Predictors of Response to Adjuvant Chemotherapy for Colorectal Cancer

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By

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Dedication

I dedicate this work to my late father,

Ormond James Thomas

who would have been very proud

and to my mother,

Robyn Arlene Thomas

who is such an inspiration

Abstract

Background:

It is well recognized that not all patients with stage C colorectal cancer (CRC) derive a survival benefit from adjuvant chemotherapy. It would therefore be advantageous to identify factors that define a target group for treatment. It has been suggested that those most likely to benefit are women with proximal tumours. Recent work has suggested microsatellite instability (MSI) may be a useful marker however the limited studies performed are conflicting.

Aim:

To determine if gender, site, tumour histology or microsatellite (MSI) status predict survival benefit from 5FU-based adjuvant chemotherapy in stage C CRC.

Method:

Data was collated on stage C colorectal cancer cases that underwent curative resection over a 20-year period (inclusive of years prior to standard chemotherapy). Pathology was re-evaluated, DNA extracted from the formalin fixed paraffin specimen and MSI status established. Primary endpoint was cancer-related death. Kaplan-Meier curves were constructed for univariate analysis and differences analysed by log rank test. Multivariate analysis was performed using Cox proportional hazard model adjusting for age, gender, site, distinct pathological variables and MSI. A compounding effect between these factors and chemotherapy benefit was measured by interaction testing

Results:

811 unselected cases were included in the study. Thirty-seven percent received chemotherapy. Chemotherapy significant improved cancer-specific survival (HR of dying 0.66 (95% CI 0.52-0.83 p=0.0003). Female gender offered a survival advantage overall (HR 0.81 95% CI 0.68-0.97; p=0.02) however site did not influence outcome (HR 1.03). On interaction testing, gender, site and tumour histology did not significantly influence the survival effect of chemotherapy.

802 cases were included in the MSI analysis of which 77 exhibited MSI. MSI status did not influence prognosis (HR of cancer death 1.45, 95% CI 0.90-2.21; p= 0.13). However, in the non-chemotherapy cohort, MSI conferred a significantly less favourable outcome (HR 1.89, 95%CI 1.13-3.16; p= 0.02). Chemotherapy produced a survival benefit in both the MSI (HR 0.08 95% CI 0.02-0.27; p=<0.0001) and the microsatellite stable (MSS) cohort (HR 0.62, 95% CI 0.47-0.81; p=0.001). On interaction testing, neither compounded the benefit of chemotherapy, however of all the tested parameters, MSI came closest to significance (p=0.08).

Conclusion:

These results suggest that 5FU-based adjuvant chemotherapy for stage C colorectal cannot be targeted using gender, tumour site, histological characteristics or MSI.

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List of Abbreviations

5FU	5-fluorouracil
ACPS	Australian clinicopathological stage
AJCC	American Joint Committee on Cancer
APC	Abnormal in polyposis coli
Ams	Amsterdam criteria
C	Colon
C/R	Colon/rectum
CA	Cancer specific survival
CIMP	CpG island methylator phenotype
CpG	Cytosine phosphate bonded to guanine
CRC	Colorectal cancer
DCC	Deleted in colorectal cancer
DNA	Deoxynucleic acid
FAP	Familial polyposis coli
FFPE	Formalin fixed paraffin embedded
GIT	Gastrointestinal
GITSG	Gastrointestinal Study Group
HNPCC	Hereditary non-polyposis colorectal cancer
HR	Hazard ratio
ICG	International Collaboration Group
IDL	Insertion/deletion loop
IDS	International documentation system
IGF	Insulin like growth factor
JPS	Juvenile polyposis coli
LN	Lymph node
LOH	Loss of heterozygosity
MGMT	O6-methylguanine methyltransferase
MMR	Mismatch repair
MNNG	N-methyl-N-nitro-N-nitrosoguanidine
MSI	Microsatellite instability
MSI-H	Microsatellite instability high
MSI-L	Microsatellite instability low

MSS	Microsatellite stable
MV	Multivariate
n	Number
N/S	Not stated or specified
NA	Not assessable
NCI	National Cancer Institute
NS	Not significant
O/C	Outcome
OR	Odds ratio
OS	Overall survival
PJS	Pertz Jegher Syndrome
R	Rectal
RR	Relative risk
Ref	Reference
RER	Replication error
SIG	Significant difference detected
TGF	Transforming growth factor
TILs	Tumour infiltrating lymphocytes
TME	Total mesorectal excision
TS	Thymidate synthetase
UICC	International Union Against Cancer
uk	Unknown
UV	Univariate

Declaration

I declare that this work contains no material that has been accepted for award of any other degree or diploma in any university or other tertiary institution to Michelle Thomas and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Is Microsatellite Instability a Useful Molecular Marker to Target Chemotherapy in Colorectal Cancer?

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Is Microsatellite Instability a Useful Molecular Marker to Target Chemotherapy in Colorectal Cancer?

Annual Scientific Congress, Royal Australasian College of Surgeons 2005

Is Microsatellite Instability a Useful Molecular Marker to Target Chemotherapy in Colorectal Cancer?

Awarded the Mark Killingback prize

Annual Scientific Congress, Royal Australasian College of Surgeons 2005

Pathological Predictors of Outcome in Colorectal Cancer

Annual Scientific Congress 2004

The Influence of Gender and Site on the Response to Adjuvant Chemotherapy in Colorectal Cancer

1 INTRODUCTION

1.1 Background

Colorectal cancer remains a significant burden on society‟s health with around half a million cases diagnosed worldwide each year [1]. Despite best treatment, only 50% of patients will survive long term [2, 3]. Surgical resection remains the therapeutic mainstay and the best chance of achieving cure. Current regimens of adjuvant chemotherapy offer an additional small improvement in outcome and the greatest potential for further improvement in colorectal cancer survival is likely to emanate from improvements in adjuvant treatment and better targeting of these therapies.

The exact target group to benefit from chemotherapy following curative resection for colorectal cancer remains unclear. Currently, 5-fluorouracil (5FU) based regimens are offered to patients on the basis of tumour stage. This approach was implemented following studies that showed a small but significant improvement in survival for stage C (lymph node positive) disease but no significant benefit for less advanced disease [4-11]. Stage, however, is a crude measure of potential benefit given that patients with stage C disease represent a heterogeneous group, both clinically and biologically. It is becoming apparent that a blanket approach to treatment may not be appropriate. Given the modest survival advantage afforded by adjuvant chemotherapy, it is indeed likely that only a subgroup of the stage C group is benefiting. However, factors to identify this group and narrow the target field have thus far remained elusive.

Identification of a responsive subgroup involves challenging traditional notions that the colon is a single entity and that colorectal cancer is a uniform disease. There are both clinical and tumour factors that theoretically vary the response to chemotherapy.

Clinical factors that have been shown to influence the outcome from CRC include patient age and gender [3, 12-20]. Recent work suggests female gender confers a survival benefit and possibly a better response to chemotherapy [21]. The anatomical site of the tumour within the colon is important to outcome, there being evidence of behavioural disparity between right and left sided colon cancers, in terms of epidemiology and prognosis, as well as limited work suggesting a differing response to chemotherapy [21-28]. Many intrinsic and luminal differences exist across the colon that may explain these observations [22, 24, 29] however, the evidence increasingly suggests that it is tumour biology that varies.

Tumour biology undoubtedly influences prognosis. Colorectal tumours are histologically diverse. Many parameters have recognised prognostic significance and thus may be useful in predicting chemotherapy response. Attempts to define a target group for treatment based on histological indicators, however, have not been made. It is often assumed that tumours with unfavourable histology have the most to gain from chemotherapy but this has not been formally tested.

With our improving understanding of molecular biology, much recent emphasis in cancer therapy has been on pharmacogenomics (the use of genetic markers to target treatment). Recent encouraging advances suggest this may be the way forward in CRC. In the last decade the traditional notion of a stepwise adenoma-carcinoma sequence of genetic mutations (chromosomal instability) as the only mechanism of CRC development was challenged and an alternative pathway has been established [30, 31]. Approximately 15% of colorectal cancers exhibit microsatellite instability (MSI). This is a result of defective mismatch repair (MMR) either due to mutation of MMR genes (germline [HNPCC] or somatic) [32-34] or transcriptional silencing due

to epigenetic inactivation of the MLH1 gene by methylation of the promoter region [35]. Defective repair leads to genomic instability (including in microsatellite sequences) and inevitably, cancer [32, 36].

This difference in tumour biology could be predicted to lead to variation in tumour behaviour. Compared to traditional colorectal cancers, MSI tumours show a female preponderance [37, 38], a predilection for the proximal colon [21, 37, 39-43], different pathological characteristics [37, 38, 42, 44-50] and improved outcome [39, 46, 48, 51-53].

What remains unclear is whether MSI status influences adjuvant chemotherapy response and as such whether it is a marker that will be useful in targeting therapy. Initial in vitro work predicted resistance in MSI tumours [54-56]. Cell lines with defective mismatch repair demonstrated tolerance to most chemotherapeutic agents, including 5-Fluorouracil. Whether these findings translate to the clinical setting remains unclear; the opposite may in fact be the case. Early studies suggested a trend to improved survival for cases of MSI positive tumours given chemotherapy [21, 43, 51, 57] but further work has produced conflicting results [48, 58-60].

Therefore the question of who best benefits from adjuvant chemotherapy is yet to be resolved. Since tumour biology is the greatest predictor of tumour behaviour, it is likely that molecular biology will hold the answer. However, until a molecular marker can be identified, it is useful to identify all predictive parameters.

There are clear treatment implications from this research. An improved ability to target 5FU chemotherapy to those who will benefit will save a significant number of

patients from undergoing ineffective, deleterious treatment. Emphasis can be placed on identifying alternative effective treatments for this group, therefore not only improving the outcome in these cases but also potentially for colorectal cancer overall.

1.2 Aims

1.2.1 General Aim

The aim of this study is to identify which clinicopathological features of colorectal cancer predict response to adjuvant chemotherapy by undertaking a study of stage C colorectal cancer cases treated by curative resection. It involves an initial examination of the prognostic significance of selected factors, then comparing survival between those who received adjuvant chemotherapy and those that did not. Consequently, which of the studied parameters exerted an influence will be determined.

1.2.2 Specific Aims

1.2.2.1 Prognostic Influences

The first logical step in the identification of factors that may influence chemotherapy effect is to recognise the variables that affect prognosis in CRC. It is likely that useful predictors will be sourced from within this pool. The first aim of this study is therefore to identify the prognostic influence of selected factors on the outcome following curative resection for stage C colorectal cancer. The range of factors selected will be as comprehensive as possible within the constraints of this study.

Aim 1 - To determine the prognostic influence of gender on survival following curative resection for stage C CRC.

Null hypothesis: That survival following resection of stage C CRC will not be influenced by gender.

Aim 2 - To determine the prognostic influence of tumour site on outcome in this group.

Null hypothesis: That tumour location within colon will not influence prognosis.

Aim 3 - To comprehensively re-examine the known histological variants in CRC and determine which have prognostic significance, in particular whether recently described parameters will be prognostically useful.

Aim 4 - To determine the prognostic influence of MSI on survival from stage C CRC

Null hypothesis: MSI status will not influence survival in this group.

1.2.3 Chemotherapy Response

Whether the above selected parameters influence the magnitude of the effect adjuvant chemotherapy has on survival following curative resection for stage C CRC will then be tested.

Aim 1 - To determine whether gender influences the survival benefit conferred by adjuvant chemotherapy following curative resection for stage C CRC.

Null hypothesis: Benefit from chemotherapy will not be influenced by gender.

Aim 2 - To determine whether tumour site influences response to chemotherapy in the above group.

Null hypothesis: Proximal tumours will gain the same survival benefit from adjuvant chemotherapy as distal tumours.

Aim 3 - To determine if histological variables (or combinations) can identify a responsive target group for adjuvant chemotherapy.

Aim 4 - To determine whether MSI tumour status influences survival benefit from chemotherapy in this same group.

Null hypothesis: The survival gain from adjuvant chemotherapy for CRC will be independent of MSI status.

1.2.4 Secondary Aim

1.2.4.1 MSI associations

Given that MSI is a relatively recent discovery, there is much to be contributed to the emerging story. The breadth of this study allows further elucidation of the tumour features associated with microsatellite unstable tumours, and thus the potential to contribute to further understanding of their unique tumour biology.

Aim - To investigate the clinical features and tumour histology associated with MSI colorectal cancers

1.3 Thesis plan

In chapter 2 the related literature will be reviewed.

Chapter 3 will detail the methodology used in this thesis. At the commencement of each results chapter, a brief summary of the methods relevant to that chapter will be reiterated.

Chapter 4 will provide overall results of the study as a foundation for the more specific chapters. Study group and cohort characteristics will be detailed, cohorts compared and overall results presented. At the commencement of the subsequent chapters, relevant results will be briefly reiterated.

Chapter 5 will examine the clinical and pathological factors associated with MSI cancers. The comparison of the MSI and MSS cohorts will be detailed further in this chapter.

In chapters 6 and 7 the main issues of this thesis are addressed; prognostic influences and influences on adjuvant chemotherapy effect. The discussion relating to each of the main broad questions will be included in the relevant chapter.

Concluding comments will be made in chapter 8.

2 LITERATURE REVIEW

2.1 Overview

Initially, relevant aspects of CRC epidemiology and pathology will be reviewed to place the disease in context and provide a point of reference with which to compare subgroups, specifically MSI cancers. Familial CRC will be discussed including a more detailed clinical overview of hereditary non-polyposis colorectal cancer (HNPCC) given that this condition is inherently part of the MSI story.

As this is an outcome-focused study, the literature relevant to clinical and histological predictors of prognosis will be explored. All factors with potential prognostic significance in CRC will be examined. The main clinical emphasis of this study is on site and gender and these two areas will be discussed in more detail. Much prognostic weight is placed on histological aspects of CRC and consequently these will be comprehensively reviewed.

Microsatellite instability will be explained including historical aspects and genetic basis. How MSI cancers vary from microsatellite stable tumours will be explored, including gender and site variations. Evidence for an influence on survival will be presented.

Finally, adjuvant chemotherapy will be considered including justification for adjuvant treatment and the influence of clinical and histological factors on response. The interaction between the MMR system and chemotherapy agents will be examined, concluding with the evidence thus far on the interaction between MSI and 5FU-based chemotherapy.

2.2 Colorectal Cancer General

2.2.1 Epidemiology

The latest global estimates of colorectal cancer incidence indicate that every year close to half a million cases of CRC are diagnosed worldwide [1] and that the 5-year prevalence is over 1 million [1, 61]. In South Australia, the estimated incidence is 40/100,000 (personal correspondence, Dr Rodda, State Cancer Registry).

Gender distribution is near equal although worldwide figures suggest a slight male predominance [1]. This has been the finding of some population studies but not all. Ratto et al."s population study from Italy of 8690 CRCs following curative resection found 40% were in women [3]. DeCosse reviewed 134 registries of colorectal cancer and found a higher male incidence in 126 [62]. The registries in populations with a low incidence of CRC had a more equal gender split. However other large population studies disagree and show equal distribution. The UK Bowel Cancer project studied 4292 cases from 23 hospitals and included all patients diagnosed with CRC over 4 years and found 50% were in men [26].

A male predominance has been shown for rectal cancers by some groups [26, 63, 64] but again not all [19]. In the older populations the proportion of men increases and the proportion of proximal cancers cases that are female increases [62].

Mean age at presentation is the seventh to eighth decade (mean 68.47 yrs) [26, 63] and gender distribution varies according to age, with higher female representation in the older age group [16].

2.2.2 Aetiology

The aetiology of CRC is multifactorial and is yet to be fully elucidated. Both genetic and luminal factors are likely to have a role in carcinogenesis. An improved understanding of the involved molecular events in the development of CRC is aiding investigation and has helped to define the genetic events in inherited forms of CRC.

Epidemiological studies have implicated various dietary and lifestyle factors in the development of CRC although no clear aetiological links have been established [65-67]. Low vegetable intake is associated with CRC and there is some evidence that cruciferous vegetables are protective [65-67]. There is a widely held belief that low fibre may be contributory and some epidemiological evidence to support this. However, prospective work has not shown an association [66, 67]. Studies have suggested that high fat intake, high energy intake and red meat are associated with CRC formation but the evidence is tenuous and inconsistent [65, 67, 68].

Certain underlying gastrointestinal conditions predispose to CRC, in particular Crohn‟s disease and ulcerative colitis [66].

2.2.3 Pathology

Most colorectal cancers arise in the left colon (70-80%) [3, 26, 63]. Of 1117 consecutive cases of CRC studied by an Australian group, 40% were in the rectum, 34% in the left colon and 26% on the right [63]. A population study from Italy of 690 cases, found that only 20% were right sided while left and rectal both accounted for 40% [3]. The bowel cancer project from the UK of 4292 CRC cases found 47% were rectal while the remainder were split equally between left and right [26].

Stage B cases represents the largest group at diagnosis (40-50%), followed by stage C (30%) and around 10% will have distal metastases at presentation [26, 63]. Over ninety percent are adenocarcinoma [2] with a small percentage being carcinoid or stromal.

2.2.4 Survival

Around half the people diagnosed will die from their disease [2, 3]. Interestingly, survival has not greatly improved from 40 years ago [69, 70]. With global annual incidence estimate at around half a million, the annual mortality is just over 250, 000 [61]. In most countries, CRC is the second most common cause of cancer death [2, 66].

2.3 Familial CRC

It is clear that there is an increased propensity to develop colorectal cancer in some families. While in most cases a definite inheritance cannot be established, there are several well-defined syndromes and these are summarised below.

2.3.1 Familial Adenomatous Polyposis (FAP)

Familial adenomatous polyposis (FAP) is due to an autosomal dominant inherited mutation of the APC gene [66]. The syndrome is characterised by hundreds of colonic polyps with the inevitable development of CRC in the second or third decade of life [66]. Extracolonic manifestations include retinal lesions, osteomas, desmoid tumours and brain tumours [71]. FAP families are heterogeneous with the clinical syndrome being dependent on the specific genotype (mutation type or codon location).

Phenotype variation includes numbers of polyps, malignant potential and tendency to develop extra colonic disease.

2.3.2 Other Polyposis Syndromes

The hamartomatous polyp syndromes including Peutz Jeghers‟ syndrome (PJS), juvenile polyposis syndrome (JPS) and Cowden‟s syndrome, have a low but definite risk of carcinoma [66]. Recently the germline defects have been elucidated for PJS and JPS [72].

2.3.3 HNPCC

The most prevalent familial CRC syndrome is HNPCC – hereditary non-polyposis colon cancer ("non polyposis" to distinguish this syndrome from the above polyposis syndromes). The concept of familial clustering of intestinal and endometrial cancer was first recognised by Warthin in his observation of Family G published in 1913 [73]. Lynch furthered this work in the 1950‟s, publishing the concept of a "cancer family syndrome" [74]

2.3.4 Clinical Definition

An exact definition of HNPCC remains elusive and relies on a combination of genealogy, clinical criteria and molecular markers.

Prior to our current understanding of the genetics, a diagnosis of HNPCC was made on clinical observations and family history. The original "cancer family syndrome" was diagnosed on the basis of a combination of suggestive factors within the studied families: frequent occurrence of CRC and endometrial cancer; CRC development at a

young age; proximal site; high rate of synchronous and metachronous lesions; and tendency to extracolonic cancers [74]. Lynch et al. demonstrated that despite the relative frequency of CRC and endometrial cancer in society, the chance of multiple proximal CRCs and endometrial cancer at a young age is rare [75]. The calculated probability of developing two proximal CRCs under the age of 40 within a family was two in a million; three CRC"s was one in a billion; and the probability of a woman developing CRC and endometrial cancer before 40 was three in 10 million. Thus, when these scenarios occur repeatedly in families, a genetic propensity is likely [75].

Other cancers were subsequently included in Lynch et al."s definition. Gastric, small bowel, ovarian, biliary tract, renal pelvis and ureteric cancers were found to have a higher than expected rate in these HNPCC families and thus were included in the description [75]. Subsequent work has added pancreatic, skin (sebaceous tumours) and, debatably, brain tumours to this group. Lynch et al. divided the syndrome into Lynch I (patients with predominantly colonic disease) and Lynch 2 (those with extracolonic tumours) [75]. This nomenclature fell from favour for a period but is again in use.

The criteria for HNPCC was formalised at a meeting of the International Collaborative Group (ICG) on HNPCC in 1990 in Amsterdam (Table 1) [76]. These criteria was criticised due to the exclusion of extracolonic cancers and was reassessed at the 9th meeting of the ICG HNPCC in 1999 [77]. The literature was reviewed and the greatest relative risk increase was found for cancers of the endometrium, ureter, renal pelvis and small bowel. Consequently, these were identified as "HNPCC associated cancers" and included in the modified criteria. Stomach cancer was excluded due to its frequency in Asian countries and thus the risk of over-diagnosing

HNPCC. It was deemed no longer essential to have one CRC case given that there is documentation of families with a HNPCC genetic mutation that exhibit extracolonic cancers but no CRC.

While the condition was recognised to have a clear autosomal dominant pattern of inheritance with high penetrance, the exact genetic defect eluded researchers until the last decade. As the genetics of HNPCC is an integral part of this study, these recent advances, the genetics and pathology will be covered in detail later in this section.

2.3.4.1 CRC Risk in HNPCC

The clinical syndrome of HNPCC is signified by development of tumours early in life with 70% of cases developing CRC by age 65 with an average age 44 years [75].

The lifetime risk of developing at least one colorectal cancer in HNPCC cases is 80-100% and most accurately assessed in studies using molecular markers for diagnosis. Overall evidence for this is summarised in Table 2.

2.3.4.2 Clinicopathological Characteristics

Much of the work on clinical and pathological characteristics of HNPCC cancers was carried out prior to the recognition of genetic markers and, as such, inclusion criteria for studies vary. Nevertheless, most studies consistently find that HNPCC cancers occur at a median age of 44-46 years [75, 78], with around two thirds occurring in the proximal colon [78, 79] and 15% in the rectum [77]. Rates of poor differentiation, mucinous component and signet ring cells are higher than normal [24]. Presentation is usually at an earlier disease stage [80] (though around 40% will be stage B and 30%

stage C or D [81]) and stage-adjusted prognosis is better than for CRC in general (HR 0.67 [80]).

2.3.4.3 Metachronous CRC Rate

The rate of metachronous CRC in HNPCC varies between 1.7 and 3.6% per year (compared with 0.33% sporadic) [78, 79, 81]. The most quoted rate is 40% per 10 years, which originates from work by Lynch et al."s group (using clinical criteria for HNPCC) [79]. Most studies found that following the first metachronous tumour, the relative risk of subsequent tumours increased even further [78, 82]. One study found that the rate of metachronous lesion was higher in patients who initially had synchronous lesions [82]. There is a proximal tendency to metachronous lesions but less than primaries [78]. An ICG collaboration found rectal metachronous lesions in 11% of 71 post-colectomy HNPCC cases but at a median of 158 months and a median age of 51 years, giving an annual rate of 1% [83].

2.3.4.4 Extracolonic Cancers

Rates of extracolonic cancers in HNPCC are summarised in Table 3. The risk of endometrial cancer is highest (40-60% risk by age 70 years) and may be more common than CRC in female HNPCC cases [84]. Around 50% of HNPCC female carriers will have endometrial or ovarian cancers as their index tumour [85]. Median age of diagnosis is 46 years (compared to normal 60 years) [86]. Ovarian cancer risk is higher than normal (cumulative risk by 70 of 12%) and also presents at an earlier age, mean 42.7 years [87].

Most studies show an increased relative risk of renal pelvic tumours, ureteric TCC, gastric cancer, small bowel and CNS tumours but in absolute terms the risk is still low. While MSI has been recognised in pancreatic cancer, an increased incidence in HNPCC is not consistently observed. Sebaceous gland tumours develop with CRC in a subgroup of HNPCC called Muir-Torre syndrome. Interestingly, cases of lung or bronchial cancer are less common in HNPCC without observed risk aversion.

Table 1 Amsterdam criteria

At least 3 relatives with CRC
One should be first degree relative to the other two
At least two successive generations affected
At least one CRC should be diagnosed before age 50
Tumours should be verified by pathological examination
FAP excluded

Table 2 Lifetime risk of CRC in HNPCC

	Vasen et al. [88]	Aarnio et al. [89]	Dunlop et al. [84]
Overall	80%	82%	
Men	92%	100%	74%
Women	83%	54%	30%

[88] 210 mutation carriers, risk by 70 years
[89] Cumulative risk by 70 years in 360 mutation carriers.
[84] 67 "mutation" carrier (some putative based on inheritance).

Table 3 Extracolonic cancer rate

	Watson and Lynch [86]	Aarnio et al. [89]	Aarnio et al. [89]	Vasen et al. [88]	Vasen et al. [88]	Dunlop et al. [84]
	RR	SIR*	%	MLH1	MSH2	
Endometrial		62	60% by 70 (vs 1.3%)	42% lifetime risk	61%	42% lifetime
Ovarian	3.5	13	12%	6.4 RR	8 RR	
Renal	17 (pelvis)	4.7				
Ureter	22	7.6		0	75.3	
Stomach	4.1	6.9	13%	4.4	19.3	
Small bowel	25	ns		292	103	
Hepatobiliary	4.9	9.1				
Brain	ns	4.5				
Pancreas	ns	ns				
Skin	ns	ns				
Brest	ns	ns				
Prostate		ns				
Lung/Bronchus	(0.4 less)	ns				

* standardized incidence ratios (observed to expected)
[86] 1300 high risk members (≥3HNPCC tumours, ≥2 CRC <50), matched to age, gender and period specific rates
[89] 1763 patients, 360 mutation carriers, age, gender and era specific.
[88] Study of phenotypic differences between MSH2 or hMLH1in 210 mutations carriers
[84] 67 "mutation" carrier (some putative based on inheritance)

2.4 Prognostic Indicators in CRC

2.4.1 Overview

In any investigation of outcome, it is important to include all prognostic influences as confounding factors in analyses. This is one of the limitations of many outcome studies. Accuracy of results will depend on whether confounding factors were considered in analysis and how comprehensively. For this reason studies that perform multivariate analysis will be more useful than those that determine outcome by univariate analysis alone.

Other considerations when reviewing prognostic studies include consideration of the study cohort. Any selection bias, such as the age restriction that may occur in cases sourced from trials, may influence outcome. The survival endpoint used is important. Studies that consider overall survival need to account for factors that influence general survival, such as age and gender. Cancer-specific survival is more indicative of what influences tumour behaviour (although it may not necessarily be the most important survival determinant). Using 5-year survival may provide erroneous results. This figure is a point determination; what the survival curve does on either side may be more informative and change the impression given at 5 years.

This review will endeavour to comprehensively include all factors that influence outcome in CRC.

2.4.2 Stage

Stage remains the greatest predictor of outcome in CRC [12, 90]. Various staging systems have been proposed over time and several different systems are still in use. These will be discussed in more detail, including the system used for this study.

The aim of a staging system in CRC is to define a cohort of patients with similar prognosis, thus providing the clinician with tools for assessing an individual‟s prognosis, information for discussion of outcome, a basis for management decisions and to allow comparison between studies. Progression of disease is the basis of CRC staging systems and has been shown to correspond well with prognosis [91, 92]. The more advanced the local spread, the greater the chance of distal spread, occult or otherwise, and therefore less chance of surgery being curative.

Three aspects of progression are inherent in most CRC staging systems – degree of involvement of bowel wall, involvement of lymph nodes and distant metastases. Each staging category should have a progressively worse prognosis. Various systems have been created and refined but all have limitations and not all are comparable. The simpler systems comprise a heterogeneous mix within each staging category while the more refined systems are complex and often misused. Not all factors that influence prognosis can be included and there are variations in how classifications deal with local invasion and residual disease.

Lockhart-Mummery is credited with the first staging classification, published in 1926, which was specific for rectal cancers and based on operative findings [93]. In his personal series of 200 rectal cases, three stages were recognised to correspond with

outcome (Table 4). Dukes and Bussey from St Mark‟s Hospital furthered this staging in 1932, again based on degree of bowel wall invasion and lymph node involvement (Table 5), which was validated by the authors‟ analysis of 2447 rectal cancer cases [94]. The classification was based on pathological review of resected specimens and as such did not incorporate clinical information. The exact description of the bowel wall involvement is often misconstrued, adding some confusion to the classification [92, 95, 96]. In 1939, Simpson and Mayo broadened the classification to include colon cancer and in 1935 Gabriel, with Dukes and Bussey, divided the lymph node involvement into C1 and C2 depending on the level of involvement [91, 95].

Astler and Coller advocated further breakdown of the Dukes system in 1954, placing importance on the degree of bowel wall involvement combined with lymph node involvement. They argued that depth of invasion through the bowel wall was predictive of outcome even with lymph node involvement (Table 6) [97].

Predicting survival based on Dukes classification may be inaccurate. These systems are based on pathological findings without consideration of whether the surgery was curative or if distant metastases are present [92]. As such, a patient with a pathological report of Dukes A may in fact have liver metastases and obviously a worse outcome than what the clinician expects of Dukes stage A disease. This issue was initially addressed in 1967 when Turnbull et al. from the Cleveland Clinic published their paper on the no-touch technique [70]. They introduced the concept of a “Dukes” D category to signify distal spread and local invasion. This was further developed in 1983 when the Australian Clinicopathological Staging (ACPS) system was proposed (Table 7) to incorporate clinical staging with pathological findings (based on the Concord Hospital Clinicopathological Staging System) [92, 98].

Consideration was given as to whether surgery was curative or palliative (involved margins, residual disease or distant disease). The system was validated on 709 consecutive patients with colonic or rectal cancers from the Concord Hospital. Crude survival data is shown in Table 8. It is interesting that the degree of bowel wall involvement is not as prognostically significant as previously suggested and not surprising that lymph node involvement significantly worsens outcome.

The classification was further subdivided in 1987 (Table 9) and validated in 1117 cases of CRC (included the previous 709 cases) [63]. Crude survival was similar to the previous study for the early stages but better for the stage D cases (no explanation was given for this difference between the two studies) (Table 8). Detailing apical node involvement was useful prognostically, with survival following a positive apical node not dissimilar to that seen in the stage D cases.

In the above study, further subdivision of bowel wall involvement did not add a great deal to prognostic information except if the free mesothelial surface was invaded by tumour, in which case prognosis significantly worsened. The importance of involvement of the serosal surface by tumour as an independent adverse outcome predictor for CRC is supported by the AJCC [99] and has been shown to be applicable to rectal cancers [100].

The TNM system, proposed in 1966 and used by the American Joint Committee on Cancer (AJCC) and International Union Against Cancer (UICC), is based on the same principles of the above systems (Table 10) [95]. While more precise than other systems, it is cumbersome and therefore not applied universally. The Concord group compared the ACPS and TNM systems in 1011 colorectal cases [101]. Both systems

showed progressive worsening of outcome as stage increased, except for TNM stage 3, where outcome was similar to stage 2. TNM did not offer additional prognostic information over ACPS and had several limitations in particular in stage 1 disease. TNM stage 1 includes both T1 and T2 and would therefore appear to inadequately tier early stage disease (given the differing survival between T1 and 2 cases). Also, several cases classified as early stage on TNM had residual disease and were therefore incurable.

Subsequent studies examining the various staging systems have reconfirmed that increasing stage correlates with worsening outcome [90]. Both the Dukes system [19, 39, 102-104] and TNM system [64, 105, 106] have been revalidated. It is apparent, however, that two points remain contentious. Debate continues as to the importance of degree of bowel wall involvement once lymph nodes are involved. The Dukes, ACPS and TNM systems do not differentiate bowel wall layers in lymph node positive disease while the Astler-Coller system does. Advocates of this latter system believe that the sub-classification of node positive disease according to the degree of bowel wall involvement offers additional prognostic information [97], including in rectal disease [64] and in particular for T4 cases [63, 107].

The other controversy is the importance of quantifying lymph node involvement. While all agree that tumour spread to lymph node is significant, there remains debate as to the whether the number involved offers additional prognostic information as proposed by some [63, 97]. It should be noted that the finding or degree of lymph node involvement might be influenced by lymph node yield (with low yield potentially under-staging disease [108]). For this reason, it was recommended that at

least 12 nodes be examined [91]. It should be further noted that this has not always been the case in earlier studies.

Despite the importance of staging in predicting outcome, each stage category represents a heterogeneous population with varied outcomes, hence the importance in defining additional prognostic markers. This particularly applies to stage B and stage C disease. Stage A cases have such favourable prognosis it is unlikely that additional factors will have a role and in stage D cases little is going to influence the poor course. All staging systems were reviewed by a working party at the World Congress of Gastroenterology in Sydney, 1990 [91]. An International Documentation System (IDS) was proposed to unify data collection and broaden collated data to incorporate stage information as well as other prognostic influences. Those prognostic factors deemed to be significant upon review of the literature were included (Table 11). The following sections will examine these potential prognostic influences.

Table 4 Lockhart-Mummery stages

[93]

A	Very favourable cases not invading muscle coat
B	Medium cases invading muscle but not beyond
C	Very bad cases fixed or glandular invasion

Table 5 Dukes Classification

[94]

A	Growth limited to the rectum
B	Growth spread by direct continuity into extra rectal tissues
C	Lymph node metastases

Table 6 Aster Coller classification

[97]

A		Carcinoma in situ
B	1	Involving submucosa and muscularis propria
	2	Though muscularis propria
C	1	B1 plus regional nodal involvement
	2	B2 plus regional nodal involvement

Table 7 ACPS Stage

[98]

NOTE:
This table is included on page 26
of the print copy of the thesis held in
the University of Adelaide Library.

Table 8 ACPS crude survival by stage compared to Dukes

Stage	System	Median Survival (mths)	5yr surv %	p	1987 expansion 5yr surv %
A	ACPS	90	63		89
	Dukes	90	63	ns	
B	ACPS	69	55		75
	Dukes	51	47	P=0.018	
C	ACPS	29	28		49
	Dukes	18	20	P=0.008	
D	ACPS	12	8		27

Table 9 Modified ACPS

[63, 92]

A	1	Not beyond mucosa
	2	Into submucosa not beyond
	3	Into muscularis propria but not beyond
B	1	As before without mesothelial surface invasion
	2	With mesothelial surface invasion (not applicable to distal rectal cancers)
C	1	Lymph node involvement
	2	Apical node involvement
D	1	Margin positive
	2	Distal metastases

Table 10 TNM stage

T (Bowel wall involvement)	1	Invades submucosa
	2	Invades muscularis propria
	3	Through muscularis propria
	4	Direct local invasion
N (nodal involvement)	1	1-3 nodes positive
	2	≥4 nodes positive
M (metastases)	0	No distal metastases
	1	Distal metastases
Stage	1	T1 and 2 (equivalent ACPS A)
	2	T 3 and 4 (equivalent ACPS B)
	3	Any nodal involvement
	4	Distal metastases

Table 11 International Documentation System

[91]

Basic information	Clinical Pathological	Country, Hospital, Patient ID, race and tumour history Tumour number, size, serosal appearance, associated pathology, tumour type
Variables with proven prognostic significance	Clinical	Surgeon, patient gender and age, tumour extent clinically, residual tumour
	Pathological	Stage, venous invasion, infiltrating margin, grade
Variables with probable prognostic significance	Clinical	Pre and postoperative treatment, tumour site, mobility, technique for mobilisation ("no touch"), perforation and procedure type.
	Pathological	Inflammatory cell infiltrate, lymphoid aggregates

2.4.3 Gender

2.4.3.1 Gender Survival Difference

It remains unclear whether gender significantly influences survival from colorectal cancer. While it has been suggested that women have an improved outcome, not all studies concur (Table 12). Nineteen studies have been identified that examined gender survival differences. Nine studies found women to have a better outcome from CRC [3, 12-18, 109], while nine found no significant gender difference [19, 20, 104-107, 110-112] and one showed a difference in overall survival but not cancer-specific survival [113]. These will be explored in more detail and to explain the disparity between these studies, methodology was considered. Differing statistical methodology and inclusion criteria may explain some of the results variation. Given that gender potentially correlates with other prognostic factors such as site, analysis should ideally be multivariate adjusting for these factors. Survival endpoint used is also important given that overall survival may be more gender dependent than cancer-specific survival.

Five large studies (totalling over 8000 cases) examined outcome following colorectal cancer resection and performed multivariate analysis [3, 12, 13, 109, 113]. Results varied, as did survival endpoint. In the first study, Chapuis et al. prospectively collated data on 709 patients who underwent resection of CRC over an 11-year period to assess the prognostic significance of selected clinicopathological features [12]. On univariate analysis, 5-year overall survival for men (n=506) was 34% compared to women (n=203) at 39% (p=0.029). Gender remained significant on multivariate

analysis adjusted for pathological factors (including stage and grade) and clinical factors (age, site and presenting symptoms) (p=0.013).

In the second study, Ratto et al. performed a population study in Italy involving 690 consecutive CRC cases following curative resection [3]. Forty percent were women. On univariate and multivariate analysis female gender was also found to significantly improve overall survival (p<0.005). Thirdly, Griffin et al. performed multivariate analysis on 400 cases following curative CRC resection (adjusting for gender, age, site, stage and grade) and found overall survival for men was worse (hazard ratio of dying of 1.59) relative to women [113]. However, there was no significant difference in outcome when cancer-specific survival was examined, highlighting the importance of endpoint used.

In contrast, the last study involving 2355 curatively-resected CRC cases showed an improved outcome for women in both overall survival for women (HR 0.76) and cancer-specific survival (HR 0.84) on analysis adjusted for age, site and stage [13]. The advantage was seen for both colon and rectal cases.

A local population study reviewing 4387 cases of CRC (all stages) showed a marginally better cancer-specific survival in men (p= 0.050) for colon cancer but no difference for rectal cancer [109]. Analysis adjusted for age, stage, site and treatment.

In the studies using only univariate analysis, results varied regardless of outcome measure. Cancer-specific survival was the endpoint in three studies. Two of these were population-based studies totalling 409 patients and no gender difference was observed [105, 106]. The third was a single surgeon‟s experience of 1939 cases of

CRC that showed a better cancer-specific outcome in women (p=0.02 on survival curves, 57% vs 52% 5-year survival) [14]. All stages were included without adjustment for stage

When overall survival was the examined endpoint, results still varied. A population study from New Zealand of 2450 patients found that crude and relative survival was better for women (39% vs 46% and 50% vs 55% respectively, significance not stated) [15]. Groups were not matched and analysis was not adjusted. Wichmann et al. also found overall survival was better in women in their study of 894 CRC cases following curative resection [16]. However, subgroup analysis narrowed the advantage to rectal cancer cases only. In contrast, Garcia-Peche et al. found no significant gender difference in overall survival in 191 colorectal cases [110].

Three studies specifically examined rectal cancers using multivariate analysis (Table 13). Bokey et al. analyzed prognostic factors in 709 consecutive patients following curative surgery for rectal cancer over a 23-year period [17]. No patient received adjuvant therapy and after adjusting for stage, age and other potential confounding factors, gender was found to significantly influence survival. Men had a poorer survival than women with a hazard ratio of dying of 1.44 (CI 1.16-1.80). Knudsen et al. found a non-significant trend (HR 1.2) to worse overall survival in men after resection in 682 rectal cases of all stages [19]. Analysis was adjusted for age, stage and neurovascular invasion. Finally, Ueno et al. studied pathological factors in 638 rectal cancers adjusting for other variables including gender, which did not significantly influence cancer-specific survival [111].

Wied et al. studied colon cancer alone and did not find a difference in overall survival [104]. In New Zealand, the mortality following colon cancer is the same for both sexes but women have a better outcome from rectal cancer [114].

Stage specific studies failed to demonstrate a convincing gender difference in survival. Newland et al. analysed 579 colorectal stage C cases and while univariate analysis suggested women had a better outcome (45% vs 37% 5 year survival), adjusted analysis showed no difference [20]. Nanni et al. in their study of biological factors in 263 stage B and C colon cancer cases, found women had a better 4-year disease-free survival but no significant difference in 4-year overall survival (on univariate analysis) [112]. Chapuis et al. reported on 378 stage C colon cancer cases following curative resection (none had adjuvant chemotherapy and rectal cancers were excluded) [12]. Multivariate analysis did not show a gender influence on cancer-specific survival.

It is therefore apparent that considerable confusion exists over the influence of gender in determining outcome following CRC resection with evidence both for [3, 12, 13, 15, 17, 113] and against [19, 20, 104, 106, 110, 112] a role in overall survival and both for [13, 14, 18, 109] and against [105-107, 111, 113] in cancer-specific survival.

Table 12 Gender variation in prognosis

Study Type	Author	Year	Ref	n	C/R*	Analysis†	Endpoint‡	Sig**	Study group
Multivariate	Chapuis et al.	1985	[12]	709	CR	MV	OS	Y	Consecutive cases
	Griffin et al.	1987	[113]	400	CR	MV	OS	Y (M Hr 1.59)	Population, curative resection
			As above				CA	N	Same study- Cancer specific
	Ratto et al.	1998	[3]	690	CR	MV	OS	Y	Population
	McArdle et al.	2003	[13]	2235	CR	MV	OS	Y (F HR 0.76)	Consecutive, curative resection
			As above				CA	Y (F HR 0.84)	Same study- Cancer specific
	Luke et al.	2005	[109]	4387	CR	MV	CA	Y (marginally worse for M)	Population, all stages
Univariate	McDermott et al.	1981	[14]	1939	CR	UV	CA	Y	Consecutive, all stages. One surgeon
	Koch et al.	1982	[18]	1522	C	UV	CA	Y	Consecutive
	Isbister and Fraser	1985	[15]	2450	CR	UV	OS	Y	Population
	Garcia-Peche et al.	1991	[110]	191	CR	UV	OS	N (trend)	All stages
	Ponz de Leon et al.	1992	[105]	134	CR	UV	CA	N	Population
	Ronucci et al.	1996	[106]	275	CR	UV	CA & OS	N	Population
	Wichmann et al.	2001	[16]	894	CR	UV	OS	Y (only in rectal)	Curative resection

*C=colon, R=rectal
†MV=multivariate analysis UV=univariate analysis, If both analyses performed only MV detailed unless varied from UV
‡ OS=overall survival, CA = cancer-specific survival
**SIG = significant difference detected

Table 13 Gender variation in prognosis – select groups

Study Type	Author	Year	Ref	*n*	C/R	Analysis	Endpoint	Sig	Comment
Rectal only	Bokey et al.	1997	[17]	709	R	MV	OS	Y (M HR 1.44)	More men
	Ueno et al.	2002	[111]	638	R	MV	CA	N	All stages
	Knudsen et al.	1983	[19]	682	R	MV	OS	N (F HR 1.2)	Consecutive, all stages
Colon only	Wied et al.	1985	[104]	442	C	MV	OS	N	Curative resection
Specific Stage	Newland et al.	1994	[20]	579	CR	UV	OS	Y (marginal)	Stage C
			As above			MV	OS	N	
	Nanni et al.	2002	[112]	263	C	UV	OS	N (trend F better)	Trial pts stage B & C
	Chapuis et al.	2004	[107]	378	C	MV	CA	N	Stage C, no chemo

2.4.3.2 Cause for Gender Variation in CRC

There are several mechanisms by which gender may influence CRC. Most studies have focussed on gender variation in incidence rather than outcome. Nevertheless, these are a useful starting point to address the influence of gender on tumour biology.

Some gender variation in incidence may be explained by gender differences in lifestyle. Environmental factors that have been implicated in the causation of CRC include dietary factors such as high fat intake, red meat, high protein, low fibre, low fruit and vegetable diet, smoking, alcohol abuse, lack of exercise and high body mass index [62, 67, 68]. Behavioural differences between the sexes exist, with women being more aware of health issues and consuming less alcohol, while significant variations in dietary intake and exercise have not been shown [62].

It may be the effect of these environmental factors varies according to gender. West et al. performed a case control study of 231 cases of colon cancer [67]. They showed that body mass index, high fat, high energy and high protein increased the odds ratio of colon cancer and that fibre and cruciferous vegetables were protective. However their influence was predominantly observed for men. For women, the only significant association was decreased risk with fibre and B-carotene and to a degree an increased risk with high fat.

Given that the influence of these lifestyle factors is slight and gender variation not substantial, a difference in CRC prevalence between the sexes would not be explained by this mechanism alone. Variations in bile exposure of the right colon have been

implicated in CRC and the incidence of symptomatic biliary disease varies between genders. Theoretically this could contribute to gender differences, however any link is unsubstantiated [115, 116].

The most obvious and likely cause for gender variation in tumour biology in the colorectum is hormonal. Evidence is circumstantial and at this point, there is no adequate explanation for how female hormones actually influence CRC. Oestrogen and progesterone receptors have been identified on a proportion of CRCs by some groups (24% - 23% ER, 12% - 43% PR) [62, 117-119], while others have failed to demonstrate any expression of these receptors [120]. A role for these receptors in CRC is unknown and whether they are related to pathogenesis remains unclear. Detection of these receptors makes a role for female hormones biologically plausible, however a link has not been established. Therefore a role in influencing tumour aetiology or behaviour and hence outcome is unconfirmed.

To investigate for a hormonal influence on CRC, the effect of reproductive factors has been studied as a clinical surrogate for oestrogen exposure. Studies often examine for an influence on incidence rather than survival, however as this endpoint probably reflects an effect on tumour biology they are worth considering. A large study from Norway assessed 63,090 women undergoing breast cancer screening by interview and prospectively followed them for at least 20 years during which time 831 cases of CRC were diagnosed [119]. The influence of parity, age of first and last pregnancy, age of menarche and age of menopause on incidence was assessed. Overall no significant association was found. In the patient group diagnosed with CRC at a young age, there was a trend towards increased risk if the first and last pregnancy were later in life. However, this trend was not significant and a large confidence interval reflects the

low numbers in this subgroup. There were no significant differences on subsite analysis.

In contrast, Howe et al. found that first pregnancy at an early age decreased the risk of colon and rectal cancer in a case control study of 229 cancers, although the number of pregnancies did not further influence incidence [121]. Again site risk was equivalent. Similarly, Peters et al. in their case-control study of 327 women with colon cancer found that, after adjustment for other lifestyle factors, ever being pregnant was protective (RR 0.56, 95% CI 0.33-0.97) [122]. A second and third pregnancy decreased the risk further but beyond four, the risk again increased. Two further papers questioning the effect of parity on CRC incidence found that only half of studies reviewed showed an effect [62, 119].

Studies of effect of parity on survival are limited. Koch et al. studied 1522 consecutive cases of colon for an effect of gender and parity on survival [18]. Overall, women had a better outcome on Kaplan-Meyer analysis (p=0.0008). Groups were age matched only. Interestingly, 5-year survival for nulliparous women (35.4%) was similar survival to men (36.9%) and women with children had a comparatively better survival (51.5% p=0.02). There was, however, no correlation between number of children and survival or any influence emanating from marital status. In contrast, Howe et al. found that parity did not affect survival or anatomic site of the cancer in their 229 CRC cases [121].

If hormones do affect CRC, menopausal status should have an impact on CRC. There is variation in gender distribution between age groups [116] with a tendency towards increasing incidence in older women [13, 123]. An influence is also suggested by

data from South Australia where proximal tumour rates are higher in women than men at all ages [115]. Distal cancers (sigmoid and descending colon) were more prevalent in women before menopause but showed a male dominance in the older age group. Rectal cancers had a similar distribution in the young but a male predominance later. The authors found their results correlated with seven other similar studies of Caucasian populations.

The role of exogenous hormones on CRC is mixed. DeCosse et al. reviewed the literature and found only one of five studies suggested a protective effect from oral contraception and three of seven showed a benefit from hormone replacement therapy [62].

None of these studies, however, explain the reason parity or other markers of oestrogen exposure affect outcome following CRC resection. It is possible there is a genetic susceptibility to tumorigenic mechanisms, for instance methylation and hence MSI. While tumour biology is unlikely to be unique to either gender, it is possible that women are over represented in the "good" biology group. This may be the case with MSI tumours and will be explored in a later section. However this is only postulated and no variation in gender genetics has been identified to explain a difference in susceptibility.

2.4.4 Site

2.4.4.1 Definition Proximal/Distal

The splenic flexure is the usual differentiator between proximal (right) colon and distal (left) colon, being the area of transition of vascular supply and embryological

origin [22]. The splenic flexure provides a convenient point of reference despite the fact there is no exact point of change and some contention that the embryological division actually occurs at the distal transverse colon [24]. Studies vary in the cut-off point used, ranging anywhere from the hepatic flexure [13, 124] to the descending colon [98] but most studies use the splenic flexure and consider splenic flexure cancers as proximal [38, 39, 46]. Division of the colon in this way is slightly artificial and oversimplified, with overlap likely. It is, however, a useful starting point for broad site comparisons and to make study data comparable.

2.4.4.2 Cellular and Functional Differences across the Colon

The colon is not a heterogeneous organ. Embryological origin varies. The proximal colon is derived from the midgut while the distal colon is derived from the hindgut [125]. The blood supply origin varies. The superior mesenteric artery supplies the midgut while the inferior mesenteric artery supplies the hindgut [126]. Function varies across the colon. Water absorption increases proximally to distally [22]. Transit slows, potentially increasing exposure to carcinogens in the left colon [22]. The metabolism of bile acids differs across the colon and there is greater fermentation in the proximal colon leading to higher concentrations of short chain fatty acids [22] [29].

There is cellular variation across the colon. Enteroendocrine cells increase distally [24]. Blood group antigens A, B, H and Le are only found in adult colon on the right [22, 29]. Interestingly there may be re-expression of these antigens in distal cancers and lost in proximal cancers [22, 24]. There is also varied expression of glycoconjugates and differing isoforms of P-450 [29].

Biochemical differences exist. Mucin is acidic in the rectum but neutral in the proximal colon and production is greater in the rectum [22, 29]. Activity of ornithine decarboxylase, which reflects cellular proliferation, declines distally in colorectal cancer [24]. Lectin binding varies according to site as does short chain fatty acid absorption [22, 29].

2.4.4.3 Luminal Fluctuations

Luminal contents and hence colonocyte exposure varies across the colon. A few epidemiological studies have considered environmental influences on CRC with subsite breakdown. Findings are generally inconsistent and there is insufficient correlation to draw any conclusion [29]. Dietary influences on CRC have been studied extensively but any site correlations are slight [22, 29]. West et al in their case control study of diet in CRC did find minor site variations [67]. The increased relative risk of colon cancer association with high BMI and high protein diet was more pronounced on the left, while a high fat diet tended to produce more right-sided tumours. The protective effect of fibre was seen across the colon.

The most obvious luminal difference is the increased exposure to bile acids in the proximal colon and the potential for upset if normal bile metabolism is disrupted. Studies are inconsistent as to whether CRC incidence increases after cholecystectomy (leading to increased bile acids in the right colon) [22]. The observed proximal shift was initially blamed on a higher cholecystectomy rate but this has been largely discounted [24]. A more recent meta-analysis showed a slight overall increased risk following cholecystectomy (RR 1.34) and the increased risk was most marked for proximal cancers (RR 1.88; 95% CI = 1.54-2.30) [127]. Whether any influence is due

to changes in bile acid metabolism, cholelithiasis or removal of the gallbladder is unclear.

2.4.4.4 Clinical Differences

There are epidemiological variations in site distribution of CRC. In areas of high incidence, most CRCs occur on the left while in low incidence areas, the percentage in the right colon increases [22, 24]. Migration to a high incidence area changes distribution towards that of the adopted country [22, 24, 29], suggesting environmental exposures may influence these differences [24].

The pattern of distribution is changing over time. The rate of right-sided cancers is increasing, even in high incidence nations while there is a corresponding decrease in left-sided lesions [23-25].

CRC distribution varies between the sexes. Societies with a high CRC incidence (and hence a predisposition to distal cancers) tend towards equal gender distribution, whereas areas of low incidence have a higher proportion of proximal cancers in older women [22, 24]. The increase in right-sided cancers in high incidence areas is observed in older women [24, 25]. Women are more likely to develop proximal cancers and a higher proportion of proximal cancers cases occur in women [26, 27]. A New Zealand group studied the sex distribution in 4678 consecutive cases and found age-standardised incidence higher in women for proximal lesions [128]. The Large Bowel Cancer Project, totalling 4292 cases, reported that right-sided lesions were more common in older women [26].

Furthermore, Gonzalez et al. performed a population-based study of 9550 CRC cases (all stages) to determine predictive factors of a proximal lesion [28]. Four factors proved to be significant on logistic regression analysis: female gender (OR of proximal lesion 1.38); older age (OR 1.02 p=<0.001); comorbidities (OR 1.28); and black non-Hispanic (OR 1.24). Women accounted for 53.9% of right-sided cancers but only 45.3% of distal lesions. In another study, Elsaleh et al. found a similar breakdown in 656 cases of resected stage C CRC [21]. Of the proximal lesions, 56% occurred in women compared to 44% of the distal lesions and in women, 58% of cancers were proximal compared to only 42% in men.

A cause for the variation in gender distribution has not been identified. As discussed in the previous section, genetic or hormonal differences may influence CRC and these may be site-specific.

2.4.4.5 Molecular Differences in CRC across the Colon

MSI cancers are over-represented in the proximal colon and may account for the observed pathological variation in CRC according to site. This will be discussed later in this chapter. Prior to the recognition of MSI, molecular differences according to tumour site had been noted (see Table 14) [22, 24, 129]. Right-sided tumours were more likely to be diploid and therefore not show loss of heterozygosity as had been observed in CRC. Loss of alleles on chromosomes 17, 18 and 5 were less commonly present in proximal tumours compared to distal tumours.

Delattre et al. quantified some of these differences in their study of DNA from 152 cases (Table 15) [129]. All the studied alleles except kras showed significantly greater

loss in the distal tumours, strongly suggesting that distal tumours are more likely to form via chromosomal instability with the traditional genes being lost. As expected, usually two or more of these genes were lost. These authors showed foresight in suggesting that different molecular mechanisms were involved in proximal carcinogenesis.

The finding of diploidy or lack of allele loss in proximal lesion was confirmed by other groups [22, 24]. This is consistent with the assertion that different tumourigenic mechanisms exist according to site.

Table 14 Features of Proximal Cancers

[24, 29, 129]

More mucin
More diploidy
Less LOH (Loss of heterozygosity)
Less allelic loss chromosome 17p, 18, 5q
Less p53 loss
Less c-myc abnormalities
Less over expression COX-2 (compared to rectum)

Table 15 Delattre et al

[129]

Allele loss	Proximal	Distal
None	58%	3%
17p (p53)	30%	74%
18	30%	85%
5q (APC)	11%	45%
Kras	41%	31% ns

2.4.4.6 Prognosis

Whether the anatomical site of a colorectal cancer influences prognosis remains debated. Traditionally it was believed that right-sided cancers presented later and a worse outcome was presumed. There was some evidence to support late presentation. A population-based study of 9550 CRC cases found that the proximal cancers presented at a later stage relative to distal lesions [28]. The Large Bowel Cancer Project of 4292 cases found the proportion of Dukes A higher in rectal and rectosigmoid cancers, but that the other stages were equally distributed through the colon [26].

The current literature does not support a variation in outcome from CRC according to anatomical site [3, 104, 105, 107, 112-114, 130, 131]. As Table 16 indicates, all ten studies reviewed, involving over 8000 cases, failed to show tumour site influenced prognosis. The findings were consistent regardless of the distinction between proximal and distal cancer and the survival endpoint used. At most, one study shows a trend towards better outcome following resection of transverse colon cancers [131]. When comparing colonic and rectal cancers (Table 17), one study showed a difference in stage C disease alone [20], while the other studies making the same comparison do not concur [12, 14, 106].

The early trials of adjuvant chemotherapy performed subsite prognostic analysis to determine if site needed to be included in analyses of chemotherapy affect. Findings suggested that site did have an influence. The Intergroup study of 318 stage C cases found a significantly worse survival for cancer arising in the transverse colon and at the flexures (39% 7-year survival) compared to caecal cancers (47%) while left-sided

cancers had the best outcome (54%) [7]. Laurie et al. performed multivariate analysis in poor prognosis stage B and C cancers, also as part of a trial of adjuvant treatment, and found significant subsite variation in survival. Five-year survival for ascending and transverse colon cancers was 63% compared to 58% for sigmoid and 39% for rectal lesions [4]. However, the IMPACT trial, which looked for potential confounding prognostic factors to be considered in analysis, did not find site to be a significant prognostic indicator [10]. These studies did not address the influence of site on chemotherapy effect, only variation in prognosis according to site. They were not designed specifically to do either and as such, subgroups were small and prognostic analysis basic.

Table 16 Prognostic influence of site

Study	Year	Ref	No	C/R	Analysis	End point	Sig	Definition	Comment
Jolly et al.	1982	[114]	455	CR	UV	CA	N	Subsite	Consecutive, all stages
Wied et al.	1985	[104]	442	C	MV	OS	N	Subsite	Consecutive, curative resection
Steinberg et al.	1986	[130]	572	C	UV/MV	OS	N	HF cut off	Trial pts, stage B2 & C
Griffin et al.	1987	[113]	400	CR	UV*	OS	N	R inc SF, L beyond	Population, all stage
Wiggers et al.	1988	[131]	350	CR	UV*	CA	N	R, Trans, L, Rectal	From 2 unrelated trials
Ponz de Leon et al.	1992	[105]	134	CR	UV*	CA	N	R inc SF, L beyond	Population, all stages
Ratto et al.	1998	[3]	690	CR	UV*	OS	N	R, L, rectal	Population, curative resections
Nanni et al.	2002	[112]	263	C	UV	OS	N	NS	Trial pts advanced stage B and C
Chapuis et al.	2004	[107]	378	C	UV*	CA	N	R inc SF, L beyond	Consecutive, no chemotherapy, curative resection
Luke et al.	2005	[109]	4387	CR	MV	CA	N	Subsite	Population, all stages

*Did not include site in MV as not significant on UV

Table 17 Prognosis colon versus rectum

Study	Year	Ref	*n*	Analysis	Endpoint	Sig	Comment
McDermott et al.	1981	[14]	1939	UV	CA	N	Consecutive, all stages
Chapuis et al.	1985	[12]	709	MV	OS	N	Consecutive, all stages
Newland et al.	1994	[20]	579	UV/MV	OS	Y	Stage C
Ronucci et al.	1996	[106]	275	UV	CA & OS	N	Consecutive, all stages

2.4.4.7 Speculation on Site Influence on CRC

The observed clinical and pathological differences between right- and left-sided colorectal lesions led to speculation that different carcinogenic mechanisms existed across the colon. Given the biological and luminal variations, a site-specific influence on tumour biology would be plausible.

The variation in environmental exposure (luminal contents) across the colon may vary exposure to potential carcinogens (i.e. bile acids on the right). Alternatively, given the cellular variations, colonocyte susceptibility to environmental carcinogens may differ. As the formation of cancer involves cumulative gene mutation or inactivation, it is plausible that there is an interaction between luminal factors and cellular DNA (i.e. as a cause of methylation). As yet, no link between environmental factors and tumour biology has been established.

Different mechanisms of carcinogenesis may be triggered by different factors or, possibly, CRC develops through an environmental carcinogen effect on a genetically predisposed cell. It is possible that environmental factors are more important in the formation of CRC in high-risk areas and in the left colon, while genetic factors may be more important on the right [24]. In HNPCC, cancers tend to be proximal, however in FAP, distal lesions are more common.

The observed differences between proximal and distal colon cancers suggest that they should be considered, and possibly treated, separately. What remains unclear is

whether these site differences are wholly explained by differing tumour biology or if the site variations detailed above differentially influence tumour behaviour.

2.4.5 Other Clinical Prognostic Factors

2.4.5.1 Age

To determine the true influence of age on CRC outcome, study methodology is important. The survival endpoint used is important as overall survival will be more age dependent than cancer-specific survival. Perioperative deaths would be expected to be higher in an older population and thus should be excluded to minimize bias. Furthermore, an analysis needs to consider confounding factors. Clinical factors related to age include gender, co-morbidities and adjuvant therapy. Gender may influence prognosis and given the increasing incidence of CRC in older women, analysis for age effect should adjust for gender. Comorbidities increase with age and ideally should be considered. Failure to commence or complete chemotherapy may be due to comorbidities or age. Thus a lack of adjuvant therapy may account for a worse outcome observed in an older patient group. Unfortunately, many studies that attempt to determine clinical prognostic indicators do not detail or adjust for adjuvant treatment.

Studies investigating the effect of age on outcome are detailed in Table 18. The four studies that performed adjusted analysis and used cancer-specific survival as an endpoint (included a total of 1500 patients), showed that age did not influence cancer outcome [105, 107, 111, 131]. One of these studies found significance on univariate analysis highlighting the importance of adjusting for confounding factors [105]. There were no obvious confounders with neither gender nor symptomatology significantly

influencing outcome. Three other studies used cancer-specific survival as their endpoint but only univariate analysis and still failed to show that age wielded an influence on survival [106, 113, 114].

Two studies examined the effect of age on both overall and cancer-specific survival in large populations including all stages [106, 113]. Neither found an effect on cancer-specific survival but one found that overall survival was affected, highlighting the importance of endpoint used [113].

Four of nine studies using only overall survival as the study endpoint found age to be significant on adjusted analysis [12, 17, 19, 20]. Three of these studies come from the same Australian group [12, 17, 20]. Two included colon and rectal cases, analysed age as a continuous variable but did not exclude perioperative deaths, which may account for the positive finding [12, 20]. The third Australian study which included only rectal cases and did not exclude perioperative deaths, found a significantly worse outcome in the over 75-year group [17]. Knudsen et al. also studied rectal cases only but did exclude perioperative deaths and found age significantly influenced outcome on multivariate analysis (though specifics are not given) [19].

The five other studies to examine overall survival did not show an effect [104, 106, 110, 112, 132]. Two were stage-specific [112, 132]. Newland et al. investigated only men with stage B cancers and found significance on univariate but not multivariate analysis [132]. Probable confounders included complications and whether surgery was emergency, both of which were also significant on univariate and thus may have negated the age influence on multivariate analysis. Nanni et al included only stage B and C [112].

The weight of evidence would suggest age does not consistently influence overall outcome and does not influence cancer-specific survival.

Table 18 Prognostic influence of Age

Study	Year	Ref	*n*	C/R	O/C	Analysis*	Sig	Grouping	Study population	Periop death†
Jolly et al.	1982	[114]	455	CR	CA	UV	N		Consecutive, all stages	
Knudsen et al.	1983	[19]	682	R	OS	MV	Y	10 yr groups	Consecutive, all stages	Exc
Chapuis et al.	1985	[12]	709	CR	OS	MV	Y	Continuous	Consecutive, all stages	Inc
Wied et al.	1985	[104]	442	C	OS	MV	N	>70	Curative	
Griffin et al.	1987	[113]	400	CR	OS	MV	Y		Population, all stages	
					CA	UV	N			
Wiggers et al.	1988	[131]	350	CR	CA	MV	N		From two unrelated trials, no noted bias relating to age	
Garcia-Peche et al.	1991	[110]	191	CR	OS	UV	N		All stages	
Ponz de Leon et al.	1992	[105]	134	CR	CA	UV	Y		Consecutive, all stages	
						MV	N			
Newland et al.	1994	[20]	579	CR	OS	MV	Y	Continuous	Stage C	
Newland et al.	1995	[132]	467	CR	OS	UV	Y		Men, stage B	
						MV	N			
Ronucci et al.	1996	[106]	275	CR	CA & OS	UV	N		Consecutive, all stages	
Bokey et al.	1997	[17]	709	R	OS	BV	Y	>75 yrs	Population, all stages	Inc
Ueno et al.	2002	[111]	638	R	CA	MV	N		All stages	
Nanni et al.	2002	[112]	263	C	OS	UV	N	<>60 yrs	Trial pts advanced stage B and C	
Chapuis et al.	2004	[107]	378	C	CA	MV	N		Consecutive, no chemo, curative resection	

*If NS on univariate, most did not include in MV even if performed.
†Perioperative deaths if stated

2.4.5.2 Obstruction

Between 10 and 20% of CRC cases present with obstruction [26, 133]. In these cases, it has been postulated that survival may be compromised by disease dissemination facilitated by the obstructive process. Physiological disturbance and compromised bowel wall integrity may be contributory [91]. Consistent with this theory, failures have been shown to be due to both local and distal recurrence [124, 130].

