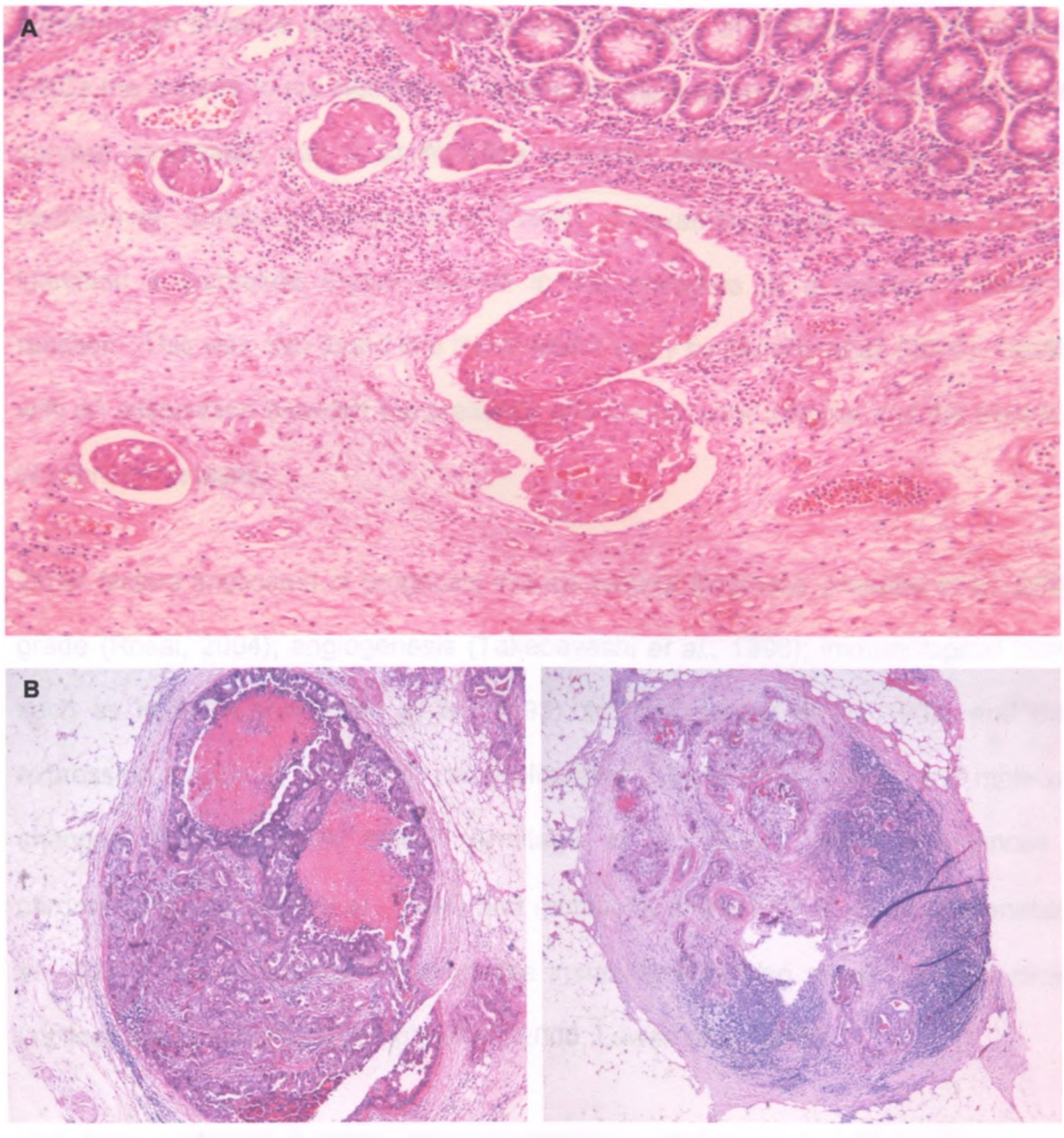


Figure 1.7: Lymphatic space invasion (A) and lymph node metastasis (B).

lamina propria (Jass and Morson, 1987). Graham and Appelman (1990) subsequently focused attention to the lymphoid aggregates (called the Crohn's-like infiltrate),

typically at the junction of the muscularis propria externa and the pericolic fat bordering an invasive adenocarcinoma (Figure 1.8b). In looking at 100 consecutive CRCs the authors found significantly less nodal metastases and an increased 10 year survival associated with a Crohn's-like lymphoid reaction in transmurally invasive carcinomas. Similarly Harrison *et al.* (1995), on examining 344 right-sided tumours, identified a increased 5-year survival in the presence of a marked Crohns'-like reaction. This was also found in a more recent study by Murphy *et al.* (2000), together with an association between the presence of a Crohn's-like infiltrate and the clearance of micrometastases.

Other factors implicated in prognosis include tumour thickness (Hasebe *et al.*, 2000); grade (Rosai, 2004); angiogenesis (Takebayashi *et al.*, 1996); immunological factors such as HLA-DR (Anderson *et al.*, 1993), hCG (Connelly *et al.*, 1993) and Bcl-2 expression (Leahy *et al.*, 1999); cell proliferation (Bauer *et al.*, 1987); and molecular changes such as DNA ploidy (Armitage *et al.*, 1991), allelic imbalances of chromosome 18q (Jen *et al.*, 1994) and mutations of the TGF-β_1 gene (Watanabe *et al.*, 2001). More recently, microsatellite instability was also shown to have clinical implications for prognosis (Wright *et al.*, 2000; Lawes *et al.*, 2003).

The tumour staging systems most commonly used include the Dukes (as well as variations thereof), TNM and Jass classification systems. The A, B, C classification system proposed by Dukes has been successfully used for many years, and is still used to this day in the staging of CRC (although it could ultimately be replaced by the TNM system) (Table 1). Dukes' "*A*" demarcates tumour growth confined to the

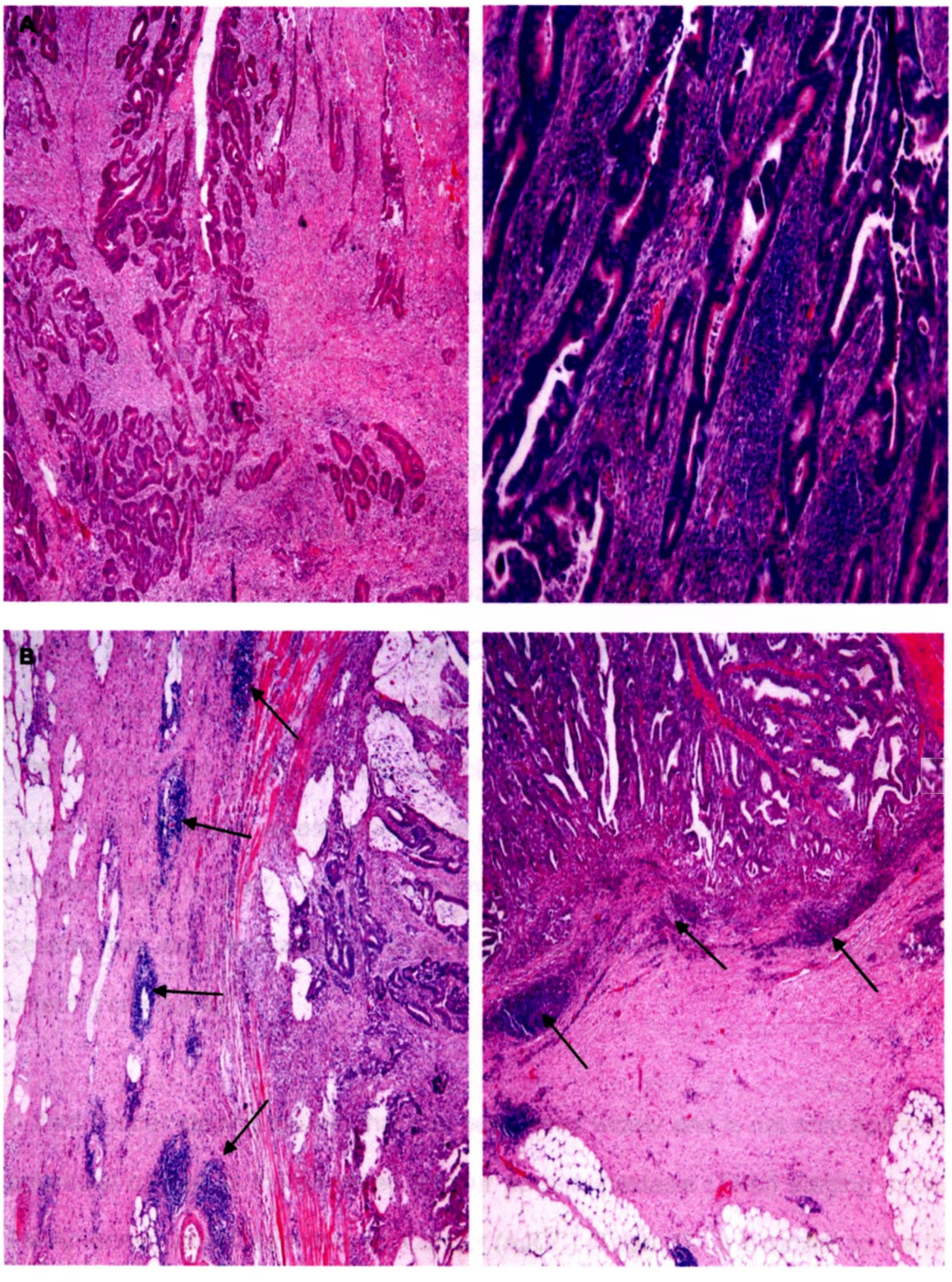


Figure 1.8: Lymphocytes at the advancing edge of the tumour (*a*) and Crohn's-like infiltrate (*b*).

submucosa or muscularis propria, whereas "*B*" cases are associated with spread beyond the muscularis propria. Originally, Dukes characterized "*C*" cases as those with lymph node metastasis (Dukes, 1932), but after some revision he divided them into two categories: "*C1*", in which only regional lymph nodes are affected, and "*C2*", in which the apical lymph node also contain metastasis (Jass, 2001). After receiving much attention from other pathologists, the Dukes' classification system has undergone various modification. The most recognised variation, the Astler-Coller modification of Dukes' classification (Table 1), further subdivides the "*B*" cases. Overall, their system classifies "*A*" cases as carcinoma *in situ*, "*B1*" cases as those where the tumour infiltrates the bowel wall and "*B2*" cases where the spread extends beyond the muscularis propria. The "*C*" cases are subdivided into "*C1*" and "*C2*", where there is lymph node metastasis but tumour is confined to the bowel wall or when tumour has penetrated the wall of the organ. Other modifications have been noted in the literature where clinical and pathological data are mixed, but as Jass and Morson noted in 1987, the comparison of *A*, *B* and *C* cancers between different centres may result in inaccuracy when a proportion of these cancers are placed within different categories.

A third cancer staging system that has been used widely and is not only applicable to colorectal cancer, is the so-called tumour node metastasis (TNM) staging system of the American Joint Committee on Cancer (AJCC) (Table 1). It is similar to the Dukes classification system, in that it defines the extent of tumour spread and lymph node

involvement, but does so on a more clearly defined basis. Although not based on research, this system describes a method of encoding pathological and clinical data (Jass and Morson, 1987). According to this system the direct tumour spread is said to be limited to the mucosa (Tis), invading the submucosa (T1), invading the muscularis propria (T2), extending beyond the muscularis propria (T3) or invading serosa or adjacent organs (T4). As the level of lymph node involvement is of great prognostic value, the TNM system defines the number of positive lymph node metastasis: N0: no positive lymph nodes; N1: up to three positive lymph node metastasis; N2: four and more lymph nodes affected; and N3: apical node metastasis. The extent of distant metastases is also noted: M0: no distant metastases noted; M1: pathologic examination documented distant metastases. In the case where distant metastases could not be determined, a score of Mx is given. Of importance is the inclusion of distant lymph node metastases that is given as the M-score (Hamilton, 1996). The TNM system appears very useful in describing the specific tumour in terms of direct spread, and, with the more detailed description of the number of lymph nodes involved, may prove to be more clinically useful. The TNM system score correlates to a specific stage: stage I tumours have a TNM score of either T1N0M0 or T2N0M0; stage II correlates to T3N0M0 or T4N0M0; whereas stage III tumours has a TNM score of T1N1-2M0, T2N1-2M0, T3N1-2M0 or T4N1-2M0. Stage IV tumour implies any distant metastasis regardless of T or N status.

Table 1.1: The most commonly used pathologic staging systems for curatively resected colorectal carcinoma.

Dukes' classification system	Score / Stage
Tumour spread confined submucosa/muscularis propria	A
Tumour spread beyond muscularis propria	B
Regional lymph nodes contain metastasis	C1
Apical lymph nodes contain metastasis	C2
Astler-Coller modification of the Dukes' classification system	
Carcinoma *in situ*	A
Tumour infiltrate bowel wall	B1
Tumour spread beyond muscularis propria	B2
Bowel wall lymph nodes contain metastasis	C1
Lymph nodes beyond muscularis propria contain metastasis	C2
Tumour-Node-Metastasis (TNM) classification system	
Tumour growth limited to mucosa	Tis
Tumour invading sub-mucosa	T1
Tumour invading muscularis propria	T2
Tumour spread beyond muscularis propria	T3
Tumour invading serosa	T4
No lymph node metastasis	N0
1 - 3 lymph nodes contain metastasis	N1
4 or more lymph nodes contain metastasis	N2
Apical lymph nodes contain metastasis	N3
Requirements to assess distant metastasis not met	Mx
No distant metastases	M0
Distant metastases noted	M1

As grading systems ultimately aim to determine tumour aggressiveness and the ability to invade, metastasize and kill a patient (Jass, 1988), it has been proposed that the ideal classification system would distinguish between those likely to be cured and those likely to die of their disease (Jass *et al.*, 1987). Using the Dukes' classification system, patients are grouped within the most confident prediction of clinical outcome, i.e. excellent (stage A) versus poor (stage C_2) prognosis, at an average rate of 97% and 4% respectively (Jass *et al.*, 1987). In an effort to increase the rate of confident

prediction of clinical outcome, Jass *et al.* (1987) then proposed the use of four variables that have an important and independent influence upon survival, including tumour spread, invasion, the involvement of lymph nodes and tumour infiltrating lymphocytes. The Jass classification system is based on the scoring of these pathological variables, as depicted in Figure 1.9 below. Differentiation into these groups allows for the association to be drawn between the Dukes' and Jass classification systems. Stages I, II, III and IV of the Jass classification system correlate to stages A, B, C1 and C2 of the Dukes' system respectively (Hamilton, 1996).

1.4. Colorectal carcinogenesis:

With the advent of modern molecular biology, it has become apparent that cancer is in large part a genetic disease. This includes the genetic factors that predispose certain individuals to cancer, as well as the specific targets, albeit genetic, that confer an altered growth capacity of normal cells during neoplastic progression. Similarly to certain hereditary colon cancers, sporadic CRC arises from pre-existing adenomatous lesions. The progression of adenoma to carcinoma involves a complex set of events that includes the inactivation of tumour suppressor genes and the activation of oncogenes. Studies based on the well-known Knudson's two-hit hypothesis (Knudson, 1977) have identified a number of alternative pathways in colorectal carcinogenesis that involve alterations to the tumour suppressor and/or oncogenes.

Growth	*Score*
Limited to bowel wall	0
Through bowel wall	1

Invasion	*Score*
Expansile	0
Infiltrative	1

Lymph node involvement	*Score*
0 nodes involved	0
1 – 4 nodes involved	1
>4 nodes involved	2

Tumour infiltrating lymphocytes	*Score*
Present	0
Absent	1

Score	*Group*
0 – 1	I
2	II
3	III
4	IV

Figure 1.9: A diagrammatic representation of the Jass classification system for grading of rectal cancers. Selected variables are given a weighted score and the total score is then converted into one of 4 prognostic groups.

The first pathway involves the loss of portions of chromosomal material leading to aneuploidy and the loss of specific genes. Chromosomal instability (CIN) typically

involves allelic loss (loss of heterozygosity; LOH), with concomitant inactivation of the tumour suppressor gene when the second allele becomes dysfunctional due to a separate mutation. With the progressive loss of cell growth checkpoints, clonal expansion of the mutated progenitor will occur (Robbins and Itzkowitz, 2002). The further evolution of adenoma to carcinoma, or morphological changes such as well to poorly differentiated tumour histology has been shown to be accompanied by LOH (Boland, 1997).

Genes that are affected by chromosomal instability include the adenomatous polyposis coli (*APC*) tumour suppressor gene (Kinzler and Vogelstein, 1996), which is the principal molecular initiator of both sporadic CRC and familial adenomatous polyposis (FAP); the Kirsten-ras (*KRAS*) proto-oncogene that is thought to play a role during the intermediate stage of tumourigenesis (Robbins and Itzkowitz, 2002); the *p53* gene that is essential in the transition of late adenoma to early carcinoma (Robbins and Itzkowitz, 2002); and the "deleted in colon cancer" (*DCC*) gene involved in the late stages of the adenoma-carcinoma sequence. An example of such a pathway is shown in Figure 1.10, illustrating the adenoma-carcinoma sequence evident in familial adenomatous polyposis (FAP).

The second major pathway in colorectal carcinogenesis involves defects in the mismatch repair (MMR) system responsible for correcting replication errors within the highly repetitive microsatellite regions. Due to the highly replicative state of DNA synthesis, several errors inevitably occur primarily during the elongation phase (Chung

and Rustgi, 2003). These errors mainly involve mis-pairing of nucleotides and slippage of the DNA polymerase complex, resulting in either base-base mismatches or loop-structures within the microsatellite sequences. This phenomenon is termed microsatellite instability (MSI). Within the DNA polymerase complex itself, an intrinsic exonuclease activity will recognize and excise the mispaired nucleotides from the newly synthesized strand. Errors not immediately corrected by the DNA polymerase will promptly be rectified through an elaborate array of mismatch repair genes.

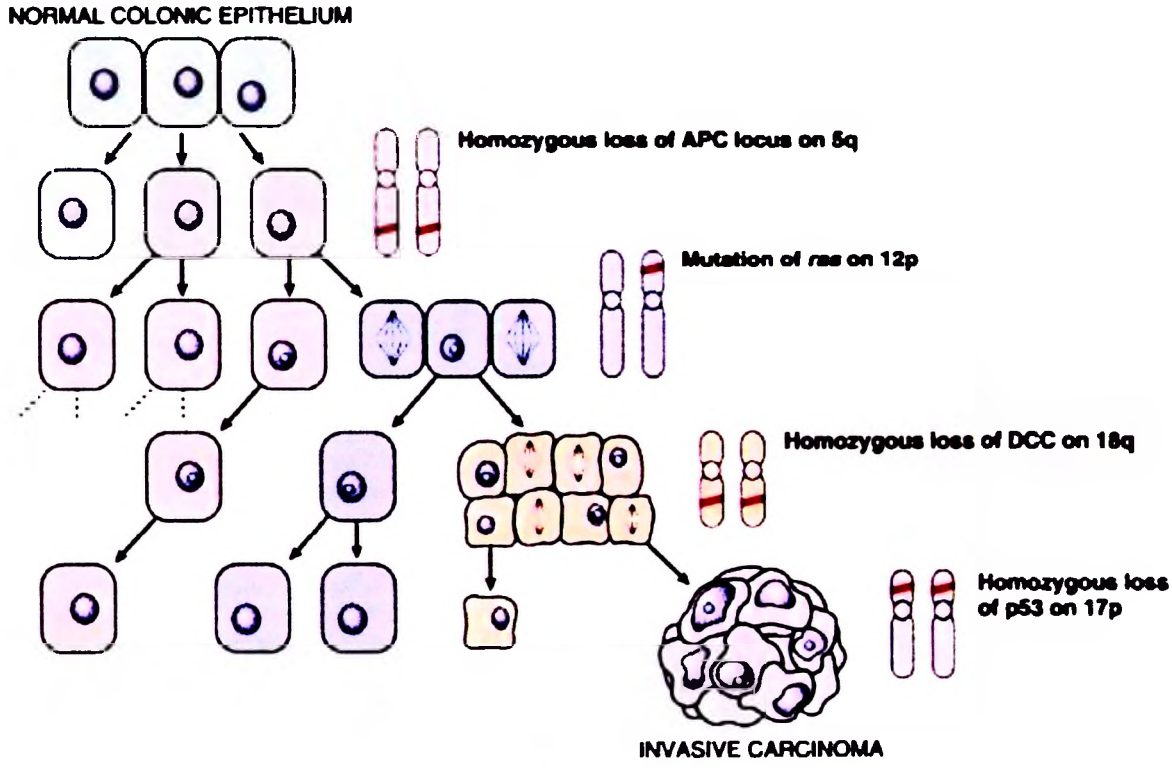


Figure 1.10: Genetic changes involved in the conversion of normal colonic epithelium, through adenoma formation to an invasive carcinoma. The homozygous loss of *APC* expression is responsible for the initiation of adenoma formation through the uncontrolled interaction with β-catenin and several other transcription factors. Point mutations in the *KRAS* gene result in gain-of-function permitting continued signal transduction and the progression of early to intermediate adenomatous

lesion. The homozygous loss of the *DCC* gene is said to be responsible for the conversion of intermediate to late adenoma. The critical transition of the late adenomatous lesion to early carcinoma is controlled through the homozygous loss of *p53* expression.
APC – adenomatous polyposis coli ; *ras* – KRAS, Kirsten-ras ; DCC – deleted in colon cancer.
Adapted from www.geneticengineering.com

The DNA mismatch repair system involves the activity of several genes of the mutS (*hMSH2, hMSH3, hMSH6*) and mutL (*hMLH1, hMLH3, hPMS1* and *hPMS2*) families. Briefly, as depicted in Figure 1.11, the process of mismatch repair involves the recognition of and binding to mismatched DNA sequences by hMSH2 (Fishel, *et al.*, 1994). Depending on the number of mismatched nucleotides, hMSH2 will form a heterodimeric complex with either hMSH6, when a single base-pair mismatch is encountered, or hMSH3 if a larger insertion or deletion is recognised. A second heterodimeric complex of hMLH1 and hPMS2 or hPMS1 is then recruited to excise the mismatched nucleotides (Chung and Rustgi, 2003). Peltomäki (2001) suggested that base-base mismatches that result in single base substitutions primarily affect non-replicative DNA. In contrast, gains or losses of short repeat units (e.g. CA repeats) generally result from insertion-deletion loops in repetitive microsatellites (Peltomäki, 2001).

Mutation in any of the mismatch repair genes will result in a deficiency in the mismatch repair system and, hence, a mutator phenotype. As a high proportion of the DNA mismatches appears within the microsatellite regions, the extent of repair deficiency depends on the specific gene that is mutated. *hMSH2* and *hMLH1* mutations

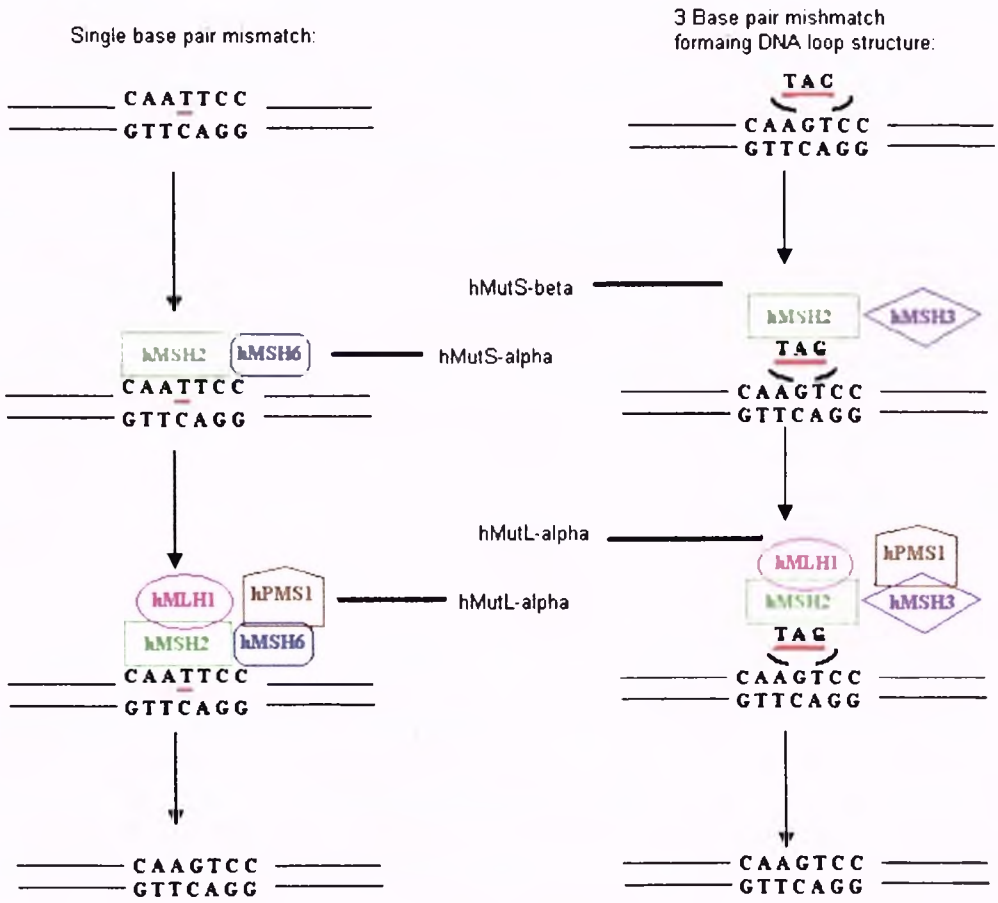


Figure 1.11: The DNA mismatch repair system that functions to eliminate single base-pair mismatches or larger loops of mismatched DNA. hMSH2 scouts the newly replicated DNA and recognizes and binds to mismatched DNA. In turn, it forms a dimeric complex with either hMSH6 in response to base-base mismatches (called hMutSα) or hMSH3 in response to larger loops of mismatched DNA (called hMutSβ). A second heterodimeric complex consisting of hMLH1 and hPMS1 or hPMS2 (called hMutLα) is subsequently recruited to excise mispaired nucleotides. Adapted from Chung and Rustgi, 2003.

generally result in a high level MSI (MSI-H), while mutations in *hMSH6* result in either partial mismatch repair and low level MSI (MSI-L) or MSI-H. Although most microsatellites occur in noncoding regions, certain genes involved in cell growth have

been found to contain microsatellites within the exons, thereby rendering them susceptible to mutation and loss of function (Robbins and Itzkowitz, 2002). These include the type II transforming growth factor β receptor (TGF-βRII) that downregulates tumour proliferation through its interaction with SMAD4. When mutated, the TGF-β molecule is unable to bind to the receptor, and results in increased tumour proliferation (Myeroff *et al.*, 1995). Other genes affected by MSI are the tumour suppressors *IGFIIR* (Souza *et al.*, 1996) and *PTEN* (Guanti *et al.*, 2000), the pro-apoptotic *BAX* gene (Rampino *et al.*, 1997) and *TCF4* involved in *Wnt* signalling (Duval *et al.*, 1999).

The MMR system not only corrects incidental biosynthetic errors, but also acts on DNA damage caused by heterocyclic amines (Li *et al.*, 1996), oxidative stress (Jackson *et al.*, 1998) and alkylating agents (Karran and Hampson, 1996). The dietary origin of many heterocyclic amines makes it of particular importance in colonic cancer (Moonen *et al.*, 2004), whereas the activity of particularly cyclo-oxygenase 2 (COX2) in response to inflammatory processes and cancer may be of importance in the reduction of polyp formation in hereditary nonpolyposis colorectal cancer (HNPCC) (Ruegg *et al.*, 2003). The acquired characteristic of tolerance to alkylation results in inhibition of programmed cell death and has been suggested to be significant in contributing to the HNPCC spectrum (de Wind *et al.*, 1998).

It has been identified that both hereditary, in particular within the HNPCC setting, and a subset of sporadic colorectal carcinogenesis arise from a defective DNA mismatch repair system (Shia *et al.*, 2003). However, the molecular mechanisms underlying the

MSI phenotype within these settings differ. In HNPCC the mismatch repair genes are inactivated through a germline mutation in predominantly the *hMLH1* or *hMSH2* genes (Lynch and de la Chapelle, 2003). In contrast to the high percentage of HNPCC cases that show MSI, only approximately 15% of sporadic CRC exhibit the MSI phenotype. These are the result of somatic alterations to the DNA mismatch repair genes, present only in the tumour cells (Chung and Rustgi, 2003). Transcription of mainly the *hMLH1* gene is blocked in sporadic CRC through the hypermethylation of the gene's promoter region (Herman *et al.*, 1998). The typical MSI-H (high level MSI) phenotype results when the second allele is inactivated.

In an attempt to establish universal guidelines for determining the level of MSI in colorectal tumours, the National Cancer Institute (NCI) introduced a panel of five microsatellite markers (Boland *et al.*, 1998). These include two mononucleotide repeats (Bat25 and Bat26) and three dinucleotide repeats (D2S123, D5S346 and D17S250). It was recommended that if instability is demonstrated in >30 – 40% of the loci, then the tumour is classified as MSI-H. Instability in <30 – 40% of the loci demarcates a MSI-L tumour, while stable tumours (MSS) demonstrate no instability in any of the loci.

A related pathway involves the methylation and subsequent gene silencing of cytosine bases within densely packed CpG sites that may occur in the promotor region of genes (Jass *et al.*, 2002), resulting in a CpG island methylator phenotype (CIMP). This frequently, but not exclusively, occurs in MSI cancers. Genes silenced in this

way include amongst others *hMLH1* (Guttmacher and Collins, 2003), *APC* (Xiong *et al.*, 2001) and the DNA repair gene *O*-6-methyl-guanine DNA methyltransferase (*MGMT*) (Esteller *et al.*, 1999). Jass *et al.* (2002) noted that extensive methylation of these genes may underlie the establishment of hypermutability and tolerance to DNA damage, thus providing a possible alternative pathway to the development of CRC.

Recently there have been considerable data forthcoming on the concept of serrated neoplasia. The hypothesis of serrated adenomas as a distinct entity, that combines the architecture of hyperplastic polyps with glandular luminal serration and the cytology of adenomas, was first described by Longacre and Fenoglio-Preiser in 1990. Initially conceived as a variant of adenoma with superimposed serrated architecture, with a mixed polyp representing a chance collision between adenoma and hyperplastic polyp, it is currently argued that hyperplastic polyps and serrated adenomas are related lesions (Jass *et al.*, 2006). Subsequent molecular analysis of serrated and classical adenomas confirms this finding (Hiyama *et al.*, 1998; Iino *et al.*, 1999) but raises the question as to whether serrated adenomas, and the colorectal cancers arising from them, develop through a pathway unique from the classical adenoma-carcinoma sequence. Jass and colleagues then developed the concept of the serrated neoplasia pathway, which encompasses hyperplastic aberrant crypt foci (ACF), hyperplastic polyps, mixed polyps and serrated adenomas (Jass, 2001) (Figure 1.12).

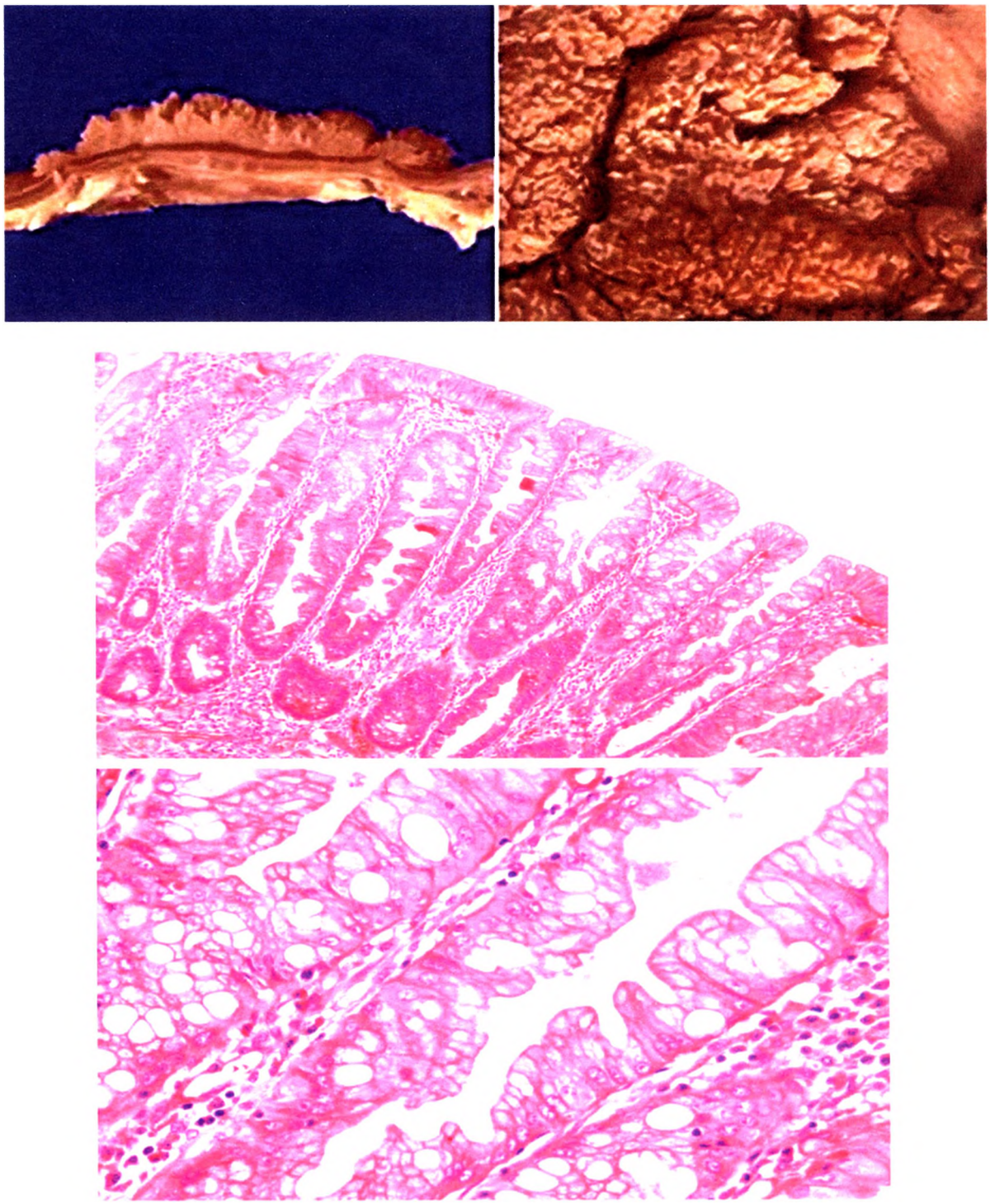


Figure 1.12: Serrated neoplasia. Macroscopic and microscopic examples of the serrated pathway. Taken from http://medlib.med.utah.edu/WebPath/ORGAN.html

DNA microsatellite instability and the loss of MMR gene expression are often identified in hyperplastic polyps (Whitehall *et al.*, 2001; Iino *et al.*, 1999). However, a bewildering genetic variability in these serrated neoplasms exists (Jass *et al.*, 2002). In mixed polyps and serrated adenomas, *KRAS* mutation is uncommon (Rashid *et al.*, 2000), whereas *KRAS* mutation occurs with a high frequency in serrated polyps (Jass *et al.*, 1999). This led to the formulation of a unifying two-step mechanism for the development of carcinoma from serrated neoplasia. The development of carcinoma from serrated neoplasia is thought to be initiated through a combination of genetic alterations and aberrant methylation (Jass *et al.*, 2000; Jass *et al.*, 2002). According to the pathway proposed by Jass and colleagues (2001, 2002, 2003), depending on the morphologic entity and the specific gene methylated, different levels of MSI may result, thus making a distinction between MSI-H and MSI-L serrated neoplasia possible (Figure 1.13).

Overall, the first step inevitably involves the inhibition of apoptosis. In mixed polyps and serrated adenomas, this involves the early silencing of the putative anti-adhesion / pro-apoptotic gene *HPP1* through methylation (Young *et al.*, 2001). In contrast, mutation of the *KRAS* gene is associated with hyperplastic and serrated polyps and nondysplastic aberrant crypt foci (ACF) (Fenton *et al.*, 1998). Once a resistance to apoptosis is established, Jass *et al.* (2002) proposed that a loss of DNA mismatch repair functioning would result in a growth advantage. It should be noted again that both hyperplastic polyps and serrated adenomas show a high degree of genetic heterogeneity (Park *et al.*, 2003). Therefore mixed polyps and serrated adenomas

have been shown to harbour loss of hMLH1 expression due to silencing through promoter hypermethylation, resulting in MSI-H cancers (Hawkins and Ward, 2001). MSI-L, in contrast, occurs in hyperplastic and serrated polyps and nondysplastic ACF, as a result of *MGMT* silencing through promoter hypermethylation (Whitehall *et al.*, 2001; Chan *et al.*, 2002). A similar diverse mutational spectrum occurs downstream to loss of DNA repair mechanisms. These include mutations of the genes *Bax*, *IGF2R*, *TGFβRII*, *TP53* and *CRAC1* on 15q.