There are potential confounders in assessing the importance of obstruction on prognosis. There are varying degrees of obstruction (solids, liquid, complete, partial) but no clear classification. Therefore studies vary in the definition of obstruction and have different criteria for establishing the diagnosis. Most studies are retrospective and reliant on clinical documentation, which is notoriously inaccurate, particularly when trying to establish if obstruction was present. Most obstructed cases present as emergencies and patient compromise often increases perioperative mortality [26]. If perioperative deaths are included in analysis, results that suggest obstruction influences prognosis may be misleading. Previous surgical management of obstruction has included the three-stage procedure, with a defunctioning stoma prior to definitive resection. This was largely abandoned when outcome was found to be worse [134]. Many of the earlier studies reviewed include a significant number of defunctioned cases, which may have contributed to any perceived reduction in survival.

Table 19 summarises the studies that investigated the influence of obstruction on outcome. Five groups performed adjusted analysis and three of these found

obstruction not to be significant on multivariate analysis [110, 113, 135]. Interestingly, all three found an effect on univariate analysis, again highlighting the inaccuracy of unadjusted analysis. Korenaga et al. confirmed obstruction in 20% of 113 patients (symptomatology, AXR, plus Ba enema or colonoscopy) and found a statistically significant association with advanced stage and older age group [135]. On univariate analysis, 5-year survival was impressively worse (24.4%) compared to the non-obstructed group (65.5%). However there was no difference in survival on adjusted analysis. (Whether perioperative deaths were included in the univariate 5-year survival figures is unclear and may have influenced the outcome). Griffin et al. performed a population study of 400 cases excluding perioperative deaths and concluded that obstruction did not influence outcome [113]. The third multivariate study of 191 CRC cases failed to find obstruction to be significant but was scant on detail [110].

In contrast, two studies found that obstruction conferred a worse outcome on multivariate analysis [3, 12]. Ratto et al. studied 690 consecutive CRC cases following curative resection [3]. Obstructed cases (clarification of obstruction is not given) had a worse outcome. Five-year survival was 52% compared to 77% for non-obstructed and 10-year survival was 47 % compared to 61% ($p=<0.005$). Death in these patients was due to a higher rate of distant metastases. Chapuis et al found obstruction to be the only symptom that was significant on multivariate analysis of 709 patients with CRC [12]. Five-year survival for obstructed cases (definition not given) was 19% compared to 38%.

Univariate analysis alone was performed in three other studies [114, 130, 133]. Two found obstruction to be significant, although there were methodological concerns. The

largest, reviewed all CRC patients seen at the Cornell Medical Centre over 33 years [133]. Of 1815 patients, 210 had obstruction (11.5%), diagnosed clinically, radiologically or on pathology. Ninety-eight patients had a curative resection and of these, overall 5-year survival was poor at 19.5%. Stage A, B and C were 57%, 39% and 30% respectively. These findings would support a worse outcome. However, as there was no comparison group and perioperative deaths were not excluded, only cautious conclusions can be drawn. Many of the cases date from early last century and around half had an initial defunctioning colostomy, which may have contributed to poorer outcome.

The second was the GITSG study of 572 patients with stage B2 or C colon cancer which found obstruction to be the only significant clinical factor influencing survival [130]. The influence of any symptoms was negated by obstruction. One third of patients had initial diverting colostomies and no mention of perioperative deaths is made, both of which may have contributed to the worse outcome. Finally, these patients were all part of a clinical trial and may not be a representative group. In contrast, in the third of these unadjusted analyses, Jolly et al. found obstruction did not influence cancer specific survival in a study of 455 consecutive CRC cases [114].

A further large population study (1159 consecutive cases curative CR, not tabled) from St Mary‟s Hospital London ranked the prognostic significance of various clinical and pathological factors [90]. When perioperative deaths were excluded, staging factors remained most significant, followed by obstruction. An unusual statistical methodology had been used.

Thus, obstruction is not consistently found to be associated with a worse outcome and the balance of evidence suggests against a prognostic influence. Furthermore, the variations above highlight the importance of methodology in any study of prognosis including consistent definitions, exclusion of perioperative deaths and adjustment for confounding factors.

2.4.5.3 Perforation

Perforation occurs in around 5 to 10% of CRC cases [26] and has been implicated in a worse long-term outcome. What is considered as prognostically significant perforation can vary. Perforation may be obvious as evidenced by faecal peritonitis or it may be only suggested, such as in the setting of a paracolic abscess. A perforation may occur at the site of the tumour or in the colon proximal to an obstructing cancer. Whether both scenarios are prognostically comparable is not clear.

Studies examining for an influence of perforation on outcome from CRC are detailed in Table 20. On simple analysis, the Cornell Medical Centre study (discussed in the previous section) showed a poor outcome for the cases with perforation drawn from over 1800 CRC cases [133]. The rate was 5.5% and 5-year outcome was 47.8% for stage B and 31.6% for stage C. Around half of these cases were free perforations. However, without more detailed breakdown or analytical comparison, little can be concluded. Similarly, Willett et al found a poor outcome in the 34 perforated cases (from 533 patients presenting with CRC) [124]. Five-year survival in the perforated group was 44% compared to 59% (significance not stated). In the four who had proximal perforations due to obstruction, the 5-year survival was 50% but this number is too small to draw conclusions.

However, more convincingly, the two studies that performed multivariate analysis comprising nearly 600 cases of all stages of CRC, found perforation did not influence overall survival [110, 113]. Highlighting again the importance of adjusted analysis, they both found significance on univariate analysis.

A large study of patients from Boston conducted during the 1970"s compared types of perforation in 2004 consecutive colorectal cancer cases [136]. The rate of perforation was 5.9% of which half (118) were free perforation (11 of which were proximal), 36 had a fistula and the rest comprised abscesses. Long-term survival was similarly poor for all types of perforation. Unfortunately as there is no adjustment (even for stage) or a control group little conclusion can be drawn from the study. Of interest was the much higher perioperative death rate for perforations through the tumour compared to proximal perforations.

Table 19 Prognostic influence of obstruction

Study	Year	Ref	*n*	C/R	O/C	Analysis	Sig	Study population	Periop deaths*
Glenn and McSherry	1971	[133]	1815	CR	OS	UV	Y	Consecutive, all stages	Inc
Jolly et al.	1982	[114]	455	CR	CA	UV	N	Consecutive, all stages	uk (Ca deaths)†
Chapuis et al.	1985	[12]	709	CR	OS	MV	Y	Consecutive, all stages	Inc
Steinberg et al.	1986	[130]	572	C	OS	UV	Y	Trial pts, stage B2/C	uk
Griffin et al.	1987	[113]	400	CR	OS	UV	Y	Population, all stage	Exc
						MV	N		
Garcia –Peche et al.	1991	[110]	191	CR	OS	UV	Y	Consecutive, all stages	uk
						MV	N		
Korenga et al.	1991	[135]	113	CR	OS	UV	Y	Population study, curative resections	uk
						MV	N		
Ratto et al.	1998	[3]	690	CR	OS	MV	Y	Population, curative resections	uk

*"uk" periop death status presumed to be included as not otherwise stated
† as cancer deaths was the endpoint, management of perioperative deaths less relevant

Table 20 Prognostic influence of perforation

Study	Ref	Year	No	CR	O/C	Analysis	Sig	Periop deaths	Study population
Willett et al.	[124]	1985	533	CR	OS	UV	5yr surv 44% vs 59% †	exc	Consecutive, curative resection
Steinberg et al.	[130]	1986	572	C	OS	UV	N	uk	Trial pts, stage B2/C
Griffin et al.	[113]	1987	400	CR	OS	UV	Y	exc	Population, all stages
						MV	N		
Garcia-Peche et al.	[110]	1991	191	CR	OS	UV	Y	uk	Consecutive, all stages
						MV	N		

†significance not stated

2.4.5.4 Surgeon/Hospital

Studies have been conducted on the influence of hospital volume, surgical volume and the expertise of the operating surgeon on outcome with variable results. The Memorial Sloan –Kettering Cancer Centre in the US investigated the influence of hospital and surgeon volume on outcome by retrospectively studying 24,166 cases of colon cancer cases [137]. On multivariate analysis, hospital volume was found to be most significant and the outcome advantage of "high volume" surgeons was lost in high volume hospitals. A previous study from this centre of over 27,000 cases showed a minor improvement in 5-year overall survival related to number of colon cancer resections per hospital [138]. The absolute difference was 4% between the very high volume hospitals and the lowest, of which half was due to a difference in 30-day mortality.

In contrast, data from the North Western Regional Cancer Registry from the UK found that on adjusted analysis of 927 cases of CRC, neither hospital throughput, consultant workload or operator grade influenced overall survival [139]. A further study from the US of 3161 cases of stage 2 and 3 CRC found that hospital volume influenced overall survival (HR 1.16) but not disease recurrence. This suggests that factors involving the patients" general health may be influencing the observed survival advantage [140].

Several local studies have addressed the issue of colorectal specialisation on outcome. In 1985, data on 378 sequential stage C colon cancer cases was published [12]. Those cases operated on before 1980 were found to have a worse outcome than those after

1980. The main recognised difference between the two time periods was the implementation of colorectal specialisation. There are other potential reasons for improvement over time including better hospital facilities and treatment. Adjuvant chemotherapy was standard for the latter 6 years of the study, however only non-chemotherapy cases were included to focus on non-treatment prognostic factors. Any resultant selection bias (towards presumably older and less well patients) would have only diluted their findings and hence the real difference may have been greater.

A further study from this group was published in 1997 and involved 709 cases of rectal cancer (all stages) [17]. On multivariate analysis, outcome was worse when a non-specialist surgeon performed the surgery (HR 1.23). This difference is less surprising with rectal cancers given the potential complexity of the surgery and the increased emphasis on accurate dissection (TME) that has coincided with establishment of specialist units. A greater rate of involved margins and breach of the mesorectal fascia in the earlier cases may have accounted for outcome differences (this is not able to be determined from the study).

Multivariate analysis of 1264 CRC cases from Adelaide also showed that survival improved in the time period that corresponded to the establishment of the colorectal specialist unit at the study hospital [141]. Treatment variables with time did not significantly vary (including chemotherapy) to account for this difference.

It can be concluded that the influence of surgical volume, either hospital or operating surgeon and surgical expertise on cancer outcome from CRC, is relatively minor.

2.4.5.5 Presenting Symptoms and Complications

The influence of presenting symptoms on outcome following resection for CRC has been examined in a small number of studies (see Table 21). Two studies considered the effect of symptomatology on cancer-specific death rate in all stages of consecutive cases and found no influence [105, 114]. The larger study from Dunedin, which consisted of 455 consecutive cases found no correlation between duration of symptoms and stage or duration of symptoms and 5yr survival [114]. The study by Steinberg et al. included only stage B2 and C cases and even when analysing overall survival, without obvious exclusion of perioperative deaths, found no effect following adjustment [130].

In their research, Chapuis et al. found several symptoms that proved to be significant on univariate analysis including rectal bleeding (better outcome) and abdominal pain (worse) but this significance disappeared on adjustment for other variables [12]. Pain was possibly related to obstruction, which did confer a worse survival. The better prognosis seen if rectal bleeding was present may be due to earlier presentation and thus disappeared once adjustment is made for stage (though correlation with stage is not given).

Bokey et al. found that cardiovascular and respiratory postoperative complications worsened overall survival (including perioperative deaths) on multivariate analysis of rectal cases [17]. When perioperative deaths were excluded only respiratory complication continued to have an influence. However, as overall survival was the endpoint, this may not be unexpected.

The same institution studied 910 lymph node negative cases [132]. Only stage B males (n=467) were considered for analysis because the stage A cases and women with stage B disease had been found to have outcomes not dissimilar to the general population. Overall survival (inclusive of perioperative deaths) was worse on univariate and multivariate analyses in the group that developed postoperative respiratory complications. No explanation or further analysis was performed. This study also found emergency surgery to confer a worse survival on multivariate analysis.

Thus there is no consistent influence of presenting symptoms on outcome and postoperative respiratory complications may confer a worse overall survival.

Table 21 Prognostic influence of presenting symptoms and complications

Study	Year	Ref	*n*	C/R	O/C	Analysis	Sx* Sig	Cx† sig	Periop deaths	Group
Jolly et al.	1982	[114]	455	CR	CA	UV	N			Consecutive, all stages
Chapuis et al.	1985	[12]	709	CR	Act OS	UV	Y		Inc	Consecutive, all stages
						MV	N			
Steinberg et al.	1986	[130]	572	C	OS	UV	N		uk	Trial pts, stage B2/C
Ponz de Leon et al.	1992	[105]	134	CR	CA	UV	N			Consecutive, all stages
Newland et al.	1995	[132]	467	CR	OS	UV	Y‡	Y	Inc	Male stage B
						MV		Y	Inc	
Bokey et al.	1997	[17]	709	R	OS	BV	Y	Y	Inc	Consecutive, all stages
						MV		Y		

*Sx = symptoms
†Cx = complications
‡ worse outcome for emergency cases

2.4.6 Pathological

2.4.6.1 Positive Resection Margin

As indicated by the ACPS staging system (and studies that validate the system) involvement of the surgical margin ("palliative" or "non-curative resection") is associated with a worse outcome, not far removed from stage D cases [63, 91, 142]. The importance of documenting this fact is now widely recognised [91, 99]. Staging classification now includes the R classification: R0 if there is no residual tumour, R1 for microscopic residual tumour and R2 for macroscopic residual tumour [99].

2.4.6.2 Differentiation

Several histological factors contribute to the grading of a tumour including degree of differentiation (gland formation), cytological atypia, nuclear pleomorphism and mitotic activity. The WHO classification recommends that well differentiated tumours demonstrate greater than 95% glandular formation, moderate 50-95%, poor 5-50%, undifferentiated <5% [91]. Jass et al proposed a more comprehensive system involving the three parameters (glandular formation, invasive margin and lymphocytic infiltration) that were demonstrated to be the most predictive of survival in 447 rectal cancer cases [102]. Use of the system is problematic due to its complexity. Classification remains very subjective [102, 143], leading to potential problems with study comparison and limited prognostic usefulness. Generally there is less observer variation in defining a tumour as poor grade, compared to differentiating well from moderate.

The influence of grade on prognosis is controversial. Poor grade - distinguished by lack of gland formation - is a sign of dedifferentiation and aggressive tumour behaviour and should be associated with a worse outcome but this is not always the case. The evidence regarding the role of tumour grade on outcome is detailed in Table 22, Table 23 and Table 24. Three of the six studies that examined colon and rectal cases and adjusted for pathological confounders, found differentiation to be independently predictive of poor outcome [12, 20, 110], while three did not [3, 106, 113]. Two of the latter found significance on univariate analysis but not multivariate analysis [106, 113]. Two studies performed only univariate analysis, one finding an influence on outcome [131], the other not doing so [105]. A correlation between differentiation, depth of wall penetration and stage has been demonstrated and thus these factors may negate the influence of differentiation in adjusted analysis [94, 96].

Some studies suggest that grade may be more predictive of outcome in colon cancer compared to rectal cancer, where other factors may assume greater importance. The three studies that focussed on colon cancer cases found differentiation to be significant in predicting outcome (Table 23) [104, 107, 112], whereas only two of the five studies that specifically examined rectal cancers showed significance (Table 24) [17, 19, 64, 102, 111].

The evidence suggests, contrary to popular belief that grade is not consistently predictive of outcome and that other associated factors are important.

2.4.6.3 Tumour Type

Within the classification adenocarcinoma, several subtypes exist. Mucinous tumours is characterised by pools of extracellular mucin, and a tumour is labelled as such if the mucinous component comprises greater than 50% of the tumour [91]). Signet rings represent intracellular mucin and are infrequently observed and usually, though not always, seen within mucinous adenocarcinoma. Other tumour subtypes described include tubular, medullary and cribriform.

Table 25 summarises studies that have examined tumour type as a predictive factor in CRC. It can be seen that none of the seven studies reviewed that included tumour type in their analysis of prognostic factors found tumour type to have an independent effect. The numbers of cases in these subgroups were small. Mucinous type and signet ring cell tumours are usually associated with poor differentiation [45] and often, as will be seen, with MSI. It is possible these associated factors assume greater prognostic importance.

Table 22 Prognostic influence of differentiation

Study	Year	Ref	*n*	C/R	Analysis	Sig
Chapuis et al.	1985	[12]	709	CR	MV	Y
Griffin et al.	1987	[113]	400	CR	UV	Y
					MV	N
Wiggers et al.	1988	[131]	350	CR	UV	Y
Garcia-Peche et al.	1991	[110]	191	CR	MV	Y
Ponz de Leon et al.	1992	[105]	134	CR	UV	N
Newland et al.	1994	[20]	579	CR Stage C	MV	Y
Roncucci et al.	1996	[106]	275	CR	UV	Y *
Ratto et al.	1998	[3]	690	CR	MV	N

*Significant for rectal cases only

Table 23 Prognostic influence of differentiation – colon cases

Study	Year	Ref	*n*	C/R	Analysis	Sig
Wied et al.	1985	[104]	442	C	MV	Y (just)
Nanni et al.	2002	[112]	263	C	MV	Y
Chapuis et al.	2004	[107]	378	C Stage C	MV	Y HR 1.8

Table 24 Prognostic influence of differentiation – rectal cases

Study	Year	Ref	*n*	C/R	Analysis	Sig
Knudsen et al.	1983	[19]	682	R	MV	N
Jass et al.	1986	[102]	447	R	MV	N
Hermanek et al.	1989	[64]	597	R	MV	N
Bokey et al.	1997	[17]	709	R	MV	Y HR 2.02
Ueno et al.	2002	[111]	638	R	MV	Y

Table 25 Prognostic influence of tumour type

Study	Year	Ref	*n*	C/R	Analysis	Sig
Jass et al.	1986	[102]	447	R	MV	N
Hermanek et al.	1989	[64]	597	R	MV	N
Ponz de Leon et al.	1992	[105]	134	CR	UV	N
Newland et al.	1994	[20]	579	CR Stage C	UV/MV*	N
Roncucci et al.	1996	[106]	275	CR	UV/MV*	N
Ratto et al.	1998	[3]	690	CR	MV	N
Ueno et al.	2002	[111]	638	R	MV	N

* due to non-significance on univariate not included in subsequent multivariate model

2.4.6.4 Vascular, Lymphatic and Neural Invasion

There is much subjectivity in diagnosing lymphovascular invasion and variability in the manner of reporting [99]. Since it can be difficult to distinguish between venous or lymphatic structures (as both have similar mural muscular component) reporting them separately may be invalid. Whether either is more important than the other is unknown. Most studies refer to venous invasion, which potentially includes either. Venous invasion may be intramural (confined to muscularis propria) or extramural. Intuitively, extramural invasion would have the greater significance, particularly in earlier stage disease. Most studies do not clarify their definition. The diagnosis of endo or perineural invasion is less prone to error and while not always addressed separately from vascular invasion, probably has the same significance [19, 104].

It is assumed that lymphovascular and neural invasion are associated with disease progression and thus would be expected to correlate with stage and potentially aggressive tumour features. A study by Knudsen et al specifically examined neurovascular invasion correlates and, as expected, found both progressively increased with stage and that venous invasion correlated with grade [19]. Despite this correlation, both had independent prognostic significance on multivariate analysis. Assigning a HR of 1 for no neurovascular invasion, HR of dying if venous invasion was present was 1.59, neural invasion was 1.71 and if both were present HR was 2.72. Phillips et al. confirmed the correlation with deeper bowel wall invasion and poor differentiation [96].

Even within a given stage, lymphovascular invasion is probably associated with occult spread and thus a worse prognosis. Ten studies that considered the prognostic

significance of vascular invasion and that adjust for other pathological variables were reviewed (Table 26). Seven found vascular invasion to be an independent predictor of survival [12, 17, 19, 20, 104, 107, 110]. Six studies included rectal cancers only and four failed to find significance [64, 111, 143, 144], whereas all the studies that included colonic cases suggested significance [12, 20, 104, 107, 110]. The weight of evidence, at least for colonic cancer, suggests that lymphovascular invasion is an independent predictor of prognosis.

It is worth noting that reliance on pathology reports may lead to erroneous conclusions given that a negative finding is not always reported. Up to 30% of pathology reports in some studies made no comment on lymphovascular invasion and thus were not included [26, 96]. It is probable that lack of comment was due to a negative finding and hence the reviewed group (with a comment in the report) would have a selection bias towards a higher rate.

Table 26 Prognostic influence of neurovascular invasion

Study	Year	Ref	*n*	C/R	Sig	Comment
Chapuis et al.	1985	[12]	709	CR	Y	
Garcia-Peche et al.	1991	[110]	191	CR	Y	
Newland et al.	1994	[20]	579	CR	Y	Stage C
Wied et al.	1985	[104]	442	C	Y	Most significant. Neural borderline
Chapuis et al.	2004	[107]	378	C	Y	Stage C
Rectal only						
Knudsen et al.	1983	[19]	682	R	Y	Neural also sig
Jass et al.	1987	[143]	331	R	N	
Hermanek et al.	1989	[64]	597	R	N	
Bokey et al.	1997	[17]	709	R	Y	HR 1.4
Ueno et al.	2002	[111]	638	R	N	

All multivariate

Table 27 Prognostic influence of growth pattern of margin

Study	Year	Ref	*n*	C/R	Anal	Sig	Comment
Jass et al.	1986	[102]	447	R	MV	Y	
Ponz de Leon et al.	1992	[105]	134	CR	UV	Y	
					MV	N	
Ronucci et al.	1996	[106]	275	CR	UV	Y	
					MV	N (Y for R)	
Ueno et al.	2002	[144]	627	R	MV	Y	LN negative
Ueno et al.	2002	[111]	638	R	MV	N	

Table 28 Prognostic influence of lymphocytes

Study	Year	Ref	*n*	C/R	Analysis	Sig	Comment
Jass et al.	1986	[102]	447	R	MV	Y	St Marks Hospital
Jass et al.	1987	[143]	331	R	MV	Y	Same pts as above
Di Giorgio et al.	1992	[145]	361	CR	MV	Y	Curative resections
Ponz de Leon et al.	1992	[105]	134	CR	UV*	N	Only stage sig
Ronucci et al.	1996	[106]	275	CR	UV	N	
Ueno et al.	2002	[111]	638	R	MV	Y	St Marks again – Four more years
Ueno et al.	2002	[144]	627	R	MV	Y	LN negative, overlap with pts above
Nanni et al.	2002	[112]	263	C	MV	N	

*Only sig put in MV

2.4.6.5 Tumour Margin

Recent evidence suggests that the nature of the invasive margin (or advancing edge) of a tumour has prognostic significance. Tumours have two distinct margin types, a circumscribed margin (pushing or expanding) or an infiltrative margin whereby tumour extends irregularly into the surrounding tissues [102, 143, 146]. The infiltrative pattern was initially described in gastric cancers and in 1986 was reported in rectal cancers where it was found to confer a worse outcome in 447 rectal cancer cases [102, 143]. Ueno et al. subsequently examined the same patient set, with a further four years follow-up and did not find significance on adjusted analysis when all stages were included [111]. However, a separate paper (including stage B rectal cases only) found that the margin type was prognostically significant, suggesting that this feature may be more important before the tumour has metastasised [144].

Similarly, Roncucci et al found on multivariate analysis of 275 patients that an infiltrative margin conferred a worse survival in rectal but not colonic cancers [106]. However, Ponz de Leon et al found the margin had no effect on the outcome in 134 colon and rectal cases (Table 27) [105]. Thus, at the time of writing there was some evidence that the nature of the tumour margin may be important in rectal cancers, but little to suggest an influence on outcome for colonic lesions.

2.4.6.6 Tumour Budding

Budding is defined as clusters of undifferentiated cells at the invasive front of a tumour [147]. It is viewed as a sign of aggressive tumour biology and may be a prelude to lymphatic invasion [147]. There is a correlation with other aggressive tumour characteristics such as poor grade [111, 147], infiltrating margin [38, 111],

lack of lymphocyte infiltration [111] and advanced stage [111]. Despite these associations, budding has been shown to be independently associated with a worse outcome [111].

Hase et al. studied 663 colorectal cancer cases following curative resection and categorised the degree of budding as none/mild or moderate/severe [147]. It was more a feature of latter stage tumours with few stage A cases demonstrating significant budding. Recurrence rates were higher and survival worse in patients with moderate or severe budding. Unadjusted five-year survival for stage B cases with significant budding was 29.1% compared to 68.3% if there was little or no budding. The predictive value of this feature was observed in both rectal and colonic tumours and was independent of stage. However, as analysis was not adjusted for other potential prognostic indicators, the importance of budding as an independent predictor could not be stated.

Ueno et al. studied budding in 638 rectal cases and confirmed it to be independently predictive of outcome (adjusting for infiltrating margin, differentiation and stage) [111]. Interestingly, the association between infiltrating margin and budding was weak. Okuyama et al. also found budding to be prognostically significant on multivariate analysis of 317 stage 2 and 3 CRC cases following curative resection [148]. Significance was observed for both stages. They adjusted for standard pathological factors but not infiltrating margin.

The exact classification of budding and what is significant budding is yet to be clarified and as such there exists a degree of observer variation. Even within studies,

reproducibility was only moderate but did improve when simplified to two categories [111]. Whether it is a clinically useful prognostic marker is still to be determined.

2.4.6.7 Tumour Stroma

Tumour stroma is believed to be important in tumour viability and progression. The degree of tumour fibrosis has been found in some studies to affect survival [102] but not by all [106]. Ueno et al categorized the nature of tumour stroma into type A (fibroid), type B (keloid) or type C (myxoid) and studied its influence on outcome in 627 cases of rectal cancer [144]. Type A was the most common (63%) followed by B and C (25% and 12%). The type of stroma correlated with stage and grade. The percentage of cases with type B and C stroma increased in the more advanced tumours and as grade worsened. In contrast, lymphocytic infiltration was less prominent in type C stroma. Despite these associations, stroma was found to have independent prognostic value for cancer-specific deaths.

To date, the influence of this stromal classification had not been studied in colon cancer.

2.4.6.8 Lymphocytes

Several types of lymphocytic invasion have been described. Peritumoral lymphocytic infiltration describes conspicuous lymphocytes at the advancing tumour margin [143, 149, 150]. Crohn‟s-like lymphocytic infiltration is defined as lymphoid aggregates, often with germinal centres at the periphery of a tumour [150, 151]. Tumour infiltrating lymphocytes (TILs) are lymphocytes within the tumour epithelium (as

distinct from most tumour associated lymphocytes, which are found in the stroma) [50].

Lymphocytic infiltration at the advancing tumour edge is thought to represent the human body‟s immune response to the neoplastic process [149] [102] [50] though this is not firmly established. A tumour deplete of lymphocytic infiltrate might be expected to more readily progress and metastasise and hence be associated with a poorer survival. This has been demonstrated in several studies on adjusted analysis as detailed in Table 28 [91, 102, 111, 143-145]. Four of these five papers are from the same institution and included only rectal cases. Three of the four groups with cohorts inclusive of colon cancer failed to find lymphocytic infiltration to be significant in [105, 106, 112]. Possibly this factor is of greater prognostic significance in rectal cancers.

Research on the specific types of lymphocytic invasion is limited. Crohn‟s-like lymphocytes were first studied in 100 curatively-resected CRC cases, categorised as absent, mild or intense [151]. Of 100 cases, 78 demonstrated some degree of Crohn‟s–like infiltration. Their presence correlated with peritumoral lymphoid infiltration, negative nodes and was less likely in rectal cases. There was no correlation to vascular invasion or grade. Overall survival on univariate analysis was better in the cases with intense Crohn‟s-like lymphocytic invasion. In another study, the presence of TILs was assessed in 276 cases of CRC and found to correlate with earlier stage and to be independently predictive of improved survival on multivariate analysis [152]. TILs are common in MSI cancers and this association will be discussed later in this chapter [44, 50].

2.4.6.9 Apical Node

It may seem logical that if the node at the apex of vascular supply (and dissection) is positive, occult distal spread is likely and the apical node status should be prognostically significant. This was recognised as early as Dukes and Bussey‟s original study of rectal cancers, which showed that 5-year survival dropped from 40.9% to 13.6% once the apical node was involved [94]. Subsequent studies show that a positive apical node is associated with a significantly worse prognosis (Table 29). Wiggers et al. determined a hazard ratio of 3.59 on multivariate analysis for cancer related death if the apical node was involved [131]. Four Australian studies concur [12, 20, 63, 107]. Of these, Newland et al studied staging data on 1117 cases of CRC and found that cases with a positive apical node had a similar survival to stage D disease [63].

Two studies from St Mark‟s Hospital focussed on rectal cases (with some patient overlap). One of these studies showed that the apical node status was prognostically significant even on multivariate analysis [111] while the other did not [143].

The reliability of apical node status depends on the accuracy of identifying the apical node. Surgical technique varies including height of ligation and less clearance may diminish the usefulness of what is perceived to be the apical node. Apical node status is also not consistently reported. Despite these limitations, the weight of evidence suggests that it is prognostically valuable to determine the apical node status.

Table 29 Prognostic influence of apical nodal status

Study	Year	Ref	*n*	C/R	Analysis	Sig of apical node	Comment
Chapuis et al.	1985	[12]	709	CR	MV	Y	
Jass et al.	1987	[143]	331	R	MV	N	St Marks
Wiggers et al.	1988	[131]	350	CR	MV	Y HR 3.59	
Hermanek et al.	1989	[64]	597	R	MV		
Newland et al.	1994	[20]	579	CR	UV	Y	Stage C only
					MV	Y	
Ueno et al.	2002	[111]	638	R	MV	Y	St Marks again – Four more yrs
Ueno et al.	2002	[144]	627	R	MV		Overlap with above pts, LN negative cases
Chapuis et al.	2004	[107]	378	C	MV	Y HR 1.8	Stage C only, no chemo

2.4.6.10 Lymph Node Harvest

Lymph node harvest is important to accurately stage CRC. It places a case in the appropriate prognostic category and potentially improves outcome by ensuring adjuvant therapy is offered if applicable. This is certainly the case for lymph node negative disease and there is much evidence that inadequate lymph node harvest understages stage B CRC [153-156].

Lymph node harvest is dependent on two factors, thoroughness of pathological process and extent of surgery. There is no doubt that thorough examination of lymph nodes is important in histological assessment of the tumour to avoid understaging [108]. Better surgical clearance may affect outcome in two ways, by its associations (higher volume hospitals, better access to multidisciplinary care and increased likelihood of appropriate adjuvant therapy) and secondly, by potentially being therapeutic in clearing disease. It is this later mechanism that is postulated to account for any improved outcome in stage C disease [156].

Whether this is the case is unclear. One study drawing data from the Intergroup chemotherapy trial found that on multivariate analysis (with node number entered as continuous data) that there was a significant association between higher numbers and better outcome (HR 0.97) [156]. On univariate analysis, harvest of greater than 35 or 40 nodes for N2 and N1 disease respectively significantly affected outcome. However, numbers in these subgroups were relatively small, and there was little difference between the subgroups with less than 40 nodes. A similar analysis of the INTACC chemotherapy trial data found node harvest had no impact on outcome in

the stage C subgroup [154]. These studies involved large numbers but were not specifically designed to address this issue. Three other studies have failed to show that number of lymph nodes harvested was prognostic in stage C disease [153, 155, 157]. Thus, the prognostic importance of high lymph node harvest once lymph node positivity has been established is debatable.

2.4.6.11 Size

The assessment of tumour size as a prognostic factor has been complicated by the fact that many studies fail to distinguish between fresh and formalin fixed specimens and by the treatment of size as a dichotomised variable (i.e. greater than 5 cm). Table 30 shows ten reviewed studies that considered tumour size. On adjusted analysis, size was not found to be independently predictive of outcome by all but one study [12, 17, 20, 105-107, 113, 144]. The one study that did find significance is worth examining [131]. Size was divided into 3 groups. The hazard ratio of dying if the tumour was small was 1.0 (<3.5 cm), for medium tumors (3.5-6 cm) 0.66 and for large tumours (>6 cm) 1.68. It is hard to reconcile a survival advantage for medium size tumours. On balance, the weight of evidence suggests that size is not a useful predictor of outcome.

2.4.6.12 Morphology

Some studies have considered the prognostic role of tumour morphology (exophytic, sessile, ulcerative). Most have not found it to be significant [20, 91, 110, 131]. In most studies, any relevance associated with morphology was negated by adjusting for stage [110, 131]. Wiggers et al. studied 350 patients drawn from two prospective trials and found unadjusted disease-related death rate was less for polypoid lesions

compared to sessile or ulcerative lesions but that the effect was less pronounced after adjustment for stage [Wiggers, 1988 #95).

Table 30 Prognostic Influence of Tumour Size

Study	Year	Ref	*n*	C/R	Analysis	Size category†	Sig	Comment
Chapuis et al.	1985	[12]	709	CR	MV	<5 cm >	N	
Griffin et al.	1987	[113]	400	CR	MV	<4 cm >	N	
Wiggers et al.	1988	[131]	350	CR	MV	3 grps	Y	
Garcia-Peche et al.	1991	[110]	191	CR	UV	<5 cm >	Y	
					MV		N	
Newland et al.	1994	[20]	579	CR	UV*		N	Stage C
Ronucci et al.	1996	[106]	275	CR	UV		N	
Bokey et al.	1997	[17]	709	R	MV	<3 cm >	N	
Ueno et al.	2002	[144]	627	R	MV		N	LN neg
Ponz de Leon et al.	2004	[105]	134	CR	UV*		N	
Chapuis et al.	2004	[107]	378	C	MV		N	

*only factors significant on univariate analysis were entered into multivariate model
†Size category if given

2.4.7 Summary of Clinical and Pathological Prognostic Factors

While studies are mixed, it is possible that gender influences outcome from colorectal cancer, whereas the evidence suggests that neither tumour site nor patient age plays a part. Hospital and surgical operative volume and surgical expertise may have a minor influence on outcome in operative cases. Obstruction does not affect prognosis, while perforation significantly worsens outcome.

Pathological factors that have been shown to be associated with a worse outcome include vascular invasion, apical nodal status and poor differentiation, although tumour grade is not consistently predictive when other factors are considered in analysis. Tumour type does not independently influence outcome. There is some evidence that several of the newer factors (stroma type, budding, invasive margin and lymphocytic invasion) may be prognostically significant, possibly more so in rectal cancers. However, research on these factors is currently limited. Tumour size and morphology are not prognostically important.

2.5 MSI

2.5.1 Traditional Understanding of CRC Genetics

Colorectal tumourgenesis has been traditionally understood to be a multistep process. It was recognised that a series of genetic mutations and sequential clonal expansions were necessary for the development of CRC [158]. In 1990, Fearon and Vogelstein reviewed the genetics of CRC and proposed a "genetic model for colorectal tumourgenesis" the so-called adenoma carcinoma sequence [159]. Loss or mutation of the APC gene was recognised as an early event in tumourgenesis that, combined with DNA hypomethylation, predisposed to adenoma formation. Cumulative genetic mutations of kras and DCC cause cells to become increasingly dysplastic and p53 has been shown to be lost late in the process towards carcinoma formation. The genetic changes in tumourgenesis are in fact far more complex, though the principle that at least four or five mutations are required for carcinogenesis is well accepted and that it is the accumulation of defects rather than the order in which they occur that is important [159, 160].

The mutation rates of the more commonly affected genes include APC in 20-50%, kras in 50%, DCC in 70% and p53 in 75% [158, 159]. Mutations of these genes usually involve loss or alteration of one or both alleles (LOH, loss of heterozygosity), leading to either activation of an oncogene or inactivation of a tumour suppressor gene [160, 161]. As the process involves chromosomal alteration, tumours formed by this mechanism are said to exhibit chromosomal instability. They have abnormal numbers or parts of chromosomes and are thus aneuploid [161, 162].

The first colorectal familial syndrome to have its genetic basis defined was FAP. One defective APC allele is inherited from the affected parent along with a normal allele from the unaffected parent. All cells have this genotype but the one functioning allele is sufficient for normal cellular function. Cancer develops in cells that lose the normal (or so called wild type allele) and hence lose APC‟s gatekeeper function (Knudson‟s second hit phenomenon) [162]. It is postulated that environmental factors cause the loss of the normal allele, which would explain the propensity for development of GIT tumours over other organs [71]. It is the predisposition to polyposis/cancer development that is inherited in an autosomal dominant manner, with a subsequent event required for neoplastic transformation.

Different mutations occur in different families and the phenotype varies according to the genotype with respect to rate, extent and site of polyp formation, transformation and tendency to develop extracolonic manifestations

2.5.2 Historical Aspects of MSI

In the early 1990‟s several research groups simultaneously made discoveries that significantly changed our understanding of the molecular biology of colorectal cancer. HNPCC had been recognised as a familial subgroup of CRC with unique properties and much work was being undertaken to identify the genetic abnormality. In May 1993, three significant papers were published.

Peltomaki et al mapped HNPCC to an abnormality on chromosome 2 by linkage analysis, though the role of this gene was unknown [163]. The same international group noted an unusual genotype associated with HNPCC associated CRC. They

found variations in the lengths of dinucleotide (CA) fragments, suggesting errors had occurred in the replication process. The phenomenon was labelled “RER” or replication error. The authors proposed that this was the cause of carcinogenesis in HNPCC and proposed it as an alternative path to colorectal cancer [30]. The idea of genetic instability predisposing to cancer was not new and had previously been described in xeroderma pigmentum, ataxia telangiectasia and Bloom‟s syndrome [162]. Interestingly, they not only found this genotype in their HNPCC cancers but also in a subset of sporadic CRC‟s.

The same instability in dinucleotide repeats was independently and simultaneously reported by Thibodeau et al. [31]. They termed this “microsatellite instability” (MSI). They noted that the presence of MSI was inversely related to abnormalities in chromosomes 5q, 17p and 18q within a tumour, again supporting a different mechanism of carcinogenesis from the classical adenoma carcinoma sequence

In view of the fact that much of the instability is in unrelated genes, they suggested that unstable microsatellites are a secondary event rather than the cause of tumourgenesis. They noted also the tendency for these tumours to occur in the proximal colon and to have a better prognosis.

Later in this same year, Ionov et al. published similar work demonstrating “ubiquitous somatic mutations in simple repeated sequences”. They suggested that an inherited mutation leads to decreased replication fidelity (mutator mutation), instability in microsatellites and cancer [32]. Finally, Peltomaki et al. noted MSI in gastric and endometrial cancers but not in other cancers tested, adding to the association with HNPCC [164].

At this stage, while microsatellite instability had been linked to HNPCC, the responsible genes and their function in HNPCC remained unknown. Similar instability had been detected in bacteria and yeasts with mutations in MutL and MutS (leading to defective mismatch repair) [165, 166]. Yeast homologues of these genes - MSH2, MLH1 and PMS2 - were investigated by Strand et al and confirmed to be associated with tract instability [166]. Leach et al. looked for a homologue of these genes in humans and found a locus homologous to MutS (MSH2) on the previously recognised HNPCC gene on 2p, thus suggesting that the HNPCC gene defect led to a defect of mismatch repair [33]. As cancers were not found in all tissue, they concluded that the inherited mutation causes one defective allele and the second allele must be inactivated for a cancer to develop (a two hit phenomena) [33]. Parsons et al. went on to confirm biochemically that the increase in mutability of repeat sequences (RER) is indeed due to lack of mismatch repair [34].

Shortly after, a locus on chromosome 3 was linked to HNPCC and found to be homologous to MutL (MLH1) [167] and, at the time of writing, a further five genetic loci were linked to HNPCC (detailed shortly).

While the genetic cause for microsatellite was being elucidated, it became increasingly apparent that a proportion of sporadic CRCs demonstrated MSI and yet had intact MMR genes. Thibodeau et al found only 7 of 19 MSI tumours had mutations in MLH1 or MSH2 despite lacking protein expression of either MLH1 or MSH2 [168]. They suggested another mechanism may be responsible, possibly an alteration in the promoter region of the MMR gene. It was not until 1997 that Kane et al demonstrated methylation of the promoter sequence of MLH1 caused transcriptional silencing and thus loss of MMR function [35]. This epigenetic

phenomenon was recognised as an alternative to HNPCC (genetic mutation) as a cause of the same phenotype of microsatellite instability and CRC.

To summarise, microsatellite instability is an alternative to chromosomal instability as a pathway to CRC. MSI may be due to either genetic mutation (germline or acquired) of a mismatch repair gene or acquired methylation of the gene, both resulting in lack of mismatch repair, leading to genomic instability and cancer.

2.5.3 Definition of Microsatellite Instability

Microsatellites are sequences of repeated bases occurring throughout the genome, predominantly in non-coding (intronic) DNA. They have no known function. Between one to six bases may be repeated producing mononucleotide repeats (i.e. PolyA– AAAA, with corresponding TTTT), dinucleotide repeats (i.e. CACACA), trinucleotide (i.e. GGCGG), etc. CA repeats are the commonest, occurring approximately 50,000 to 100,000 times throughout the genome [31, 169]. There are two alleles of each microsatellite that are inherited by Mendelian inheritance. Some microsatellite sequences are identical between individuals (monomorphic); others vary slightly (quasimonomorphic - variation not exceeding two nucleotides), while most are highly variable (polymorphic). Given the Mendelian inheritance, these polymorphic sequences are individually unique, which is the basis for DNA fingerprinting.

These sequences are duplicated during replication as per any DNA sequence, by the action of DNA polymerases. However, during strand replication they are particularly prone to error as the repeats appears to „confuse" the process [32, 34, 166, 170]. While

the DNA polymerase is creating a template strand from the primer, it is common for slippages to occur, whereby bases do not align appropriately [36, 166]. The common errors are base/base mismatch or insertion/ deletion loops (IDL) [171]. The risk of replication error appears to be proportional to the length of the repetition. Microsatellites do occur in coding as well as non-coding DNA but the sequences are shorter and hence less prone to error [170, 172].

Replication fidelity is maintained by the mismatch repair system (MMR) [166]. MMR proteins detect mismatched bases, excise the defective template sequence and initiate the creation of a new strand. If this system is faulty and the mishap not repaired, the mismatch or IDL causes the microsatellite sequence to be either extended or, more usually, truncated [30, 169, 170, 173]. Microsatellite instability is therefore defined as a change of length of the microsatellite [174]. With the repair system malfunctioning, escalating microsatellite sequences will be affected resulting in ongoing genetic mutation, termed "mutator phenotype". Hence, due to their susceptibility to error, microsatellites are useful indicators of MMR function. This causal link between MSI and deficient MMR has been established experimentally. Cell cultures deficient in MMR proteins show MSI, but become stable on addition of either chromosome 2 or 3 [175, 176].

As microsatellite instability occurs predominantly in non-coding DNA, it is unlikely to be responsible for carcinogenesis. Defective mismatch repair has wider genomic consequences and it is the generalised genomic instability and the lack of repair in vital genes that is responsible for tumourgenesis (discussed further in subsequent sections) [36]. Hence microsatellite instability is a marker for tumours associated with defective MMR but not the cause.

What was initially unclear was the extent or spectrum of instability that was required before becoming associated with clinical significance (i.e. indicative of a pathological MMR defect and thus tumour phenotype). Dietmaier et al. attempted to answer this question by testing 58 CRCs using 31 markers and correlated MSI status with MMR immunohistochemistry (IHC) [170]. MSI was defined as instability in greater than 20% of markers. Fourteen of the 15 tumours meeting this criterion showed corresponding loss of MMR protein on IHC and 10 had characteristics of HNPCC tumours. In contrast, none in the group with less than 20% unstable markers demonstrated loss of the MMR protein and only four had characteristics associated with HNPCC. This suggests that instability in more than 20% of markers correlates with a particular phenotype and therefore is a valid percentage cut-off. The most frequent and reproducible, unequivocal length shifts occurred in mononucleotides especially BAT25, 26 and 40.

In 1997, the third workshop of the National Cancer Institute established uniform criteria for diagnosing MSI. The recommended panel consisted of 5 markers, 2 mononucleotides (BAT26 and BAT25) and 3 dinucleotides (D5S346, D2S123, and D17S250) [174]. The presence of instability in two or more (≥ 30%) of markers represents MSI high (MSI-H). If only 1 of 5 (< 30%) is unstable, this is labelled MSI low (MSI-L).

From within this panel, BAT26 has proved particularly useful. It is quasimonomorphic and as such the sequence has a similar number of base pairs (length) in all individuals [177]. There is a bell curve distribution of variation. This

uniformity makes BAT26 a particularly accessible marker because, once standard length is established on testing, no normal tissue is required for comparison.

BAT26 instability has been established as highly specific for MSI high [40, 41, 45, 52, 170, 177, 178]. Dietmaier et al. studied 58 CRCs using 31 markers and correlated MSI status with MMR immunohistochemistry (IHC) [170]. They found the most frequent, reproducible and unequivocal length shifts occurred in mononucleotides especially BAT25, 26 and 40. Loukola et al. established MSI status for 497 CRC cases using the Bethesda criteria and compared this with BAT26 results [178]. BAT26 instability was found in all 27 cases in which a mutation was identified. Interestingly, two other cases that were determined to be MSI-H using the NCI panel did not show a mutation in MLH1, MSH2 or MSH6. Hoang et al. established "RER" status in 160 colorectal tumours and cell lines using 32 microsatellite loci [177]. BAT26 correctly determined MSI in 159 of the 160. Other researchers support the sensitivity of BAT26 for MSI-H [40, 41, 45, 52].

There is much debate as to whether MSI-L tumours are phenotypically different from MSS tumours. It is likely that if enough microsatellites are examined, at least one will be unstable, the significance of which is unclear. MSI-L tumours in fact express MMR proteins and thus are MMR proficient [41, 170]. The term was retained until further research clarifies the clinical relevance of this finding. It should be noted this criteria applies to CRC‟s and the significance of markers in other HNPCC cancers was not evaluated.

Many pseudonyms have been used for microsatellite instability since its inception. These include RER (replication error), MIN, MI, MMP (microsatellite mutator

phenotype) and USM (ubiquitous somatic mutations). The above working party deemed MSI should be the standard nomenclature [174].

2.5.4 MMR Genes

There are now many loci identified as mismatch genes in humans and the number is escalating (see **Table 31**) [33, 167, 179-182].

Much of our understanding of the function of the mismatch repair proteins in humans extends from our understanding of their function in bacteria and yeast [166, 167]. The mismatch repair genes MutL, S, H, and U were recognised in prokaryotes and yeast [165, 166]. MutS is important for mismatched base recognition and binding. MutL binds this complex and triggers the endonucleolytic activity of MutH and excision of faulty strand, which is then replaced by the action of DNA polymerase and completed by DNA ligase [36]. The relevant humans homologues of MutS are hMSH2, hMSH3, and hMSH6 and the homologues of MutL are hMLH1, PMS1 and PMS2. MSH2 and MLH1 are considered vital for mismatch repair and interact with the others to complete their function.

As per the prokaryocyte model, MSH2‟s function is one of recognition and binding [181]. MSH2 forms a heterodimer with either MSH3 or MSH6 (MutSα equivalent is a dimer of MSH2 and MSH6 and MutSβ a dimer of MSH2 and MSH3) [180, 181]. Both complexes (MSH3 or MSH6) have different roles, recognising different types of base mismatch or IDLs [54, 181, 183]. Each is considered partially redundant with overlapping function. Therefore loss of either does not produce full instability**,**

whereas a combined defect of MSH3 and MSH6 in cell lines causes dysfunction of mismatch repair, with widespread instability consistent with a full mutator phenotype.

Replacement of either gene leads to reduced instability [36, 181]. MSH6 mutation creates predominantly mononucleotide instability [180] while MSH3 affects more di, tri and tetranucleotides [181].

The causal link between deficiency in the above MMR genes and a microsatellite unstable phenotype was confirmed in human cell cultures [181, 184]. Umar et al found that cell cultures deficient in either MSH2 or MSH6 demonstrated microsatellite instability. Restoration of MSH2 and MSH6 by addition of chromosome 2 corrected the instability and restored sensitivity in both cultures, confirming that the MMR gene and their proteins are responsible for the phenotype (i.e. rather than a result of subsequent gene mutation due to genomic instability) [176]. Koi et al. confirmed the role of MLH1 in strand repair and microsatellite stability by demonstrating that MLH1 deficient cell lines showed lack of repair of intentionally damaged strands as well as exhibiting MSI [184]. Once chromosome 3 was added both repair and stability were restored.

MutLα equivalent is a heterodimer of MLH1 and PMS2. This complex binds the involved MSH2 dimer and initiates repair [36, 167, 179, 183 119]. The other two dimers of MLH1 (PMS1 and MLH3) are yet to have MMR function demonstrated in humans [171]. MSH 4 and 5 (homologues MutS 4 and 5) form a hetero-oligomer but have yet to have their role established [179]. Many homologues of the PMS genes exist and have mismatch repair function but disease association has not been identified [179]. A MutH equivalent has not been identified.

Table 31 Mismatch repair genes

Bacterial gene	Human homologue	Chromosomal location	Comment
MutS	hMSH2	2p22 – 2p21	Dimerizes with MSH3 or 6
	hMSH3	5	
	hMSH4	1p31	Dimerises with MSH5
	hMSH5		
	hMSH6	2p16	Also called GTBP- G/T mismatch binding protein, close to MSH2
MutL	hMLH1	3p21.3	Dimerises with PMS1 or PMS2 and MLH3. Only PMS2 complex has a MMR role
	hMLH3	14q24.3	
	hPMS1	2q31-33	MMR role not established
	hPMS2	7p22.2	Main MutL complex
	hPMS4		Multiple homologues of PMS4 and 5 exist and have MMR function
	hPMS5		

2.5.4.1 MMR Mutation Rates in CRC

Determining the clinically relevant mutation rate from the literature is difficult for several reasons. Detection rates in studies will vary according to how comprehensively mutations are sought. The pathogenicity of some mutations has not been established (i.e. polymorphisms or intronic mutation). The proportion of familial cases in studies varies. Some have higher rates due to a referral bias while population studies will have lower rates. Surviving patients are required for serological genetic testing and as such may represent a specific (better prognosis) phenotype and genotype.

One study from Finland of 509 consecutive cases of CRC in nine regional hospitals revealed a 12% "RER" (MSI) rate [185]. The markers used were appropriate and the cases not obviously selected. All MSI tumours were examined for mutations in MLH1 and MSH2. Ten cases were positive, giving a rate of MMR mutation in CRC of 2%. Despite only testing for two gene mutations, the mutation rate closely correlated with positive family history. No MSI mutation negative or MSS tumours had significant family history, suggesting all familial cases were detected and were caused by these two genes. Cunningham et al. found a similar rate in their study of MLH1, MSH2 or MSH6 expression in 257 unselected patients with CRC [41]. Seven germline mutations (4 MLH1 and 3 MSH2) were identified but only five were pathogenic (caused defective protein) giving a rate of mutation in CRC of 1.9%.

Dunlop et al. calculated population mutation carriage rates using previously determined CRC rates in carriers and mutation rates in CRC along with CRC

prevalence figures from Finland and US [84]. The population mutation carriage rate was calculated to be 319 per million or 1 per 3139 (0.03%, 95% CI; 1 per 1247 to 7626) [186].

2.5.4.2 Mutation Spectrum and Variants in CRC

MLH1 or MSH2 account for the majority of germline mutations in CRC [41, 170, 187, 188]. A small number of inherited cases are attributed to the other MMR genes with germline mutations detected in MSH6, PMS1 and PMS2 but not MSH3 or MLH3 [41, 189]. The largest study of gene mutation spectrum comes from John Hopkins Oncology Centre [190]. An analysis of 5 genes was performed on 48 cases from HNPCC kindred (non-modified Amsterdam criteria). There may be some selection bias given only surviving cases could be tested for mutation. Mutations were detected in 34 (71%) - 15 MSH2 (31%), 16 MLH1 (33%), 1 PMS1 (2%), 2 PMS2 (4%) and no GTBP (MSH6). This confirms the preponderance of MLH1 and MSH2 mutations in HNPCC. Similarly, Cunningham et al. determined of the 20% of 257 unselected cases of CRC that were MSI cancers, 94% lacked MLH1 and 6% lacked MSH2 [41]. Four patients were deficient of MSH6, but this coexisted with a lack of MLH1 (n=1) or MSH2 (n=3).

Somatic mutations of MSH3 and MSH6 have been demonstrated in CRC and cancer cell lines. Clinical cases of germline mutations of MSH6 have been reported but no germline defect of MSH3 [41, 171, 174, 191, 192]. Vogelstein‟s group identified MSH6 mutation in cancer cell lines in 1995 but found no such mutations in 20 HNPCC kindred (previously found to be negative for MLH1, MSH2, PMS1 or PMS2 mutation) [180]. Cell lines deplete of MSH6 showed predominantly mononucleotide

instability. Given the lack of clinical findings, they concluded that mutation in this gene alone was insufficient for clinically significant genomic instability. Countering this, a study of 146 MSH6 mutation carriers (other MMR genes were intact) showed an increase in relative risk of CRC, albeit less than that seen with MLH1 and MSH2 mutations [193]. Interestingly, only 6 of the 20 families from which the cases were sourced fulfilled the Amsterdam 2 criteria. While the rate of CRC cancer was lower, the rate of endometrial cancers was twice that seen in other mutations.

Additionally, a Japanese group found no MSH6 mutation in 6 classic HNPCC cases but did demonstrate germline mutation in one "atypical" case (three colorectal cancers in the index case, both parents having GIT cancers but all were over 50) [191]. In this case, the cancers showed predominantly mononucleotide instability and some dinucleotide instability. They suggested the specific phenotype caused by MSH6 mutation has reduced tumorigenic tendency.

Furthermore, Wu et al. demonstrated MSH6 mutation in 5 of 21 HNPCC cases but found most were MSI-L [194]. With this knowledge, Parc et al. studied 41 patients with MSI-L CRC. They identified several mutations but all were either intronic, polymorphisms or silent. MSH6 immmunohistochemistry was normal in all (thus there was functioning protein) and therefore the MSH6 mutations were not deemed pathogenic [192].

MSH3 mutation alone has been shown to cause microsatellite instability in yeast but has not been shown to be pathogenic in humans [171].

A combined defect of MSH3 and MSH6 may be of greater clinically significance. Cell lines with mutation of both show widespread instability, consistent with a full mutator phenotype. Replacement of either gene lead to reduced instability, supporting the fact they are partially redundant but in combination may be pathogenic [181]. Further study in endometrial cancer and cancer cell lines demonstrated the presence of a combined mutation in one of 16 of the cancers and in one cell line [195] [36].

PMS2 mutations have been demonstrated in a very few cases of CRC and PMS1 in one [171]. Partial redundancy of the PMS genes may explain this low rate [36].

It remains unclear if MLH3 is associated with CRC. Mutations have been demonstrated in normal and colorectal cancer tissue but overlap in both and therefore are not clearly pathogenic. Wu et al found that MLH3 mutation was associated with a specific phenotype (more tri and tetra nucleotide instability) but again its significance was unclear. Hienonen et al. analysed 30 CRC cases with a suspicious phenotype (17 familial, 7 MLH1 and MSH2 mutation negative HNPCC and 6 sporadic MSI)[196].. They identified 5 missense MLH3 mutations in the cancer cases, however, all but one also occurred in controls, as well as 3 mutations in controls only. None of the previously recognised mutations variants were detected. A pathogenic role for MLH3 was not demonstrated.

The types of mutations that occur within these genes are numerous [190, 197]. The International Collaborative Group on HNPCC commenced a database of mutations in 1994 [197]. In 1997 they published 126 recognised mutations but this has now escalated to hundreds [197]. Most pathogenic mutations are frameshifts or missense

mutations in MSH2 and MLH1 with a small number of variants in PMS2 and one mutation type only in PMS1.

2.5.4.3 Genotype/Phenotype Correlation in HNPCC

Unfortunately, there is no established genotype/ phenotype correlation between MMR gene mutations. Cases sharing the same mutation do not demonstrate a specific phenotype [190]. Within the ICG database, no clinical subgroup correlated with gene mutation type [188]. This is supported by a comparison of MSH2 and MLH1 phenotype by Vasen et al. that showed no difference in age-specific CRC risk, gender, site distribution and synchronous or metachronous tumour rate. There was, however, a trend to greater risk of endometrial cancer in older MSH2 cases [88]. A similar study by Lin et al. generally concurred but found a higher rate of rectal lesions and a significant increase in extracolonic tumours in the MSH2 cases (33% compared to 12% $p<0.001$) [81]. Minor clustering in families has been observed with ureteric and renal pelvis cancers as well as endometrial and ovarian cancers (though not necessarily together within the same family) while stomach and small bowel tumours are homogenously spread [86].

Some variance in the clinical manifestation of the rare gene mutations has been observed (as mentioned previously in this section). Akiyama et al. demonstrated MSH6 mutation in one patient with atypical HNPCC and suggest a specific phenotype with a lower tumorigenic tendency [191]. More recent work is showing a stronger correlation between MSH6 mutation and endometrial cancer [198]. Risk of CRC by age 70 was 69% for men versus 30% for women and 71% for endometrial cancer. Lui

et al. examined genotype/phenotype correlation in 48 HNPCC kindred and found no specific correlation including no ethnic or geographical correlation [190].

2.5.4.4 Inheritance of MMR Mutation

HNPCC (germline mutation of MMR gene) is inherited in an autosomal dominant pattern. One mutated allele is inherited from the affected parent along with a wild type (normal) allele from the other parent. Every cell contains the mutation but the wild type allele provides sufficient mismatch repair function for the cell to be phenotypically normal [199]. An epigenetic (acquired) event leads to inactivation or loss of the normal allele and therefore loss of mismatch repair function in that cell. This leads to microsatellite and genomic instability and cancer ensues [33]. This process has occurred independently in every cell that has undergone neoplastic transformation. Hence, it is the propensity to form cancers that is inherited in an autosomal dominant fashion. Alternatively, MMR gene mutation may arise as a de novo somatic mutation in a tumour.

Normal cells in HNPCC cases do show minor abnormalities. Parsons et al studied the heterozygote cells of several HNPCC patients and two controls [199]. Biochemical analysis of cells with one mutated allele and one normal allele (lymphoid and colonic epithelium) showed abnormal mismatch repair function. However, the cells were non-neoplastic, suggesting one allele provides adequate mismatch repair function. Several novel microsatellite alleles in BAT26 and BAT40 were detected but only on increased sensitivity PCR. This suggests that cells with an inherited MMR mutation will be phenotypically normal despite a decrease in mismatch repair function and will demonstrate MSI but at an insignificant level to be detected by normal methods. Koi

et al. supported this finding by demonstrating that MSI was no longer detectable once a normal allele was restored to MLH1 deficient cell lines [184].

2.5.4.5 HNPCC – Clinical and Molecular Correlations

All MMR gene mutation cancers are considered to be HNPCC cancers however the definition of HNPCC encompasses clinical and molecular criteria and there is considerable variability in correlation between clinical presentation and molecular findings. Several factors may contribute to this incongruity. Clinical criteria are often inconsistent and not all studies adhere to the Amsterdam criteria and some use the modified criteria. A lack of family history or inadequate family size may increase false negative rate. Mutation detection rates will vary, being dependent on the number of genes tested and how comprehensively each of those genes is tested (i.e. were all loci examined). Many groups only test MLH1 and MSH2 and therefore miss less common mutations (though this is likely to represent only a small number given that the majority of recognised mutations occur within these two genes). Additionally, there may be as yet unidentified mutations.

2.5.4.5.1 Mutation Rate if Amsterdam Positive

Not all cases meeting the Amsterdam criteria have an identified mutation. Generally, 70% of Amsterdam criteria positive cases have detectable mutations (Table 32). Cunningham et al. studied 225 cases of unselected CRC (where family history was available). Seven patients met Amsterdam criteria and three had a mutation (43%) when tested for MSH2, MLH1 or MSH6, while six of the nine who met the modified Amsterdam criteria had a mutation (67%) [41].

Lui et al. in their analysis of 48 cases fulfilling Amsterdam criteria (non-modified) performed molecular analysis on 5 genes (MSH2, MLH1, PMS1, PMS2 and MSH6) and detected mutation in 71% [190]. In another study, Thibodeau et al determined mutation status in 12 Amsterdam (non-modified) criteria and found only 3 (25%) mutations in either MLH1 or MSH2 [168]. In the 13 patients with ≥1 familial case, 31% had a mutation; why this rate is lower than others is unclear. Their mutation testing was limited to MLH1 and MSH2 but this should not significantly reduce the rate and only one of the mutation negative cases was actually MSI.

2.5.4.5.2 MSI Rate in HNPCC Clinical Cases

If the Amsterdam criteria were specific for HNPCC then all cases would be expected to show MSI (even when a mutation was not demonstrated). This is close to the observed findings in two reports but not all (Table 33). Lui et al. in their study of 48 Amsterdam positive cases found a MSI rate of 92% [190]. The NCI Working Party reported a similar rate, with one member‟s series showing a 95% MSI rate in Amsterdam positive cancers [174]. However, an earlier and smaller study indicated a lower rate of MSI of 25% (despite using 34 markers) [168] and Lothe et al. found a rate of 31% [200]. Strict clinical criteria were not used and might be presumed to account for this lower rate.

2.5.4.5.3 Fulfilment of Amsterdam criteria if Mutation Positive

On the other hand, not all families with recognised mutations fit the Amsterdam criteria implying insensitivity (high false negative). Studies vary between 40-70% correlation (Table 34). Correlation is heavily dependent on completeness of family history and availability, which is not always detailed. In 8 cases with mutations of either MLH1 or MSH2, Thibodeau et al. found only 3 (38%) met Amsterdam (non-modified) criteria (details of family history or completeness not given) [168].

Aaltonen et al. found 10 germline mutations (MLH1 or MSH2) in 509 CRC cases of which 7 met non-modified Amsterdam criteria [185]. However, nine of the ten did have one or more first degree relative with a HNPCC tumour. None of rest of the 63 MSI cases (or 509 overall) met the Amsterdam criteria, which suggests that mutation testing detected all HNPCC cases as defined by clinical criteria. The finding of three mutation positive cases that did not meet Amsterdam criteria but had a significant family history, suggests the criteria are too strict even in their modified form.

Cunningham et al. studied 257 consecutive unselected patients with CRC. Family history was available in 225 and of these, seven had a germline mutation and three met Amsterdam criteria [41]. Therefore, according to the Amsterdam criteria, over 50% were missed; possibly more if those without verified family history were included.

These results imply that the Amsterdam criteria are too restrictive and will miss a significant number of HNPCC cases. The belief that the majority of HNPCC cases

will exhibit MSI led to the creation of a broader set of guidelines (the Bethesda criteria) to prompt tumour testing for MSI (Table 35) [201]. If a patient with CRC has a suspicious family history or their tumour demonstrates certain characteristics, MSI testing should be undertaken. If positive, current best clinical practice suggests genetic counselling and screening for a mutation is warranted. It was anticipated that 15-20% of CRC would warrant testing. The criteria have been subsequently assessed to be 87% sensitive for MSI-H and 29% specific (i.e. 30% of those tested will be MSI-H) [202]. Further refinement of the criteria occurred in 2004 (see Table 36).

Table 32 Amsterdam and mutation correlation

Study	Ref	Amsterdam	% mutation	Tested
Cunningham et al.	[41]	9 Ams (mod)*	67%	MSH2. MLH1, MSH6
Lui et al.	[190]	48 Kindred	70%	MSH2, MLH1, PMS1, PMS2 and MSH6
Thibodeau et al.	[168]	12 Ams (NM)†	25%	MLH1 MSH2
As above		13 Fam Hx (≥1case)	31%	MLH1 MSH2

*modified
†non modified

Table 33 Rate of MSI in Amsterdam cases

Study	Year	Ref	Amsterdam	% MSI
Lothe et al.	1993	[200]	13 strong fam Hx	31%
Thibodeau et al.	1996	[168]	12 Ams NM	25%
Lui et al.	1996	[190]	74 kindred Ams NM	92%
Boland et al.	1998	[174]	394 Ams NM	95%

Table 34 Amsterdam criteria in mutation positive cases

Study	Year	Ref	Mutation *n*	Amsterdam Criteria met
Thibodeau et al.	1996	[168]	8	3 (37.5%)
Aaltonen et al.	1998	[185]	10	7 (70%)
Cunningham et al.	2001	[41]	7	5 (71%)

Table 35 Bethesda Criteria

[201]

Individuals with	
1	Cancer in families that meet the Amsterdam criteria
2	Two HNPCC-related cancers including synchronous and metachronous CRC or associated extracolonic cancers
3	CRC and a first degree relative with CRC and/or HNPCC-related extracolonic cancer and /or colorectal adenoma; one of the cancers diagnosed at age < 45 yrs and the adenoma diagnosed < 40 yrs
4	CRC or endometrial cancer diagnosed < 45 yrs
5	Right sided CRC with an undifferentiated pattern (solid or cribriform) on histopathology diagnosed at age< 45 years
6	Signet –ring cell type CRC diagnosed < 45 years
7	Adenomas diagnosed at age < 40 years

Table 36 Revised Bethesda criteria

[203]

Tumours from individuals should be tested for MSI if any of the following criteria is met	
1	CRC diagnosed in a patient who is < 50 years of age
2	Presence of synchronous, metachronous colorectal or other HNPCC-associated tumours regardless of age
3	CRC with the MSI-H histology diagnosed in a patient who is <60 years of age.
4	CRC diagnosed in one or more first-degree relatives with an HNPCC-related tumour, with one of the cancers being diagnosed under age 50 years.
5	CRC diagnosed in two or more first- or second-degree relatives with HNPCC-related tumours, regardless of age.

2.5.5 Methylation

It is apparent that, of all CRC cases around 15% will exhibit MSI and yet only approximately 2% are due to germline MMR gene mutation. There is now significant evidence that these non-germline MSI cases are caused by transcriptional silencing due to methylation of the promoter region of MLH1.

2.5.5.1 Normal Methylation State

Of the 3 billion base pairs in humans, 40% are CpG (cytosine phosphate bonded to guanine along DNA rather than hydrogen bonded across DNA strands). Normally 2-7 % are methylated (CH3 group attached to cytosine). CpG islands are CpG rich areas, 1-2 kb long found in the 5' region of about 30,000 (half) human genes [204]. They are rarely methylated.

Methylation of a gene promoter sequence causes gene silencing by preventing transcription. This process occurs normally in humans for X inactivation and gene imprinting (suppression of gene according to parentage i.e. in some genes only the allele from one parent will be expressed) [204]. Aberrant CpG methylation occurs in such conditions as Fragile X and acquired methylation is normally seen with ageing and may be associated with carcinogenic exposures [204]. Methylation can be reversed with demethylating agents 5-aza-2-prime-deoxycytidine or 5-azacytidine, which reactivates gene expression [205].

2.5.5.2 Methylation in CRC

In the mid-1990s, it became apparent that some MSI CRC cancers were in fact diploid (complete chromosome compliment with no allele loss) suggesting that genetic mutation was not the cause of the MMR defect [30]. As mismatch repair protein expression was lacking, an alternative mechanism of gene inactivation was assumed [168]. In 1997, Kane et al. were the first to identify the cause. They isolated four tumour cell lines that failed to transcribe MLH1 protein but had no mutation [35]. They sequenced the promoter region of MLH1 and demonstrated reactivity consistent with methylation that was not observed in the normal cells.

Other researchers have confirmed that hypermethylation of cytosine at the 5′ CpG promoter region of MLH1 is responsible for the epigenetic silencing seen in sporadic MSI tumours [205-208]. The association was confirmed by demonstrating lack of protein expression and repair when MLH1 gene is methylated and that the protein is re-expressed when methyl group is removed with 5'-aza-2'-deoxycytidine and repair function returns [205, 206].

MLH1 methylation has also been demonstrated in MSI endometrial cell lines but not other cancers [205] and does not occur to any degree in normal cells (colon or other) [35, 209]. Biallelic methylation is required for the phenotype [206]. Herman et al did find MLH1 methylation in 2 non-MSI cancers [205] but assumed this was partial or single allelic methylation, insufficient to cause MSI phenotype. (Accuracy of MSI status could not be determined, as the markers used were not detailed; these may have in fact been MSI on other measures).

The only MMR gene to be involved in methylation-caused CRC is MLH1 [41]. Methylation of MSH2 and the other MMR genes has not been demonstrated [205]. MLH1 methylation has been demonstrated in 70-84% of sporadic MSI tumours [41, 205, 210]. Why all "sporadic" cases do not show methylation is unclear. Most studies determine cases to be sporadic by testing for and excluding genetic mutations, however it is likely that a percentage of these non-methylated MSI cases are still due to mutation. Methods of MSI determination and methylation detection vary and presumably insensitivity of tests accounts for some of the non-detection along with the possibility of yet to be defined mutations.

A percentage of cases that do have a demonstrable mutation also show MLH1 promoter methylation suggesting this may be responsible for the "second hit" inactivation of the normal allele [41, 205]. This is debated by others, who failed to find any MLH1 methylation in CRC cells from HNPCC families [210].

2.5.5.3 Multi Loci/CIMP

Shortly after MLH1 methylation was described, Ahuja et al detailed the methylation pattern of MSI cancers [211]. They found that many genes other than MLH1 were methylated in CRC and at a rate higher than seen in MSS tumours. The main genes were p16 (60% vs 22%), Thrombospondin-1 (angiogenesis inhibitor, 27% vs 0%), insulin like growth factor 2 (60% vs 6%) and hypermethylated in cancer-1 gene (80% vs 38%). Herman et al. supported this finding, showing methylation of P16 gene in 5 of 23 MSS tumours compared to 60% in 15 MSI tumours [205]. Given the nature of the genes involved (P16 is a tumour suppressor gene and THBS1 an angiogenesis inhibitor) their silencing may be important in tumourgenesis.

Toyota et al. furthered the concept of multiloci methylation in 2000 when they described the entity CpG island methylator phenotype (CIMP) [207]. They identified 26 newly methylated CpG islands in CRC (MINT loci), 19 of which were also found in normal cells and therefore attributed to the ageing process. However, seven were unique to CRC and they used these to identify a distinct subgroup they labelled CIMP (≥3 methylated genes from a panel of 6). This sub group was examined for correlation with other gene mutation and methylation. Not unexpectedly, methylation of MLH1, p16 and THBS1 were much more common in CIMP. Only 1% of 21 CIMP negative cases showed MLH1 methylation. However, not all cases of CIMP were observed to have MLH1 methylation or MSI. Of 29 CIMP cases only 41% had MLH1 methylation [204]. Of MSI tumours, 75% have been shown to be CIMP positive [204]. In short, there is considerable overlap in these conditions but not complete concordance.

As the pattern was not consistently found in all CRCs they proposed two mechanisms of tumourgenesis based on the pattern of methylation. Type A methylation as seen in ageing cells and some CRC and type C seen in CIMP (not observed in any normal cells). Tumours formed by CIMP did not correlate with age, gender or stage but had a proximal tendency. Regarding proximal cancers, 82% were CIMP positive compared with 37% of distal cancers [204]. These results were not tested for confounding influences and given the overlap with MSI the significance of the findings may be questioned. The importance of CIMP is yet to be established.

To further highlight the differences in tumour characteristics, the same group investigated the methylation rates of genes associated in 41 unselected CRC cases of

which 51% were CIMP positive [207]. Kras mutations were significantly higher in CIMP positive cases (68% vs 30%) while p53 mutation was higher in CIMP negative cases (60% vs 24%), regardless of MSI status. Contrary to previous studies, MSI rates were similar whether CIMP positive (24%) or negative (21%). However, within the CIMP positive cases, MSI positive status did correlate with high rate of TGF methylation (88%) whereas kras was more prominent in the MSS/CIMP positive tumours. Stronger support for varying mechanism of tumourgenesis came from the finding of only a 5% overlap between P16 and P53 inactivation. Similar rates were found in adenoma suggesting extensive methylation occurs early in tumourgenesis.

Whether CIMP is indeed a distinct entity or part of the spectrum of global methylation is yet to be established. What is evident is that methylation of MLH1 is usually associated with multiloci methylation and appears to occur early in tumourgenesis. Methylation may be the mechanism of tumourgenesis in some MSS tumours mediated by a different set of genes such as kras and the genes involved in non-methylated tumours are different again (i.e. p53 versus P16).

2.5.5.4 Cause of Methylation

It is believed that epigenetic silencing of MLH1 by methylation is the initiating event in sporadic MSI tumourgenesis. What causes this methylation is unknown. Neither ageing nor carcinogen exposure are likely to fully provide the explanation. Ageing does cause widespread methylation (i.e. IGF2 and ER) but as discussed previously, the pattern is different than that observed in MSI tumours [204].

The aberrant methylation at multiple other gene loci may be a consequence of the mutator phenotype rather than related to the initiating cause. MMR deficient cells have a propensity to methylate genes that is not observed in MMR proficient cells [211]. Introduced exogenous DNA (via retrovirus) becomes methylated only in MMR deficient cell lines [212]. Methylation of these other genes has been shown to be an acquired process rather than inherent in the original tumour cell clone [212].

How the mutator phenotype could lead to aberrant methylation is unclear. Given the extent of methylation, a defect of regulation seems likely and could be caused by genomic instability. The nature of the defect is not known. DNA methyltransferase (methylating enzyme) has been implicated. An abnormality of this enzyme through mutation due to the genomic instability is possible though levels and activity have been shown to be normal in methylator phenotypes [204, 211]. Other explanations include lack of protection against denovo methylation [204] or lack of removal. It may be that methylation occurs but is normally corrected by MMR proteins [211]. Alternatively, methylation may be triggered by microsatellite alterations [211].

Challenging these theories is the previously discussed concept of CIMP. If extensive methylation occurs in a subgroup of CRC not always related to MSI and early in tumourgenesis, then mechanisms other than defective MMR must be involved but have not yet been determined.

2.5.6 Tumourgenesis in MSI

The exact mechanism of tumourgenesis in MSI cancers has not been established. It is assumed the loss of MMR function initiates the genomic instability that then affects

genes with a regulatory or tumour suppression role and hence leads to neoplastic change. These genes vary from those traditionally found to be abnormal in CRC. It is possible that in sporadic MSI cases methylation of genes other than MLH1 may play a part in tumourgenesis.

2.5.6.1 Precursor Lesions

If MMR malfunction is the cause of cancers, then MSI would be expected to be an early event in carcinogenesis given that microsatellites are the most susceptibly part of the genome. It would therefore be expected that this would occur in precursor lesions and polyps should demonstrate MSI. Limited work has been done in this area and microsatellite instability has been detected in adenoma but to a lesser extent than CRC [32, 174, 191]. At the second meeting of the NCIO, a research group from Japan (Baba in [174]) presented work showing MSI in 3% of sporadic adenoma but in all the HNPCC adenoma (details not given though all families were high risk). Akiyama et al. in their study of MSH6 found both alleles mutated in the CRC of the one patient with inherited MSH6 mutation. In contrast, two adenomata from the same patient showed only subtle dinucleotide instability [191].

2.5.6.2 Correlation MSI and Traditionally Abnormal Markers

MSI tumours have been shown to have a negative correlation with factors involved in the traditional CRC tumourgenesis pathway (Table 37). It has been demonstrated that MSI tumours are usually diploid (Table 37) suggesting that allelic loss (in traditional genes or otherwise) is not the mechanism of tumourgenesis [31, 51, 173, 200, 213].

Examination of the correlation between MSI and traditional markers in CRC is important in the study of prognostic significance and chemotherapy effect. It may be that the effects we are attributing to MSI are in fact due to a negative correlation with other molecular markers, which may be the more important determinants of outcome.

2.5.6.3 Factors in MSI Tumourgenesis

Several alternative factors have been implicated in the tumourgenesis process in MSI. The most important are transforming growth factor-β type II receptor gene (TGF-β-R11), BAX gene, insulin-like growth factor gene (IGF) and p16. Increased mutation frequency in all these genes has been found in HNPCC tumours [174]. Repetitive sequences do occur within the exonic sequences of these genes, although they are usually shorter and therefore less prone to error [170].

The TGF-β receptor gene is a tumour suppressor gene that contains a 10 repeat poly A tract in exon 9 BAT R 11 [172] and inhibits cellular proliferation by blocking the cell cycle [214]. A frameshift has been observed in 90% of HNPCC tumours [169] and mutation of TGF- β- R11 has been often demonstrated in MSI tumours [173, 214, 215] and infrequently in MSS tumours (86% vs 0.6% [216]).

The BAX gene contains a (G)8 microsatellite in its coding region, which has been found to be truncated and inactivated in 37% of MMR deficient tumours leading to disruption of the apoptotic pathway [169, 214].

Methylation of genes may have significance in tumourgenesis. There is high concordance between methylation of both P16 (tumour suppressor gene) and insulin-like growth factor (regulatory and growth role) and MSI [209, 211].

Much ongoing work in this field will hopefully elucidate the molecular events of tumourgenesis in MSI cancers in the near future.

Table 37 Rates of mutation of other factors in MSI vs MSS

Study	Year	Ref	*n*	p53	APC	kras	Association between diploid state and MSI	Study population†
Ionov et al.	1993	[32]	*	Neg correlation		Neg correlation		N/S**
Lothe et al.	1993	[200]	252				Sig ‡	N/S
Thibodeau et al.	1993	[31]	90				Sig	N/S
Kim et al.	1994	[42]	137	18% vs 44%				Stage B and C
Konishi et al.	1996	[173]	227	13% vs 55%	20 vs 60%	7 vs 50	Sig	HNPCC cases
Halling et al.	1999	[51]	508				Sig	Chemotherapy trial patients
Feeley et al.	1999	[213]	50	NS			Sig	Sporadic
Salahshor et al.	1999	[216]	191	4.5% vs 60%	23% vs 57%	18 vs 35% ns		Consecutive, sporadic
Gafa et al.	2000	[45]	216	22.7% vs 54.1%			17.7% vs 82.5%	N/S
Ward et al.	2001	[37]	302	18% vs 55%		16% vs 29% ns		Consecutive, sporadic
Wang et al.	2003	[217]	396			29% vs 40% ns		Stage 2
Watanabe et al.	2004	[214]	460	27% vs 52%				Stage 2 or 3 chemotherapy trial patients

Not all data was detailed in studies.
* Number non-specified but >200
†Study population was included in comments if specified.
**NS = not specified
‡ Significant

2.5.7 MSI Rates in CRC

The cited percentage of CRC that are MSI positive varies between 9 to 20% (Table 38 and Table 39). Testing variation may partially explain study differences. Prior to the establishment of a panel, inconsistent markers were used for diagnosis. Selection bias is also likely to contribute. Many groups studying MSI tumours have an interest in HNPCC that may affect referral patterns and produce a higher rate of MSI cases. In others, HNPCC cases are not always identified or excluded. Even the unselected groups usually comprise surgical cases and therefore may represent a select group (possible less aggressive case therefore higher MSI).

The more robust studies confirm a rate of between 10 and 20%. Ward et al. determined the MSI rate in 302 consecutive cases of sporadic CRC cases undergoing curative resection [37]. Family history was obtained, verified and HNPCC excluded. The NCI panel was used for diagnosis. MSI-H was found in 10.6% (and MSI-L in 6.8%). The Mayo Clinic prospectively analysed 257 CRCs from unselected consecutive patients and found MSI-H in 51 (20%) using a slightly extended panel [41]. A large population study from Finland of 509 consecutive cases of CRC from 9 regional hospitals revealed a 12% “RER” (MSI) rate, again with a slightly extended panel [185].

Table 38 MSI rate in sporadic CRC

Study	Year	Ref	*n*	%MSI	Inclusion*	Markers
Ionov et al.	1993	[32]	†	12%	Not stated	Various loci
Lothe et al.	1993	[200]	207	17%	No family Hx subgroup	7 loci
Konishi et al.	1996	[173]	227	17%	Sporadic	>3 of 5 loci
Aaltonen et al.	1998	[185]	509	12%	Consecutive population	≥2 of 7 or BAT26 (13% positive)
Jass (in Boland et al.)	1998	[174]	303	9%	Sequential	2 mono, 3 dinuc, 1 tetra
Salahshor et al.	1999	[40]	191	12%	Unselected	3 mono, 3 dinuc, ≥3
Feeley et al.	1999	[213]	50	10%	Sporadic	2 mono, 2 dinuc
Malkhosyan et al.	2000	[218]	511	12%	Unselected	2 mono, 1 dinuc
Gryfe et al.	2000	[46]	607	17%	Population, < 50 yo	5-10 loci
Ward et al.	2001	[37]	302	10.6%	Unselected (curative resection) prospective, known HNPCC excluded	NCI panel
Cunningham et al.	2001	[41]	257	20%	Unselected, consecutive	6 dinuc loci and BAT26
Wright et al.	2003	[38]	458	19.4%	Consecutive	Immunohistochemistry MLH1 and MSH2
Lim et al.	2004	[53]	248	9.3%	Consecutive	BAT26

*Inclusion criteria as stated in studies
†Number not specified but > 200

Table 39 MSI rates in select groups

Study	Year	Ref	*n*	%MSI	Inclusion	Markers
Kim et al.	1994	[42]	137	13%	B & C sporadic, consecutive	≥2 dinuc
Halling et al.	1999	[51]	508	15%	Stage B2 and C chemotherapy trial pts	11 dinuc
Wright et al.	2000	[52]	255	9%	Stage C	Panel, >40%
Hemminki et al.	2000	[43]	282	12%	Stage C	Varied > 7 or BAT26
Elsaleh et al.	2000	[21]	656	9%	Consecutive, stage C	BAT26
Ribic et al.	2003	[48]	570	16.7%	B and C, multicentre	Mixed testing – 2 to 11 markers, some BAT 25 and 26 only as no normal tissue

2.5.8 Clinicopathological Characteristics of MSI CRC

Given the different molecular events in MSI and MSS tumours it is not surprising their phenotype varies. Most studies report findings for MSI-H only or include MSI – L with the MSS cases and this can be assumed to be the case in this review unless otherwise stated. As expected, there is overlap in the clinical features of HNPCC (germline) and sporadic MSI tumours with some notable differences. Not all studies of MSI tumour characteristics separate the HNPCC cases, which may distort findings. Even if an attempt is made to exclude HNPCC cases, a significant number go unrecognised. However, given that the proportion of HNPCC is low, the impact on results should be small.

2.5.8.1 MSI CRC and Age

The evidence for an association between MSI and age in sporadic CRC is mixed. Table 40 shows eight studies that found no significant age variation between MSI cancers cases and MSS, although the MSI cases tended to younger [37, 38, 41, 42, 47, 48, 53, 213].

Not all these studies recognised or excluded HNPCC cases, which may have led to a lower median age for MSI cases overall, whereas sporadic MSI cases may be older. This is supported by two studies. Malkhosyan et al. studied 511 unselected CRC cases and determined 12% were MSI [218]. Of these around half were due to mutation and methylation was confirmed in the remainder. The methylated group was 18 years older on average (70.8 versus 52.9 p=0.0001). The second study was from Salahshor

et al. who found a non-significant trend to a higher MSI rate in the older patient group compared to the younger cohort (16% vs 7%) [40].

A large study from the Mayo Clinic found a correlation between older age and sporadic MSI cases [219]. The rate of MLH1 loss in different age groups was studied in 867 patients. The group consisted of predominantly young (<40 years) or aged (>90 years) patients but there was no selection bias within each age category. No MLH1 loss was found in tumours of the younger group while 29% of older cohort was found to be MLH1 deficient. The correlation persisted on multivariate analysis. Jass came to a similar conclusion after reviewing the literature, despite the fact that not all studies concurred [220].

Alternatively there are a significant number of studies that do not suggest an association between MSI and age. Wright et al. showed no increase in MSI with advancing age (though few in the study were in the younger group) [38]. Gryfe et al. in their research found MSI in 17% of 607 patients under 50 years old [46]. However, 15% of the MSI group met Amsterdam criteria and if excluded the MSI rate was 10%. Lukish et al., in contrast, reported a high rate in their young cohort (47% of 36 cases <40 years old) [57]. There was a male predominance due to being a naval medical centre and non-standard markers were used for "RER". Despite this, there was no obvious explanation for their findings.

HNPCC cases do occur in a younger population with a median age of 44-46 years [75] [78]. A difference in age of onset between HNPCC and sporadic MSI would not be surprising given that a germline MMR defect requires only one further event for tumourgenesis whereas sporadic MSI tumours require two. Methylation (the main

cause of sporadic cases) is an acquired process that presumably occurs later in life. Studies that do not separate HNPCC cases (most do not) will cause a skew in results to a younger median.

Jass proposed that the varied results between study groups (other than inclusion of HNPCC cases) might be due to the influence of environment, race and familial factors on rate of methylation [220].

2.5.8.2 MSI CRC and Gender

For CRC generally there is near equal gender distribution but a trend to male predominance in some populations (as previously discussed). In contrast, there is usually a female predominance in MSI-H tumours. Ward et al. in a study of 302 consecutive cases found 68% of cancers in women exhibited MSI compared to only 40% of tumours in men [37]. Wright et al. studied 458 consecutive cases and found 70% of female cases were MSI compared to 50% in men [38].

Some studies have found a higher MSI rate in women compared to men in the older patients [45, 219]. Kakar et al. in an older cohort found 24.3% of female cases were MSI compared to 11.5% of male cases [219]. Significance persisted on multivariate analysis with the odds ratio of MLH1 loss for women being 1.85 (95% CI, 1.24-2.75). Gafa et al. found no gender variation except in the patient group over 75 years old, where MSI rate was higher in women [45]. Within each gender group, the rate of MSI positive cancers is similar (of the order of 10-20%) [40, 45, 46, 52, 53, 213].

Selection in studies, in particular regarding age, may produce biased results given the predominance of women in late onset CRC. As above, the female predominance in MSI was more noted in the older age group [45, 219]. Gryfe et al. only included patients less than 50 years of age in their population study of 607 CRC cases and found equal gender distribution [46]. Notably, studies that use trial cases tend to exclude older cases (either because of an age cut off or indirectly by excluding unwell patients), which may explain a lack of female predominance [47].

2.5.8.3 MSI CRC and Site

There is a right-sided preponderance for MSI CRCs. Overall, around 30% of CRC occur in the right colon and MSS cases tend to be left-sided while most MSI cancers are proximal (71% to 94%)(Table 41). MSI is detected in around one third of proximal cancers (20%-36%) compared to less than 10% of distal cancers (1-12%) (Table 42). The difference is even more pronounced in women. Wright et al. found 41.7% of right-sided cancers in women were MMR deficient compared to 25.3% in men [38].

This proximal trend is also observed in HNPCC but is less pronounced [52]. As discussed in section 2.4.4, there is currently no adequate biological explanation to explain CRC site variations. The same is true for the MSI predilection.

Table 40 Age variation MSI and MSS

Study	Year	Ref	*n*	MSI %	MSI age years*	MSS age	Sig	Group
Kim et al.	1994	[42]	137	13%	60±5	66±1	N	Sporadic, consecutive
Feeley et al.	1999	[213]	50	10%	66.3	67.8	N	Sporadic
Alexander et al.	2001	[47]	323	28%	63	64	N	Sporadic
Ward et al.	2001	[37]	302	10.6%	No difference (no figures)		N	Unselected, HNPCC excluded
Cunningham et al.	2001	[41]	257	20%	% <50yo same in both grps		N	Unselected, inc HNPCC cases
Ribic et al.	2003	[48]	570	16.7%	60.7	59.7	N	Stage B and C, trial patients
Wright et al.	2003	[38]	458	20%	73	75	N	Consecutive
Lim et al.	2004	[53]	248	9.3%	56.6	60.4	N	Curative, sporadic, consecutive

*Age as median or average

Table 41 Proximal location in MSI cases

Study	Year	Ref	*n*	MSI %	MSI % proximal	MSS % proximal	Study group
Kim et al.	1994	[42]	137	13%	94%	34%	B & C sporadic, consecutive
Bubb et al.	1996	[39]	215	10.5%	74%		All stages
Jass et al.	1998	[44]	90	30%	74.1%	28.6	All stages
Salahshor et al.	1999	[40]	191	12%	81%		Unselected
Gryfe et al.	2000	[46]	587	17%	71%	26%	Population <50 yrs
Gafa et al.	2000	[45]	216	20%	90.9%	60.5%	Select group, 50% proximal
Elsaleh et al.	2000	[21]	656	9%	93%		Consecutive, stage C
Hemminki et al.	2000	[43]	282	12%	82%	36%	Stage C
Cunningham et al.	2001	[41]	257	20%	86%	47%	Unselected, consecutive inc HNPCC cases
Ward et al.	2001	[37]	302	11%	79%	33%	Unselected sporadic
Wright et al.	2003	[38]	458	19%	83.1%		Stage C
Ribic et al.	2003	[48]	570	16.7%	89%	36%	B and C, trial pts
Lim et al.	2004	[53]	248	9.3%	74%	19%	All stages

Table 42 MSI rate per site

Study	Year	Ref	*n* prox	% MSI	Distal % MSI	Comments
Thibodeau et al.	1993	[31]	37	35%		Not stated
Lothe et al.	1993	[200]	77	25%		Proximal tumours only
Kim et al.	1994	[42]	58	29%		Consecutive Stage B & C, sporadic
Bubb et al.	1996	[39]	86	27%	12%	Unselected sporadic
Elsaleh et al.	2000	[21]	260	20%	1%	Consecutive, Stage C
Gryfe et al.	2000	[46]	198	36%	5%	Population, <50
Ward et al.	2001	[37]	115	29%	4%	Unselected, known HNPCC excluded
Kakar et al.	2003	[219]	867	32.7%	5.2%	More young and very old
Wright et al.	2003	[38]	207	35.7	6%	Consecutive

2.5.8.4 MSI and Pathological Factors

There are several pathological features that are over-represented in MSI tumours relative to MSS tumours.

2.5.8.4.1 Differentiation

There are a greater proportion of poorly differentiated or undifferentiated tumours in MSI cases (Table 43*).* Between 33-64% of MSI tumours exhibit poorly differentiated histology compared to 11-30% of MSS tumours [38, 42, 44-47]. Ribic et al. in a study of 507 cases found the rate of poor differentiation or undifferentiated tumours to be 30% of MSI cases compared to 12% in MSS cases [48]. The proportion that was well differentiated in the MSI group was 9% compared to 19% in the MSS group.

2.5.8.4.2 Tumour Type

Mucinous CRC requires the mucinous component to represent greater than 50% of the tumour (though lesser degrees may still be significant). Signet ring cells represent intracellular mucin. Much overlap exists between mucinous type and poor differentiation [45] and by definition signet cell tumours are poorly differentiated. These features have reasonably good reproducibility [47].

A greater percentage of MSI CRC exhibits mucinous pattern and signet ring cells (Table 44). Mucinous pattern was found in between 15-78% of MSI CRC compared to 4-17% of MSS cancers [37-39, 42, 44, 45, 47]. Wright et al. demonstrated mucinous, signet ring or medullary type in 43.8% of MMR deficient tumours

compared to 10.6% of MMR intact tumours [38]. MMR deficient tumours represented around half (44.8%) of the mucinous CRC but only 13.2% of the non-mucinous adenocarcinomas. This association was stronger for right-sided tumours.

Medullary type (a subgroup of poor differentiation) has been associated with MSI-H tumours [45, 47]. It is a rare variant, relatively specific for MSI-H tumours [47]. The significance of the sometimes-reported cribriform architecture is unclear. It has been found to be more prevalent in MSS tumours by some [47] while others have found the reverse, with a non-significant trend to a higher rate in MSI cancers [44]. Observer variation may account for this difference.

2.5.8.4.3 Lymphocytes

Another distinct pathological feature of MSI cancers is the increased presence of tumour lymphocytes (Table 45) [37, 38, 42, 44, 45, 47, 49, 50]. Various forms of lymphocytic infiltration are described - Crohn"s-like lymphocytes [42, 45], intraepithelial [38, 47], tumour infiltrating [42, 50] and peritumoral lymphocytes [38, 42, 44, 149]. What these features truly signify is unclear. While there are indications that the lymphocytes represent an up regulation of the immune response [47, 50], this has not been established. This seemingly activated immune response has been credited with the earlier stage and better prognosis of these tumours [42] but again this is yet to be confirmed [47]. Whether this feature translates into improved survival has yet to be determined.

The reproducibility of these features is moderate and again definitions vary, including numbers of specific lymphocytes require per field to be considered positive.

Table 43 Rate of poor differentiation in MSI

Study	Year	Ref	*n*	MSI	MSS	p
Kim et al.	1994	[42]	137	56%		
Jass et al.	1998	[44]	303	33.3%	11.1%	0.02
Gafa et al.	2000	[45]	216	63.6%	24.4%	<0.0001
Gryfe et al.	2000	[46]	587	42%	18%	<0.001
Alexander et al.	2001	[47]	323	38%	13%	<0.001
Wright et al.	2003	[38]	458	65%	30%	
Ribic et al.	2003	[48]	570	Well 9% Mod 53% Poor 26% Undiff 13%	Well 19% Mod 69% Poor 9% Undiff 3%	

Table 44 Mucinous type MSI

Study	Year	Ref	*n*	MSI	MSS	Signet ring
Kim et al.	1994	[42]	137	78%		
Bubb et al.	1996	[39]	215	41%	17%	
Jass et al.	1998	[44]	90	48.2%	7.9%	
Gafa et al.	2000	[45]	216	36.4%	17.4%	
Ward et al.	2001	[37]	302	31%	15%	
Alexander et al.	2001	[47]	323	15%	5%	13% in MSI vs 5% in MSS
Wright et al.	2003	[38]	458	43.8%	10.6%	

Table 45 Lymphocytes in MSI

Study	Year	Ref	*n*	Lymphocytes MSI vs MSS
Ward et al.	2001	[37]	302	Crohn‟s 33% vs 12% Peritumoral 48% vs 19%
Alexander et al.	2001	[47]	323	Crohn‟s 49% vs 36% Intraepithelial lymphocytes, 21% vs 3%
Jass et al.	1998	[44]	90	Pertumoral 29.6% vs 14.3%
Gafa et al.	2000	[45]	216	“Conspicuous” Crohn‟s 50% vs 10.5%
Michael-Robinson et al.	2001	[49]	102	TILs 72% vs 12.5%
Wright et al.	2003	[38]	458	More IEL 78.7% MSI, TILS + 29%, peritumoral 64% vs 38% , Crohn‟s 57 vs 32%
Kim et al.	1994	[42]	137	89% Crohn‟s

2.5.8.4.4 Tumour Margins and Stroma

A pushing margin of tumour (as distinct from an infiltrating margin) has been associated with MSI cancers [44, 45, 47, 213] and demonstrated in 60% of MSI tumours compared to 26% of MSS [38]. Lack of budding relative to MSS tumours has also been observed [38]. These may both account for a decreased tendency to metastasize.

Stromal pattern was not found to be distinct in MSI tumours by Wright et al. [38]. This is the only study to examine this histological feature in MSI.

2.5.8.4.5 Morphology and Stage

Large size [38, 42, 45, 52, 213] and exophytic growth [213] have been associated with MSI tumours, which would be consistent with tumour biology of low metastatic tendency. Gafa et al. found 45% of MSI-H tumours were greater than seven centimeters compared to 14.5% of MSS cancers [45].

Despite the seemingly aggressive histological features, there appears to be a tendency for MSI tumours to remain localised. MSI tumours generally present at an early stage [38, 40, 46]. Ward et al. found 52 % of the MSI tumours presented at stage B compared to 35% of MSS [37]. Wright et al. found a higher proportion of stage 1 or 2 tumours (72.2% vs 51%) and a decreased tendency to lymph node metastasis (72.2% vs 52.1%) [38]. Gryfe et al. demonstrated a hazard ratio of lymph nodes metastases for MSI CRC of 0.33 and distal organs 0.27 in their study of 607 young CRC patients [46]. Lim et al. in a study of 248 consecutive cases of CRC found a non-significant

trend to earlier TNM stage (stage 1-4 MSI % 9:48:39:4 compared to MSS 13:34:29:23) [53]. Despite the remaining localised, MSI cancers have been shown to have higher than expected T stage by some groups [46] though not by others [38, 53].

The rate of MSI in CRC metastases has been shown to be low. Schneider et al. failed to demonstrate MSI in 29 liver metastases compared to 6 of 39 primaries [221]. Salahshor et al. found no MSI in the 24 stage D cases compared to a rate of 12% in the other 157 unselected cases [40]. Rosty et al. found one (1.8%) MSI case in 56 CRC liver metastases cases which was HNPCC [222].

2.5.8.4.6 Predictive Combinations

Attempts have been made to group pathology tumour features to predict MSI status. At the second meeting of the NCI working party, pathological correlations of MSI tumours were discussed. Jass reported on 303 sequential tumours, 9% of which were MSI-H [174]. There was significant tendency to proximal site, mucinous, undifferentiated histology, presence of tumour infiltrating lymphocytes (TILs), less liver metastases and expansile growth pattern and a combination of these findings was 93% predictive of MSI status [174].

Ward et al. studied 302 consecutive sporadic CRCs of which 33 were MSI [37]. They also found a tendency to more mucinous, higher grade, more intraepithelial and peritumoral lymphocytes and earlier stage. However, no one factor had a predictive value. In combination, right-sided and intraepithelial lymphocytes were the most predictive with positive predictive value of 57% and a negative predictive value of 95%.

Alexander et al. examined the histological features of 323 sporadic CRC found a higher rate of signet rings (13% MSI vs 5% MSS), mucinous (15% vs 5%), poor differentiation (38% vs 13%), Crohn‟s-like lymphocytes (49% vs 36%), “marked‟ intraepithelial lymphocytes (21% vs 3%)[47]. The most predictive feature for MSI was intraepithelial lymphocytes then mucinous type with a sensitivity of 74% and specificity of 83%. Intraepithelial lymphocytes as a marker would have only missed 10% of cases. They specify “marked” but do not define this quantity. This feature occurred independently of mucinous type.

2.5.8.5 Rate of Synchronous and Metachronous Tumours in Sporadic MSI CRC

It is debatable whether the rate of synchronous and metachronous tumours is higher in patients with sporadic MSI tumours, a feature clearly associated with HNPCC (MSI) CRC. Wright et al. found that 6% of 458 consecutive CRC cases had synchronous cancers and of these 37.7% were MMR protein deficient (on immunohistochemistry) [38]. Put another way, MMR deficient tumours had a synchronous rate of 22.5% compared to 8.9%. It is interesting to note that not all the synchronous lesions were MMR deficient. Metachronous lesions occurred in 4% of the 458 patients with a trend to a greater rate amongst the MSI group, 23.5% compared to 18.2% (not significant but small numbers). This study did not exclude HNPCC cases but only four were recognised and 90% of the MSI tumours showed loss of MLH1 suggesting the familial group would be too small to significantly skew the results.

Gryfe et al. also showed a higher synchronous rate for MSI tumours, 11% compared to 4% in MSS cases [46]. The 587 patients, however, represented a young group and familial cases were not excluded. Ward et al. in their study of 302 consecutive cases

excluded HNPCC and did not find an increased rate of synchronous or metachronous lesions associated with MSI [37].

2.5.9 Variation between MSI Sporadic and Germline

Since some features of tumour biology are shared, it is not surprising that many clinicopathological features of sporadic MSI tumours are shared with HNPCC tumours. The main differences appear to be age, gender, metachronous tumour rate and possibly site.

Age would be expected to vary between sporadic and germline mutation. While the cause of methylation is not understood, it is known to be an acquired event, probably related to aging or exposure, and as such the risk would be expected to increase with time. A study of 500 unselected CRCs found that of the 12% that were MSI, 46.4% were methylated (therefore sporadic) and the rest were assumed to be caused by mutation [218]. The methylated group had a higher average age of onset (70.8 years vs 52.9 years) and a female preponderance. Whether all the non-methylated were truly mutations could be questioned, as it is unlikely that germline mutations would account for over half of the MSI tumours when the figure is usually around 10%.

Wright et al. in their study of 458 consecutive stage C CRC cases found 20% were MMR deficient on IHC and 10% of these were MSH2 deficient and therefore germline [38]. There was no difference in age distribution between either the MSH2 and the MLH1 deficient cases compared to the MMR intact cases. The MLH1 cases had a proximal tendency but the MSH2 were evenly distributed in a similar manner to the tumours with intact MMR protein. Wright et al. also found a lower proportion of

poor differentiation and mucinous tumours in the MSH2 cases but a similar tendency to earlier stage and the same proportion with lymphocytic infiltration.

2.5.10 Influence of MSI on Prognosis

Studies that addressed outcome in MSI tumours are detailed in Table 46 and Table 47. Most studies find MSI confers a survival advantage, however, these represent a very mixed group. The accuracy of studies to determine the prognostic significance of MSI will rely on the same study limitation as discussed previously. To achieve the best result, multivariate analysis must include all potentially confounding factors. Stage is important in MSI outcome analysis. As shown, MSI tumours tend to be less advanced and unless analysis is adjusted for stage, survival will be erroneously better. Whether surgery was truly curative or only palliative should be considered (i.e. margin status must be accurately assessed). Overall survival will be influenced by age and possibly gender, both of which may be influenced by MSI status and therefore need to be accounted for in analysis. Cancer-specific survival may counter some of these factors as a more accurate reflection of outcome from cancer. Perioperative deaths should be excluded (unless only cancer deaths are examined following curative surgery). Use of adjuvant therapy must be considered in analysis.

Of 13 tabulated studies, four did not find a significant better survival outcome in the MSI cohorts. Ward et al. showed a trend to better survival in MSI cases but this disappeared when adjusted for stage [37]. Feeley et al. found median time to death was actually shorter in the MSI cases, however they included only 50 patients and only univariate analysis was used [213]. The two other non-significant studies both showed a trend to better survival in MSI. Both performed multivariate analysis on

reasonable numbers of patients and included either all stages [40] or stage B cases only [217].

Of those that performed multivariate analysis, six of nine studies found an improved survival in the MSI cohort with hazard ratios of dying varying between 0.39 and 0.61 [39, 46, 48, 51-53]. Inclusion criteria varied, mainly in relation to stage, but this was adjusted for in analysis. Two of the three studies that failed to find a significantly worse prognosis with MSI had fewer patients and did indicate a trend towards a better outcome [40, 217]. The final study demonstrated a trend that disappeared upon adjustment for stage [37].

The studies that focussed on outcome predictors (including MSI) in stage B and C disease will be the most relevant when attempting to identify those who will benefit from chemotherapy and the indicators that are useful in targeting treatment. Most studies of stage B and C disease have found that MSI conferred a survival advantage. Halling et al. studied 508 stage B2 or C cancers from patients enrolled in various chemotherapy trials. On univariate analysis, significantly improved survival was seen in the 14% of stage C cases demonstrating MSI compared to the equivalent stage MSS cases [51]. No survival advantage was observed in the stage B group despite 26% being MSI-H [51]. Wright et al. in their study found a better outcome for MSI cases in their multivariate analysis of 255 unselected, sporadic stage C cases [52]. The hazard ratio of dying was 0.44 in the MSI cohort compared to the MSS. Ribic et al also found an improved outcome in the MSI group of 257 stage B and C cases from various chemotherapy trials (HR 0.61) [48].

Lukish et al. included patients 40 years of age or less with stage B and C and showed the 5-year survival for MSI cases was 68% compared to only 32% for MSS cases [57]. Rates for each stage were not detailed and analysis did not adjust for other factors. Outcome for the MSS cases was unaccountably poor. Elsaleh et al. included stage C cases and showed a five-year survival of 58 compared to 33% [21]. Again analysis was unadjusted.

Because MSI tumours are biologically distinct from MSS cancers, it is not surprising that prognosis varies. Why outcome should be better, despite seemingly poorer prognostic features such as poor differentiation and mucinous type, is unclear. It has been postulated that the high mutational load may be detrimental to metastatic potential [45] or that the lymphocytic infiltration represents an up-regulation of the immune response, thus improving outcome [45]. The inverse relationship with p53 and other markers has been implicated but studies investigating the prognostic value of p53 and MSI found that MSI was significant but not p53 [39, 51].

Table 46 MSI Prognosis – Unadjusted studies

Study	Year	Ref	O/C*	*n*	MSI	5yr surv MSI	5yr surv MSS	Sig/HR of dying	Study group
Lukish et al.	1998	[57]	OS	36	47%	68%	32%		Young pts, stage B & C
Feeley et al.	1999	[213]		50	10%			NS MSI shorter median survival time	. Unselected
Elsaleh et al.	2000	[21]		656	9%	58%	33%	P= 0.043	Stage C, mixed chemo
Gafa et al.	2000	[45]		216	20%	82%	57%	Better DFS MSI	All stages, not adjusted

*O/C = outcome measure, either overall survival or cancer specific survival if stated or determinable

Table 47 MSI Prognosis – Multivariate studies

Study	Year	Ref	O/C	*n*	MSI	5yr surv MSI	5yr surv MSS	Sig/HR of dying	Study group	Comment
Bubb et al.	1996	[39]	OS	169	10.5%			0.39 (95% CI 0.19-0.82) p=0.0051	All stage	Non-standard MSI panel and unexplained high MSH2. Survival data not available on all 215.
Salahshor et al.	1999	[40]	OS	181	12%			NS (trend) 1.81 HR MSS (0.73-4.44)	Mixed stage & adjuvant therapy	
Halling et al.	1999	[51]	OS	508	9%	76%	63%	0.51 (0.31-0.82 p=0.006)	Mixed B and C trial pts, mixed chemo	
Wright et al.	2000	[52]	CA	255	9%			0.44 (0.23-0.85) p=0.015	Unselected, sporadic stage C curative surg, no chemo (‘86-‘92)	
Gryfe et al.	2000	[46].	OS	607	17%	76%	54%	0.42 (0.27-0.67 p<0.001)	<50 yo, All stage & chemo	Young group but adjusted for age
Ward et al.	2001	[37]	OS	302	10.6%			NS	Sporadic, curative surg	Trend disappeared when stratified by stage
Wang et al.	2003	[217]		154	23%			NS (trend) RR 0.66 (0.31-1.40 p=0.27	Consecutive stage B, only prox compared	
Ribic et al.	2003	[48]	CA	570	17%			0.61 p=0.03	B & C, mixed chemo	
Lim et al.	2004	[53]	OS	248	9.3%	90.7	59.2	Sig on MV, HR ns P=0.038	Curative, sporadic, consecutive	

2.6 Chemotherapy

2.6.1 Adjuvant Chemotherapy

Implementation of standard adjuvant chemotherapy for CRC began in the early 1990s. Adjuvant 5 fluorouracil (5FU) as a single agent had not improved survival in CRC [5], however in 1989, Laurie et al. studied the combination of 5FU and Levamisole (a antihelminthic drug discovered to be an immunostimulator). They randomised 401 patients with resected locally invasive stage B or stage C to one of three groups; no adjuvant therapy, Levamisole alone or 5FU / Levamisole for one year [4]. Groups were matched and cases stratified by site. Follow-up was adequate (median 7 years, 9 months). Tumour recurrence was significantly decreased for combined therapy (31% RR 95%CI 8%-48%). The advantage was only observed in the stage C group, with a trend to improved overall survival with combined therapy (p=0.07) and only slight advantage with Levamisole alone. On multivariate analysis, distal site and high grade had significantly higher recurrence overall and worsened outcome in the stage C group. Gender did not have an influence. An advantage was not demonstrated for the (poor prognosis) stage B cancer group.

This work led to the Intergroup trial published in 1990 [5]. The same selection criteria were used to randomise 1296 patients to 5FU plus Levamisole (for 1 year) or observation with the exclusion of rectal cancers. Results were initially published at median follow-up of 3 yrs due to the significance of the findings. Significant advantage was shown for stage C cases (929 patients) with a 41% reduction in risk of recurrence (95% CI 23%-54%, multivariate analysis) and overall death rate reduction of 33% (85% CI 10%-50%); 3.5 yr survival 71% versus 55%). Survival was

independently influenced by site (worse for proximal), differentiation, the number of involved nodes and obstruction. Advantage persisted after adjustment for these factors. Subset analysis showed greater advantage for men (regarding recurrence and survival). However, no advantage in recurrence or survival was shown for stage B disease. Levamisole alone did not significantly change outcome.

The final report of the stage C cases from this trial was published in 1995 after median follow-up of 6.5 years with similar results [6]. The recurrence rate was reduced by 40% and death rate reduced by 33% (from Kaplan-Meier curves, 5-year survival was not detailed). Levamisole did not offer any outcome benefit over no-treatment. Prognostic factors for recurrence and survival were examined. Advanced T stage, local invasion, obstruction, poorer differentiation and higher number nodal involvement all had a detrimental effect on survival. Gender had no influence. Patients with transverse and splenic flexure lesions faired best, followed by right-sided lesions while left colon cancers did worst ($p=0.025$). Significant factors were included in a proportional hazards model and the benefit of combined therapy persisted.

These subgroup results should be treated with caution. They are based on univariate analysis for prognostic significance. The study was not set up or powered for subgroup analysis nor does it attempt to test for outcome interaction with treatment. The study does not detail if any factors (other than treatment) maintained significance on adjusted analysis. Therefore the subgroup analysis reveals prognostic factors but not indicators of a responsive subgroup.

The results from locally advanced stage B cases was published separately [7]. Analysis included 318 cases with median follow-up of 7 years. Recurrence rate was reduced by 31% but this was not significant (RR 0.69; 95% CI 0.44 to 1.08) nor was there a significant difference in overall survival. Multivariate analysis revealed only age, obstruction and site (better outcome for right-sided) had independent prognostic significance and no factors changed the magnitude of treatment effect (i.e. no compounding interactions). The study was underpowered and there were more non-cancer deaths in the treatment arm. Nevertheless no support for adjuvant treatment of stage B disease with 5FU/levamisole can be drawn from this study.

In 1994, a small trial from Italy of 239 stage B and C cases examined the benefit of 5FU combined with Folinic acid over no adjuvant therapy [8]. Inclusion criteria were similar to the intergroup study. Five-year recurrence rate decreased by 35% (41% to 26%; 95% CI 18%-52%) and 5-year survival was 79% compared to 65% (rate reduction 35%; 23%-45%; p=0.0044). Estimated 5-year survival for stage C was 69% compared to 43% whereas rates in the stage B2 group were not significantly affected by treatment (89% versus 86%).

In 1995, pooled data from three randomised trials were published as the IMPACT trial [9]. 5FU combined with Folinic acid (leucovorin) was compared to no adjuvant therapy. Again, stages B and C cancers following curative resection were included. 1493 patients were analysed and mortality was reduced by 22% (95%CI 3-38; p=0.029), recurrences by 35% (22-46; p<0.0001) and 3-year overall survival from 83% to 78%. Benefit persisted on multivariate analysis. The only other factor that remained significant was stage and notably not gender or site. Analysis of the stage C cohort separately increased the magnitude of the affect. Overall 3-year survival was

76% compared to 64% for controls (HR of death 0.7 (0.53-0.92)). However, when stage B cases were analysed separately, no significant benefit was found (HR 0.91, 2% difference overall survival).

The IMPACT group also separately published the results of the pooled data on stage B cases, the results of which failed to support adjuvant treatment [10]. 1016 patients were analysed (study design as per above). Overall survival HR with treatment was 0.86 (90% CI 0.68-1.07), a 2% difference in absolute 5-year survival. Multivariate analysis showed age and grade to be the only independent predictors of survival (not gender or site). Testing for interaction was not performed.

A further large trial from the Netherlands investigated the case for stage C disease and in this case 1029 patients were randomised to 5FU /Levamisole or no further treatment [11]. Curatively resected stage 2 and 3 colon and rectal cases were included. Overall 5-year survival improved from 58% to 68% (25% reduction odds of death; p=0.007). Subgroup analysis showed a relative survival benefit for both stage 3 (odds reduction 27%) and stage 2 disease (19%) but both confidence intervals crossed non-significance. The trial was underpowered for subgroup analysis and as stage 3 only just failed to reach significance the trend is noteworthy. Univariate analysis of gender and age subgroups did not show significant affect. Treatment findings persisted on multivariate analysis (details regarding other factors that were included in multivariate analysis were not given).

The NSABP C-04 indicated a superiority of leucovorin over levamisole. Comparison was made between regimens of 5FU combined with levamisole, leucovorin or both in stage B and C disease for 1 year [223]. The leucovorin group had an improved 5yr

disease free survival compared to levamisole (65% v 60% p=0.04) and 5yr overall survival rate of 74% compared to 70% (p=0.07). Levamisole did not add any additional benefit to leucovorin and 5FU. Gender and site were not analysed. Their findings were supported by the Mayo Clinic trial examining the benefit of the addition of leucovorin to 5FU and levamisole in stage B and C cases that showed the leucovorin combination was superior (5yr survival 70% v 60%; $p<0.01$) [224].

Overall, a significant improvement in overall survival through the use of 5FU-based adjuvant chemotherapy has been shown for stage C CRC cases. Mortality risk reduction is in the region of 25-33%. Translation to an absolute 5-year survival difference is difficult from the data given but is probably around 10-15%. This improvement in survival justifies offering adjuvant therapy but suggests only a small percentage will actually benefit and many patients are thus treated unnecessarily.

Various factors were found to contribute to prognosis but few consistently maintained an effect on multivariate analysis (except age as expected for overall survival) and no compounding effect with chemotherapy was demonstrated. Most studies did not perform adequate analysis to truly test for an effect or interaction. Thus no indicators, clinical or pathological, were identified as defining a subgroup to narrow the target group. Hence treatment continues to be recommended largely on the basis of stage alone.

While there may be a trend to better outcome in stage B disease given adjuvant therapy, the magnitude of difference is too small to justify standard treatment. Even selection of stage B cases with currently recognised poor prognostic features (though usually only advanced T stage) fails to translate to a clinically significant difference.

2.6.2 Effect of Gender on Chemotherapy Effect

Subgroup analysis in these initial studies did not find that gender influenced chemotherapy response. However, such subgroup analysis is flawed and very little work has specifically focussed on defining a gender effect. This was addressed by a Western Australian study, which found the most benefit from chemotherapy was observed in women [21]. Survival of 656 consecutive cases of stage C CRC was retrospectively reviewed, 49% of which were women and 42% had received 5FU-based chemotherapy. A slightly higher proportion of men received chemotherapy (26% to 36%) and the chemotherapy cohort was younger. A survival benefit with chemotherapy was seen in women on univariate analysis, 5-year survival being 53% with treatment and 33% without (HR 0.37 CI 0.25-0.56 $p<0.001$). In contrast, there was no benefit observed in the male group, 26% vs 32% (HR 0.79 CI 0.58-1.07 $p=0.133$). On multivariate analysis, female gender still conferred a survival advantage regardless of whether chemotherapy was given or not. For men, comparatively the hazard ratio of dying was 2.1 (95% CI 1.4-3.2 $p=0.0003$). An interaction (or confounding) influence on chemotherapy effect was not tested.

Watanabe et al. retrospectively reviewed 460 stage 2 and 3 colon cancer patients who had received chemotherapy within several NCI intergroup trials, to investigate the significance of molecular markers [214]. As part of analysis they determined the influence of gender. Within the treated group, women had a better outcome on univariate (5yr survival 72 vs 62%) and multivariate analysis (male sex RR of dying 1.71 (1.19-2.47 $p=0.004$). As overall survival was the end point and only the chemotherapy cohort was analysed, limited conclusions can be drawn.

The little work that has been done suggests that gender may influence the affect of chemotherapy and thus further study is warranted.

2.6.3 Effect of Site on Chemotherapy Effect

As with gender, the anatomical site of the tumour was considered in the early adjuvant chemotherapy trials. Again, most emphasis was on prognostic influence, not treatment interaction. The subgroup analysis was crude and details were not always given and site breakdown was variable.

The Elsaleh et al. study also considered site [21]. Forty percent of lesions were proximal to the splenic flexure, with similar gender distribution and chemotherapy rate. In regards to univariate analysis there was a significant variance in outcome according to site with no benefit with chemotherapy observed in the left-sided cancer group (5-year survival 37% vs 36%) comparable to a significant advantage for chemotherapy right-sided lesions (48% vs 27%, $p<0.0001$). When gender was combined with site in subgroup analysis, it was found that only it was only the men with distal cancers subgroup that did not benefit from chemotherapy. However, on multivariate analysis, site was not found to independently influence outcome. This study adjusted for MSI status, which given the association with proximal site, may have negated the significant finding on univariate analysis.

Compared to this, Buyse et al. detailed the results of a meta-analysis of 10 randomised controlled trials of liver infusion in CRC [225]. Stage C cases were extracted. On univariate analysis the results contradicted those of the Elsaleh group. A significant benefit was observed in men but not women. No benefit in either gender was found

for proximal tumours while in left-sided lesions only men demonstrated a benefit from treatment. This analysis was limited and unadjusted but does highlight the lack of resolution of the issue. No other work has specifically addressed whether site influences chemotherapy benefit.

2.6.4 Interaction of the MMR system and Chemotherapy

2.6.4.1 Role of MMR in Cytotoxic Drug Affect

As will be seen, the cytotoxic actions of many common chemotherapeutic agents are at least partially dependent on the MMR system. While the pharmacological action of agents varies, the common link is disruption of DNA. It may be expected that a DNA repair system would correct DNA damage and hence render cells tolerant to such agents [226]. However, in vitro work suggests the opposite, whereby mismatch repair is actually important in maintaining sensitivity to some agents.

It is postulated that the MMR system has the capability to recognise a spectrum of DNA damage beyond mismatched bases. The system recognises the damage caused by chemotherapeutic agents and repair is attempted but not possible. Once repair is deemed futile, apoptosis is triggered and cell death ensues [226-229]. Hence, cells without effective MMR still sustain DNA damage but may forego the cytotoxic outcome. The link between the MMR system and apoptosis has been validated experimentally though the exact mechanism is unknown [226, 228]. Apoptosis in response to DNA damage has been demonstrated in MMR proficient cell lines and shown not to occur in MMR deficient cells [228]. It would appear that both steps in the MMR process are required, as cell culture deficient in either MLH1 or MSH2 fail to trigger apoptosis [226].

There is evidence that MMR also mediates growth inhibition by activating cell cycle checkpoints and causing G2 arrest, thus limiting reproduction of DNA damaged cells [175]. Hawn et al. showed MMR proficient cells caused arrest of cell cycle at G2 in response to 6 thioguanine and that this was lost in deficient cells [175]. Carethers et al. found cells deficient in either MSH2 or MLH1 failed to arrest at G2 on exposure to N-methyl-N-nitro-N-nitrosoguanidine (MNNG) but replacement of the appropriate chromosome restored cycle arrest [182]. It is likely this mechanism exists to prevent replication while repair is attempted, prior to apoptosis.

The mechanism of interaction between the MMR system and the DNA consequence is more easily explained for some agents compared to others. Many agents (including alkalating agents) form adducts to DNA thus altering a base. It is plausible that in replicating the affected strand an appropriate base can be located for the complementary strand and a “mismatch” occurs. The MMR repair system recognises the mismatch and attempts repair but cannot find a suitable candidate. Once repair is perceived as futile, the MMR system triggers apoptosis. It is also possible that strand distortion caused by the adduct is recognised directly and since removal is not possible, apoptosis is triggered [182, 226, 227].

The mechanism of MMR mediated cytotoxicity for drugs that are incorporated into DNA is less well understood. It is hypothesized that the complex is recognized in a similar manner to adducts [175]. How the MMR system functions in agents that cause cross-linking is unknown.

2.6.4.2 Specific Drug Interaction with MMR

There have been several methods used to determine if there is an interaction between the MMR system and various chemotherapeutic agents. MMR function can be examined in cells resistant to agents. MMR deficient cell cultures that demonstrate resistance can be tested for sensitivity when Chromosome 2 and 3 are added, hence restoring MMR function. Direct binding or cell cycle arrest may be demonstrated.

Alkalating agents

Methylating agents have been the most extensively studied. Methylating agents, N-methyl-N'-nitro-N-nitroguanidine (MNNG, experimental agent), procarbazine and temozolomide (active form of procarbazine) all form DNA base adducts by methylating bases [227]. In most cells the resultant methyl group is removed by MGMT (O6-methylguanineDNA methyltransferase). Cells with high MGMT (Mex+) are therefore resistant to damage by methylating agents (methylation tolerant phenotype) while cells with low MGMT levels (Mex-) are sensitive (methyl group is not removed) [230]. It is in the Mex- sensitive cells that MMR mediates cytotoxicity and loss of MMR proteins leads to tolerance. It is unclear if any additional survival benefit is conferred by loss of MMR in the already tolerant Mex+ cells [230, 231].

The O^6 methylguanine (alkalated guanine) adduct, common to all the above agents, is the most cytotoxic. It has been shown to cause miscoding [227], binding of MMR proteins [230] and subsequent apoptosis [227, 230]. Experimentally, removal of the adduct (by adding other agents) results in loss of cytotoxicity [227]. However if MMR is deficient, even large numbers of adducts do not result in cell death, confirming it is

not the effect of the adduct alone but the consequence and action of the MMR system combined with the adduct that leads to cell death [227].

The causal link between deficient MMR and resistance to methylating agents has been substantiated in colon cancer cell lines [176, 182, 184, 231]. MSI and MMR gene inactivation have been demonstrated in resistant cell lines. Hampson et al. selected resistant cells following application of methylation agent N-methy-N-nitrosourea [231]. Some cells acquired resistance through reactivation of MGMT while others (with persistently low MGMT) showed MMR gene inactivation (mostly MSH6). The authors postulated that the methylating agent caused the methylation related gene silencing.

MNNG resistance has been demonstrated in MLH1 deficient cell lines with sensitivity restored on addition of chromosome 3 [182, 184]. Similarly, cell cultures deficient in either MSH2 or MSH6 demonstrated resistance to MNNG that was reversed on addition of chromosome 2 (hence addition of both these genes) [176]. These studies further confirm that cytotoxic response is due to MMR.

Experimentally, resistance to alkalating agents due to MMR deficiency has been demonstrated in nude mice deficient in MSH2 [227, 230]. Cyclophosphamide and busulphan form covalent bonds with DNA and this cross-link prevents replication. Resistance is formed through enhanced repair of cross-linkage but the mechanism is unclear [232]. How the MMR system interacts with these agents is also unclear but there is evidence of MMR response [228]. There is a suggestion that busulphan adducts (which have not been well characterised) may be recognised by the MMR system but it is unclear if resistance forms in the setting of MMR deficiency. The

efficiency of cyclophosphamide and perfosfamide (active form of cyclophosphamide) is not influenced by MMR status [227, 233]. Neither was the cytotoxicity of melphalan in deficient cells until high dosage was used in MLH1 deficient cells, which suggests a dose dependent relationship [233].

Platinum agents include cisplatin, carboplatin and oxaloplatin. These drugs bind DNA and also form adducts. Some interact with the MMR system, although the mechanism is less well understood [230]. The same adduct is formed by cisplatin and carboplatin, which varies from other platinum agents [226]. Cells lines showing resistance to cisplatin were found to have microsatellite instability [230]. Cell cultures deficient in either MSH2 or MLH1 show tolerance to cisplatin and carboplatin but not oxaliplatin, tetraplatin or transplatin [226, 227, 232, 233]. In the latter agents, either the adduct is not recognised or the MMR complex is prevented from binding [226].

Antimetabolites such as Methotrexate inhibit a critical enzymes in folate metabolism [232]. 6-thioguanine and mercaptopurine are converted and incorporated into DNA [227]. Cross-resistance between methylating agents and 6thiooguanine is often observed. The incorporated analogue is similar and also recognised by the MMR system [228]. Resistance is significantly increased in MMR deficient cells [227].

Topoisomerase 11 inhibitors. Both etoposide and doxorubicin have been shown to have low level resistance in the presence of defective MMR [227, 228, 233]. As these drugs do not form an adduct, the mechanism of resistance is unknown [227, 228]. It is possible the “cleavage” complex of drug and enzyme is recognised [227, 233].

2.6.4.3 Ionising Radiation

Resistance and sensitivity to ionising radiation have been reported in association with MMR deficiency [228]. Ionising radiation causes an increase in oxidised bases, which accumulate in MMR deficient cells and increase DNA breakage [228]. Cells that do not have cell cycle arrest have decreased survival with ionizing radiation.

2.6.4.4 5FU

The main cytotoxic affect of 5-fluorouracil (5FU, a fluoropyrimidine) is through inhibition of thymidylate synthetase (TS). TS catalyses the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) by transfer of a methyl group from its cofactor (THF) [229]. dTMP converts to dTTP, an essential factor in folate metabolism and pyrimidine synthesis. 5FU (metabolite) inhibits this process by forming a complex with TS and co-factor CH2-THF. The presence of a fluorinated uracil impedes further reaction thus decreasing the amount of dTTP substrate for DNA and hence decreased synthesis [56, 229].

5FU is incorporated in small amounts into RNA, which is believed to interfere with normal functioning and contribute to cytotoxicity [54, 229]. While 5FU metabolites are also known to be incorporated in small amounts into DNA, this potential cytotoxic mechanism is less well established [54, 229, 232]. Excess of dUMP leads to excess dUTP, which can be mistakenly incorporated into DNA and, while often tolerated by cells, can be potentially cytotoxic at high levels [229]. The effect of this erroneous incorporation is countered by the rapid removal of the substrate by uracil glycosylase [54, 229, 233].

Folinic acid (or Leucovorin) is usually combined with 5FU and biomodulates the effect of 5FU by acting as a precursor to the folate cofactor CH2 –THF, thereby stabilising the complex and more effectively inhibiting TS [56].

It is not clear how or whether the MMR system influences 5FU affect. Given the lack of significant DNA affect, intuitively one would not expect a dependent interaction. The incorporated 5FU is much less bulky than other adducts and not obviously recognised by the MMR system. It does not cause the significant DNA distortion seen with other agents. It is possible the subtle changes that have been observed are sufficient to trigger a response [56]. Any incorporated metabolite is rapidly removed but even the process of removal has been proposed as a recognisable event [54]. It is also postulated that the disturbance of bases in the pool caused by low dTTP leads to mis-incorporation of bases into DNA (by DNA polymerase) which increases error rate, thus involving MMR [56]. Furthermore, 5FU treatment has been observed to cause double strand breaks (DSB) that trigger cell cycle arrest. While the cycle arrest is not attributed to the MMR system, the DSBs may be a consequence of fragility caused by massive attempted repair [56].

2.6.4.5 Invitro Studies of 5FU Effect in MMR Deficiency

Despite no clear explanation, most (but not all) invitro studies suggested cells deficient in MMR proteins have resistance to 5FU. To confirm an interaction, Carethers et al. treated MLH1 deficient human colon cancer cell lines with 5 FU and measured growth by clonogenic assays, as well as determining cell cycle integrity by flow cytometry and looked for DNA and RNA incorporation. The MMR proficient cells showed a 28% reduction in clonal survival (much higher sensitivity) 10 days

after treatment with 5FU compared to MMR deficient cells, suggesting MMR is integral to the cytotoxic effect [54]. Analysis of cell cycling failed to show arrest suggesting that unlike other agents, this is not a mechanism of MMR mediated toxicity in 5FU. DNA incorporation of 5FU was demonstrated but did not cause the disruption or the miscoding seen with other agents and the degree of incorporation did not vary between proficient and deficient cells lines.

Further work by Carethers et al. on methylation-caused MLH1 deficient cell lines demonstrated resistance to 5FU [55]. Demethylation with 5-Aza-2'-deoxycytidine (therefore reactivating the MLH1 gene) led to restored sensitivity with a significant decrease in colony counts. It was conceded that treatment with a demethylating agent might cause some toxicity of its own and thus lower colony counts. The authors raise the interesting thought that demethylating agents may have future therapeutic value in rendering resistant tumours sensitive to standard therapies.

Meyers et al. further supported these findings. They studied the effect of 5FU and its derivative 5-Fluoro-2-deoxyuridine (FdUrd) on human cell lines using clonogenic survival assays [56]. MLH1 deficient cell lines were 18 –fold more resistant to 5FU than proficient cells and 17-fold more resistant to FdUrd. Unlike Carethers, they were able to demonstrate cell cycle arrest in cells sensitive to 5FU (at a higher dose) though they attribute this only indirectly to the MMR system. The predominant role of TS inhibition in cytotoxicity was reconfirmed. Addition of thymidine substrate replaced the diminished dTTP pool and reversed the 5FU cytotoxic effect [56].