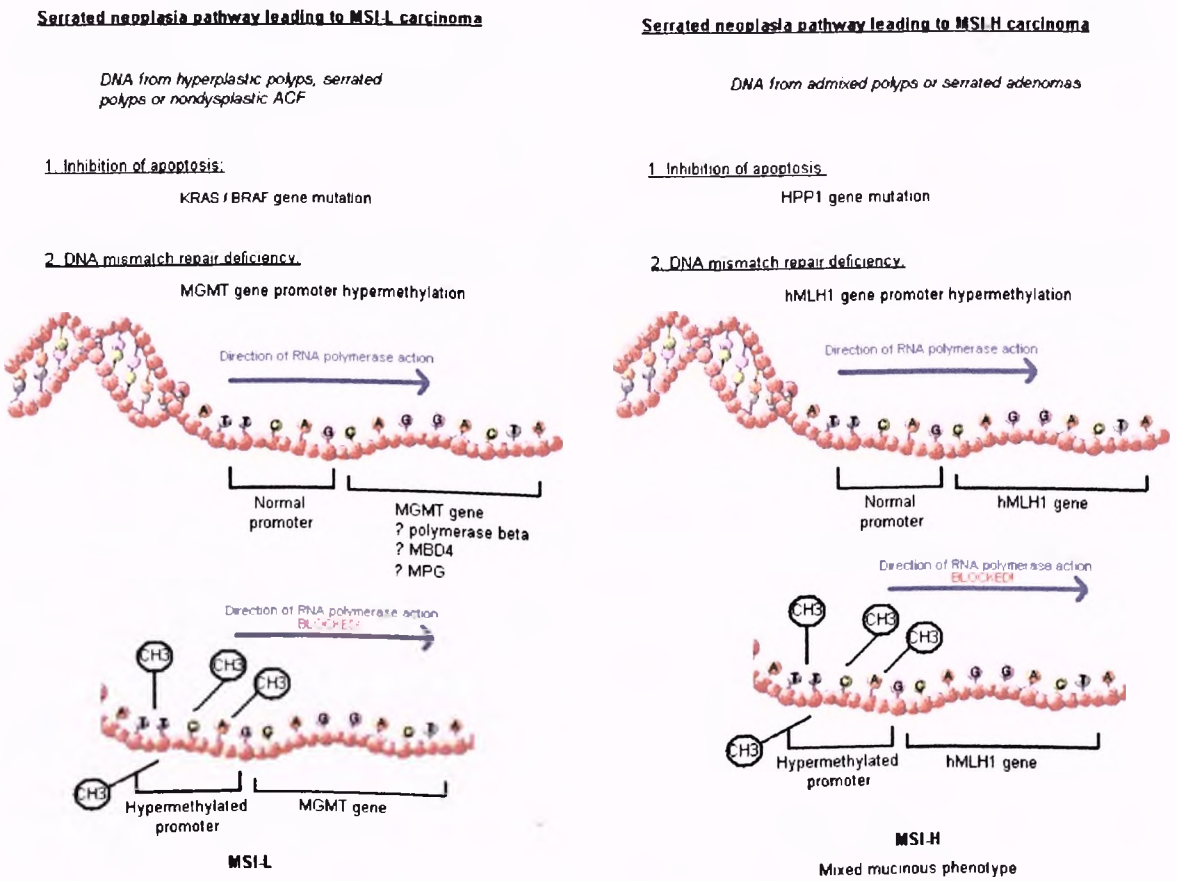


Figure 1.13: Genetic changes involved in the initiation of the serrated neoplasia pathway. Details are discussed in the text.

It is thus not surprising that the genetic variability observed in these serrated neoplasms is matched with an even higher degree of morphologic variability (Jass 2002). These include serrated adenomas that are flat or sessile (similar to the hyperplastic polyp) (Jass, 2003), or show either a tubulovillous or villous architecture (and often mistaken for their traditional counterparts) (Longacre and Fenoglio-Preiser, 1990). It has also been shown that their precursor lesions differ. Some arise within pre-existing hyperplastic polyps, others within a pre-existing adenoma (Jass, 2003). Even microscopic hyperplastic lesions (i.e. ACF) may give rise to serrated neoplasia (Jass *et al.*, 2001). The majority, however, seem to arise *de novo*.

A very low frequency of serrated adenomas is observed during endoscopy and 6% of CRCs show residual serrated adenomas adjacent to the colorectal cancer (Mäkinen *et al.*, 2001). The progression of serrated adenoma to carcinoma therefore seems to occur at an accelerated rate. This is a similar finding to the rapid conversion from normal through adenoma to carcinoma observed in HNPCC. Jass (2003) recently argued for the rapid conversion in the serrated neoplasia pathway on the basis of hypermutability resulting from a loss of DNA mismatch repair proficiency. It therefore seems likely that the role of the serrated pathway in colorectal carcinogenesis is presently underestimated.

At present time, various well documented and defined variants of CRC are identified, often demonstrating and autosomal dominant heredity. They are summarised below.

1.5. Familial adenomatous polyposis (FAP)

1.5.1 Clinical and molecular features

Familial adenomatous polyposis (FAP), characterized by several hundred to thousands of benign (adenomatous) polyps spread throughout the large bowel (Figure 1.14), presents at a very early age (between 7 and 36 years) without a gender bias and almost inevitably develops into colon cancer at a younger age (40 to 50 years) if left untreated (Grady, 2003). There is also an increased risk for extracolonic tumours, including cancers of the stomach, duodenum, pancreas, thyroid and even central nervous system (Grady, 2003; Lynch and de la Chapelle, 2003). FAP lesions may primarily evolve from microscopic adenomatous lesions, some even smaller than 3mm in size (Ichii *et al.*, 1992), termed aberrant crypt foci (ACF) and present with either a tubular, tubulovillous or villous glandular architecture (Robbins and Itzkowitz, 2002).

The ACF precursor lesions show chromosomal instability due to germline mutations of either tumour suppressor genes (primarily *APC* and, secondarily, *p53*) in dysplastic ACF (homologous to microadenomas) or oncogenes (*KRAS*) in non-dysplastic ACF (microscopic epithelial lesions observed in animal models following carcinogen exposure). These mutations are inherited in an autosomal dominant fashion, with a 50% risk to the patient's first-degree relatives (Möslein *et al.*, 2003).

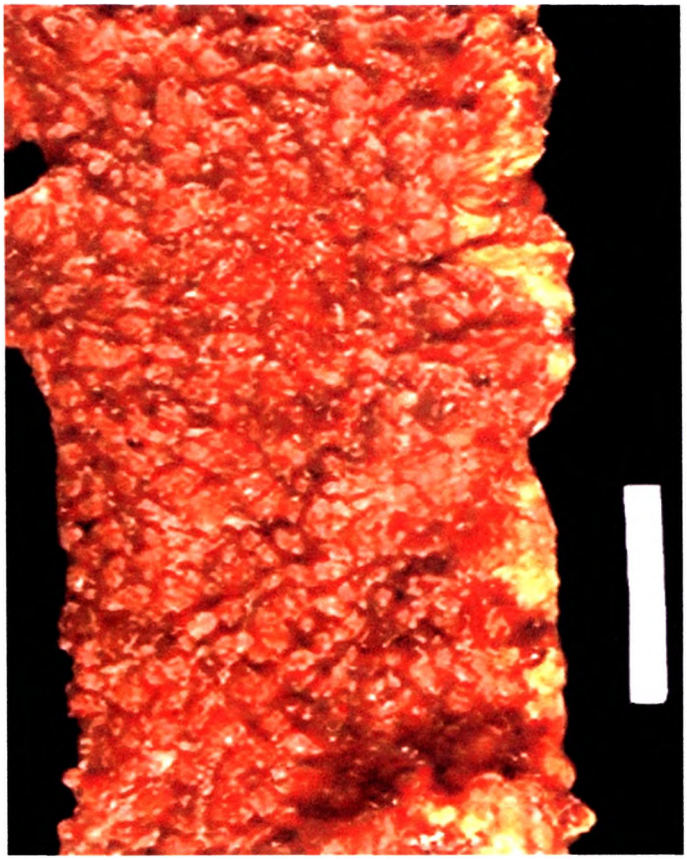


Figure 1.14: Illustration of familial adenomatous polyposis (FAP). FAP presents with thousands of adenomatous polyps within the large bowel wall. Patients with FAP have a near 100% lifetime risk of developing colorectal cancers (Grady, 2003). Taken from http://medlib.med.utah.edu/WebPath/ORGAN.html

Proceeding along the well-established adenoma-carcinoma sequence, FAP is initiated through mutation of the *APC* gene located on chromosome 5q21-22 (Groden *et al.*, 1991) (Figure 1.15). Several mutations have already been identified within the large *APC* gene especially within the region between codon 1194 and 1392 (Robbins and Itzkowitz, 2002). In addition, 20 - 25% of FAP cases present with "new" *APC*

mutations (Bisgaard *et al.*, 1994). These mainly involve small insertions or deletions and single base substitutions. Mutations result in a truncation of the multifunctional, 2843 amino acid protein (309kDa) involved in cellular proliferation, differentiation, migration and apoptosis through the phosporylation and subsequent down-regulation of β-catenin (Robbins and Itzkowitz, 2002). The majority of mutations result in the abolition of the β-catenin binding sites. Increased cytoplasmic β-catenin levels then increase their association with T-cell factor 4 (TCF-4) or its homologues, forming a bipartite transcription factor that promotes adenoma formation through the up-regulation of proto-oncogenes (Robbins and Itzkowitz, 2002, Bright-Thomas and Hargest, 2003).

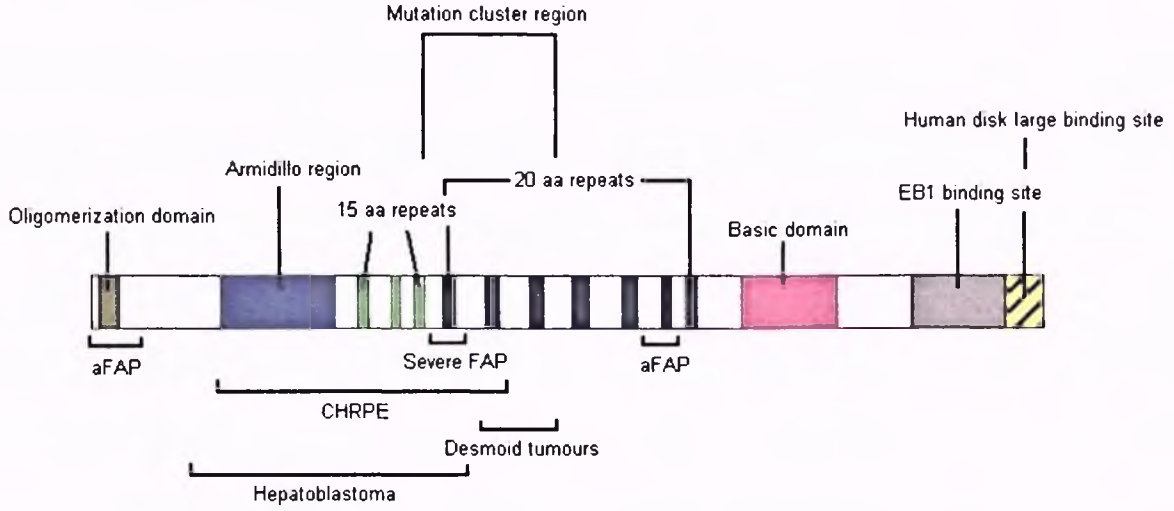


Figure 1.15: Schematic representation of the *APC* gene structure indicating the approximate exon regions, functional domains and associated phenptypic mutations in colorectal carcinoma (CRC). Situated on chromosome 5q21-22, this large gene encompasses 21 exons, of which exon 15 comprises more than 75% of the coding sequence. aFAP, attenuated

familial adenomatous polyposis; CRC. (Adapted from Fearnhead *et al.*, 2001; Bright-Thomas and Hargest, 2003; Grady, 2003; Robbins and Itzkowitz, 2002)

Diagnosis of FAP is, however, made regardless of family history or *APC* mutational status (Möslein *et al.*, 2003) as a subset of patients with multiple adenomas does not present with any *APC* mutations. In a recent study by Sieber and colleagues (2003), it was shown that germ-line mutations in the DNA glycosylase *MYH* gene predispose individuals to FAP. Similarly, *APC* mutations have been described in patients presenting with either early CRC or multiple adenomas, but without FAP. These are specifically seen in a sub-population of Ashkenazi Jews, where a simple T to A conversion results in a polymorphism that doubles the risk of colorectal cancer in these families (Laken *et al.*, 1997).

Although clear genotype-phenotype correlations have been observed, this phenomenon is not uniform and the role of modifier genes should not be excluded. Nevertheless, congenital hypertrophy of the retinal pigment epithelium (CHRPE) has been associated with the presence of mutations between *APC* codons 463 and 1387 (Olschwang *et al.*, 1997), whereas mutations between codon 1445 and 1578 show an increased risk of desmoid tumours (Caspari *et al.*, 1995). In addition, mutations located on the 5' and 3' ends are associated with attenuated FAP which, unlike classic FAP, presents with less adenomatous polyps at a more advanced age and with a predilection for the proximal colon (Leppert *et al.*, 1990). As noted, the Ashkenazi Jewish polymorphism, I1307K, presents with a unique mutation that dramatically increases the risk of colonic adenomas and adenocarcinomas (Laken *et al.*, 1997).

1.5.2 Clinical management

As it is commonly accepted that *APC* mutations form the genetic basis for FAP, the management of suspected FAP includes genetic testing in consultation with a genetic counsellor, the protein truncation test and linkage analysis (Grady, 2003). This should be followed by regular endoscopic surveillance (Grady, 2003). Due to the vast numbers of colonic polyps occurring in FAP, regular polyp removal cannot be considered as a treatment option. Several trials are currently ongoing to determine the effect of chemoprevention on polyp and cancer occurrence and its possible application in delaying prophylactic colectomy (Lynch and de la Chapelle, 2003).

1.5.3 Variants of the familial polyposis syndrome

In contrast to classical FAP, attenuated FAP is characterized by smaller numbers of adenomas with a flat rather than polypoid architecture (Robbins and Itzkowitz, 2002). Molecular features include germline mutations in the proximal and distal regions of the APC gene (Figure 1.15) that result in a truncated APC protein (Reale and Fearon, 1996). Although the clinical presentation of attenuated FAP is consistent with the classical form of the disease, they are indeed distinct, with attenuated FAP developing into cancer at a mean age of 57 years (Reale and Fearon, 1996).

Gardner syndrome is a variant of FAP that exhibits multiple osteomas, epidermal cysts and fibromatosis in addition to intestinal polyps (Crawford, 1999). It has been suggested that FAP and Gardner syndrome are synonymous, and that the difference

in polyp numbers and extracolonic presentations are due to the variable penetrance of the *APC* mutation (Jass, 2000). Hepatoblastomas are also frequent in the FAP kindred, with *APC* mutations located between codon 767 and 1513 (Bisgaard and Bulow, 2006). Although only rarely diagnosed, Turcot syndrome is a variant of FAP that presents in the early teens, and is marked by the primary occurrence of gliomas (Crawford, 1999). The phenotypic spectrum of this disease is very broad. Hamilton and colleagues (1995) studied the molecular features of 14 families affected by Turcot syndrome and concluded that two distinct genotype-phenotype associations existed. The first showed germline *APC* mutations and medulloblastomas, whereas the second group presented with glioblastoma multiforme tumours associated with MMR gene mutations.

1.5.3.1 Juvenile polyposis syndrome

A similarly appearing, yet genetically different disease occurs mainly in children and presents as 5 or more juvenile polyps. Juvenile polyposis (JPS) includes solitary rectal hamartomas (Robbins and Itzkowitz, 2002) that result as a consequence of germline mutations in several genes including the tumour suppressor gene, *SMAD4* (Huang *et al.*, 2000). Further tumour suppressor genes (p53 and p21) have also been implicated, thereby supporting a hamartoma – adenoma sequence (Wu *et al.*, 1997).

1.5.3.2 Peutz-Jeghers syndrome

Peutz-Jeghers syndrome (PJS) consists of multiple hamartomatous polyps in the gastrointestinal tract. Carcinomas in association with PJS have been reported from

the oesophagus to the colon (Robbins and Itzkowitz, 2002). Truncating mutations in a serine-threonine-kinase gene on chromosome 19p (*STK11-LKB1*) are responsible for most of the cases of this syndrome (Jenne *et al.*, 1998), and it has hence been termed "*gatekeeper to carcinogenesis*" (Robbins and Itzkowitz, 2002). Mutations of *KRAS* and overexpression of p53 have, in addition, been described (Entius *et al.*, 1999), again implicating a hamartoma – carcinoma sequence.

1.6. Hereditary non-polyposis colorectal cancer (HNPCC)

1.6.1 Clinical features

The adenoma-carcinoma sequence was accepted for some time as the major model in the development of CRC due to its simplicity, the lack of evidence for an alternative pathway and the opportunity for prevention and intervention that this model has afforded (Jass *et al.*, 2002). However, Pollock and Quirke (1991) had previously noted that the adenoma-carcinoma ratio is close to 30:1. When applied to the FAP situation, this would amount to extremely large numbers of adenomas that need to be removed in order to prevent a single carcinoma. Although adenoma removal does prevent cancer (Winawer *et al.*, 1997), the adenoma-carcinoma model for the development of CRC does not explain the time required for adenoma to carcinoma conversion. The possibility of an alternative and rapidly evolving pathway can therefore not be excluded (Jass *et al.*, 2002) and hereditary non-polyposis colorectal cancer (HNPCC), otherwise known as Lynch Syndrome, may offer evidence for this pathway.

First described in 1913 by Alfred Warthin, HNPCC is an autosomal dominant cancer susceptibility syndrome that accounts for a small percentage of all CRC cases (Samowitz *et al.*, 2001). It is recognised at an early age (~45yr) (Chung and Rustgi, 2003), in multiple individuals within a family (Grady, 2003) and arises from adenomas with an advanced transformation rate. HNPCC affects males and females equally, and the tumours are predominantly found proximal to the splenic flexure. The clinical diagnosis of HNPCC depended largely on the Amsterdam (I) criteria. This has, however, undergone some modification due to its strict criteria resulting in the exclusion of certain families. Thus the Amsterdam II and Bethesda criteria have subsequently been developed. Therefore, HNPCC is clinically defined according to the Amsterdam I, Amsterdam II and Bethesda criteria (Table 1.2) (Grady, 2003). Furthermore, a broad spectrum of extracolonic tumours may present during HNPCC, including endometrial, ovarian, gastric, urinary tract, renal, bile duct, central nervous system and small bowel tumours. When all these features are included and molecular screening is employed, the estimated incidence of HNPCC ranges from between 1% to 6% (Samowitz *et al.*, 2001; Lynch and de la Chapelle, 2003).

1.6.2 Pathologic characteristics

The most prominent feature discriminating between FAP and HNPCC, as its name implies, is the absence of the extensive polyposis observed during FAP. The lesser numbers of HNPCC adenomas found predominantly proximal to the splenic flexure are histologically more advanced and show a high level of dysplasia (Jass and Stewart, 1992) when compared to FAP adenomas. The villous architecture of these

Table 1.2: Diagnostic criteria for hereditary non-polyposis colorectal cancer (HNPCC)*.

A. Amsterdam criteria

1. One member diagnosed with colorectal cancer before age 50
2. Two affected generations
3. Three affected relatives, one of them a first-degree relative of the other two
4. FAP should be excluded
5. Tumours should be verified by pathologic examination

B. Amsterdam criteria II

1. There should be at least three relatives with an HNPCC-associated cancer (colorectal cancer or cancer of the endometrium, small bowel, ureter or renal pelvis)
2. One should be a first-degree relative of the other two
3. At least two consecutive generations should be affected
4. At least one should be diagnosed before age 50
5. FAP should be excluded from the colorectal cancer cases
6. Tumours should be verified by pathologic examination

C. Bethesda guidelines

1. Individuals with cancer in families that meet the Amsterdam criteria
2. Individuals with 2 HNPCC-related cancers, including synchronous and metachronous colorectal cancers or associated extracolonic cancers (Note: endometrial, ovarian, hepatobiliary, or small bowel cancer or transitional cell carcinoma of the renal pelvis or ureter)
3. Individuals with colorectal cancer and a first-degree relative with colorectal cancer and/or HNPCC-related extracolonic cancer and/or a colorectal adenoma; one of the cancers diagnosed at age younger than 45 years, and the adenoma diagnosed at age younger than 40 years
4. Individuals with colorectal cancer or endometrial cancer diagnosed at age younger than 45 years
5. Individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed at age younger than 45 years (Note: solid/cribriform defined as poorly differentiated or undifferentiated carcinoma composed of irregular, solid sheets of large eosinophilic cells and containing small gland-like spaces)
6. Individuals with signet-ring cell type colorectal cancer diagnosed at age younger than 45 years (Note: composed of >50% signet-ring cells)
7. Individuals with adenomas diagnosed at age younger than 40 years

*Taken from Grady (2003)

carcinomas generally shows poor differentiation and a marked mucinous component (Robbins *et al.*, 2002; Chung and Rustgi, 2003). Furthermore, these lesions show a

high degree of lymphocyte infiltration (Smyrk *et al.*, 1997) and a Crohn's-like reaction involving the confluence of a heterogeneous population of B and T cells at the periphery of the tumour (Jass *et al.*, 1994). The presence of these histopathological features in a young patient (<45yr of age) with CRC is, according to the Bethesda criteria, sufficient to make a diagnosis of HNPCC.

1.6.3 Molecular features

HNPCC adenomas progress to carcinomas at an accelerated rate due to mutation and the subsequent inactivation of DNA mismatch repair genes, especially *hMLH1* and *hMSH2*, through the so-called mutator pathway. To a lesser extent, *hMSH6*, the pseudogene *hPMS1* and *hPMS2* have also been shown to contribute to HNPCC (Lynch and de la Chapelle, 2003). As discussed earlier, under normal circumstances the mismatch repair system functions through the co-operation of several genes to correct single base-pair mismatches and mispaired DNA loops within microsatellites (Chung and Rustgi, 2003). When MMR genes become mutated or silenced, errors accumulate (mutator phenotype) within the microsatellites, resulting in microsatellite instability (MSI) – a trademark feature of HNPCC, which can be used in its molecular diagnosis (Grady, 2003; Chung and Rustgi, 2002). A mismatch within microsatellite regions in intron sequences has little or no effect, while gene exons containing microsatellite regions are sensitive to MSI when mismatch repair genes are inactivated. Genes implicated in HNPCC to contain MSI include those coding for TGF-βRII (Markowitz *et al.*, 1995), insulin-like growth factors (Souza *et al.*, 1996), regulators of cell cycle (Yoshitaka *et al.*, 1996) or apoptosis (Rampino *et al.*, 1997), as

well as some mismatch repair genes themselves (Malkhosyn *et al.*, 1996). Mutations in *hMSH2* or *hMLH1*, accounting for 90% of HNPCC mutations (Chung and Rustgi, 2003), typically result in high-level MSI (MSI-H), whereas *hMSH6* mutations cause only partial mismatch repair deficiency and are found in approximately 10% of HNPCC cases (Lynch and de la Chapelle, 2003).

Through the use of the NCI panel of 5 microsatellite markers (containing 2 mononucleotide markers and 3 dinucleotide markers), MSI can effectively be detected (Boland *et al*, 1998). When at least two of the five microsatellite markers show instability, tumours are to be classified as MSI-H and analysis of mismatch repair gene mutations is recommended (Grady, 2003), usually in *hMLH1* and *hMSH2*. A high frequency of bandshifts occurs within the mononucleotide markers such as BAT25, BAT26, BAT34 and BAT40 in MSI-H tumours, whereas the dinucleotide markers (such as D5S346, D2S123 and D17S250) are frequently affected in MSI-H tumours but are also involved in MSI-L CRC (Dietmaier *et al.*, 1997).

1.6.4 Surveillance

The frequency of colonoscopy in patients with HNPCC is a controversial issue. These patients are generally recommended to undergo a full colonoscopy annually, especially where there is a strong evidence of germline mutations in *hMLH1*, *hMSH2* or *hMSH6*. Recently, Burke *et al.* (1997) suggested colonoscopy every three years in HNPCC patients with a known mutation in a mismatch repair gene. However, as Lynch and de la Chapelle (2003) noted, this may lead to the delayed diagnosis of

HNPCC. Furthermore, extracolonic screening of sites known to be susceptible to HNPCC-related cancers, is also indicated.

1.7. CRC without an inherited susceptibility: Sporadic CRC with MSI

1.7.1 Clinical and pathological features

Sporadic CRC shares many of the clinical, pathological and molecular characteristics with hereditary CRC. Several studies have shown that both sporadic and hereditary CRC develop from pre-existing adenomatous polyps (Robbins and Itzkowitz, 2002), but the time required to progress from normal mucosa to carcinoma seems to be prolonged (Wong *et al.*, 2002). The majority (~90%) of CRCs do not have an identifiable inherited basis and the distinctions between the hereditary and sporadic forms are very subtle. Morphologically, sporadic CRC are found in the proximal colon of essentially female patients at an advanced age (Malkhosyan *et al.*, 2000). Although the polyps appear to be of larger size, sporadic CRCs are also poorly differentiated and mucinous with the presence of tumour infiltrating lymphocytes and a Crohn's-like lymphocytic reaction (Jass *et al.*, 1998). It has been suggested that sporadic CRC develops from right-sided hyperplastic polyps and serrated adenomas (Hawkins and Ward, 2001). Recently, Wong *et al.* (2002) showed that the expansion of the proliferative compartment of sporadic human adenomas and hyperplastic polyps occurs through the process of crypt fission. These features are said to be consistent with a deregulation of cell cycle control (Wong *et al.*, 2002).

1.7.2 Molecular features

Although sporadic CRCs and HNPCC share the MSI phenotype, the molecular mechanisms leading to MSI may differ (Robbins and Itzkowitz, 2002). Sporadic CRC is characterized by the absence of a germline mutation in a DNA mismatch repair gene. Rather, sporadic CRC MSI is thought to be caused by an alternative method to gene inactivation through methylation of gene promotor sequences, commonly those of the *MLH1*, *TGF-βRII*, *IGFIIR* and *Bax* genes (Jass *et al.*, 2002; Lawes *et al.*, 2003). Additionally the DNA repair gene *O*-6-methylguanine DNA methyltransferase (*MGMT*), which, under normal conditions, functions to repair the methylation of guanine residues, is frequently inactivated through promoter methylation in CRC (Esteller *et al.*, 1999). Under normal conditions, DNA methylation functions to silence gene expression (Jones and Baylin, 2002) and to keep noncoding DNA in a transcriptionally inert state (Herman and Baylin, 2003). Abnormal patterns of loss or gain of DNA methylation has, however, been identified in several cancers.

Methylation occurs in regions rich in CpG sequences that cluster in the 5' region (promoter region) of many genes, and results in a CpG island methylator phenotype (CIMP) (Jass *et al.*, 2002). When many of these loci (methylated in tumour, MINT loci) are silenced through promoter methylation, the cancer is said to be CIMP positive. Recently it has been established that the biallelic methylation of the *MLH1* promotor sequence is linked to the development of MSI (Herman *et al.*, 1998; Cunningham *et al.*, 1998; Hawkins *et al.*, 2001), specifically MSI-H (Kuismanen *et al.*, 1999). In contrast, MSI-L tumours most commonly show *MGMT*-methylation (Whitehall *et al.*,

2001). Jass *et al.* (2002) propose that CIMP establishes hypermutability and tolerance to DNA damage, thereby resulting in the methylation and inactivation of multiple promotor sequences and their respective genes (Haydon and Jass, 2002). If this proves to be the case, silencing through methylation will result in clonal expansion, as is seen in ACF of the serrated neoplasia pathway (discussed earlier).

Two pathways have been proposed for MSI sporadic colorectal neoplasia, both of which involve promoter methylation (Jass *et al.*, 2000; Jass, 2001). In the first pathway, MSI-H results from the methylation of *MLH1* promoter sequences in mixed polyps and serrated adenomas (Jass *et al.*, 2000). This is thought to be an early, rate-limiting step (Jass *et al.*, 2002) with subsequent molecular events that include mutation of *TGFβRII*, *IGF2R* and *BAX* (Jass *et al.*, 2000). These findings have been confirmed through studies showing, amongst others, serrated adenomas in close proximity to MSI-H tumours (Iino *et al.*, 2000); through the increased frequency of hyperplastic polyps in patients with MSI-H cancer (Hawkins *et al.*, 2001); and through the absence of the classical mutational spectrum in MSI-H tumours (Olschwang *et al.*, 1997).

The second route involves silencing of the *MGMT* gene, again through promoter methylation. This occurs within hyperplastic polyps, serrated polyps and nondysplastic ACF (Jass *et al.*, 2002), but is not necessarily the initiating step. Other genes that may be implicated include the base excision repair genes methyl purine

glycosylase (*MPG*) (Vickers *et al.*, 1993), thymine DNA glycosylase (*MBD4*) (Bader *et al.*, 1999) and polymerase β (Wang *et al.*, 1992).

1.7.3 Improved prognosis for MSI positive tumours

Although several authors have suggested an improved prognosis for HNPCC when compared to stage-matched sporadic CRC (Lynch *et al.*, 1981; Albano *et al.*, 1982; Sankilo *et al.*, 1996; Watson *et al.*, 1998), some studies failed to show such a trend (Bertario *et al.*, 1999). Recently MSI has been shown to be a more reliable independent predictor of improved prognosis (Halling *et al.*, 1999). Lawes *et al.* (2003) put forward three theories to explain this phenomenon. As MSI positive tumours seldom show loss of heterozygosity, especially on chromosomes such as those harbouring DCC, p53 or KRAS genes, they proposed that it might be the presence of these proteins rather than MSI that confers the improved prognosis. They further speculated that MSI positive tumours might express aberrant proteins that mark these cells for destruction through the host immune system. This seems plausible as MSI positive cancers are often associated with a marked lymphocytic infiltration and a Crohn's-like response (Jass *et al.*, 1998). It has, however, been demonstrated that MSI positive cells fail to express the immunologically important major histocompatibility complex (MHC) class I molecule, β_2 microglobulin (Branch *et al.*, 1995), that masks these cells against immunological detection. It may also be possible that the accumulation of mutations within these MSI positive cells renders them susceptible to dying, as the capacity to perform basic functions needed for survival is lost (Lawes *et al.*, 2003).

1.7.4 Response of MSI positive tumours to chemo- and radiotherapy

Deficient mismatch repair plays a key role in the response of MSI positive tumours to the therapeutic effect of chemotherapy. Methylating agents commonly used in the treatment of cancer will be less effective during treatment of these tumours, as these cells readily become resistant to such agents (Humbert *et al.*, 1999). Similarly, MSI positive tumours are resistant to platinum compounds (Nehme *et al.*, 1997), which led to the discovery of 5-fluorouracil that has been reported to cause cell cycle arrest in MMR deficient cells (Carethers *et al.*, 1999). Although this is the preferred compound for the treatment of CRC, MSI positive tumours are less responsive to this treatment than MSS tumours (Niv, 2005).

Radiotherapy offers an alternative treatment regimen that leads to cellular apoptosis. The damage induced by radiation includes strand breaks. Lawes and colleagues thereby commented that, although its effect on MMR deficient cells is likely to be small, it might be clinically significant and suggest that further evaluation in this regard is required.

CHAPTER 2
CANCER OF THE COLORECTUM IN AFRICA

2.1 Introduction

Developing countries contribute more than half of the burden of newly diagnosed cancer cases each year (WHO, 2002). It is further expected that within the next 16 years, sixty percent of the world's cancers will be diagnosed in developing countries. Pisani *et al.* further propose a 71% increase in cancer deaths in developing countries for the period 1990 to 2010, in contrast to a 30% increase put forward for developed countries (Pisani *et al.*, 1999). This expected increase in incidence is thought partly to be due to HIV/AIDS, with AIDS-related Kaposi's sarcoma (KS) increasing from less than 5% of all cancers in central and southeast Africa during the 1960s to approximately a third in recent studies (Wabinga *et al.,* 1993; Banda *et al.*, 2001). Further research will most likely shed more light on the association of HIV/AIDS with other cancers. A stricter control on communicable diseases and changing lifestyles coupled with redress of existing cancer risk factors may ultimately contribute to the decrease in cancer incidence in developing countries.

South Africa houses a diverse population with dramatic differences in socio-economic circumstances, lifestyle and other environmental factors. However, access to health care has improved and several national initiatives to prevent and control the incidence of certain cancers have been established. The burden of hepatocellular cancer in children is expected to be reduced with the increase in hepatitis B immunization cover. A national PAP smear campaign is currently underway and stringent anti-

tobacco policies are progressively enforced, all holding promise to reduce cancer incidence.

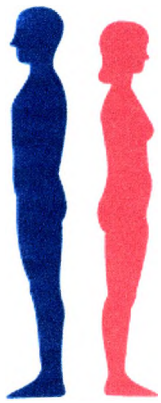
The National Cancer Registry of South Africa (NCR) was founded at the South African Institute for Medical Research (SAIMR) in 1986. The register is based on histopathological diagnosis of all cancers reported by both public sector and private laboratories throughout the country. It is considered an accurate indication of cancer rates but suffers from a significant lag phase in that the most current publication records malignant disease diagnosed during the period 1996 to 1997. For those years, prostate, lung, oesophagus, bladder and colorectal cancer were identified as the leading malignancies in males of all races. Amongst female patients, cancers of the cervix, breast, colorectum, oesophagus and lung were the leading cancers.

Of particular interest is an increase in colorectal cancer observed in the South African population, both male and female, although these rates were still considerably lower than those observed in western countries (Figure 2.1). In 1989, CRC was the tenth most common cancer diagnosed in both males and females in South Africa but is more recently ranked amongst the top 5 cancers (fifth amongst males and third amongst females) (NCR, 2003). Similarly, the lifetime risk of developing CRC increased from 1 in 139 for males and 1 in 176 for females during 1989, to 1 in 91 for males and 1 in 134 for females during 1997. A graphical depiction of age standardised incidence rates during 1989, 1992 and 1997 is illustrated (Figure 2.2).

Percentage distribution of the 10 most common cancers in the United States (2002)

Lung 31%	25% Lung
Prostate 11%	15% Breast
Colorectal 10%	**11% Colorectal**
Pancreas 5%	6% Pancreas
Non-Hodgkin's Lymphoma 5%	5% Ovary
Leukemia 4%	4% Non-Hodgkin's Lymphoma
Oesophagus 3%	4% Leukemia
Liver 3%	2% Uterus
Bladder 3%	2% Brain
Kidney 3%	2% Melanoma

Percentage distribution of the 10 most common cancers in South African populations (1996/7)



Prostate 12%	18% Cervix
Lung 6%	17% Breast
Squamous cell carcinoma of the skin 6%	16% Basal cell carcinoma
Oesophagus 6%	4% Squamous cell carcinoma of the skin
Bladder 4%	**3% Colorectal**
Colorectal 4%	3% Oesophagus
Stomach 3%	3% Uterus
Melanoma 3%	3% Lung
Larynx 2%	3% Melanoma
	2% Ovary

Figure 2.1: The estimated percentage of cancers in the United States and South Africa for the year 2002 and 1996/77 respectively, showing the 10 leading cancer sites by gender. Note that basal and squamous cell cancers of the skin are note registered in the USA (Adapted from Jemal *et al.*, 2002 and The National Cancer Registry of South Africa, 1996 – 1997)

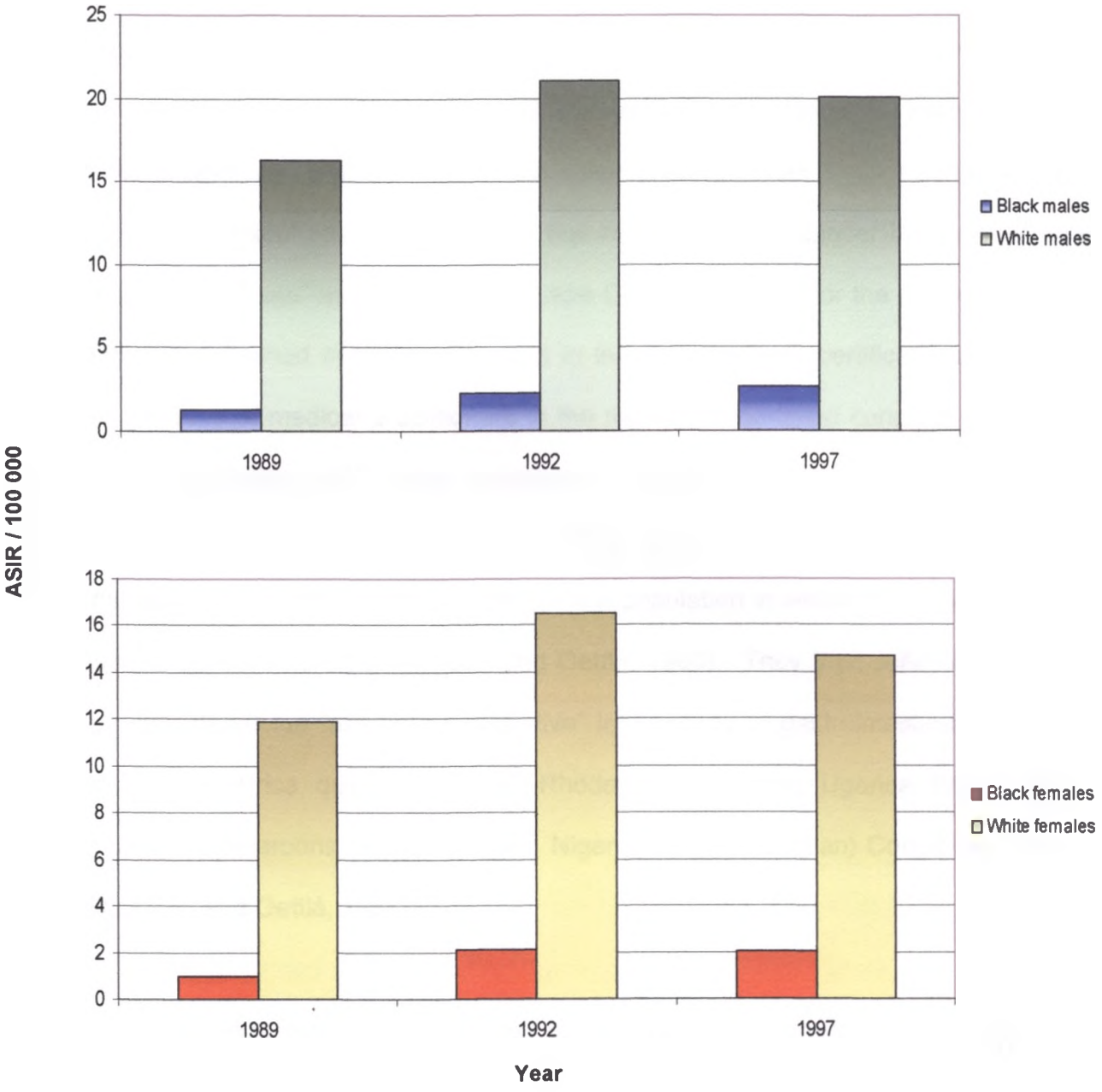


Figure 2.2: Age standardised incidence rate (ASIR) (per 100 000) for colorectal cancer diagnosed in South African populations. Results for black and white, male and female patients are shown. Adapted from the National Cancer Registry of South Africa for 1989, 1992 and 1996-1997.

2.2 Epidemiology of CRC in black South Africans

Modern cancer epidemiology in South Africa had its origins in the pioneering work of John Higginson, an anatomical pathologist, and George Oettlé, a histologist and epidemiologist, working at the SAIMR in Johannesburg. After smaller series relating to liver and oesophageal cancer, their first major study on cancer incidence in the Transvaal in "Bantu" and mixed race ("Cape Coloured") races for the periods 1953 to 1955 was published in 1960. This was in the form of death certificate surveillance and a survey of medical practitioners in the region. It identified cancer of the colon and rectum having $1/10^{th}$ of the incidence in "United States whites and Negroes or Danish whites". They postulated a dietary reason for this anomaly (a simple "unspiced natural diet" high in "roughage" in a population in which "constipation in the Western sense is rare") (Higginson and Oettlé, 1960). They then reflected on what they considered the "absolute not relative" infrequency of gastrointestinal cancer in sub-Saharan Africa, quoting data from Rhodesia (Zimbabwe), Uganda, French West Africa, the Cameroons, French Guinea, Nigeria, Ghana, (Belgian) Congo and Kenya (Higginson and Oettlé, 1961).

In 1967, Oettlé re-examined all tumours derived from the gastrointestinal tract in white and black patients from amongst 80,000 histopathology specimens identified in the former Transvaal province and seen at the SAIMR from 1949 to 1953 (Oettlé, 1967). Again noting the "exceedingly rare" incidence of CRC in black patients, he also hypothesised stool bulk and fibre as protective factors, quoting the early studies and personal communications of ARP Walker. Walker, a humble and self-effacing

scientist also employed by the SAIMR, had previously analysed frequency of bowel motions and stool volume in this population although tradition credits Dennis Burkitt with the original observation regarding dietary fibre, some years after Oettlé's publication (Burkitt, 1971; Burkitt *et al.*, 1972).

An association between CRC and adenomatous poyps had long been identified in western society. In 1970, Bremner and Ackerman, working at Baragwanath Hospital, first called attention to the rarity of colonic polyps in the black population. At that time, the hospital was admitting an increasing number of patients, from 37547 annually in 1955 to 77997 in 1968 and, in a review of the twelve-year period from 1956 to 1958 they identified only 47 polyps, 41 thereof being retention (juvenile) polyps. For the same period, CRC was histologically confirmed in 96 patients but not associated with polyps (two cases of "familial polyposis" being excluded). Furthermore, polyps were not identified in 1000 serial autopsies from the same period (Bremner and Ackerman, 1970). The rarity of polyps in black patients with CRC was again highlighted in a second study at Baragwanath Hospital from 1969 to 1978 when 6 polyps (2.9%) were identified, associated with or separate from 206 CRC (Segal *et al.*, 1981). In this study, the presence of "flat dysplastic lesions" in the colon in this population was illustrated (Figure 2.3) but this line of investigation was unfortunately never followed up.

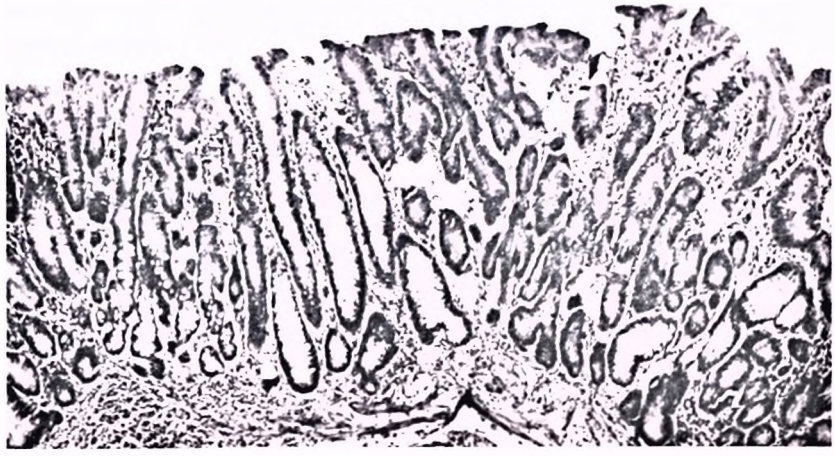


Figure 2.3: Colonic displasia as indicated in the paper by Segal *et al.* 1981.

A study from Hillbrow Hospital in Johannesburg from 1987 to 1990, serving what was then considered a more affluent black African population than the Soweto-based Baragwanath patients, identified a 14% incidence of adenomas over three years (Mayor-Davies *et al.*, 1992). However, a third series from Baragwanath Hospital for the five-year period 1990 to 1994 reported polyps in only 5.5% of 127 patients with CRC (Degiannis *et al.*, 1995) and a survey based on records of the South African National Cancer Registry for the entire Witwatersrand over two periods, 1991 and 1996, identified a similar frequency (Boytchev *et al.*, 1999) (Table 2.1).

Table 2.1: Percentage of associated adenomas in cases with large bowel cancer from earlier studies in Africa.

Town or region	Percentage
Soweto (1956 – 68)	0
Bulawayo (before 1969)	0
Sudan (1963 – 72)	0
Uganda (1964 – 76)	3.6
Soweto (1969 – 78)	8
Nigeria (1971 – 90)	1.5
Harare (1978 – 82)	3
Nigeria (1982 – 84)	0
Zimbabwe (1985 – 1986)	0
Hillbrow (1987 – 90)	14
Uganda (1990 – 95)	17
Kenya (1991 – 5)	4
Witwatersrand (1991 & 96)*	5.5

*** From: Boytchev *et al.*, 1999**

A constant theme over ensuing years was the rarity of African CRC with numerous postulates regarding protective factors. Although, as noted, Burkitt had originally identified the high fibre diet as having a protective effect leading to the paucity of primary bowel diseases in low risk populations (Burkitt, 1971), this was subsequently challenged by South African researchers (Oettlé and Segal, 1985; Segal and Walker, 1986; O'Keefe *et al.*, 1999). They re-affirmed the continuing low CRC incidence despite a decline in dietary fibre in the urban Black population and postulated that the low occurrence was due to a diet with continuing high resistant starch content associated with effective colonic microflora acquired in early infancy (Segal *et al.*, 2000). O'Keefe and co-workers also identified a change in the modern African diet to one low in fibre content as well as other suboptimal "protective" factors but with on-

going rarity of CRC with incidence rates less than 1:100,000 (O'Keefe *et al.*, 1999). These authors proposed that the rarity of CRC in the African population was due to low animal product consumption.

For the period 1993 through 1995, CRC was rarely diagnosed in black African patients (NCR, 1993-1995), but more recent reports have indicated a change in the incidence of CRC in this cohort. Population comparisons revealed an interesting trend. When comparing the percentage contribution to the total number of CRC cases diagnosed, no difference was observed during 1989 and 1992 in the frequency distribution of black patients *vs.* white patients (26% black patients *vs.* 74% white patients respectively). During 1996, however, black patients contributed on average an increased percentage of 2.65% to the total number of CRC cases (NCR, 1989, 1992, 1996-1997). Furthermore, gender comparisons between different racial groups are also notable. In 1989, black female patients were approximately twelve times less likely to develop colorectal cancer when compared to their white counterparts. Similarly during the same year black male patients were fourteen times less likely to develop CRC than white male patients. In 1996, however, this likelihood halved, making white females only 6 times and white males only 7 times more likely to develop CRC than their black counterparts. Therefore, the probability of black patients developing CRC doubled within a relatively short span of seven years. If the current trend persists, it can be expected that, within the next 10 years, black patients would have similar incidence rates for colorectal carcinoma as those white patients that are traditionally associated with a western lifestyle.

Subsequent to the most recent Cancer Registry figures being published, Angelo and Dreyer also described an increase in the number of black patients with CRC in the Pretoria/Tshwane region. Comparing the periods 1986 to 1987 and 1996 to 1997, they noted an increase in average age of the black cohort but increased numbers of young black females under 40 years of age, and an increase in the number of associated adenomatous polyps (10%) (Angelo and Dreyer, 2001).

2.3 Epidemiology of CRC elsewhere in the African continent

Although the first cancer registry outside of South Africa was set up in Uganda fifty years ago by JNP Davies (Davies, 1957), declining health standards and loss of health professionals and infrastructure across the continent over the ensuing half-century have led to the demise of formal and reliable large-scale cancer reporting. (This had not escaped the South African Registry over the past three years where certain private laboratories have withheld data, citing "ethical" issues pertaining to patient confidentiality.) Nevertheless, publications from 1935 to 1957 in (then) Rhodesia, and the French West African colonies, as summarized by Higginson and Oettlé (Higginson and Oettlé, 1961), all noted the infrequency of CRC. Publications documenting CRC were largely confined to smaller series and case reports, these being mostly submitted to local and regional medical journals in later years.

These reports, published over a period of 30 years, emanated from countries as diverse as Mozambique, Zimbabwe, Zambia, Zaire, Congo, Nigeria, Ghana, Kenya,

Uganda, Ethiopia, the Sudan and Egypt (Prates and Torres, 1967; Elmasri and Boulos, 1975; Kenda, 1976; Adekunle and Abioye, 1980; Owor, 1983; Murphree and Nkanza, 1988; Ojo *et al.*, 1991; Naaeder and Archampong, 1994; Ajao *et al.*, 1994; Iliyasu *et al.*,1996; Ashenafi, 2000; Adesanya and da Rocha-Afodu, 2000; Akute, 2000; Ashenafi, 2000; Sule *et al.*, 2001; Seleye-Fubara and Gbobo, 2005). They mostly identified relatively small numbers of cases, an exception being 526 patients identified over a 19-year period (average 27 patients annually) from Ibadan in Nigeria (Iliyasu *et al.*, 1996). Noteworthy is that data from almost identical number of patients (504 cases) from the same period and hospital were then published some four years later (Akute, 2000). Total patient numbers were invariably not identified and it was not possible to identify true incidence and prevalence statistics. Only the largest series, the "second" from Ibadan (Akute, 2000) "confirmed" a rise in incidence although some other studies alluded to this issue. Conversely Ashenafi reviewed 255 cases from Ethiopia over two five-year periods with intervening ten year interval and noted no significant differences (Ashenafi, 2000). Common themes were identified, however, including dominant rectal involvement but significant number of patients with right-sided disease (Table 2.2), a generally late presentation and poor prognosis when (limited) follow-up was available. It can only be speculated as to whether any rise in incidence is real or relates, at least in part, to increased surveillance or availability of medical care. However, another theme common to many studies, as well as most of the South African series, is an average age younger than would be expected in western society.

Table 2.2: Percentages of tumours occurring at different sites in the large bowel, from earlier studies in Africa.

	Caecum	Ascend/trans/desc*	Sigmoid	Rectum
Johannesburg (1949 – 53)		38		62
Nigeria (1954 – 67)		56		44
Soweto (1956-68)	19	13	13	54
Nigeria (1958-65)	22	14	15	49
Nigeria (1960-63)		52		48
Zaire (1960-74)	19	27		55
Uganda (1964-76)	13	7	12	67
Nigeria (1970-77)	10	0	6	67
Nigeria (1971-90)	13	14	7	61
Durban (1972-77)	12	28	24	37
Zimbabwe (1975-78)		10		87
Garankuwa (1976-91)		41		59
Ethiopia (1976-95)		21		61
Zimbabwe (1978-82)	17	24	5	54
Zimbabwe (1980-81)		43		57
Nigeria (1981-90)	10	22	41	28
Harare (1985-86)		35	14	51
Johannesburg (1987-90)	8	30	14	49
Ghana (1987-91)	25	15	10	51
Kenya (1991-95)		48		52
Witwatersrand (1991-96)#	13	15	16	55

* Ascend = ascending; transv = transverse; desc = descending
# From: Boytchev *et al.*, 1999

2.4 CRC in young black patients

In 1978, Pillay and co-workers from Natal identified eight cases of young South African black patients less than 25 years of age with CRC (Pillay *et al.*, 1978). At the same time, numbers of young patients, both black and mixed race (coloured), were identified in the Western Cape (Elliot and Louw, 1979; Elliot and Steven, 1984). The latter possibly pre-empted the later identification of HNPCC in a mixed race kindred as discussed later (Goldblatt *et al.*, 1990), although a subsequent study from the Western Cape identified a purported protective effect from the diet of coloured fishermen from the same region (Schloss *et al.*, 1997). Eleven years later Skalsky and Jaques described an "epidemic" of CRC in young black patients with 8 cases presenting over a 7-month period. This was within a defined geographic region of South Africa (Gazankulu) that is now incorporated into the Limpopo province in the north-east of the country (Skalsky and Jaques, 1989). In 2003 Stones and McGill reported signet ring adenocarcinomas identified over a five-year period in three black children residing in the Free State province. Two males and a female aged 13, 10 and 14 years had tumours of the ascending colon, rectum and transverse colon respectively (Stones and McGill, 2003).

Critical review of almost all South African publications documenting CRC in black patients confirms this phenomenon and identifies it as both long-standing and constant. Reappraisal of Oettlé's data for the former Transvaal province of South Africa over 50 years ago (1949 to 1953) identifies 4.84% of white patients less than 35 years of age compared to 24.93% of black patients, the latter notable for the

presence of "colloid" carcinomas (Oettlé, 1967). Similarly, 58% of black patients with CRC in Bremner's study at Baragwanath Hospital were at or under the age of 50 years while 27% were less than or equal to 40 years (Bremner *et al.*, 1970). In 1997, Bonnet and Grobler reviewed all CRC in black patients over a 7-year period (1986-1992) at Pelonomi Hospital, which serves as the academic referral centre for Bloemfontein in the Free State. These authors identified 121 patients (17 annually) of which 44 (32.4%) were under the age of 40 years. No differences were identified with respect to tumour sidedness and mucin production in younger *vs.* older patients (Bonnet and Grobler, 1997). Interestingly, Walker & Segal, who had published in the field for many years, identified for the first time in 2002 the occurrence of CRC in younger Black patients in Soweto, a "population in transition," despite its presence for at least the preceding 50 years (Walker and Segal, 2002). These authors considered the phenomenon to represent a "cohort effect" indicating a rising occurrence of the tumour in the urban African population. Finally, the publication by Angelo and Dreyer, noted previously, also identified disproportionately large numbers of younger black patients for both periods of their study (Angelo and Dreyer, 2001).

This paradigm furthermore appears common to countries elsewhere in the African continent. Papers documenting CRC in young African patients have appeared regularly but almost entirely in medical journals originating from Africa (Adekunle and Ajao, 1986; Ajao *et al.*, 1988; Ajao *et al.*, 1994; Sule *et al.*, 1999; Ameh and Nmadu, 2000). In a recent paper by Edino and co-workers from Nigeria, the mean age was noted to be 42.9 years with 72% of patients being below the age of 50 years.

Thirty percent of their patients also had mucinous adenocarcinomas (Edino *et al.*, 2005).

Tumour differentiation and type were often difficult to analyse. Many series only specified the tumours as being adenocarcinomas and some studies differentiated "adenomucinous" from mucinous carcinomas. In studies where histology was more specific, younger age and sometimes site (right colon) appeared to relate to mucinous tumours. In Oettlé's early series from 1947 to 1953, mucinous tumours were more prevalent in the often younger Black patients, these having caecal dominance (Oettlé, 1967). Degiannis and co-workers also emphasized the frequency of mucinous tumours in their series, 40% of patients under the age of 40 harbouring mucinous CRC *cf.* 14% of those above 55 years (Degiannis *et al.*, 1995). Boytchev and colleagues also commented on the frequency of mucinous and signet ring tumours, identified in a third of their 172 tumours. They did not differentiate these according to age, however (Boytchev *et al.*, 1999). Even in Egypt, mucinous tumours appear to predominate in younger patients. In their review of 50 patients with rectal carcinoma, 26 thereof being under 40 years of age, El-Hennawy observed greater numbers of mucinous tumours in the younger cohort (38.5% *cf.* 8.3%) (El-Hennawy *et al.*, 2003).

Again, critical review of other publications identified earlier in this chapter identifies, virtually without exception, excessive numbers of patients under the age of 50 years when compared with western CRC. In the series from Ethiopia, for example, 61.4% of cases occurred below the age of 50 years, 36% below 40 and 16% below 30 years

(Ashenafi, 2000). In 1997, Professor GJ Oettlé, a surgical gastroenterologist based at the Helen Joseph Hospital of the University of the Witwatersrand academic complex (and son of George Oettlé) wrote an invited commentary on the paper by Bonnet and Grobler, previously alluded to (Bonnet and Grobler, 1997). Oettlé analysed the age characteristics of 20 previous publications from Africa (including South Africa) over a 40-year period. He noted a frequency of patients under 30 years of age that ranged from 4 to 48% of the total, stating that "in Europe, typically less than 1% of cases occur in patients under 30 years old" (Oettlé, 1997). He also commented on the increased frequency of mucinous and signet ring tumours as well as the high frequency of caecal cancers although noting that "overall there are more rectal than colonic cancers". Oettlé also co-authored the last paper concerning CRC in black patients from the Witwatersrand for the current period (Boytchev *et al.*, 1999). Using the files from the National Cancer Registry for the periods 1991 and 1996, they identified 172 patients, 6% thereof presenting before 30 years of age and 22% before 40 years (Table 2.3). Mucinous or signet ring tumours were identified in a third of cases, this more often in males. Again adenomas were rare and the authors suggested a pathogenetic pathway different to the classical adenoma-carcinoma sequence.

Table 2.3: Cumulative percentages of cases of large bowel cancer occurring at different ages from earlier studies in Africa.

	<30 years	<40 years	<50 years	Mean age (years)
Transvaal (1953-55)	4%	15%	33%	51.7
Soweto (1954-63)	19%	31%	45%	50.3
Nigeria (1954-67)	11%	22%		47.6
Ibadan (1958-65)		46%		44.4
Ibadan (1960-75)	18%	42%	64%	44
Kinshasa (1960-74)	33%	58%	77%	38.9
Uganda (1964-76)	8%	24%	48%	50.8
Bulawayo (before 1968)	9%	27%		48
Nigeria (1970-77)		22%	56%	
Zimbabwe (1978-82)	16%	35%	63%	45.6
Nigeria (1981-90)	2%	7%	37%	53
Zimbabwe (1985-86)	19%	29%	39%	51
South Africa (1986)	8%	22%	41%	53.9
Hillbrow (1987-90)	10%	18%	43%	52.1
Ghana (1987-91)	5%	18%	43%	58.7
Witwatersrand (1991/96)*	6%	22%	39%	54.3

*** From: Boytchev *et al.*, 1999**

Finally, and of interest in the African experience of CRC, is the existence of the disease in young Egyptian patients, although this nation has a different phylogenetic origin to that of the sub-Saharan black population. In their review of 50 patients with rectal carcinoma, 26 thereof under 40 years of age, El-Hennawy and colleagues observed greater numbers of mucinous tumours in the younger cohort (38.5% *cf.* 8.3%) with more advanced disease at time of presentation and lower survival rates when compared with patients greater than 40 years (El-Hennawy *et al.*, 2003).

Analysis of most recent data provided by the South African National Cancer Registry also notes age-related differences. Amongst patients younger than 35 years of age,

an increase in the number of cases reported per year was noticed for black female and white male patients. In contrast, black male, and white female patients showed slightly less CRC cases diagnosed in 1996, when compared to the number of cases reported in 1989 (Figure 2.4). Older patients (above 50 years of age), specifically female patients of both races and black male patients, presented with CRC at an increasing rate, while white male patients did not show a difference in CRC incidence between 1989 and 1996 (Figure 2.4) (Mqoqi *et al.* 2004). It is not known how much these figures relate to circumstances including increased access to basic health care

The Division of Anatomical Pathology at the Johannesburg Hospital, within the School of Pathology of the University of the Witwatersrand and National Health Laboratory Service, is the successor to and serves largely the same patient population as the SAIMR. As opposed to data reported by the NCR, which reflects cases seen throughout South Africa, the cases seen by this department include those from the academic hospitals in Johannesburg (Johannesburg and Helen Joseph Hospitals), as well as all public sector hospitals in the Northern regions of the country, namely Gauteng, Mpumalanga, and North-West Province as well as a portion of Limpopo Province. Health services in these regions cover an estimated population of 14 million, one third of the national total. The patient population served by the academic and public sector hospitals has been predominantly black since the late 1980s as the majority of white patients have private medical insurance. Thus figures emanating from this department do not represent the incidence figures of the Cancer Registry.

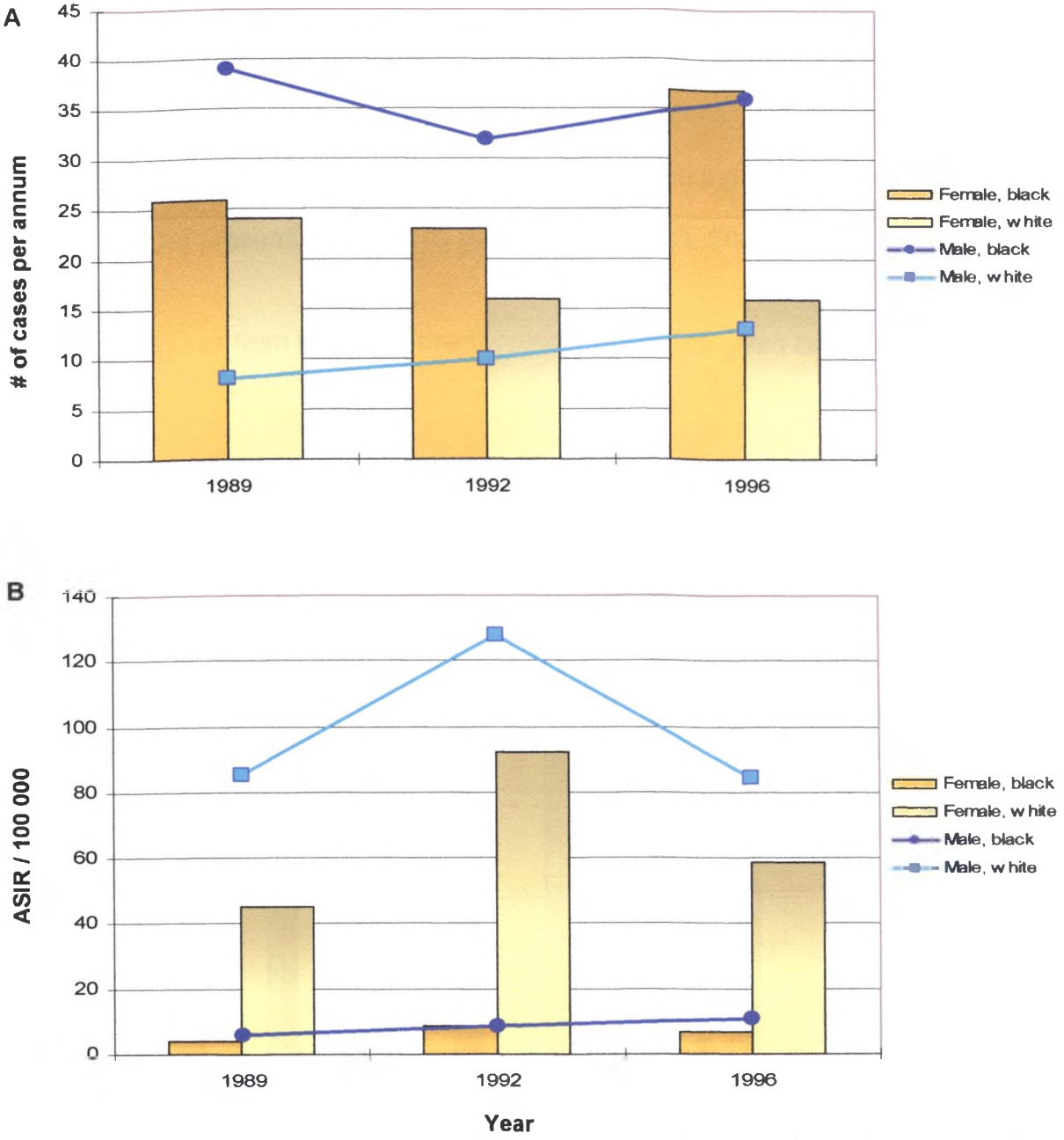


Figure 2.4: Frequency of histologically diagnosed colorectal cancer (CRC) (A) and age standardised incidence rate (ASIR) (per 100 000) (B) during 1989, 1992 and 1996 in South Africa. *A* represents the frequency of CRC diagnosed amongst patients 35 years of age and younger. *B* represents the incidence rates of CRC amongst patients 50 years and older. Results for black and white, male and female patients are shown. Adapted from the National Cancer Registry of South Africa for 1989, 1992 and 1996-1997.

Nevertheless, the prevalence of unexpectedly large numbers of younger black patients with CRC has been identified in this department since computerised records became available in 1990. Preliminary comparisons between black and white patients found a disproportionately high number of young black South Africans (< 35 years of age) presenting with CRC (Muc and Paterson, 2000).

Updated figures from this department (1990 – 2002) are given below (Figure 2.5).

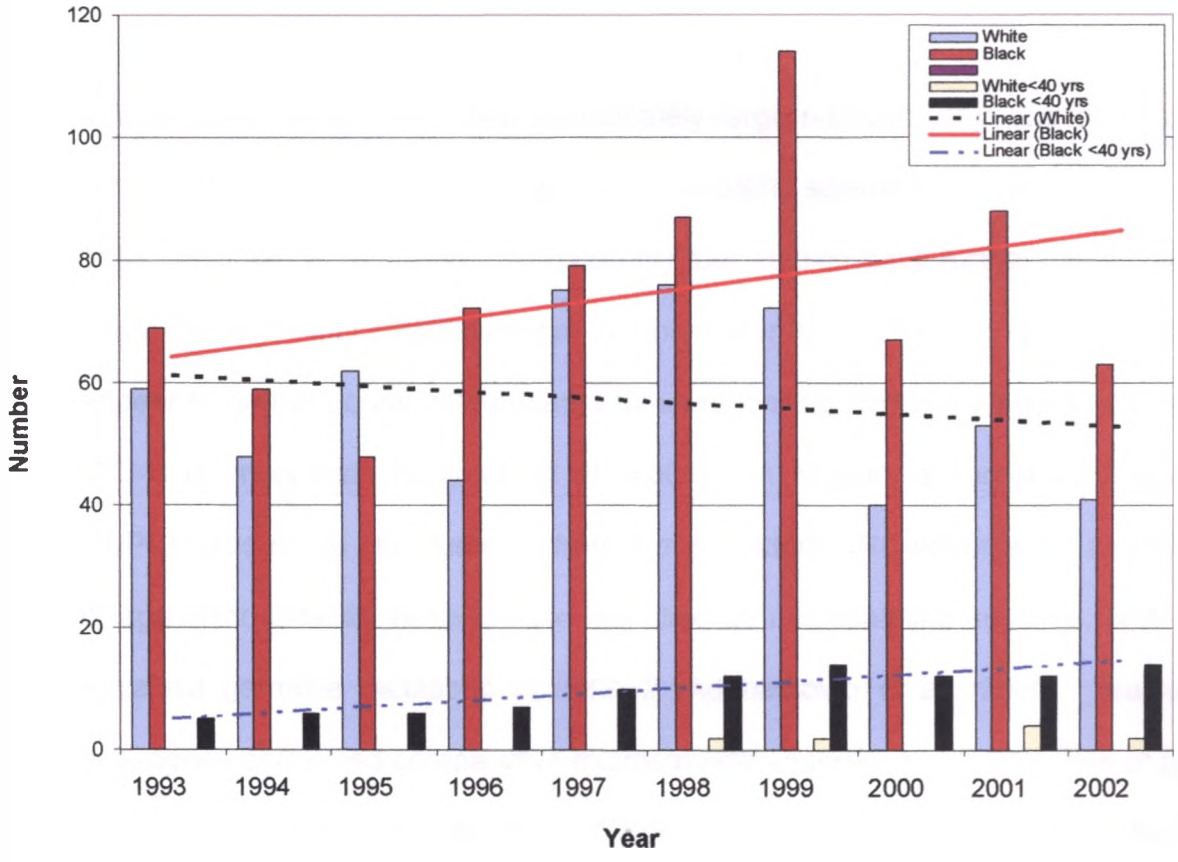


Figure 2.5: Updated figures for the number of black and white South African patients presenting with colorectal cancer (CRC) in the Department of Anatomical Pathology, University of the Witwatersrand , 1993 to 2002.

2.5 Summary and hypothesis

The review of literature accumulated over a period of nearly 60 years from the African continent identifies a constant and sustained number of young (< 50 years and often much younger) patients presenting with CRC, a phenomenon not identified in Western society. These tumours are often mucinous or poorly differentiated and possibly increased numbers occur on the right side of the colon although the literature is less certain thereof, with the majority of tumours overall occurring in the left side of the bowel. This situation may be slightly different in younger patients.

The presence in Africa of disproportionately large numbers of young black patients with CRC appears virtually unnoticed in western scientific literature (Rubin *et al.*, 1998; Soliman *et al*, 1999). To date only four studies have further investigated this event at an immunohistochemical or molecular level. Young Egyptian patients appear to harbor poorly differentiated tumours lacking *KRAS* mutations and in whom MSI-H is uncommon (Soliman *et al.*, 2001). In Nigeria, a purported diagnosis of HNPCC was made after tumours from 2 of 5 random CRC patients, 51 and 67 years of age without family history, were identified as microsatellite unstable, both having apparent germline mutations in *hMSH2* (Adebamowo *et al.*, 2000). Naidoo and colleagues performed comparative microsatellite analysis in a small series of patients in the Natal region of South Africa (Naidoo *et al.*, 2000). These authors found that 31% of younger patients showed MSI-H when compared to 12% of older individuals. Race was not specifically identified in their study although the majority was black African (Naidoo, personal communication). Finally, a mixed-race kindred has been

identified across three generations in the Namaqualand region of South Africa, and confirmed as showing HNPCC (Goldblatt *et al.*, 1990; Ramesar *et al.*, 2000).

It is thus hypothesised that CRC as occurring in younger black African patients is the result of primary genetic events rather than dietary factors or cohort effect. As is often the situation in relatively poor developing populations, family history is not always provided, which makes the diagnosis of heredity-based diseases problematic. Thus further morphological, immunohistochemical and molecular analysis may provide insight into whether these cases are indeed familial-based or represent a novel pathway to the development of colorectal carcinoma.

CHAPTER 3

A PATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF COLORECTAL CARCINOMA

3.1. Introduction:

Classically, CRC is associated with chromosomal instability and mutations of multiple oncogenes, including *APC*, *K-ras* and *p53*. Alternatively, cancer may develop through microsatellite instability (MSI), which may be sporadic (10% of cases) or hereditary (90% of cases) (Liang *et al.*, 2003). MSI is associated with mutations in the DNA mismatch repair genes *hMLH1* and *hMSH2*, and less frequently *hMSH6* and *PMS2* that lead to the rapid development of neoplasms through the accumulation of mutations (Lynch & de la Chapelle, 2003). Both these pathways follow the well-known adenoma-carcinoma sequence of events.

Recently, however, concepts relating to a "serrated-adenoma" pathway were proposed, that involve the formation of a tumour from hyperplastic polyps and adenomas through intermediate serrated adenomas (Jass *et al.*, 1999). These tumours show a low level of MSI (MSI-L), together with a methylation phenotype, characterized by the methylation of CpG islands within the promoter regions of genes such as *hMLH1* (Park *et al.*, 2003) and O^6-methylguanine DNA-methyltransferase (*MGMT*) (Jass, 2005). This is less common, and could possibly explain the occurrence of CRC amongst young patients

As discussed in Chapter 2, the incidence of CRC in South Africa appears to have increased over the past decade. Furthermore, disproportionate numbers of young patients have long been identified both in South Africa and elsewhere in the continent. The tumour, as identified in white patients from this country, has not

been comprehensively investigated but, empirically, it would appear highly likely that these patients follow a westernised trend with aetiogical factors common to the First World.

There has been no comprehensive systemic investigation of the aetiology of CRC in black patients, particularly the younger individuals. These patients could present with CRC that is more compatible with that identified in hereditary syndromes.

3.2. Materials and Methods:

3.2.1 Patient selection

The material was derived initially from a retrospectively collected series of biopsies and resection specimens (the period 1990 – 2003), retrieved from the records of the Division of Anatomical Pathology, School of Pathology, University of the Witwatersrand. These included cases seen at the Johannesburg academic hospitals (Johannesburg General and Helen Joseph) as well as all of the public sector hospitals in the provinces of Gauteng, Mpumalanga and North-West Province. The records of Chris Hani-Baragwanath Hospital were not included in this study as computerized records for the period under review were unavailable. Cases were selected when they fulfilled the diagnostic criteria of adenocarcinoma of the colon or rectum. Cases of familial adenomatous polyposis were excluded from the study. All the cases were stratified by age (<35 years, 35 – 50 years, >50 years), gender and ethnicity (black or white) for statistical purposes. All blocks were anonymous and this study was approved by the Ethics committee of the

University of the Witwatersrand for retrospective work on archival material (clearance number 9/11/88).

Due to the large numbers of cases identified (1 732 cases), all cases were initially subjected to analysis of age, sex, race, and tumour site based on original histopathology reports. Thereafter a subset of patients for the five-year period 1998 to 2002 were subjected to formal histopathological review. Haematoxylin and eosin-stained slides were reviewed without the knowledge of patient demographics or tumour site. This was done in consultation with one study supervisor, Prof A C Paterson, an experienced gastrointestinal and liver pathologist.

3.2.2 Pathologic assessment

3.2.2.1 Morphological features

The following criteria were used to evaluate the tumour :

1. Site

   The site of the tumour was recorded as rectum, sigmoid, descending colon, transverse colon, ascending colon or caecum. Right-sided tumours were recorded as those proximal to the splenic flexure.

2. Differentiation

   a. *Well differentiated (low grade)*

      Tumour was graded as well differentiated when it was composed of complex or simple tubules, nuclear polarity and nuclei of uniform size with a distinct resemblance to adenomatous epithelium.

b. *Moderately differentiated (low grade)*

A grade of moderately differentiated was awarded when tubules were complex, simple or irregular with little or no nuclear polarity. A percentage of tumours were also classified morphologically as having a cribriform apprearance.

c. *Poorly differentiated (high grade)*

Criteria for poor differentiation included poor or highly irregular gland development with epithelial cells being arranged in small and irregular clusters showing a complete loss of nuclear polarity.

d. *Mucinous differentiation*

Mucinous tumours were those classified with >50% area showing extracellular mucin. Cases showing <50% extracellular mucin were classified as having focal mucinous differentiation.

e. *Signet-ring cells*

Tumours were classified as signet-ring cell carcinomas if >50% of the area showed the presence of signet-ring cells. If the tumour contained <50% signet-ring cells, it was classified as having focal signet-ring cell differentiation.

Signet-ring carcinomas were considered high grade tumours according to WHO criteria (Hamilton *et al.*, 2000).

3. Histologic heterogeneity

Tumours with at least two distinct growth patterns were classified as showing histologic heterogeneity.

4. Growth pattern of tumour at advancing edge

   The advancing edge of the tumour was examined under low power and classified as either expansile or infiltrative. Tumours advancing by a process of outward infiltration through the normal structures of the bowel wall, was said to *infiltrative*. A sharply delineated growing margin was said to be *expansile*. If the edge was not visible, this field was coded as unknown.

5. Tumour necrosis

   The presence of necrosis within the tumour was noted (tumoral necrosis) when the entity was identified within glandular lumina. Care was taken to distinguish between tumoral necrosis and necrosis secondary to ulceration of the tumour.

6. Crohn's-like host response

   The tumour advancing edge was assessed for the presence of a Crohn's-like inflammatory response. This was considered prominent if a minimum of three lymphoid aggregates was visible per section. If the advancing edge of the tumour was not present, this field was graded as unknown.

7. Tumour infiltrating lymphocytes (TIL)

   Tumour infiltrating lymphocytes (TIL) were identified on haematoxylin and eosin-stained sections as mononuclear inflammatory cells infiltrating between tumour cells. This included a distinct inflammatory margin at the tumour edge in which lymphocytes were well represented. This was occasionally confirmed using immunohistochemistry for CD8 expression.

8. Adenoma

The presence of adenomas was noted and grouped into sessile, tubular, villous, tubulovillous and serrated based on established morphological features.

9. Tumour stage

The haematoxylin and eosin-stained slides of resected specimens for the period 1998-2003 (n = 246) were staged according to the Dukes classification (1932), Astler-Coller modification of Dukes' classification, TNM classification (1997) and the Jass classification system (See Chapter 1, Table 1.1). As very little or no patient history was available the number of metastases could not be determined and was universally scored as *Mx* in the TNM classification. Grading based on the Jass classification system involved calculating a score based on specific parameters. Spread limited to the bowel wall scored zero, while spread beyond the bowel wall scored one. An expansile growth pattern scored zero, while an infiltrative pattern scored one. The presence of infiltrating lymphocytes scored zero, whereas the absence thereof scored 1. Based on the number of lymph nodes involved with tumour, a score of zero was given when no nodes where involved, one when up to 4 nodes were positive, and two when more than 4 nodes were involved. After calculation, a total score of up to one demarcated Group I; a score of two was grouped into Group II; Group III had a score of three and a score of four to five was placed in Group IV (Jass *et al*, 1987).

3.2.2 Immunohistochemical analysis

Table 2.1 summarises the antibody specifications. Immunohistochemical staining was performed on 4μm sections of formalin-fixed, paraffin-embedded tissue sections on 2% APES coated microscope slides, using the DakoCytomation Autostainer (DakoCytomation, Denmark). Sections were deparaffinized and rehydrated through immersion in xylene and graded alcohol. Heat-induced antigen retrieval was performed using a combination of microwaving and the use of a pressure cooker. This entailed heating 10mM citrate buffer (pH6) in the pressure cooker using the microwave oven prior to the addition of slides. After cooling, endogenous peroxidase was blocked through the incubation of slides in 3% H_2O_2 in dH_2O for 15 minutes. Slides were then thoroughly washed in Tris-buffered saline (TBS) (Dakocytomation, Denmark) (pH7.6) containing 0.1% Tween20, before blocking any non-specific antigen activity through immersion of slides in 5% normal goat sera to block non-specific antigen activity. Sections were subsequently exposed to the monoclonal antibodies (dilutions prepared in antibody diluent; DakoCytomation, Denmark) in a humidified atmosphere at room temperature for 1 hour. After rinsing thoroughly with TBS, slides were treated with a peroxidase-conjugated polymer and antibody (i.e. secondary antibody) directed against rabbit and mouse immunoglobulin for 30 minutes (ChemMate Dako EnVision Detection kit, DakoCytomation, Denmark). Slides were washed and incubated with 3,3'-diaminobenzidine (DAB) chromogen and H_2O_2 for 7 minutes. Sections were then washed in dH_2O, counterstained with haematoxylin, dehydrated and mounted with a cover slip.

Table 3.1: Details of immunohistochemistry antibodies

Antigen	Antibody clone	Dilution	Source
APC	H-290	1:200	Santa Cruz
p53	PAb240	1:75	Dako
hMLH1	G168-15	1:100	BD Pharmingen
hMSH2	G219-1129	1:200	BD Pharmingen
hMSH6	Clone 44	1:500	BD Transduction
hMGMT	MT23.2	1:75	Zymed

3.2.3 Statistical analysis

Statistical comparisons between groups were completed using the two-sided Fisher's exact test. Patients were analyzed based on ethnicity (black vs. white), age (<50 years vs. >50 years), race (male vs. female) and tumor site (as described above). Multinomial logistic regression analysis was performed to calculate odds ratios. All statistical analyses were completed using Stata Intercooled 7.0 (Stata, College Station, TX, USA). With $p < 0.05$, the differences were considered statistically significant.