In contrast Aebi et al. did not find a significant interaction. Cell lines deficient in MSH2 or MLH1 did not show significant resistance to 5FU (as measured by colony number) compared to cells following addition of either chromosome [233].

2.6.5 Clinical Studies of MSI Influence on Chemotherapy Effect

It was assumed from the in vitro work that MSI and MMR deficiency would also lead to tolerance to chemotherapy agents in the clinical setting. Furthermore, the better prognosis seen in MSI tumours implied there would be little to gain from chemotherapy. There is, however, some conflicting evidence that this may not be the case and that MSI cancers have a better outcome with 5FU-based chemotherapy. Studied thus far are few and contradictory (Table 48).

Initial studies suggested that MSI cancer cases had improved survival with adjuvant chemotherapy following curative resection for CRC. In 1998, Lukish et al. studied cases presenting with CRC to a naval medical centre over a 22 yrs period [57]. Thirty-six mainly male patients less than 40 years of age were included. Half of the cases received adjuvant 5FU chemotherapy. MSI was found in 47% (established using 6 non-standard markers, 5 dinucleotide and 1 mononucleotide gene TGFβ2R). Family history was established confirming 4 HNPCC cases, which were included though the high MSI rate implies further MMR germline cases were missed. Table 48 details the 5-year survivals. The MSI cases trended towards a better outcome than the MSS cases, in both the chemotherapy and non-chemotherapy cohort (also on Kaplan – Meier curves) but the difference was not significant. While it was suggested this might indicate that MSI cases have a better outcome with chemotherapy, the findings only support a better prognosis in MSI cancers over MSS, with or without

chemotherapy, not that the effect of chemotherapy depends on this factor. The magnitude of the survival difference did not vary significantly (and, if anything, it appears to be less in the MSI group).

Halling et al. studied 508 patients with stage B2 or C tumours drawn from several chemotherapy trials [51]. Seventy-six (15%) were MSI positive. An improved overall survival was seen in the MSI group relative to the MSS cases, mainly in stage C but not B2 cases. Subgroup analysis examined survival with or without treatment in the MSI-H, MSI-L and MSS groups. There was no significant treatment benefit in any of these subgroups. The study primarily investigated prognosis and did not detail analysis method or numerical data for the treatment effect. Also, subgroup numbers were small and inclusion of stage B disease may have diluted any perceived benefit to stage C cases. It is therefore difficult to draw any conclusions from this study.

Hemminki et al. selected the 95 patients who received 5FU-based chemotherapy (based on clinician"s choice) from 282 prospectively collated stage C CRC cases [43]. Eleven were MSI (12%); four of these had a germline mutation (HNPCC). Three-year recurrence-free survival was better in the MSI group (90% vs 43%) as was the 3-year overall survival (90% vs 62%). On multivariate analysis, MSI remained significant and this suggests that MSI cancers have a better outcome than MSS when given chemotherapy. However, no comment can be made on an interaction with chemotherapy or difference in treatment effect given the lack of control group. Thus this finding may be consistent with the better prognosis seen in MSI cancers but does not suggest MSI signifies a subgroup with better response.

Elsaleh et al. studied the influence of gender, site and MSI on 656 stage C cases [21] (as detailed earlier). Just under half received 5FU-based chemotherapy. MSI (established by BAT26 status) was only detected in 56 (9%). A similar proportion of MSI and MSS received chemotherapy. Univariate analysis of 5-year survival showed that overall, chemotherapy was beneficial (58% vs 33%). However, in the MSS group there was no significant effect (37% vs 32%) compared to the MSI cases where there was a marked improvement in 5-year survival (90% vs 35% p= 0.0007). Survival was better in the MSI group and significant benefit was observed with chemotherapy. In contrast, no benefit was seen in the MSS group, strongly suggesting that only the MSI cases responded to adjuvant treatment. Multivariate analysis of the chemotherapy group (including gender and site) showed MSI independently conferred a survival advantage (HR 0.07 [0.01-0.53] $p<0.0001$). Again this suggests a better prognosis with MSI but not necessarily that chemotherapy effect varied according to MSI. The subgroup analysis is unadjusted and the cohorts not matched but the difference is so marked that it would be difficult to imagine the results are accounted for by other factors. However, this study did not test for an interaction between chemotherapy and MSI survival effect to confirm significance.

The same group published similar work in 2001 and included p53 status in the analysis [234]. The same patient group was extended to 891 stage C cases. Chemotherapy use was similar between the MSI and MSS groups (younger in both) and only 7% were MSI positive. A strong inverse relationship was observed between p53 and MSI. In the non-treatment group, P53 was prognostic while MSI was not (which goes against the previous studies findings). The reverse was true in the treatment group; only MSI influenced prognosis. Of most interest was the finding via

multivariate analysis that p53 was an independent predictor of outcome while MSI was not (its influence probably negated by the inverse relationship with p53).

Watanabe et al. retrospectively reviewed 516 patients from several NCI intergroup trials [58, 214]. Only the chemotherapy cohort of 460 patients was included for analysis. MSI testing methodology varied and, of 516 cases tested, MSI status could only be established in 329 of which 31% were MSI positive. Other molecular markers tested included LOH at 18q, 17p and TGFβ1 type II receptor. Analysis of the 229 stage C cases showed that MSI cases had an improved 5-year disease-free survival (64% vs 49% p=0.02) but there was no significant difference in 5-year survival (68% vs 56% p=0.2). On adjusted analysis, MSI cases RR of dying was 1.39 but this was not significant (p=0.18). Mutation of TGF alone did not influence response, however if neither TGF mutation nor MSI were present, RR of recurrence was 2.90 (1.14-7.35 p=0.03) and dying 1.83 (ns p=0.07). Thus, adjusted analysis suggests MSI tumours have a better outcome than MSS in the group receiving chemotherapy, especially if TGFβ1 type II receptor mutation co-exists. However, without a control group no comment can be made on a lesser benefit from chemotherapy. Again this may only reflect that MSI has prognostic significance, not that MSI influences chemotherapy effect. The high rate of MSI and low success of testing despite having tumour tissue also suggests methodological problems.

Barratt et al. drew from the UK AXIS trial (portal vein fluorouracil infusion vs control) and examined MSI status (amongst other molecular markers) in 368 colon cancer cases of which 24% were “RER” positive [59]. They used an interaction model and found that MSI status did not influence chemotherapy effect (p=0.54).

Three studies examined for an influence of MSI tumour status on chemotherapy effect in the palliative setting and both suggested a better outcome in the MSI cases [222, 235, 236]. Liang et al. studied 244 stage 4 CRCs with liver metastases [235]. Twenty-one percent were MSI-H based on NCI panel. Chemotherapy was non-randomly assigned to 69% but groups were well matched. In the treatment group, the MSI cases had a significantly longer median survival compared to the MSS (24 months vs 13 months p=0.0001) and better response rate on subsequent CT scan. In the non-treatment group, MSI status did not influence outcome; all did poorly. Thus chemotherapy had a benefit in both groups but greater in the MSI cases. Rosty et al. studied 56 patients with CRC liver metastases treated with 5FU chemotherapy [222]. Only 1 patient (1.8%) was found to be MSI positive and this patient had no residual tumour on subsequent liver resection.

Bruecki et al. studied 43 palliative cases for MSI influence on treatment response [236]. All patients were initially given a 5FU-based regimen followed by either irinotecan or oxaliplatin for the non-responders. MSI was established by NCI panel and immunohistochemistry and was positive in 16%. Of the MSI cohort, 72% showed complete or partial response compared to 41% of the MSS cases (p=0.072). The median survival was significantly better in the MSI cases at 33 months (95% CI 20-46) compared to 19 months (95% CI 10-28: p=0.021). On multivariate analysis adjusted for patient status, tumour response, organs involved, age, gender and site MSI was independently predictive of increased survival (p=0.032).

In contrast to the above studies, a group from Toronto published their research in 2003 that suggested MSI tumours actually do poorly with chemotherapy [48]. From five chemotherapy trials, 570 stage 2 and 3 cases were selected. Half had received

treatment as per randomisation. MSI was detected in 95 (16.7%). MSI-L cases were included with MSS and this group was well matched with the MSI group, with similar proportions receiving chemotherapy. Five-year survival in the MSI group was better than the MSS group (75.3 vs 64.1%) and remained so on multivariate analysis (HR 0.61 (95% CI 0.38-0.96 p=0.03)). On further breakdown, within the non-treatment group, MSI was predictive of better prognosis but in the treatment group, MSI status had no influence (in contrast to Elsaleh et al.).

Overall there was no survival advantage with chemotherapy, probably due to inclusion of stage B cases. However, on subgroup analysis, chemotherapy led to a significant better 5-year survival in the MSS group (75.5% vs 68.4% p=0.02), which persisted on multivariate analysis (HR of death 0.72 (95% CI 0.53-0.99) p=0.04). This contrasts to the MSI group that appeared fair worse with adjuvant treatment. Univariate analysis showed a worse 5-year survival in the MSI cases that received chemotherapy (70.7% vs 88% p=0.07) and on multivariate analysis (adjusted for stage and grade), the cohort of MSI cases who received chemotherapy had a HR of dying of 2.14 though the confidence interval was wide and crossed non-significance (95% CI 0.83-5.49 ns).

A trend in the same direction was observed with stage subgrouping. Both stage B and C MSS cases trended to a better outcome with chemotherapy (HR of death 0.67 (95% CI 0.39-1.15) and 0.69 (95% CI 0.47-1.01) respectively. For both stages the MSI cases did worse (HR of death chemotherapy vs none 3.28 (95% CI 0.86-12.48) for stage 2 and 1.42 (95% CI 0.36-5.56) for stage 3). Again confidence intervals are wide and cross non-significance. However, on formal interaction testing this perceived variance was confirmed (p= 0.02) suggesting that there was a detrimental effect on

survival from chemotherapy in MSI cases. Despite the non-significance of subgroup findings, this study does question conclusions made in previous work.

Carethers et al. published findings on 204 consecutive stage 2 and 3 CRC cases [60]. MSI-H (NCI panel) was found in 36 (17.6%) and MSI-L in 43 (which were included in MSS group). On unadjusted subgroup analysis, the MSI group did not have a significant survival benefit from chemotherapy (p= 0.52) whereas the MSS group just reached significance (P= 0.0478) (significance was calculated from survival curves with no percentage or HR stated). With multivariate analysis, MSI status did not affect overall survival, in either the treatment or non-treatment group, which suggests no prognostic significance. An interaction was not tested but with this negative finding it is unlikely that a prognostic interaction would be observed on adjusted analysis for interaction.

The numbers are very small in this study and events few; as Watanabe et al. point out in related correspondence, one further death in the MSI group would have changed survival rate by 20% [214]. Group mismatching may have distorted the unadjusted analysis (more stage B cases in the MSS group, though this would have skewed results towards less chemotherapy benefit). Other limitations include the use of overall survival and a similar selection bias as in Elsaleh et al. with the non-random allocation of chemotherapy. It is difficult to draw the same conclusions as the authors- that MSS cases benefit from chemotherapy while MSI cases do not. At most this study suggests equivalence rather than varied effect.

The only study to explore the effectiveness of chemotherapy in HNPCC comes from the Netherlands [237]. A retrospective review of 92 stage 3 HNPCC patients with

colon cancer showed that the 30% who received chemotherapy had the same 5-year cancer-specific survival as those that did not (70% 5-year survival for both). However, numbers are probably too small to show an effect and there was potential bias in who received chemotherapy, with a younger mean age in the treatment group. Nonetheless, if anything this should have exaggerated any perceived benefit from chemotherapy (which was none). Furthermore as cancer-specific survival was used as the endpoint, the effect of age (or comorbidities) was unlikely to be significantly contributory. The lack of any trend to benefit raises the interesting thought that methylated MSI cases may need to be considered separately from HNPCC cases.

On the other hand, Van Rijnsoever et al. examined for the influence of methylator phenotype on chemotherapy response, suggesting this may be more important than MSI status [238]. One hundred and three cases of curatively resected Stage 3 CRC were compared to 103 who had surgery and 5FU-based adjuvant chemotherapy. CIMP status was established by examining for methylation in p16 promoter, MINT-2 clone and MDRI promoter and was positive in 33% (2 or greater were methylated). Of these, 31% were microsatellite unstable (9.7% overall). On Kaplan Meyer analysis, the CIMP +ve cases that did not receive chemotherapy had a worse survival compared to the chemotherapy cohort where there was a trend towards a better outcome. The CIMP +ve cases accounted for any improved survival from chemotherapy with no benefit observed in the CIMP –ve group. On multivariate analysis CIMP +ve independently predicted improved survival, negating the effect of MSI though interaction testing for chemotherapy effect was not performed.

In conclusion, the issue is unresolved. Several studies suggest that MSI cases have a better survival than the MSS in those that receive chemotherapy [43, 51, 57, 58, 235,

236], but this may only reflect the better prognosis observed in MSI case, with or without chemotherapy and the studies are not set up to determine otherwise. The two larger studies are conflicting. Elsaleh et al."s subgroup analysis points to a dramatic difference in outcome, suggesting MSI cancers are the only ones to benefit from chemotherapy but this conclusion relies on unadjusted subgroup analysis [21]. Ribic et al. suggest the opposite: that MSI cases are doing worse if given chemotherapy but this conclusion is based on trends in results only [48]. Further clarification on this issue is required.

Table 48 Chemotherapy/MSI trials

Study	Year	Ref	*n*	MSI*	MSI Chemo	MSI No chemo	MSS Chemo	MSS No chemo	Suggested effect‡	Group	Comments
Lukish et al.	1998	[57]	36	47% (17)	85%	73%	55%	30%	MSI better	Young	NS
Halling et al.	1999	[51]	508	15% (76)					NS (details not given)	Stage B & C	
Hemminki et al.	2000	[43]	95	12% (11)	90%		62%		MSI better	Stage C	3-year survival p=0.10
Elsaleh et al.	2000	[21]	656	9% (56)	90%	37%	35%	32%	MSI better	Stage C	
Watanabe et al.	2001	[58]	229†	32%	68%		56%		MSI NS	Stage B & C all chemo	
Barratt et al.	2002	[59]	368	24%					No interaction RER status	Stage B and C	UK AXIS trial pts
Ribic et al.	2003	[48]	570	17% (95)	71%	88%	76%	68%	MSI worse	Stage B & C	
Carethers et al.	2004	[60]	204	17.6% (36)	No benefit		Benefit		MSI NS	Stage B & C	

‡Effect as suggested by authors in text (this is not always the conclusion of candidate)
*% rounded to nearest whole for all
†Watanabe (of 460 only 229 in MSI analysis)

3 METHODS

3.1 Overview of Study Methodology

This study is a retrospective cohort study of stage C colorectal cancer cases, where a comparison is made regarding survival in those who received chemotherapy with those who did not. The compounding effect of various clinical and pathological parameters was tested to determine whether any of these influenced response to treatment and hence might be used to identify the target subgroup for adjuvant chemotherapy.

All available stage C cases from three hospitals over the last 20 years were included. Demographic, operative, pathological and death data were collated and checked. Pathology was independently evaluated. Archived tissue was accessed for MSI processing. A database was constructed for recording the retrieved information.

Each chosen parameter was tested for prognostic significance and entered into a multivariate model to determine if there was an effect independent of chemotherapy (i.e. indicating prognostic influence). An interaction model was then used to test for a compounding effect with chemotherapy (that the factor increased magnitude of the benefit from chemotherapy). A subgroup analysis was performed to determine if one subgroup identified by a combination of factors responded while another did not.

3.2 Design

While prospective randomised studies are optimal, this would no longer be an ethical or practical approach to fulfil the aims of this thesis given established benefit, albeit small of chemotherapy in stage C disease. While the limitations of a retrospective study need to be addressed, in this study there were several advantages to a

retrospective approach. Firstly, there was extended follow-up allowing for better determination of survival differences. Secondly, selection bias was minimised by sampling cases from the era prior to standard chemotherapy (discussed below).

The disadvantages of a retrospective study include inaccuracy of information and cohort mismatch. Non-prospective collation of information is prone to error and mechanisms of countering inaccuracy are discussed in subsequent method sections. Cohort mismatch in this study warrants further attention.

The non-randomised allocation of chemotherapy to either cohort leads to potential mismatching of groups and hence selection bias. There are varied reasons why patients do not receive adjuvant therapy, including comorbidities (and indirectly older age), patient choice and logistical problems (especially in South Australia given the rural catchment area). Whether these comorbidities and demographic factors were informative is unknown and evidence that they influence prognosis is weak or lacking (see Chapter 1). Retrospectively it is not possible to accurately determine why treatment was not given and therefore the reason could not be included in analysis.

This bias was minimised in three ways. Most cases in the non-chemotherapy cohort were drawn from years prior to standard chemotherapy thus minimising any selection bias. Secondly, adjustment was made for age and lastly, cancer-specific survival was used as an endpoint, which is less dependent on comorbidities or age.

The study aimed to achieve as close to consecutive cases as possible by including all identified cases for the years included (see subsequent case identification section).

3.2.1 Eligibility

3.2.1.1 Inclusion Criteria

Patients were eligible for the study if they underwent curative resection of ACPS stage C colon or rectal adenocarcinoma. The study focused on stage C cases, being the predominant recipient group for adjuvant chemotherapy. Some stage B cases are offered chemotherapy but in insufficient numbers to gain meaningful comparative results. Any case with lymph node spread without distal metastases was eligible. This included micrometastases because these are considered stage C for treatment purposes. Mesenteric nodules (usually tumour replaced lymph nodes) were considered equivalent to lymph node positive disease and included.

Only cases that underwent resection with a potentially curative resection were included. Cases with residual disease either macroscopically (residual at operation) or microscopically (positive margins) are considered palliative and excluded.

Colon or rectal cancer cases were included. Anal cancers were excluded given the variation in biology and treatment.

3.2.1.2 Exclusion Criteria

Exclusion criteria are listed in Table 49. In-hospital deaths were excluded rather than 30-day deaths, being a better reflection of perioperative mortality. Only adenocarcinomas were included because they are the main target of 5FU-based chemotherapy regimens and to achieve a relatively homogenous group. Other histological variants have different biology, different natural history and received different chemotherapy regimens. Subtypes of adenocarcinoma were included (see

pathology section). Patients with additional non-colorectal cancers were excluded if they died from cancer and the responsible primary could not be assured (CRC or other).

Any cases where information was deemed to be inadequate were excluded, usually due to uncertainty over chemotherapy status (i.e. treatment planned on return home to distant area but commencement unable to be confirmed). Cases where site was unclear following review of casenotes, operative report and pathology report were also excluded (this was usually in the setting of previous resection). If slides were unable to be reviewed and therefore pathology unable to be verified, the case was excluded. If archived blocks were available, repeat H&E slides were produced and the case was included (see more in pathology process).

People who had surgery performed outside of the study hospitals were excluded. The registries at the study hospitals aim to include any cancer patients (past or current) that present to the hospital. Most of the cases that had surgery elsewhere were included retrospectively in the hospital databases when they presented to one of these tertiary referral centres for ongoing treatment. Many of these presented for liver resection for subsequently developed metastases. As the original pathological stage was C, these cases were recorded as such on the hospital registry. However they represent a select poor prognosis group and inclusion would have contributed to a selection bias. Cases that presented simply for adjuvant therapy were also excluded, as their pathology was not available for validation (having usually been sent to private laboratories).

Initially it was planned to exclude HNPCC cases, however it became apparent that such status was not available in most cases and did not influence decisions regarding adjuvant chemotherapy. Given the intention to treat type analysis, it was appropriate not to pursue the diagnosis further if it had not been appreciated at the time of treatment. This may influence results (in particular MSI rate) but given the low proportion of mutation positive cases within the MSI subgroup (overall rate 2%) this is unlikely to significantly distort findings.

The inclusion and/or exclusion of cases of multiple CRC warrants more detailed discussion. Metachronous and synchronous CRCs were included if the stage C lesion could be clearly established as the index cancer and main influence on outcome. A five-year period was considered reasonable "cure" time given that most cancer-related deaths occur during this interval. Metachronous cases were included if they were alive at the study endpoint (i.e. neither cancer caused death) or suffered a non-cancer death (neither cancer contributed to death, in which case the earlier cancer was considered for analysis), or if they suffered a cancer death but the other cancer was stage A (in which case it was unlikely to have contributed to outcome) or a stage B over 5 years from the stage C cancer. Cases were excluded if the second cancer was stage B or C and occurred within 5 years of the first and the patient died from colorectal cancer, in which case either may have caused the death.

Synchronous lesions were included if they: occurred on the same side of the colon (in which case the larger or more locally advanced was considered for analysis); were across the colon but the positive lymph nodes could be clearly attributed to one lesion; and were across the colon but the second cancer was stage A (again accepting a small margin of error) or stage B but the patient had not died from CRC (in which case the

second lesion had not contributed to outcome). Cases were excluded from site and pathological analysis if they were across colon and both stage C or across the colon and stage B and C and the patient died of cancer, in which case the responsible cancer was not able to be determined. These cases were suitable to include in the MSI analysis if both tumours had the same MSI status (i.e. it did not matter which was primarily responsible as they had the same status for analysis)

Table 49 Exclusion criteria

1. Perioperative death
2. Non-adenocarcinoma (SCC, carcinoid)
3. Other non-colorectal cancer/s
4. Inadequate information
5. Inability to localise site
6. Pathology not available for review
7. Operation performed elsewhere
8. Recognised HNPCC
9. Multiple CRC – if index stage C case could not be established – see separate section on management of multiple CRCs

Table 50 Standard Adjuvant chemotherapy regimen

5FU

370mg/m^2 IV bolus Days 1-5

If no toxicity increase to 425mg/m^2

Repeat every 28 days

6 month regimen

Leucovorin (calcium folinate)

IV 20 mg/m^2 days 1-5

Given immediately prior to 5FU every 28days

3.2.2 Cohorts

3.2.2.1 Chemotherapy

Only patients who received 5FU-based regimens were included, being the main adjuvant therapeutic option and that which has proven benefit in the adjuvant setting. The Mayo regime has been used as the standard practice in the study hospitals (Table 50). Occasionally, non-significant regimen variations occurred (i.e. with or without Leucovorin or panorex). Any major deviations were excluded (oxaloplatin/capcitebine/ CMF/panorex alone).

3.2.2.2 Subgroups

One of the main aims of this study was to validate recent work suggesting gender, site and MSI combinations may be important for predicting a responsive subgroup. These three parameters were therefore used for subgroup analysis. While subgroup analysis should be interpreted with caution, *a priori* declaration of subgroups improves validity of results.

Analysis was based on "intention to treat". If the patient at least commenced treatment (though may not have completed the course), the case was included in the chemotherapy cohort. If there were discrepancies in adjuvant therapy details in the registry data, case notes were reviewed to clarify type of treatment and if treatment was actually received. As stated above, if any non-resolvable uncertainty existed as to whether planned chemotherapy had commenced, the case was excluded.

Adjuvant therapy generally commences within three months of surgery. Cases where treatment commenced after three months were reviewed and included only if treatment was clearly adjuvant (not for recurrence). Documentation in notes usually clarified the nature of treatment and reasons for delay (usually distance or patient-related factors i.e. failure to attend initial appointments).

3.2.3 Outcome Measures

Survival was used as the study endpoint as this represented an unbiased, objective measure of response and the main objective of the intervention. Any other assessments such as disease-free survival or recurrence are unable to be determined accurately in retrospect. Overall survival and cancer-specific survival were determined but cancer-specific survival was chosen as the primary endpoint being a better reflection of cancer influence on outcome and as it is not influenced by factors that affect general survival such as age and co-morbidities.

3.3 Case Identification

Potential cases were identified using hospital and state cancer registry information and pathological databases. In the initial part of this section, how these registries work will be discussed. Following this, the actual years included will be detailed and the method of sourcing the included cases will be discussed.

South Australia has a major advantage in performing this type of study due to hospital and state registry data collection. The logistics of sourcing data in other ways would have limited the scale of the project and the years that could have been included. As will become evident, case sourcing using pathology records is difficult for all but the most recent cases. The thoroughness of checking and thus accuracy of death data from

the state registry is commendable and allows confidence to be placed in survival information.

3.3.1 State Cancer Registry

The state of South Australia commenced a registry in 1977 to include all cancer cases. Information for this registry includes the following:

- Demographic – name, hospital number, address, gender, race occupation
- Date of diagnosis and admission details
- Cancer – site, type, but not stage. Each cancer site is uniquely coded.
- Chemotherapy (if actually commenced)
- Death and cause or last contact date

Case information for the registry is sourced from the hospital coders, hospital registries and pathology reports. All cancer admissions have a data sheet generated by the hospital coders, which is forwarded to the state registry within one month of discharge. Pathology departments also forward all cancer pathology reports. Cross-checking between state and hospital registries improves completeness of case detection. Any inconsistent or missing information is actively sought by contacting the treating doctors and by casenote retrieval.

Death data is retrieved from Births, Deaths and Marriages. Information from the death certificate is used for coding of death. If the related cancer is specified in section A, B or C of the death certificate as cause of death, then the death is registered as being due to that cancer. Other causes are registered as “other”. Any discrepancies are checked, again by direct contact with treating physician or casenote review. Intermittently (usually annually) data are sent to a central registry in Canberra for interstate collation

and national cross-checking to detect deaths that have occurred interstate and this information is fed back to the hospital registries.

3.3.2 Hospital Registries

Cases from three hospitals were considered for the study: Royal Adelaide Hospital (RAH), The Queen Elizabeth Hospital (TQEH) and The Lyell McEwen Hospital (LMHS). Each hospital established cancer registries at different times.

RAH established its cancer registry in 1987. Some earlier CRC cases were retrospectively entered. Registry staff actively sought cases that had surgery in 1980 and data for this year are deemed complete. Entry of patient data for those who had surgery between 1981 and 1987 are incomplete. Limited resources meant that registry staff were unable to actively search for these cases. Some patients were entered retrospectively when they presented to the hospital subsequently. From 1987 to 1999, data entry improved but staff turnover meant accuracy could not be guaranteed. From 1999, the current staff have run the registry and accuracy is much improved.

The TQEH registry commenced in 1992 and has been maintained by dedicated staff motivated to maintaining an accurate record. LMHS was initially separate but subsequently incorporated into the TQEH registry.

Thus for the years included, cases were as close to consecutive as possible. For the period 1981 to 1985 there is potential for selection bias in those cases included in the study. Patients included in the registry for these years were often those who presented

with recurrence and hence may be a poorer prognostic group. To remedy this, cases for at least some of this period were retrieved from other sources (see below).

Data recorded for hospital registries on CRCs includes the following:

- Demographic information – hospital number, name, gender, postcode
- Operative procedure and date
- Chemotherapy – if administered, type and date commenced
- Radiotherapy – if administered and date commenced
- Tumour subsite, type, differentiation
- ACPS Stage
- Lymph node – status, total number examined, number positive
- Multiple primaries
- Other cancers
- Other procedures
- Intent of treatment – curative/palliative

(Further case details not relevant to this study were also recorded).

The registries receive data from several sources. Cases are identified from pathology reports and the state registry. All pathology reports are forwarded to the registry and listings are sent from the state registry to hospitals regularly for any missed cases (data fed to state level being more exhaustive). Demographic and further information is sourced from hospital computer records and case notes. At registry inception, clinically generated forms were also used but it quickly became apparent that this system did not work and it was abandoned after a few years.

3.3.3 Registry Accuracy

It is assumed that the registries detect most cases of cancer. Pathology laboratories are vigilant in forwarding reports and forms from coders increase the chances of case detection and include non-resected cases. However, both methods rely on the motivation and astuteness of staff. The accuracy of clinical information depends partially on what data are reported to the registries. Coders are non-medical and therefore may misinterpret information. However, the form from coders mostly details demographic information, which is sourced from records with little room for error. Some clinical judgement may be required in stating the site of the cancer but this is cross-checked with the relevant pathology report. Completeness is enhanced by further cross-checking of notes by registry staff (at both hospital and state level). Contact is made with treating doctors when required.

Interpretation of pathology reports has potential for error. Some aspects are straightforward while others require interpretation by non-medical staff. For example, it is easy for staff to determine a cancer is adenocarcinoma but not necessarily subtype. A tumour may be recorded as mucinous if there is any mention of this in the report, rather than only if the proportion is greater than 50% (as per pathological definition).

The main difficulty lies in combining pathology and clinical data. Pathology reports of lymph node positive cases often state “Dukes C” in the summary. However, without clinical correlation to ensure there were no distal metastases, this may be inaccurate. Hence, some cases are staged as “C” in the registry when they are in fact “D”. Registry staff attempt to cross-check with operative notes but not always successfully.

Potential limitations of registry information are therefore:

- Incomplete early data – particularly at hospital level
- Reliance on non-medical staff information (though usually checked)
- Interpretation of pathology reports
- Linking pathology with clinical – incorrect staging

Despite this, many reviews of the state registry accuracy have been performed over the years with reassuring findings. For this study, registries were used for case identification but not all data were used directly. The data extracted and crosschecking process is detailed later.

3.3.4 Pathology Databases

All pathology was processed through the Institute of Medical and Veterinary Science (IMVS). Within this institution, the source of pathology reports varied for the years included. All pre-1991 pathology records are in hard copy form only. Cases are listed on microfiche and data are organised alphabetically and includes patient"s name, hospital number, laboratory number and first line of report. The actual pathology report is stored separately on microfilm, organised by year and laboratory number. It is not possible to search data by disease or screen for colorectal cancer cases.

Since 1991, data have been computerised in various formats and it is possible to perform key word searches for colorectal cancer. Staging information is less readily available, and usually requires reading all reports. Some have Dukes stage in report summary (hence Dukes C can be used for keyword search) but not all. Pathology

databases were therefore not used for primary case identification but as an adjunct to the registries.

3.3.5 Case Sourcing

As the state registry does not include stage data, the hospital registries were used as the starting point for case identification. A search was conducted for all stage C CRC cases for all the years since the databases" inception. Some additional cases were detected by searching computerised pathology databases. Most of the cases detected by this method were from country areas (other hospitals using the IMVS service and hence not detected by hospital registry) and as information could not be verified (i.e. whether there were distal metastases at time of operation or if chemotherapy was received) they were not included. Hence, only a few cases were added using pathology databases.

Data from the state registry were used for cross-checking but this registry was a difficult source for initial case identification due to the lack of information on tumour stage. For the years covered by the hospital registries this was not an issue. Stage specific lists could be generated and then data sourced from the state registry (and subsequently from pathology).

Identification of cases from the years not covered in entirety by the hospital registries was extremely difficult. It was considered important to increase the number of cases from the pre-standard chemotherapy era and therefore, the state registry was used to identify early cases. It was only possible to access hard copy state registry lists of all the CRC cases (all stages) for a given period. A print-out of all CRC cases from 1986

and 1987 was made. To determine stage, pathological data were sought. Microfiche records were searched by patient name to find laboratory numbers. Microfilm records were then searched by laboratory number to access pathology report. From these, the lymph node positive cases were extracted. Casenotes were retrieved and checked for evidence of metastases at time of surgery (operative note, investigations, and documentation) to ensure the cases were actually stage C. While all cases in the study had their pathology reports retrieved, including many from microfilm, using this method for case identification was much more labour intensive than using stage-specific searches of the hospital registries. Hence the number of cases included using this method was limited.

Any cases sourced from outside of the hospital registries (and may have been missed in the hospital death data update) were checked separately with state information to ensure death information was accurate.

3.3.6 Years Included and Dates for Follow-Up

All stage C CRC cases that could be identified from 1980 to 2003 were included and the years included depended on each registry commencement date. The final inclusion dates were determined by when the last registry search was conducted for that hospital. RAH cases were included from 01/01/1980 to 06/11/2003 (limited numbers for 1981-1985 as mentioned previously). TQEH and LMHS cases were from 01/01/1992 until 16/06/2003.

Updated hospital death data were sought from the state registry up to 04/05/2004. The accuracy of death data in the registry ensures that essentially all South Australian

deaths to this time would have been detected. The last national crosscheck was performed in 2002 and it is possible a small number of interstate deaths may have gone undetected (estimated to be at most two from demographic data). As such, "follow-up" (or alive status) can confidently be recorded to this date in the patients not recorded as dead.

Date of operation was taken as the starting date for follow-up.

3.4 Database Construction

A database was designed using Microsoft Office Access (Microsoft, 2000, Redmond, WA, USA). Three relational tables were constructed (as a single patient may have had multiple procedures and multiple pathology). The fields included are listed in the appendix. As the database is a potential source of information for subsequent work, fields were exhaustive and not all were ultimately used in this study. The covariates relevant to this study and those that were included in analysis are detailed more thoroughly in the next sections, including definitions.

3.5 Clinical Information

3.5.1 Data

The data extracted from hospital registries for the study included the following:

- Hospital number
- Demographic details – gender, date of birth
- Procedure and date
- Chemotherapy and radiotherapy- if received and starting date
- Death data

Several other clinical variables have potential prognostic significance but could not be assessed adequately for inclusion. The operating surgeon‟s experience, specialisation and operative volume may be prognostically significant, however the actual operator could not be accurately determined from retrospective review of case notes. The operation reports often named both trainee and consultant as the primary surgeon and computer operative recording by nurses often defaults to the consultant as the operator.

Pathology data, while recorded on the hospital registries, were not extracted from this source due to concerns regarding accuracy.

3.5.2 Cross-Checks

Registry data were considered accurate but cross-checked with casenotes and the state registry in several circumstances, which were as follows. Any incomplete death data was completed with information from case notes and the state registry. If a patient died within six months of operation the possibility of understaging was considered. Casenotes, operative note and investigation reports were reviewed to exclude the presence of metastasis at the time of operation. Any death that occurred within three months of surgery was specifically examined to ensure it was not an „in-hospital death‟.

Cross-checks were carried out if there were discrepancies in data such as tumour site not correlating with operative procedure (i.e. right colon cancer undergoing an anterior resection) or dates not corresponding such as date of death preceding operation date. If the “intention to treat” field in the hospital database was marked

palliative the case was reviewed to ensure that the case was in fact stage C and thus curative.

Any cases with therapy inconsistencies were checked, such as adjuvant radiotherapy being purportedly given for non-rectal cancer. Any unusual chemotherapy regimens were examined to ensure inclusion was appropriate. If chemotherapy or radiotherapy commenced three months or more after surgery, notes were checked to ensure that treatment was of adjuvant intent not given for recurrence or metastases missed at diagnosis.

Checks were made to ensure there was no duplication of cases. All repeated surnames and hospital numbers were checked to ensure they were in fact separate cases or from separate hospitals. Any case with multiple cancers or other cancers or multiple operations was examined more thoroughly.

3.5.3 Clinical Covariates used in Analysis

The definition of the clinical parameters included for analysis were as follows:

- Age – at time of operation in years, continuous variable
- Gender
- Adjuvant chemotherapy
    - Yes = commenced
    - No = no treatment
- Adjuvant radiotherapy
    - Yes = commenced (pre and post operative)
    - No = no treatment
- Obstruction

- Yes = proximal dilatation on the pathology report
- No = no pathological evidence

Obstruction was determined from the pathology report and required documented proximal dilatation indicating a significant degree of obstruction

- Perforation
  - Yes = perforation through tumour, clearly evident on the pathology review
  - No = no evidence of perforation at tumour

Perforation was only considered to be positive if the perforation clearly occurred through the tumour according to our evaluation. Proximal perforation was not considered as a "positive". Pericolonic abscess was not considered as positive unless definite perforation could not be established. It is possible the abscess formation is secondary to a localised perforation but may also be due to translocation of bacteria. Perforation did not always indicate stage T4 tumour and may occur in relation to an area of localised necrosis caused by tumour.

3.6 Pathological Assessment

3.6.1 Process

Once cases were identified, pathology was reviewed. In summary, this process involved retrieval of reports and slides and an independent evaluation of histology. Laboratory (specimen) numbers were retrieved, from either computerised records or from microfiche, then reports printed, either from computer or microfilm. Reports were read and any apparent exclusions noted and clinical information rechecked (i.e. site and procedure). If laboratory number was not located, notes were retrieved to find the report and number.

Reports alone were not relied upon. Reporting varies over time and between pathologists. Current protocols require more detail than even a few years ago. Many parameters that were evaluated in this study were not recorded until recently and others not at all, as their role is still being established. Much observer variation exists between pathologists and thoroughness varies. For these reasons, a single pathologist specialising in colorectal cancer and the candidate reviewed all pathology. Every slide from each case was re-examined methodically according to current protocols. Accuracy of original report was assessed and if the specialist review varied from the original, the amended assessment was used for the study. Histological variables not originally reported upon were assessed for each case. If slides were unable to be located or inadequate, further sections were cut from the original formalin fixed blocks. The pathologist was blinded to the patient‟s MSI result.

3.6.2 Pathological Parameters Recorded and Definitions

- Size - Continuous

Size was recorded as the maximal dimension on the pathological report. Formalin fixation causes a decrease in tumour size but as all measurements were following fixation (rather than from clinical or operative findings) cases were comparable. In a small number of cases size was not reported and was estimated from H & E slide.

- TNM staging criteria
    - **T** – Bowel wall involvement

        1 – Invades submucosa

        2 – Invades muscularis propria

        3 – Through muscularis propria

        4 – Direct local invasion

- **N**

  1 – 1-3 nodes positive

  2 - ≥4 nodes positive

All cases analysed were ACPS C and as such M stage 0

- Proximal/distal
  - Proximal - cancers from the caecum to the splenic flexure inclusive
  - Distal – beyond splenic flexure including rectal
- Subsite
  - Caecum
  - Ascending colon
  - Hepatic flexure
  - Transverse colon
  - Splenic flexure
  - Descending colon
  - Sigmoid colon
  - Rectosigmoid junction
  - Rectum

Site was determined by a combination of inputs including registry data, case notes (operation record/radiology checked if there was any discrepancy) and pathology reports.

- Differentiation
  - Moderate
  - Poor

Tumours were classified as per the Jass et al. classification [102]. Differentiation was classified as per the least differentiated area [102]. The leading edge, where lack of glandular formation may not necessarily indicate poor differentiation, was not used to determine grade. Tumours were not described as well differentiated given the subjectiveness of this determination [99]. Well and moderately differentiated tumours are thus grouped together (as per WHO classification [91]).

- Type
    - Tumours were classified as NOS, adenomucinous (mucinous component < 50%), mucinous (>50%), signet ring, undifferentiated
    - Signet ring component was noted separately

Mucinous tumours comprise greater than 50% mucinous component [91].

- Lymphocytes
    - Peritumoral - at leading edge
    - Crohn‟s-like - aggregates
    - Tumour infiltrating lymphocytes (TILs)

Lymphocytic invasion was divided into the above three categories if it was present. Peritumoral lymphocytic invasion was classed as positive if there was prominent lymphocytic invasion at the leading edge [143, 149, 150]. Crohn‟s-like lymphocytes were considered positive if aggregates were present [150, 151], at least four per low powered field (4x objective). TILs was considered positive if intraepithelial lymphocytes were present [50], again at least four per low powered field.

- Stroma type
    - Fibroid – multiple fine mature layers, stratified into layers
    - Keloid – broad bands of brightly eosinophilic hyalinized collagen intermingled with cancer stroma
    - Myxoid – basophilic stroma

Stroma type was assessed as that infiltrating the tumour and not the supporting stroma. Keloid was the most distinctive type, indicated by thick collagen bands of eosinophilic bundles, similar to the appearance of the collagen in keloid scar formation.

- Invasive margin
    - Pushing – reasonably well-circumscribed edge
    - Infiltrative – poorly defined edge, finger like projections into surrounding tissue

Margin type was determined as described by Jass et al. [102, 143, 146]. An infiltrating margin invades in a diffuse manner with widespread penetration of normal tissues [143]. There must have "streaming dissection" of muscularis propria and dissection of mesenteric adipose tissue by small glands or irregular clusters or cords of cells on microscopic assessment. On macroscopic assessment the limits of the tumour are difficult to define and host tissue is difficult to resolve from malignant [146].

- Budding

Classified as the presence of clustered of undifferentiated tumour cells just beyond the advancing tumour margin [147].

- Margin involvement
    - Yes or No
    - Proximal/Distal/Radial

Resection margin involvement was recorded as either microscopic or macroscopic (margin positive cases were excluded).

- Neurovascular invasion
    - Mural vascular - involving vessels within mucosa or muscle propria
    - Extramural vascular - involving vessels outside of the muscle layer
    - Perineural

As discussed previously, differentiation of venous from lymphatic invasion was not attempted given the inaccuracy of this distinction. Any vessel invasion was labelled vascular invasion and site relative to muscular propria noted.

- Nodal status
    - Apical node status
    - Total number examined
    - Number positive
    - Micrometasases

- Polyps
    - Residual
    - Other adjacent polyps

3.6.3 Pathology Parameters used in Analysis

The pathological parameters used in analysis are detailed in Table 51. T and N stage criteria were dichotomised because of small subgroup numbers. T stage was divided into no muscular bowel wall breach or beyond muscularis propria (i.e. T1/T2 or T3/T4). Nodal involvement was divided into N1 (up to 3 nodes involved) or N2 (more than 3 involved nodes). The apical node was only recorded in a small proportion of reports and therefore status was not deemed sufficiently accurate for inclusion.

Tumour type was dichotomised for analysis due to small numbers. Tumours were divided according to whether there was any mucinous component, thus "mucinous component" includes adenomucinous, mucinous and signet ring tumours.

Table 51 Histological parameters

Parameter	Definition for analyses
Size	Continuous
Site	Proximal or distal Subsite
T stage	T1/T2 or T3/T4
N stage	1-3 nodes or >3 nodes
Differentiation	Moderate or Poor
Type	Mucinous component or NOS
Vascular Invasion	Mural - Present or absent Extramural - Present or absent
Perineural invasion	Present or absent
Advancing edge	Pushing or Infiltrative
Stroma	Fibroid Keloid Myxoid
Lymphocytic infiltration	Peritumoral Crohn‟s-like TILs
Budding	Present or absent

3.7 MSI Analysis

3.7.1 Markers used

In this study, MSI was established by identification of instability in the mononucleotides sequences BAT26 and BAT40. As discussed in the literature review, BAT26 instability accurately diagnoses MSI and as there is uniformity within individuals, normal tissue is not required for comparison. This translates to easier and less expensive research as well as increased clinical availability, which is particularly relevant for this study. Readily available and simpler testing means using this marker to plan treatment would be a viable option in most centres.

For research purposes BAT40 was also tested to increase the sensitivity of testing. Normal tissue was processed in conjunction to determine BAT40 status, as it is a polymorphic microsatellite (varies between individuals).

3.7.2 Laboratory Process

3.7.2.1 Summary of Laboratory Process

Tissue was extracted from formalin fixed paraffin embedded (FFPE) archived specimens. DNA was extracted. PCR was performed to amplify microsatellite sequences of interest. Samples were transferred to the DNA automatic sequencer and results then translated by computer software to produce a readable tracing from which deviations from normal were detected.

3.7.2.2 Sampling and DNA Extraction

Four-micron thick slices were taken from FFPE blocks using a microtome and applied to a slide. One sample of tumour and one sample of normal tissue were taken. Slides were dewaxed and the tissue removed by applying sterile water with a pipette to lightly cover the area and then using a scalpel-cut pipette end to dislodge tissue into the liquid by scraping. Sample was then transferred to a 1.5 ml tube and vacuum centrifuged and dried.

DNA was extracted by the following method (IMVS procedure number MPM.21D-11-9-03). The DNA was digested by addition of DNA buffer and proteinase K solution (50µl DNA buffer 1 and 50µl DNA buffer 2 (Table 52) and 20µl 10 mg/ml proteinase K solution). This was incubated for two days at 55 ^{0}C. On day 2, further proteinase K was added if necessary. Samples were agitated several times during incubation.

For salt extraction 40µl 6M NaCl was added. Samples were placed in the vortex for 15 seconds then centrifuged for 10 min on maximum speed. The supernatant was removed and transferred to a clean 1.5 ml tube. The above steps were repeated as necessary. 350µl of 100% ethanol was added and tubes inverted. At this stage the DNA strand should be visible. Samples were then centrifuged again for 10 minutes at maximum speed. The supernatant was discarded. 0.5 ml of 70% ethanol added and samples centrifuged for 10 minutes at maximum speed. Again residual supernatant was discarded. 50µl of sterile water was added and samples incubated at 37^{o}C for 15 minutes.

Table 52 DNA buffers

DNA buffer 1

- 0.01M Tris-HCl, pH 7.4 0.158g
- 0.10M NaCl 0.0584g
- 0.01M EDTA 0.372g
- Made up to 100ml with Milli Q water

DNA buffer 2

- 0.01M Tris-HCl, pH 7.4 0.158g
- 0.10M NaCl 0.0584g
- 0.01M EDTA 0.372g
- 1% SDS Natriumalauryl-sulphate 10ml of 10% SDS
- Made up to 100ml with Milli Q water

3.7.2.3 PCR Amplification

PCR amplification was carried out as per IMVS procedure number MDM.13D-1/8/01 (see below), using UV irradiated equipment and a clean environment.

Master mixes comprising 0.4µl Primer, 25 mM $MgCl_2$, 2 mM Deoxynucleoside triphosphates, Taq GOLD and Buffer (x10) were prepared for each locus to be amplified. 49 µl of this mix was combined with 1µl of extracted DNA. Samples were then placed in the thermal cycler. The MSI program for the thermal cycler was 2 min at 95^{o}C to denature the DNA (break H bonds), 2 mins at 55 ^{o}C to anneal (create template), 2 min at 68 ^{o}C extension (for incorporation), 45 cycles of 1 min at 95 ^{o}C (for repeats to form) and an extension cycle of 7 mins to finish.

3.7.2.4 MSI Sequencing

An automatic capillary sequencer was used to diagnose aberrant microsatellites. The process is essentially one of electrophoresis with fluorescent markers (IMVS procedure number is MDM.56.C 04-03-03). 1 µl PCR product of each locus was aliquoted into a microtitre plate. 4µl of sterile water was added (to dilute degree of fluorescence). 1µl of this solution is then transferred to the optical plate wells. Ninety-six wells per optical plate allowed 96 samples to be run at a time.

A mixture of 1000µl HiDi Formamide and 40µl ROX 400HD Size Standard was prepared and 10 µl of this added to each sample. Formamide denatures DNA to single strand and size standard produces a peak for comparison. Samples were placed in the thermal cycler for 5 mins at 95 ^{o}C to denture DNA, placed on ice to cool, then

centrifuged and checked to ensure the sample was in the base of the tube without air bubbles.

The samples were then processed through the ABI 3700 capillary sequencer. BAT25 and BAT40 were multiplexed in the same lane.

3.7.2.5 Gene Scan

The results of sequencing were read using ABI prism Genotyper software (Version 3.5, PE Corporation, 1999). An example of output data is shown in Figure 2. Allele length variation can be seen in the tumour sample compared to the standard length as seen in the normal tissue sample. This allelic truncation shows instability, which is indicative of MSI.

Figure 1 Gene Scan

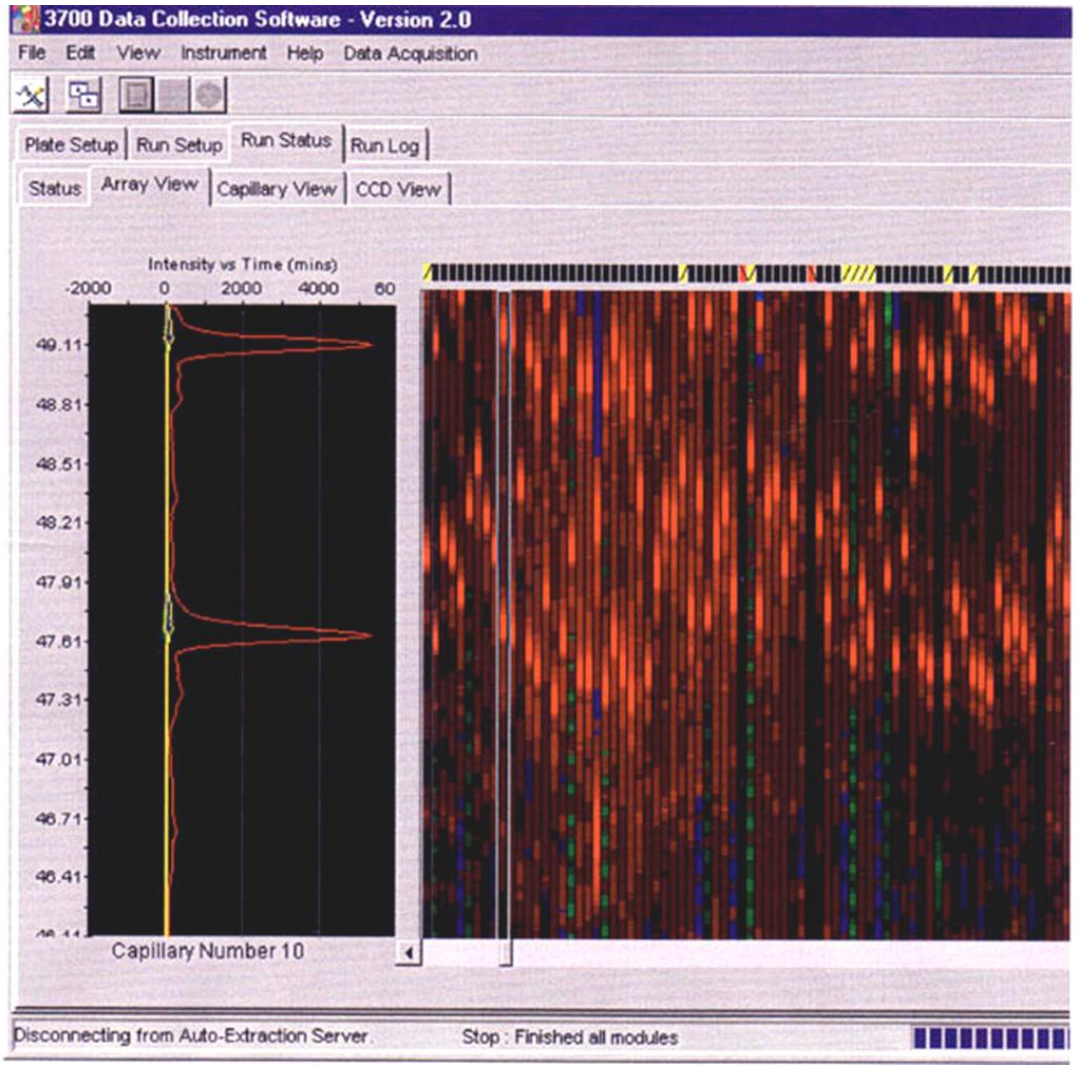
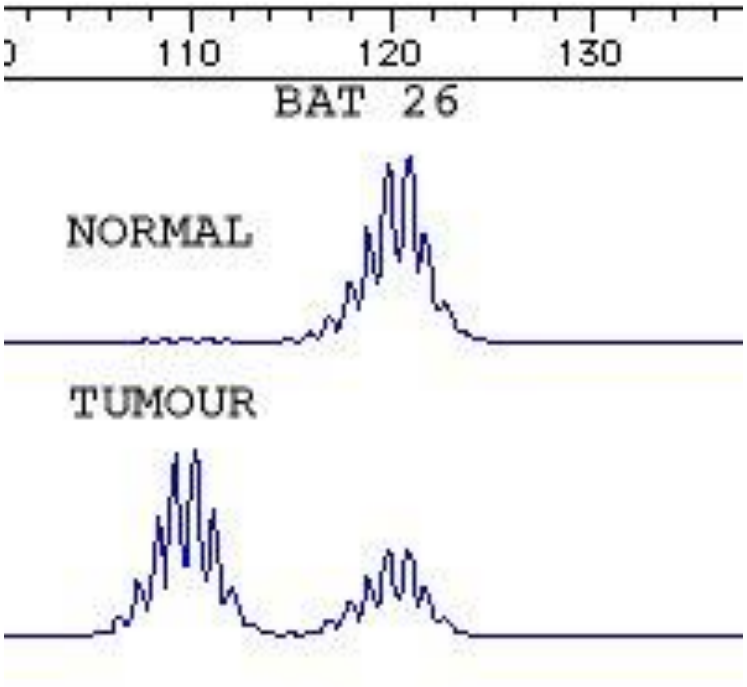


Figure 2 Computerised output from genescan



3.7.2.6 Assessment

Graphic deviation from established standard BAT26 length and deviations in BAT40 from the individual‟s normal were manually assessed. The complete NCI panel was run on equivocal cases.

A separate database was constructed for this DNA bank (Microsoft Office Access, 2000, Microsoft, Redmond, WA) and each sample was given an identifying number. Each patient could have several DNA numbers.

3.7.2.7 MSI Information Recorded

- Overall status (stable or unstable)
- BAT26 result
- BAT40 result
- Tumour block used and identifying number on DNA database
- Normal block used and identifying number on DNA database

3.7.2.8 MSI for Analysis

MSI status was analysed as positive or negative. “Positive” in this study is equivalent to MSI high (given the specificity of the markers used). MSI low (equivocal NCI panel result) was not applicable in this study.

3.8 Statistical Analysis

3.8.1 Statistical Analysis

Follow-up was calculated in months from time of operation to time of death or to 05/04/04. Non-cancer deaths were censored for analysis of cancer-specific survival.

Variable rates between groups (i.e. chemotherapy rate) were compared using the Chi square test. If the expected number for any group was underpowered (<5), Fisher exact test was used for dichotomised variables and proportion testing for variables with multiple categories. Continuous data were compared using the Mann-Whitney test. Median was used for age comparison.

Prognostic significance and chemotherapy effect were initially determined by univariate analysis. Kaplan-Meier survival curves (cumulative event curves) were constructed and compared using logrank testing. The significance of each variable and combination subgroups was tested. Significance was set at 0.05%. Graphs were not truncated but numbers remaining at intervals were recorded if subgroups were small. Both overall survival and cancer-specific survival curves were created for the main analysis to detect any obvious variation and to allow comparison to other studies (in particular to determine if variation in results could be accounted for by differing endpoint measure). Non-cancer deaths were censored for cancer-specific survival. For both analyses, cases that had not had an event at time of follow-up were censored. For further subgrouping, only cancer specific analysis was conducted, being this study‟s main outcome measure. Five-year overall survivals were determined from Kaplan-Meier survival life tables.

The Cox Regression proportional hazard model Multivariate analysis was used for multivariate analysis. Main effects models were calculated with backward stepwise exclusion using log likelihood ratio test. Factors with significance over 0.20 were included in the equation. Gender and site, being two of the foci of the study, were forced into the model despite lack of significance on univariate. Elimination from the model was set at 0.10. Final significance was set at 0.05 and results rounded to two decimal places. Goodness of fit was checked using Cox-Snell residuals, linearity of covariates was checked using Martingale residuals and the proportional hazard assumptions were tested.

A compounding effect with chemotherapy was tested by regression interaction modelling, as multivariate analysis alone will not determine an effect on chemotherapy survival. A variable that has prognostic significance on multivariate analysis will influence survival in the chemotherapy and non-chemotherapy group, i.e. it will still have an effect independent of other prognostic factors. For example if presence of factor X is associated with a 10% better outcome, then the patient group with factor X will tend to do 10% better than those without X in either chemotherapy or non-chemotherapy cohort (all other variables being equal). However, there may be no interaction as such with chemotherapy – the effect of chemotherapy is the same regardless of factor X, i.e. if chemotherapy improves survival by 15% then all patient groups (with or without factor X) improve by 15% but those with factor X will still be ahead 10%. On the other hand, if factor X has a positive interactive survival effect with chemotherapy, it would still be expected that this group do 10% better in the non-chemotherapy group but the outcome in the chemotherapy group with factor X

would be greater than the expected 10%. The effect of either (factor X and chemotherapy) is compounded, as though they work synergistically.

This is an important distinction, as multivariate analysis is often used in studies to imply one group does better than another with treatment and hence they are the ones gaining benefit. The outcome may be better in that group but it does not mean the other group is not gaining equal benefit; it is just that they have a worse prognosis with or without treatment.

Interaction testing looks for effects-modification by a co-variate on the effect of the main study variate (the survival benefit from adjuvant chemotherapy). The equation for interaction incorporates all variables of interest into an adjusted analysis model. Each covariate is then tested individually for an association by examining for an effect at different strata of that variable. If the relative risk across the strata of a potential effect-modifier is homogenous then there is no interaction between that covariate and chemotherapy. P values less than 0.05 suggest a significant interaction, which can be either synergistic or antagonistic.

Association between factors was tested using logistic regression.

Most analysis was performed with Statistical Package for the Social Sciences (SPSS) for Windows; version 12.0 (Copyright 2003, SPSS Inc., Chicago, IL, USA). Interaction testing was performed using Intercooled Stata for Windows; version 8.2 (Copyright 2004, StataCorp LP, Texas, USA). Statistical help was sought to ensure the most appropriate analysis was carried out. Manual models were also created by

the assisting statistician, the results of which correlated with those produced by the candidate with SPSS.

3.8.2 Power Calculations

Power calculations determined that 330 patients were required per group to achieve 80% power to show a 10% survival difference with significance set at 0.05. Ninety-eight patients per group had an 80% probability of showing a 20% difference with significance set at 0.05%.

3.9 Ethics

Ethics approval was sought and gained from each of the participating institutions and the State Cancer Registry.

4 RESULTS - STUDY GROUP CHARACTERISTICS

4.1 Overview

This chapter discusses general results including inclusions, exclusions and their justification, study group demographics and characteristics. It will produce a results base that will be used in subsequent chapters. Study cohort matching will be examined and used for consideration in later analyses. Variations in study parameter frequencies across subgroups will be investigated as a starting point to explore differences between cohorts.

4.2 Inclusions

From the 23-year study period, 1166 potential cases were identified and entered into the database. Of these, 352 were excluded (see below) and 814 included for analysis. Analysis of MSI was not possible in 12 cases (11 blocks not located, 1 insufficient material on block) thus 802 cases were included in MSI analysis. Three cases were included in MSI analysis but not site and pathology analyses. They were all cases of synchronous stage C cancers occurring across colon, thus the index cancer could not be determined for site and pathology analysis. MSI status, however, was the same for both tumours.

4.3 Exclusions

352 cases were excluded (**Table 53**).

Ninety-seven patients had surgery outside of the study hospitals. These cases had been included retrospectively onto the registry when referred to one of the study hospitals for further treatment, for example subsequent liver resection. Pathology was sent to private laboratories and not available for review. As detailed in methods, these

cases were not included as to do so would lead to selection bias towards a worse prognosis group.

Laboratory number could not be located or pathology was unavailable in 26 patients. There were several reasons, namely:

- 16 - slides and blocks not in file and unable to be located
- 14 - laboratory number could not be located.
- 1 - no slides in file and insufficient material to assess in retrieved block

Forty-two patients died during the hospital admission for their initial resection and consequently they were excluded.

Eighty-two cases had metastases at the time of surgery and were as follows:

- 35 metastases were established by reviewing the casenotes. Particular attention was paid to patients who died within 6 months of operation. Many of these cases had recognised spread at initial diagnosis but were incorrectly staged in the registry.
- 19 had metastases on the clinical history on the pathology report
- 28 had clear metastases on review of the pathology (body of report and slides)

Thirty-one cases were in fact not stage C (they were lymph node negative). Most of these had been mistakenly entered in the hospital databases as stage C, a few were incorrectly reported on pathology.

Thirty-one patients underwent palliative resection. Twenty-two of these had positive margins on pathology review and nine others had residual disease noted on the operation report. Most of these cases were detected while reviewing "deaths within 6 mths" or were indicated on state registry data (the field for "residual disease" was marked).

Seven of the cases with multiple cancers were excluded for the following reasons:

- Two cases had metachronous disease across the colon.
    - One case had numerous operations of which at least two were for stage C cancers and he subsequently died of his cancer.
    - The other had a stage C cancer followed by a stage B cancer within one year and died from cancer.
- Five had synchronous disease across the colon without a clear index case and incongruous MSI status.

Management of multiple cancers in this study is detailed in the methods.

Only two patients were excluded due to inadequate clinical information. One patient was to have chemotherapy on return interstate and commencement could not be confirmed. In the other case the chemotherapy type was listed as unknown and details were not in casenotes.

Three patients did not have an operation as they died before receiving any treatment. How they came to be recorded as stage C on registry is unclear. There may actually be more cases that fall into this category, as several cases for which pathology was unable to be located did have a biopsy result registered and whether or not surgery

followed (maybe elsewhere) is unclear. These cases were excluded also as having “no pathology”.

Seven cancers were not adenocarcinoma (despite being listed as such on the registry). Search of registry data was conducted for “colorectal cancer” but recognised non-adenocarcinomas were excluded before transfer to the database. These seven exclusions were detected on subsequent pathological review.

Eleven patients had non-standard therapy. Ten had 5FU combined with oxaloplatin and one had CMF for concurrent breast Cancer.

Seven patients had secondary non-colorectal primaries and died of cancer with the responsible cancer unable to be determined (i.e. liver metastases could have been from either primary). These were excluded.

Table 53 Exclusions

Reason	Subtotal	Total
Operation elsewhere		97
Pathology not located		31
In hospital deaths		42
Metastases		
• Operation note	20	
• Clinical notes	15	
• Pathology report	19	
• Pathology	28	
Total		82
Lymph node negative (not stage C)		32
Non curative		
• margins positive	22	
• residual at operation	9	
Total		31
Multiple cancers (non index C)		
• metachronous across colon	2	
• synchronous across colon	5	
Total		7
Inadequate information		2
No operation		3
Non adenocarcinoma		7
Non 5FU		11
Other cancer		7
TOTAL		352

4.4 Study Population

Of the 814 analysed, median age was 71.1 years (range 30.3 - 96.1 years) (Table 54). Median follow-up was 36.30 months (range 0.6 to 290) (Table 54). The overall median time was significantly shortened by early deaths. The median time in the surviving patients was 72 months (6 years). Overall, 469 (57.6%) of cases died during the study period (Table 56). Of these, 76% were cancer-related deaths. Gender distribution was near equal (men 49.6%, women 50.4%) (Table 57). Chemotherapy was commenced in 37.7% and radiotherapy in 11.7% (Table 58). Cases prior to 1993 (year of implementation of adjuvant chemotherapy) comprised half of the chemotherapy cohort. Potential reasons for the later cases not receiving chemotherapy are discussed later when the cohorts are compared.

Table 54 Age

Median age	71.1yrs
Mean age	69.7yrs
Alive median	68.8yrs
Died median	73.2yrs
Range age	30.3-96.1 yrs

Figure 3 Age distribution

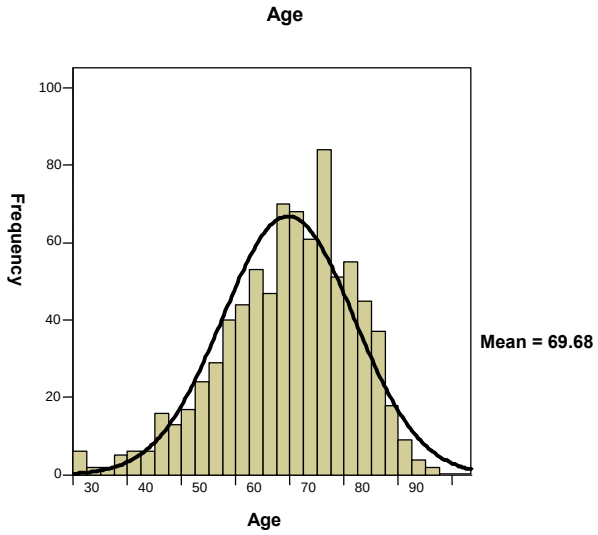


Table 55 Follow- up

Follow-up	
Median	36.3 mths
Range	0.59– 290 mths
Alive median	72 mths (6 yrs)
Died median	22.9 mths

Table 56 Deaths

Deaths	***n***	**%**
Died	469	57.6%
Alive	345	42.4%
Cancer deaths	355	43.6%

Table 57 Gender distribution

Gender	***n***	**%**
Men	404	49.6%
Women	410	50.4%

Table 58 Adjuvant therapy

Adjuvant therapy	***n***	**%**
Chemotherapy	307	37.7%
Radiotherapy	95	11.7%

4.5 Tumour Characteristics

4.5.1 Site

60.8% of cancers were located distal to or at the splenic flexure and 39.2% proximal (Table 59). Subsite breakdown was similar to expectations (Table 59).