3.3. Results:

3.3.1 Patient demographics

For the period 1990 to 2003, 1 732 black and white patients with colorectal cancer were identified according to pathology records (Figure 3.1A). These reports were not informative for all the parameters in every case which led to differences in the

number of cases compared. Being a retrospective study, no family history was obtainable, and patients therefore could not be classified according to the Amsterdam or Bethesda criteria. Cases were subsequently stratified into 3 groups according to age at diagnosis, with 73% (1259/1731) being older than 50 years of age, 21% (361/1731) between 31 and 49 years of age, and 6% (111/1731) below 30 years of age (age range 14 to 100yrs, mean 59) (Figure 3.1B). For ease of statistical analyses the age groups <30yr and 31 – 49yr were combined into a <50yr age category, thereby accounting for 27% (472/1731) of cases. One case report did not indicate patient age (0.06%).

The overall distribution between male and female was slightly skewed: 52% (838/1619) of the patients were male, while 48% (781/1619) were female (Figure 3.1C). One hundred and fourteen case reports (7%; 114/1733) did not give any indication of gender.

Sidedness of the tumour was noted in 1240 of the case reports with 78% (971/1240) of tumours occurring distal to the splenic flexure and 22% (269/1240) occurring proximal to the splenic flexure (Figure 3.1D). Twenty eight percent (494/1734) of reports did not state tumour location.

Distinct differences were noted between blacks and whites. Black patients diagnosed with colorectal cancer were predominantly male (55%), while 53% whites were females ($p = 0.001$) (Figure 3.2). Similarly, 83% black patients were younger than 50 years of age, compared to only 16% of whites falling into the same category ($p = 0.000$) (Figure 3.2). No significant difference was noted between ethnicity and tumour location ($p = 1.000$).

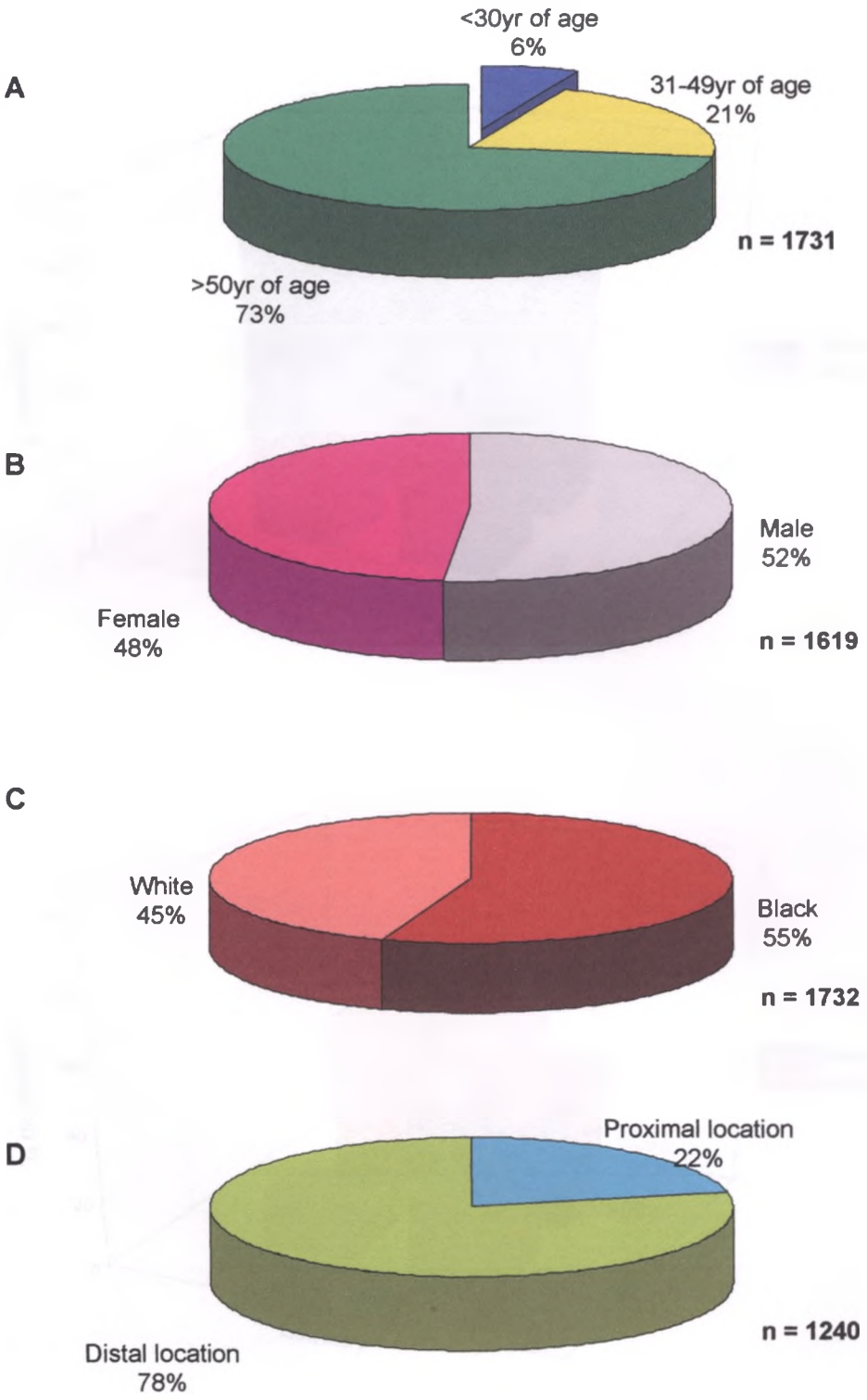


Figure 3.1: Percentage distribution of demographic characteristics of South African patients diagnosed with colorectal carcinoma (CRC) during 1990 – 2003. Data was retrospectively collected from pathology reports and stratified according to age (A), gender (B) and ethnicity (C). Tumour location was noted as proximal or distal to the splenic flexure (D).

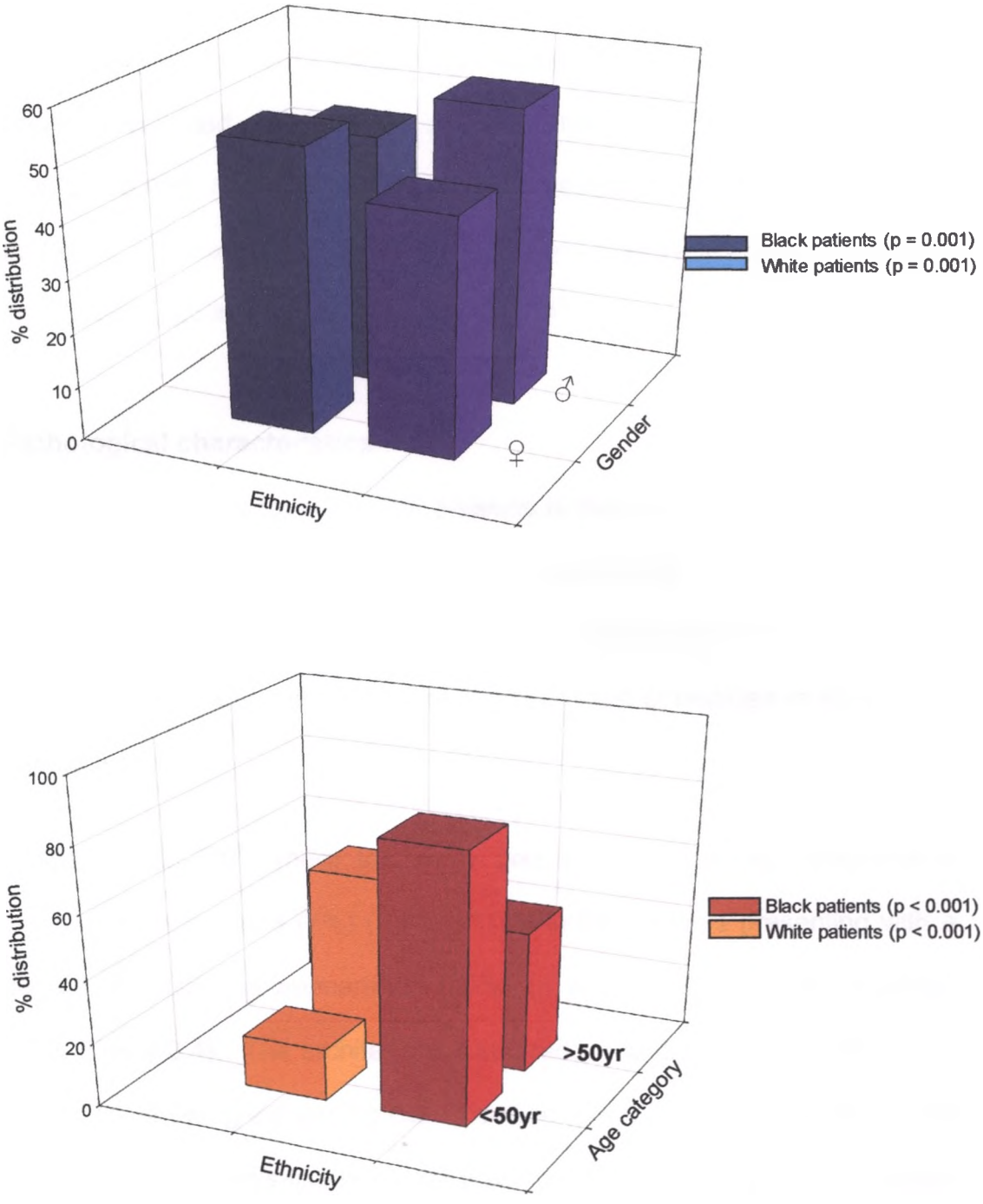


Figure 3.2: Blacks with colorectal cancer (CRC) were more frequently observed in young, male patients. Demographic data was obtained from retrospectively collected records. Values are given as percentages and associations were determined through the Fisher's exact test at a 95% confidence interval.

Significant associations (95% CI) were found between age category and ethnicity, gender and tumour location. These included an association between young patients and gender, with 56% being male and 44% female (p = 0.026). Gender was equally distributed amongst the patients older than 50 years of age at diagnosis (p = 0.026) (Figure 3.3). Young patients had predominantly right-sided tumours (32%), compared to the 26% which presented with left-sided tumours (p = 0.064; marginally significant at 95% CI) (Figure 3.3).

3.3.2 Pathological characteristics

A summary of the results together with p-values is depicted in Table 3.2. It must be noted that p-values were calculated for associations between each main variable, for example the association between tumour location in either black or white patients or, similarly, the association between level of necrosis in either male or female patients.

Black patients with CRC more frequently presented with poorly differentiated (15%) or mucinous tumours (7%) (p = 0.001) with the tumours presenting with a high degree of mucinous appearance (50%) (p = 0.006), compared to whites (Table 3.2, Figure 3.4). The architecture was predominantly glandular (68%) (p = 0.011), with 7% showing signet-ring cell formation (p = 0.011). In both blacks and whites low levels of Crohn's-like response was noted (p = 0.771) and 40% showed marked tumoral necrosis (p = 0.388). Whites in contrast had predominantly moderately differentiated tumours (85%) (p = 0.001) with lower levels of mucin expression (Table 3.2, Figure 3.4). A cribriform architecture was more evident in whites than in blacks (p = 0.011).

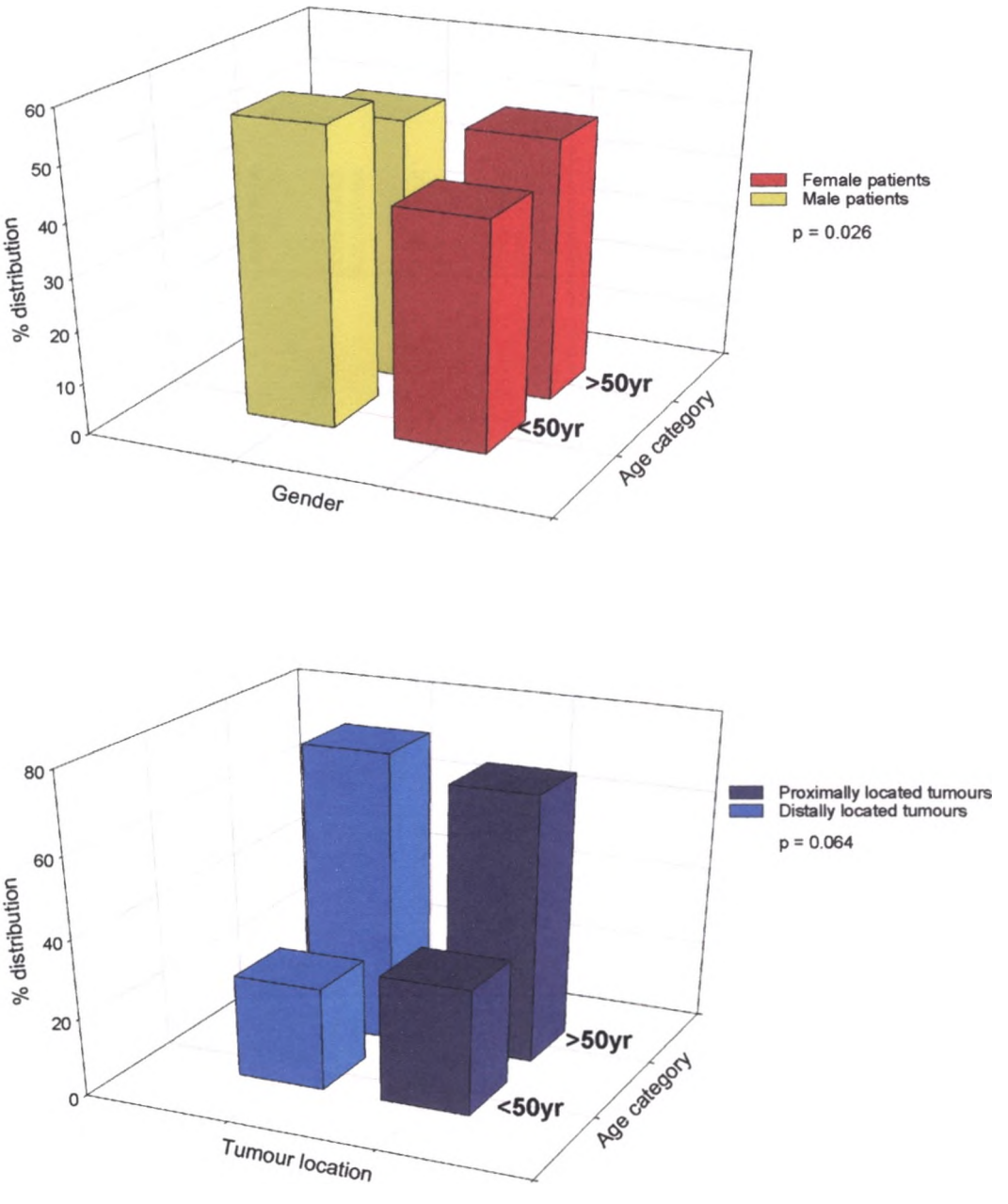
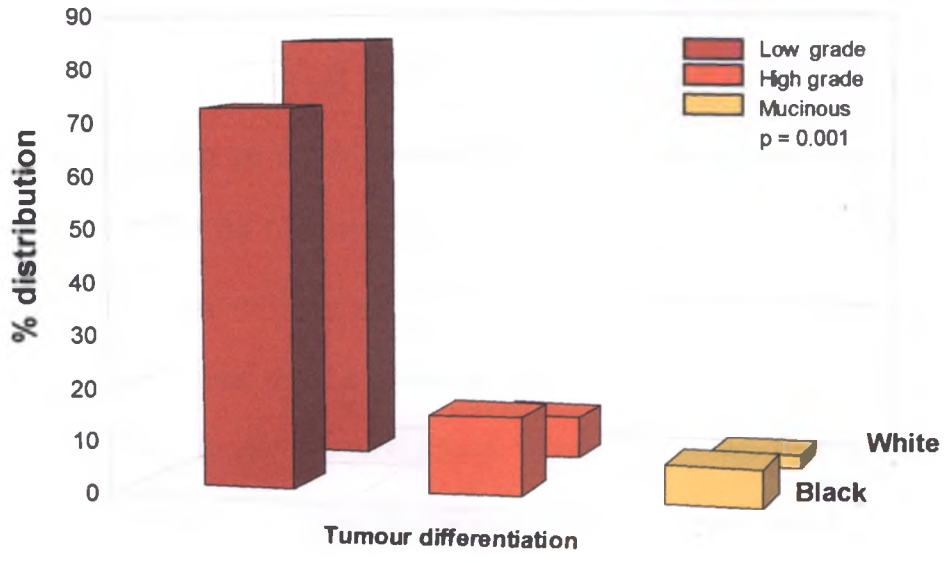


Figure 3.3: Young patients (<50 years of age) with colorectal cancer (CRC) are predominantly found in male patients with tumours located proximally to the splenic flexure. Demographic data was obtained from retrospectively collected records. Values are given as percentages and associations were determined through the Fisher's exact test at a 95% confidence interval.

Table 3.2: An overview of the association between ethnicity, age category and tumour location to pathological characteristics. Values are given as a percentage of the overall distribution between the different variable. P-values printed in bold represent significant association at 95% confidence interval.

	Ethnicity			Age category			Gender			Tumour location		
	Black	White	p-value	<50yr of age	>50yr of age	p-value	Female	Male	p-value	Caecum - transverse colon	Descending colon - rectum	p-value
Tumour differentiation:			**0.001**			**0.000**			0.119			**0.000**
Well differentiated	3.81% (14/367)	4.33% (11/245)		4.73% (8/169)	3.76% (17/452)		3.77% (11/292)	4.38% (14/320)		6.14% (7/114)	3.34% (15/449)	
Moderately differentiated	72.48% (266/367)	84.65% (215/245)		64.50% (109/169)	82.30% (372/452)		81.16% (237/292)	74.06% (237/320)		59.65% (68/114)	82.63% (371/449)	
Poorly differentiated	14.99% (55/367)	8.27% (21/245)		18.93% (32/169)	9.73% (44/452)		8.56% (25/292)	15.31% (49/320)		22.81 (26/114)	9.58% (43/449)	
Mucinous tumour	7.36% (27/367)	2.76% (7/245)		9.47% (16/160)	3.98% (18/452)		5.82% (17/292)	5.31% (17/320)		11.40% (13/114)	3.56% (16/449)	
Signet ring tumour	1.36% (5/367)	0		2.37% (4/160)	0.22% (1/452)		0.68% (2/292)	0.94% (3/320)		0	0.89% (4/449)	
% Mucin expression:			**0.006**			0.105			0.427			1.000
<50% mucin	39.64% (44/111)	64.00% (32/50)		38.71% (24/62)	52.53% (52/99)		51.43% (36/70)	44.44% (40/90)		48.98% (24/49)	48.00% (48/100)	
>50% mucin	60.36% (67/111)	36.00% (18/50)		61.29% (38/62)	47.47% (47/99)		48.57% (34/70)	55.56% (50/90)		51.02% (25/49)	52.00% (52/100)	
Chron's infiltrate:			0.771			0.520			0.777			1.000
Low level infiltrate	90.67% (68/75)	88.68% (47/53)		86.49% (32/37)	91.21% (83/91)		90.77% (59/65)	88.89% (56/63)		91.49% (43/47)	90% (63/70)	
High level infiltrate	9.33% (7/75)	11.32% (6/53)		13.51% (5/37)	8.79% (8/91)		9.23% (6/65)	11.11% (7/63)		8.51% (4/47)	10.00% (7/70)	
Necrosis:			0.388			0.806			0.590			**0.001**
Low level necrosis	27.56% (35/127)	36.26% (33/91)		28.79% (19/66)	32.24% (49/152)		34.74% (33/95)	28.33% (34/120)		18.18% (12/66)	36.03% (49/136)	
Moderate level necrosis	32.28% (41/127)	29.67% (27/91)		30.30% (20/66)	31.58% (48/152)		28.42% (27/95)	33.33% (40/120)		25.76% (17/66)	34.56% (47/136)	
High level necrosis	40.16% (51/127)	34.07% (31/91)		40.91% (27/66)	36.18% (55/152)		36.84% (35/95)	38.33% (46/120)		56.06% (37/66)	29.41% (40/136)	



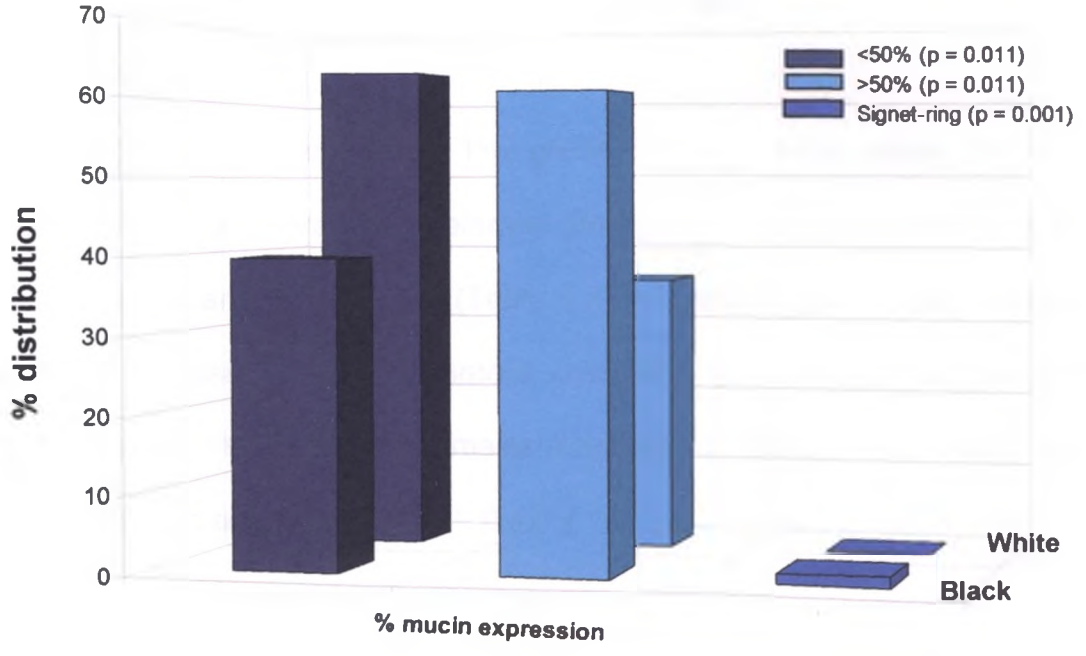


Figure 3.4: Colorectal tumours from black patients as compared to whites are predominantly high grade with a high degree of mucin expression. Demographic data was obtained from retrospectively collected records. Values are given as percentages and associations were determined through the Fisher's exact test at a 95% confidence interval.

Poorly differentiated and mucinous tumours were more common amongst young patients (<50yr of age) than old patients (>50yr of age) ($p = 0.000$), which was mirrored by the high degree of mucin expression in younger patients (61% *vs* 47% in older patients) ($p = 0.105$) (Table 3.2, Figure 3.5). Similarly, young patients more frequently showed a signet-ring cell morphology as opposed to older patients who more commonly presented with a cribriform architecture ($p = 0.011$). A glandular architecture was seen in similar proportions between the two age categories. The tumour architecture was significantly different between the two age groups ($p = 0.001$) (Table 3.2, Figure 3.5). Both young and old patients rarely had a distinct Crohn's infiltrate ($p = 0.520$) and no significant association was found between age category and level of tumoral necrosis present ($p = 0.806$).

Females frequently presented with low grade tumours, while poorly differentiated (high grade) tumours were more common amongst males. This was, however, not statistically significantly ($p = 0.119$) (Table 3.2). An equal percentage of males and females had a classic mucinous tumour, and although not statistically significant, males expressed more mucin (55% males expressed more than 50% while only 49% of females showed similar levels) ($p = 0.427$).

Tumours located proximal to the splenic flexure were most often moderately differentiated (60%) with a considerable percentage of poorly differentiated (23%) and mucinous tumours (11%) compared to those located distally ($p = 0.000$). Twelve percent of proximal tumours showed a signet-ring cell architecture ($p = 0.005$), and high levels of necrosis was observed in 56% ($p = 0.005$). Distal tumours

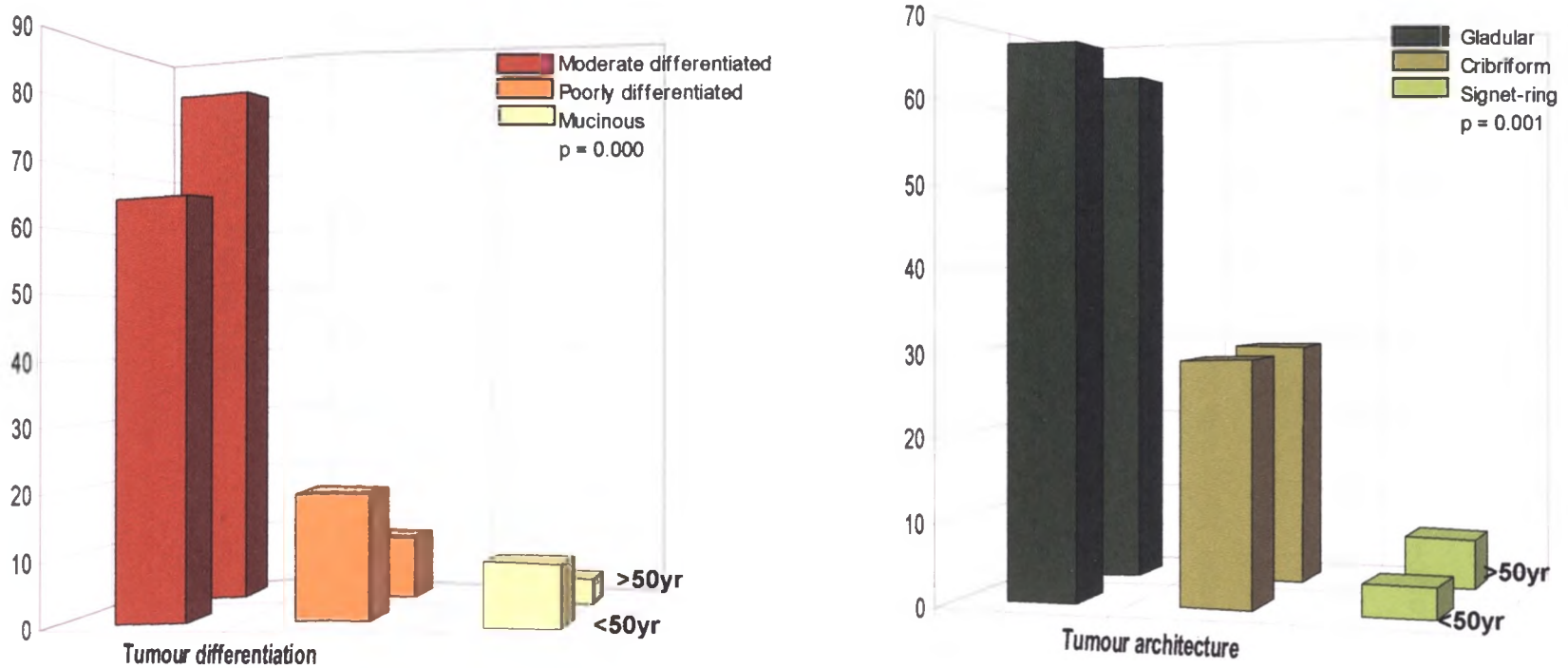
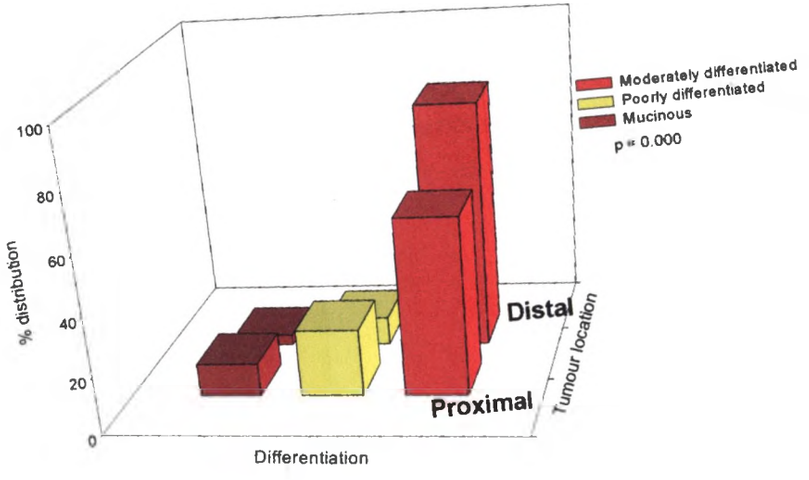


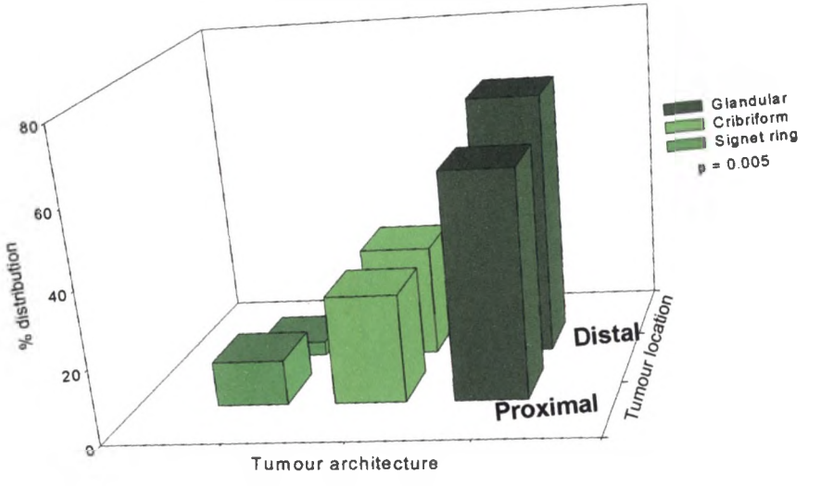
Figure 3.5: Young patients frequently present with moderate to poorly differentiated tumours with either a glandular or signet-ring architecture. Demographic data was obtained from retrospectively collected records. Values are given as percentages and associations were determined through the Fisher's exact test at a 95% confidence interval.

were poorly differentiated in 10% of the cases ($p < 0.000$), and 4% showed a signet-ring cell architecture ($p = 0.005$) compared to those located proximally. Necrosis was rated as low to moderate in distal tumours ($p = 0.001$).

No significant difference was observed between the tumour architecture of the different genders, although a higher percentage of males had a signet-ring cell architecture (6% males *vs* 4% females) ($p = 0.448$) (Table 3.2). Similarly, both males and females showed low levels of Crohn's infiltrate ($p = 0.777$) and similar levels of tumour necrosis ($p = 0.590$) (Table 3.2).

Tumours located proximal to the splenic flexure were more frequently poorly differentiated or mucinous compared to distally located tumours ($p = 0.000$) (Table 3.2, Figure 3.6). Distally located tumours showed a predilection for a lower grade ($p = 0.000$), while no significant association was found between tumour location and level of mucin expression or Crohn's infiltrate ($p = 1.000$). Tumour architecture was predominantly glandular in distally located tumours and signet-ring cell in proximal tumours ($p = 0.005$) (Table 3.2, Figure 3.6). Proximal tumours also showed a high degree of tumour necrosis, while distal tumours showed little tumour necrosis ($p = 0.001$) (Table 3.2, Figure 3.6).


Moderately differentiated
Poorly differentiated
Mucinous
p = 0.000
% distribution
100
80
60
40
20
0
Distal
Proximal
Tumour location
Differentiation


Glandular
Cribriform
Signet ring
p = 0.005
% distribution
80
60
40
20
0
Distal
Proximal
Tumour location
Tumour architecture

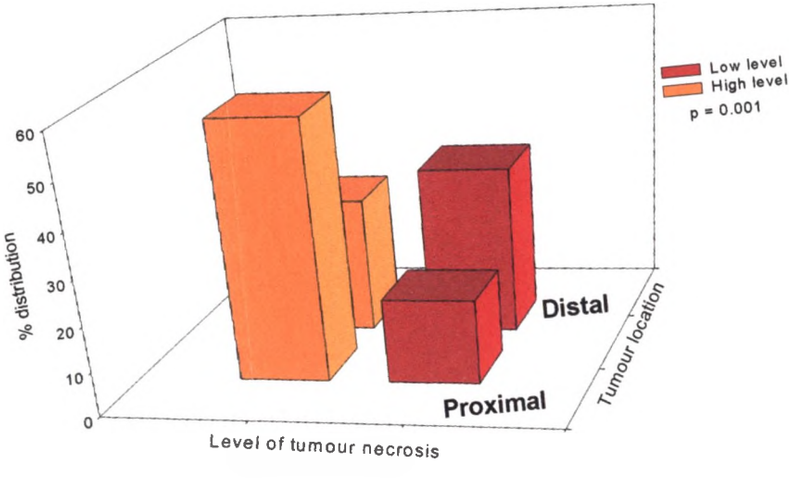

Low level
High level
p = 0.001
% distribution
60
50
40
30
20
10
0
Distal
Proximal
Tumour location
Level of tumour necrosis

Figure 3.6: Proximally located tumours are frequently poorly differentiated or mucinous, and have a signet-ring cell architecture and high level tumour necrosis. Demographic data was obtained from retrospectively collected records. Values are given as percentages and associations were determined through the Fisher's exact test at a 95% confidence interval.

3.3.3 Adenomas associated with CRC

The presence of contiguous or separate adenomas was noted in all patients having surgical resections for CRC. Colonic adenomas were identified in 22% of all patients who had undergone resection for CRC for the period 1998-2002. With respect to racial differences, overall 9% of black patients were identified as having adenomas *cf.* 10% of white patients ($p = 0.523$). When stratified according to race and age, adenomas were identified in resected specimens in 39% of black patients < 50 years of age when compared to 61% of patients > 50 years ($p = 0.458$). In white patients adenomas were identified in 11% of young patients *cf.* 89% of older patients ($p = 0.340$).

Whites more frequently presented with a single adenoma (84% whites *vs* 79% blacks), while more than 2 adenomas were more likely to be found in blacks (21% blacks *vs* 16% whites) ($p = 0.523$) (Figure 3.7). Young patients were more likely to harbour several adenomas (26%) compared to older patients, who presented more frequently with single adenomas (85% *vs* 74% in young patients) ($p = 0.115$) (Figure 3.7). Although females had more cases of single adenomas (85%) compared to males (78%), the presence of several adenomas was more evident in male patients (22% *vs* 15% in females). Adenomas located distally to the splenic flexure were more often single adenomas than those found located proximally (81% distal *vs* 76% proximal) (Figure 3.7). Cases with several adenomas, however, were more often proximally located (24% proximal *vs* 19% distal) ($p = 0.618$). It is noteworthy that only two adenomas in this series were identified as having serrated features although increasing numbers of polyps having a serrated

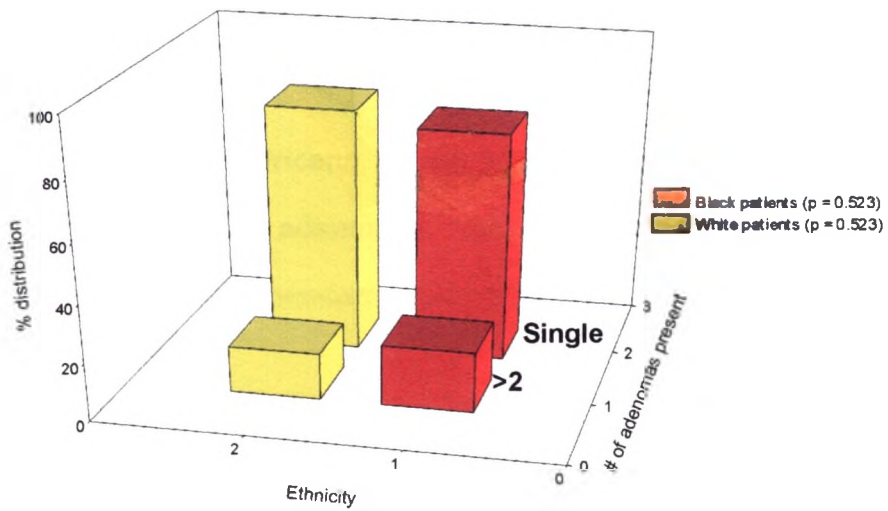
or mixed conventional/serrated appearance are more recently identified (Paterson AC: personal communication). It is postulated that the low percentage of serrated adenomas in our series might be indicative of a low detection rate rather than a low incidence and calls for the careful identification of the entity in future.

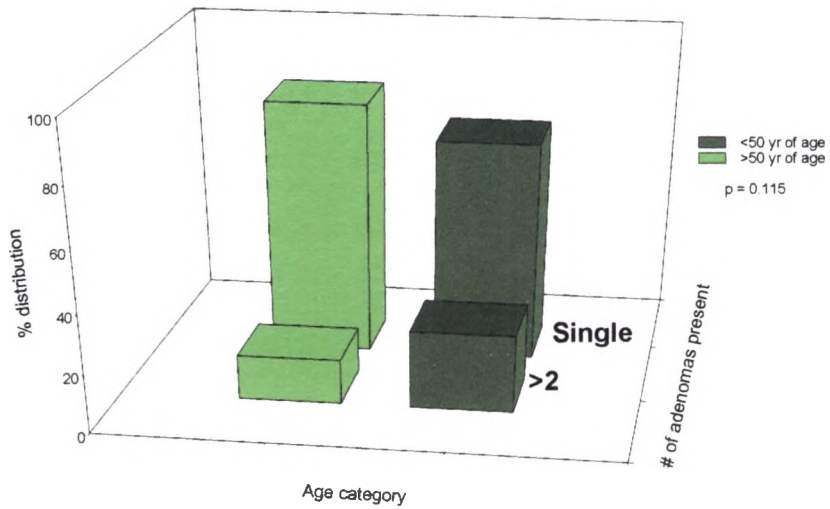
3.3.4 Tumour staging

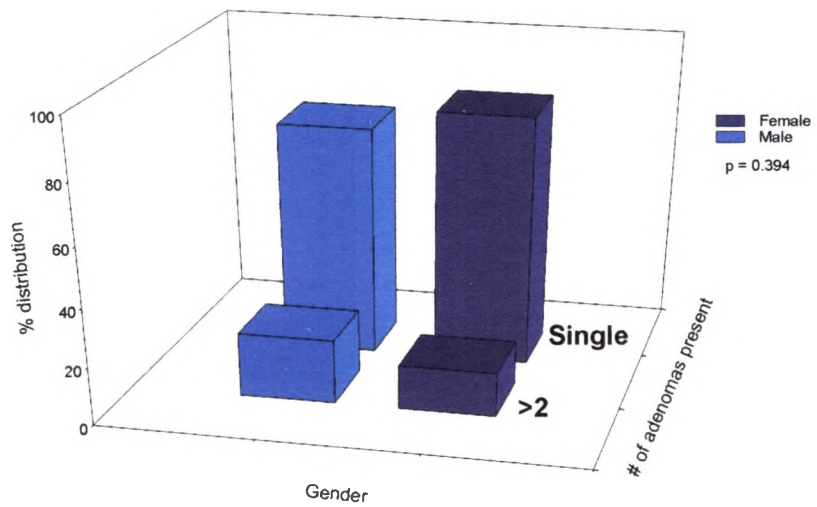
Four different staging methods were employed to classify the cases of resected CRC. The Dukes staging method did not reveal a significant association with ethnicity, age category, gender or tumour location (Table 3.3). Similar results were obtained using the Astler-Coller and TNM methods of staging, although tumour location revealed an association with TNM staging ($p = 0.004$) (Table 3.3). Tumour staging using the methods proposed by Jass and co-workers showed an association with ethnicity ($p = 0.049$) and gender ($p = 0.050$) (Table 3.3). Jass score 1 was found to be associated with white and female patients, while score 2 was associated with male patients ($p = 0.050$). Similarly Jass score 3 was associated with black and female patients, and score 4 with white and male patients. The significance of this trend is uncertain.

3.3.5 Immunohistochemistry

Black patients more frequently showed loss of expression of the mismatch repair gene proteins hMLH1 (23%) ($p = 0.121$), hMSH2 (12%) ($p = 0.013$) and hMSH6 (43%) ($p = 0.210$) in comparison to whites (13%, 2% and 33% respectively) (Table 3.4, Figure 3.8). In contrast, whites more frequently showed loss of APC protein expression (15% *vs* 12% blacks) ($p = 0.648$) and accumulation of p53 protein


100
80
60
40
20
0
% distribution
2
1
0
Ethnicity
Single
>2
3
2
1
0
# of adenomas present
Black patients (p = 0.523)
White patients (p = 0.523)


100
80
60
40
20
0
% distribution
Single
>2
# of adenomas present
Age category
<50 yr of age
>50 yr of age
p = 0.115


100
80
60
40
20
0
% distribution
Single
>2
# of adenomas present
Gender
Female
Male
p = 0.394

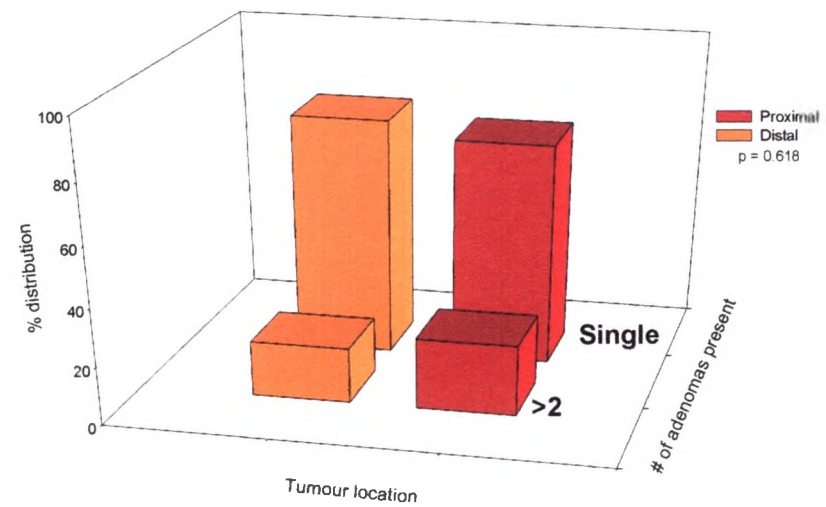

100
80
60
40
20
0
% distribution
Single
>2
# of adenomas present
Tumour location
Proximal
Distal
p = 0.618

Figure 3.7: **Black Africans, young patients and males, together with tumours located proximally to the splenic flexure more often present with several adenomas.** Data was obtained from retrospectively collected patient records. Values are given as percentages and associations were determined through the Fisher's exact test at a 95% confidence interval.

Table 3.3: An overview of the association between ethnicity, age category and tumour location and pathological staging. Values are given as a percentage of the overall distribution between the different variable. P-values printed in bold represent significant association at 95% confidence interval.

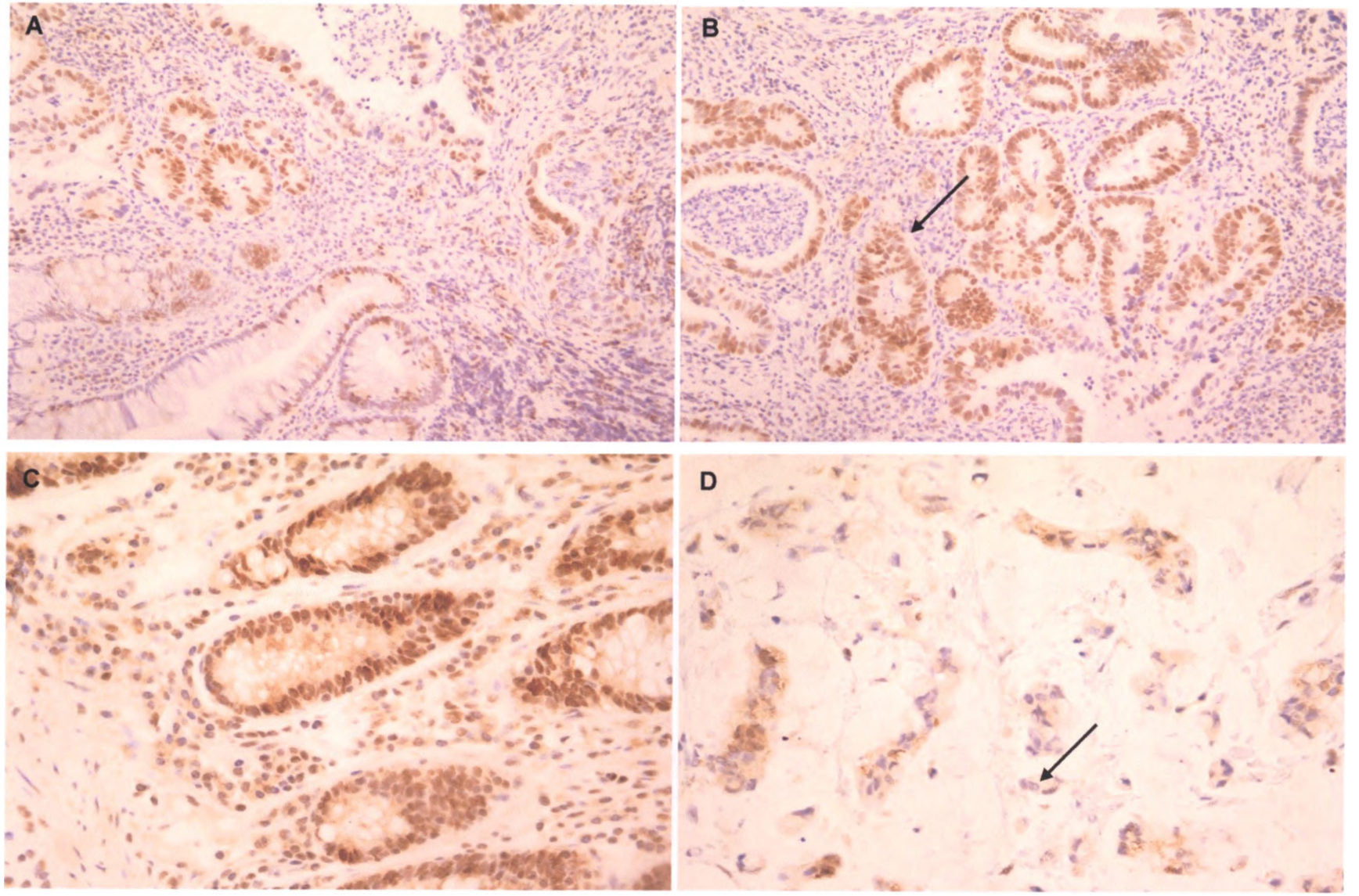
	Ethnicity			Age category			Gender			Tumour location		
	Black	White	p-value	<50yr of age	>50yr of age	p-value	Female	Male	p-value	Caecum - transverse colon	Descending colon - rectum	p-value
Dukes staging:			1.000			0.721			0.858			0.149
Stage A	8.90% (13/146)	8.00% (8/100)		8.45% (6/71)	8.57% (15/175)		9.40% (11/117)	7.81% (10/128)		3.85% (3/78)	11.35% (16/141)	
Stage B	44.52% (65/146)	45.00% (45/100)		40.85% (29/71)	46.29% (81/175)		43.59% (51/117)	46.09% (59/128)		47.44% (37/78)	40.43% (57/141)	
Stage C1 and C2	46.58% (68/146)	47.00% (47/100)		50.70% (36/71)	45.15% (79/175)		47.01% (55/117)	46.09% (59/128)		48.72% (38/78)	48.23% (68/141)	
Astler-Coller staging:			0.581			0.713			0.853			0.218
Stage A	1.37% (2/146)	2.00% (2/100)		0	2.29% (4/175)		2.56% (3/117)	0.78% (1/128)		0	2.84% (4/141)	
Stage B1	8.90% (13/146)	12.00% (12/100)		11.27% (8/71)	9.71% (17/175)		10.26% (12/117)	10.16% (13/128)		6.41% (5/78)	12.06% (17/141)	
Stage B2	43.15% (63/146)	40.00% (40/100)		38.03% (27/71)	43.43% (76/175)		41.03% (48/117)	42.97% (55/128)		44.87% (35/78)	37.59% (53/141)	
Stage C1	15.75% (23/146)	10.00% (10/100)		15.49% (11/71)	12.57% (22/175)		11.97% (14/117)	14.06% (18/128)		10.26% (8/78)	15.60% (22/141)	
Stage C2	30.82% (45/146)	36.00% (36/100)		35.21% (25/71)	32.00% (56/175)		34.19% (40/117)	32.03% (41/128)		38.46% (30/78)	31.91% (45/141)	
TNM staging:			0.347			0.450			0.490			**0.004**
Level T1	3.42% (5/146)	5.05% (5/99)		1.41% (1/71)	5.17% (9/174)		5.98% (7/117)	2.36% (3/127)		1.28% (1/78)	6.38% (9/141)	
Level T2	13.70% (20/146)	15.15% (15/99)		18.31% (13/71)	12.64%(22/174)		12.82% (15/117)	15.75% (20/127)		5.13% (4/78)	19.15% (27/141)	
Level T3	76.03% (111/146)	77.78% (77.99)		76.06% (54/71)	77.01% (134/174)		76.92% (90/117)	76.38% (97/127)		88.46% (69/78)	68.79% (97/141)	
Level T4	6.85% (10/146)	2.02% (2/99)		4.23% (3/71)	5.17% (9/174)		4.27% (5/117)	5.51% (7/127)		5.13% (4/78)	5.67% (8/141)	
Jass staging:			**0.049**			0.754			**0.050**			0.410
Stage 1	19.82% (22/111)	23.46% (19/81)		22.22% (12/54)	21.01% (29/138)		24.47% (23/94)	18.37% (18/98)		16.67% (10/60)	24.77% (27/109)	
Stage 2	28.83% (32/111)	29.63% (24/81)		31.48% (17/54)	28.26% (39/138)		23.40% (22/94)	34.69% (34/98)		31/67% (19/60)	25.69% (28/109)	
Stage 3	36.04% (40/111)	19.75% (16/81)		33.33% (18/54)	27.54% (38/138)		36.17% (34/94)	22.45% (22/98)		28.33% (17/60)	32.11% (35/109)	
Stage 4	14.41% (16/111)	25.93% (21/81)		12.96% (7/54)	21.74% (30/138)		14.89% (14.94)	23.47% (23/98)		21.67% (13/60)	17.43% (19/109)	
Stage 5	0.90% (1/111)	0		0	0.72% (1/138)		0	1.02% (1/98)		1.67% (1/60)	0	

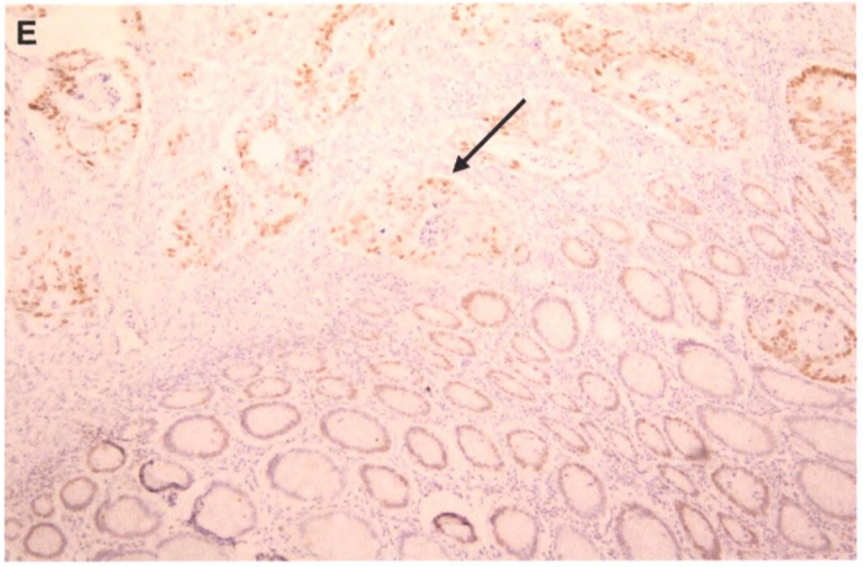
cancer cells (58% *vs* 42%) (p = 0.047). Loss of MGMT protein expression was observed in similar percentages in both ethnic groups, i.e. 27% blacks and 26% whites (p = 1.000). Although frequently used elsewhere, immunohistochemical detection of PMS2 was problematic and it was decided to exclude from the panel of markers.

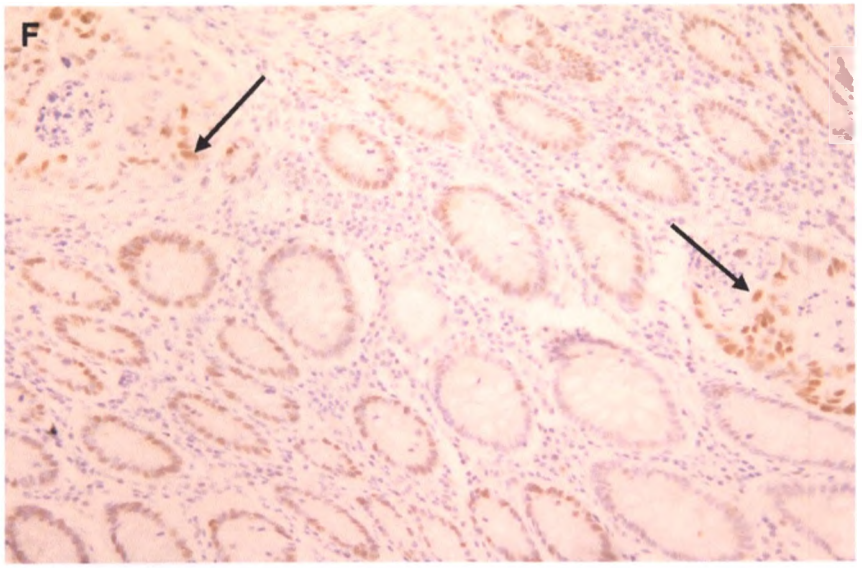
Table 3.4: Differences between blacks and whites in the loss of protein expression, as monitored by immunohistochemistry. Values are given as a percentage of the overall distribution between the different variables. P-values printed in bold represent significant association between ethnicity and loss of protein expression at 95% confidence interval.

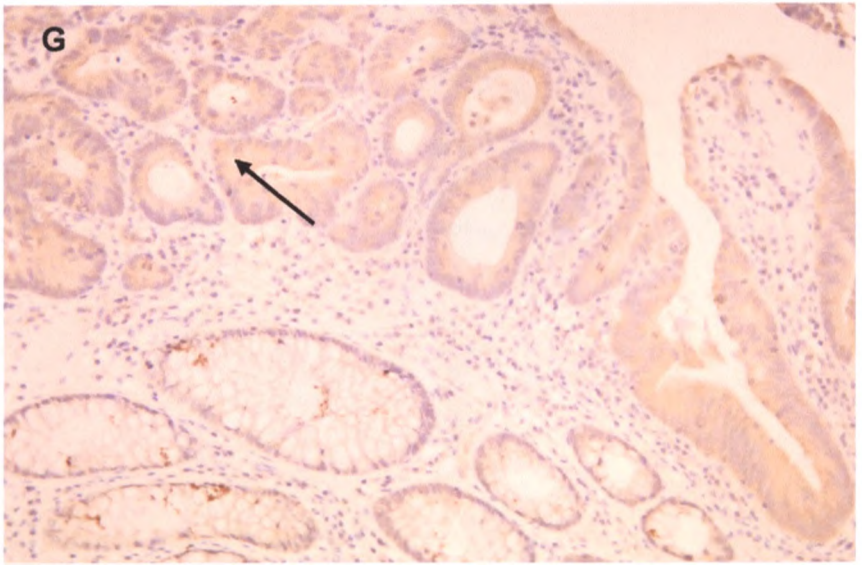
	Blacks	Whites	p-value
hMLH1	23% (29/128)	13% (8/63)	0.121
hMSH2	12% (16/129)	2% (1/65)	**0.013**
hMSH6	43% (54/126)	33% (21/64)	0.210
APC	12% (15/124)	15% (9/62)	0.648
p53	42% (54/128)	58% (37/64)	**0.047**
MGMT	27% (33/123)	26% (16/62)	1.000

Through multinomial regression analysis the risk of young (<50yr of age) male black patients presenting with tumours with specific characteristics was calculated (Table 3.5). These patients showed a significant risk for developing poorly differentiated tumours (OR 1.9, p = 0.014) (Figure 3.9). The risk of developing a mucinous CRC was significant for young (OR 1.9, p = 0.011) and black patients (OR 2.5, p = 0.002) (Table 3.5, Figure 3.9). No significant risk or protection was found for tumoral necrosis or the presence of adenomas. Amongst the tumour staging methods employed, male patients showed a borderline risk for developing a Jass 4 tumour (OR 2.3, p = 0.079) (Table 3.5). The loss of hMSH2 protein expression was a significant risk for young patients (OR 6.5, p = 0.003) (Table 3.5, Figure 3.9).


A
B
C
D


E


F


G

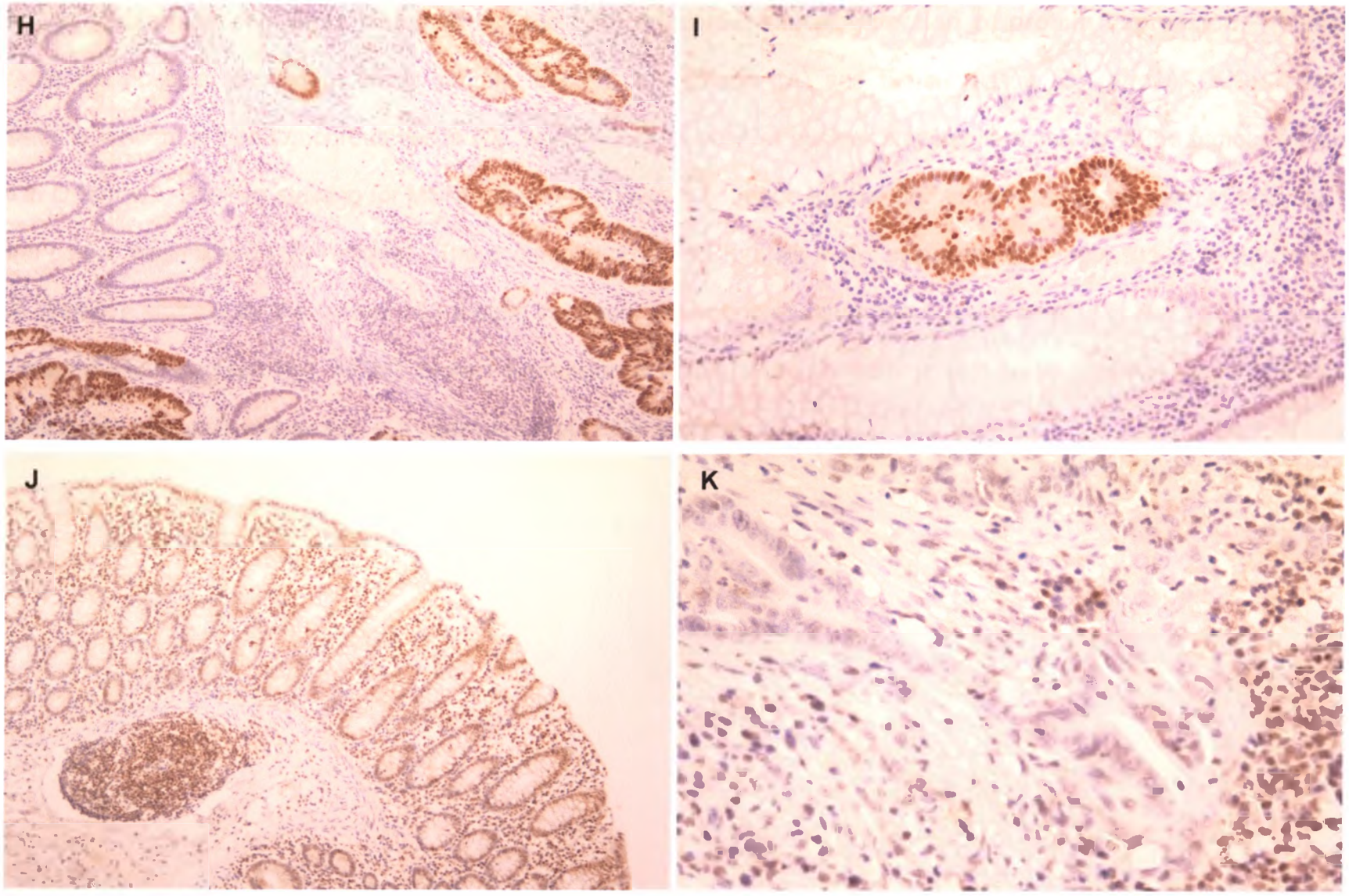

H
I
J
K

Figure 3.8: Examples of results obtained for the immunohistochemical detection of protein expression. **A** and **B** represents the positive staining of MLH1 protein in both non-tumours (**A**) and tumour tissue (**B**) (arrow). MSH2 protein expression is evident in non-tumourous tissue (**C**), while a lack of positive nuclear staining is observed in the tumour example shown in **D** (arrow). Positive staining for MSH6 protein is shown in both non-tumour and tumour tissue, as seen at both low (**E**) and high power magnification (**F**) (arrows). The accumulation of APC protein in the cytoplasm of tumour tissue is depicted in **G** (arrow). Positive staining for the mutant p53 protein in tumour but not non-tumour tissue are evident in **H** and **I** at low and high power magnification respectively. MGMT protein expression is visible in the non-tumour tissue and lymphoid aggregate shown in **J**, while a loss of protein expression is evident in tumour cells shown in **K**.

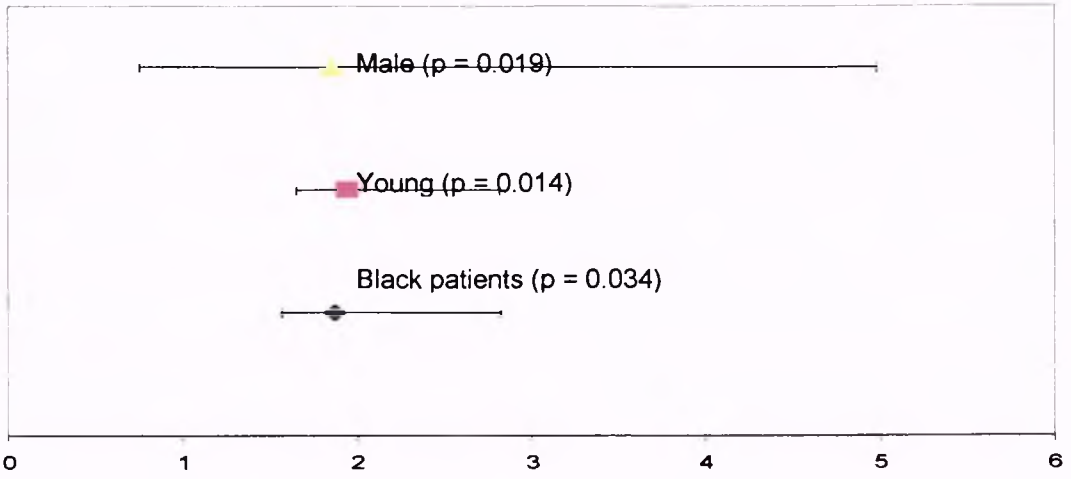
Table 3.5: Interaction between patient demographics (ethnicity, age and gender) and pathological characteristics. Values are given as odds ratios with p-values indicated in brackets. Values printed in bold represent significant odds in the association between variables.

	N	Blacks	<50yr	Male
Poor differentiation	607	**1.872**	**1.95**	**1.859**
Cribriform architecture	475	0.703	0.762	1.074
>50% mucinous	609	**2.513**	**1.927**	1.33
Necrotic tumour	215	1.538	1.023	1.251
Presence of Adenomas	649	1.045	1.279	0.862
Staging:				
Dukes C	245	0.815	1.174	1.194
Astler-Coller C2	245	1.115	1.114	1.075
TNM - $T_4N_2M_x$	244	4.008	1.888	2.803
Jass IV	190	0.709	0.581	2.28
Loss of protein expression:				
MLH1	190	1.728	1.792	0.626
MSH2	193	4.03	**6.519**	2.098
MSH6	189	1.577	0.904	0.939
APC	185	0.523	2.316	1.185
p53	191	0.576	0.859	0.914
MGMT	184	1.087	1.133	0.585

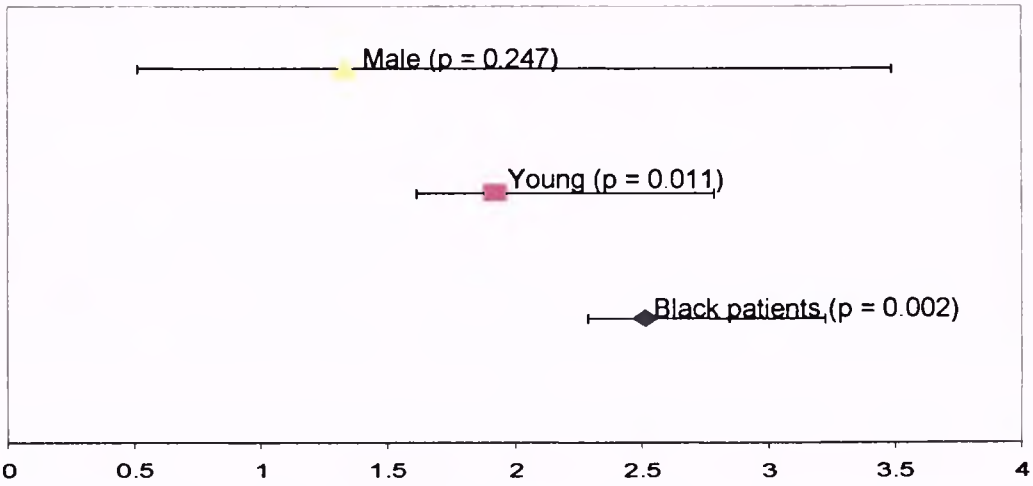
3.4. Discussion:

This study investigated the occurrence and morphological features of colorectal cancer (CRC) amongst black and white patients presenting to academic and public sector hospitals in the most heavily populated regions of South Africa. It must be noted that this does not represent a primary epidemiological survey. In the most recent National Cancer Registry (Mqoqi *et al.*, 2004), reporting on all cancers diagnosed in both the public and private sector during 1998 and 1999, it was reported that the vast majority of South African patients with CRC are of European origin (Mqoqi *et al.*, 2004). However, in the current series 55.4% of the

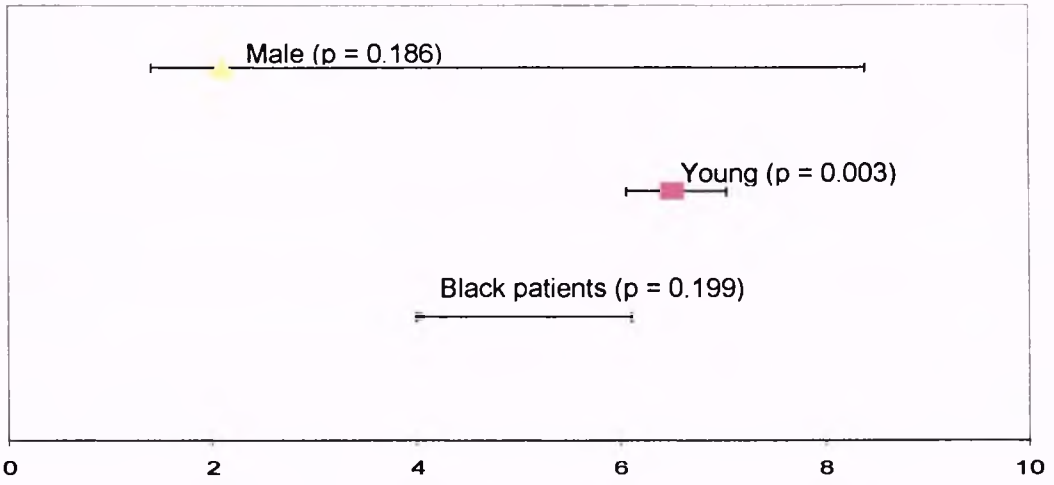
A: Poor differentiation



B: High mucin expression



C: Loss of hMSH2 protein expression



Odds ratio

Figure 3.9: **Young, black and male patients' risk to develop poorly differentiated tumours (A), a high mucin expression (B), and loss of hMSH2 protein expression (C).** Odds ratios for the development of these pathological characteristics were calculated through

regression analysis. Values are given as odds rations together with 95% confidence interval indicated as error bars.

patient population was black. This is attributable to historical inequalities within the South African health service. The majority of patients using academic and public sector hospitals represent a lower socio-economic stratum that is currently biased towards a black population. Nevertheless, the study serves to confirm previously smaller series that have suggested that disproportionately large numbers of young South African black patients present with CRC (Pillay *et al.*, 1978; Bonnet *et al.*, 1997; Boytchev *et al.*, 1999; Walker & Segal, 2002), as well as series from the rest of Africa, as previously alluded to in Chapter 2. Amongst the young patients (<50 years of age at diagnosis) a disproportionately high number (83%) of patients diagnosed with CRC were black, while only 16% were white. These patients were predominantly male, with poorly differentiated to mucinous proximal tumours. A high percentage contained a signet-ring cell architecture (11%). There is a significant increased risk of young, male, black patients developing poorly differentiated tumours, with more than 50% mucin expression and loss of hMSH2 protein expression. These patients could, however, not be classified according to the Amsterdam or Bethesda criteria, as no family history was available in this retrospective study.

The increased incidence of colorectal cancer amongst young patients is seen both locally and abroad (NCR, 1998 – 1999; O'Connell *et al.*, 2003). In a recent nationwide study in the USA, O'Connell reported a higher proportion of black and Hispanic patients between 20 and 40 years of age, compared to older patients (60

– 80 years) of the same ethnicity (O'Connell *et al.*, 2004). Similar results were obtained in this study (Figure 2.1). Young patients comprised 52% male and 48% female, and there were more cases that were poorly differentiated compared to their older counterparts. The percentage of mucin expression and the presence of a signet-ring cell architecture was also more frequent in young patients. With regard to stage, 56% of the young patient group presented with an American Joint Committee on Cancer (AJCC) stage III or IV, while 47% of our young patients were stage III of IV. O'Connell *et al.* (2004) noted that the overall 5-year cancer-specific survival of young patients was worse than for older patients, but that this effect is reversed in a stage for stage comparison: young patients with an advanced stage tumour had a better 5-year survival than older patients. This may be indicative of different chemotherapeutic approaches and/or responses to treatment. A follow-up study regarding the survival rates of patients in our cohort could provide more insight into the situation in South Africa.

Microsatellite unstable tumours show distinct clinical and pathological features that include proximal location, mucinous histology, poor differentiation, the presence of tumour-infiltrating lymphocytes and most distinctively the demonstration of mismatch repair gene deficiency, most frequently in the *hMLH1* and *hMSH2* genes (Jass *et al.*, 1998; Smyrk *et al.*, 2001). Young, male, black patients present with a morphology similar to that observed in microsatellite unstable tumours (Table 2.2, Figure 2.4), including a risk for absent hMLH1 protein expression (Table 2.5) and an increased possibility of loss of hMSH2 protein expression (Table 2.5). Hereditary nonpolyposis colorectal cancer (HNPCC) shows similar features (Robbins *et al.*, 2002; Chung and Rustgi, 2003) and this includes presentation at

an average age of younger than 50 years (Rowley, 2005). Penetrance is purportedly higher in males than in females (Mitchell *et al.*, 2002). Diagnosis of HNPCC, however, relies on fulfilment of the Amsterdam and Bethesda criteria, which is mainly based on a family history of colon cancer (Grady, 2003). The strict Amsterdam criteria may lead to an underdiagnosis of HNPCC and it was since suggested in the Bethesda guidelines (1997) that CRC diagnosed at a young age, even without a family history, may indeed have a genetic component to cancer development and should be tested for HNPCC (Rodriques-Bigas *et al.*, 1997). Velayos *et al.* (2005) recently showed in preliminary data that early screening for HNPCC actually failed to identify new HNPCC cases and concurred with the revised Bethesda guidelines (Umar *et al.*, 2004) that excluded microsatellite instability testing in all adenomas from patients younger than 40 years of age. The authors did, however, comment on the possibility of using direct germline testing as an alternative, as this shows increased sensitivity (Velayos *et al.*, 2005). In the present study no family history was obtainable. In order to confirm young patients as being HNPCC in the future, obtaining a family history is obligatory.

Microsatellite unstable tumours, however, also occur in 10 to 15% of sporadic CRCs (Young *et al.*, 2001). The difference in the development of sporadic versus hereditary CRC is thought to involve the so called "methylator pathway". Promoter regions are rich in CpG islands, and therefore susceptible to hypermethylation, which causes the transcriptional silencing of genes. The promoter regions of both the *hMLH1* and O^6-methylguanine DNA-methyltransferase (*MGMT*) genes have been shown to be silenced in such a way (Herman *et al.*, 1998; Whitehall *et al.*, 2001). Microsatellite instability in sporadic CRC has been shown to be due to

methylation of the *hMLH1* gene and to be common in proximal tumours (Miyakura *et al.*, 2001). Compared to older whites, young black patients showed an increased risk for undetectable hMLH1 protein expression (Table 2.5). Further studies into the methylation status of this promoter region would provide insight into the CpG island methylator phenotype of these tumours.

Methylation of the *MGMT* gene is characteristic of CRC with low-level microsatellite instability (Whitehall *et al.*, 2001) and has since become associated with the "serrated adenoma pathway", first described by Jass *et al.* in 1999. This pathway essentially involves the formation of carcinoma from hyperplastic polyps and adenomas through intermediate serrated adenomas. Cancers associated with this serrated pathway have been shown to secrete abundant mucin (Mäkinen *et al.*, 2004). We report 26% (49/185) of cases showing loss of expression of MGMT which could imply hypermethylation of this promoter region.

In general, the occurrence of serrated neoplasia is rare. In the present study, only 2 serrated adenomas (0.3% of all adenomas examined) were identified during the period 1990 to 2003, the first in 1998 and the second in 2002. A third patient, female aged 28 years, was identified in 2003 with CRC associated with numbers of serrated polyps and was thus not included in the 1998-2002 review (Minoo *et al.*, In press). As noted, increasing numbers of this entity are being identified in this unit, possibly due to increased awareness of the condition, as is noted elsewhere. Matsumoto *et al.* identified 43 serrated adenomas amongst the 3305 adenomas studied (1%) (Matsumoto *et al*, 1999), while Bariol *et al.* showed a similar 1% serrated adenoma occurrence in an Australian study involving 919

individuals (Bariol *et al*, 2003). More recently, however, Rubio and co-workers identified as many as 7% serrated adenomas amongst the 1552 adenomas identified in an Italian cohort (Rubio *et al*, 2005).

The serrated adenomas, observed in two older female whites, showed loss of hMSH6 protein expression in one adenoma, while another showed accumulation of p53 and absence of MGMT protein expression. As previously mentioned, the serrated adenoma pathway is associated with low level microsatellite instability and methylation within the *MGMT* promoter region that leads to the functional silencing of this gene (Jass *et al.*, 1999; Whitehall *et al.*, 2001). These lesions are often found in the sigmoid colon and rectum (Hawkins & Ward, 2001) and through the associated *p53* gene mutation might indicate the involvement of *APC* gene mutations.

Black patients were less likely than whites to show an accumulation of p53 in cancer cells (Table 3.4). p53 mutations are associated with the well-known adenoma-carcinoma sequence of events, that is initiated by mutations in the *APC* gene. The reduced biological activity of mutated p53 protein contributes to uninhibited cell proliferation and the accumulation of mutations (Vesculescu *et al.*, 1992). Paluszkiewicz *et al.* (2004) have shown that the accumulation of p53 in tumour cells frequently occurs within distal tumours and is associated with a poor prognosis. However, proximal tumours developed in a p53-independent manner and this may indicate a preference towards to mutator phenotype with the accumulation of mutations due to mismatch repair deficiency.

Despite the limitation of not having a family history, we were able to analyze 1734 colorectal cancer cases for patient demographics as well as smaller numbers of patients for pathological and immunohistochemical characteristics. On the basis of the morphological features of CRC in young black patients it seems likely that colorectal cancer develops predominantly through either the mutator or serrated pathway. This is associated with the accumulation of mutations due to a deficiency in the function of mismatch repair, which could be due to germline mutations in the mutator pathway or promoter methylation in the serrated pathway. Further analysis of microsatellite instability and methylation status would aid in discriminating these two pathways involved in the formation of colorectal tumours in young patients of African descent.

CHAPTER 4
MOLECULAR PATHOLOGY OF CRC IN SOUTH AFRICA

4.1. Introduction

Colorectal cancer is a genetic disease, although not all cases show a familial basis. Colorectal carcinogenesis involves the stepwise accumulation of mutations due to either an inherited or an epigenetic alteration that leads to the transformation of normal colonic epithelia. This process may develop and progress over a period of 10 to 15 years and has lead to the comprehensive study of both histological and molecular changes associated with the initiation and progression of colorectal cancer. A wealth of knowledge has thus far been accumulated and has lead to the detailed classification of four distinct pathways.