4.5.2 Histological Profile

Breakdown of histological features is detailed in Table 60. Most cases were T3 (invading beyond muscularis propria). Lymph node involvement was confined to three or less nodes in the majority (71%). In the 28.9% of the cases with more than three involved nodes (N2), 79% were T3. Seventeen percent of tumours were poorly differentiated. Mucinous component was present in 39.3%, although only 47 (5.8%) were true mucinous type (>50% mucinous). When type was broken down further, very few were undifferentiated (2%) or signet ring type (0.4%). Radiotherapy influenced the ability to assess type, with radiotherapy-affected cells appearing vacuous and at times difficult to distinguish from mucinous.

No lymphocytic infiltrate was identified in 11.8% of our patients. These cases did not necessarily have a higher rate of other poor features. Grade was poor in 17%, which was similar to the overall rate. Peritumoral lymphocytes were detected in most patients (84.5%). Crohn"s-like lymphocytes were observed in 21.6% and TILs in 4.3%. Stroma was described as fibrous in 66.5%, keloid in 32.3% and myxoid in 1%.

An infiltrating margin was found in 31.6% and pushing in 67.8%. Budding was observed in 38.7%. If the margin was infiltrative, not surprisingly, it was more likely

that budding was present (63%). However, 28% of those with a pushing margin also demonstrated budding, suggesting the correlation is not complete. Similarly, of those that had budding, 52% had an infiltrative margin, compared to 20% of those when no budding was seen. Mural invasion was observed in 68.6%, extramural in 55.8% and perineural in 17.5%. Twenty percent of cases with extramural vascular invasion did not have mural vascular invasion. Obstruction was determined to be present in 15.8% and perforation in 3.7%.

4.5.3 MSI

Of the 814 cases, twelve could not be analysed either due to extraction difficulty (one insufficient tissue) or missing blocks (these cases had adequate archived slides for histological assessment). Hence, 802 cases were included in MSI analysis. MSI was evident in 77 (9.6%) cases.

Status was established by various methods. Seventy-three cases had MSI status established prior to this study and analysis was not repeated (either unrelated research, recent cases where MSI has been tested on all or clinician request). For these cases a panel of markers had been used comprising BAT25, BAT26, BAT40, DSS123, D10S197, D17S579, D18S34, D5S346 D17S250 and LMYC, with >30% constituting MSI-H. For the remainder (729), BAT26 and BAT40 were analysed and for BAT40, the results were compared to normal tissue. In 720 there was concordance of BAT26 and BAT40 and status confirmed. In one case BAT40 failed to amplify and the BAT26 result was used (negative). In four cases interpretation was equivocal and the full panel was run (Table 61). All were MSI negative. Four discordant results were analysed using the full panel, with criteria for positive being >30% unstable (Table

61). Three of these were BAT 26 negative but BAT 40 positive. The full panel determined two cases to be positive and the other negative. In one other case BAT26 was positive while BAT40 was negative and the panel result was positive.

Sensitivity of BAT26 cannot be commented upon without having performed the full panel on all cases. However from the above discordant results it can be seen that BAT26 falsely determined two positive cases to be negative (false negatives). Without knowing the sensitivity of BAT40 it is unclear if all false negatives were detected. There were no known false positives from these observations but the true rate was unable to be determined without running the panel on all the BAT26 determined positive cases. From the discordant results BAT40 determined one false positive and one false negative. Again true accuracy could not to be determined without more extensive testing.

Table 59 Site

	n	**%**
Proximal	320	39.5%
Distal	491	60.5%
Subsite		
Caecum	135	16.7%
Ascending colon	78	9.6%
Hepatic Flexure	35	4.3%
Transverse colon	48	5.9%
Splenic flexure	24	3.0%
Descending colon	29	3.6%
Sigmoid	170	21.0%
Rectosigmoid	73	9.0%
Rectum	219	27.0%

Table 60 Histology parameters

Overall	Subcategory	*n**	%†	*Comments*
T stage	1	10	1.2%	
	2	55	6.8%	
	3	638	78.0%	*More later stage as C only*
	4	105	13.0%	
	X	3		
N stage	1	577	71.1%	
	2	234	28.9%	*186 (79%) T3, 40 (17%) T4*
Micro metastases		30	3.7%	
Differentiation	Mod	667	82.2%	
	Poor	143	17.6%	
	NA	1	0.1%	*Radiotherapy affected*
Type	NOS	484	59.7%	
	Adenomucinous	259	31.9%	
	Mucinous	47	5.8%	
	Undifferentiated	11	1.4%	
	Undiff/Mucinous	5	0.6%	
	Signet	3	0.4%	
	NA	2	0.2%	
	Sig ring comp	*26*	*3.2%*	*12 adenomucinous, 13 mucinous, 1undifferentiated/mucinous*
Mucinous Comp	Yes	314	39.3%	
Lymphocytes	Peritumoral	685	84.5%	*6 NA‡*
	Crohn"s	175	21.6%	*4 NA*
	TILs	35	4.3%	*2 NA*
	None	96	11.8%	*16 (17%) poor, 39%- one of 27 adenomuc, 9 muc, 1 signet*
Stroma	A	539	66.5%	
	B	262	32.3%	
	C	8	1.0%	
	NA	5	0.6%	
Infiltrating margin	yes	256	31.6%	*160 (63%) budding,*
	no	550	67.8%	*154 (28%) budding*
	NA	5	0.6%	
Budding	yes	314	38.7%	*160 (51%) infiltrating margin*
	no	492	60.7%	*96 (20%) infiltrating margin*
	NA	5	0.6%	
NeuroVasc Invas	Mural	558	68.6%	*361(65%) extra also, (36% did not). 2 NA*
	Extramural	453	55.8%	*361 (80%) also mural, 93 not (21%) suggests sampling error 2NA*
	Perineural	142	17.5%	*100 both vasular as well (70%), 11(8%) just mural, 21 (15%) extra mural only. 7 NA*

Overall	Subcategory	*n**	%†	*Comments*
Apical node	Positive	36	19%	*Only 190 known*
Obstructed		129	15.8%	
Perforated		30	3.7%	

*Denominator 811, 3 had MSI but excluded from path as synchronous lesions,
†Percentage calculated from valid inclusions (minus unknowns)
‡NA = not assessable, usually following radiotherapy

Table 61 Full panel MSI results

ID	MSI Status	Bat25	Bat26	Bat40	D2S123	D10S197	D17S579	D18S34	D5S346	D17S250	LMYC
1424	neg	neg	neg	neg	neg	pos	neg	neg	nd	neg	neg
1578	pos	pos	neg	pos	neg	pos	pos	pos	neg	neg	pos
1764	pos	neg	pos	neg	neg	pos	pos	pos	neg	neg	neg
2273	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg
1943	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
1895	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	pos
2198	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
183	pos	neg	neg	pos	pos	neg	pos	pos	nd	neg	pos

4.6 Cohort Matching

4.6.1 Chemotherapy

The chemotherapy versus no chemotherapy groups were compared (Table 62) to assist in interpretation of unadjusted results and ensure all significant differences were accounted for in adjusted analysis. Various discrepancies were observed with the chemotherapy cohort having the following characteristics:

- Younger age (median 65.4 years vs 75.1 years)
- Male predominance (56 % vs 46%)
- Higher nodal stage (N2 34% vs 26%)
- Higher rate of extramural vascular invasion (60% vs 53%)
- Higher rate of mural vascular invasion (73% vs 67%)
- Higher rate peritumoral lymphocytic invasion (94% vs 80%)

To determine a reason for these differences further analyses were performed. To determine if the male bias was due to a higher rate of rectal cancers (and therefore a greater tendency for referral), an association was investigated. While the percentage of men with rectal cancer was greater than women (32.0% vs 22.1% Table 63), the percentage of rectal cancers cases that received chemotherapy is not significantly different from other sites (Table 64).

There were a greater proportion of cases with four or more involved nodes (N2) in the chemotherapy group. This could be explained by selection bias (those with more involved nodes would have been assessed to have advanced cancers prompting a lower threshold for offering chemotherapy in patients that may otherwise had been

deemed unsuitable i.e. older or comorbidities). To test this theory, the dataset was divided into pre-and post-standard chemotherapy years (Table 65). The discrepancy should be pronounced in the later years whereas the earlier cohort should provide a baseline comparison (given the lack of selection bias as most cases did not receive chemotherapy). Variance in administration of treatment according to involved node number was only significant in the post-1993 subgroup suggesting that selection bias accounted for the difference.

Cancers with vascular invasion have been perceived as more aggressive, possible prompting a stronger recommendation for chemotherapy. The same method as above was used to test this theory (Table 66). The findings do not support selection bias but the smaller size of the subgroups makes interpretation difficult (the difference between cohorts was no longer significant). To test if this aberration was due to association with another disproportionately distributed factor logistic regression analysis was performed (Table 67 and Table 68). There was an observed association between extramural vascular invasion and male gender as well as higher nodal status. Extramural invasion was associated with mural invasion. .

The chemotherapy group had a higher rate of peritumoral lymphocytes. There is no immediately apparent reason for this. The finding is consistent across eras (Table 69) and as this feature has only recently been included in reporting, there would be no influence on patient selection for treatment. Logistic regression was also carried out to test for associations for peritumoral lymphocytic invasion (Table 70). The only two significantly associated factors were infiltrating margin (decreases likelihood of having peritumoral lymphocytes) and budding (increases likelihood) neither of which explains the variation in chemotherapy administration rate.

4.6.2 Gender

As subgroups of interest in this study, gender and site were examined to determine if there were any obvious mismatching (that should be considered in analysis) or associations (that may explain any differences). Obvious differences in tumour characteristics between genders and, more so, site were examined to determine if the speculation that cancer biology varied according to these factors was supported.

There were several significant differences observed between the two gender groups (Table 71). The female group was significantly older (72.5 yrs vs 69.8 yrs, P=0.004), had more proximal lesions (in women 45.3% were proximal compared to 33.0% in men) and a much higher rate of MSI (13.4% vs 5.8%). The other pathological differences are thought to be due to this variation in MSI rate (higher poor differentiation and TILs) and this is supported by logistic regression analysis with these factors losing significance (Table 72). Interestingly, the association with proximal lesions also disappears and thus, may also be explained by an association with MSI. The other factors independently associated with women with CRC were higher T stage (without higher nodal status) and extramural vascular invasion. A cause for the associations is not readily apparent. There is a persistent slight association with older age.

4.6.3 Site

Site variations in CRC contributed to the discovery of an alternative path to cancer and many of the differences we observed appear to be related to the higher rate of MSI (20.4% vs 2.7%). This would be expected to explain the higher rate of poorly differentiated tumours and mucinous type and would also be sufficient to explain the

gender variation with women comprising 58.2% of the proximal cancers compared to 45.2% of distal cancers (actual difference from expected was 26 cases, easily accounted for by 51 extra MSI cases) (Table 73). A higher rate of rectal cancers in men was observed (32.0% vs 22.1%) (Table 74).

To determine which factors were significantly associated with site, independent of MSI, logistic regression analysis was conducted (Table 75). The strongest association remained MSI. Female gender just reached significance independent of MSI, as did mucinous type and poor differentiation. There was a strong association with advanced T stage, which may be consistent with later presentation but the lack of association with size or nodal status does not support this.

4.6.4 MSI

The MSI and MSS cohorts are compared in Table 76. Adjuvant therapy was delivered at the same rate between the two cohorts and there was no significant difference in death rates. Median age was significantly younger on unadjusted analysis in the MSI group (70.0 vs 72.8) and there was a preponderance of women (70.1%). Most of the MSI cancers were proximal (83%), which is markedly higher than the percentage for MSS cancers (35%).

Comparative tabulation of the histological variables according to MSI status will be provided in the next chapter where pathological differences between the MSI and MSS cohorts will be explored further and associations discussed.

Table 62 Chemo vs non-chemo cohort

Subgroup	Subcategory	Chemo (307)	*n*	No Chemo (507)	*n*	P value*	Proportion test
Median age		65.4 yrs		75.1 yrs		**<0.0001**	
Gender	Men	56.0%	172	45.8%	232	**0.005**	
	Women	44.0%	135	54.2%	275		
Site	Proximal	37.3%	114	40.4%	204	0.37	
	Distal	62.7%	192	59.6%	301		
T stage	Confined to wall	8.2	25	8.0	40	0.90	
	Breached	91.8	280	92.0	463		
N stage	N1	66.4	204	74.2	376	**0.02**	
	N2	33.6	103	25.8	131		
Differentiation	Mod	84.3%	258	81.2%	409	0.25	
	Poor	15.7%	48	18.8%	95		
Type	Mucinous comp	39.4	119	39.3	195	0.98	
	NOS	60.6	183	60.7	301		
Subtype	NOS	59.6%	183	59.4%	301	0.54	0.95
	Adenomucinous	30.6%	94	32.5%	165		0.57
	Mucinous	7.5%	23	4.7%	24		0.10
	Signet	0.0%	0	0.6%	3		0.29
	Undifferentiated	1.0%	3	1.6%	8		0.47
	Undiff/Mucinous	0.7%	2	0.6%	3		0.92
Lymphocytes	Peritumoral	93.7%	283	79.9%	402	**<0.0001**	
	Crohn˝s	22.8%	69	21.0%	106	0.53	
	TILs	3.9%	12	4.6%	23	0.68	
Stroma	a	64.0%	194	68.0%	342	0.14	0.21
	b	35.6%	108	30.6%	154		0.16
	c	0.3%	1	1.4%	7		0.14
Obstructed		14.1%	43	17.2%	86	0.26	
Perforated		3.6%	11	3.8%	19	0.89	
Infitrating margin		34.7%	105	30.0%	151	0.17	
Budding		38.9%	118	39.0%	196	0.995	
NeuroVascular invasion	Mural	73.4%	223	66.5%	335	**0.04**	
	Extramural	60.3%	184	53.4%	269	**0.05**	
	Perineural	19.5%	59	16.6%	83	0.30	
MSI pos		9.0%	27	10.0%	50	0.66	

*Chi test for significance of categorical data, Mann Whitney for scaled data (age), Proportion testing used to compare individual

Table 63 Subsite Gender distribution

Subsite	Men	Women
Caecum	13.2%	20.1%
Ascending colon	8.2%	11.0%
Hepatic flexure	3.5%	5.1%
Transverse colon	5.2%	6.6%
Splenic flexure	3.5%	2.5%
Descending colon	3.0%	4.2%
Rectosigmoid	10.9%	7.1%
Sigmoid	20.6%	21.3%
Rectum	**32.0%**	**22.1%**

Table 64 Chemotherapy/subsite

	AC	C	DC	HF	Rec	RS	SF	Sig	TC
No Chemo	65.4%	67.4%	79.3%	60.0%	60.3%	50.7%	70.8%	63.5%	52.1%
Chemo	34.6%	32.6%	20.7%	40.0%	**39.7%**	49.3%	29.2%	36.5%	47.9%

Table 65 Chemo nodal status correlation pre- and post-standard chemotherapy (1992 and earlier)

	N stage	Chemo	*n*	No Chemo	*n*	p
Pre 1993	1	60.9%	14	71.6%	184	0.28
	2	39.1%	9	28.4%	73	
Post 1993	1	66.9%	190	76.8%	192	0.011
	2	33.1%	94	23.2%	58	

Table 66 Chemo vascular invasion correlation pre- and post-standard chemotherapy

		Chemo	*n*	No Chemo	*n*	p
Pre 1993	Mural	52.2%	12	64.2%	165	0.25
	Extra mural	60.9%	14	52.5%	135	0.44
Post 1993	Mural	75.1%	211	68.8%	170	0.11
	Extra mural	60.3%	170	54.3%	134	0.16

Table 67 Extramural vascular associations

	OR	95% CI		p
		Lower	Upper	
Female gender	0.71	0.51	0.98	0.04
Breached bowel wall	6.57	3.07	14.09	<0.0001
N2	1.83	1.25	2.66	0.002
Poor Differentiation	2.33	1.40	3.88	0.001
TILs	0.35	0.13	0.89	0.028
Perineural	4.18	2.45	7.12	<0.0001
Mural	2.83	2.00	4.04	<0.0001
Stroma - keloid	1.97	1.37	2.83	<0.0001

Table 68 Mural vascular associations

	OR	95% CI		p
		Lower	Upper	
Mucinous component	0.62	0.45	0.86	0.005
Budding	1.70	1.20	2.40	0.003
Extramural	2.97	2.15	4.12	<0.001

Table 69 Chemo peritumoral lymphocyte correlation to year

	Chemo	*n*	No chemo	*n*	p
Pre 1993					
Peritumoral	91.3%	21	72.0%	185	0.04
1993 on					
Peritumoral	93.9%	262	88.2%	217	0.02

Table 70 Associations with peritumoral lymphocytes

	OR	95% CI		p
		Lower	Upper	
Infiltrating Margin	0.57	0.36	0.88	0.01
Budding	2.00	1.27	3.16	0.003

Table 71 Gender cohort comparison

Subgroup	Subcategory	Men	*n*	Women	*n*	P value	Proportion test
Median age		69.8yrs	404	72.5yrs	410	**0.004**	
Died	Overall	58.4%	236	56.8%	233	0.65	
	Cancer	45.5%	184	41.7%	171	0.27	
Chemotherapy		42.6%	172	32.9%	135	**0.005**	
Size		40mm		40mm			
Site	Proximal	33.0%	133	45.3%	185	**<0.0001**	
	Distal	67.0%	270	54.7%	223		
	uk		1		2		
Differentiation	Mod	85.1%	342	79.7%	325	**0.04**	
	Poor	14.9%	60	20.3%	83		
Type	NOS	61.1%	247	57.8%	237	0.18	0.33
	Adenomucinous	32.4%	131	31.2%	128		0.71
	Mucinous	4.7%	19	6.8%	28		0.19
	Signet	0.2%	1	0.5%	2		0.57
	Undifferentiated	0.5%	2	2.2%	9		0.04*
	Undiff/Mucinous	0.2%	1	1.0%	4		0.18
	uk	0.7%	3	0.5%	2		
Lymphocytes	Peritumoral	84.9%	338	85.3%	347	0.89	
	Crohn‟s	20.5%	82	22.9%	93	0.42	
	TILs	2.2%	9	6.4%	26	**0.004**†	
Stroma	a	66.4%	265	66.6%	271	0.42	0.88
	b	33.1%	132	31.9%	130		0.77
	c	0.5%	2	1.5%	6		0.16
	uk				1		
Obstructed		14.4%	58	17.7%	71	0.21	
Perforated		3.3%	13	4.2%	17	0.47	
Inf margin		34.7%	138	28.9%	118	0.08	
Budding		39.2%	156	38.7%	158	0.89	
NeuroVasc	Mural	71.8%	287	66.4%	271	0.10	
	Extramural	58.6%	235	53.4%	218	0.14	
	Perineural	18.3%	73	17.0%	69	0.64	
MSI pos		5.8%	23	13.4%	54	**<0.0001**	
	uk	1.5%		1.5%			

*small number
† Presumably MSI related

Table 72 Female CRC associations

	OR	95% CI		p
		Lower	Upper	
Age	1.01	1.002	1.03	0.02
T3/T4	2.07	1.18	3.64	0.11
Extramural	0.66	0.49	0.90	0.008
MSI	2.25	1.31	3.86	0.003

Logistic regression, forward likelihood ratio for elimination at 0.10, all variables were included

Table 73 Site cohort variations

Subgroup	Subcategory	Distal	*n*	Proximal	*n*	p	Proportion test
Median age		70.3	493	71.7	318	0.05	
Died	Overall	57.6%	284	57.9%	185	0.94	
	Cancer	44.2%	218	42.8%	136	0.68	
Gender	Men	54.8%	270	41.8%	133	**<0.0001**	
	Women	45.2%	223	58.2%	185		
Chemotherapy		38.9%	192	35.8%	114	0.37	
Differentiation	Mod	89.0%	438	72.0%	229	**<0.0001**	
	Poor	11.0%	54	28.0%	89		
	uk		1		2		
Type	NOS	66.5%	328	49.1%	156	**<0.0001**	<0.0001
	Adenomucinous	28.0%	138	38.1%	121		0.003
	Mucinous	4.9%	24	7.2%	23		0.16
	Signet		0	0.9%	3		0.06
	Undifferentiated		0	3.5%	11		<0.0001
	Undiff/Mucinous	0.2%	1	1.3%	4		0.06
	uk	0.4%	2		0		
Signet rings		2.0%	10	5.0%	16		
Lymphocytes	Peritumoral	84.8%	414	85.5%	271	0.80	
	Crohn‟s	18.8%	92	26.1%	83	**0.01**	
	TILs	1.6%	8	8.5%	27	**<0.0001**	
Stroma	a	68.1%	333	64.0%	203	0.41	0.28
	b	31.1%	152	34.7%	110		0.26
	c	0.8%%	4	1.3%	4		0.53
	uk		5		1		
Obstructed		14.5%	71	18.6%	58	0.12	
Perforated		3.3%	16	4.5%	14	0.38	
Inf margin		31.9%	156	31.5%	100	0.92	
Budding		41.5%	203	35.0%	111	0.065	
NeuroVasc	Mural	68.6%	336	69.8%	222	0.71	
	Extramural	55.0%	270	57.5%	183	0.47	
	Perineural	19.4%	95	15.0%	47	0.11	
MSI pos		2.7%	13	20.4%	64	**<0.0001**	
	uk	1.6%	8	1.5%	4		

Chi test for comparisons unless expected numbers <5, then Fisher exact. Mann Whitney test for scaled data.

Table 74 Subsite Gender variations

Subsite	Men	%	Women	%
Caecum	53	13.2%	82	20.1%
Ascending colon	33	8.2%	45	11.0%
Hepatic Flexure	14	3.5%	21	5.1%
Transverse Colon	21	5.2%	27	6.6%
Splenic Flexure	14	3.5%	10	2.5%
Descending Colon	12	3.0%	17	4.2%
Rectosigmoid	44	10.9%	29	7.1%
Sigmoid	83	20.6%	87	21.3%
Rectum	129	32.0%	90	22.1%

Table 75 Associations of site

	OR	95.0% C.I.		p
	of proximal lesion	Lower	Upper	
Gender - female	1.38	1.009	1.89	0.04
Bowel Wall – T3 or 4	3.47	1.62	7.46	0.001
Poor differentiation	1.80	1.17	2.77	0.008
Mucinous type	1.43	1.03	1.98	0.03
MSI	5.83	2.99	11.38	<0.001

Logistic regression, forward likelihood ratio for elimination at 0.10
All variables were included

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Table 76 MSI/MSS cohorts

Subgroup	Subcategory	MSI *n*	MSI %	MSS *n*	MSS %	p
Number		77		725		
Died	Overall	48	60.8%	416	57.5%	0.40
	Cancer	33	42.9%	318	43.9%	0.87
Chemo		27	35.1%	273	37.7%	0.65
Median age		72.83*		70.70		**0.02**
Gender	Men	23	29.9%	375	51.7%	**<0.0001**
	Women	54	70.1%	350	48.3%	
Proximal		64	83.1%	250	34.6%	**<0.0001**

*Age analysed as ranked data

4.7 Multiple Cancers

Cases of multiple colorectal cancers were dealt with in more detail. The rate of metachronous and synchronous lesions is of interest, especially the rates in MSI cases given the propensity for multiple cancers in familial cases. Furthermore, the method with which multiple cancers were dealt with for the study is important for reproducibility.

4.7.1 Metachronous Cancers

Of the 1166 cases included in the database, 25 patients had metachronous tumours (2%). Metachronous lesions were detected by several methods. Both state and hospital registries record the presence of multiple primaries and on pathology reports there is often suggestion of previous surgery, either in the clinical history or the histological examination (i.e. evidence of previous resection). All these cases were confirmed by reviewing casenotes and searching for past pathology reports. Despite these checks, it is likely this rate is an underestimation.

Of these 25 cases with metachronous tumours, 6 were excluded from analysis for other reasons (two palliative, 1 distal metastases, 1 other cancer, 1 in hospital death, 1 no pathology). Two were excluded from analysis as the lesions were across the colon and the index C cases could not be identified and the patient subsequently died of cancer. One of these patients had both a stage B and stage C cancer within 5 years, the other had four resections for at least five cancers and staging was unclear. This was the only case of more than two metachronous lesions.

Thus, 17 cases were included in analysis because it was determined that the index stage C cancer could be identified. In ten of these the cancer was unlikely to be contributory to outcome, the first having occurred more than 5 years prior, and in five the other cancer was stage A (4 subsequent, 1 prior). In four cases the other cancer was stage B but the patient had not died (hence neither cancer contributed to outcome). In one case, the other cancer was stage B and patient died of cancer but the responsible cancer was clearly the first stage C cancer because it was found to have recurred locally at the time of the subsequent operation.

4.7.1.1 MSI in Metachronous Cases

Only five cases had MSI status established on both tumours. In four cases both tumours were MSS. The fifth patient had a stage C caecal cancer that was MSS, followed 16 months later by a MSI tumour in the transverse colon. One other case had a MSI positive tumour but the status of the second CRC was unknown. Hence of 77 MSI positive lesions, 2 had recognised metachronous CRCs giving a rate of 2.5%.

4.7.2 Synchronous Cancers

Five percent of patients from the original dataset had synchronous lesions (63). Fifteen were excluded for other reasons (3 palliative, 2 other cancers, 3 metastases, 1 inadequate information, 4 in-hospital deaths, 1 operation elsewhere and one was already excluded for multiple metachronous lesions). Five others were excluded because the cancer responsible for outcome could not be determined. Three of these patients had synchronous stage C lesions across the colon and the index C case could not be established. Two other patients had stage B and C cancers and subsequently died of cancer, therefore the cancer contributing to outcome could not be determined.

Thus, 43 cases of synchronous CRCs were included in the final analysis (rate 5%, 43 of 814). In 16 of these cases both cancers were distal, in 18 both proximal and 9 were across the colon. Interestingly, there was not a higher rate in the proximal colon. The nine cases where the synchronous cancers occurred across the colon were considered in the following way. In six cases the second cancer was an earlier stage (four stage B and two stage A) and patient had not died hence neither contributed to outcome. Three cases were included for MSI analysis but not site, as index C cases could not be established but MSI on both cancers concurred.

4.7.2.1 MSI in Synchronous Cases

Twelve cases with synchronous lesions had evidence of MSI. In eight cases both lesions were microsatellite unstable. In two other cases the primary cancer was MSI high but tissue was unavailable for the second lesion. Two further cases had discordant lesions. The larger cancer (therefore considered the primary lesion by our definition) in both cases was MSS but both second cancers were MSI. One of these had a 25 mm MSS Mucinous lesion and a 5 mm invasive adenomucinous component in an adenoma that was MSI. The other cases had a 50 mm mucinous MSS cancer and a 15 mm adenomucinous cancer. It is possible the extent of mucin in the larger lesions has led to an erroneous negative result. Both were grouped with the MSS cohort for analysis. Thus the MSI rate in synchronous lesions was 23% (and possible 28% if mucinous two were in fact positive). Of the 77 MSI positive cases, 10 had synchronous lesions (13%). Of the 12 MSI positive synchronous cases (one or both cancers), three were distal while nine were proximal, consistent with the proximal tendency seen in MSI cases.

4.8 Discussion

The clinical profile of the study group was similar to that reported in the literature with median age in the eighth decade and near equal gender distribution [19, 26, 63]. There was no trend towards a male predominance. Forty percent of tumours were located in the proximal colon, which is slightly more than reported in studies inclusive of all stages of colorectal cancer [3, 26, 63].

As the group comprised stage C cases, it is not surprising that most tumours were T3. Of the cases that were N2 (3 or more involved node), the percentage that were T3 was not significantly different from the N1 cases suggesting extent of bowel wall involvement is not predictive of number of nodes involved. Only 190 of the included cases had apical nodal status established. It is not known if selection bias existed (that this factor was more likely to be commented upon in the report or sampled if positive). For both these reasons this parameter was not considered meaningful for further analysis.

The rate of poorly differentiated tumours was 17%. This is similar to studies inclusive of all stages (10%-28%) [12, 105, 131] but less than work inclusive of only stage C disease (32%) [20]. In the current study, the inter observer variation often observed when tumours are graded was eliminated as all histology was re-evaluated by a single pathologist. Categorisation was limited to moderate or poor to improve reproducibility. Minimal sampling may influence grading, as degree of differentiation tends to vary within a tumour. However, the leading edge was assessed to minimise error. Mucinous type was difficult to distinguish in tissue affected by neoadjuvant

radiotherapy but comprised only a small number of cases (including two where no assessment was possible).

The high prevalence of peritumoral lymphocytes and the subjectiveness of this measure raises questions as to the significance and usefulness of this measure. This will be addressed further when the prognostic significance of tumour lymphocytes is examined.

The rate of fibrous and keloid stroma is similar to the literature but myxoid has been found to be present in up to 12% of rectal cases [144], a much higher rate than we observed in colon and rectal cancer. Previous work has found that the presence of keloid and myxoid stroma is more pronounced in advanced stage tumours [144] and as such we should have detected an even higher rate. The age of the slides probably contributed to this variation and may have led to inaccuracy in our study. Fading of H and E staining made accurate determination of stroma type difficult and thus it is likely we have under-called keloid and myxoid type.

Given this study included only stage C cases, the rate of infiltrative margin and budding might be expected to be high given the presumed related tendency for spread. Given both are reported to indicate aggressive histology, it was interesting that there was not a closer correlation. Budding was more likely if the tumour margin was infiltrating, however only around half of those with budding had an infiltrative margin.

Rate of neurovascular invasion was high as might be expected given the advanced stage of the cohort. It would be expected that all those with extramural invasion

would also have mural invasion however this was not the case in 20%. This leads to suspicion that the result for mural invasion may be erroneous due to lack of detection, possibly because of sampling error and questions the usefulness of this measure.

Our strict criterion for obstruction may have led to false negatives. However this number is unlikely to be large. The observed frequency of obstruction of 15.8% is not dissimilar to other studies [26, 133]. Our definition of perforation was also strict, which may explain our lower rate of 3.7% compared to other reports (5-10%)[26].

We detected MSI in 9.6% of cases, which is similar to the rate observed in other studies inclusive of stage C disease [21, 52]. It is less than the rate observed in studies inclusive of all stages [37, 38, 40, 41, 173, 185, 200, 218] and this may be explained by the tendency for MSI colorectal cancers to remain localised and present at an earlier stage.

Some of the discrepancies observed between the chemotherapy cohort and the group that did not receive adjuvant chemotherapy may be explained by selection bias. This is likely to account for the age variation given that older patients may be precluded from treatment for medical reasons. No other explanation was apparent. This cohort mismatch is likely to affect overall survival more than cancer-specific survival and any influence will be removed in adjusted analysis. Of the men, 43% received chemotherapy compared to 33% of the women. This gender variance is possibly explained by the younger median age of the male cohort and hence selection bias in offering treatment. It was thought this might also be due to a male predominance in the rectal cancers cases. Because of the addition of radiotherapy to treatment and management within specialist units, there is possibly greater vigilance in

administration of chemotherapy in rectal cases. However while there was a male predominance in the rectal cancer group, the rate of chemotherapy administration did not vary from colonic cases.

Selection bias is the likely explanation for the higher nodal status in the chemotherapy group as shown. Selection bias could not be shown to account for the increased rates of aggression tumour features in the chemotherapy cohort, however an association between extramural vascular invasion and higher nodal status may explain the variance. The association between male gender and extramural vascular invasion may also contribute to this variance. It is unclear why these two factors should be associated.

The variances in rates of histological features between the two cohorts highlights the need for adjusted analysis ensuring that all these factors are taken into consideration.

Most of the gender cohort variations are explained by the different rate of MSI tumours and there is little to suggest varying biology between genders apart from MSI. Similarly, most of the variations associated with tumour site can be accounted for by MSI. There were, however, weak histological site associations independent of MSI that may suggest that tumour biology is not consistent across the colon.

The MSI cancer cohort was older than the MSS cases. There was a preponderance of proximal colon cancers and a higher percentage of women, findings consistent with most research groups. These and other associations will be discussed in the subsequent chapter.

Metachronous colorectal cancers occurred in 2% of the patients. Of the MSI cases, 2.5% developed metachronous cancers, not dissimilar to the rate for the whole group. Synchronous cancers occurred in 5% of the patients. Of these cases, 23% had an MSI cancer, much higher than the rate of 9.6% overall and the rate of synchronous cancers in the 77 MSI positive cases was 13%. This suggests a tendency for multiple lesions in MSI cases. Excluded cases were unlikely to have been informative or to have skewed findings but given two were due the presence of other cancers, HNPCC was a possibility.

5 RESULTS - MSI ASSOCIATIONS

5.1 Overview

MSI is a relatively recent discovery and as such, defining the exact characteristics of these cancers is continuing. It is important to determine clinical indicators and predictive histological features so that suspicious tumours may be investigated for microsatellite instability. Further delineation of related histology will improve our understanding of the biology of these tumours.

5.2 Aim

To investigate the clinical features and tumour histology associated with stage C microsatellite unstable colorectal cancers.

5.3 Specific Method

Detailed methodology is given in chapter 3. The statistical analysis used in this chapter to determine factor association was logistic regression. Factors with significance over 0.1 were included and assessed by backwards likelihood ratio. Significance was set at 0.05.

5.4 Results

5.4.1 Overview

Of the 814 stage C colorectal cancer cases included in the study, MSI status was successfully established in 802 patients of whom 77 (9.6%) were positive. Histology was independently reviewed and details are given in the previous chapter.

5.4.2 Frequencies of Clinicopathological Variable According to MSI

The MSI and MSS cohorts are compared and tabulated in Table 77. On this unadjusted analysis, median age was significantly higher in the MSI group (72 vs 70 years) and there was a preponderance of women (70% of MSI cohort were women compared to the near equal split of the MSS cases). Most of the MSI cancers were proximal (83%), markedly higher than the rate of proximal cancers in MSS cohort (35%).

The unadjusted comparison of histological features revealed an association between MSI cancers and poor differentiation and mucinous component, indicating potentially aggressive tumour behaviour. However countering this, there was significantly less budding and most tumours had a pushing margin, both factors potentially associated with better prognosis. There were fewer cases with perineural invasion though similar rates of extramural vascular invasion. TILs and Crohn‟s-like lymphocytes were over-represented in the MSI cases, while peritumoral lymphocytes were not. MSI cases tended to be larger and were proportionally higher T stage. There was no difference in rates of obstruction of perforation, nor stroma type.

5.4.3 Logistic Regression

On adjusted analysis several factors continued to have a significant association with MSI (Table 78). Interestingly this analysis (inclusive of all variables) indicated that older age and female gender were not independent predictors of MSI. Proximal site was strongly indicative (low hazard ratio of distal lesion), suggesting site may override the other clinical factors. When only clinical variables are included (Table 79), age remained non-significant and site strongly significant but interestingly gender

was also significant with twice the likelihood of being female in the MSI cohort. It is unclear why more comprehensive analysis ablates this significance. When associations for female gender are investigated (Table 80), MSI is almost twice as likely to exist, while site is only weakly associated.

MSI cases tended to be larger in size (median 53 mm vs 40 mm for MSS cancers $p<0.0001$). Within this specific stage C group, they did not vary from MSS in relation to T or N stage. They were more likely to be poorly differentiation (55.8% vs 13.6% $p<0.0001$) and much more likely to be associated with mucin production (66.2 vs 34.7%). Signet rings were prominent in 1.3% of MSI cancers compared to 1% of MSS. This was not significantly different but too few cases had signet rings to draw definite conclusions. There was a very strong association with tumour infiltrating lymphocytes, which were present in 31.2% of MSI cancers but were rarely seen in MSS cancers (1.5%). This suggests TILs may be a useful marker of MSI. There was not a significant independent association with the other lymphocytes types and type of stroma did not significantly vary according to MSI status. The negative association with infiltrating margin persisted (tendency to pushing margin in MSI cases) but budding did not vary, most likely negated by its association with margin type.

5.4.4 Combinations

Using these MSI associations, an attempt was made to combine factors to usefully predict the likelihood that a tumour was MSI positive. A site/gender combination revealed a strong tendency to MSI in the women with proximal lesions (Table 81), with around 30% of proximal tumours in women being microsatellite unstable (compared to 20% in proximal cancers overall).

In Table 82, predictive histological factors are combined. Tumour lymphocytes were included in all combinations having the highest odds ratio for MSI. This did make for small subgroups, which means TILs is not a useful screening tool, given most MSI cases do not show this feature. However if it is present, especially combined with poor differentiation and mucin, the cancer is almost certainly a MSI cancer. The addition of pushing margin did not add value to the prediction. While lesser combinations of TILs and mucinous component or poor differentiation were indicative but less specific.

When these features were examined in the clinical subgroups (Table 83), it can be seen that gender may not add significant value over site alone. The combination of TILs and poor differentiation in a proximal tumour would be highly indicative of a MSI cancer (over 90% MSI), as is TILs in a proximal tumour (over 70%). Even poor differentiation in a proximal tumour should raise suspicion; especially in women given 50% will be MSI.

Table 77 MSI/MSS cohort comparison

Subgroup	Subcategory	MSI	MSI	MSS	MSS	p	Proportion
		n	%	*n*	%		test
Number		77		725			
Median age		72.8*		70.7		**0.016**	
Gender	Men	23	29.9%	375	51.7%	**<0.0001**	
	Women	54	70.1%	350	48.3%		
Proximal		64	83.1%	250	34.6%	**<0.0001**	
Median size			53 mm		40 mm	**<0.0001**	
T stage	1	0		10	1.4%		
	2	0		55	7.6%		
	3	64	83.1%	562	78.2%		
	4	13	16.9%	92	12.8%		
N stage	1	55	71.4%	516	71.2%		
	2	22	28.6%	209	28.8%		
Differentiation	Mod	34	44.2%	623	86.4%	**<0.0001**	
	Poor	43	55.8%	98	13.6%		
Type	NOS	16	20.8%	461	64.0%	**<0.0001**	<0.0001
	Adenomucinous	40	51.9%	216	30.0%		<0.0001
	Mucinous	11	14.3%	34	4.7%		0.0007
	Signet	1	1.3%	2	0.3%		0.17
	Undifferentiated	6	7.8%	5	0.7%		<0.0001
	Undifferentiated/ Mucinous	3	3.9%	2	0.3%		0.0002
	uk	0		2	0.3%		
Lymphocytes	Peritumoral	69	89.6%	606	84.5%	0.234	
	Crohn"s	32	41.6%	141	19.6%	**<0.0001**	
	TILs	24	31.2%	11	1.5%	**<0.0001**	
Stroma	a	55	71.4%	472	65.8%	0512	0.20
	b	21	27.3%	238	33.2%		0.25
	c	1	1.3%	7	1.0%		0.80
	uk			5			
Obstructed		13	17.1%	112	15.6%	0.732	
Perforated		2	2.7%	28	3.9%	0.590	
Infiltrating margin		12	15.6%	241	33.6%	**0.001**	
Budding		13	16.9%	298	41.5%	**<0.0001**	
NeuroVascular invasion	Mural	46	59.7%	506	70.3%	0.057	
	Extramural	42	54.5%	403	56.0%	0.811	
	Perineural	7	9.1%	134	18.7%	0.036	

*Age analysed as ranked data

Table 78 All variables - associations with MSI

Covariate*	HR	95% CI		p
		Lower	Upper	
Age	1.03	0.999	1.06	0.06
Female Gender	1.89	0.95	3.48	0.07
Distal Site	0.16	0.08	0.33	<0.0001
Size	1.02	1.004	1.03	0.01
Differentiation	2.29	1.08	4.86	0.03
Mucinous	3.82	1.90	7.67	<0.0001
Signet rings	3.00	0.90	9.97	0.07
TILs	11.92	3.97	35.72	<0.0001
Infiltrating Margin	0.36	0.16	0.82	0.02

*Variables entered on step 1 for backward LR analysis: Age, Gender, Prox/Dist, T stage, N stage, Size, Differentiation, Mucinous type, Signet rings, Peritumoral, Crohn″s, TILs, Stroma, Obstruction, Perforation, Infiltrating Margin, Budding, Mural, Extramural, Perineural invasion.

Table 79 Clinical associations with MSI

Covariate	OR	95% CI		p
		Lower	Upper	
Age (older)	1.02	0.998	1.04	0.08
Gender (female)	2.02	1.18	3.43	0.01
Site (distal)	0.12	0.06	0.22	<0.0001

Backward LR logistic regression. Age, gender, site only variables entered

Table 80 Female gender associations

Covariate*	OR	95% CI		p
		Lower	Upper	
Age (older)	1.02	1.003	1.03	0.01
Site (distal)	0.73	0.54	1.00	0.05
Extramural	0.71	0.53	0.96	0.03
MSI	1.92	1.11	3.32	0.02

*Variables entered on step 1: Age, Prox/Dist, Size, Differentiation, Type Muc, Signet, Peritum, Crohn″s, TILs, Stroma, InfMargin, Budding, Mural, Extramural, Perineural, Nstage, MSI, Tstage.

Table 81 Site gender correlation with MSI

Clinical combination	MSI %	*n*	MSS %	*n*
Men Proximal	11.4%	15	88.6%	117
Men Distal	3.0%	8	97.0%	257
Women Proximal	26.9%	49	73.1%	133
Women Distal	2.3%	5	97.7%	215

P= <0.0001

Table 82 Histological predictive combinations

Histological Combination	MSI %	*n*	Total
Poor differentiation, TILs, mucinous component	92.3%	12	13
Poor, TILs, mucinous component	92.9%	13	14
TILs, mucinous component	71.4%	15	21
TILs, poor differentiation	81.5%	22	27

Table 83 Clinical and histological predictive combinations

Histological Combination	Proximal			Women/proximal		
	MSI %	*n*	Total	MSI %	*n*	Total
TILs, mucinous component	60%	3	5	60%	3	5
TILs, poor differentiation	81.8%	18	22	82.4%	14	17
TILs	74.1%	20	27	72.7%	16	22
Poor differentiation	42.7%	38	89	50.9%	29	57

5.5 Discussion

We selected a stage-specific group of CRCs and as such we can only state the factors that are associated with MSI in stage C disease. We detected no significant difference in age distribution between MSI and MSS cases on adjusted analysis. Despite the suggestion that sporadic MSI cases occur more frequently in the older female population, the weight of evidence in the literature is against any age variance according to MSI [37, 38, 41, 42, 47, 48, 53, 213]. Median age of the MSI cases was higher when simply comparing our groups, however on adjusted analysis this was negated by other factors and no significant difference was found. Thus studies that compare cohorts without considering compounding factors may erroneously conclude there is an age variance.

The association between female gender and MSI cancers shown in this study concurs with the literature [37, 38, 45, 219]. Seventy percent of our MSI cases were women compared to the near equal split for MSS cases. Studies report rates between 50 and 70% [37, 38]. Logistic regression analysis of our group suggested an association between MSI and female gender. This concurs with the literature. Kakar et al. found an odds ratio for being female in the MSI group of 1.85 (95% CI, 1.24-2.75), which proved to be very similar to our 2.02 [219].

There is a strong association between proximal site and MSI. We found that 80% of MSI tumours develop in the proximal colon, which is similar to most reports of all stages (74% - 86%) [37, 39-41, 44, 53] and stage C (82-94%) [21, 38, 42, 43, 48]. Reported rates of MSI cancers in the proximal colon vary between 20-36% (compared to 10-16% rate overall) [21, 31, 37-39, 42, 46, 200, 219]. The contrast in our study

was even more marked, with only 2.7% of distal lesions being MSI compared to 20% of proximal lesions. This association was maintained on adjusted analysis. The association was even stronger in women with proximal lesions with 30% being microsatellite unstable. Site was also useful to predict MSI status when combined with indicative histological features. Female gender was less so.

As with most groups, we found there was a strong association between MSI and poor differentiation and mucinous component to the tumour [38, 42, 44-48]. The rate of poor differentiation in the MSI cohort was 56% compared to 14% and this is comparable to the literature. If the tumour demonstrated a tendency to mucin production, we found the tumour was 3.8 times more likely to be microsatellite unstable. We did not find an increased rate of signet rings but the percentage of tumours detected to have this feature was very small (only three patients overall) therefore it is difficult to draw any conclusions. However as only one of 77 MSI cases had detectable signet rings, it was not useful predictive factor in this group. Others have found it to be more prevalent in MSI cases as might be expected due to its association with mucinous tumour component [47].

The other two histological features associated with MSI in our group were tumour infiltrating lymphocytes and pushing margin (or lack of infiltrating margin). These seemingly good prognostic features would seem at odds with the above findings of poor differentiation and mucin production but this contradictory finding is similar to that observed by others [37, 47, 174]. The percentage of MSI cases we found with TILs was 31.2% compared to 1.5% of MSS and this is consistent with the adjusted analysis finding that tumours with TILs were 12 times more likely to be MSI tumour. We did not find Crohn‟s-like aggregates to be independently associated with MSI as

has been reported [37, 38, 42, 45, 47]. They were, however, present twice as often in MSI cases (42% vs 20%). An infiltrating margin was observed at half the rate in our MSI cases compared to MSS cases (16% vs 34%) and budding showed an even greater variance (12% vs 42%), similar to what has been demonstrated by others [38, 44, 45, 47, 213]. Budding, however, did not maintain significance on adjusted analysis while margin did, which was probably due to their mutual association.

MSI tumours tend to remain localised and therefore often present as large tumours. We sampled a specific stage of disease and did not find within this specific group that either bowel wall invasion or diseased lymph node number varied according to MSI status. Larger size was associated with MSI but the lower confidence interval approached unity.

In conclusion, we found that the most useful indicator of an MSI tumour was tumour-infiltrating lymphocytes. This feature is not common but when present it is highly indicative of MSI. If it is present in combination with poor differentiation and mucin, then the tumour is almost certainly a MSI cancer. This is similar to the opinion of Jass in Boland et al. [174]. Proximal poorly differentiated tumours should raise suspicion of MSI especially in women.

6 RESULTS - PROGNOSTIC INFLUENCES

6.1 Overview

Stage remains the greatest predictor of outcome from CRC. However, each staging category contains a heterogeneous population of patients and cancers with a wide range in survival. Many factors have been investigated for prognostic significance but not all are firmly established and many emerging indices are yet to be validated.

An understanding of what influences survival from CRC and recognition of prognostic factors is important for several reasons. An improved ability to predict outcome is important when considering treatment options and individualising patient management plans. On a larger scale, a contribution to the greater understanding of tumour biology and behaviour aids in furthering research, potentially towards better treatments.

Specifically for this study, recognition of prognostic factors is the first step in identifying which factors are likely to influence chemotherapy effect.

6.2 Aim

The aim of this section is to determine the prognostic significance of selected clinical and pathological variables, with a focus on gender and site, and MSI.

6.3 Specific Method

Unadjusted survival was determined for each parameter by crude overall 5-year survival (from life tables) and Kaplan–Meier survival curves, which were compared by logrank testing. All factors were entered in a Cox regression proportional hazards model for multivariate analysis with inclusion into the equation at significance of 0.2,

and elimination at 0.10 by backward stepwise elimination, based on likelihood ratio. Final significance was set at 0.05.

6.4 Results

6.4.1 Overview

For this analysis, 814 stage C colorectal cancer cases were included. Median follow-up was 36.3 months. Median age was 71.1 years (range 30.3-96.1 years). Gender distribution was equal (men 49.6%, women 50.4%). Sixty percent were located distal to the splenic flexure and 40% were proximal. Thirty-seven percent received chemotherapy.

6.4.2 Multivariate Analysis

Each subgroup will be considered separately, however the multivariate analysis included all variables and as such is applicable to all sections. The results will therefore be detailed first. Covariate categorisation is given in Table 84. All hazard ratios refer to the second variable relative to the first. The main analysis refers to cancer-specific survival. All variables were entered in the proportional hazards model and an unadjusted hazard ratio and significance determined (Table 85). The factors that maintained prognostic significance on multivariate analysis are given in Table 86, while the non-significant variables are listed in Table 87. Multivariate analysis of prognostic factors for overall survival is given in Table 88 and Table 89. Individual results will be discussed in each section. Subsequent sections will refer to these tables.

Table 84 Covariates for multivariate analysis

Covariates		*n*
Age	Continuous variable	
Gender	1=male	384
	2=female	378
Site	1=proximal	290
	2=distal	472
Chemotherapy	0=no	474
	1=yes	288
Radiotherapy	0=no	674
	1=yes	88
Bowel Wall breach	0=Confined to bowel wall	63
	1=Breached bowel wall	699
N stage	1	542
	2	220
Size	Continuous variable	
Differentiation	1=moderate	635
	2=poor	127
Type	0=NOS	465
	1=Mucinous component	297
Mural vascular invasion	0=no	234
	1=yes	528
Extramural vascular invasion	0=no	337
	1=yes	425
Perineural invasion	0=no	627
	1=yes	135
Infiltrating Margin	0=no	521
	1=yes	241
Budding	0=no	458
	1=yes	304
Stroma	1=a	504
	2=b	250
	3=c	8
Peritumoral lymphocytes	0=no	116
	1=yes	646
Crohn's lymphocytes	0=no	596
	1=yes	166
TILs lymphocytes	0=no	735
	1=yes	27
Obstruction	0=no	643
	1=yes	119
Perforation	0=no	733
	1=yes	29
MSI	0=negative	693
	1=positive	69

762 cases included analysis as unknown variables omitted
Reference = first variable - i.e. HR of second variable relative to first

Table 85 Initial step multivariate analysis – entry of all variables

	HR	95.0% CI		p
		Lower	Upper	
Age	1.01	1.00	1.02	**0.02**
Gender	0.85	0.68	1.07	0.16
Site	1.04	0.80	1.33	0.79
Chemotherapy	0.52	0.39	0.70	**<0.0001**
Radiotherapy	1.31	0.88	1.94	0.18
Bowel Wall	1.46	0.82	2.63	0.20
N stage	2.11	1.66	2.67	**<0.0001**
Size	1.01	1.00	1.01	0.13
Differentiation	1.38	1.03	1.87	**0.03**
Type - Mucinous	1.23	0.96	1.57	0.10
Peritumoral	1.50	1.08	2.07	**0.02**
Crohn's	0.83	0.62	1.10	0.20
TILs	0.49	0.22	1.11	0.09
Stroma- fibrous				0.53
Stroma - keloid	0.86	0.67	1.12	0.26
Stroma - myxoid	1.07	0.39	2.92	0.90
Obstruction	1.29	0.97	1.71	0.08
Perforation	2.28	1.45	3.57	**<0.0001**
Inf Margin	1.73	1.34	2.23	**<0.0001**
Budding	1.07	0.83	1.38	0.62
Mural	.77	0.59	0.99	0.04
Extramural	1.67	1.28	2.19	**<0.0001**
Perineural	1.48	1.12	1.96	**0.01**
MSI	1.41	0.90	2.21	0.13

Table 86 Significant variables at last step

	HR	95.0% CI		p
		Lower	Upper	
Age	1.01	1.003	1.02	0.01
Chemotherapy	0.58	0.44	0.75	<0.0001
N stage	2.11	1.67	2.66	<0.0001
Differentiation	1.40	1.05	1.87	0.02
Type	1.29	1.02	1.63	0.03
Infiltrating Margin	1.73	1.37	2.18	<0.0001
Peritumoral	1.48	1.08	2.04	0.02
Mural	0.76	0.60	0.98	0.04
Extramural	1.78	1.37	2.31	<0.0001
Perineural	1.45	1.10	1.91	0.01
Perforation	2.27	1.46	3.53	<0.0001

Table 87 Non significant variables

Covariate	Final p
Gender	0.24
Prox Dist	0.66
Radio	0.16
Bowel Wall	0.21
Size	0.15
Crohn's	0.24
TILs	0.10
Stroma - fibroid	0.64
Stroma keloid	0.34
Stroma Myxoid	0.96
Obstruction	0.17
Budding	0.82
MSI	0.15

Table 88 Significant variables- overall survival,

	HR	95.0% CI		p
		Lower	Upper	
Age	1.02	1.01	1.03	<0.0001
Chemotherapy	0.52	0.41	0.67	<0.0001
N stage	1.80	1.47	2.22	<0.0001
Differentiation	1.37	1.06	1.77	
Infiltrating Margin	1.47	1.19	1.82	<0.0001
Peritumoral	1.31	1.001	1.71	0.05
Extramural	1.32	1.06	1.64	0.01
Perineural	1.41	1.09	1.82	0.01
Perforation	1.72	1.11	2.64	0.01
MSI	1.57	1.08	2.27	0.02

Table 89 Non significant variables – overall survival

	p
Gender	0.07
Site (prox/dist)	0.63
Radiotherapy	0.24
Bowel Wall invasion	0.07
Size	0.12
Mural	0.45
Crohn's	0.07
TILs	0.07
Fibroid stroma	0.47
Keloid stroma	0.26
Myxoid stroma	0.72
Obstruction	0.30
Budding	0.80

6.4.3 Survival

During the study period, 469 (57.6%) patients died. Of these 76% were due to cancer. The five-year overall survival was 46%. Figure 4 and Figure 5 show the survival curves.

Chemotherapy conferred a survival advantage across the whole group, improving 5-year survival from 40% to 58% Kaplan Meier curves confirm a significant survival difference across the study period ($p<0.0001$) (Figure 6 and Figure 7). On adjusted analysis, considering all other clinical and pathological factors, the chemotherapy cohort had a hazard ratio of dying of 0.52 (95% CI 0.39-0.70 $p<0.0001$) for overall survival and a HR of 0.66 (95% CI 0.52-0.83 $p=0.0003$) for cancer-related death. It is interesting to note from the cancer-specific survival curves that most cancer deaths occurred within the first five years, particularly in the group that received chemotherapy.

Radiotherapy did not significantly improve survival (Figure 8 and Figure 9) even when rectal cases alone were analysed (Figure 10 and Figure 11). Five-year survival was 53% in the radiotherapy group and 46% otherwise. However, numbers within the radiotherapy group were small, possibly causing a type 2 error.

Advancing age had a detrimental effect on both overall (HR 1.02 95% CI 1.01-1.03 $p<0.0001$) and cancer-specific survival (HR 1.01 95% CI 1.003-1.02 $p=0.01$). While significant, both lower limits of the confidence interval approached 1.00 and an age effect was less apparent for cancer-specific survival.

Figure 4 Study group overall survival

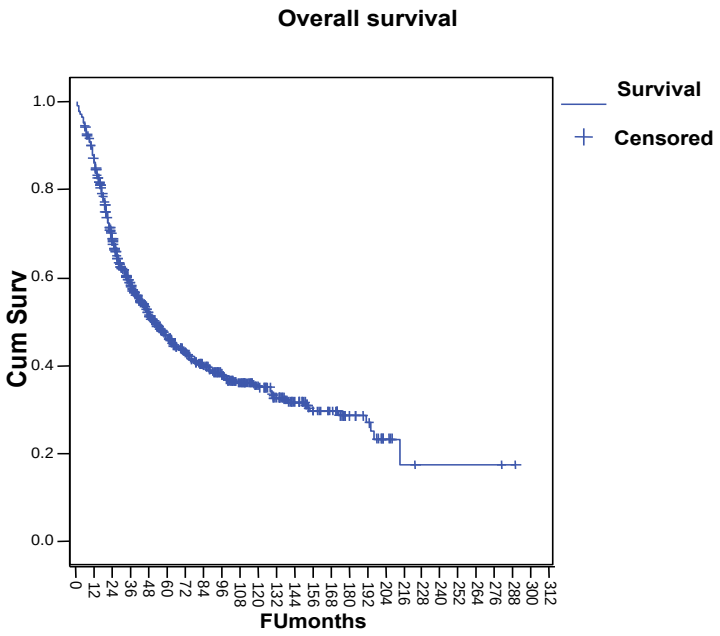


Figure 5 Study group cancer-specific survival

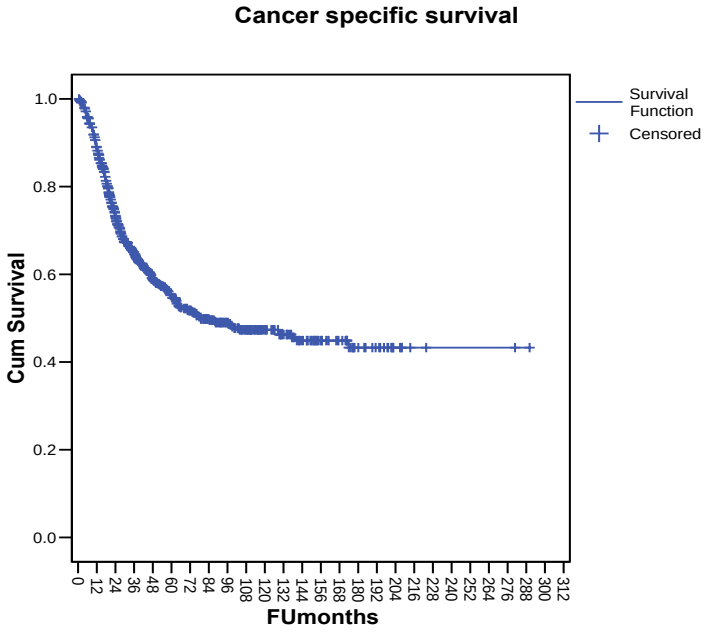
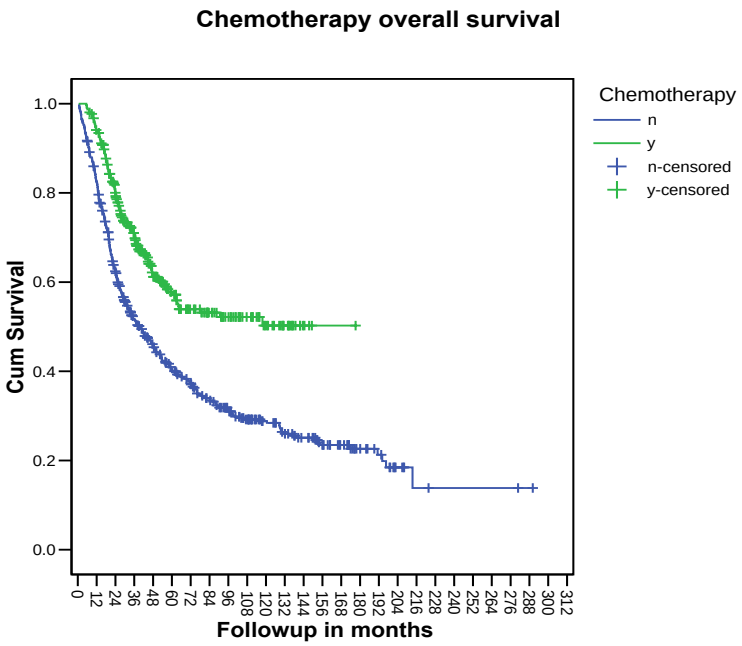
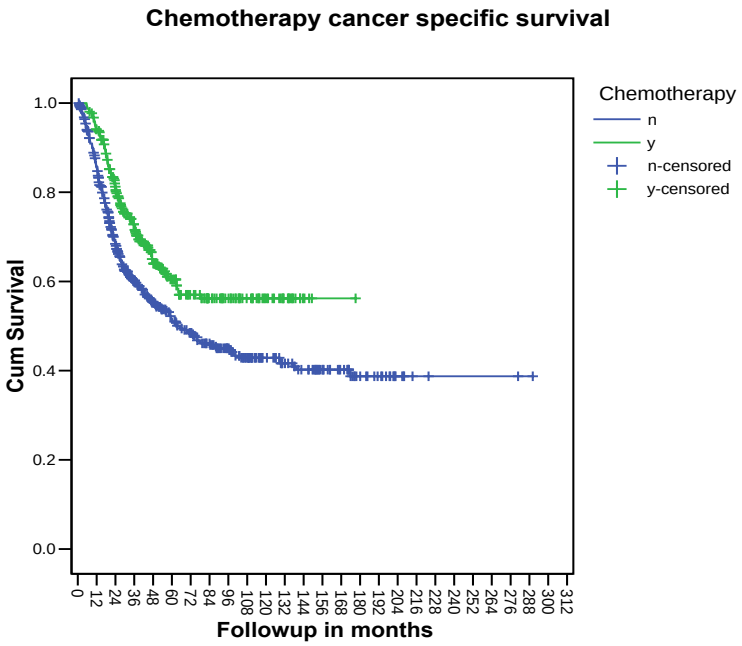


Figure 6 Chemotherapy effect on overall survival



p <0.0001

Figure 7 Chemotherapy effect on cancer-specific survival



p= 0.0003

Figure 8 Effect of radiotherapy on overall survival

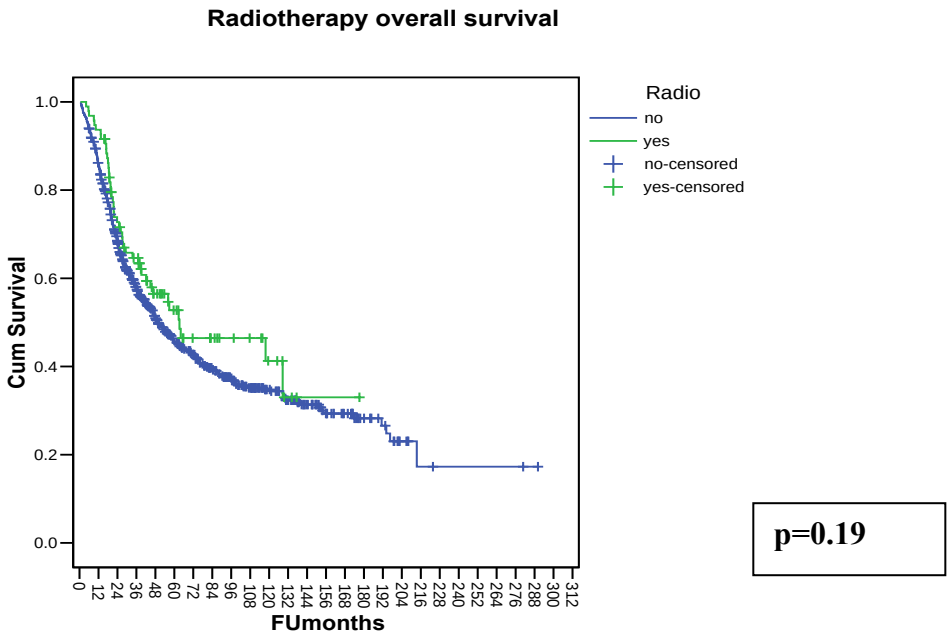


Figure 9 Effect of radiotherapy on cancer-specific survival

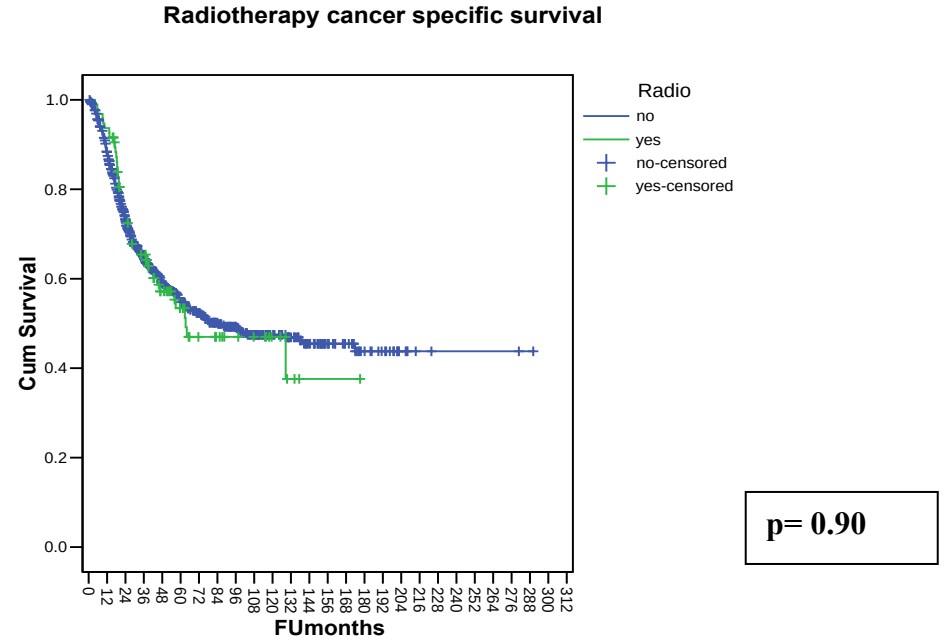
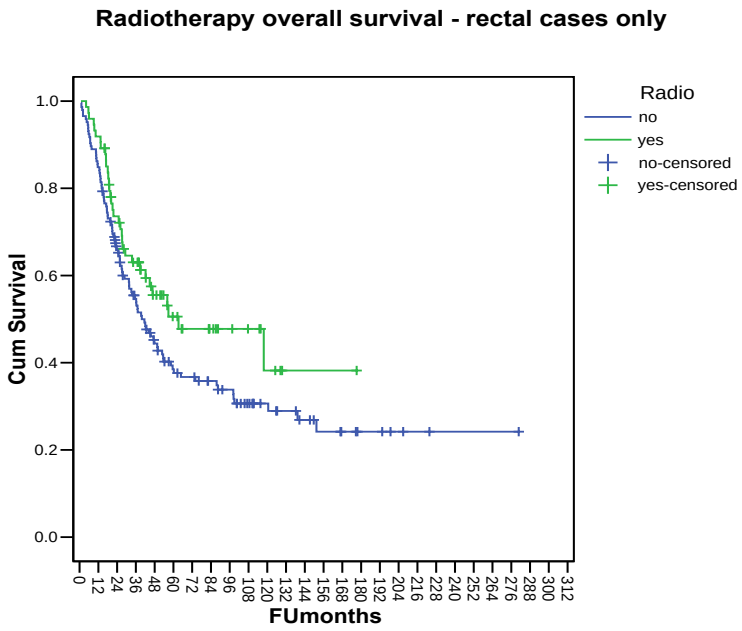
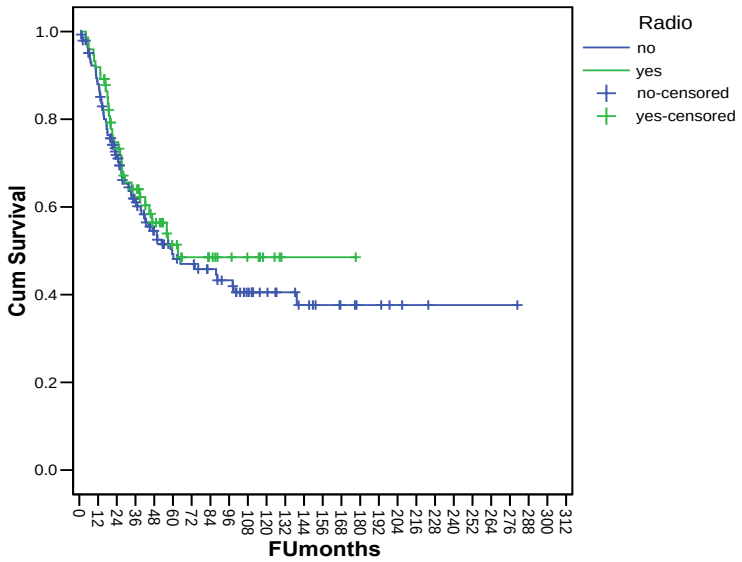


Figure 10 Effect of radiotherapy on overall survival of rectal cases



p= 0.08

Figure 11 Effect of radiotherapy on cancer-survival of rectal cases



p=0.48

6.4.4 Gender and Site

Neither gender nor site influenced prognosis in this group of stage C cases. Crude 5-year survival by sex and site is given in Table 90. Survival was not significantly different in any of the subgroups, ranging between 43 and 49%. Univariate analysis for gender is shown in Figure 12 and Figure 13 (overall and cancer-specific) as well as unadjusted hazard ratio of female to male risk of overall death (HR 0.92, 95% CI 0.77-1.11) and cancer-specific death (HR 0.88, 95% CI 0.72-1.09). No significant difference is observed in either overall or cancer-specific survival and this is further confirmed on multivariate analysis (Table 87).