The classical or "environmental" pathway, which seems to be involved to a certain extent in all of the pathways described, involves mutational inactivation of the adenomatous polyposis coli (*APC*) gene in colorectal epithelial cells (Hamilton *et al.*, 2000). This leads to a cascade of events including amongst others β-catenin translocation to the nucleus to act as a transcription factor (Bright-Thomas *et al.*, 2003) as well as the interference with E-cadherin homeostasis during tumour initiation to *p53* gene mutations during tumour progression.

The familial counterpart of this pathway is the autosomal dominant cancer susceptibility disease familial adenomatous polyposis (FAP) that is characterized by germline mutations in the *APC* gene (Fearnhead *et al.*, 2001), and by the presence of adenomatous polyps that inevitably develop into colorectal cancer if left untreated.

A third pathway involves the accumulation of mutations due to a mismatch repair (MMR) deficiency, resulting in microsatellite instability (MSI) in the coding regions of genes implicated in tumour progression, such as *TGF-βRII*, *IGFIIR*, *hMSH3* and *BAX* (Souza *et al.*, 1996; Rampino *et al.*, 1997; Raut *et al.*, 2004). This may lead to different levels of MSI, distinguished by the number of tested loci displaying instability (Boland *et al.*, 1998). Tumours with high levels of instability (MSI-H) may develop through a hereditary basis, involving the MMR genes *hMSH2*, *hMLH1*, *hMSH6*, *hPMS2* and *hPMS1* (Peltomäki and de la Chapelle, 1997) and predispose to hereditary nonpolyposis colorectal cancer (HNPCC).

The fourth and somewhat related pathway involves the silencing of MMR genes through promoter hypermethylation that may result in either high or low levels of MSI (Jass *et al.*, 2002). This so-called "serrated" pathway is initiated in serrated neoplasia including hyperplastic, admixed or serrated polyps, serrated adenomas and non-dysplastic aberrant crypt foci (ACF) (Jass, 2001) through the inhibition of apoptosis and followed by the disruption of DNA repair mechanisms through epigenetic silencing (Jass *et al.*, 2002).

As mentioned previously (Chapter 1 and 2), there is a trend in South Africa for a disproportionately large number of young black patients to present with CRC (Pillay *et al.*, 1978; Bonnet *et al.*, 1997; Boytchev *et al.*, 1999; Walker & Segal, 2002). The epidemiology of CRC in South African whites follows the classical western trend although the molecular pathology has not been comprehensively investigated. Furthermore, the large number of young Black CRC patients is

postulated not to involve dietary or lifestyle-related factors and investigations into the pathogenesis of these tumours might prove to be useful to establish molecular markers for early detection and treatment. This study aimed to comprehensively investigate CRC in black and white patients to identify the possible molecular pathways involved in each cohort. Features such as overall methylation phenotype, MSI and *BRAF* and *KRAS* gene mutation status were correlated with morphological traits. Results were obtained through methylation specific polymerase chain reaction (MSP-PCR), microsatellite analysis, real time PCR and DNA sequencing, and now reported.

4.2. Materials and Methods:

4.2.1 Patient selection

As mentioned previously (Chapter 2, Section 2.2) the original material was derived from a retrospectively collected series of samples (1990 – 2003) collected from the archives of the Division of Anatomical Pathology, School of Pathology, University of the Witwatersrand, and included all specimens originally diagnosed as adenocarcinoma of the colon and/or rectum. Cases were stratified according to age (<35yr, 36 – 50yr or >50yr), gender and ethnicity (black or white). For DNA extraction and subsequent molecular analysis, cases were limited to subset of the more recent cases (1999 – 2003). In total, 80 cases were selected, broken down into patients aged less than 50 years (n=41) and a cohort above 50 years of age (n= 39).

4.2.2 DNA extraction

Initially, DNA from tumour and normal mucosa was initially extracted separately by laser microdissection. This was performed at P.A.L.M. Microlaser Technologies (Bernried, Germany) on a system that comprised the P.A.L.M. Robo version 3.0 system (P.A.L.M. Microlaser Technologies, Bernried, Germany), consisting of an Olympus 1x81 microscope (Olympus, Hamburg, Germany) coupled to an Olympus 1x2 – UCB laser (Olympus, Hamburg, Germany). For each case, formalin fixed, paraffin embedded tissue blocks were serially cut, mounted and stained with haematoxylin and eosin (H&E) for histological assessment. The subsequent serial sections were mounted on glass slides that were lightly washed with 100% ethanol to remove any surface dust, and lightly stained with H&E. Laser microdissection and pressure catapulting (LMPC) with the PALM Microbeam technology allowed for the non-contact isolation of either individual cells or cell populations without contamination from surrounding or unwanted cells. The neoplastic component of interest was highlighted using the computer software, and the laser used to cut and catapult the section into the PALM AdhesiveCaps collection tube (P.A.L.M. Microlaser Technologies, Bernried, Germany). Subsequent DNA extraction was performed using the commercial extraction kit ChargeSwitch® Forensic DNA purification kit (Invitrogen, Carlsbad, CA) according to manufacturer instructions.

Insufficient yields of quality DNA for further molecular analysis were, however, obtained. This necessitated the re-evaluation of the usefulness of LMPC in the current context. Thus for the present study, DNA was extracted from sections of the original paraffin embedded tissue blocks.

For each selected case, two blocks were identified that contained exclusively either non-tumourous or tumourous material. Separate specimen tubes were marked accordingly and serial sections of 10μm were collected. In some cases, however, the tumour block contained small amounts of normal mucosa. In these cases, the available block was used, as the subsequent molecular methods used, if analyzed appropriately, could reflect the presence of normal tissue within "tumour-only" tissue blocks.

DNA extraction was performed through the well-known phenol-chloroform method of extraction. Briefly, serially cut sections were dewaxed by three changes of xylene through centrifugation at 13 200 rpm (16.1x g) for four minutes at room temperature. Lysis buffer, consisting of 50mM Tris base (Saarchem, Wadeville, South Africa), 5mM EDTA (Saarchem, Wadeville, South Africa), Tween20 (Sigma Chemical Company, St Louis, MO) and 10% proteinase K (Roche Diagnostics, Penzburg, Germany) (pH 8) was subsequently added, and incubated overnight at 56°C (Thermolyne Type 17600). The following morning, the proteinase K was inactivated by incubation at 80°C for 10 min, followed by the addition of phenol-chloroform, pre-mixed in a 1:1 ratio. The mixture was briefly vortexed (FineVortex, FinePCR) to ensure thorough mixing of the separate layers. Following centrifugation (13 200rpm, four minutes) (Eppendorf, model 5415R) the top organic layer was removed and added to a separate tube containing chloroform (Sigma Chemical Company, St Louis, MO). Again this was briefly vortexed centrifuged at 13 200rpm for four minutes, before removing the top layer and adding it to a mixture of iso-propanol (Saarchem, Wadeville, South Africa) and 3M sodium acetate (Saarchem, Wadeville, South Africa). This was carefully inverted

to assure thorough mixing, before being centrifuged at 13 200rpm for 30min at 4°C. Subsequently the supernatant was discarded, the DNA-pellet washed with 100% ethanol (Saarchem, Wadeville, South Africa), and dried at 65°C for two minutes. The pellet was then dissolved in 200µl TAE buffer, pH 8.4 (0.04mM Tris base, 0.1% glacial acetic acid, 0.05M EDTA pH8), incubated at 65°C for ten minutes and stored at 4°C until further use. Yield was determined using the NanoDrop ® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

4.2.3 Molecular assessment of DNA

To determine the integrity and performance of the isolated DNA during a polymerase chain reaction (PCR), amplification of the β-globin gene was routinely carried out on all DNA samples. The primer sequences used were PCO4/GH20 and are described in Table 4.1. Appropriate positive and no-template reaction controls were included in each PCR setting. The cycling conditions are described in Table 4.2. PCR products were run on a 3% agarose (Saekem LE, Cambrex BioScience, Rockland, ME) gel (80V, 45min) and visualized under UV illumination.

Table 4.1: Primer sequences for molecular analysis of colorectal cancer, including probes used during real time polymerase chain reaction (PCR).

Primer name[1]	Sense primer (5' – 3')	Antisense primer (5' – 3')	Product size (bp)
β-globin	CAA CTT CAT CCA CGT TCA CC	GAA GAG CCA AGG ACA GGT AC	268bp
MINT1 (U)[2]	TTT TGA TGT AGA TGT TTT ATT AGG GTT GT	ACC ACC TCA TCA TAA CTA CCC ACA	~100bp
MINT1 (M)[3]	ACG TAG ACG TTT TAT TAG GGT CGC	AAA AAC CTC AAC CCC GCG	~90bp
MINT2 (U)	GAT TTT GTT AAA GTG TTG AGT TTG TT	CAA AAT AAT AAC AAC AAT TCC ATA CA	~100bp
MINT2 (M)	TTG TTA AAG TGT TGA GTT CGT C	AAT AAC GAC GAT TCC GTA CG	~90bp
MINT31 (U)	TAG ATG TTG GGG AAG TGT TTT TTG GT	TAA ATA CCC AAA AAC AAA ACA CCA CA	~90bp
MINT31 (M)	TGT TGG GGA AGT GTT TTT CGG C	CGA AAA CGA AAC GCC GCG	~80bp
hMLH1 (U)	TTT TGA TGT AGA TGT TTT ATT AGG GTT GT	ACC ACC TCA TCA TAA CTA CCC ACA	124bp
hMLH1 (M)	ACG TAG ACG TTT TAT TAG GGT CGC	CCT CAT CGT AAC TAC CCG CG	115bp
MGMT (U)	TTT GTG TTT TGA TGT TTG TAG GTT TTT GT	AAC TCC ACA CTC TTC CAA AAA CAA AAC A	93bp
MGMT (M)	TTT CGA CGT TCG TAG GTT TTC GC	GCA CTC TTC CGA AAA CGA AAC G	81bp
KRAS	GTA TTA ACC TTA TGT GTG ACA TGT TC	ATG GTC CTG CAC CAG TA	225 bp
KRAS probe	LCred640- TTG CCT ACG CCA CCA GCT C	AAA TGA TTC TGA ATT AGC TGT ATC GTC AAG GCA C –Fl	
BRAF	CCT AAA CTC TTC ATA ATG CTT GCT C	GAC TTT CTA GTA ACT CAG CAG CAT C	263bp
BRAF probe	LCred640- TCG AGA TTT CAC TGT AGC TAG	CAG ACA ACT GTT CAA ACT GAT GGG ACC CAC TCC –Fl	

[1] Primer sequences obtained from references as depicted in Table 3.2
[2] U – primers specific for unmethylated DNA, i.e. C modified to T
[3] M – primers specific for methylated DNA, i.e. C not modified

Table 4.2: Polymerase chain reaction (PCR) reaction conditions.

Gene	PCR program	Reference
β-globin	94°C for four minutes, followed by 35 cycles of 94°C for one minute, 55°C for two minutes and 78°C for one minute. Final extension was for ten minutes at 72°C.	This study
MINT1	95°C for ten minutes, followed by 37 cycles of 95°C for 30sec, 55°C for 45sec and 72°C for 45 sec. Final extension was for four minutes at 72°C	Chan *et al.*, 2002
MINT2	95°C for ten minutes, followed by 40 cycles of 95°C for 30sec, 60°C for 45sec and 72°C for 45 sec. Final extension was for four minutes at 72°C	Chan *et al.*, 2002
MINT31	95°C for ten minutes, followed by 38 cycles of 95°C for 30sec, 60°C for 45sec and 72°C for 45 sec. Final extension was for four minutes at 72°C	Chan *et al.*, 2002
hMLH1	95°C for ten minutes, followed by 40 cycles of 95°C for 30sec, 60°C for 45sec and 72°C for 45sec. Final extension was for four minutes at 72°C	Hong *et al.*, 2005 Herman *et al.*, 1998
MGMT	95°C for ten minutes, followed by 38 cycles of 95°C for 30sec, 59°C for 45sec and 72°C for 45 sec. Final extension was for four minutes at 72°C	Hong *et al.*, 2005 Esteller *et al.*, 2002 Esteller *et al.*, 1999
MSI	94°C for 2min, followed by 40 cycles of 94°C for 10sec, 55°C for 30sec and 72°C for 30sec. Final extension was for seven minutes at 72°C	Roche kit, cat # 2 041 901
KRAS	95°C for ten minutes, followed by 45 cycles of 95°C for 0sec, 55°C for 10sec and 72°C for 15sec. Ramping rate was 20°C/sec	This study
BRAF	95°C for ten minutes, followed by 45 cycles of 95°C for 0sec, 55°C for 10sec and 72°C for 15sec. Ramping rate was 20°C/sec	Ikenoue *et al.*, 2004

4.2.3.1 Bisulfite treatment of DNA and methylation-specific PCR

The methylation status of MINT1, MINT2, MINT31, *hMLH1* and *MGMT* was determined by bisulphite treatment of DNA that chemically modified unmethylated, but not methylated, cytosines to uracil. Methylation status of both non-tumour and tumour tissue of each case was determined. These loci were chosen based on published data showing that they offered a means of discriminating the CpG island methylator phenotype (CIMP) and that the MINT loci were unmethylated in normal tissues (Toyota *et al.*, 1999). Bisulphite modification was performed using a commercially available kit (CpGenome^{TM} DNA modification kit, Chemicon Int, Temecula, CA). Briefly, 1μg of genomic DNA was denatured in 3M NaOH for ten

minutes at 55°C. The denatured DNA was incubated in the kit reagent I containing sodium bisulphite, at 58°C for 16hours.

The bisulphite treated DNA was then bound to a micro-particulate carrier (reagent III) in the presence of another salt contained in regent II, and desalted by repeated centrifugation (5000xg; 15sec) and resuspensed in 70% ethanol (Saarchem, Wadeville, South Africa). The conversion to uracil was completed by alkaline desulfonation and desalting was repeated in 90% ethanol (Saarchem, Wadeville, South Africa). The DNA was finally eluted from the carrier by heating in TE buffer (pH8). The subsequent PCR used primers specific for either the methylated (M) or modified unmethylated (U) DNA. In the unmethylated DNA, the C nucleotides would have been converted to U's, which are replaced by T's during subsequent PCR. Methylation-specific PCR (MSP) was performed using primers described in Table 4.1. The cycling conditions are described in Table 4.2. Appropriate positive and no-template DNA reaction controls were included in each PCR experiment. The PCR product (10μl) was visualized on a 3% agarose gel (Saekem LE, Cambrex BioScience, Rockland, ME; 3% MS-4 agarose for *MGMT* reaction products, Whitehead Scientific, Cape Town, South Africa) (80V, 1h20min) stained with ethidium bromide. The loci were classified as unmethylated if the intensity of the methylated band was visually less than that of the unmethylated band or as methylated if the intensity of the methylated band was visually more than that of the unmethylated band. CIMP status was determined as CIMP-negative if none of the evaluated loci were methylated, CIMP-low if one locus was methylated, and CIMP-high if two or more loci were methylated.

4.2.3.2 Microsatellite instability (MSI) testing

The loci recommended as the reference panel by the American Joint Commission on Cancer and the International Collaborative Group on HNPCC include BAT25, BAT26, D5S346 (APC), D17S250 (Mfd15CA) and D2S123 (Boland *et al.*, 1998). The MSI status of these loci was detected using a multiplex PCR system developed by Roche Diagnostics (HNPCC Microsatellite Instability Test, Catalogue number 2 041 901) (Roche, Mannheim, Germany) that generates fluorescence labelled PCR products and was developed for analysis on an automated ABI sequencer. Briefly, matching tumour and normal tissue DNA from each patient was extracted as described previously (See DNA extraction, section 3.2.2). One hundred nanograms of DNA was used to amplify the MSI loci in a single multiplex reaction using the provided primer pairs labelled with different fluorophores (6-FAM, TET or HEX). The cycling conditions are described in Table 4.2 and were slightly modified from the original protocol by increasing the number of cycles to 40. Analysis of PCR products was performed on the ABI PRISM automated sequencer model 377 (Applied Biosystems, Foster City, CA) according to manufacturer instructions. In brief, 1.5µl deionized formamide were combined with 0.5µl GeneScan-350 [TAMRA] size standard (Applied Biosystems, Foster City, CA) and 1µl PCR product. The samples were denatured in a heat block for 2min at 90°C and chilled on ice before loading 1.5µl of each sample onto a 0.2mm 4% polyacrylamide gel. The run was started and completed in accordance with the supplier's protocol. MSI was defined by the presence of novel peaks, following the PCR amplification of tumour DNA, which was not present in non-tumour DNA. A tumour was classified as high-MSI (MSI-H) if at least 40% (2/5) of the examined loci showed unequivocal instabilities (Boland *et al.*, 1998). Microsatellite stable

(MSS) tumours were those where no microsatellite instability was found, while tumours with only one marker showing instability was declared microsatellite instability low (MSI-L).

4.2.3.3 Real time PCR analysis for *KRAS* and *BRAF* gene mutation status

Detection of mutations in codon 12 and 13 of exon one of the *KRAS* gene (GGT to GAT transition), and exon 15 of the *BRAF* gene (T1796A or V599E mutation) was performed using real-time PCR and melting curve analysis. DNA from a papillary thyroid carcinoma was used as a positive control for *BRAF* V599E mutation (Xing, 2005) (Figure 4.1), while an oesophageal carcinoma was used as a control for *KRAS* mutation (Lord *et al.*, 2000) (Figure 4.1). The primers and fluorescently labelled probes (MetaBion International, Martinsried, Germany) were designed using the LightCycler Probe Design Software (Version 2.0, Roche, Mannheim, Germany) and are specified in Table 4.1. PCR was performed in 20 μl volumes consisting of 2μl DNA, 1.6 μl $MgCl_2$ (3mM final concentration), 1μl of each primer (0.5μM), 0.4μl of each probe (0.2μM), 2μl of LightCycler FastStart DNA master HybProbe (Roche, Mannheim, Germany) and 11.6μl of PCR-grade distilled water (Roche, Mannheim, Germany) and resulted in a product size of 263 bp for the BRAF reaction and a 225 bp product for the KRAS reaction The cycling conditions for the Roche LightCycler thermal cycler (Software Version LCS4 4.0.0.23, Roche, Mannheim, Germany) are described in Table 4.2. Following amplification, the reaction mixtures were denatured at 95°C for 0sec, held at 45°C for 30sec and then slowly heated to 80°C at a ramping rate of 0.1°C/sec. During this process the decline in fluorescence was monitored continuously, while constructing melting curves using the LightCycler software. Melting curves were subsequently

converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature (-d*F*/d*T*). Results were randomly verified through cycle sequencing using the BigDye Terminators v3.0 cycle sequencing kit using the Applied Biosystems 3130 Genetic analyzer utilizing capillary based technology.

4.3. Statistical analysis

Statistical comparisons between groups were completed using the two-sided Fisher's exact test. Patients were analyzed based on ethnicity (black vs. white), age (<50 years vs. >50 years), gender (male vs. female) and tumour site (as described in Chapter 2). Multinomial logistic regression analysis was performed to calculate odds ratios. All statistical analyses were completed using Stata Intercooled 7.0 (Stata, College Station, TX, USA). With $p < 0.05$, the differences were considered statistically significant.

4.4. Results:

4.4.1 Patient demographics

A total number of 80 cases were randomly selected for the period 1999 to 2002, which included 57 black and 23 white patients. An equivalent number of cases younger and older than 50 years of age at diagnosis were used: 51% (41/80) young and 49% (39/80) old. The majority of patients were female (53%), with slightly lesser numbers of males (47%).

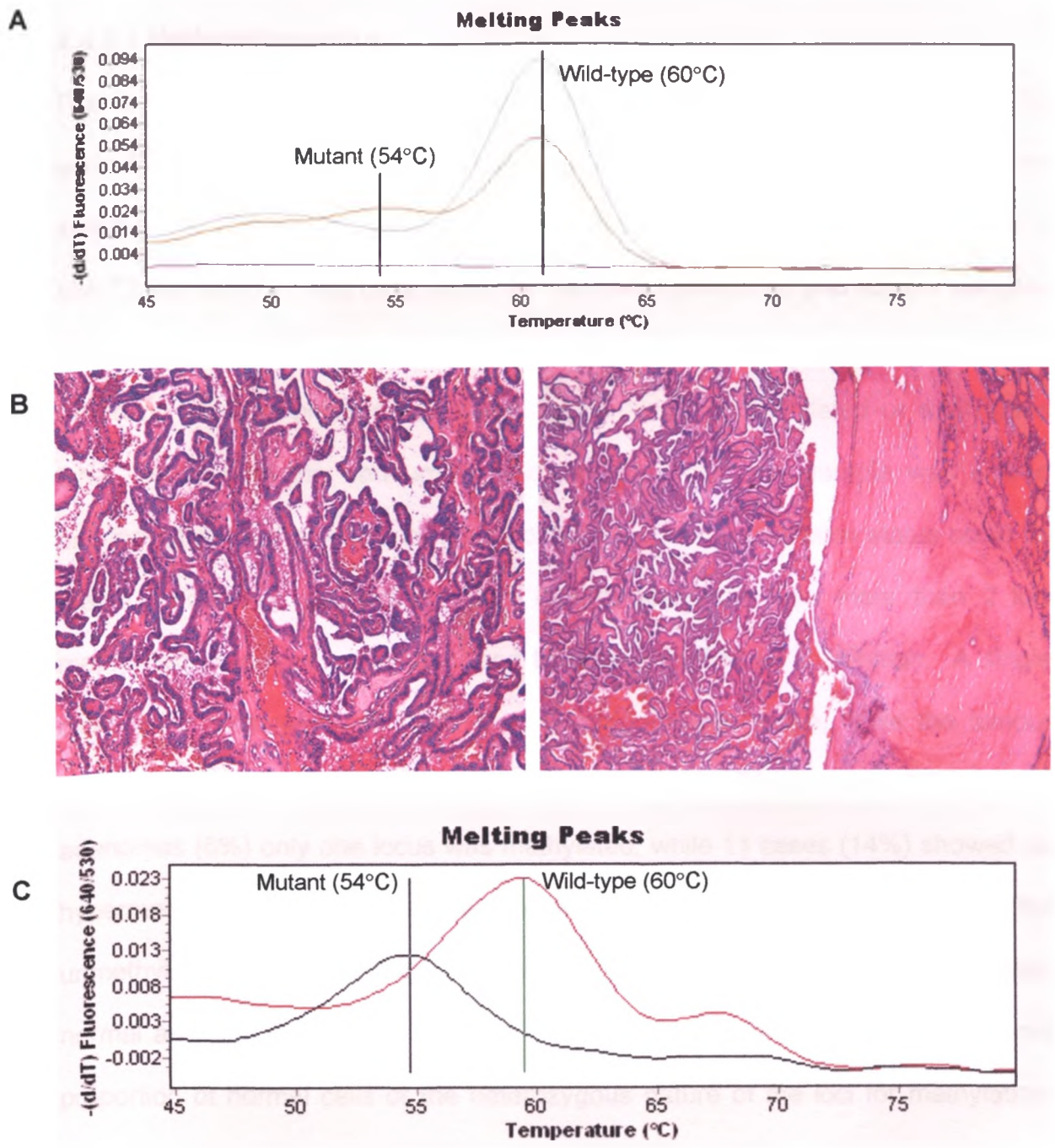


Figure 4.1: Melting curve analysis (A) for the detection of V599E mutant forms of the *BRAF* gene, using DNA from a papillary thyroid carcinoma (B), and G to A transition in codon 12 and 13 of exon 1 of the *KRAS* gene (C), using oesophageal carcinoma DNA. Melting curves were converted to melting peaks by plotting the negative derivative of the fluorescence (-d*F*/d*T*) with respect to temperature. The melting peaks for the wild-type and mutant forms of each gene are at different temperatures (54°C for the mutant and 60°C for the wild-type of both the *BRAF* and *KRAS* genes).

4.4.2 Molecular pathology

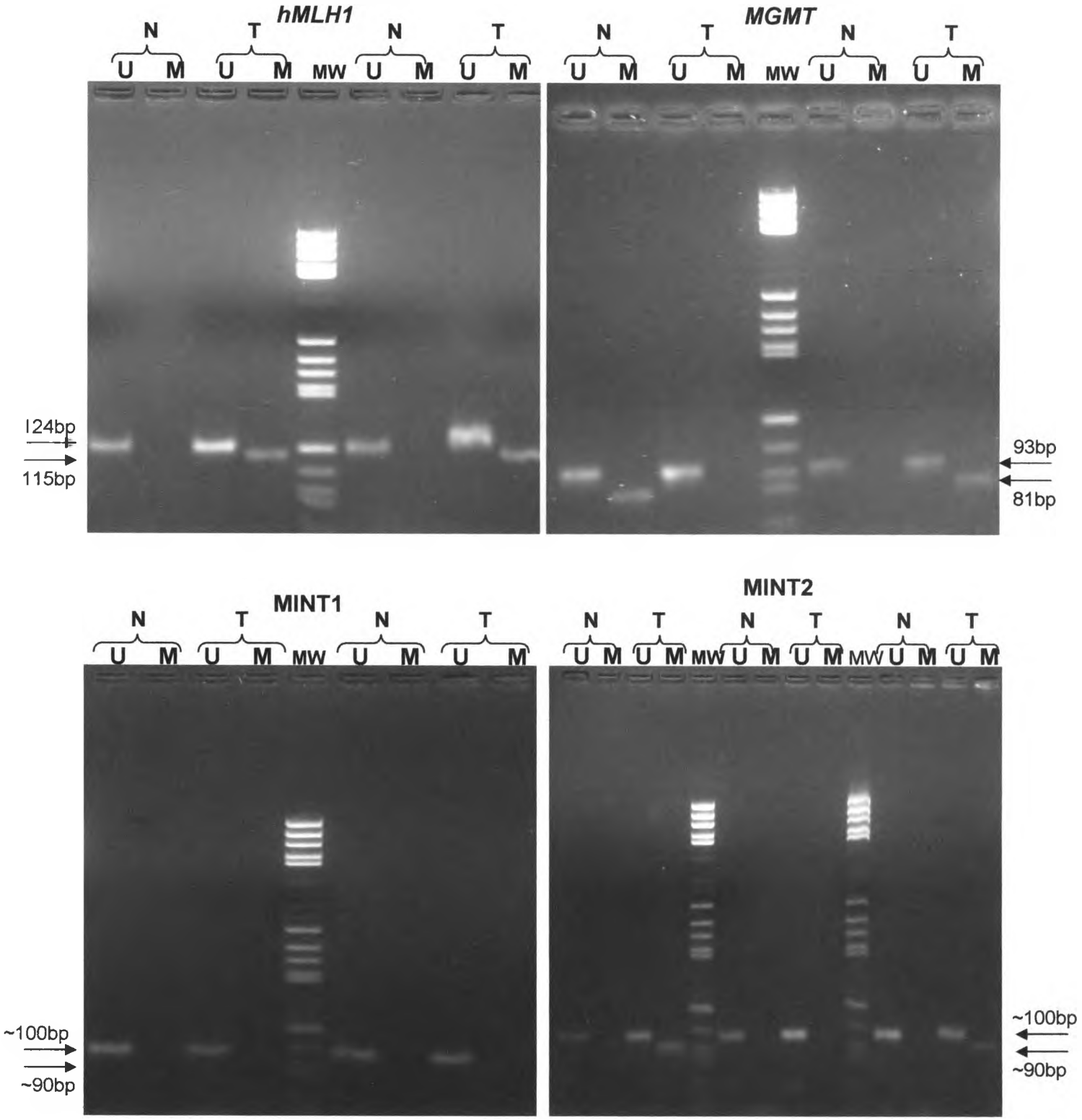
4.4.2.1 Methylation status

Figure 4.2 shows examples of the reaction products of methylation-specific PCR, while Figure 4.3 summarises the results for all cases examined. The methylation status of the *hMLH1* and *MGMT* genes and the methylation in tumour loci MINT1, MINT2 and MINT31 was determined for matched non-tumour and tumour samples of each case by bisulphite treatment of DNA followed by methylation-specific PCR. When no reaction product was observed for either unmethylated or methylated DNA, within both the non-tumour and tumour tissue, the sample was scored uninformative for that locus, as no conclusion as to its methylation status could be drawn. Forty-four percent of cases (35/80) presented with more than 2 loci methylated in tumour specimens, and thereby classified as CIMP-high. A single case (#47) presented with CIMP-high in adenomatous tissue, while the tumour tissue from the same patient showed CIMP-low. In 29 tumours (36%) and 5 adenomas (6%) only one locus was methylated, while 11 cases (14%) showed no hypermethylation in any of the examined loci. For the majority of cases, the unmethylated form of all the loci examined was detected in both non-neoplastic normal and tumour tissue. This may be interpreted as the presence of a small proportion of normal cells or the heterozygous nature of the loci for methylation. In a single case the methylated form of the *MGMT* promoter regions was observed in the non-neoplastic normal but not the tumour tissue (Figure 4.2). This could indicate early neoplastic changes observed at a molecular level that would initiate tumour development but not necessarily be involved in the later, more advanced stages of the tumour. Of the 80 cases examined, only 3 (cases 10, 16 and 36) proved to be uninformative and did not produce a reaction product for either the

unmethylated or methylated polymerase chain reactions at a minimum of 2 loci. The *hMLH1* promoter gene locus produced a PCR product in 91% of the specimens analyzed (73/80), and showed methylation in 25 of these specimens (31%). All the examined cases were informative for the *MGMT* gene locus, which was methylated in 61% of the cases (49/80). The MINT loci were informative in 97% (MINT1 and MINT2) and 98% (MINT31) of cases, with 2 cases (2%) showing hypermethylation at MINT1, 17 cases (20%) at MINT2 and 29 cases (34%) at MINT31.

Four cases with an adenoma present in addition to a colorectal tumour were examined for methylation status. In two of the four cases, the adenomas showed promoter hypermethylation, while the tumour tissue from the same patients was CIMP negative. The remaining 2 adenomas being contiguous and tubulovillous in nature were scored CIMP-low and CIMP-high respectively, while the representative tumour from the same patients was CIMP-low. In a case from an 81 year old white female containing a contiguous adenoma and a serrated adenoma, both were scored CIMP-low, showing only one methylated locus each, i.e. *MGMT* and *hMLH1* respectively. The tumour from this patient, being an infiltrative adenocarcinoma, was CIMP negative.

Examination of the data revealed a trend towards the CIMP-H phenotype in white and female patients ($p = 0.456$ and $p = 0.824$ respectively), those older than 50 years of age ($p = 0.179$), and tumours found proximal to the splenic flexure (0.813) (Table 4.3). The Fisher's exact test, however, did not find this to be statistically


hMLH1
MGMT
MINT1
MINT2
N
T
U
M
MW
124bp
115bp
93bp
81bp
~100bp
~90bp

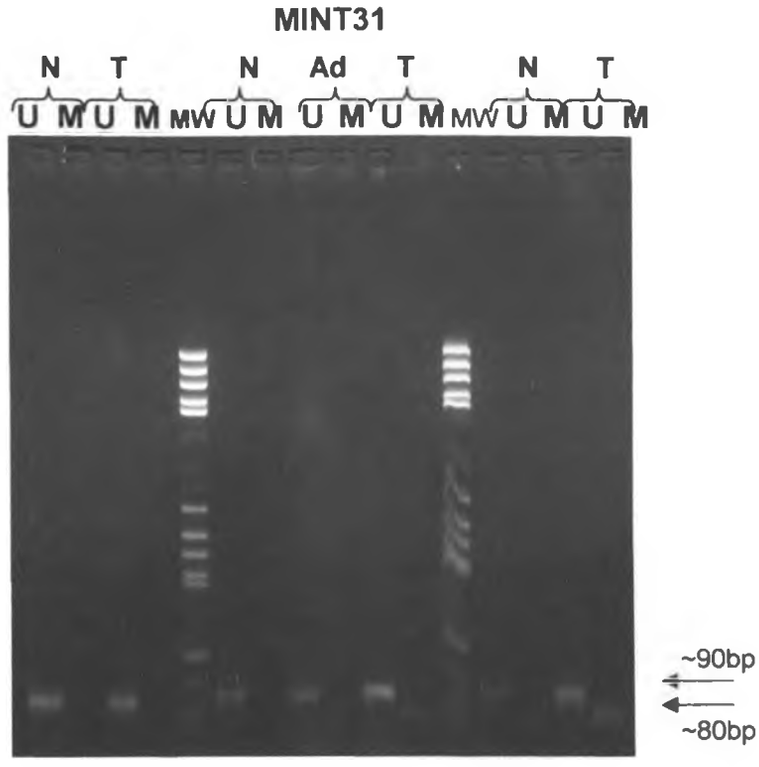


Figure 4.2: Examples of methylation-specific PCR (MSP) at *hMLH1, MGMT*, MINT1, MINT2 and MINT31 promoter region CpG island in human colorectal cancer. Methylation of the loci was evaluated by MSP using primers for unmethylated (U) and methylated (M) alleles of bisulphite-treated DNA. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated DNA; the presence of product in those lanes marked M indicates the presence of methylated DNA. MW represents lane with molecular weight marker (Marker V, Roche Diagnostics), with the expected sizes of each fragment indicated on the side. Loci examined are indicated above each gel. N = non-neoplastic normal tissue; Ad = adenomatous tissue; T = tumour tissue.

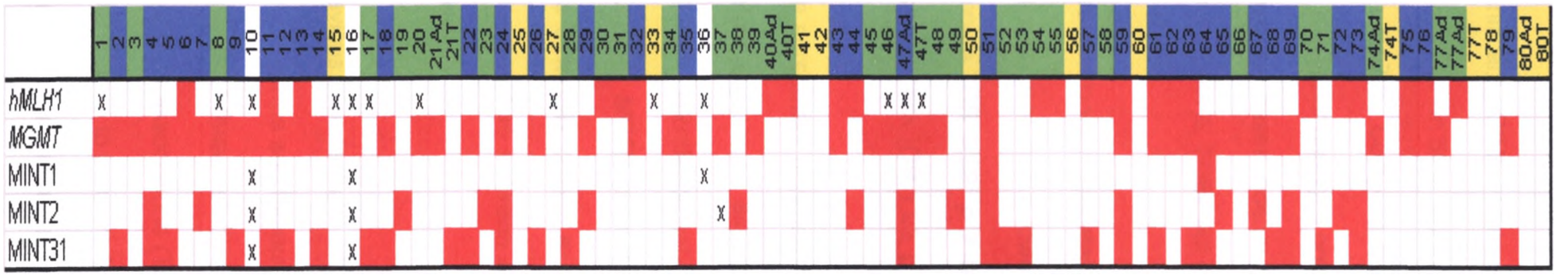


Figure 4.3: A summary of CpG island hypermethylation in tumour and adenomatous tissue, as compared to the non-tumour tissue of each case respectively. Methylation status of 5 CpG islands from the *hMLH1* and *MGMT* genes, and methylated in tumour loci MINT1, MINT2 and MINT31. Unmethylated and methylated sequences were amplified from bisulphite modified DNA through methylation-specific PCR. Each column represents the tumour (T) tissue from a single case, which was arbitrarily numbered. In instances where adenomatous (Ad) tissue in addition to tumour tissue was available, both samples were included for analysis. Boxes filled in red indicate methylated genes, while blank boxes indicate unmethylated genes. Those marked with an "X" indicate a non-informative reaction product, where no reaction product was observed for either the unmethylated or methylated reactions. Case number boxes filled in blue indicate cases that contained 2 or more methylated loci, and hence termed CpG island methylator phenotype high (CIMP-H); boxes filled in green indicate cases that contained only 1 methylated locus, termed CIMP-low (CIMP-L); boxes filled in yellow indicate cases that contained no methylated loci (CIMP-negative). Clear case number boxes indicate cases that were uninformative by producing no reaction product at a minimum of 2 loci. The methylation status of the samples was randomly repeated on 2 separate occasions and consistently confirmed the original findings.

significant. CIMP-H tumours were predominantly poorly differentiated (p = 0.078) with less than 50% mucin expression (p = 0.261) and moderate levels of necrosis (p = 0.070). Complete morphological analysis revealed 16 of the cases analyzed for molecular changes presented with an adenoma in addition to tumour tissue. In these 16 cases adenomas were not often seen in association with CIMP-H tumours (44%; 7/16), but occurred in 56% of CIMP-L cases (9/16) (Table 4.3).

Through logistic regression analysis it was shown that in relation to female patients, men had a lower risk, although not significant, for developing CIMP-H tumours (OR 0.89; p = 0.802), while older and white patients had marginally increased risk (OR 1.93, p = 0.150; OR 1.54, p = 0.395 respectively) (Figure 4.4).

The agreement between *hMLH1* promoter region methylation and loss of hMLH1 protein expression assessed through immunohistochemistry was 66% with no significant correlation between methylation status and loss of protein expression (p = 0.183). *MGMT* promoter region methylation showed only 54% agreement with loss of MGMT protein expression although a slight significance was observed in the correlation between mehtylation status and loss of protein expression (p = 0.046).

Previous reports have shown methylation to be associated with specific patient demographics and tumour characteristics (Issa *et al.*, 2004; Whitehall *et al.*, 2002). Analysis of these relationships in the present study did not show any statistically significant associations (Table 4.4). A trend towards promoter methylation of the *hMLH1* gene to be more frequent in young, white and male patients was observed. These tumours were further found predominantly proximal to the splenic flexure

Table 4.3: The association of CpG island methylator phenotype (CIMP) with patient demographics and tumour characteristics. Associations were calculated using the Fisher's exact test at a 95% confidence interval.