Survival curves according to proximal or distal site show no significant deviation, for either overall or cancer-specific survival (Figure 14 and Figure 15). Both univariate hazard ratios (proximal versus distal) are very close to 1.00 (overall death HR 1.05 (95% CI 0.87-1.26) and cancer specific death HR 1.02 (95% CI 0.82-1.27). Site was also not significant on multivariate modelling. There was no significant difference on further breakdown to subsite but subgroup numbers are small for this analysis (Figure 16 and Figure 17). Sub grouping analysis based on site and gender showed no significant survival differences (Figure 18 and Figure 19). Table 91 details subgroup sizes. As per power calculations these subgroups are underpowered to show a 10% difference but well powered to show a 20% variance. A lack of trend suggests that larger cohorts would still not have produced a significantly different result.

Table 90 5-year survival in site gender subgroups

	5yr surv
Proximal	47%
Distal	46%
Men	45%
Women	48%
Men/prox	45%
Men/dist	43%
Women/prox	47%
Women/dist	49%

Table 91 Gender site subgroup numbers

Subgroup sizes	*n*
Men/proximal	133
Men/distal	270
Women/proximal	185
Women/Distal	223

Figure 12 Overall survival by gender

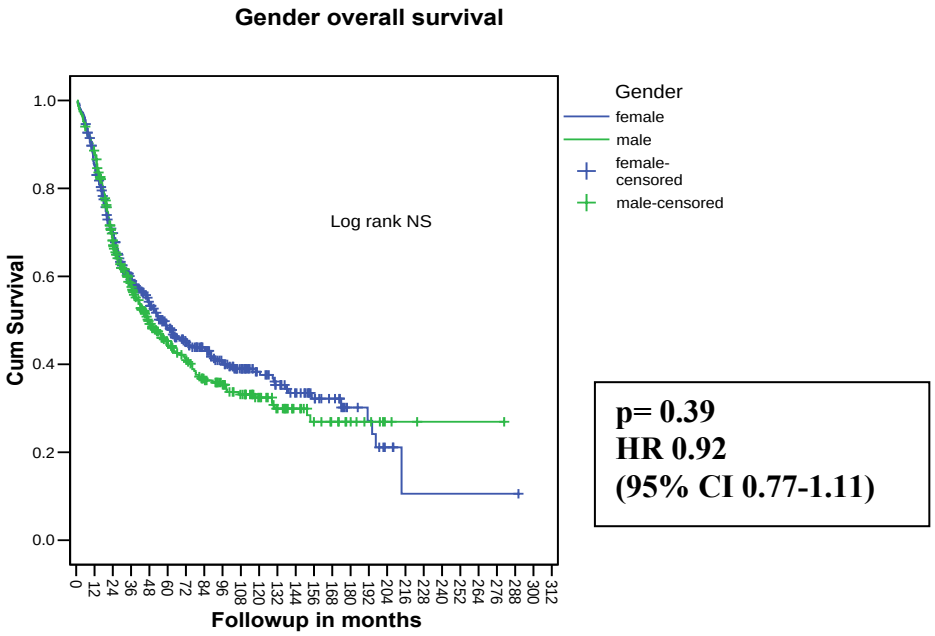


Figure 13 Cancer-specific survival by gender

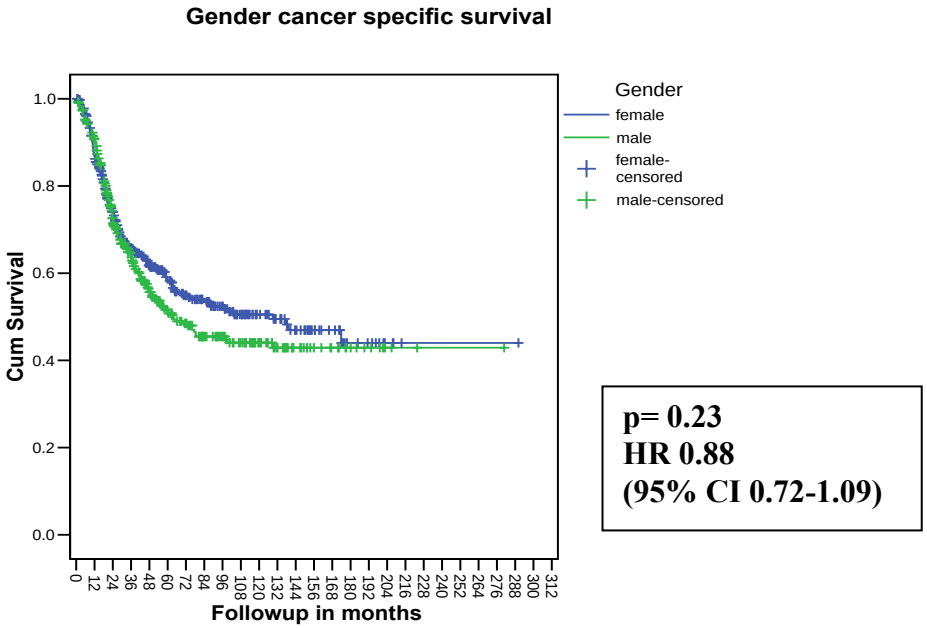


Figure 14 Overall survival according to site

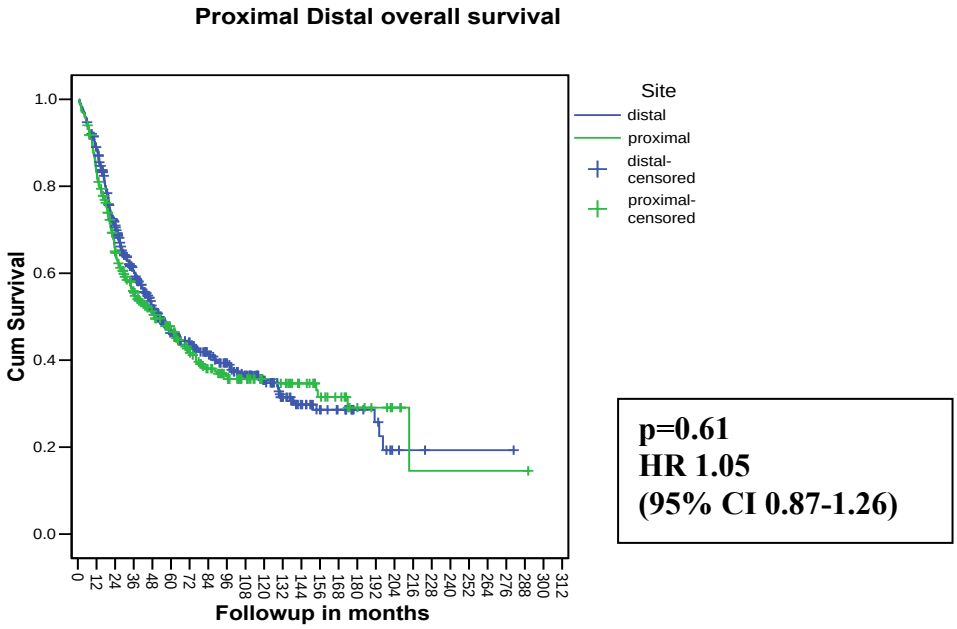


Figure 15 Cancer-specific survival according to site

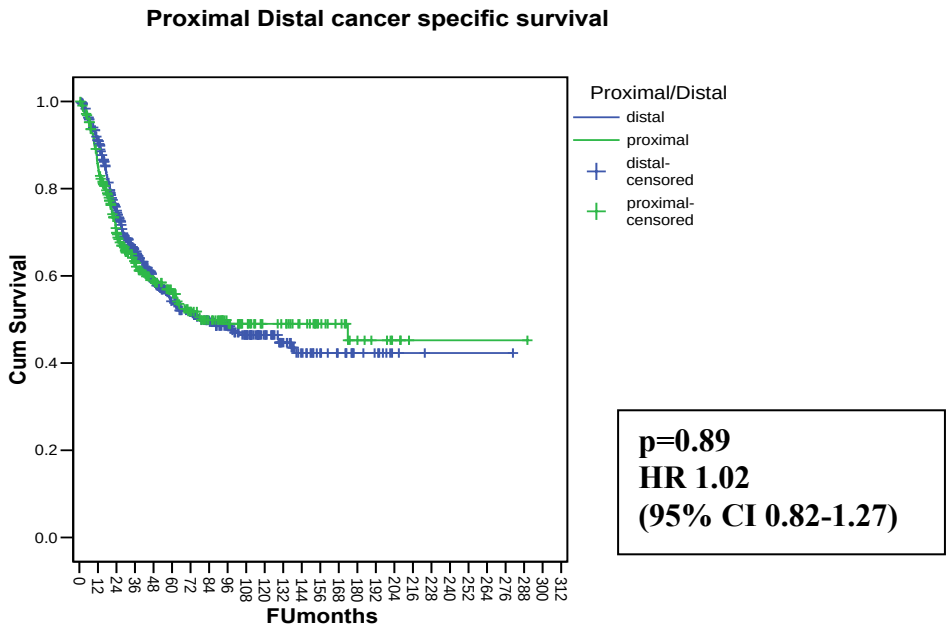
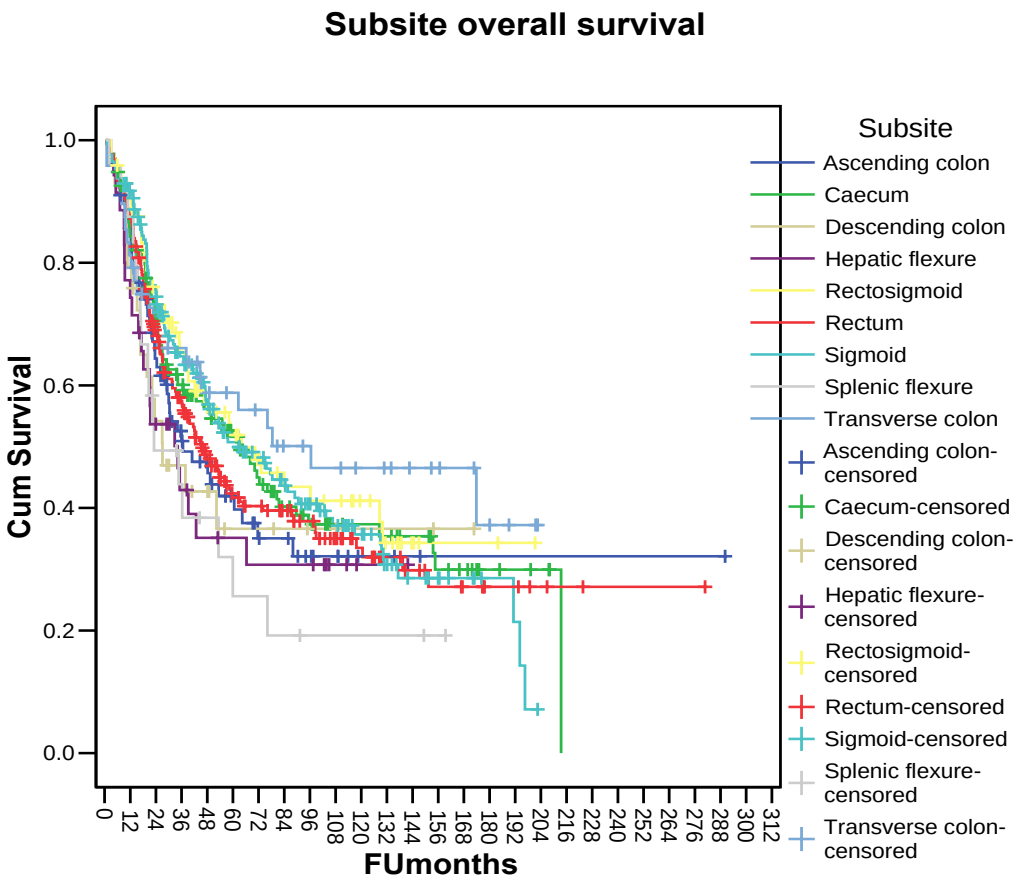
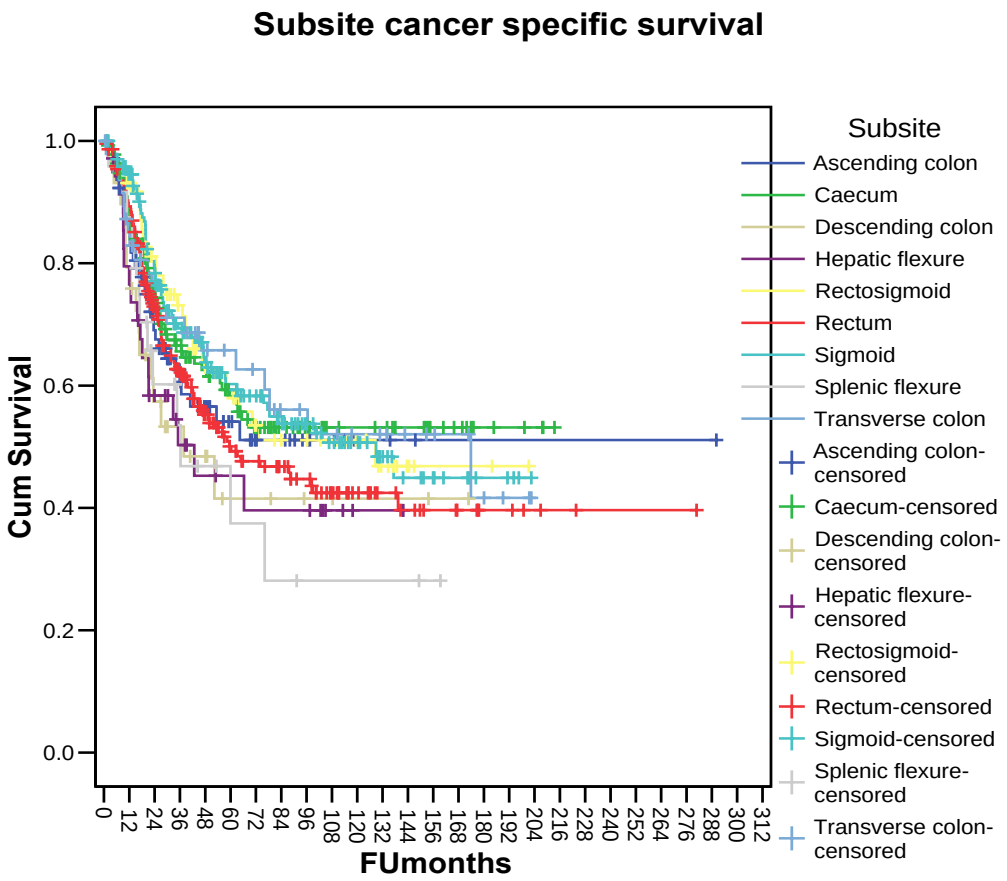


Figure 16 Overall survival per site subgroup



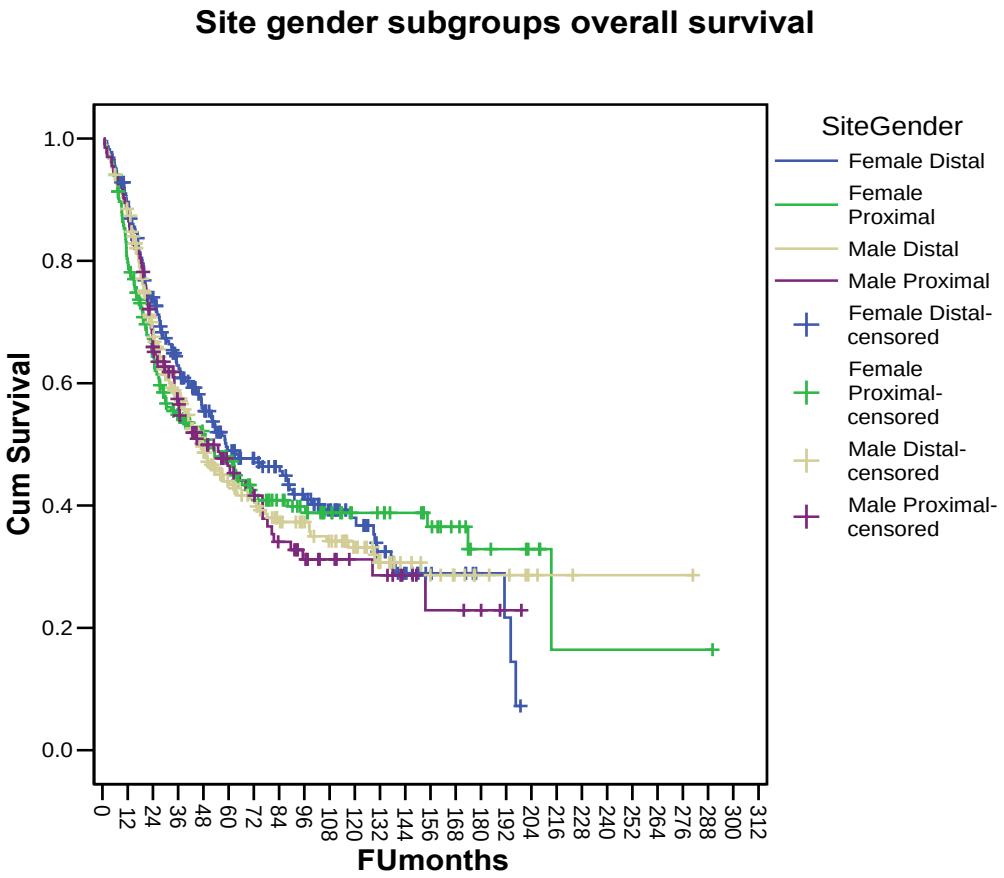
p= 0.21

Figure 17 Cancer-specific survival per site subgroup



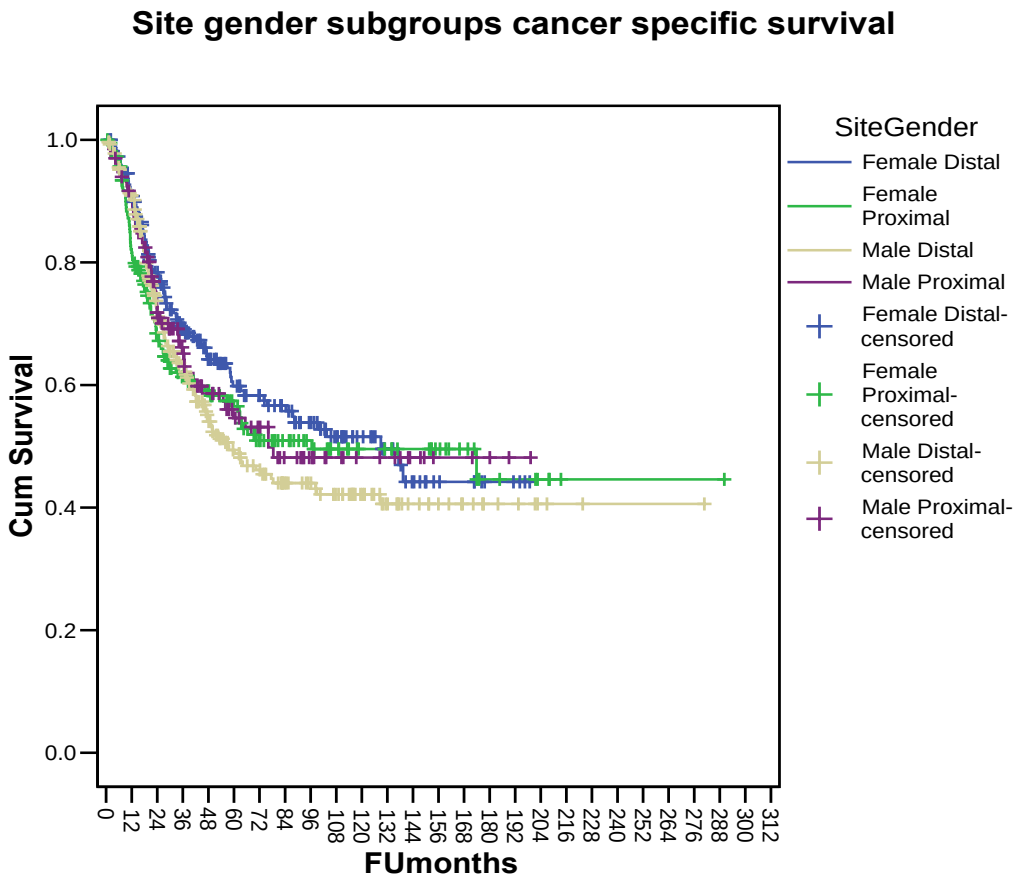
p= 0.18

Figure 18 Overall survivals per site gender subgroups



p= 0.77

Figure 19 Cancer-specific survivals per site gender subgroups



p= 0.32

6.4.5 Pathological Factors

Pathological variables were assessed individually on univariate analysis and entered into the multivariate model. On multivariate analysis the factors in Table 92 proved to be significant, while those listed in Table 93 were not. Findings were similar for overall survival except that mural vascular invasion was no longer significant (Table 88). Five-year survivals are listed in Table 94. Each pathological covariate will be detailed separately.

6.4.5.1 Staging Criteria

TNM staging criteria was assessed in addition to ACPS stage to determine if further detailing of degree of bowel wall invasion and number of involved nodes gave additional prognostic information. To attain meaningful numbers, data were also dichotomised. Degree of bowel wall involvement was divided into confined to the bowel wall (not breaching muscularis propria, T1 and T2 equivalent) and breached bowel wall (through muscularis propria, T3 and T4). Nodal status was divided into standard N1 (3 or less node positive) and N2 (greater than 3 positive).

Five-year survivals appeared quite different; 38% if muscular bowel wall is breached (T3 or T4), 61% if not and 52% for N1 against 33% for N2 (Table 94). On the Kaplan Meier survival curves, advancing T stage showed progressively worse overall and cancer specific survival as may be expected (Figure 20 and Figure 21). No T1 cases died during the study period (a good outcome given all these cases did have positive nodes) but numbers in this group were small (n=10). Not surprisingly, bowel wall breach also significantly influenced outcome (Figure 22), as did nodal status (Figure

23). However on multivariate analysis (using dichotomised data), bowel wall involvement did not significantly change outcome while nodal status remained one of the stronger independent predictors of outcome (Table 92 and Table 93). Thus, number of involved nodes is a useful measure, as it provides additional prognostic information in ACPS stage C, while degree of bowel wall involvement does not.

Thirty cases were limited to micrometastases (involvement of node < 2 mm). These cases would be expected to have better outcome but despite a deviation in survival curves the difference was not significant (Figure 24). This may however be due to type 2 error given the small number in this group (n=30).

6.4.5.2 Differentiation

The degree of tumour differentiation significantly influenced outcome on univariate analysis (overall and cancer-specific survival) (Figure 25 and Figure 26). Corresponding five-year survivals were 49% for moderate differentiation and 37% for poor differentiation. This significance was confirmed on multivariate analysis (HR 1.40 95% CI 1.05-1.87 p=0.24) (Table 92). While a difference was observed, it may be surprising that it was not greater. It is possible the importance of tumour grade has been over emphasised.

6.4.5.3 Type

Survival according to tumour type was initially considered for all tumour types and no significant difference was detected (Figure 27 and Figure 28). As subgroup numbers were small, data was dichotomised into mucinous component or NOS. Again, no significant difference in outcome was determined on this unadjusted analysis (Figure

29). Interestingly, this varies from the findings on multivariate analysis (Table 92), which shows that mucinous component confers a worse survival (HR 1.29 95% CI 1.02-1.63). Because significance was 0.096 on initial univariate model, this variable was included in the equation. This subsequent finding of significance suggests a confounding influence that is negating the detrimental effect on survival (i.e. association with a factor that improves survival).

6.4.5.4 Neurovascular Invasion

Invasion of neurovascular structures would be expected to worsen prognosis. Both extramural vascular invasion and perineural invasion were associated with a poorer survival (Figure 30, Figure 31, Figure 32 and Figure 33). Extramural invasion was associated with a HR of dying of 1.78 (95% CI 1.37-2.31, $p<0.001$) and perineural invasion with a HR of 1.45 (955 CI 1.10-1.91, $p= 0.008$). Mural vascular invasion did not have an effect on univariate analysis (Figure 34 and Figure 35) but unexpectedly was associated with a better survival on adjusted analysis (Table 92). This significance was not observed for overall survival. Without obvious reason for this difference, it calls into question to relevance of the findings on this factor. This issue will be furthered in discussion.

6.4.5.5 Invasive Margin

An infiltrating margin was the greatest determinant of poor prognosis in this study group (just above number of nodes involved). The difference in outcome compared to pushing margin is highly significant on univariate (Figure 36 and Figure 37) and multivariate analysis (Table 92) (HR 2.27, 95% CI 1.67-2.66, $p<0.001$).

6.4.5.6 Budding

The presence of budding significantly influenced cancer-specific survival on univariate analysis (just) but had no effect on overall survival (Figure 38 and Figure 39) and furthermore had no significant influence once adjusted analysis was performed (Table 93). This suggests there is a confounding interaction, which would be explained by its association with an infiltrative margin. From these results it cannot be concluded budding is a significant prognostic indicator in colorectal cancer.

6.4.5.7 Stroma

Tumour stroma was not found to influence outcome according to either analysis (Figure 40 and Figure 41), with very little variation observed between survival curves. The number of cases with type C stroma was too small for meaningful analysis. Complete lack of a trend suggests that even larger numbers would not have shown a difference.

6.4.5.8 Lymphocytes

It was predicted that lymphocytic infiltration of tumours would improve prognosis if, as postulated they represented the body"s immune response to the cancer. However, as can be seen in Figure 42 (overall survival) and Figure 43 (cancer specific survival), the presence of peritumoral lymphocytes did not influence survival on univariate analysis. However, on multivariate analysis (Table 92) presence of this parameter was associated with a poorer outcome. This result is counterintuitive and a reason for this aberrant finding is not apparent. As discussed in the previous chapter the usefulness of this parameter, as measured in this study, has been questioned.

The presence of Crohn"s-like lymphocytes did offer a slight survival advantage on univariate analysis for both overall and cancer-specific survival (Figure 44 and Figure 45) however the effect was lost in adjusted analysis (Table 93). This suggests that this finding does not significantly change outcome and any perceived influence (on unadjusted analysis) is due to association with other factors.

The presence of tumour infiltrating lymphocytes did not influence outcome on either unadjusted (Figure 47 and Figure 46) or multivariate analyses (Table 92) but numbers in this group were very small and results may therefore be erroneous.

6.4.5.9 Obstruction

Obstruction and perforation have been included in the pathological section rather than clinical as they were defined by pathological criteria.

Obstruction contributed to a worse outcome on univariate analysis but significance was lost on adjusted results (Figure 48, Figure 49 and Table 93), signifying correlation with other prognostic influences. A larger tumour may be more likely to cause obstruction, however size did not contribute to outcome (Table 93). It is probable that advanced, circumferential tumours, presumably with aggressive features, were associated with obstruction and adjustment for these factors negated the influence of obstruction. As we did not include morphology due to concerns regarding accuracy, this cannot be determined.

6.4.5.10 Perforation

The presence of a perforated tumour significantly worsened survival on both analyses (Figure 50, Figure 51 and Table 92). The definition was strict with only perforations through the tumour being included, which should be taken into account when using this information. The subgroup was small but the results significant.

Table 92 Pathological factors significantly affecting cancer-specific survival

	HR	95.0% CI		p
		Lower	Upper	
N Stage	2.11	1.67	2.66	<0.001
Differentiation	1.40	1.05	1.87	0.02
Mucinous	1.29	1.02	1.63	0.03
Peritumoral lymphocytes	1.48	1.08	2.04	0.02
Perforation	2.27	1.46	3.53	<0.001
Infiltrating margin	1.73	1.37	2.18	<0.001
Mural	0.76	0.60	0.98	0.03
Extramural	1.78	1.37	2.31	<0.001
Perineural	1.45	1.10	1.91	0.01

Table 93 Pathological factors not affecting survival

	p
Bowel wall invasion	0.21
Size	0.15
Crohn's lymphocytes	0.24
TILs	0.10
Fibroid stroma	0.64
Keloid stroma	0.34
Myxoid stroma	0.96
Obstruction	0.17
Budding	0.82

Table 94 5-year survival according to pathological factors

		5yr surv
Bowel Wall (T3 or T4)	No	61%
	Yes	38%
Nodal	N1	52%
	N2	33%
Differentiation	Mod	49%
	Poor	37%
Type	Mucinous	46%
	NOS	47%
Infiltrating margin	No	52%
	Yes	35%
Budding	No	49%
	Yes	42%
Peritumoral	No	48%
	Yes	46%
Crohn's	No	44%
	Yes	56%
TILs	No	46%
	Yes	53%
Stroma	Fibroid	48%
	Keloid	44%
	Myxoid	38%
Mural	No	49%
	Yes	45%
Extramural	No	58%
	Yes	37%
Perineural	No	50%
	Yes	33%

Figure 20 Overall survival by T stage

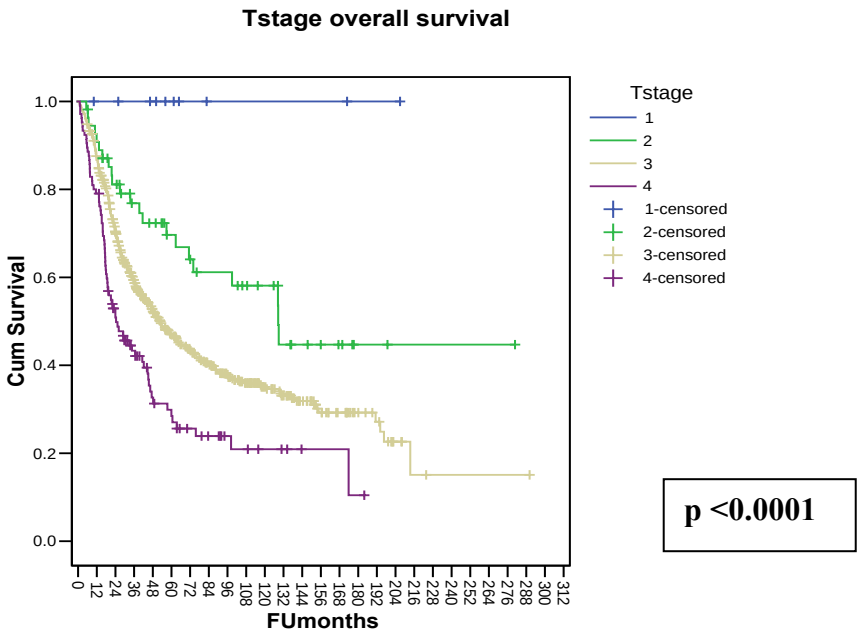


Figure 21 Cancer-specific survival by T stage

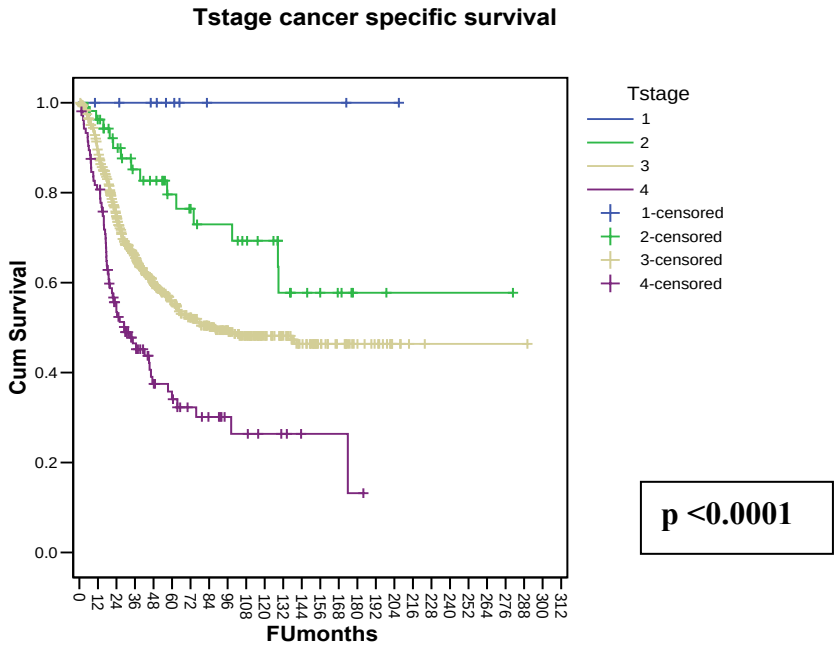


Figure 22 Cancer-specific survival according to muscularis propria breach

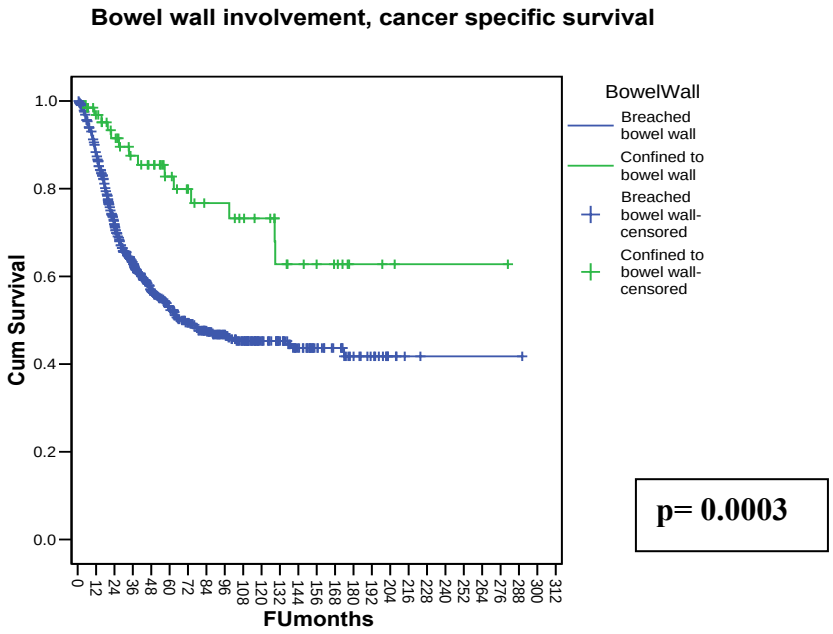


Figure 23 Cancer-specific survival according to N stage

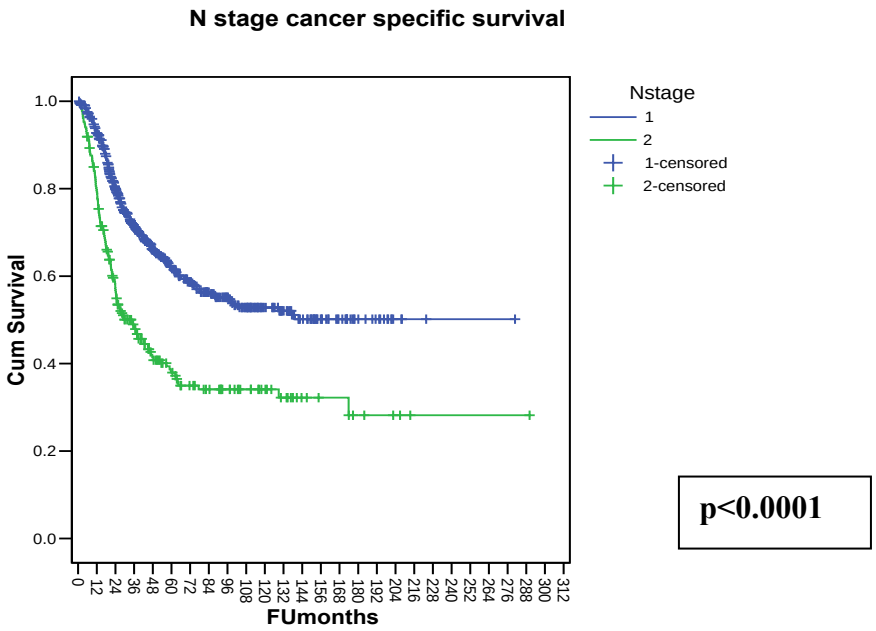


Figure 24 Cancer-specific survival if only micrometastases in LN

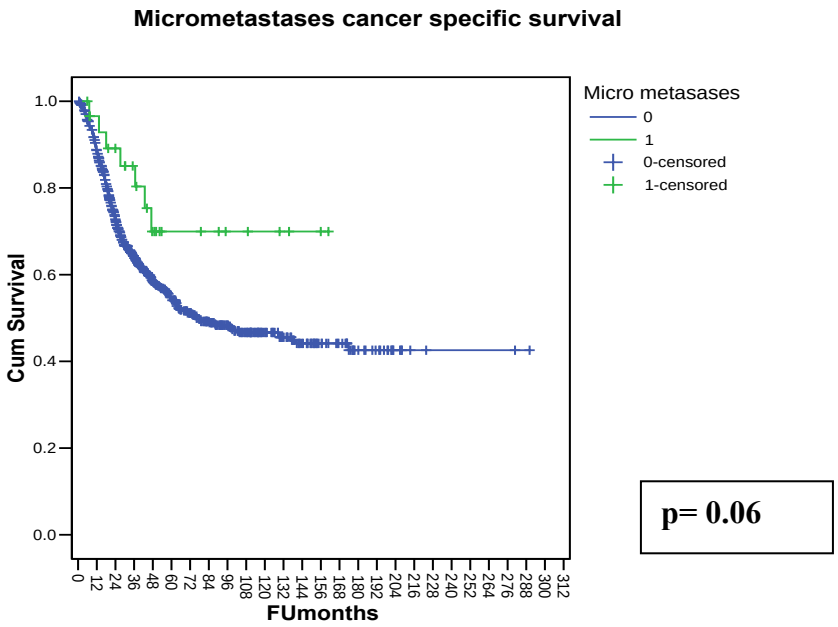


Figure 25 Overall survival effect of differentiation

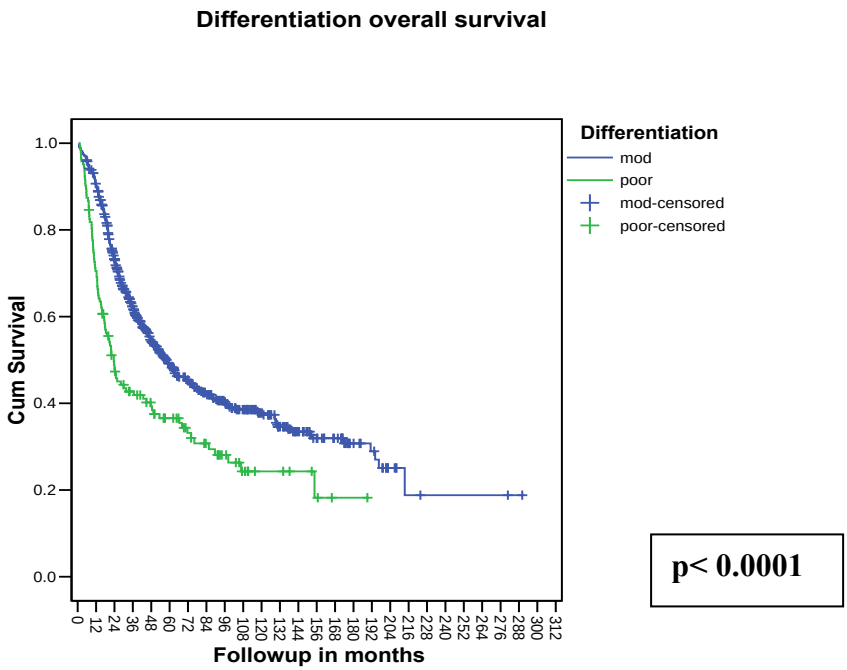


Figure 26 Cancer-specific survival effect of differentiation

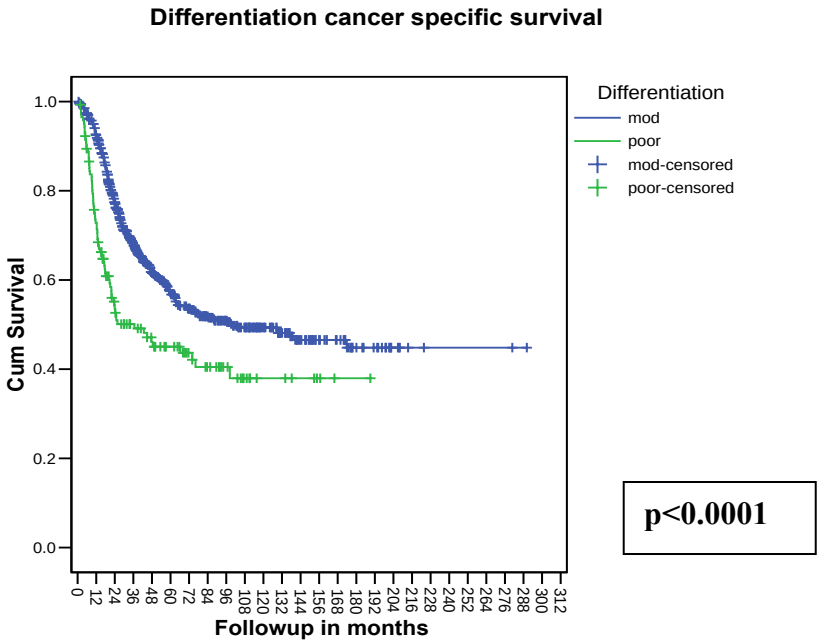
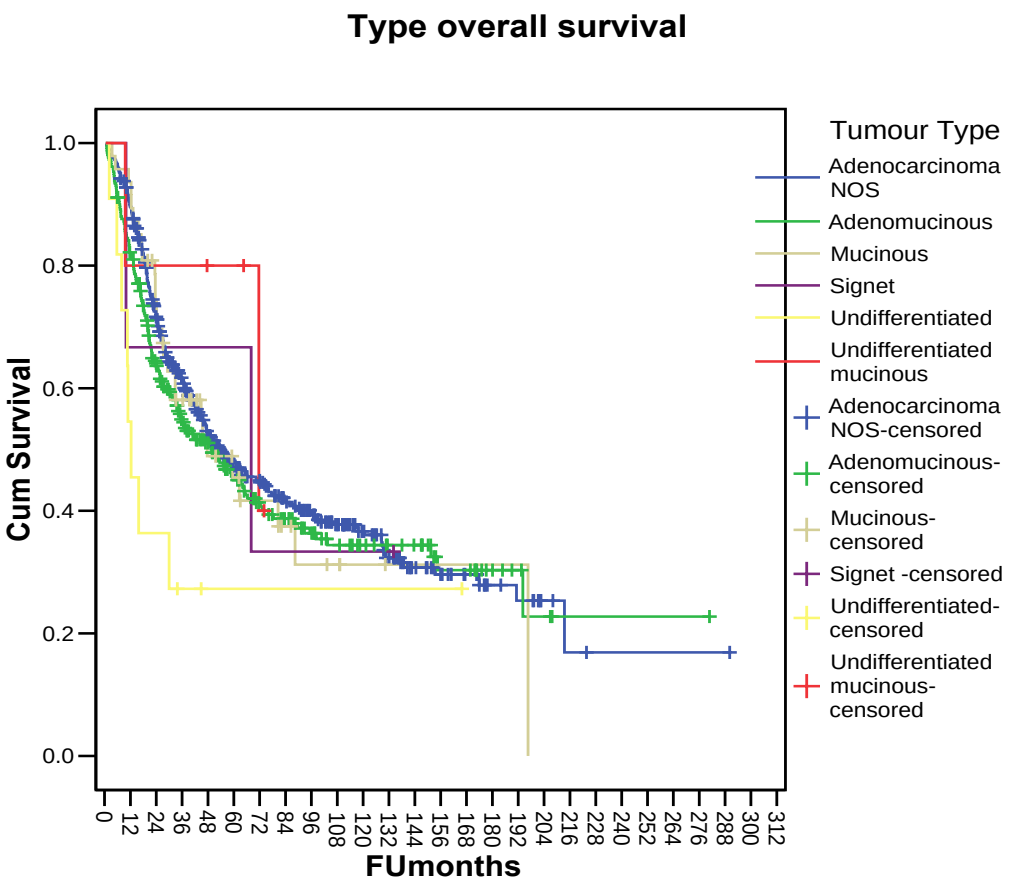
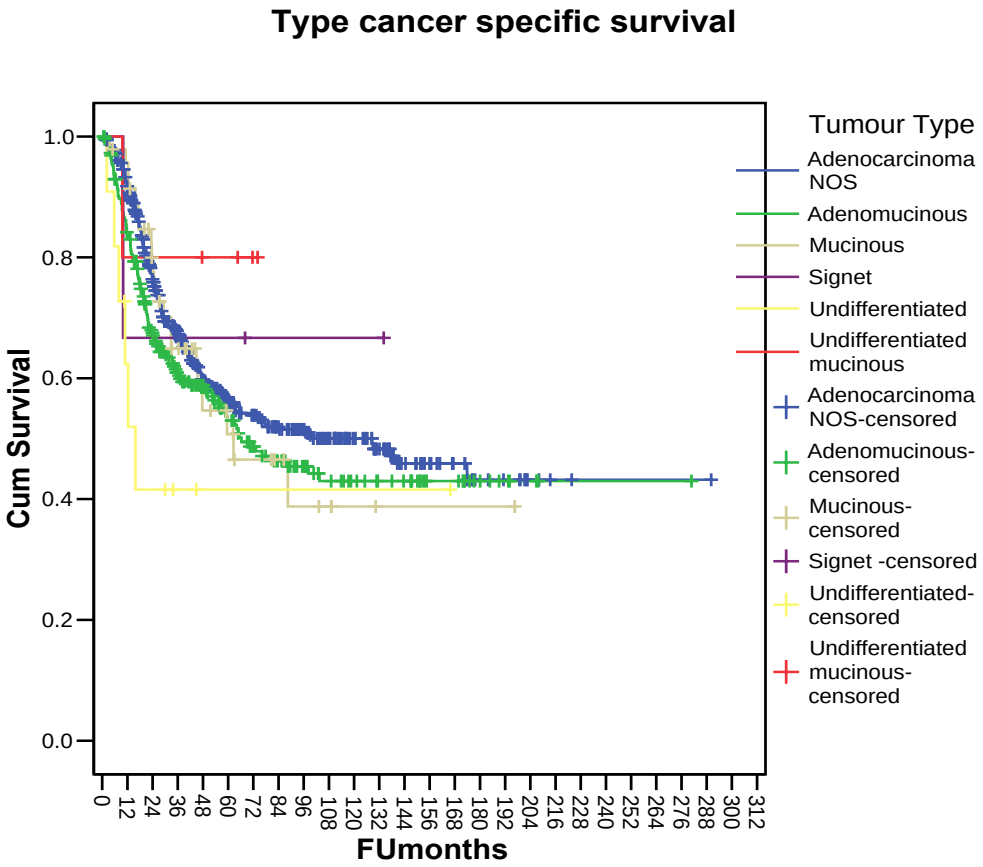