	CIMP status		
	CIMP-L	**CIMP-H**	**p-value**
Gender:			0.824
Female	51.22% (21/41)	54.05% (20/37)	
Male	48.78% (20/41)	45.95% (17/37)	
Age:			0.179
<50yr	59.52% (25/42)	43.24% (16/37)	
>50yr	40.48% (17/42)	56.76% (21/37)	
Ethnicity:			0.456
Black	56.14% (32/57)	43.86% (25/57)	
White	45.45% (10/22)	54.55% (12/22)	
Sidedness:			0.813
Caecum - transverse	33.33% (13/39)	36.11% (13/36)	
Descending - rectum	66.67% (26/39)	63.89% (23/36)	
Tumour differentiation:			***0.078****
Moderate - well	52.63% (30/57)	47.37% (27/57)	
Poor	30.77% (4/13)	69.23% (9/13)	
Mucinous	100% (3/3)	0	
% Mucin expression:			0.261
None	50.00% (29/58)	50.00% (29/58)	
<50% mucin	40.00% (4/10)	60.00% (6/10)	
>50% mucin	83.33% (5/6)	16.67% (1/6)	
Necrosis:			***0.070****
None	26.32% (5/19)	22.22% (4/18)	
Moderate level	15.79% (3/19)	50.00% (9/18)	
High level	57.89% (11/19)	27.78% (5/18)	
Presence of adenomas:			0.779
None	50.00% (28/56)	50.00% (28/56)	
Present	56.25% (9/16)	43.75% (7/16)	

**** Marginally signficant values at 95% CI***

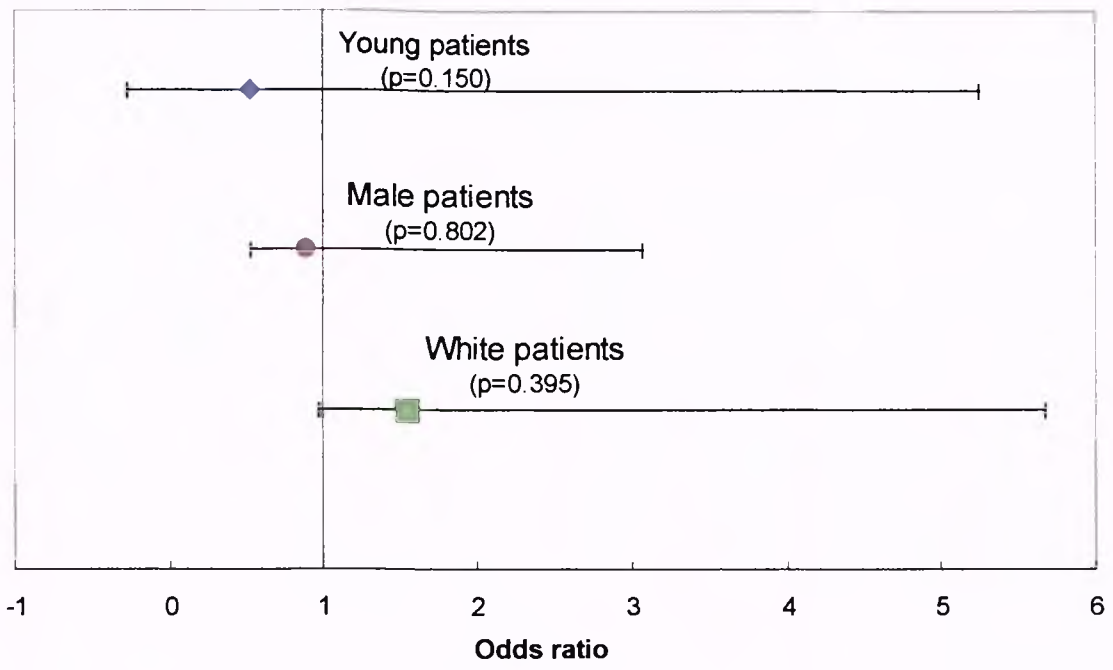


Figure 4.4: The risk of white, male patients developing colorectal cancer (CRC) with a high CpG island methylator phenotype (CIMP-H). Odds ratios were calculated through logistic regression analysis, at a 95% confidence interval.

(p = 0.197), were poorly differentiated (p = 0.171) with less than 50% mucin expression (p =0.111) and displayed moderate levels of necrosis (p = 0.804). Also *hMLH1* promoter methylation in the tumour tissue was found in 26% of cases with an adenoma in addition to the tumour, although the association was not statistically significant (Table 4.4). In contrast to *hMLh1* promoter methylation, *MGMT* promoter methylation was more frequently found in older (p = 0.261), female (p = 1.000) and white patients (p = 0.620). Tumours with *MGMT* promoter methylation were frequently found distal to the splenic flexure (p = 0.326), moderately to well differentiated (p = 0.049) with no mucin expression (p = 0.024). Although not significant, these tumours more often have low to moderate levels of necrosis when compared to those without *MGMT* promoter methylation (68% vs 44%).

Table 4.4: The association of the presence of *hMLH1* promoter methylation with patient demographics and tumour characteristics. Associations were calculated using the Fisher's exact test at a 95% confidence interval.

	hMLH1 methylation status		
	Unmethylated	**Methylated**	**p-value**
Gender:			1.000
Female	52.63% (30/57)	50.00% (11/22)	
Male	47.37% (27/57)	50.00% (11/22)	
Age:			1.000
<50yr	50.88% (29/57)	52.17% (12/23)	
>50yr	49.12% (28/57)	47.83% (11/23)	
Ethnicity:			1.000
Black	71.93% (41/57)	69.57% (16/23)	
White	28.07% (16/57)	30.43% (7/23)	
Sidedness:			0.197
Caecum - transverse	29.63% (16/54)	45.45% (10/22)	
Descending - rectum	70.37% (38/54)	54.55% (12/22)	
Tumour differentiation:			0.171
Moderate - well	81.48% (44/54)	70.00% (14/20)	
Poor	12.96% (7/54)	30.00% (6/20)	
Mucinous	5.56% (3/54)	0	
% Mucin expression:			0.111
None	80.00% (44/55)	75.00% (15/20)	
<50% mucin	9.09% (5/55)	25.00% (5/20)	
>50% mucin	10.91% (6/55)	0	
Necrosis:			0.804
None	25.93% (7/27)	20.00% (2/10)	
Moderate level	29.63% (8/27)	40.00% (4/10)	
High level	44.44% (12/27)	40.00% (4/10)	
Presence of adenomas:			0.748
None	79.63% (43/54)	73.68% (14/19)	
Present	20.37% (11/54)	26.32% (5/19)	

	MGMT methylation status		
	Unmethylated	**Methylated**	**p-value**
Gender:			1.000
Female	51.61% (16/31)	52.08% (25/48)	
Male	48.39% (1531)	47.92% (23/48)	
Age:			0.261
<50yr	59.38% (19/32)	45.83% (22/48)	
>50yr	40.63% (13/32)	54.17% (26/48)	
Ethnicity:			0.620
Black	75.00% (24/32)	68.75% (33/48)	
White	25.00% (8/32)	31.25% (15/48)	
Sidedness:			0.326
Caecum - transverse	41.94% (13/31)	28.89% (13/45)	
Descending - rectum	58.06% (18/31)	71.11% (32/45)	
Tumour differentiation:			***0.049****
Moderate - well	67.86% (19/28)	84.78% (39/46)	
Poor	21.43% (6/28)	15.22% (7/46)	
Mucinous	10.71% (3/28)	0	
% Mucin expression:			***0.024****
None	64.29% (18/28)	87.23% (41/47)	
<50% mucin	17.86% (5/28)	10.64% (5/47)	
>50% mucin	17.86% (5/28)	2.13% (1/47)	
Necrosis:			1.000
None	22.22% (4/18)	26.32% (5/19)	
Moderate level	22.22% (4/18)	42.11% (8/19)	
High level	55.56% (10/18)	31.58% (6/19)	
Presence of adenomas:			1.000
None	76.92% (20/26)	78.72% (37/47)	
Present	23.08% (6/26)	21.28% (10/47)	

**** Significant values at 95% confidence interval***

The risk of the study patients presenting with promoter methylation of either the *hMLH1* or *MGMT* genes was next analysed (Figure 4.5). The odds of young (OR 1.95, p=0.916), male (OR 1.11, p=0.834) and white (OR 1.12, p=0.833) patients to have *hMLH1* promoter methylation, was higher than older (OR 0.95, p=0.916), female (OR 0.90, p=0.834) and black patients (OR 0.89, p=0.833), although not statistically significant. The risk of having *MGMT* promoter methylation was higher in older patients (OR 1.73, p=0.237) when compared to those younger than 50 years of age. Similar to the results observed for *hMLH1* promoter methylation, white patients were at higher risk for *MGMT* promotermethylation (OR 1.36, p=0.546), while male and female patients showed moderate, but not significantly increased risks (OR 1.02 and OR 1.73 respectively, p=0.967).

4.4.2.2 Microsatellite instability (MSI)

As a subset of cases analyzed during the methylation studies proved to be uninformative, these were omitted from further analyses and hence 45 cases were subsequently reviewed. Tumour MSI was determined at five loci recommended by the American Joint Commission on Cancer, the International Collaborative Group on HNPCC and the HNPCC Cancer study group in Germany (Boland *et al.*, 1998). These included the mononucleotide markers BAT25 and BAT26, and the dinucleotide markers D5S346, D17S250 and D2S123. A summary of the results obtained is depicted in Figure 4.6. In addition to MSI loss of heterozygosity (LOH) at the D5S346 (APC) locus was determined as the disappearance of an allele when comparing normal and tumour alleles. Cases were selected based on the availability of matching non-tumour control and tumour

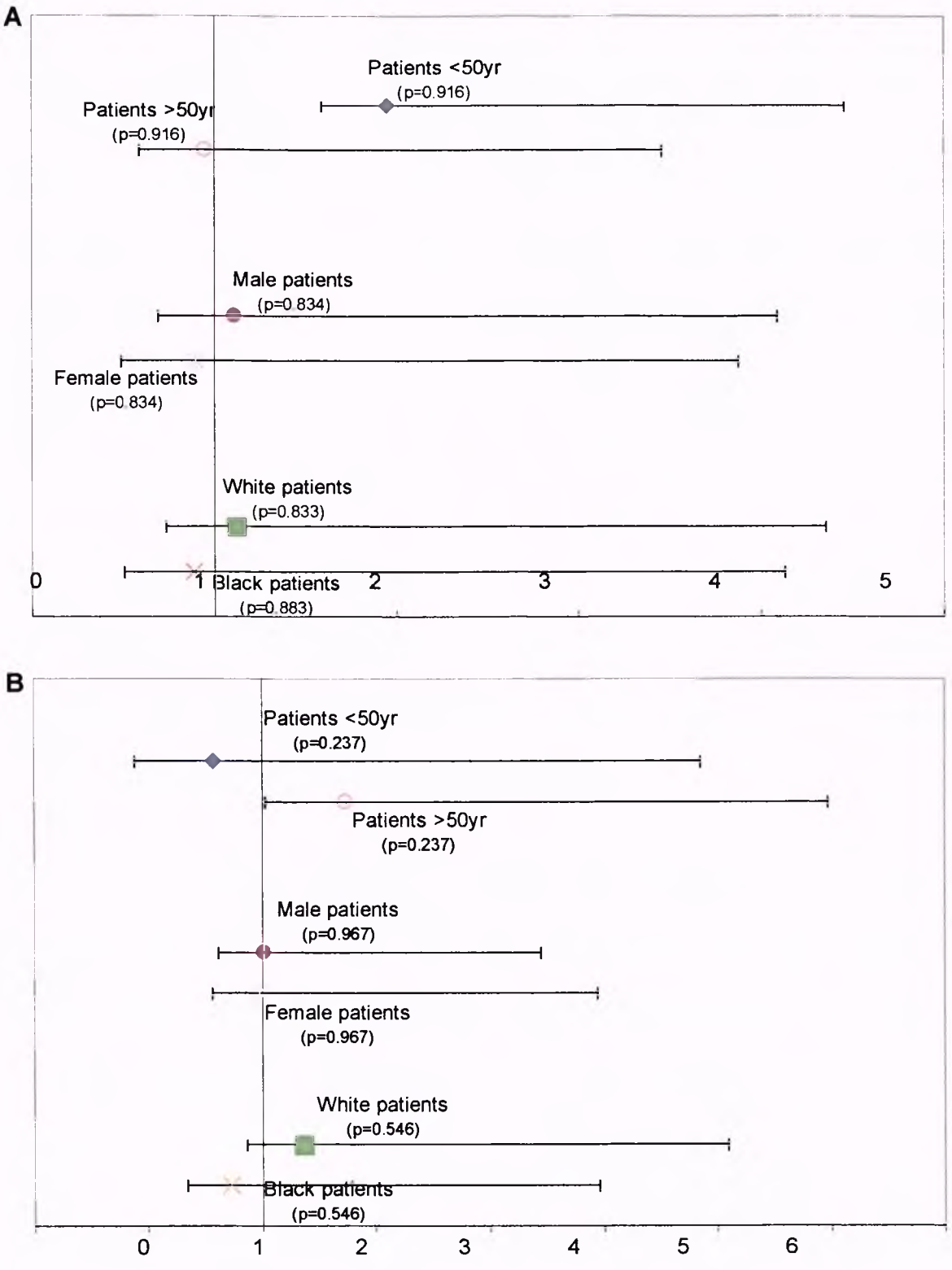


Figure 4.5: The risk of South African patients developing colorectal cancer (CRC) with methylation within the promoter regions of the *hMLH1* or *MGMT* genes. Odds ratios for tumours showing *hMLH1* (**A**) and O^6-methylguanine DNA methyltransferase (*MGMT*) (**B**) promoter methylation was calculated through logistic regression analysis, at a 95% confidence interval.

tissue, and included 44 cases consisting of 28 cases below 50 years of age (25 black and 3 white), and 16 cases above 50 years of age (8 black and white each). Of the cases analyzed 2 (4%) proved to be uninformative (case # 13 and 18) by producing no reaction product at any of the five loci analyzed after amplification (Figure 4.6). This could likely be due to the insufficient quantity or poor quality of the extracted DNA. Loss of heterozygosity at the APC locus was observed in 8 cases (18%), seven of which occurred in MSS tumours and one in a tumour with one of the five loci unstable (Figure 4.6).

Overall, 60% of the cases examined (27/45) proved to be stable at all five of the loci examined in the tumour tissue. In five cases (11%) a single locus was unstable, while 24% (11/45) were unstable at two or more loci. Tumours of cases 2 and 30 were unstable at all five loci (Figure 4.6).

For six of the cases (13%) both adenoma tissue and tumour tissue was available, and the analysis of MSI included both adenomatous and tumour tissue (Case # 8, 16, 21, 38, 41 and 45) (Figure 4.6). The majority of these cases (83%; 5/6) had the same microsatellite status in both the adenoma and tumour, while a single case (Case # 8) showed a transition from MSS in the adenoma to MSI-H in the tumour. Case number 41 presented with and had biopsies of several adenomas, including a contiguous and a single serrated adenoma. The contiguous adenoma showed instability in 2 loci (i.e MSI-H), while the serrated adenoma and tumour were MSS, which seem to indicate that the tumour in this case evolved from the serrated adenoma and not the contiguous adenoma. Interestingly, cases 16 and 21 presented with LOH at the APC locus in the tumour which was not evident in

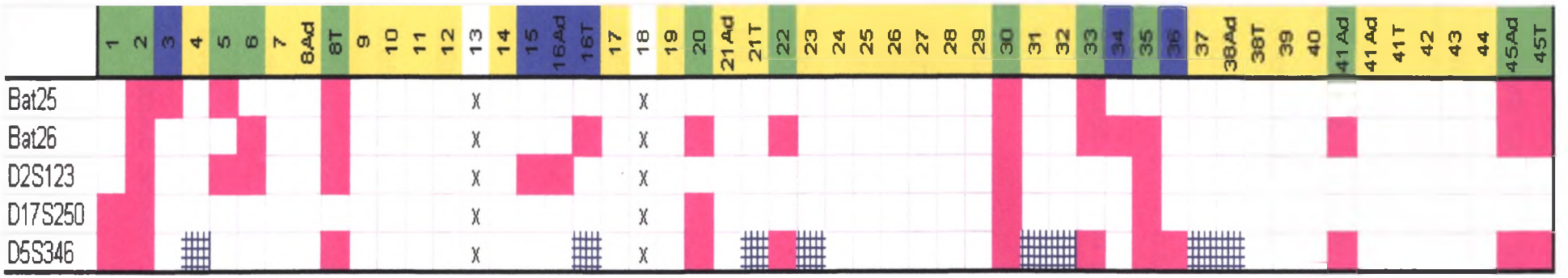


Figure 4.6: A summary of the results obtained for tumour microsatellite instability (MSI) and loss of heterozygosity (LOH) at the *APC* gene locus (D5S346). Microsatellite loci examined included the mononucleotide markers BAT25 and BAT26, and the dinucleotide markers D2S123, D17S250 and D5S346. Loci were amplified from non-tumour control and matching tumour DNA in a multiplex PCR system. Each column represents the tumour tissue (T) of a single case, and was arbitrarily numbered. In instances where adenomatous (Ad) tissue in addition to tumour tissue was available, both samples were included for analysis. Boxes filled in pink indicate an unstable loci identified by the appearance of additional peaks or novel fragments, while blank boxes indicate a stable locus. Striped boxes corresponding to the D5S346 locus (*APC*) represent cases with LOH. Those marked with an "X" indicate a non-informative case for MSI, where no reaction product was observed for either the non-tumour control or tumour DNA. Case number boxes filled in yellow indicate cases that contained no unstable loci, and hence termed microsatellite stable (MSS); boxes filled in blue indicate cases that contained only 1 unstable locus, termed MSI-low (MSI-L); boxes filled in green indicate cases that contained 2 or more unstable loci (MSi-H). Clear case number boxes indicate cases that were uninformative by producing no reaction product in either the non-tumour control or tumour DNA reactions. Samples were randomly repeated on 2 separate occasions to confirm original findings.

the adenomatous tissue. In contrast, LOH at the APC locus in case number 38 was shown in the adenoma, but absent in the tumour.

Tumours presenting with microsatellite stability (MSS) and low levels of microsatellite instability (MSI-L) are indistinguishable both clinically and morphologically (Pawlik *et al.*, 2004), and for purposes of statistical analyses these tumours were combined into a non-MSI-H group. Analysis using the Fisher's exact test revealed the MSI-H phenotype to be more often found in males, while non-MSI-H tumours were more often shown in female patients although not statistically significant ($p = 0.144$) (Table 4.5). Amongst MSI-H tumours, 82% were found in young patients (<50 years of age) and only 18% in patients older than 50 years of age ($p = 0.273$). Although the majority of non-MSI-H tumours (58%) were also found in young patients, in comparison to MSI-H tumours more than double the number of tumours is represented in older patients (42%) ($p = 0.273$). The lack of statistical significance achieved here could be related to the small size. No significant association between microsatellite status and ethnicity was observed (Table 4.5).

MSI-H tumours were predominantly found proximal to the splenic flexure (64%; $p = 0.080$) with a high degree of low grade differentiation (91%; $p = 0.440$). (Table 4.5). In contrast, non-MSI-H tumours were located in the descending colon and rectum (69%; $p = 0.080$) with 26% being poorly differentiated ($p = 0.440$). Similar levels of mucin expression were noted ($p = 0.497$), and no significant association was found between MSI status and the level of Crohn's infiltrate. Slightly more

tumours with MSI-H were found in the presence of an adenoma (30% *vs* 23% of non-MSI-H tumours), but this was not significant (p = 0.689).

There was no significant association between loss of protein expression and microsatellite status using the Fisher's exact test. Loss of hMLH1 and hMSH6 protein expression was marginally more frequent in MSI-H tumours (p = 0.574 and p = 0.697 respectively), while non-MSI-H tumours tended to present with loss of APC, p53 and MGMT protein expression (not significant) (Table 4.5).

As loss of heterzygosity (LOH) at the *APC* gene locus (5q21) is associated with mutation in the p53 gene, loss of p53 protein expression was monitored in association with measured LOH. Loss of p53 expression was found in 67% (4/6) of cases that showed LOH at the *APC* gene locus. In contrast, only 33% of cases with LOH in addition showed loss of APC protein expression.

The sensitivities of the microsatellite markers to predict an MSI-H phenotype was calculated by combining results from the mononucleotide markers (Bat25 and Bat26) and those from the dinucleotide markers (D2S123, D5S346 and D17S250) (Figure 4.7). Mononucleotide markers were sensitive and specific for MSI-H in 91% and 87% of the cases. The dinucleotide markers, however, proved to be both more sensitive (100%) and specific (94%) as a group. Mononucleotide markers gave more false positive results than the dinucleotide markers, while false negativity was limited to 9% for the mononucleotide markers. Dinucleotide markers were more sensitive to predict positivity (MSI-H) (85% *vs* 71% in mononucleotide markers), while the negative predictive value was similar between the two types of markers.

Table 4.5: The association of microsatellite instability (MSI) phenotype with patient demographics and tumour characteristics. Associations were calculated using the Fisher's exact test at a 95% confidence interval.

	MSI status		
	Non-MSI-H	**MSI-H**	**p-value**
Gender:			0.144
Female	61.29% (19/31)	30.00% (3/10)	
Male	38.71% (12/31)	70.00% (7/10)	
Age:			0.273
<50yr	58.06% (18/31)	81.82% (9/11)	
>50yr	41.94% (13/31)	18.18% (2/11)	
Ethnicity:			1.000
Black	74.19% (23/31)	72.73% (8/11)	
White	25.81% (8/31)	27.27% (3/11)	
Sidedness:			0.080
Caecum - transverse	31.03% (9/29)	63.64% (7/11)	
Descending - rectum	68.97% (20/29)	36.36% (4/11)	
Tumour differentiation:			0.440
Moderate - well	67.74% (21/31)	90.91% (10/11)	
Poor	25.81% (8/31)	9.09% (1/11)	
Mucinous	6.45% (2/31)	0	
% Mucin expression:			0.497
None	77.42% (24/31)	63.64% (7/11)	
<50% mucin	16.13% (5/31)	27.27% (3/11)	
>50% mucin	6.45% (2/31)	9.09% (1/11)	
Crohn's infiltrate:			1.000
Low level	81.82% (9/11)	100.00% (3/3)	
High level	18.18% (2/11)	0	
hMLH1 protein expression:			0.574
Present	89.66% (26/29)	77.78% (7/9)	
Absent	10.34% (3/29)	22.22% (2/9)	
hMSH2 protein expression:			1.000
Present	89.66% (26/29)	90.00% (9/10)	
Absent	10.34% (3/29)	10.00% (1/10)	
hMSH6 protein expression:			0.697
Present	68.97% (20/29)	62.50% (5/8)	
Absent	31.03% (9/29)	37.50% (3/8)	
APC protein expression:			1.000
Present	84.62% (22/26)	90.00% (9/10)	
Absent	15.38% (4/26)	10.00% (1/10)	
p53 protein expression:			1.000
Present	37.93% (11/29)	40.00% (4/10)	
Absent	62.07% (18/29)	60.00% (6/10)	
MGMT protein expression:			1.000
Present	68.97% (20/29)	71.43% (5/7)	
Absent	31.03% (9/29)	28.57% (2/7)	

Analysis of the promoter region methylation status, as well as overall methylation status in association with the microsatellite status revealed *hMLH1* and *MGMT* promoter methylation to occur more frequently in non-MSI-H tumours (p = 0.296 and 0.589 respectively) (Table 4.6). An inverse relationship between CIMP and MSI was shown, with CIMP-L occurring more frequently in MSI-H tumours, while CIMP-H was more frequent in non-MSI-H tumours.

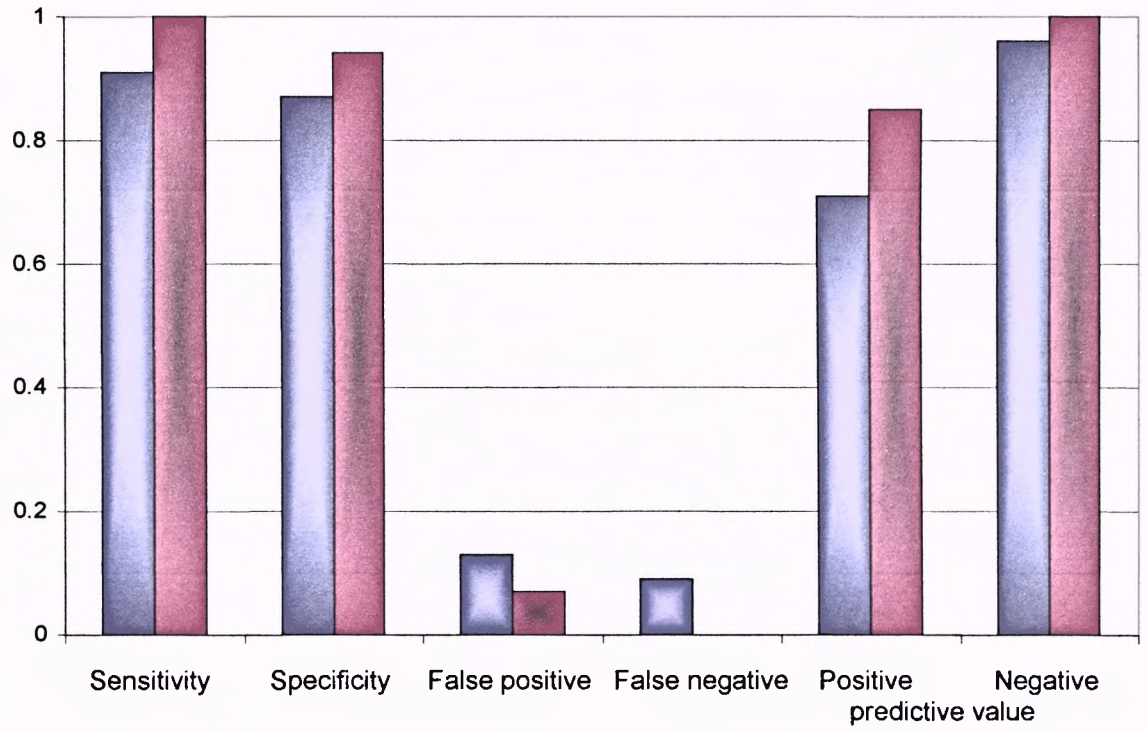


Figure 4.7: The predictive value of mononucleotide (Bat25, Bat26) and dinucleotide (D2S123, D5S346, D17S250) markers for the presence of an MSI-H phenotype. Values for mononucleotide markers are represented by blue bars, while the purple bars represent dinucleotide markers.

Table 4.6: The association between microsatellite instability (MSI) status and methylation phenotype (CIMP). Associations were calculated using the Fisher's exact test at a 95% confidence interval.

	MSI status		
	Non-MSI-H	**MSI-H**	**p-value**
***hMLH1* promoter methylation:**			0.296
Unmethylated	66.67% (20/30)	81.82% (9/11)	
Methylated	33.33% (10/30)	18.18% (2/11)	
***MGMT* promoter methylation:**			0.589
Unmethylated	43.33% (13/30)	45.45% (5/11)	
Methylated	56.67% (17/30)	54.55% (6/11)	
Methylation status:			0.272
CIMP-L	46.67% (14/30)	63.64% (7/11)	
CIMP-H	53.33% (16/30)	36.36% (4/11)	

4.4.2.3 *BRAF* and *KRAS* gene mutation status

Analysis of the mutational hot spots within the *BRAF* and *KRAS* genes was performed using real-time PCR technology in which fluorogenic probes emit fluorescence when they are bound to the target DNA in close proximity to each other. Probes bound to the wild-type sequence will be more thermodynamically stable and hence "melt off" under denaturing conditions at a higher temperature than when bound to the mutant sequence. Overall, in comparison to the papillary thyroid carcinoma used as positive control, only one of the cases analyzed contained the *BRAF* V599E mutation (case # 36) (Figure 4.8). The melting peak profile observed for cases with the *BRAF* mutation (Figure 4.1a) are likely to indicate a heterogenous population containing both wild-type and mutant DNA. Consistent with previously published reports, it is hypothesized that the wild-type to mutant DNA ratio in the *BRAF* mutation positive sample was in the region of 5:1 (Ikenoue *et al.*, 2004).

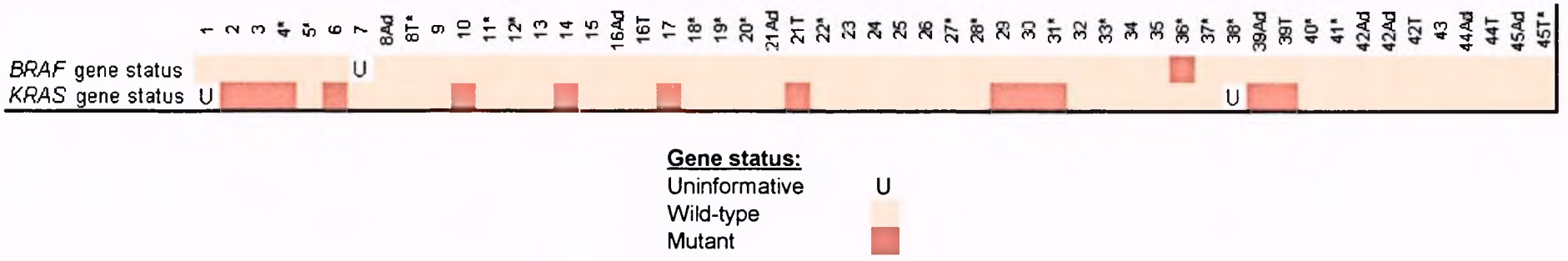


Figure 4.8: ***BRAF*** **and** ***KRAS*** **gene mutation status of colorectal carcinoma (CRC) cases in South African whites and blacks.** Gene mutation status was determined through the use of fluorogenic probes during real-time PCR analysis. Each column represents a single case, and was arbitrarily numbered in accordance with cases analyzed for microsatellite instability. Uninformative cases (U) were those that did not produce a reaction product following PCR on three separate occasions in the presence of adequate controls. Cases that were negative for the respective mutations (V599E in the *BRAF* gene and a G to A transition in codon 12 and 13 of the *KRAS* gene) yielded a single homozygous melting peak at 60°C and were classified as wild-type. In contrast, the presence of an additional or unique melting peak at 54°C signified a mutant version of the gene and was hence termed mutant. Results were randomly verified through cycle sequencing. These cases are indicated with an asterisk (*) next to the case number.

KRAS mutations in codon 12 and 13 of exon 1 were detected in 12 of the analyzed tumour samples (27%). The adenomatous material of sample number 21 did not show the same molecular profile for the *KRAS* gene mutation as the tumour material, with no mutations detected for the adenomatous material. As *KRAS* mutations are thought to be involved in the intermediate stages of tumour development, the lack of mutation observed in the adenomatous material could be indicative of tumour development through a distinct pathway not involving pre-existing adenomas. In samples 8, 16, 39, 42, 43 and 44, however, the same mutation profile was observed in both types of specimen samples, whether wild-type or mutant. These cases further showed a homozygous mutant melting peak with only a single peak observed at 54°C (Figure 4.1). Because only wild-type *KRAS* was seen in the non-tumourous samples, the mutations found in the pre-malignant adenoma and malignant tumour specimens are likely to be associated with colorectal carcinogenesis.

Real-time PCR results from selected samples were verified through cycle sequencing. In a single case, case # 11, inconsistent results were reported where sequence results appeared to indicate a *KRAS* mutation in the analyzed exon. This was opposite to that reported using real-time PCR and could be explained through preferential amplication.

4.4. Discussion:

Although the incidence of colorectal carcinoma (CRC) in the South African white and black populations has been documented (Pillay *et al.*, 1978; Bonnet *et al.*, 1997; Boytchev *et al.*, 1999; Walker & Segal, 2002), the molecular pathology has

not been comprehensively studied. Therefore, this study aimed to discriminate between the possible genetic pathways that these tumours might follow in the progression to carcinoma. Analysis of the overall methylation status revealed that high levels of methylation showed a trend to be more frequently found in older (>50 years of age), white and female patients specifically (Table 4.3). These tumours were further proximally located with a predominantly poorly differentiated architecture, and associated with low levels of microsatellite instability (MSS) (Table 4.6) and the presence of *KRAS* gene mutation (Figure 4.8). In contrast, low levels of methylation were found in young (<50 years of age), black and male patients with a purely mucinous tumour distally located (Table 4.3). These were further shown to contain high levels of microsatellite instability (Table 4.6) with wild-type *BRAF* gene status (Figure 4.8).

CpG island methylator phenotype (CIMP), characterized by the presence of hypermethylation at more than 2 of the selected loci, was observed in 45% (36/80) of the cases examined, while only 14% (11/80) showed no methylation at any of the selected loci, i.e. CIMP-negative (Figure 4.4). Similar percentages of methylation in colon cancer have been reported: van Rijnsoever *et al.* (2002) reported 32% of colorectal cancers showing CIMP-H in a study correlating CRC with CIMP. The concept of promoter hypermethylation during colorectal tumour development was first described in 1999 (Toyota *et al.*, 1999), and although this may explain an epigenetic-based Darwinian approach for a mechanistic contribution of methylation to phenotypic diversity (Issa *et al.*, 2005), it has developed into a controversial issue (Issa, 2004; Anacleto *et al.*, 2005). CIMP has been demonstrated in several malignancies, including hepatocellular carcinoma

(Shen *et al.*, 2002) and acute myeloid leukemia (Toyota *et al.*, 2001), as well as colorectal cancer (van Rijnsoever, *et al.*, 2002), but this is not a universal finding. Some investigators attribute the effects of methylation to ageing (Yamashita *et al.*, 2003) while others state that it represents a statistical artifact (Anacleto *et al.*, 2005). What remains clear is that methylation is not a random process, as seen in the significantly different rates of methylation in different cancers (Issa, 2004). Nonetheless, evidence is still forthcoming validating CIMP as significantly contributing to tumour development, as has been shown in a recent population-based study by Samowitz *et al.* (2005). These authors convincingly demonstrated the existence of the phenotype in an American population, and although no mention as to ethnicity was given, it was shown to be significantly associated with older patients and distally located tumours of the MSS phenotype. In contrast, CIMP-H in MSI-H tumours was found to be associated with proximally located, poorly differentiated tumours (Samowitz *et al.*, 2005).

Overall, a high percentage of cases (30%; 24/80) showed methylation within the promoter region of the *hMLH1* gene, while as many as 60% (48/80) were methylated within the *MGMT* promoter region (Figure 4.4). In a review describing the epigenetic changes in colorectal cancer Kondo and Issa (2004) reported that 10 to 20% of colon cancers show *hMLH1* promoter methylation, while 30% are reported to be methylated within the *MGMT* promoter region. In a study investigating the prevalence of *hMLH1* promoter methylation in African American patients with CRC, even higher percentages were reported: 85% were shown to contain hypermethylated promoter regions, although the sample size was rather small (n = 22) (Ashktorab *et al.*, 2005). Similarly, Shen *et al.* (2005) reported 46%

of sporadic colorectal cancers and 26% of normal appearing mucosa (n = 95) to show promoter methylation within the *MGMT* gene. Furthermore, Whitehall *et al.* (2001) described as many as 64% of tumours characterized by low-level microsatellite instability (MSI-L) present with *MGMT* promoter methylation. Loss of hMLH1 protein expression has been linked to the methylation of a small region within the promoter site, located at position -248 to -178 relative to the transcription start site (Deng *et al.*, 1999). In cases that showed promoter methylation within the *hMLH1* gene, but did not present with a loss of protein expression, it can be expected to find methylation within a different region. Deng *et al.* (1999) demonstrated that methylation in regions more upstream to this small proximal region did not prove to be critical for gene silencing. Another explanation could be the observation that mutant hMLH1 proteins retain their antigenicity while showing functional abrogation (Jass, 1999). A similar explanation relating to site of methylation and protein antigenicity could provide evidence for the loss of MGMT protein expression. Here it is reported that *hMLH1* promoter methylation is linked to the loss of hMLH1 protein expression in 66% of the cases, while loss of MGMT protein expression was linked to 54% of cases showing *MGMT* promoter methylation.

It has been proposed that methylation of the *MGMT* promoter region may be the basis for an epigenetic event predisposing to cancer (Shen *et al.*, 2005). In such a case it can be expected to find detectable levels of *MGMT* methylation in adjacent, normal appearing mucosa, even if at a low frequency. In an effort to use this marker as a useful method in risk assessment, Shen *et al.* (2005) showed that 72% of patients with *MGMT* methylation in tumour tissue also had detectable

levels of methylation in normal mucosa a distance of 1cm removed from the tumour. In the current study a high proportion of cases presented with promoter methylation (60%), with several cases indicating methylation within the normal appearing tissue (See Figure 4.4). The overall number of cases identified with *MGMT* methylation was substantially more than rates reported in studies utilizing microdissected material (Whitehall *et al.*, 2001; Chirieac *et al.*, 2005). Great care was taken during this study in the analysis of methylation status by only positively scoring a case as showing methylation when the methylated band was of greater intensity than the unmethylated band. Thus the high degree of *MGMT* methylation may in fact reflect "contamination" of tumour DNA with non-tumour DNA. Studies examining the methylation status of these loci in blood samples may in future prove to be useful.

CIMP has been linked to distinct clinical and epidemiological features that include older age, female gender, proximal location and mucinous poorly differentiated histology, together with high rates of *BRAF* and *KRAS* mutations but low rates of *p53* mutations (Issa, 2004; Whitehall *et al.*, 2002). In the present study, these associations with age, gender, location and differentiation were supported, although not statistically significant (Table 4.3). Contrary to published reports, however, CIMP-H tumours showed little mucin expression. Tumours showing CIMP can genetically be divided into two distinct groups: those with microsatellite instability high (MSI-H) relating to *hMLH1* promoter methylation (Chaves *et al.*, 2000) and those with microsatellite instability low (MSI-L), which is related to *MGMT* inactivation through promoter methylation. In the present study 45% (17/38) of CIMP-H tumours were methylated within the *hMLH1* promoter region

(Table 4.2). Sporadic colorectal cancers showing MSI-H due to *hMLH1* promoter methylation frequently present with mucinous carcinomas, a morphological feature shared with HNPCC tumours (Mecklin *et al.*, 1996; Jass *et al.*, 1998). Alternatively, Wright *et al.* (2005) recently reported that tumours with MSI-L were more frequently of a lower grade, while Tang *et al.* (2004) demonstrated microsatellite stable (MSS) tumours as presenting with a higher degree of low grade differentiation than high grade or mucinous architecture. It therefore seems plausible that the lack of mucin expression may be due to a low MSI phenotype related to a high degree of *MGMT* promoter methylation, observed in 89% (34/38) of CIMP-H tumours (Figure 4.4).

Male patients, those younger than 50 years of age and white patients showed more cases with *hMLH1* promoter methylation (Table 4.4). In a prospective series of unselected cases identifying the frequency of hereditary defective DNA mismatch repair, it was shown that the majority (75%) of cases characterized by defective DNA mismatch repair was due to *hMLH1* promoter hypermethylation (Cunningham *et al.*, 2001). These tumours were further found predominantly in older (>50 years of age) and female patients, presenting at a location proximal to the splenic flexure. In the present study, tumours were also predominantly proximally located, characterized by a poorly differentiated architecture with little mucin expression (<50%) (Table 4.4).

High levels of microsatellite instability (MSI-H) were shown to be associated with a CIMP-L phenotype (Table 4.6). This is in agreement with the molecular phenotype shown in hereditary non-polyposis colorectal cancer (HNPCC), where cancer

arises from an accumulation of mutations due to mismatch repair deficiency (Shia *et al.*, 2003), with infrequent loss of heterozygosity (LOH) and mutations of *APC* and *p53* (Jass *et al.*, 1999) while *KRAS* mutations are common and *BRAF* mutations absent (Oliveira *et al.*, 2004). In accordance, loss of expression of the mismatch repair proteins hMLH1 and hMSH6, but not hMSH2, was shown to occur at a higher frequency in MSI-H tumours (Table 4.5), while *BRAF* mutations were absent (Figure 4.8). HNPCC tumours are predominantly found in younger patients (younger than 50 years) in a proximal location characterized by a poorly differentiated to mucinous architecture (Samowitz *et al.*, 2001; Raut *et al.*, 2004). These features, together with infrequent LOH and mutations of *APC* and *p53*, were confirmed in MSI-H and CIMP-L tumours (Table 4.3 and 4.5) and indicate the involvement of the mutator pathway.