Figure 27 Overall survival according to tumour type



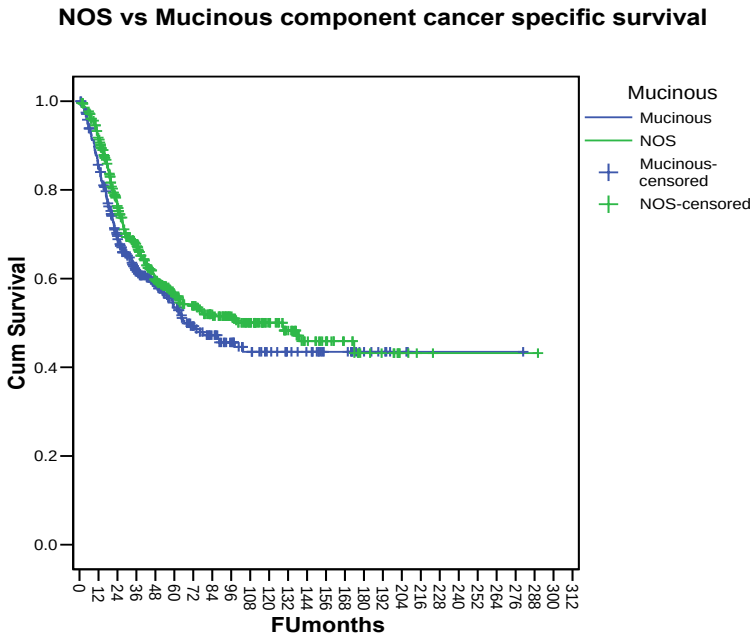
p= 0.19

Figure 28 Cancer-specific survival according to tumour type



p=0.15

Figure 29 Cancer-specific survival if mucinous component



p= 0.14

Figure 30 Effect of extravascular invasion on overall survival

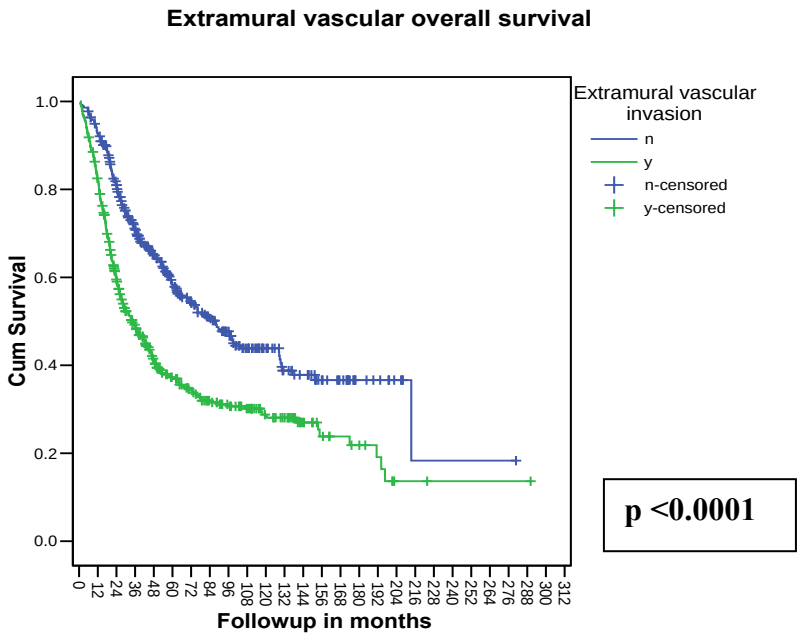


Figure 31 Effect of extravascular invasion on cancer-specific survival

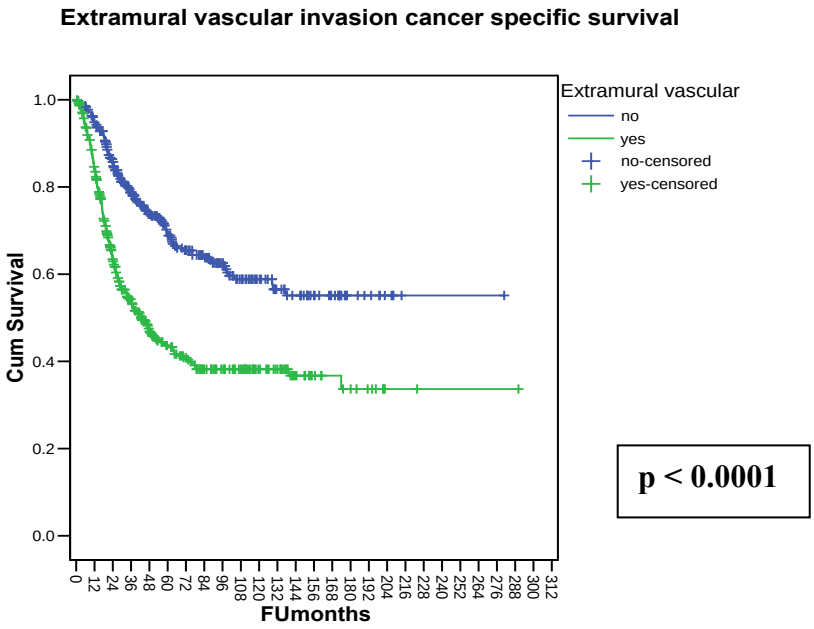


Figure 32 Effect of perineural invasion on overall survival

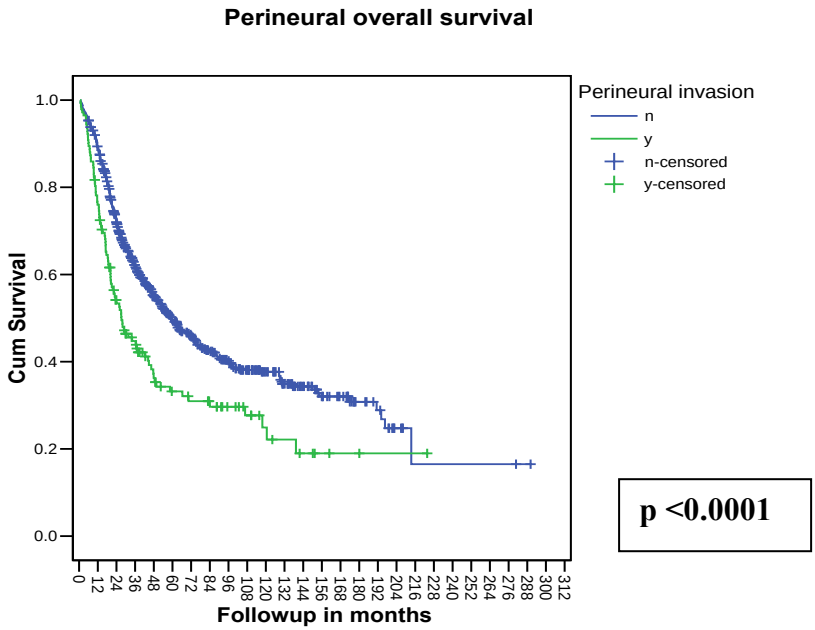


Figure 33 Effect of perineural invasion on cancer-specific survival

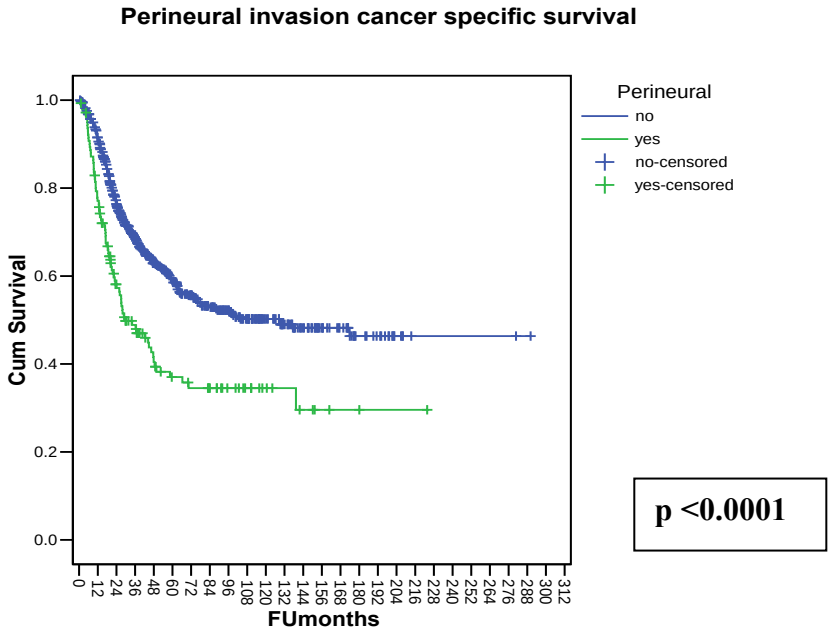


Figure 34 Effect of mural vascular invasion on overall survival

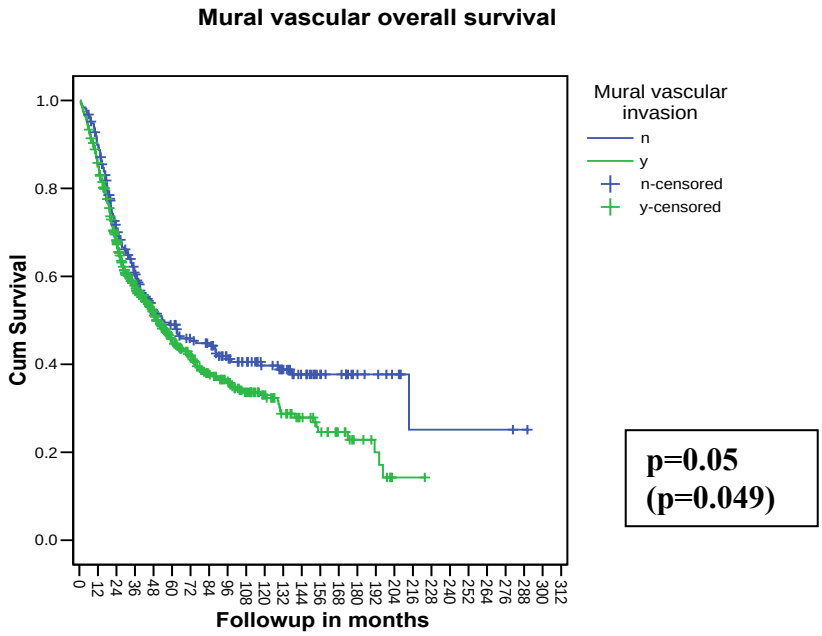


Figure 35 Effect of mural vascular invasion on cancer-specific survival

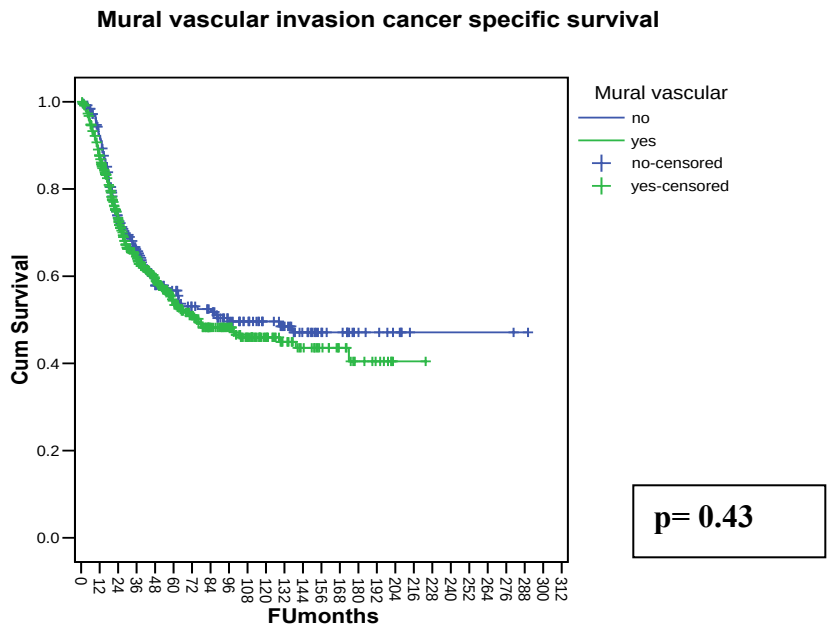


Figure 36 Effect of infiltrating margin on overall survival

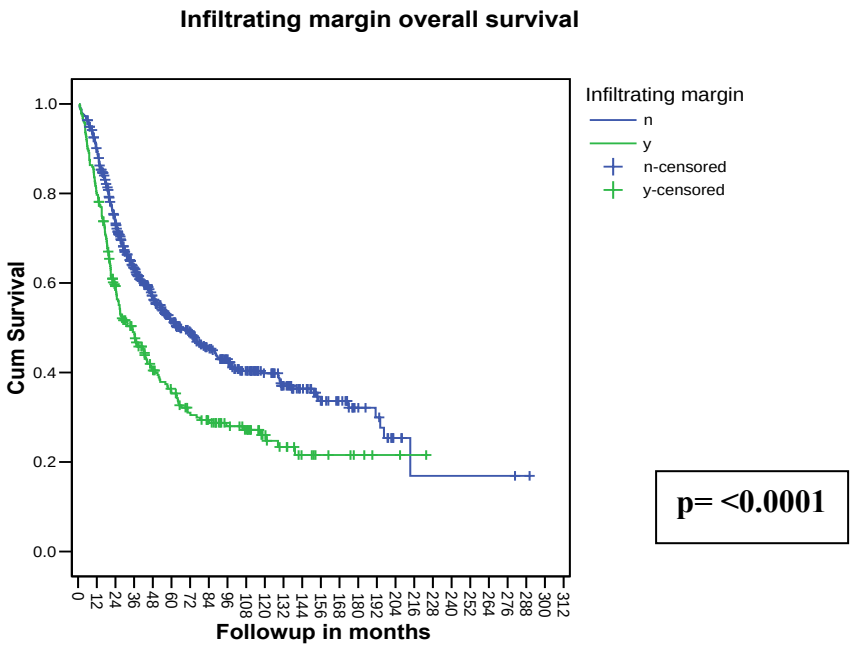


Figure 37 Effect of infiltrating margin on cancer-specific survival

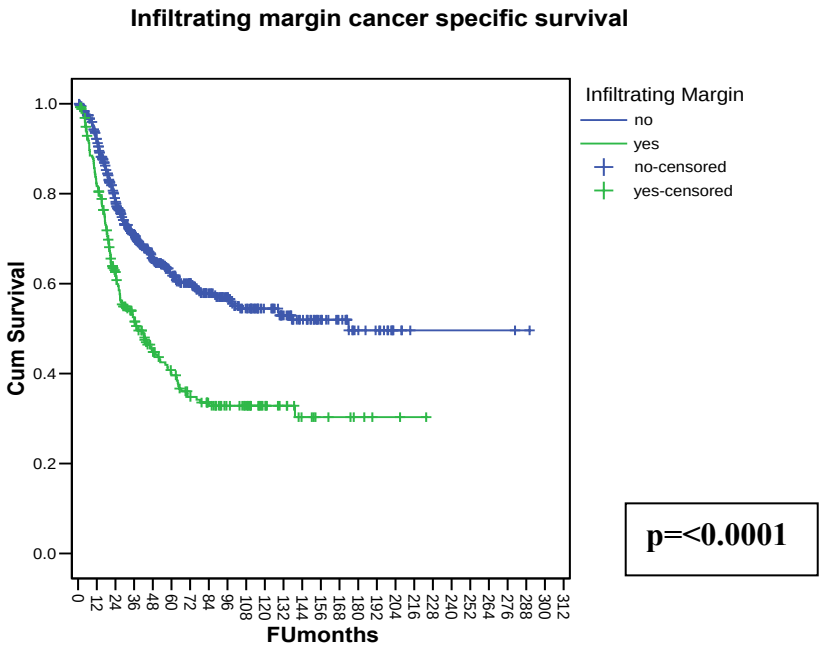


Figure 38 Effect of budding on overall survival

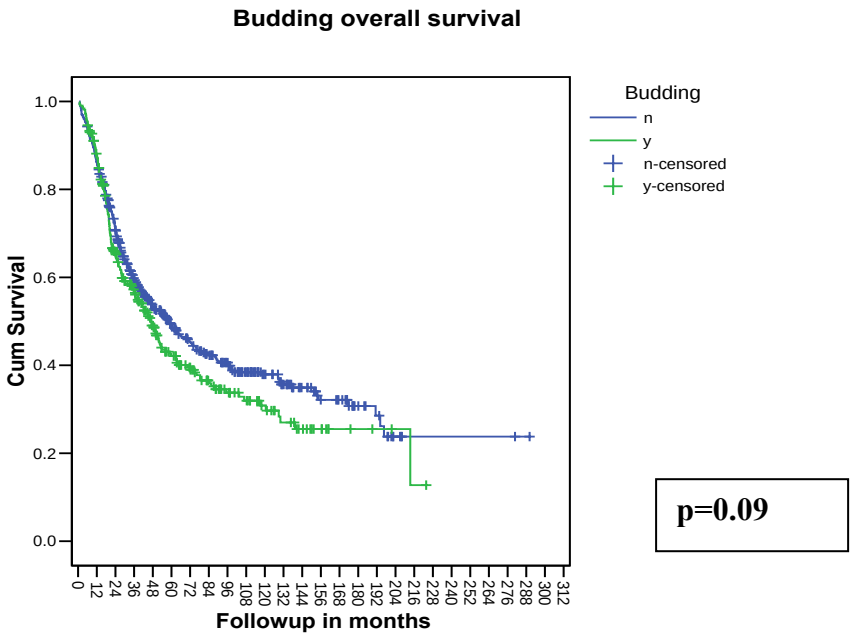


Figure 39 Effect of budding on cancer-specific survival

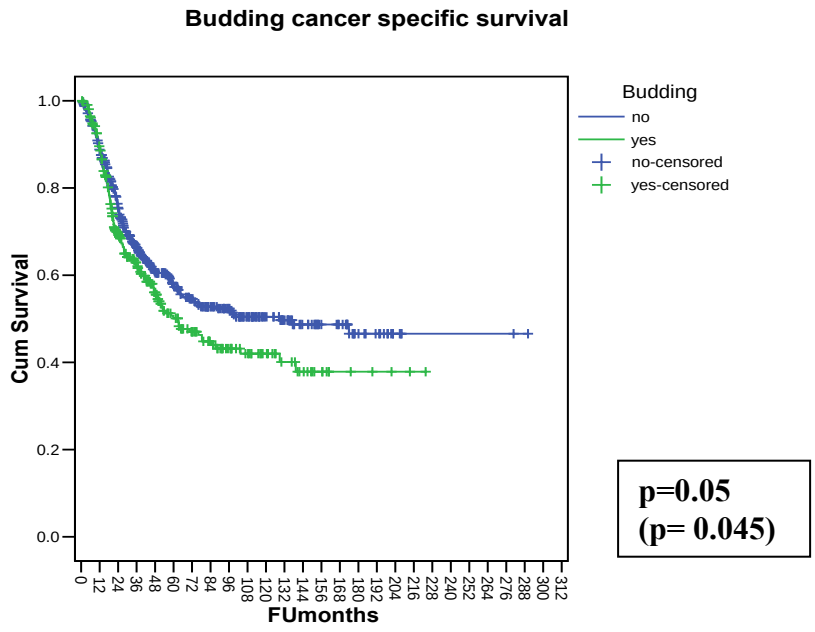


Figure 40 Effect of stroma type on overall survival

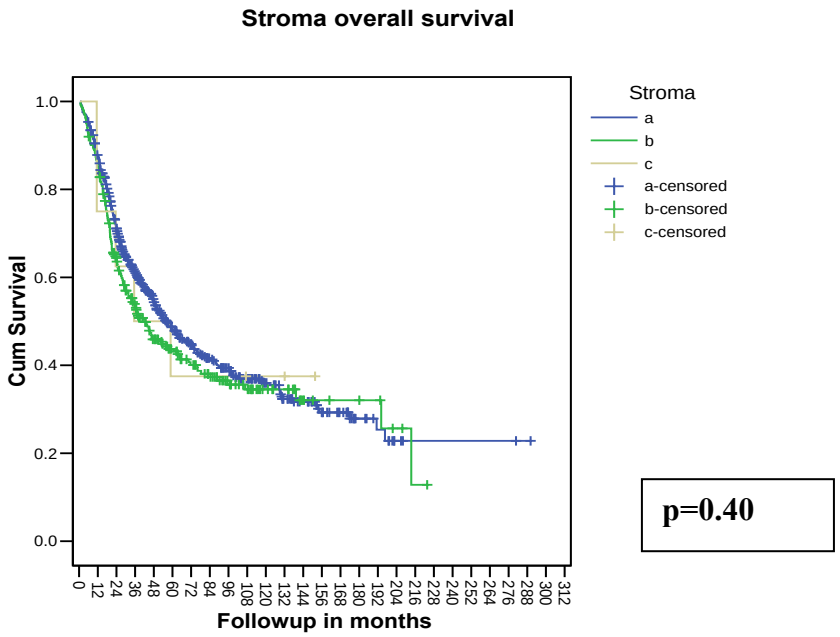


Figure 41 Effect of stroma type on cancer-specific survival

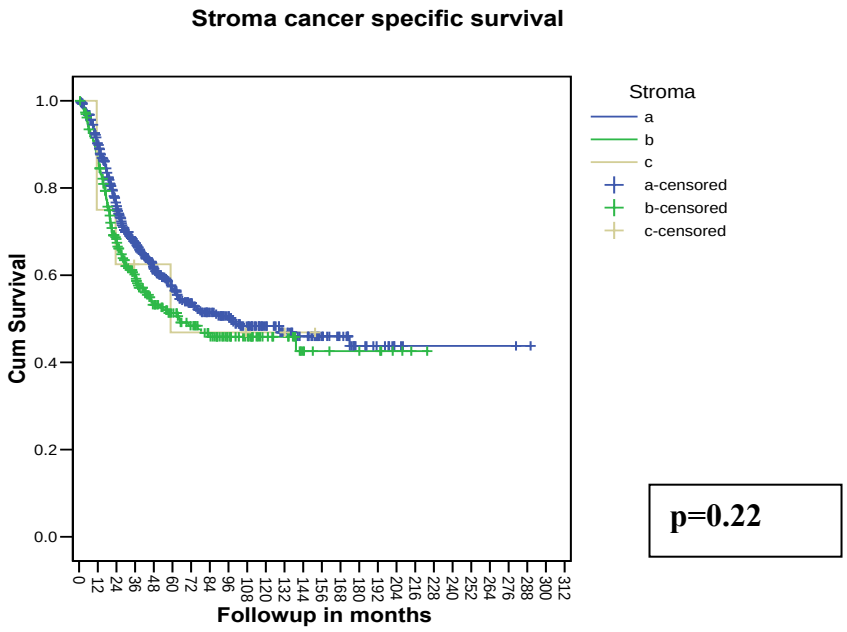


Figure 42 Effect of peritumoral lymphocytes on overall survival

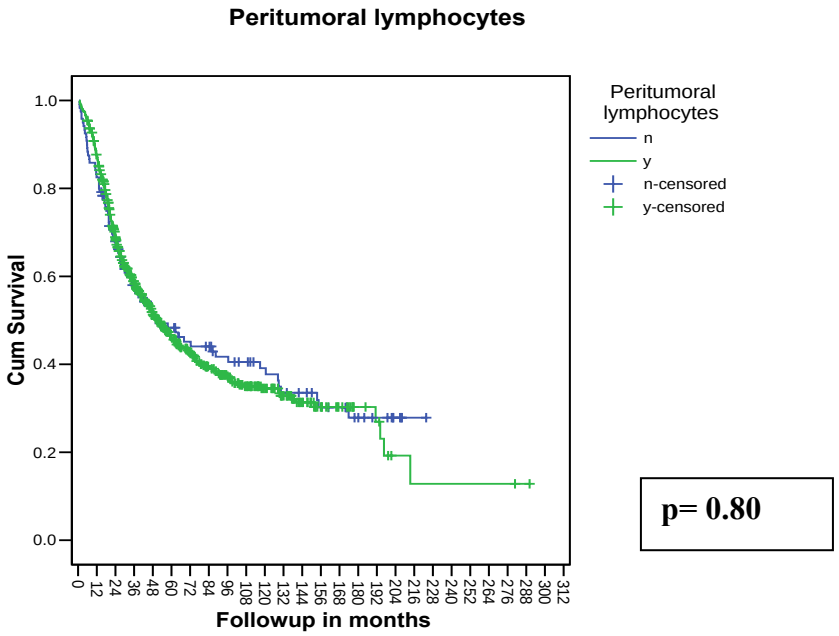


Figure 43 Effect of peritumoral lymphocytes on cancer-specific survival

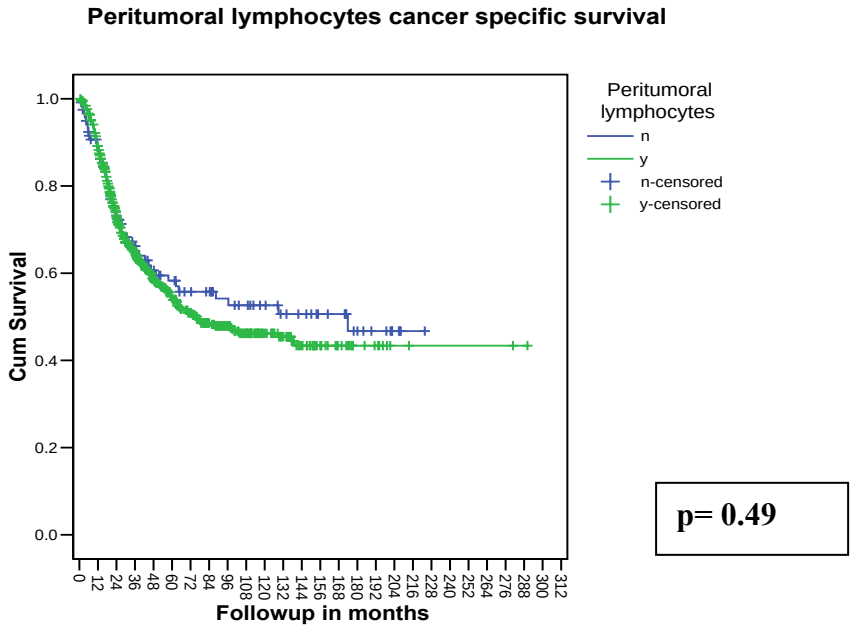


Figure 44 Effect of crohn's like lymphocytes on overall survival

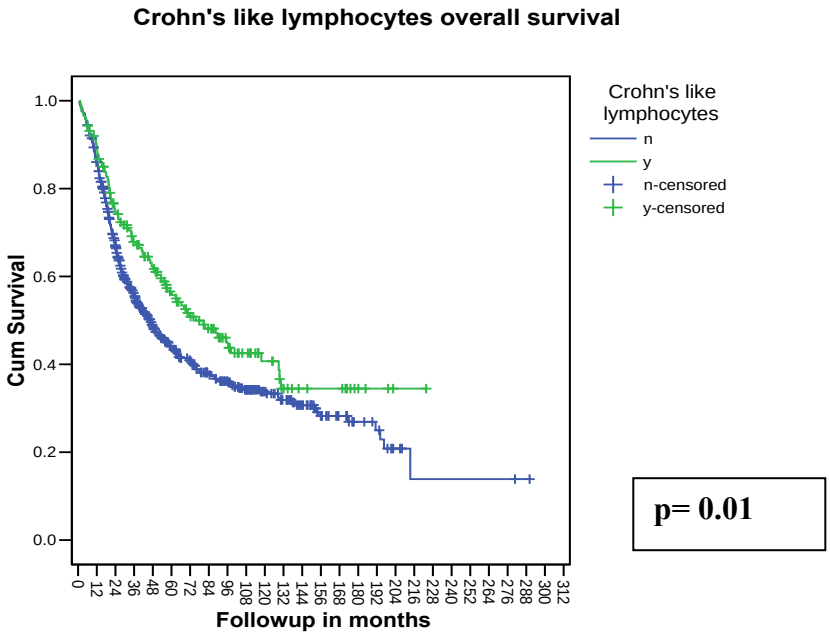


Figure 45 Effect of crohn's-like lymphocytes on cancer-specific survival

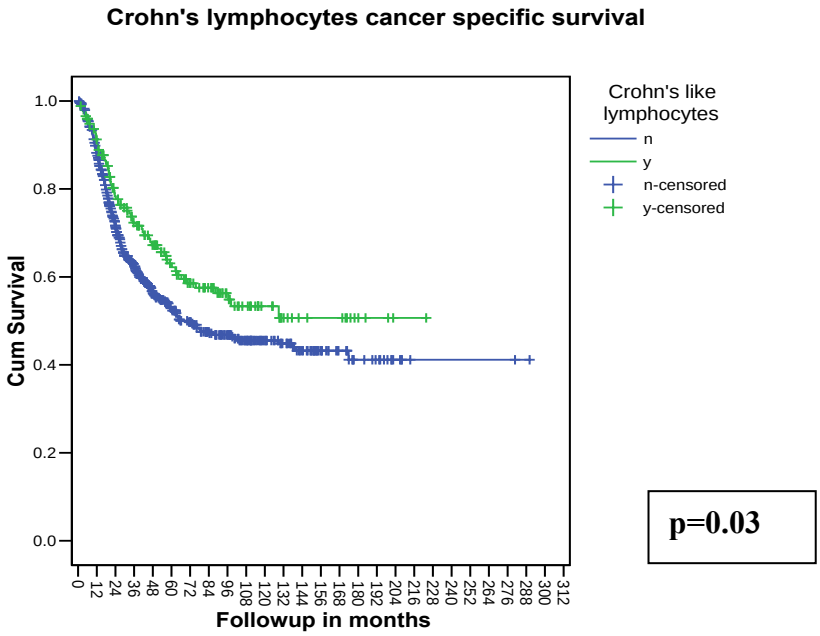


Figure 46 Effect of TILs on overall survival

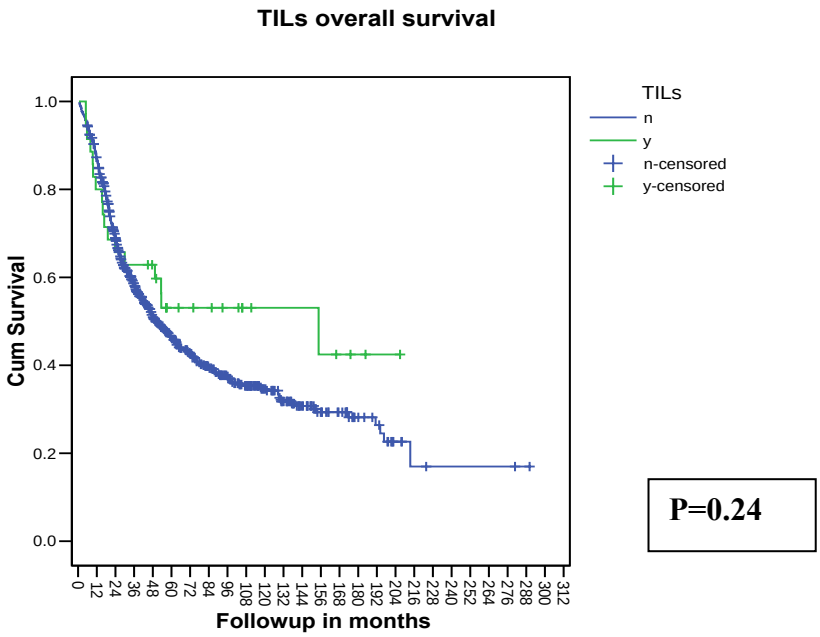


Figure 47 Effect of TILs on cancer specific survival

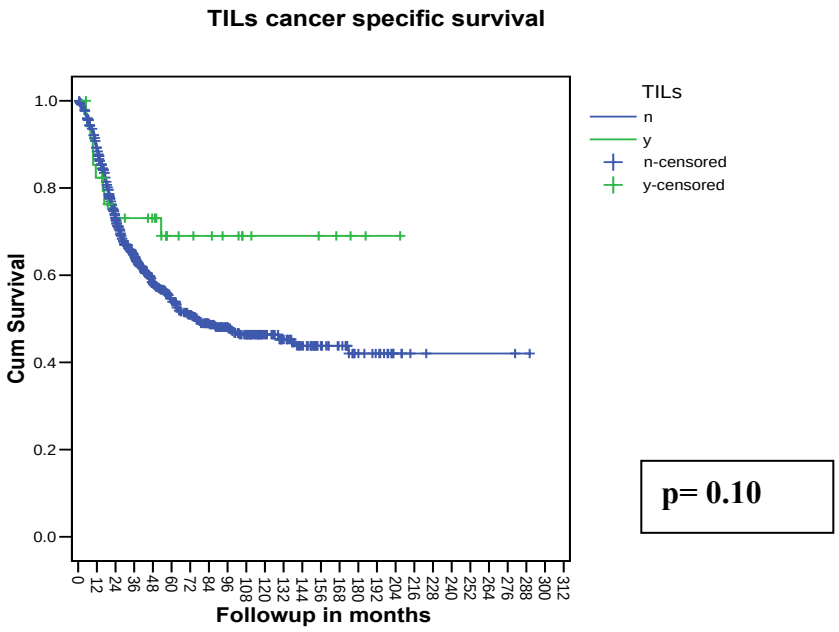


Figure 48 Influence of obstruction on overall survival

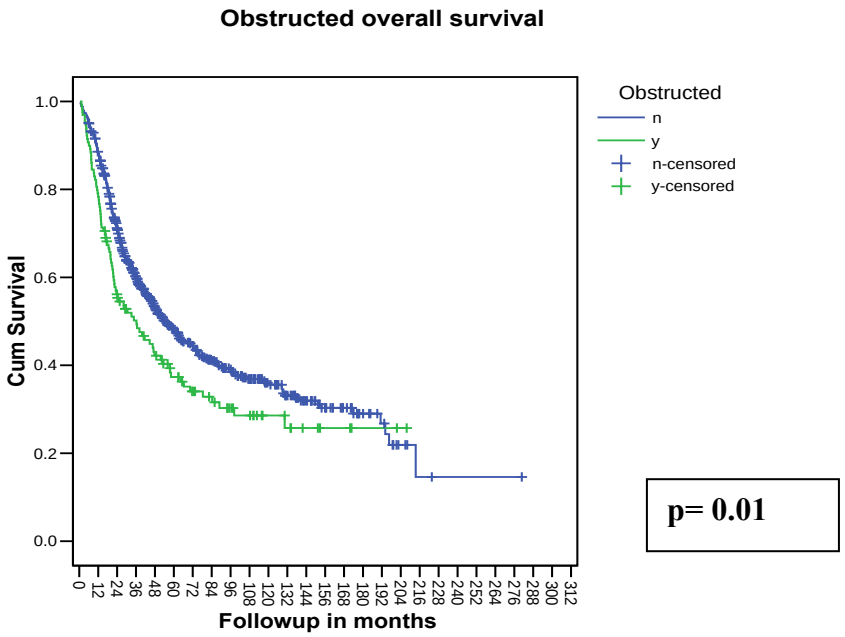


Figure 49 Influence of obstruction on cancer-specific survival

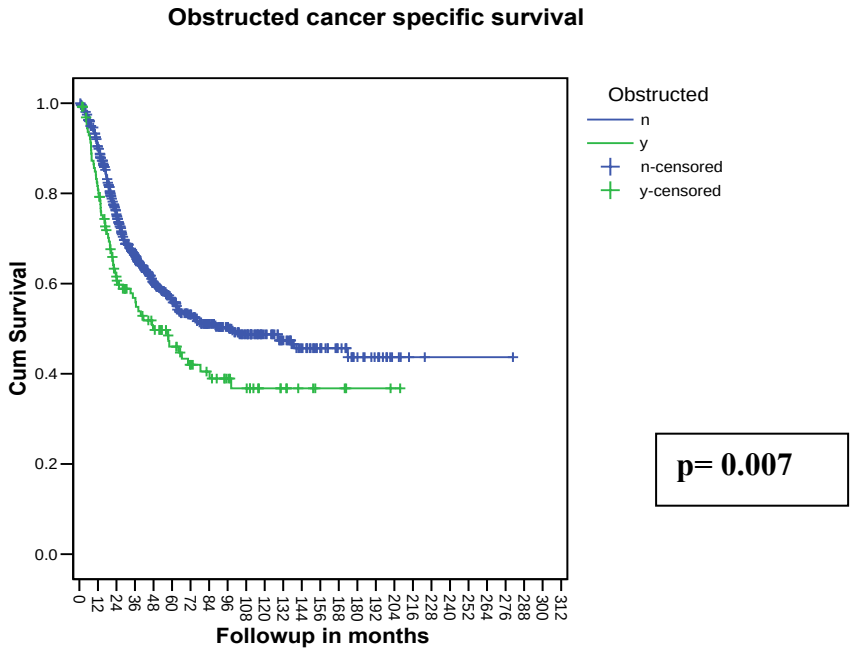


Figure 50 Influence of perforation of tumour on overall survival

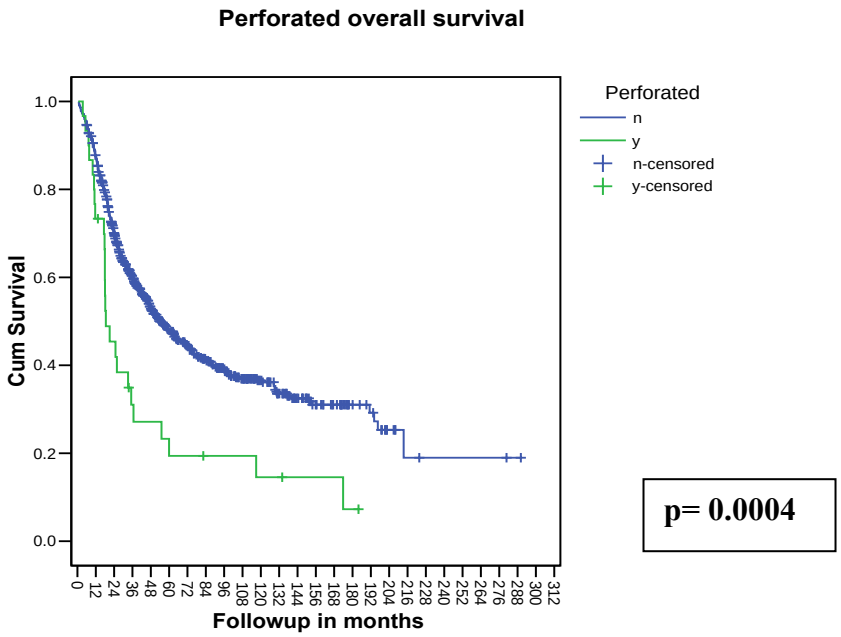
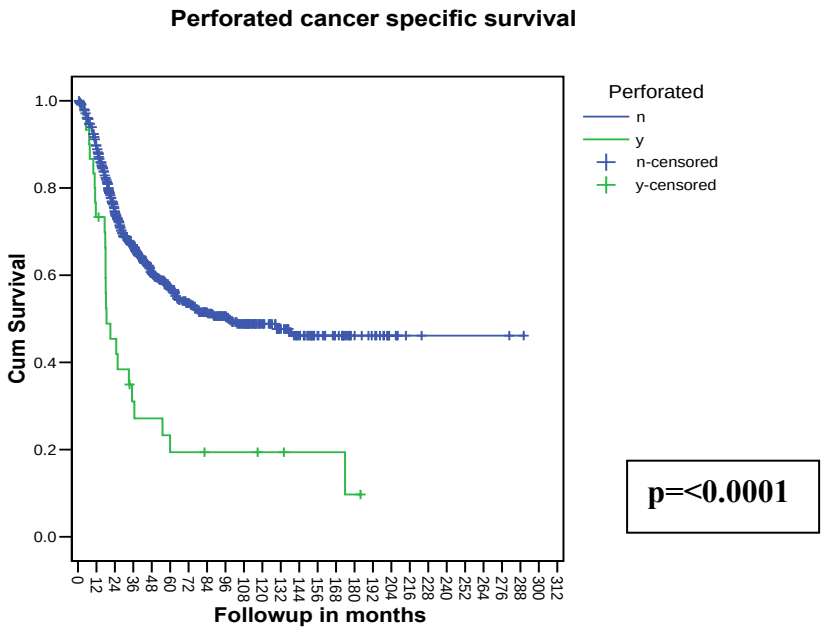


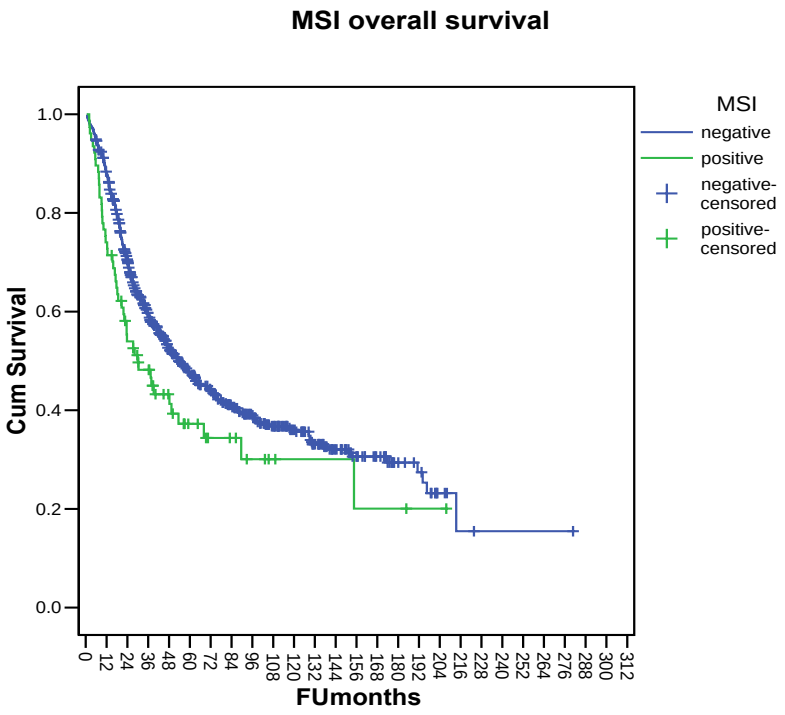
Figure 51 Influence of perforation of tumour on cancer-specific survival



6.4.6 MSI

On univariate analysis, those cases with microsatellite instability had a worse overall survival (Figure 52) (HR 1.42, p= 0.022) but not cancer-specific survival. The lower limit of confidence interval for overall survival did, however, approach non-significance (95% CI 1.05-1.91). On adjusted analysis, again MSI did not influence cancer-specific survival (Table 87) but it did have a detrimental effect on overall survival (HR 1.57 95% CI 1.08-2.27; p= 0.017).

Figure 52 Influence of MSI on overall survival

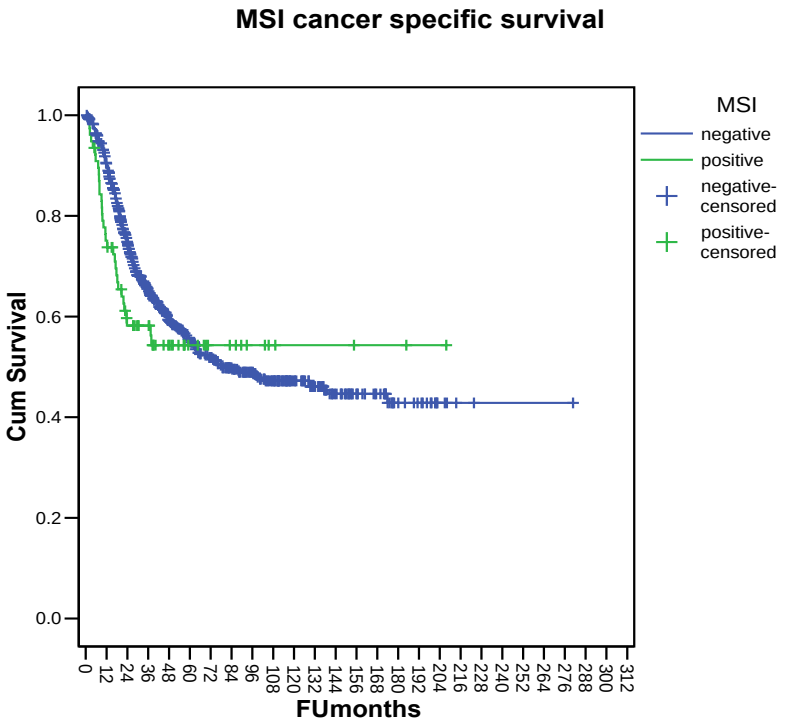


p= 0.02 HR 1.42 (95% CI 1.05-1.91)

Numbers remaining

	12	24	36	48	60	72	84	96	108	120	132	144	156	168	180	192
MSI	57	39	32	22	14	10	9	6	4	3	3	3	2			
MSS	630	479	369	296	246	213	177	151	122	94	74	54	39	31	18	14

Figure 53 Influence of MSI on cancer-specific survival



p= 0.24 HR 1.24 (95% CI 0.86-1.77)

Numbers remaining

	12	24	36	48	60	72	84	96	108	120	132	144	156	168	180	192
MSI	57	39	32	22	14	10	9	6	4	3	3	3	2			
MSS	630	479	370	296	246	213	177	151	122	94	74	54	39	31	18	

6.5 Discussion

6.5.1 Gender and Site

We found neither gender nor site to have prognostic significance in this group of ACPS C CRC. There is no agreement in the literature as to whether gender does influence survival, with only around half of the reviewed papers finding that women had a better outcome from CRC. Three large population-based studies that performed multivariate analysis, did find that women had a significantly better outcome [3, 12, 13]. These studies included all stages and adjusted for stage in analysis. They did not, however, give subgroup results to determine if the observed gender survival advantage applied to all stages. It may be that the benefit observed in women is more applicable to early stage disease, which would explain the null finding of this study. This is supported by two other studies inclusive of only stage B and C cases, neither of which found a gender variation in survival [20, 112].

We did not perform relative survival analysis (correcting for normal regional gender variations in survival). However, given there was no significant prognostic variation this would be unlikely yield a different result, especially in relation to cancer specific survival.

Not all studies adjust for other prognostic influences. It could be argued that the female survival advantage may have been negated if all factors were comprehensively accounted for in analysis. However, according to our findings this is unlikely to be the case. The main associations with female gender were older age, MSI and poor grade (possible related to MSI). If anything, these factors are associated with worse

outcome, with at least age and poor grade being detrimental to survival on our results. Thus, adjusted analysis would, if anything, further improve survival.

Equally, prognosis did not vary according to the anatomical site of the tumour. There was negligible difference in the hazard ratios of dying between proximal and distal tumours and with narrow confidence intervals this does not even suggest a trend. Subsite analysis was limited by small subgroup size, but even so, there was little to suggest any survival variation. These findings are in keeping with the literature, which does not support a prognostic role for tumour site [3, 104, 105, 107, 112-114, 130, 131].

Again, when subgroups combining gender and site were analysed, no significant prognostic variation was observed. Low subgroup numbers meant this analysis was underpowered and would not have shown a less than 10% difference. There was, however, little to suggest that even increasing the cohort size would have led to significance.

The current study was of sufficient size to show a clinically significant difference related to gender or site and was appropriately analysed. The weight of evidence suggests that gender and tumour site do not influence survival following resection of stage C CRC.

6.5.2 Pathology

The pathological factors we found to have useful prognostic significance were (in order of significance): nodal status (number involved); extramural vascular invasion;

infiltrating margin; perineural invasion; differentiation; and type (mucinous compared to NOS). Perforation was also found to be detrimental to outcome. Three of these factors reflect degree of spread (nodal status, vascular and neural invasion). While they do correlate with each other, they all influenced outcome independently. The other factors (infiltrating margin, poor grade and mucinous type) reflect aggressive biology and while this is linked to rapidity of spread, these factors also independently worsened outcome (i.e. their effect is not purely through greater propensity for spread).

The fact that greater quantitative nodal involvement is detrimental to survival is not surprising (though not all research groups agree). Our findings suggest that within a given staging system, where lymph node positive disease is grouped together (ACPS, Duke‟s), it is prognostically useful to know the number of nodes involved. The TNM system includes this information, but staging categories group all positive nodes together (stage 3). The Modified ACPS further classifies stage C into C1 or C2 depending on the involvement or not of the apical node [63]. We could not meaningfully assess the apical node status due to the retrospective nature of this study and limited reporting. Using apical nodal status in staging must be considered with caution. What constitutes the apical node may be unclear or there may be no nodal tissue adjacent to the transacted pedicle. A further modification of the ACPS system may be warranted so that the number of involved nodes is considered.

Some caution should be employed in interpreting our nodal results. Lymph node yields varied according to pathologist, hospital and across time, with possible under-calling of positive nodes. This potential down-staging, if anything, would have diluted a significant finding.

The other aspect of staging that was assessed was degree of bowel wall involvement. Degree of spread through the bowel did not completely correlate with nodal status. To increase subgroup numbers for meaningful results, data were dichotomised in relation to the muscular propria involvement (either "confined by bowel wall" or "spread beyond muscularis propria"). In contrast to some of the other staging classifications (i.e. Astler Coller, [64]) we did not find this division added prognostic information. Other groups have found that free serosal involvement is highly significant [63, 99, 100, 107]. In this study the numbers with serosal involvement were small and analysis using four T-stage categories involved subgroups too small to draw meaningful results.

The degree of tumour differentiation was prognostically significant, but not to the expected extent and with the lower limit of the confidence interval approaching non-significance. Univariate analysis suggests a strong prognostic influence, highlighting that factors associated with grade account for at least a degree of its perceived effect (as was found by others [106, 113]). Thus, studies that do not adjust for other pathological variables should be interpreted with caution. The literature review indicated that even studies including multivariate analysis produced mixed results, with many finding grade not to be prognostically important [105, 106, 113]. Our study varies from many by including only stage C cases. One other study of stage C disease (and only colon cancers) found differentiation to be important to outcome [107].

Subjective assessment of this parameter may contribute to the inconsistent results observed in the literature. In this study, limiting categorisation to moderate or poor increased the accuracy and reproducibility of the findings and thus the validity of the study. The lack of consistent findings between groups and the potential inaccuracy of

this measure means it should be used with caution and the prognostic importance attributed to grade should be limited.

Tumour subtype did not influence outcome in this study. However, categorising to include all subtypes led to inadequate numbers to draw meaningful conclusions. It would be expected that signet ring and undifferentiated tumours have a worse outcome, yet this was not apparent. Therefore, to improve the usefulness of findings, tumour type was dichotomised according to whether mucin was present or not. Mucinous type is one of the consistent types examined in most studies. There was a low number of true mucinous tumours (>50% mucin) hence we looked for the influence of any mucin. On univariate analysis, no difference was evident. On adjusted analysis, tumours with mucinous component had a worse outcome, which just reached significance (HR 1.29 95% CI 1.02-1.63 p= 0.03). As with differentiation, the lower limit of the confidence interval approached one. The relative importance of this factor in determining prognosis is debatable. Studies have consistently failed to find tumour type significantly affects outcome [3, 20, 64, 102, 105, 106, 111]. One of the difficulties when comparing studies is the variation in classification. The overwhelming negative results from other research groups and the borderline results from this work, however, suggest that type (and the presence of mucin) has little prognostic use.

As expected, we found extramural vascular invasion and perineural invasion to confer a worse outcome (extramural HR 1.78 95% CI 1.37-2.31, p<0.0001) and perineural HR 1.45 95% CI 1.10 – 1.91, p=0.008). This concurs with the reviewed studies of colorectal cancers [12, 104, 110] including two of stage C cases only [20, 107]. These findings reconfirm that these measures have independent prognostic importance and

do not simply signify increased likelihood of spread. It should be remembered that in this study, "extramural vascular" invasion may be venous or lymphatic as we do not believe they are distinguishable on routine staining.

The results for mural vascular invasion were unexpected. Multivariate analysis found that the presence of mural vascular invasion was actually associated with a better outcome, but only for cancer-specific survival. This result cannot be easily explained and is counterintuitive given that vascular invasion is usually associated with a worse outcome. There are several possibilities. As the tumour grows, the mural space may become obliterated by disease to the extent that mural invasion can no longer be visualised. This would be consistent with the finding that not all cases with extramural invasion demonstrated mural invasion. Tumour size did not correlate with mural invasion on analysis but this may be meaningless due to the above observation (that large tumours may override mural invasion). Possibly ulceration obliterates mural invasion in the more advanced tumours but as we did not record morphology, we cannot deduce this. The other possible explanation is sampling error and observer error.

From our results we can conclude that extramural lymphovascular invasion is one of the strongest predictors of poor outcome. Perineural invasion gives additional prognostic information, while the importance of mural vascular invasion is questioned from this work. This highlights the need to clarify whether vascular invasion is extramural or mural.

We found that the nature of the tumour margin had a profound influence on outcome. An infiltrating margin appears to signify aggressive biology and tendency to spread,

but remains independently useful within a given stage. Little has been published on the prognostic role of this feature. Most work has been on rectal cancers, where a detrimental effect on outcome has been demonstrated [102, 111, 143, 144]. The only two papers to examine the effect in colonic disease did not find it had a significant prognostic influence [105, 106]. Numbers were small and significance was lost on multivariate analysis when adjustment was made for other factors including stage. The current study, being larger and comprehensively adjusting for other prognostic factors, can confidently state this parameter is useful in predicting outcome from stage C CRC. It will be interesting if this is also the case with earlier stage disease. Our findings suggest further study in this regard is warranted.

Budding is thought to correlate with infiltrative margin [38, 111], and while we found some correlation, there was not complete concordance (similar to the findings of Ueno et al) [111]). We did not find budding to be independently predictive of outcome in stage C cases. Significance was found on univariate analysis but not multivariate analysis, suggesting the association with the highly significant infiltrating margin accounted for the univariate finding of significance. This is in contrast to the literature, although limited work in this area has been published. Three studies showed budding to have prognostic value, signifying more aggressive disease. One included only stage B and C cases but did not adjust for infiltrating margin [148]. The other two adjusted for stage but only one for infiltrating margin [111, 147]. As can be seen from our results, not adjusted for infiltrating margin may lead to erroneous conclusions. Again this measure is subjective [111] and the number of clusters considered to constitute significant budding varies. Our results suggest budding is not useful in predicting outcome in stage C colorectal cancer.

We did not find the stroma of the tumour to influence outcome. There were sufficient numbers in the fibrous and keloid subgroups to gain meaningful results, however the numbers of cases with myxoid stroma were too small to draw conclusion. There was no trend to indicate that larger numbers may have produced significance. Our findings contrast with the limited work on this parameter. The only study to examine the prognostic influence of stroma found this factor to be independently predictive of outcome on adjusted analysis [144]. They used the same classification as our study but included rectal cases only, which may account for the different results. There is no explanation as to why this aspect of a tumour should be more important in rectal tumours than colonic. The influence on prognosis is postulated to relate to the fibroblastic content of keloid type stroma, which may influence the tumour‟s biological behaviour (cell migration, tumour progression and differentiation) [144] and, as such, should not be site-specific.

Correct identification of stroma type (particularly keloid and myxoid) was hampered in the older slides by fading. This may have contributed to some error, diluting the (predicted) better prognosis of fibroid stroma by including undiagnosed keloid and myxoid types. However, the breakdown in subgroup frequencies is similar to Ueno et al. [144], suggesting we have not significantly under-called the non-fibroid types. Despite potential detection problems, there were a significant number of cases able to be classified as keloid and without even a trend to worse prognosis, there is little to suggest that the results are erroneous. We can conclude from our results that tumour stroma type - fibroid or keloid - does not affect outcome.

The study of tumour lymphocytes is in its infancy. What constitutes lymphocytic invasion and the classification of this parameter varies between groups. The measure

itself is very subjective, making comparisons difficult. One group (in several papers) has shown lymphocytic infiltration influenced outcome in rectal tumours [102, 111, 143, 144]), while others have found no influence in colonic disease [105, 106, 112]. We found the results on peritumoral lymphocytes to be confusing. Most cases showed these lymphocytes (only 126 did not). It was expected that their presence would confer a survival advantage, however the opposite appeared to be the case. Possibly lymphocytic infiltration is more prominent in more aggressive tumours, however this should have been negated on adjusted analysis. There was not a correlation between lack of lymphocytes and infiltration, poor grade or mucinous component as might be expected if this sign represented more aggressive tumours. What use can be made of this pathological factor is unclear and further work is required to clarify its role.

Crohn‟s-like lymphocytes and TILs are more definite entities (though often grouped with peritumoral in studies). The current study did not find either had prognostic significance. The numbers however were small especially for TILs (n=35). The results for TILs came closer to significance (p= 0.10) and it is possible a type 2 error led to the null finding. We cannot conclude that lymphocytic invasion influences outcome from our results. While these findings contribute to the knowledge on these pathological measures, further work is required to determine their true value.

Obstruction was not associated with a worse outcome in this study. The literature is inconsistent regarding the prognostic importance of obstruction [3, 12, 110, 113, 114, 130, 133, 135]. The accuracy of the current study relative to others was improved for two reasons. Many studies include perioperative deaths, which increases the likelihood of positive findings, whereas in the current study these cases were excluded. Secondly, in some studies, the method of diagnosis is not clear for

comparison whereas we relied on pathological evidence. Having a stricter criterion should have exaggerated any survival effect, therefore the lack of prognostic influence in stage C cancers is more likely to be true. It is possible that a detrimental effect from obstruction is more evident in earlier stage disease where poor prognostic factors may have a greater impact than in stage C cases where the tumour has already metastasised.

In this study, perforation was the strongest predictor of poor outcome (HR 2.27, 95% CI 1.46-3.53). The associated poor outcome was not due to advanced T stage. Adjustment analysis considered bowel wall involvement rather than T stage subgroups (hence T3 and T4 together) however most perforated cases were in fact T3 (67%) while only 33% were T4. This apparent incongruity implies that perforation is not always caused by direct invasion and may be due to overlying necrosis. The results from the literature on outcome following perforation are obscured by several methodological flaws: the fact that perioperative deaths are included; analysis is often non-adjusted; definition is not clear; and separation between proximal and tumour perforation is not frequently made. Having addressed these issues, the results from this study clearly demonstrate a long term deleterious impact if the perforation is through the tumour.

6.5.2.1 Pathology Conclusion

We have examined a select group of patients and it needs to be emphasised that our findings only apply to stage C disease. However, as good outcome is reasonably assured for stage A and B cases, it is in stage C disease that other prognostic factors have a greater role and may be of greater use, not only for patient information but also

in planning treatment. The select nature of our cohort undoubtedly contributed to variation between our results and others. Stage C cases have already declared their hand (biology or aggressiveness) by metastasising. Factors that may be more predictive in early stage disease may be less relevant in stage C. Even studies that adjust for stage do not separately examine for an effect in each stage category.

The assessment of pathological prognostic factors in this study was greatly strengthened by the re-evaluation of all pathology by a single pathologist. Inter-observer variation was removed and we did not rely on reports which were of varying thoroughness and that used protocols that varied over time. Some impediment was encountered in the form of aged slide and limited sampling.

Subgroups were predetermined and simplified to ensure adequate power. This was not always possible and some results were interpreted with caution as has been detailed. The list of included pathological parameters was comprehensive, and as such all appropriate adjustments were considered. Overall the accuracy of findings should be assured.

It can be concluded that the most clinically useful pathological factors for predicting outcome in stage C CRC are perforation, extramural vascular invasion, perineural invasion, infiltrating margin and high number of involved nodes. Differentiation and tumour type influence survival to a lesser extent. Factors that were not prognostically useful were tumour size, obstruction, extent of bowel wall invasion, stroma type, the presence of budding, TILs and Crohn‟s-like lymphocytes. The role of mural vascular invasion and peritumoral lymphocytes is yet to be established but interesting questions were raised by our aberrant findings.

6.5.3 MSI

We did not find MSI to influence cancer-specific survival in this group of stage C CRC cases but found it had a detrimental effect on overall survival, albeit close to non-significance. This varies from the dogma that MSI cases have a better outcome. The literature generally supports this assumption and, even though studies are mixed and have varying selection criteria, most find MSI decreases HR of dying by over 50% [39, 46, 48, 51-53].

The variance seen in this study may be due to the select study group. By selecting cases that have already metastasised to lymph nodes, we may have chosen a more aggressive subgroup of MSI cancers, given the usual propensity for these cancers to remain localised. However, even studies that limited inclusion to stage B and C cases generally showed a better prognosis in the MSI cohort [21, 48, 51, 52, 57]. Two of these also included only stage C cases. Wright et al. found that MSI conferred a significantly better outcome (HR 0.44 (95% CI 0.23-0.85) p=0.015) in 255 sporadic cases [52]. The variation to our study increased when the influence of chemotherapy is removed. No patient in the Wright et al. study received chemotherapy as the years sampled were prior to standard adjuvant therapy. As will be shown in the next chapter, in the non-chemotherapy cohort of the current study, MSI actually predicted a worse outcome. Other study differences are unlikely to account for any results variance. While both studies included age, gender, differentiation and venous invasion in adjusted analysis, the pathological parameters included in our study were more comprehensive. Two of the additional parameters were infiltrating margin and mucinous type, both of which were found to be associated with MSI and have prognostic significance. However, removing their influence from the MSI cohort by

including them in the multivariate analysis of the Wright et al. study would have theoretically only further improved the prognosis in the MSI group.

Furthermore, it is unclear if all resections in the Wright et al. group had curative resections but unless disproportionately distributed, this should not have made a significant difference. Lastly, the basis of MSI diagnosis varied. Wright et al. used the NCI panel, while we based a positive finding on BAT26 and BAT40. It is theoretically possible we selected a more aggressive phenotype by limiting the microsatellites analysed. Even if this was the case, given that BAT26 sensitivity is around 98% it is unlikely to be sufficient to change the overall outcome. Hence, the difference between the study findings cannot be accounted for.

The second study of stage C cases by Elsaleh et al. found a borderline improvement in 5-year survival in the MSI cases (53 vs 33% p= 0.043) [21]. Given the association of MSI with various pathological features, analysis that is not adjusted is of limited use.

Given our findings and the conflicting study results, it must be concluded that MSI cannot be used to predict prognosis in stage C cases.

6.6 Prognosis Conclusion

Overall our results suggest that in stage C CRC that has been curatively resected, gender and site do not influence outcome. Pathological features that are useful prognostically include infiltrating margin, extramural vascular invasion, perineural invasion as well as the number of involved nodes and, to a lesser extent, differentiation and type. In contrast, size and T stage do not independently influence outcome. The recently described factors - budding, stroma and lymphocytes - were

not useful in predicting prognosis in this group. Perforated tumours do worse whereas obstruction does not affect survival. MSI status did not influence outcome.

7 RESULTS - FACTORS INFLUENCING CHEMOTHERAPY RESPONSE

7.1 Overview

Given the assumption that only a subgroup of stage C CRC cases is benefiting from adjuvant 5FU-based chemotherapy, it would be beneficial to identify predictive factors to better target treatment. Limited research suggests that gender and site may be useful to indicate a responsive clinical subgroup [21] and, while it is often presumed that cancers with unfavourable pathology will have greater benefit from chemotherapy, this is unproven. Recent research suggests a role for molecular markers in particular MSI but the limited work is conflicting[21, 48]. At this stage definite predictors of response to adjuvant chemotherapy are lacking and treatment cannot be rationalised.

7.2 Aim

This chapter aims to determine which factors influence the survival advantage seen with adjuvant chemotherapy, with emphasis on gender and site, histological variables and MSI.

7.3 Specific Method

Univariate analysis of subgroups was performed using Kaplan–Meier survival curves (for overall and cancer-specific survival), examining for a compounding effect with chemotherapy. Logrank testing was used to assess differences in curves. Five-year overall survival was determined from Kaplan-Meier life tables. This unadjusted subgroup analysis was performed, in addition to adjusted analysis, so study results could be better compared to previous work.

To perform adjusted analysis, prognostic influence was initially determined using the Cox regression proportional hazard model. Then a regression model for interaction was constructed for each factor, incorporating covariates with significance over 0.2. This tested for an interaction with chemotherapy affecting survival (a compounding effect).

7.4 Results

7.4.1 Overview

To summarise the study group 814 stage C colorectal cancer cases were included in analysis, median follow-up was 36.30 months and median age was 71.10 years (range 30.3 - 96.1). Gender distribution was equal (men 49.6%, women 50.4%). Sixty percent were located distal to the splenic flexure while 40% were proximal. Thirty-seven percent received chemotherapy. MSI status was successfully established in 802 cases, of which 9.6% (77) were positive. During the study period, 469 (57.6%) patients died. Of these, 76% were cancer related deaths. The chemotherapy cohort was younger (65.4 yrs vs 75.1 yrs), had a slight male predominance (56.0% vs 45.8%) and a higher rate of poor prognostic indicators (higher nodal status and higher rate vascular invasion).

7.4.2 Site Gender Subgroups

Initially, clinical subgroups were examined. Both men and women showed a benefit from chemotherapy and the magnitude of the survival advantage was similar. Both overall and cancer-specific survival curves (Figure 54 and Figure 55) suggest that women trend towards a better outcome than men in either cohort but that the effect of chemotherapy is the same for either sex. The significant logrank value across the

curves reflects the significance of chemotherapy effect rather than a gender difference. Five-year survival figures suggest the same trend (Table 95). To test whether the trend was significant, gender survival differences within each cohort (chemotherapy or not) were examined (Figure 56, Figure 57, Figure 58 and Figure 59). It can be seen that any perceived trend toward better outcome in women is not significant – women are not doing significantly better than men in either cohort.

Both proximal and distal cancers were shown to have a survival benefit from chemotherapy on unadjusted analysis and there was no significant difference in survival according to site, either on overall or cancer-specific survival (Figure 60 and Figure 61) or on five-year survival (Table 95). The significant difference observed across the curves (and significant log rank) reflects chemotherapy effect only not a variation between the subgroups.

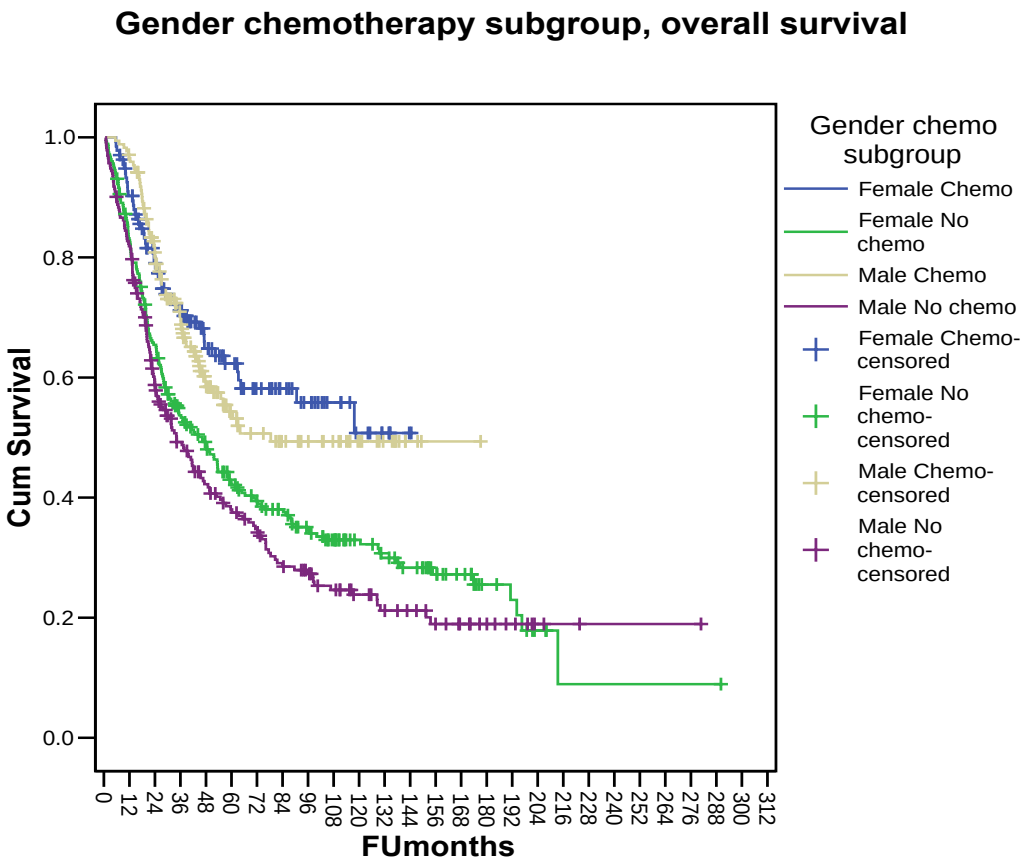
Survival curves for subgroups based on gender and site combinations (Figure 62, Figure 63, Figure 64 and Figure 65) appeared to show a trend towards worse outcome in men, with either proximal or distal cancers, especially in those that received chemotherapy but the difference is not significant ($p= 0.64$ for cancer-specific survival). There was not significant variation in the curves between the chemotherapy and non-chemotherapy group to suggest an interaction i.e. no subgroup showed proportionately greater survival advantage to indicate a potential target group based on gender or site.

Each subgroup category was then examined separately to ensure all groups benefited from chemotherapy (Figure 66). Survival curves suggest a survival advantage in all subgroups, but significance is only reached in men with distal lesions ($p= 0.006$). Sub

grouping to this extent led to small numbers and lack of power. When adjusted analysis was performed (Table 96), a significant benefit (lower HR) was observed in all subgroups upon receiving chemotherapy. Most importantly, on interaction testing there was no significant compounding effect of either gender or site on the improvement in outcome seen with chemotherapy, cancer-specific survival (gender p= 0.81 and site p= 0.80) nor overall survival (gender p= 0.67 and site p= 0.97).

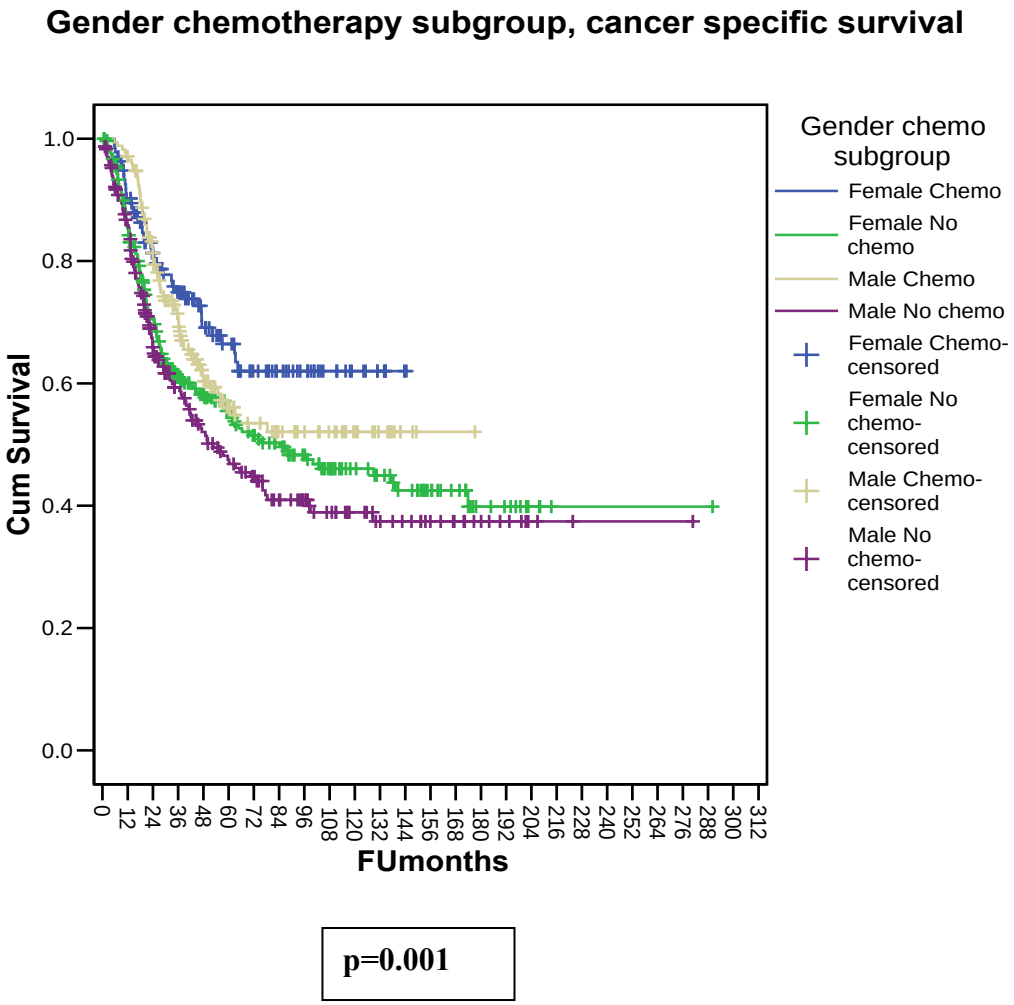
Thus, no subgroup based on gender, site or combinations can be shown to be gaining greater benefit than another. Consequently, these factors cannot be used to select a responsive subgroup.