A subset of MSI-H tumours may, however, present with the CIMP-H phenotype, as was shown in 36% of cases analyzed here (Table 4.6). These sporadic CRC cases rarely present with a family history consistent with HNPCC (Raut *et al.*, 2004) and the basis of instability has been shown to be epigenetic. Promoter methylation of the *hMLH1* gene was shown to be an alternative route to MSI-H (Herman *et al.*, 1998; Thibodeau *et al.*, 1998) and 2 of the 4 cases with both MSI-H and CIMP-H were methylated in this region (Table 4.6). *BRAF*, one of the RAF genes identified in malignant transformation, is frequently mutated in sporadic MSI-H CRC (Rajagopalan *et al.*, 2002; Wang *et al.*, 2003). In contrast, however, the immediately upstream located *KRAS* is less often mutated (Jass *et al.*, 1999), as *BRAF* and *KRAS* mutations are mutually exclusive (Rajagopalan *et al.*, 2002). When *hMLH1* promoter methylation is, however, not the mechanism leading to

MSI-H, the mutational spectrum for *BRAF* and *KRAS* is the opposite: *KRAS* mutation will be of a higher frequency in comparison to *BRAF* mutations (Oliveira *et al.*, 2004). In the present study, as *KRAS* mutation was frequent and *BRAF* mutation absent (Figure 4.8), it seems that the sporadic cases of CRC that present with CIMP-H and MSI-H, may in fact develop through a pathway independent of *hMLH1* promoter methylation. It therefore seems plausible that although tumours with MSI-H develop through the same molecular mechanism involving the *hMLH1* gene, the mode of involvement is different and involves either germline mutations or promoter methylation.

An alternative route to colorectal cancer development has been proposed and involves low levels of microsatellite instability in a setting with high levels of methylation (Jass *et al.*, 2000b). These tumours arise in precursor lesions termed "serrated" and include hyperplastic polyps, mixed hyperplastic or adenomatous polyps and serrated adenomas (Jass *et al.*, 2000c). Whitehall *et al.* (2001) have subsequently shown that methylation of the *MGMT* promoter region is associated with the MSI-L phenotype, and that loss of expression of MGMT forms the basis for this phenotype. In addition, *BRAF* mutation occurs frequently in hyperplastic polyps and serrated adenomas, especially in a setting with extensive *hMLH1* methylation (Pawlik *et al.*, 2004; Oliveira *et al.*, 2004). When methylation occurs in the promoter region of a gene other than *hMLH1*, *KRAS* mutations tend to be the initiating events and occur with a high frequency (Konishi *et al.*, 1996; Jass *et al.*, 1999), which is implicated in the current study. As Pawlik *et al.* (2004) noted, however, the divergence of the pathway increases its significance in terms of its overall contribution to tumourigenesis, instead of diminishing its importance.

In the current study only 2 serrated adenomas were identified (See Chapter 2) but this is most likely due to a low detection rate rather a low incidence rate, as these lesions were only recently shown to be important in colorectal carcinogenesis (Jass, 2001; Young *et al.*, 2001; Fogt *et al.*, 2002) and hence more attention is currently given to identifying them. Wynter *et al.* (2006) recently described the molecular features associated with sporadic and familial adenomas and concluded that familial adenomas follow a different pathway to tumourigenesis than their sporadic counterparts. It therefore seems acceptable to conclude that molecular features of a tumour correlate with that observed in the precursor lesion and may thereby be grouped within a specific tumourigenic pathway. We described 53% of tumours examined to show a high methylation phenotype within a low microsatellite instability background occurring predominantly in older patients with tumours located distally to the splenic flexure (Tables 4.5 and 4.6). Furthermore, these tumours were methylated at the *MGMT* promoter region in 57% of the cases (Table 4.6), and showed loss of MGMT expression in 31% (Table 4.5). Similarly, *KRAS* mutations were frequent in microsatellite stable tumours and those with high methylation phenotype (Table 4.7). These tumours may thus follow the serrated neoplasia pathway. Rapid progression to malignancy through the acquisition of DNA instability may occur through the loss of hMLH1 expression (through *hMLH1* methylation) after loss of MGMT expression and then the advancement to the MSI-H phenotype (Jass *et al.*, 2000; Hawkins and Ward, 2001).

Through the analysis of specific genetic alterations, it is shown that two distinct pathways to the development of CRC in South African patients are present: the first involving the accumulation of mutations due to MSI and the second pertaining to high levels of methylation. Young black patients presented predominantly with genetic alterations that include high levels of MSI and low levels of CpG island methylation, associated with loss of mismatch repair protein expression. Tumours from these patients further presented with mutations in the *KRAS* gene (G to A transition in codon 12 and 13 of exon 1), which seem to be associated with the so-called mutator pathway that is otherwise indicative of hereditary colon cancer (HNPCC) in western cohorts. In contrast, young white patients present with sporadic colorectal cancer, associated with high levels of CpG island methylation and low levels of MSI resulting from methylation of the *MGMT* promoter region. A lack of *BRAF* mutations and presence of *KRAS* mutations confirm the involvement of the serrated neoplasia pathway, which suggests that these tumours may have develop from precursor lesions with a characteristic serrated architecture. Identification of these molecular features is important in the management of the disease, as treatment and prognosis may be affected. Another factor implicated is the importance of family history. Young black patients presented with a form of CRC that may imply a hereditary basis, although unfortunately no family data were available to confirm this. In a familial cancer one expects to find at least one mutation in the genetic makeup of the individual. This could be tested by examining DNA extracted from blood for mutation in the mismatch repair genes. High risk individuals can thereby be identified. Identification of familial risk is important to initiate early screening regimens to detect cancer not only at a young age, but also at an early stage. As Müller *et al.* (2004) rightly suggested the

(2004) rightly suggested the interpretation of results in the context of both clinico-pathological findings and family history is important in the diagnosis of familial cancer.

CHAPTER 5
SUMMARY AND DISCUSSION

Colorectal carcinoma (CRC) is one of the leading cancers in developed countries but occupies a much lower position in developing countries (Hamilton *et al.*, 2000; Mqoqi *et al.*, 2004). The higher prevalence in developed countries is generally associated with older age at presentation, a western type diet high in calories and/or animal fat, and a sedentary lifestyle. A small percentage of patients (3-5%), however, have genetically defined high-risk CRC family syndromes, and a further 10 to 20% may have potentially definable inherited causes. These patients are usually younger and dietary or lifestyle-related factors do not play a major role (Grady, 2003; Lynch and de la Chapelle, 2003).

As mentioned previously, the incidence of CRC in South Africa has increased over the past decade, from being ranked tenth to currently 5th according to the most recent data supplied by the South African National Cancer Registry (Mqoqi *et al.*, 2004). Within this country, the epidemiological and pathological features of CRC amongst whites appear to follow a classical western trend. Although it is far less common amongst the black African population, there is evidence that its incidence may be increasing. Of particular interest is the disproportionately large numbers of young black patients with CRC (Bonnet and Grobler, 1998; Boytchev *et al.*, 1999; Walker and Segal, 2002). In the present study it was identified that 40% of CRC in black patients occurs under the age of 50 years, with a comparable 10% in whites. Despite the relative infrequency of these cases, this is an undesirable burden of CRC amongst such a young and productive cohort.

There are currently four main molecular pathways along which CRC is postulated to develop, as depicted in Figure 5.1.

1: The "classical or environmental" Vogelstein model of colorectal carcinogenesis, pertaining primarily to diet and lifestyle is marked by defined genetic abnormalities including KRAS, APC, p21, p53 and the catenin/cadherin interaction (Vogelstein *et al.*, 1988).

2: The attenuated disease of familial adenomatous polyposis (FAP), an autosomal dominant predisposition to CRC that is mostly characterized by germline mutations of the *APC* gene (Fearnhead *et al.*, 2001).

3: Mismatch repair deficiency associated with microsatellite instability (MSI), which is identified in the autosomal dominant non-polyposis colorectal cancer (HNPCC) (Jass, 1998).

4: The recently identified "serrated" pathway to CRC involving methylation abnormalities, with the possibility of a currently unknown hereditary basis (Park *et al.*, 2003).

Although several researchers have investigated CRC in South African populations, the information regarding the molecular aetiology is limited. Scientists at the University of Cape Town identified an extended mixed-race family with proven HNPCC (due to a mutation of the *hMLH1* gene on chromosome 3) (Goldblatt *et* al., 1990; Goldberg *et al.*, 1998; Ramesar *et al.*, 2000). A cohort of 28 unrelated South

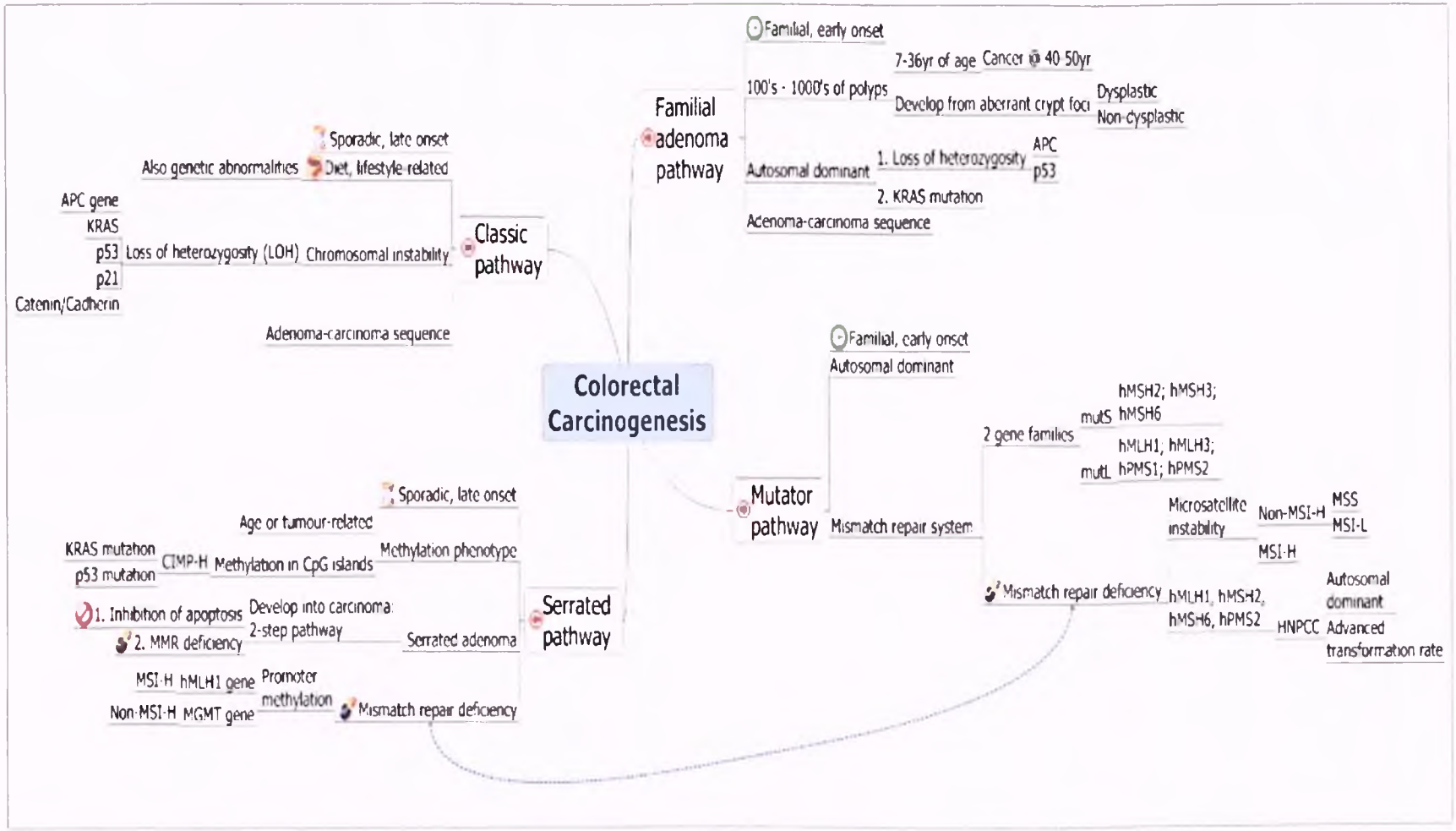


Figure 5.1: A summary of the 4 major pathways involved in colorectal carcinogenesis, as discussed in the text.

African families with FAP has been identified by the University of Stellenbosch (Grobbelaar *et al.*, 2000), while 32 cases of patients younger than 35 years of age with variable levels of MSI were studied by scientists at the University of Natal (Chetty *et al.*, 1998). The latter study did unfortunately not give a breakdown of ethnicity although most thereof were black patients (Naidoo R, personal communication).

The aim in the present study was to comprehensively study a series of young black patients in comparison to their white counterparts and to analyse their molecular pathogenesis. It was hypothesized that the tumours within this group are due to a primary genetic disease, unrelated to diet and lifestyle that could be identified by molecular means as following one or more of the set pathways. Figure 5.2 gives a summary of the complete molecular analyses of each patient, with a key to the major molecular alterations associated with the well-known pathways. Figure 5.3 recapitulates the morphological and molecular characteristics associated with young or old, black or white patients. The caveat, however, is that immunohistochemical staining of mismatch repair gene proteins may be open to a degree of over-interpretation in older paraffin blocks, an issue that has not yet been addressed in the literature.

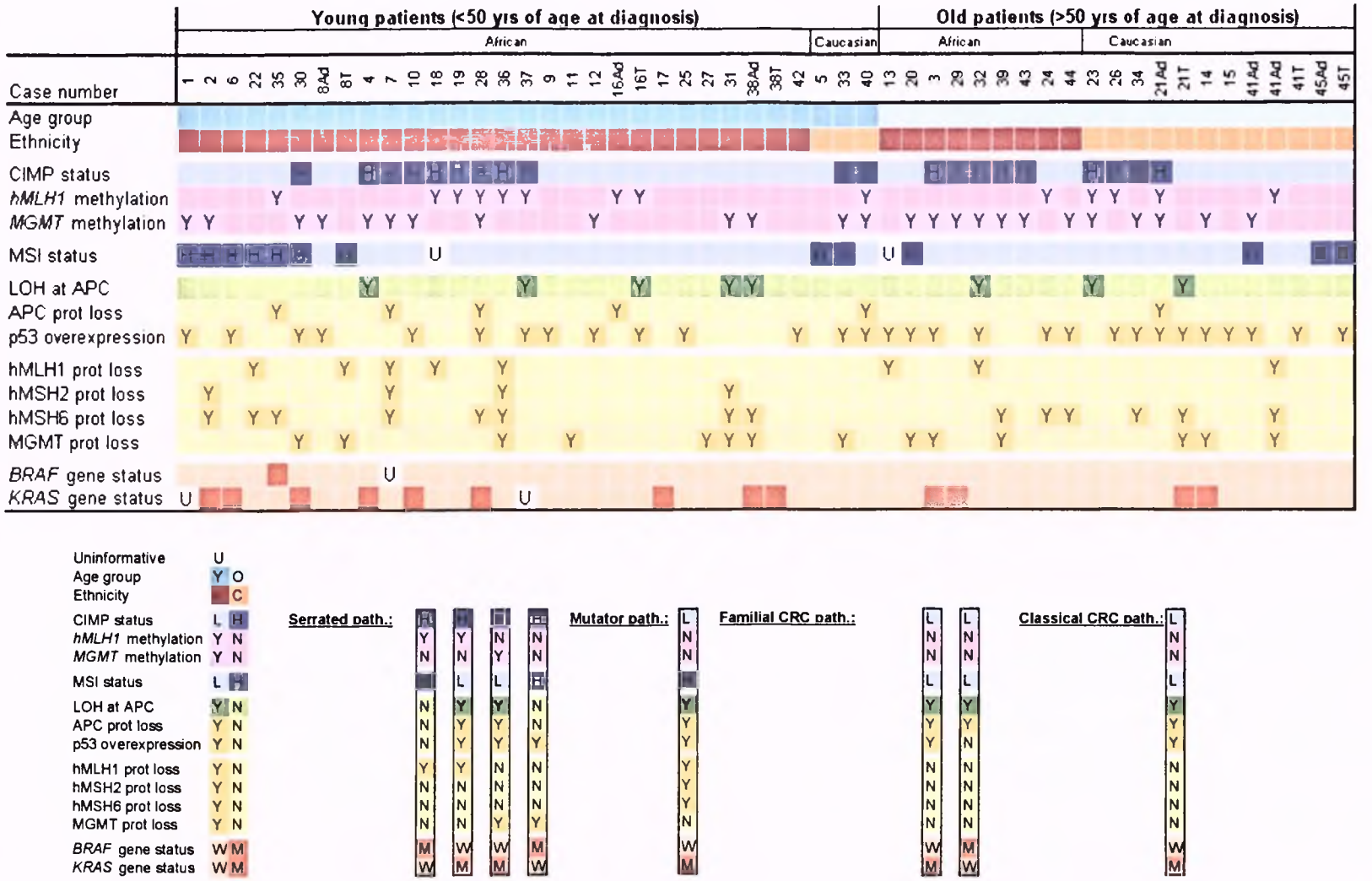


Figure 5.2: A summary of the molecular alterations in the colorectal cancers of a selected group of South African patients, as reported in previous chapters. Each column represents a single case, and was arbitrarily numbered and consistent with previous chapters. Numbers marked "Ad" represent an adenoma in addition to a tumour ("T") within the same patient. All other numbers refer to tumour tissue.

	Sidedness	Differentiation	Tumour appearance	Crohn's infiltrate	High mucin	Tumour necrosis	Adenoma present	Dukes staging	Aster-Coller staging	TNM staging	Jass staging	Loss of MLH1	Loss of MSH2	Loss of MSH6	Loss of MGMT	Loss of APC	Loss of p53	*hMLH1* methylation	*MGMT* methylation	CIMP	MSI	*BRAF* mutation	*KRAS* mutation
Young, African	R	↓	U	↓	↑	↑	↓	↓	↓	↓	↑*	→	↑	→	↑	↓	↔	↓	↓	↓	↓	↔	→
Young, Caucasian	R	↑	F	↑	↓	↓	→	↑	↑	↑	↓*	↓	↓	↓	↓	↑	↔	↑	→	↑	↑	↔	↓
Old, African	R	↓#	U	↓	↑*	↑	↑	↔	↓	↑	↓	↑	↑	↑	↓	↓	↓#	↔	↔	↓	↑	↔	↔
Old, Caucasian	L	↑#	F	↑	↓*	↓	↓	↔	↑	↓	↑	↓	↓	↓	↑	↑	↑#	↔	↔	↑	↓	↔	↔

Figure 5.3: A summary of the morphological and molecular features associated with young or old, black or white patients. Each row represents a specific cohort (Young black or Young white or Older black or Older white) with the identified association indicated as higher (↑), lower (↓) or equal to (↔) that observed for the opposite cohort. Statistical significant associations are indicated at 95% (*) and 90% (#) confidence interval. Due to the small sample size for the molecular analyses very few of the differences reached statistical significance.

Individual patterns of the molecular profiles observed in each patient could be used in directing the diagnosis towards a specific pathogenic pathway (Figure 5.1). Amongst young black patients a higher proportion follow hereditary pathways involving either a mutator phenotype through mismatch repair deficiency or the classical adenoma-carcinoma sequence of events initiated through *APC* gene mutations. In contrast, tumours from young whites tend to develop through the sporadic serrated adenoma pathway involving promoter hypermethylation, a feature that on an individually analyzed basis seems to be shared by older black patients.

When comparing the morphological and molecular features of young black and young white patients, it would appear that these tumours develop through different pathways (Figure 5.3). Overall, in comparison to young whites, young black patients may present more often with poorly differentiated tumours, ulcerating in appearance and located proximal to the splenic flexure. These tumours appear to occur in the absence of adenomas, with high percentage mucin expression and the presence of high levels of tumour necrosis. Although MSI status was restricted to low levels, loss of expression of the mismatch repair proteins was evident, while APC and p53 expression were considered normal. Furthermore, methylation status was predominantly low, with infrequent methylation of the *hMLH1* and *MGMT* promoter regions. These features are all in keeping with the mutator pathway, characterized by proximally located tumours that are poorly differentiated or mucinous in architecture with the loss of mismatch repair proficiency due to germline mutations (Lynch *et al.*, 1996; Jass *et al.*, 2000; Ward *et al.*, 2001; Peltomäki, 2001).

HNPCC cancers occur in the proximal colon of predominantly young patients (Guillem *et al.*, 1999) often as mucinous carcinomas and with frequent mutations in the *hMLH1* and *hMSH2* genes (Jass *et al.*, 1999). According to the Revised Bethesda criteria for the diagnosis of HNPCC (Umar *et al.*, 2004), the morphological and molecular features shown in young black patients may be in agreement with such a diagnosis. Furthermore, the Revised Bethesda criteria exclude individuals presenting with adenomas that are younger than 40 years of age (Umar *et al.*, 2004). Results from this study seem to confirm the notion that the presence of adenomas in young patients is not an important prerequisite for HNPCC diagnosis. It is therefore suggested that young black patients presenting with CRC be tested for MSI using the panel of markers suggested by the NCI (Boland *et al.*, 1998).

In comparison to young black patients, young whites may develop sporadic colorectal cancer through the serrated adenoma pathway involving high levels of methylation and microsatellite instability relating to promoter methylation of the *hMLH1* gene (Figure 5.3). This may reflect the serrated adenoma pathway. Sporadic MSI-H CRC and its familial counterpart, HNPCC, share similar morphological features as well as a predilection for the proximal colon (Jass, 2004). However, distinction between the subsets is shown through the high frequency of DNA methylation and *BRAF* mutation in sporadic cases (Young *et al.*, 2001; Wang *et al.*, 2003). Serrated neoplasia, including serrated adenomas, has been shown as the precursor lesion to sporadic MSI-H within serrated neoplasia (Jass *et al.*, 2002). The high incidence of adenomas in addition to carcinomas in young white patients may actually reflect and strengthen the notion that serrated neoplasia was underdiagnosed in this series (See Chapter 2).

As whites present with features that may relate to the serrated pathway, it is suggested that morphological assessment include a vigilant and comprehensive inspection for serrated neoplasia, followed by molecular inspection of methylation and microsatellite status.

Amongst the older (>50 years of age) patients, blacks present with features similar to those found in the younger cohort relating to MSI due to mismatch repair deficiency (Figure 5.3). In comparison to the black cohort, whites presented with features relating to the serrated pathway, leading to a high methylation phenotype with associated low levels of MSI, due to loss of MGMT protein expression (Whitehall *et al.*, 2001). Confirmatory features include loss of APC and p53 protein expression (Hiyama *et al.*, 1998; Thorstensen *et al.*, 2005), mutant *KRAS* (Yang *et al.*, 2004) and a prominent Crohn's-like infiltrate (Chireach *et al.*, 2005) in comparison to old black patients.

Young blacks may thus present with a form of colorectal cancer that is analogous to HNPCC due to the associated molecular features, while young whites present with the sporadic form thereof. The results have highlighted two limitations of the study. Firstly, there was a lack of family history and a low detection rate of serrated neoplasia. It is acknowledged that obtaining sufficient family history in a developing country such as South Africa is problematic and often bordering on impossible. However, knowledge of at least the immediate family members could prove to be beneficial in the diagnosis of a hereditary disease. An improved screening program within these families may lead to an earlier diagnosis and possibly improved

prognosis. The second factor relating to the low detection rate of serrated neoplasia can partially be justified by the time spectrum of this study, which included cases diagnosed as far back as 1990. Although Longacre and Fenoglio-Preiser first described these entities in 1990, it was only much later that a detailed morphological description together with increasing understanding of its importance in colorectal carcinogenesis was identified (Jass, 2001; Young *et al.*, 2001; Fogt *et al.*, 2002). It has, however, become clear that these entities are precursor lesions to the development of a percentage of "sporadic" colorectal cancer and great care must be taken to note their presence.

In this study it was shown that normal appearing tissue adjacent to tumour may already exhibit some of the molecular changes associated with tumour formation. This was particularly evident when examining the methylation status of the specified loci (See Chapter 4, Section 4.3.2.1) and corroborated with findings reported in literature: Shen *et al.* (2005) showed that 77% of normal appearing tissue as far removed as 10cm from the tumour tissue was positive for promoter methylation. Although methylation patterns are likely to be tissue specific, the presence of any germline (i.e. presence in all cells, hence inherited) mutations could be established through the use of blood samples collected at the time of biopsy or resection.

In conclusion, the following recommendations are made when diagnosing CRC in South African patients (Figure 5.4):

As is evident from the current study, it is likely that CRC in young black patients develops predominantly through the mutator pathway which could link to a hereditary CRC (HNPCC). According to the Amsterdam and Bethesda criteria, diagnosis of HNPCC relies largely on a family history and it is therefore recommended to obtain a detailed family history at the time of consultation. It is further suggested to collect a blood sample at this point for use in the molecular analysis of the tumour, as this would aid in identifying germline or inherited versus acquired alterations. Prior to surgery it is of importance to clearly document the patient's age, gender and ethnicity, together with tumour location (proximal or distal to the splenic flexure). In the case of a biopsy, it is recommended to biopsy a portion of normal-appearing tissue some distance away from the tumour (~5cm), in addition to the tumour biopsy. As indicated in this study, and reported in literature (Shen *et al.*, 2005), normal-appearing tissue might already exhibit some of the molecular changes associated with carcinogenesis. Furthermore, such normal-appearing tissue could be useful as an internal control for molecular changes observed in the tumour. Once the formal resected specimen is received and processed, it is emphasized that the anatomical pathologist meticulously reviews the sections for areas with a serrated architecture. As noted previously, these entities are of importance in the development of CRC and will direct further molecular analysis. Immunohistochemical analysis will be directed by patient demographics and tumour morphology. For black patients, with poorly differentiated tumours often expressing excess amounts of mucin, the primary panel of immunohistochemical markers should include the mismatch repair proteins hMLH1, hMSH2 and hMSH6. In addition, expression of MGMT protein will be useful to identify cases evolving towards tumour via promoter methylation. As the loss of

expression of the APC and p53 proteins was less prevalent in young black patients these markers need not be included in the primary analysis but should be used in any secondary analysis where normal MMR protein expression was found. For whites, the panel of markers should primarily include the APC and p53 protein, together with the MGMT protein for the establishment of promoter hypermethylation. Secondary markers for white patients should include the panel of MMR protein markers. Findings of these markers will then direct the further molecular analysis of the tumours. If loss of expression of any of the MMR proteins is found in young black patients, it is recommended to perform microsatellite instability analysis using the techniques established here. In cases showing low MSI, or that are microsatellite stable, methylation analysis of the *hMLH1* and *MGMT* gene promoter regions may direct the diagnosis from HNPCC to, alternatively, sporadic CRC through the serrated neoplasia pathway. Analysis of the *KRAS* and *BRAF* gene status may serve as confirmation.

For whites loss of expression of the APC and p53 proteins identified through immunohistochemistry should be followed by analysis of the overall methylation status (CpG island methylator phenotype) and specifically the *hMLH1* and *MGMT* promoter status, as well as microsatellite instability. Again, examining the *KRAS* and *BRAF* gene status may serve as confirmatory analyses in the diagnosis of sporadic CRC, as proposed by the result of this study.

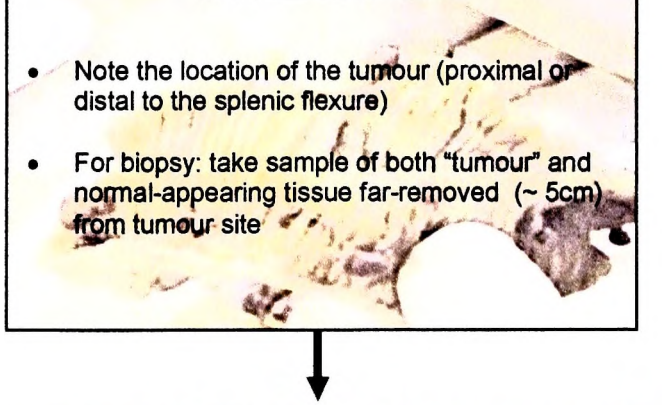
PRE-SURGERY:

- Obtain a comprehensive family history (follow Bethesda guidelines) including age, gender and ethnicity
- Take and store a blood sample

Patients <50yrs of age:

DURING SURGERY:

- Note the location of the tumour (proximal or distal to the splenic flexure)
- For biopsy: take sample of both "tumour" and normal-appearing tissue far-removed (~ 5cm) from tumour site

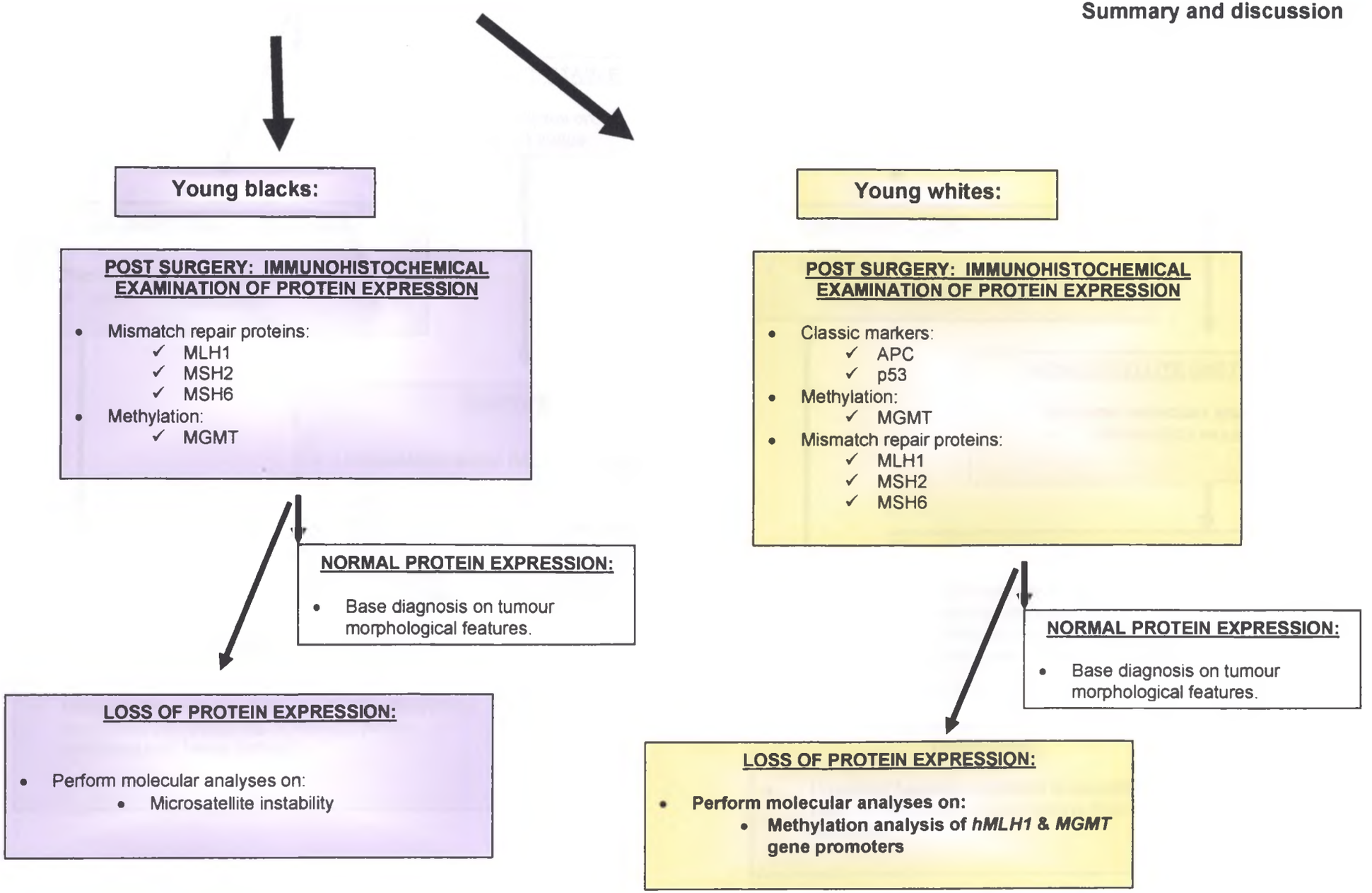


POST SURGERY: HISTOLOGICAL EXAMINATION

- Note specifically tumour differentiation and percentage mucin expression
- Take special care to identify serrated neoplasia

Patients >50yrs of age:

Follow standard procedures involved in the diagnosis of colorectal cancer, including the necessary histological and immunohistochemical analyses.


Young blacks:
POST SURGERY: IMMUNOHISTOCHEMICAL EXAMINATION OF PROTEIN EXPRESSION
Mismatch repair proteins:
✓ MLH1
✓ MSH2
✓ MSH6
Methylation:
✓ MGMT
NORMAL PROTEIN EXPRESSION:
Base diagnosis on tumour morphological features.
LOSS OF PROTEIN EXPRESSION:
Perform molecular analyses on:
Microsatellite instability
Young whites:
POST SURGERY: IMMUNOHISTOCHEMICAL EXAMINATION OF PROTEIN EXPRESSION
Classic markers:
✓ APC
✓ p53
Methylation:
✓ MGMT
Mismatch repair proteins:
✓ MLH1
✓ MSH2
✓ MSH6
NORMAL PROTEIN EXPRESSION:
Base diagnosis on tumour morphological features.
LOSS OF PROTEIN EXPRESSION:
Perform molecular analyses on:
Methylation analysis of hMLH1 & MGMT gene promoters

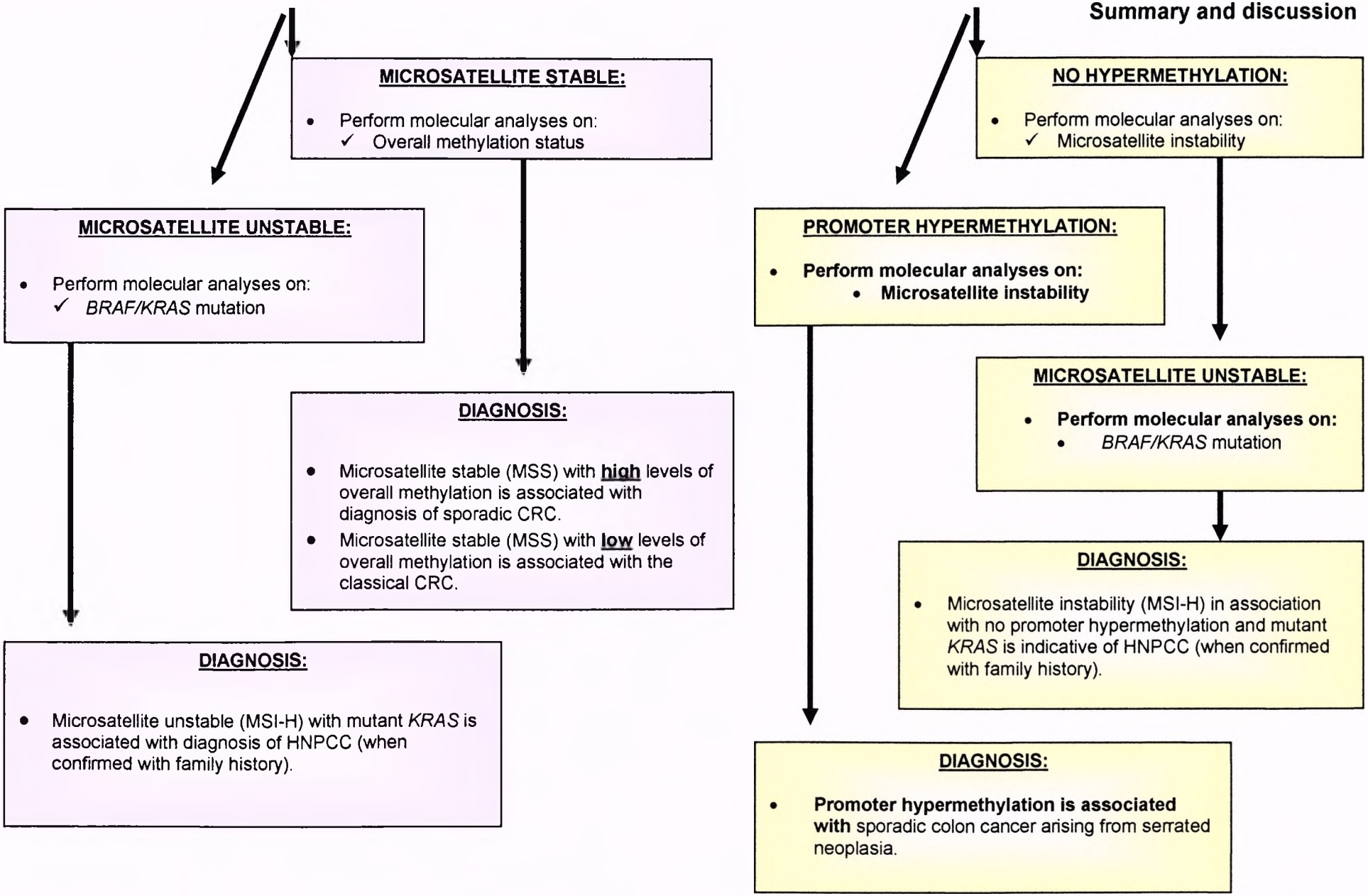

MICROSATELLITE STABLE:
• Perform molecular analyses on:
✓ Overall methylation status
MICROSATELLITE UNSTABLE:
• Perform molecular analyses on:
✓ BRAF/KRAS mutation
DIAGNOSIS:
• Microsatellite stable (MSS) with high levels of overall methylation is associated with diagnosis of sporadic CRC.
• Microsatellite stable (MSS) with low levels of overall methylation is associated with the classical CRC.
DIAGNOSIS:
• Microsatellite unstable (MSI-H) with mutant KRAS is associated with diagnosis of HNPCC (when confirmed with family history).
NO HYPERMETHYLATION:
• Perform molecular analyses on:
✓ Microsatellite instability
PROMOTER HYPERMETHYLATION:
• Perform molecular analyses on:
• Microsatellite instability
MICROSATELLITE UNSTABLE:
• Perform molecular analyses on:
• BRAF/KRAS mutation
DIAGNOSIS:
• Microsatellite instability (MSI-H) in association with no promoter hypermethylation and mutant KRAS is indicative of HNPCC (when confirmed with family history).
DIAGNOSIS:
• Promoter hypermethylation is associated with sporadic colon cancer arising from serrated neoplasia.

Figure 5.4: Recommended flow of information and laboratory investigation in the diagnosis of colorectal cancer in young South African patients.

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