Figure 54 Effect of gender on chemotherapy survival response



p= <0.0001

Figure 55 Effect of gender on chemotherapy cancer-specific survival response



*(significant across the curves but this compares chemo with no chemo, not a difference between gender curves)

Table 95 5-year overall survival according to gender site and chemotherapy

	Chemo	Non Chemo
Proximal	57%	41%
Distal	57%	39%
Women	62%	42%
Men	53%	37%
Proximal/Women	62%	42%
Proximal/Men	52%	40%
Distal/Women	63%	43%
Distal/Men	54%	36%

From Kaplan-Meier life tables (unadjusted)

Table 96 Chemotherapy effect on site gender subgroups

	HR	95% CI	p
Men Proximal	0.47	0.25 - 0.87	0.02
Men Distal	0.61	0.39 - 0.97	0.04
Women Proximal	0.43	0.24 - 0.78	0.005
Women Distal	0.40	0.24 - 0.68	0.001

Cox regression analysis all factors included, backward elimination by likelihood ratio

Figure 56 Chemotherapy cohort, influence of gender on overall survival

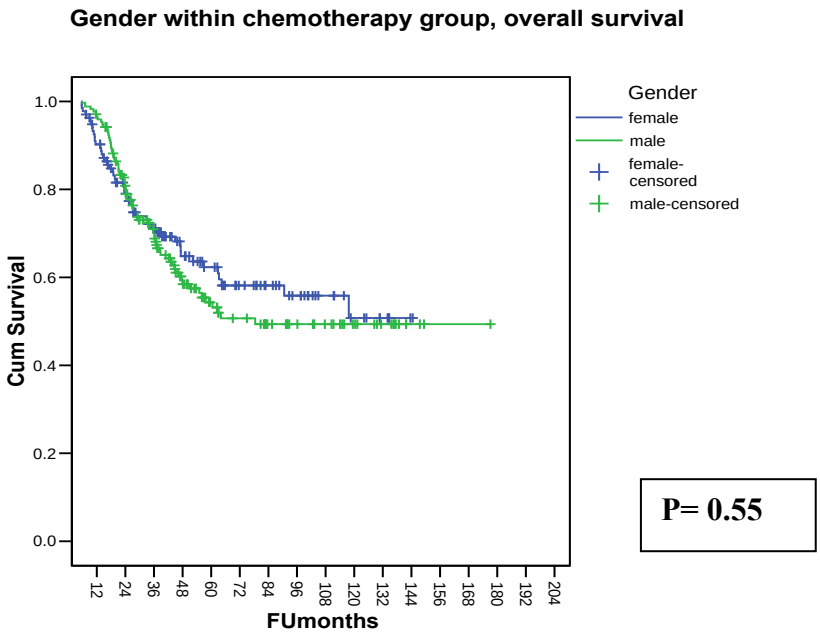


Figure 57 Non-chemotherapy cohort influence of gender on overall survival

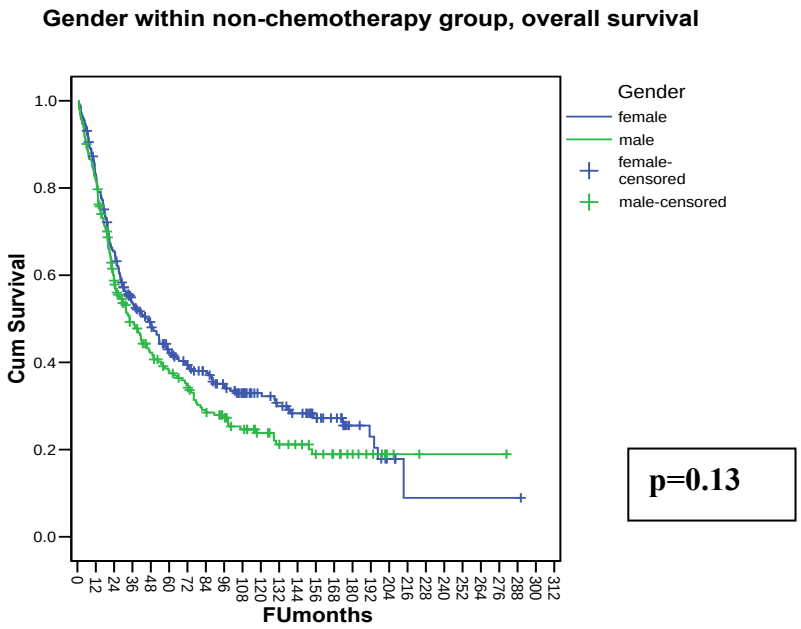


Figure 58 Chemotherapy cohort, influence of gender on cancer-specific survival

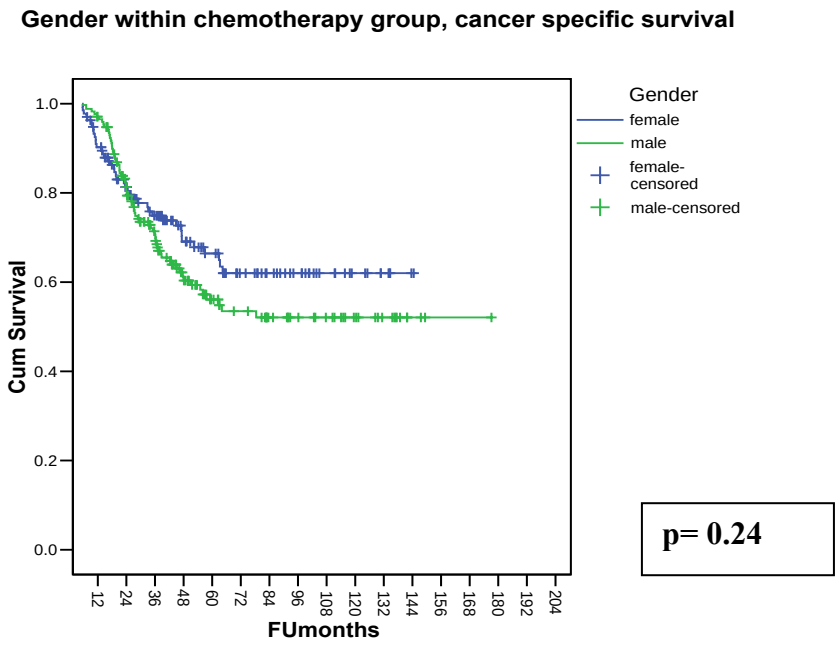


Figure 59 Non-chemotherapy cohort influence of gender on cancer-specific survival

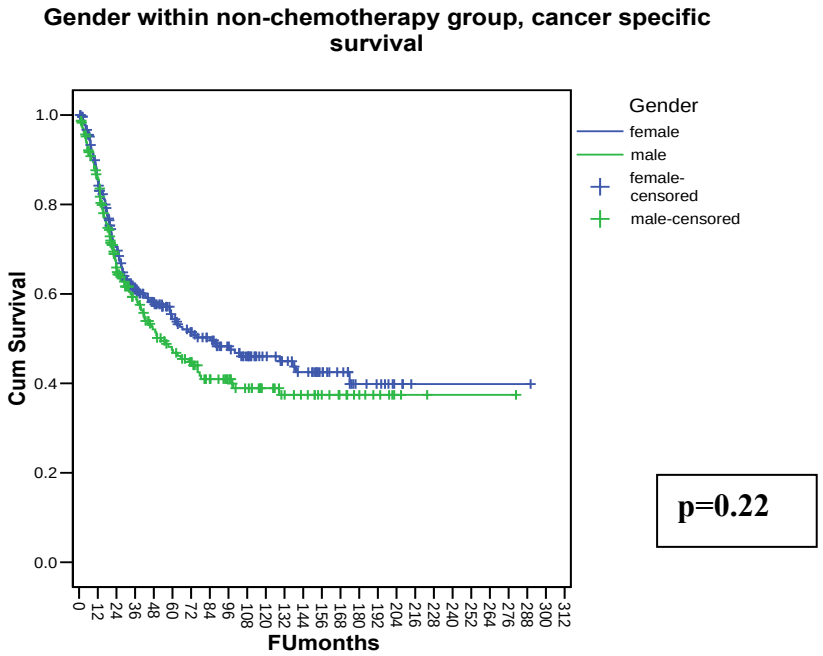
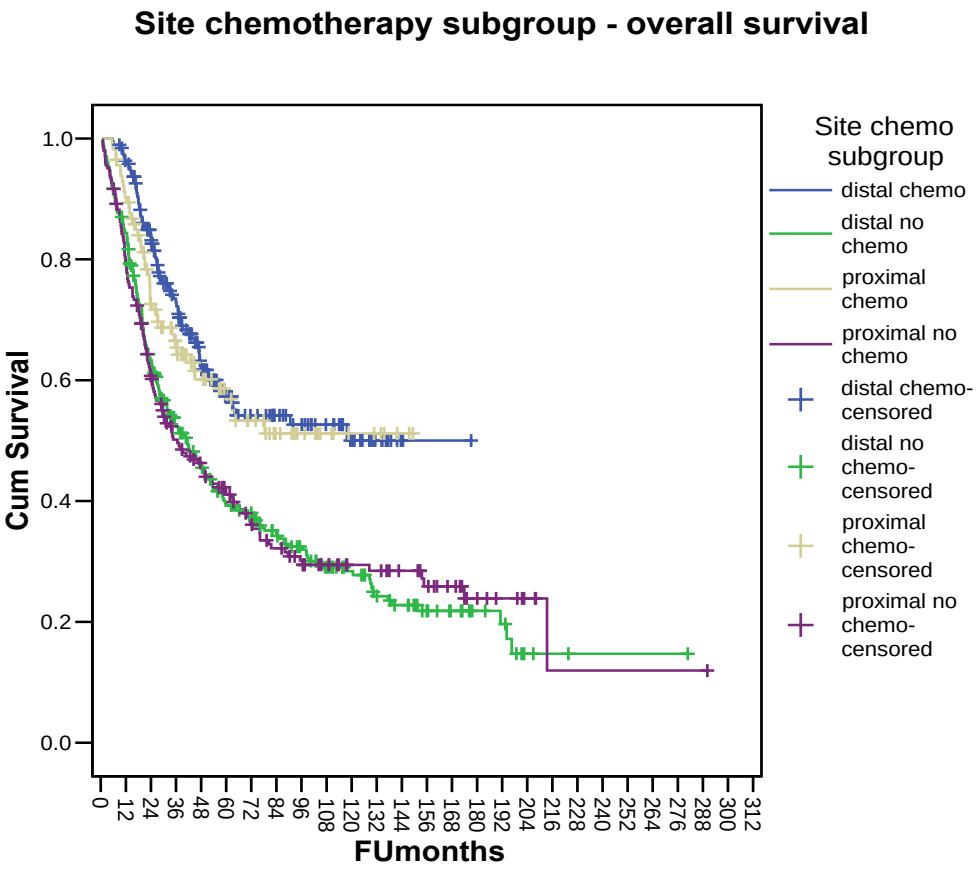
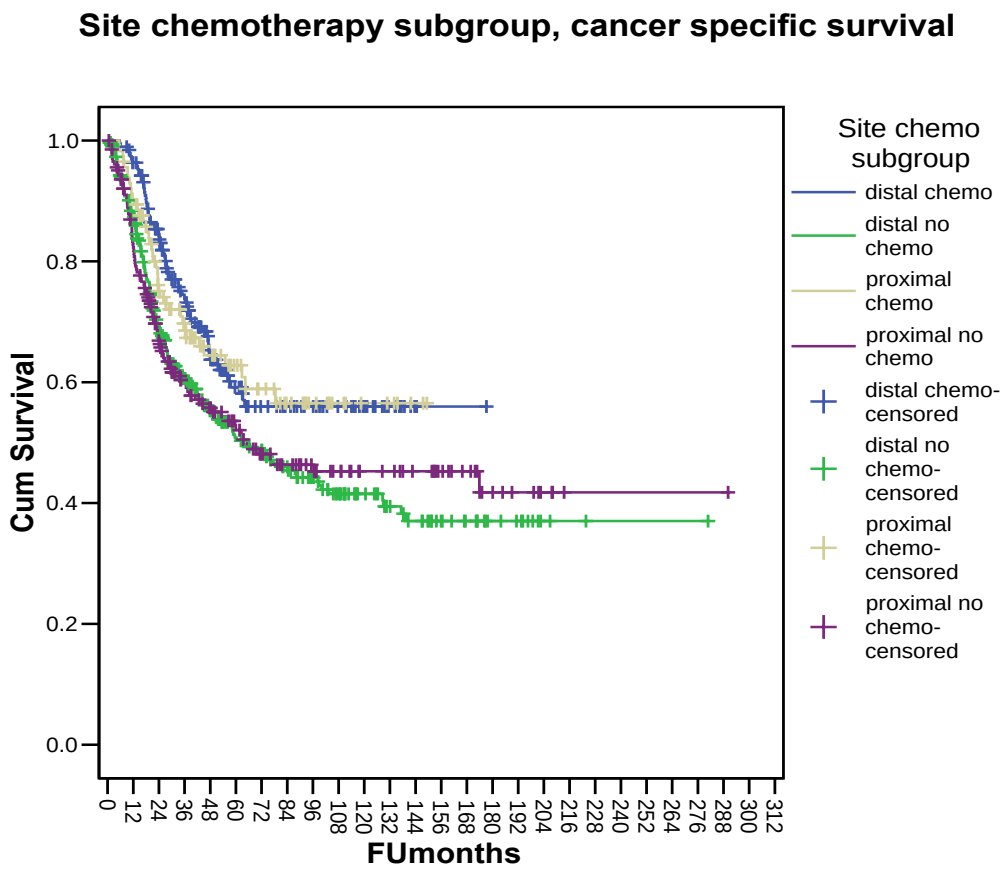


Figure 60 Chemotherapy cohort, influence of site on overall survival



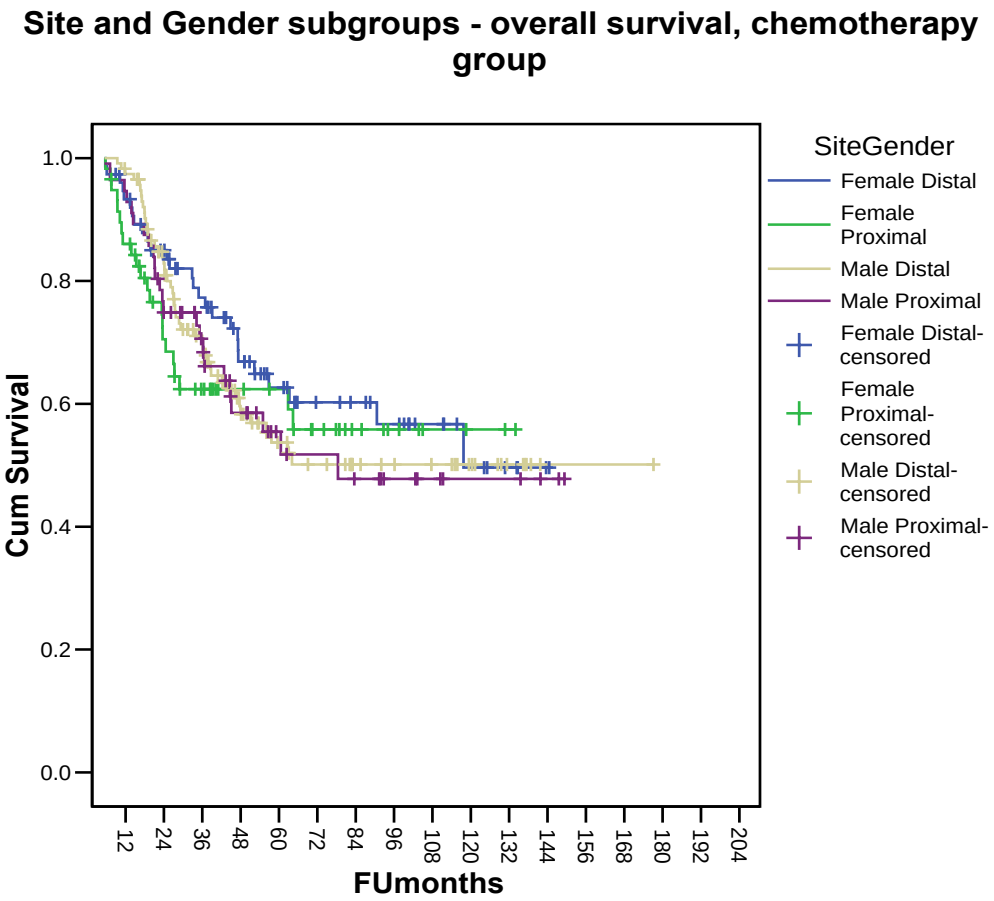
p= <0.0001

Figure 61 Chemotherapy cohort, influence of site on cancer-specific survival



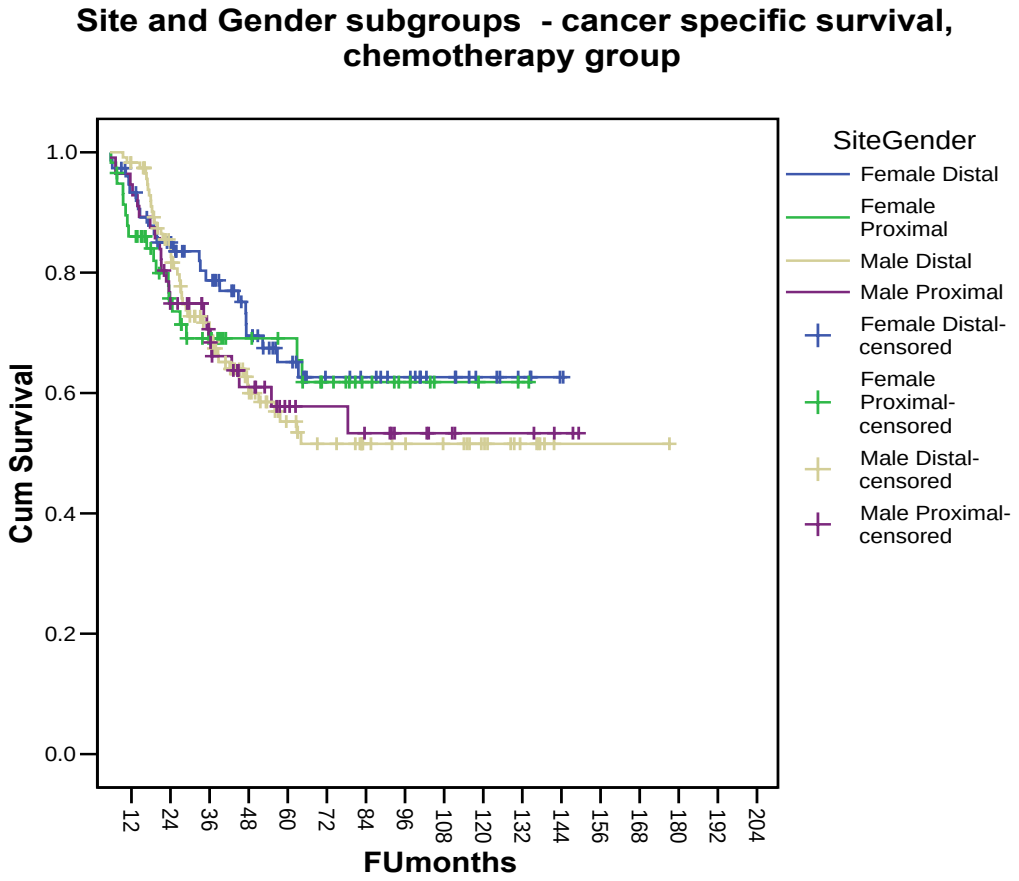
p=0.005

Figure 62 Chemotherapy cohort, influence of gender and site on overall survival



p=0.76

Figure 63 Chemotherapy cohort, influence of gender and site on cancer-specific survival

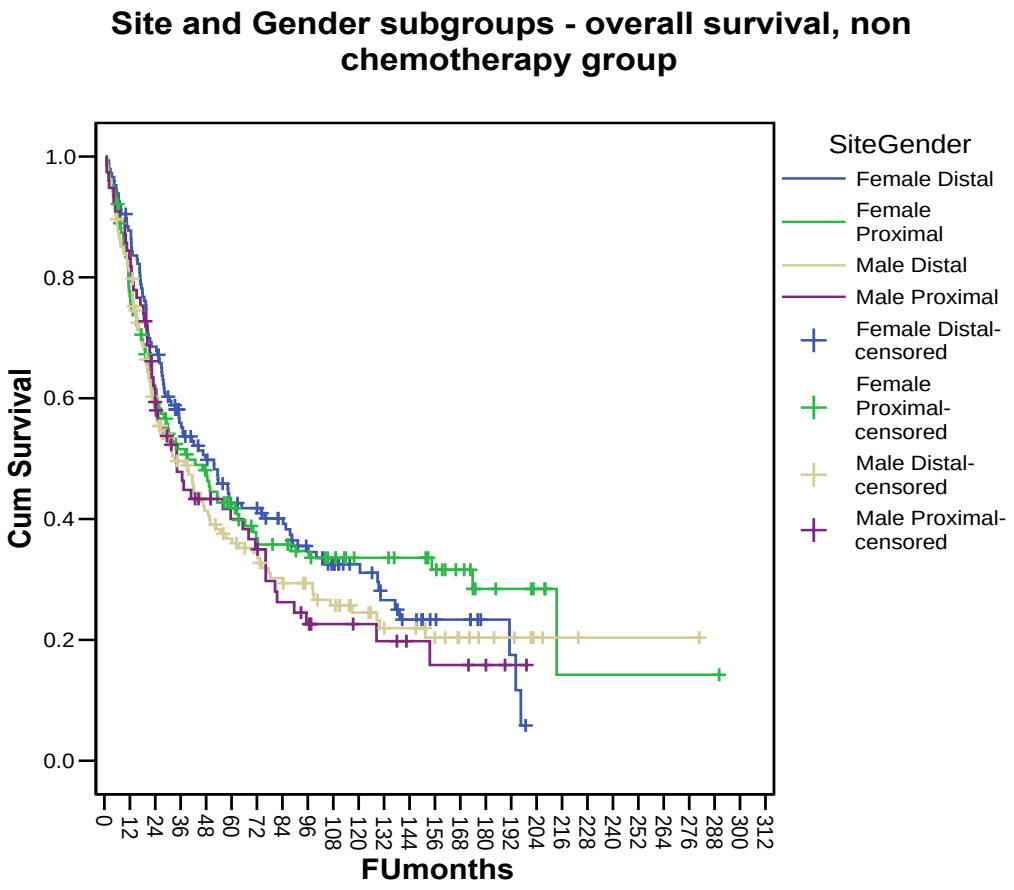


p= 0.64

Numbers at risk

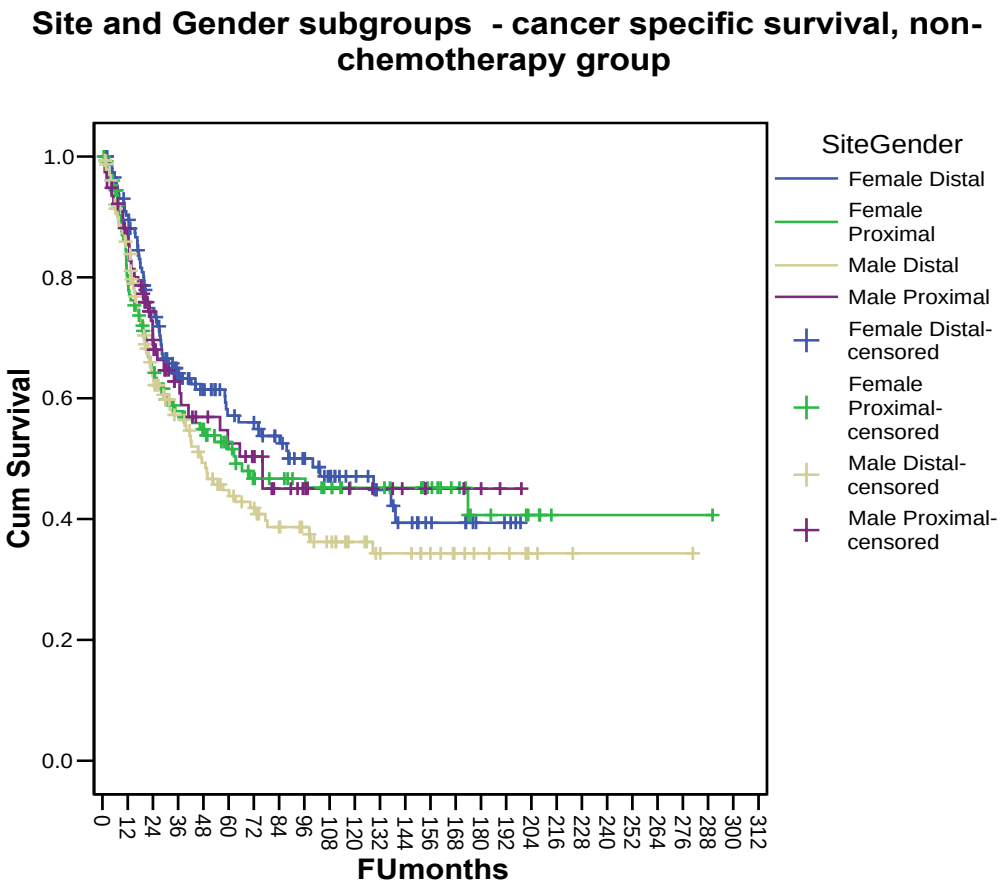
mths	0	30	60	90	120
F/Prox	58	29	19	8	2
F/Dist	76	52	28	17	7
M/Prox	56	37	15	11	4
M/Dist	116	72	32	18	11

Figure 64 Non-chemotherapy cohort, influence of gender and site on overall survival



p= 0.46

Figure 65 Non-chemotherapy cohort, influence of gender and site on cancer specific survival



p=0.30

Figure 66 Chemotherapy effect for men/proximal cancers

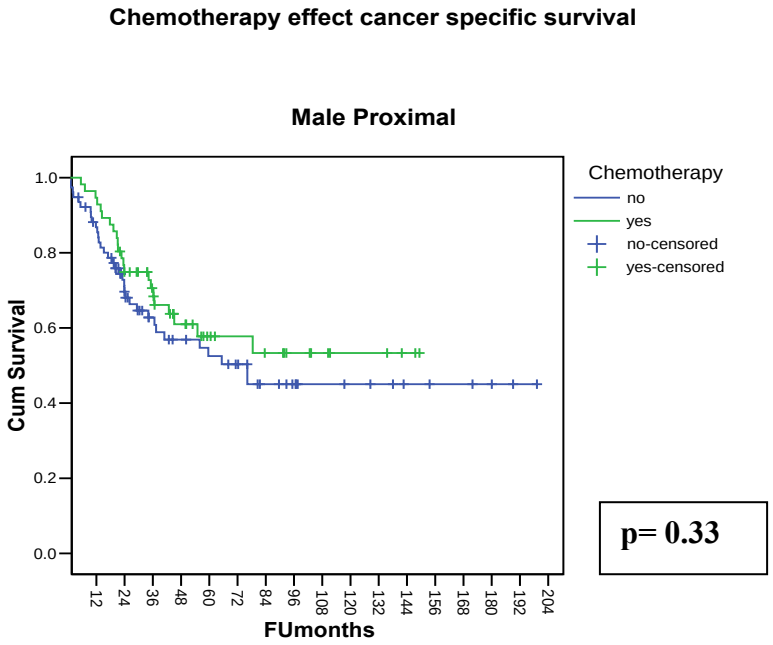


Figure 67 Chemotherapy effect for men/distal cancers

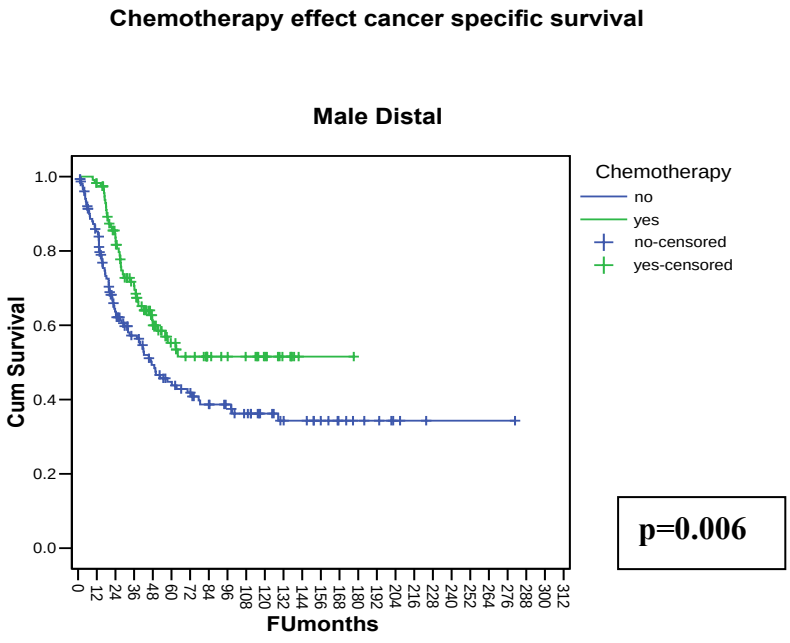


Figure 68 Chemotherapy effect for women/proximal cancers

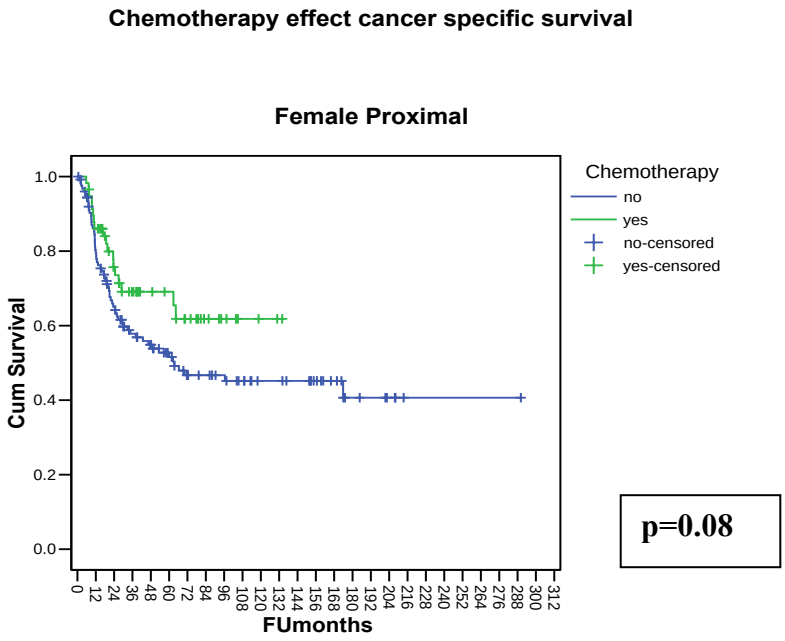
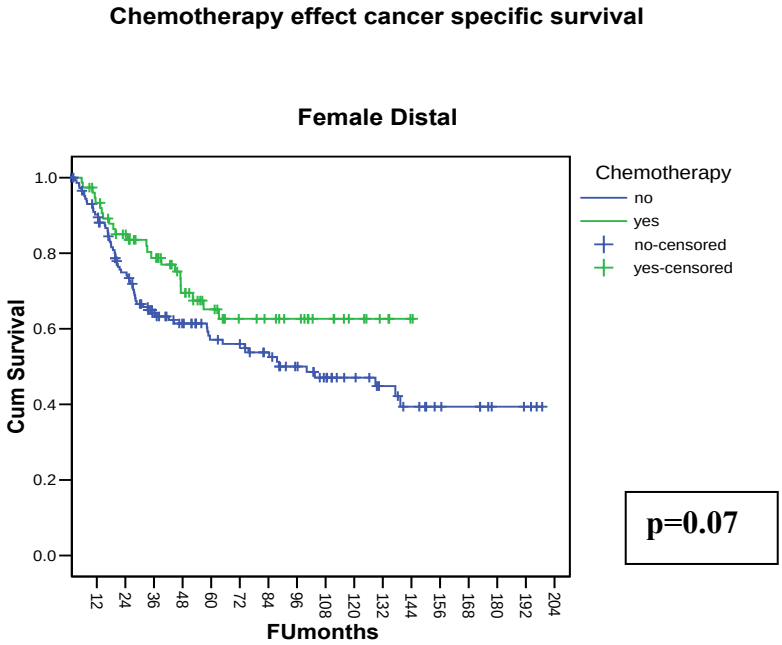


Figure 69 Chemotherapy effect for women/distal cancers



7.5 Pathology Variables

All pathological variables were tested for an interaction with chemotherapy (Table 97). No factor significantly modified the chemotherapy survival effect, suggesting than none of those variables examined could be used to indicate the responsive subgroup. Further analysis was performed to determine if there were any subtle trends that should be pursued further. The group who succumbed to cancer were compared to the alive (and non-cancer deaths) within the chemotherapy cohort to determine what characterised the survivor group (Table 98, Table 99, Table 100). The main difference was the higher rate of poor prognostic indicators in the patients who died of cancer (N2, infiltrating margin, extramural vascular invasion and perforation). This finding raised the question: Was this due to the prognostic influence of these factors only or was the patient group with these factors less likely to benefit from treatment (opposing common assumptions)?

To answer this question, the effect of chemotherapy was examined according to each of these significant variables. On univariate analysis, chemotherapy conferred a significant survival advantage in both nodal stages (N1 and N2), in the patient group with infiltrating margin and when extramural vascular invasion was present. Thus, despite the worse outcome, all these groups were still benefiting from chemotherapy (Figure 70 Figure 71 Figure 72 and Figure 73). This suggests that the over-representation in the group that died of cancer was due to prognostic influences and not due to lack of chemotherapy effect. The number of cases with perforated cancers was too small to draw meaningful conclusion and the observed lack of chemotherapy effect cannot be assumed to be true (Figure 74).

The patient group was then subgrouped into prognostic groups according to pathological factors. Small numbers thwarted this analysis and therefore the two extreme groups were examined. A poor prognostic group was established by the presence of poor differentiation, infiltrating margin and extramural vascular invasion while the good prognosis group had none of these features. Other prognostic factors were excluded so as not to further weaken the power of the analysis. As can be seen in Figure 75 and Figure 76, neither subgroup showed a significant benefit from chemotherapy. However, small numbers especially in the poor prognostic group are likely to have prevented meaningful results (Table 101). There was a trend to a benefit in the good prognosis group countering the assumption that tumours with favourable histology may not benefit from adjuvant therapy.

The above findings are consistent with the results of interaction testing and indicate that no pathological factor is useful in identifying the target group for adjuvant chemotherapy for CRC.

Table 97 Interaction between pathological variables and chemotherapy effect

Interaction	Probability value
Nodal status	0.26
Differentiation	0.77
Type (all)	0.70
Extramural vascular invasion	0.80
Mural vascular invasion	0.54
Perineural invasion	0.87
Infiltrating margin	0.94
Peritumoral lymphocytes	0.25
Perforation	0.22

Bowel wall, Size, Budding, Crohn″s lymphocytes, TILs, stroma and obstruction failed to reach adequate significance to be included in model. Accuracy of model is increased by limiting variables in this way.
Findings were similar for overall survival – no factor showed significance

Table 98 Cancer deaths vs no cancer deaths

Subgroup	Subcategory	Cancer Death	*n*	No cancer death	*n*	p
Median age		66.4 yrs	108	64.3 yrs	199	NS
Gender	Men	62%	67	52.8%	105	NS
	Women	38%	41	47.2%	94	
Site	Proximal	37%	40	37.2%	74	NS
	Distal	63%	68	62.3%	124	
Nodal stage	1	52.8%	57	73.9%	147	<0.0001
	2	47.2%	51	26.1%	52	
Differentiation	Mod	79.6%	86	86.4%	172	NS (p=0.096)
	Poor	20.4%	22	13.1%	26	
Mucinous vs NOS	NOS	60.4%	64	60.7%	119	NS
	Mucinous	39.6%	42	39.3%	77	
Lymphocytes	Peritumoral	92.6%	100	92.0%	183	NS
	Crohn"s	19.4%	21	24.1%	48	NS
	TILs	2.8%	3	4.5%	9	NS
Stroma	a	61.1%	66	64.3%	128	NS
	b	37.0%	40	34.2%	68	
	c	0.9%	1			
Obstructed		19.4%	21	11.1%	22	P=0.04
Perforated		7.4%	8	1.5%	3	P=0.007
Infiltrating margin		49.1%	53	26.1%	52	P=<0.0001
Budding		41.7%	45	36.7%	73	NS
NeuroVascular	Mural	73.1%	79	72.4%	144	NS
invasion	Extramural	75.0%	81	51.8%	103	P=<0.0001
	Perineural	28.7%	31	14.1%	28	P=0.002
MSI pos		9.4%	33	9.8%	44	NS (p= 0.87)

Chi test for significance for categorical data, Mann Whitney for continuous variables

Table 99 Predictors of cancer death within chemotherapy cohort, significant variables

	OR	95% CI OR		p
		Lower	Upper	
Perforation	3.91	1.84	8.34	<0.0001
Infiltrating margin	2.46	1.60	3.77	<0.0001
Extramural	1.79	1.12	2.85	0.02
N Stage (N2)	2.17	1.45	3.24	<0.0001

Cox regression analysis, backward likelihood ratio elimination

Table 100 Predictors of cancer death within chemotherapy cohort, non-significant variables

	p
Gender	0.37
Prox/Dist	0.94
Radiotherapy	0.67
Bowel wall invasion	0.31
Differentiation	0.18
Mucinous type	0.23
Peritumoral lymphocytes	0.95
Crohn"s lymphocytes	0.98
TILs lymphocytes	0.87
Obstruction	0.95
Budding	0.41
Mural vascular invasion	0.52
Perineural invasion	0.18
MSI	0.61

Stroma excluded as too few numbers in C

Figure 70 Chemotherapy effect if N1 nodal status

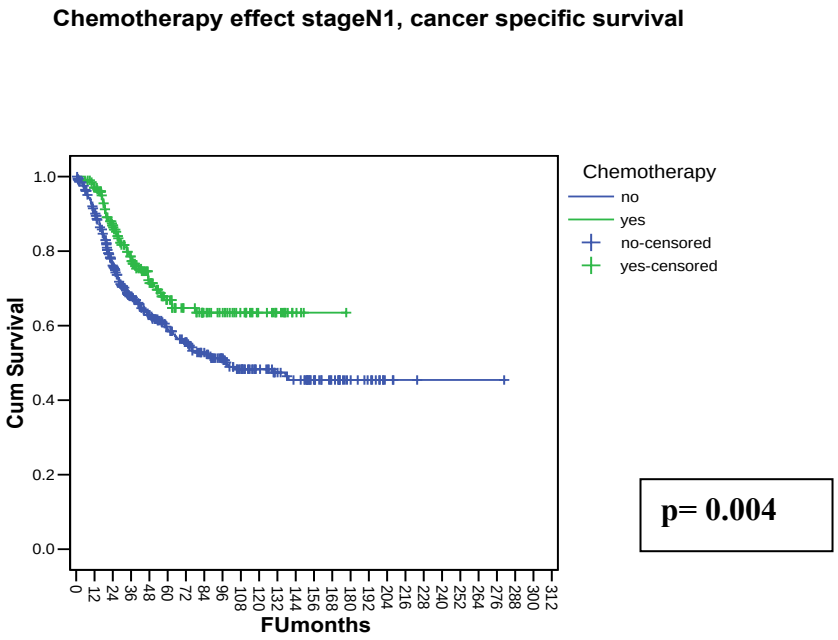


Figure 71 Chemotherapy effect if N2 nodal status

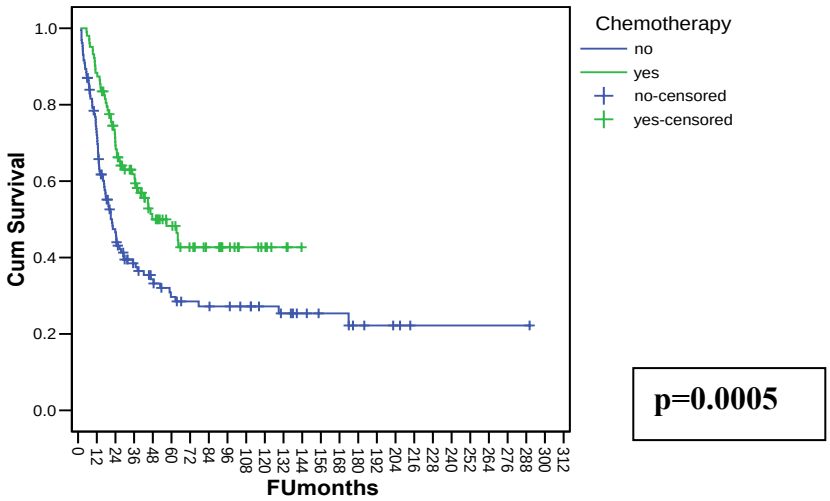


Figure 72 Chemotherapy effect according to margin

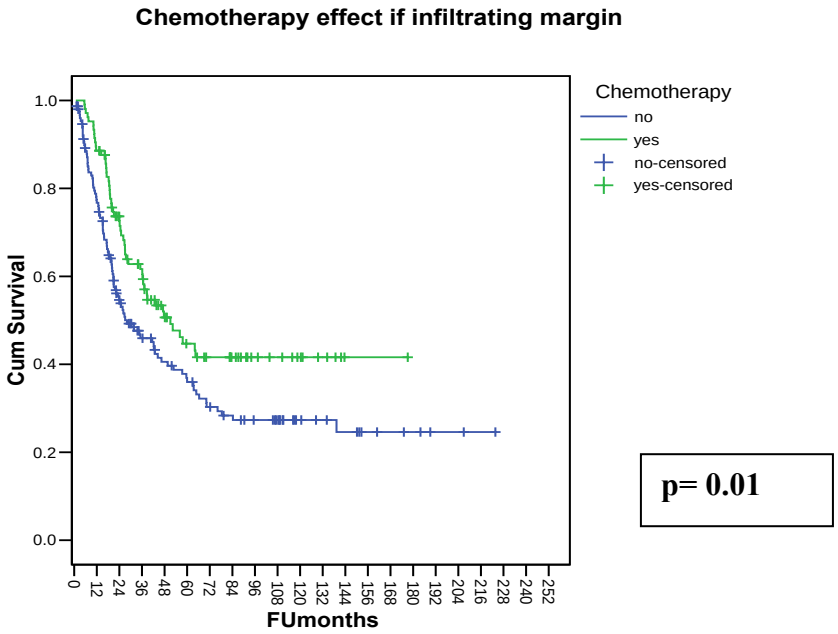


Figure 73 Chemotherapy effect if extravascular invasion

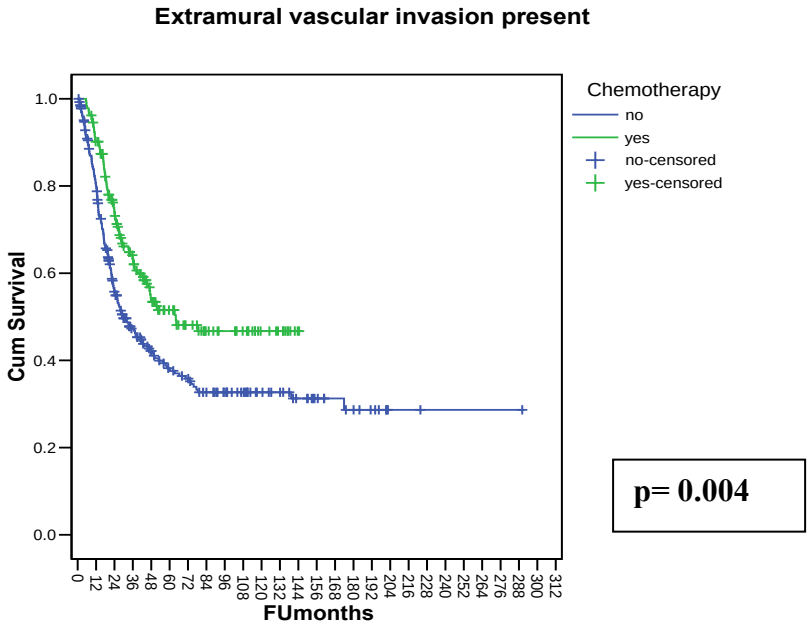
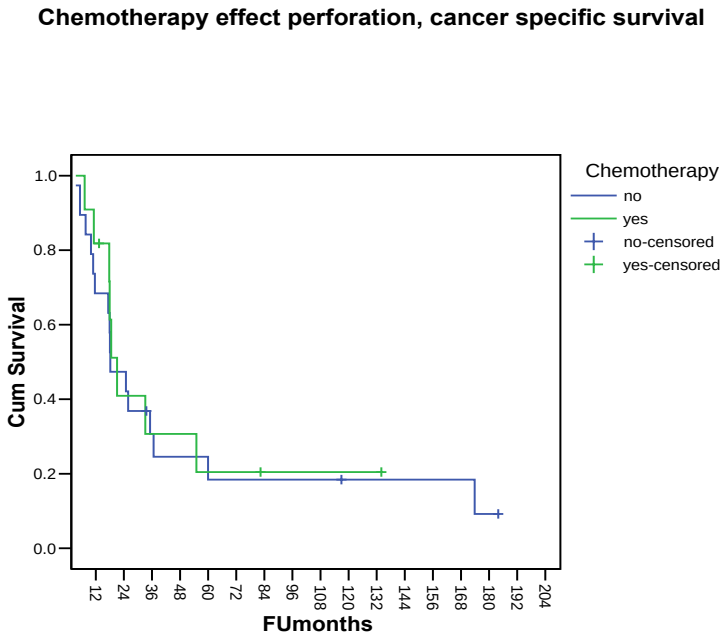


Figure 74 Chemotherapy effect if perforation



p=0.80

Numbers at risk

Months	0	30	60	90	120	150
No Chemo	19	6	3	2	2	
Chemo	10	4	1	1		

Figure 75 Prognostic groups cancer-specific survival –good prognosis

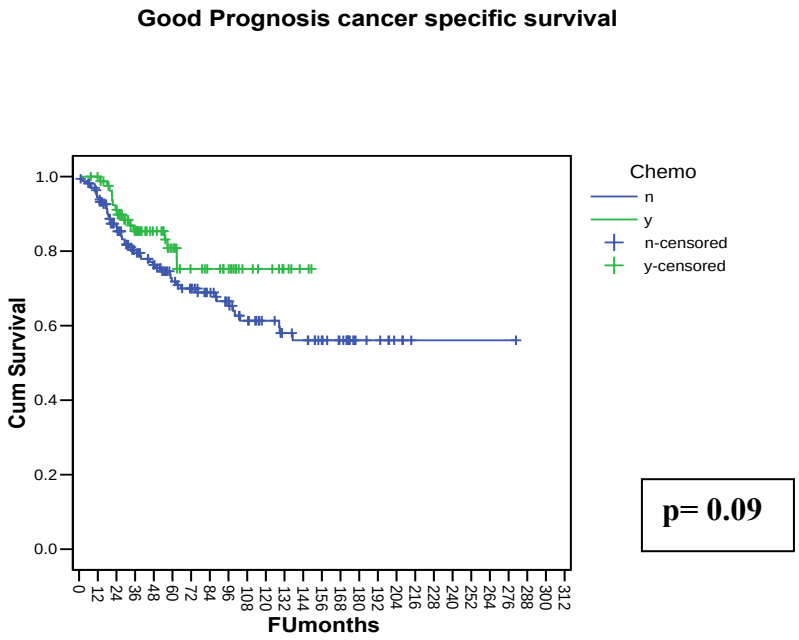


Figure 76 Prognostic groups cancer-specific survival –poor prognosis

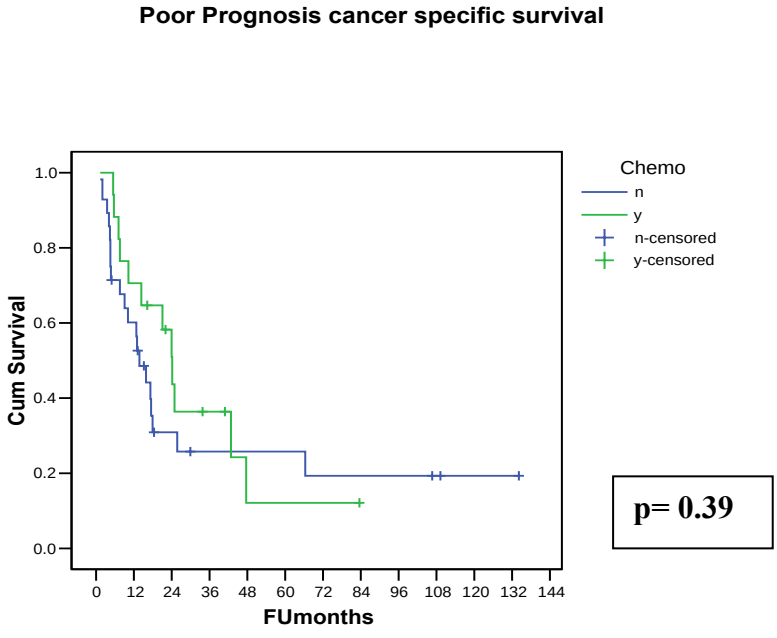


Table 101 Prognostic subgroup numbers

		Chemo		Total
		n	y	
Prognosis	good	167	83	250
	poor	28	17	45

7.5.1 MSI

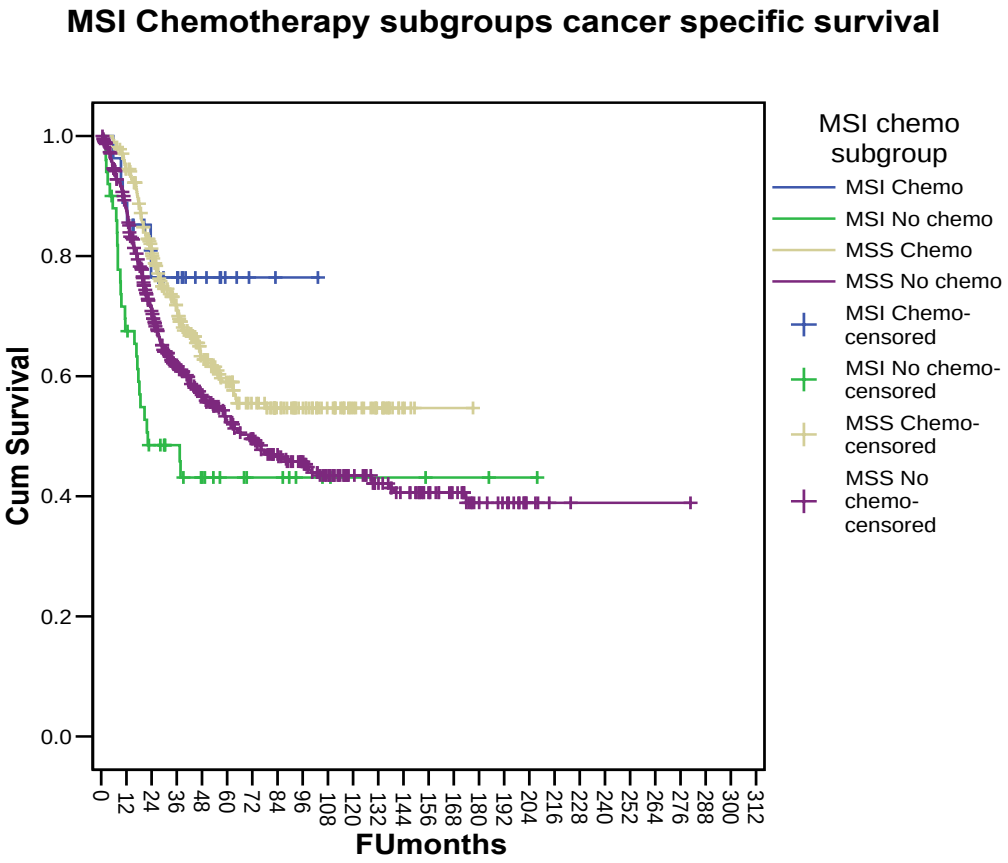
On unadjusted subgroup analysis, combined survival curves (Figure 77) indicate that the MSI cases has a similar outcome to the MSS in the non-chemotherapy cohort but appear to have a better outcome in the chemotherapy group suggesting there may be a greater benefit from chemotherapy. The cohorts were separated to explore this observation. In those that did not receive chemotherapy, the MSI group showed a trend to a poorer outcome compared to the MSS cases (p= 0.056) (Figure 78). Examination of only those who received chemotherapy however, shows that the apparent difference between the curves is not significant (Figure 79). There is no survival benefit of MSI cases over MSS in those that received chemotherapy. Furthermore, when chemotherapy effect is tested in either MSI or MSS (Figure 80 and Figure 81), it can be seen that both derive a significant benefit from treatment.

Unadjusted 5-year survivals determined from Kaplan-Meier life tables (Table 102) show the same trend as above. Both MSI and MSS are benefiting from chemotherapy. MSI confers a worse survival in the non-chemotherapy cohort, but has a similar survival to MSS in the chemotherapy cohort. This hints at a greater benefit from treatment. On adjusted analysis, these findings are upheld (Table 103). In an analysis of all patients (chemo and non-chemo), MSI cases did not have a significantly worse outcome (HR 1.45 ns) but in the non-chemotherapy cohort, the trend to worse outcome in the MSI cases reached significance (HR 1.89, 95%CI 1.13-3.16; p= 0.015). In contrast, MSI status did not influence outcome in the chemotherapy cohort (though interestingly they trended towards a better outcome, HR 0.62 (95% CI 0.22-1.72; p=0.36).

In the second part of this analysis (Table 103) it can be seen that chemotherapy improved survival in both the MSI (HR 0.08 95% CI 0.02-0.27; p=<0.0001) and the MSS group (HR 0.62, 95% CI 0.47-0.81; p=0.001). Both groups gained benefit (though seemingly more in the MSI group). However, on interaction testing, MSI status did not modify the survival benefit from chemotherapy (cancer-specific p= 0.08, overall survival p=0.49). Of note, MSI was the factor that came closest to significance.

In summary, while there appeared to be a trend towards a more marked chemotherapy effect in the MSI group, the interaction is not significant and thus MSI cases do not indicate the target subgroup for adjuvant chemotherapy, nor can it be said that chemotherapy in MSI cases is detrimental. Both MSS and MSI cases benefit and on the basis of these results neither can be excluded from treatment.

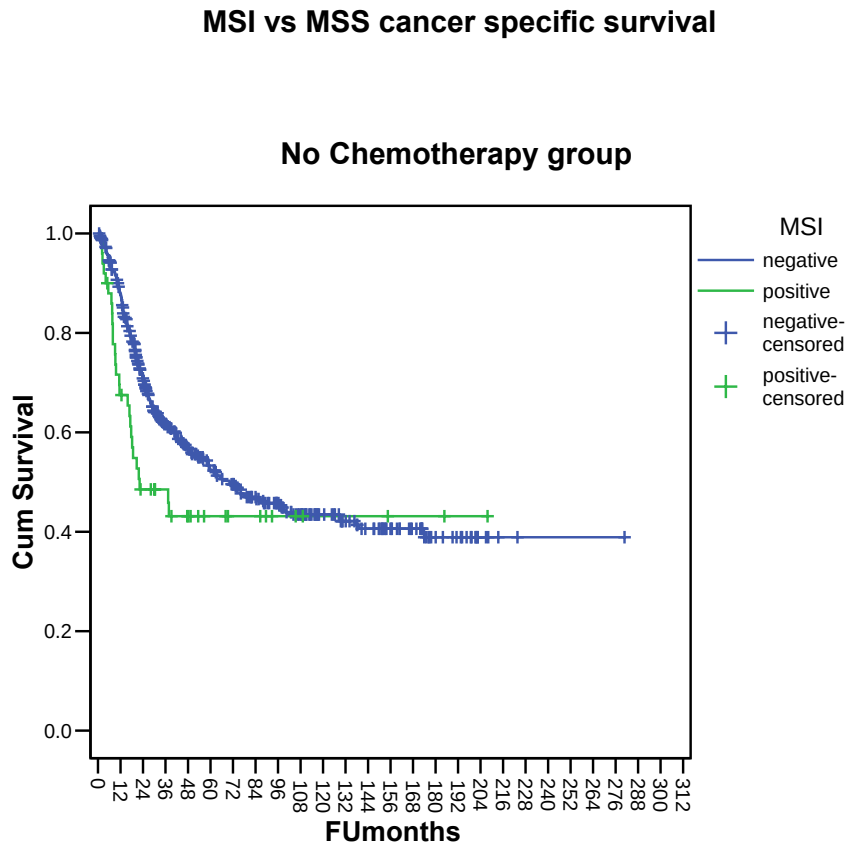
Figure 77 Cancer-specific survival according to MSI chemotherapy subgroups



p= 0.008*

*chemotherapy effect, not variation between MSI and MSS

Figure 78 Survival MSI vs MSS groups within non-chemotherapy cohort

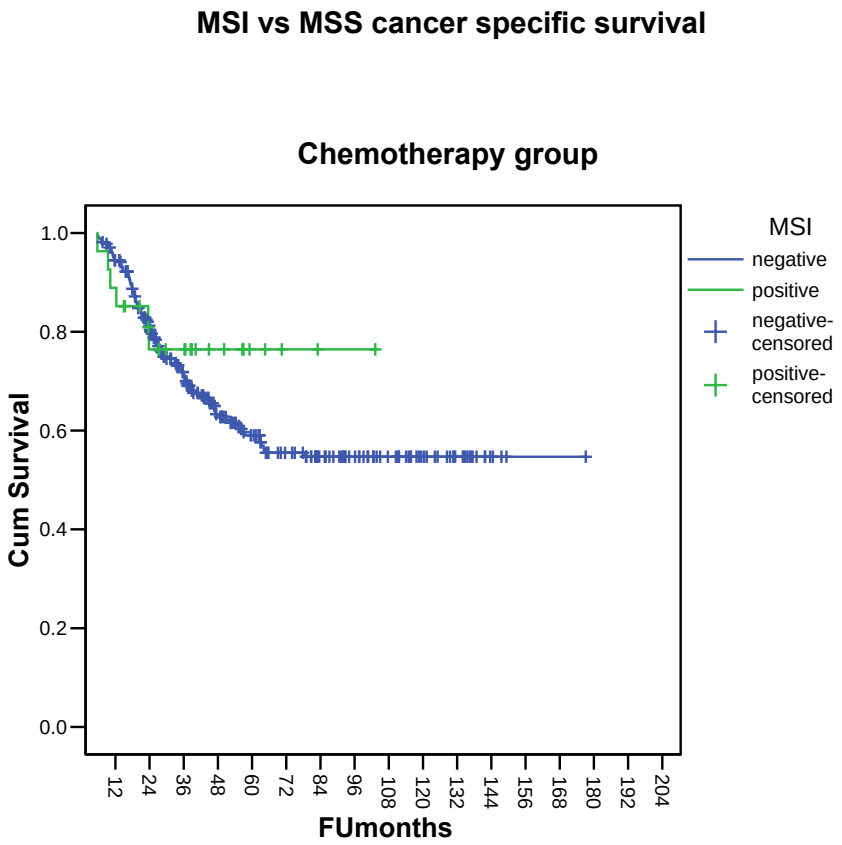


p= 0.06

Number remaining

mths	12	24	36	48	60	72	84	96	108	120	132	144	156	168	180	192
MSI	33	22	18	14	10	8	7	5	4	3	3	3	2	2	2	1
MSS	377	280	215	184	156	141	120	105	87	70	59	50	38	30	18	14

Figure 79 Survival MSI vs MSS groups within chemotherapy cohort

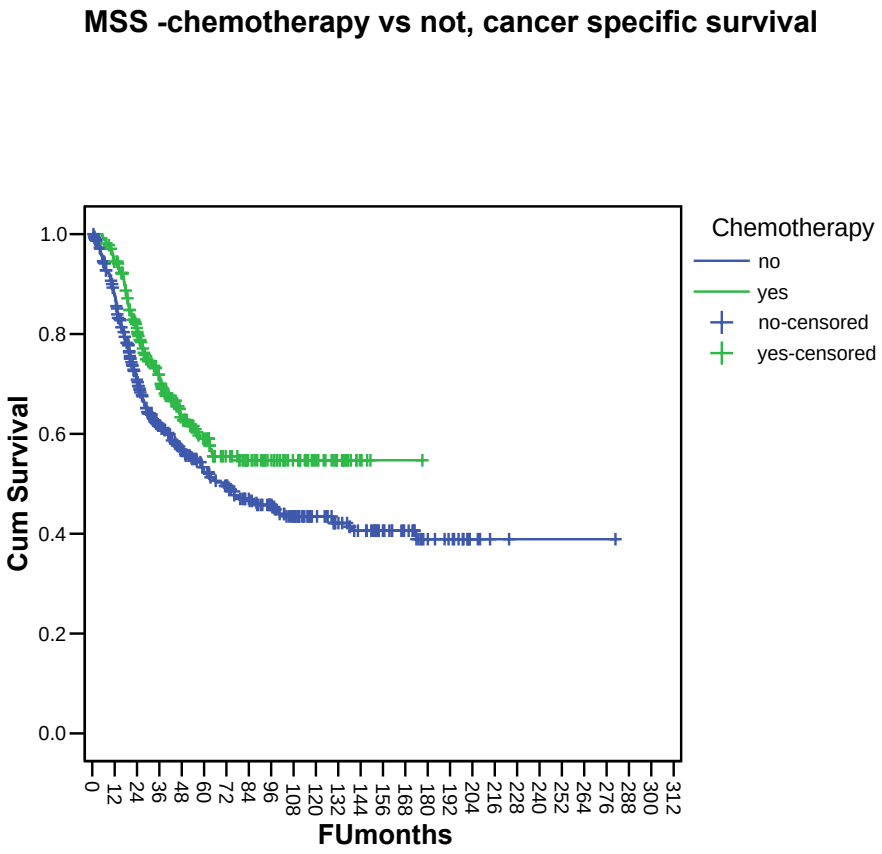


p= 0.39

Numbers remaining

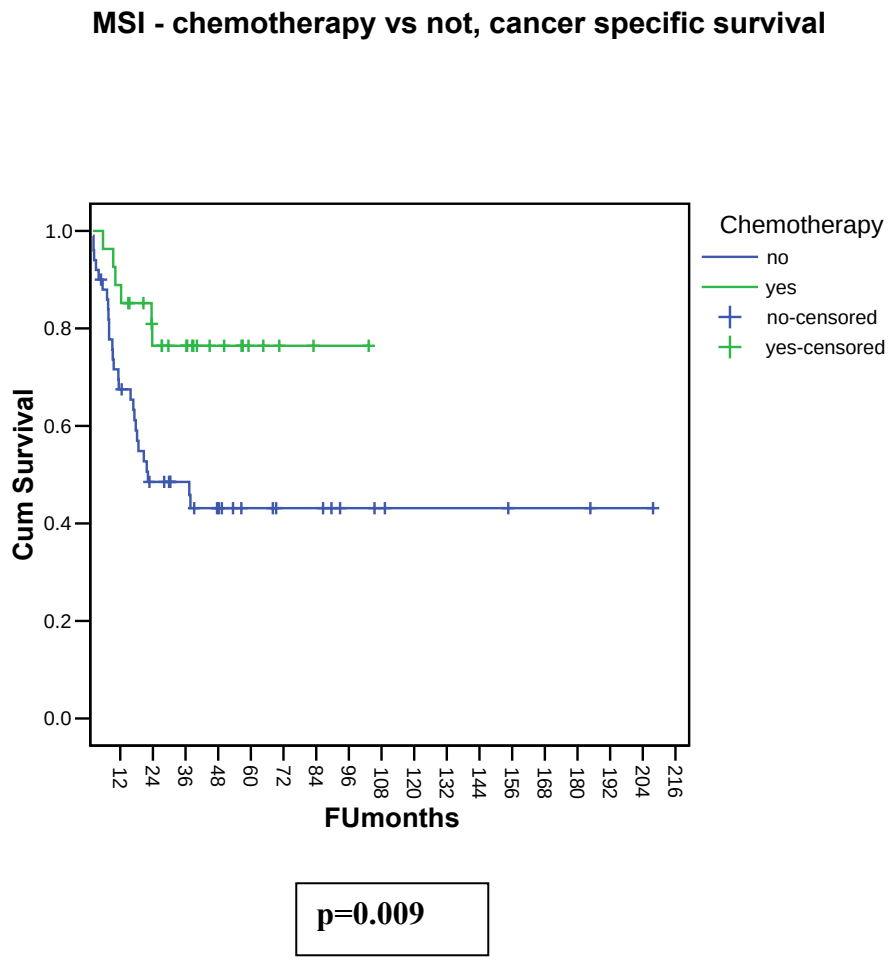
mths	12	24	36	48	60	72	84	96	108	120	132	144
MSI	24	17	14	8	4	2	1	1	0			
MSS	253	199	155	112	90	72	57	46	35	24	15	4

Figure 80 Chemotherapy effect within MSS group



p= 0.005

Figure 81 Chemotherapy effect within MSI group



Numbers remaining

mths	12	24	36	48	60	72	84	96	108	120	132	144	156	168	180	192
MSI	24	17	14	8	4	2	1									
MSS	33	22	18	14	10	8	8	5	4	3	3	3	2	2	2	1

Table 102 5-year overall survival depending on MSI and chemotherapy

	Chemo	No Chemo
MSI	65%	26%
MSS	56%	42%

Significance not stated as more accurately determined by logrank testing of survival curves rather than this single time point

Table 103 Adjusted analysis for MSI effect on chemotherapy survival response

		HR for death	CI	p
According to MSI (MSI vs MSS)	All patients	1.45	0.92-2.26	0.11
	No Chemo	1.89	1.13-3.16	0.02
	Chemo	0.62	0.22-1.72	0.36
According to chemo (chemo vs none)	All pt	0.58	0.44-0.75	<0.0001
	MSS	0.62	0.47-0.81	0.001
	MSI	0.08	0.02-0.27	<0.0001

Multivariate analysis adjusted for all factors

7.6 Discussion

7.6.1 Gender and Site

Neither gender nor site (or combination of subgroups) was shown to significantly influence response to chemotherapy. No one group gained greater benefit and all subgroups showed benefit. This contrasts with the only other sizable work specifically examining the influence of these factors. Elsaleh et al. showed that proximal cancers and women (and a combination of) benefited the most from adjuvant chemotherapy and that men with distal lesion did not derive any benefit [21]. Their results were based solely on unadjusted analysis and therefore do not take into account confounding factors. As gender and site group matching is not detailed, findings cannot be fully interpreted. There was selection bias in those that received chemotherapy cohort being significantly younger and having a male predominance. Without adjustment for these discrepancies, findings should be interpreted with caution. When we performed the equivalent analysis on our dataset (and our chemotherapy cohort is skewed the same way as their study), our results still vary. Men with distal lesions still gained significant benefit from chemotherapy.

The validity of subgroup analysis is sometimes questioned but defended by a priori determination of subgroup categories, as occurred in this study, and recognition of power limitations. The numbers within each combination subgroup were small, especially within the chemotherapy cohort, and as such our study was underpowered for this subgroup analysis. However, without even a trend towards a compounding effect with chemotherapy, it is very unlikely that larger numbers would lead to a significant finding.

Even though it was determined that these factors are not useful indices in identifying the target group for chemotherapy, it was hoped that a trend may be identified and, while not allowing one group to be excluded from treatment, may guide further research. However, there is little in our findings to suggest these indices (or related factors) are worth pursuing.

7.6.2 Pathological Factors

None of the histological features we investigated signified the responsive group for chemotherapy. As no one histological trait is indicative of a specific biology or subgroup, it was unlikely that any one factor would be useful. Unfortunately, categorizing data further based on combinations of factors was of limited use due to lack of power.

It is often assumed that cancers predicted to have a worse outcome (based on the presence of unfavourable histology as indicated by poor prognostic factors) would have more to benefit from chemotherapy. There have been no studies to support this assumption. Our work found that the patient group with poor prognostic features had a worse outcome as expected and did derive benefit from chemotherapy but not necessarily greater than those with good prognosis. As chemotherapy is offered on the basis of lymph node status and not pathological features, these findings will not have an impact on the management of stage C disease. However the above assumption is often extended to stage B cases. It would be inappropriate to use our results to predict the influence of poor prognostic factors on the effectiveness of chemotherapy in lymph node negative disease. However, they do question the assumptions made in treating stage B cases and should prompt prospective validation.

Without even a trend in our results, further investigation to identify markers for a target group for chemotherapy based on histological measures cannot even be narrowed or guided.

7.6.3 MSI

In the previous chapter it was demonstrated that MSI did not significantly affect prognosis in this cohort of Stage C cases. When the group was divided on the basis of chemotherapy and the influence of MSI was re-examined, it emerged that for those who received chemotherapy MSI status did not significantly affect outcome. However, in those that did not receive chemotherapy, the MSI cohort had a worse outcome. It is possible we sampled a more aggressive subgroup of MSI cancers by selecting lymph node positive disease, given the usual propensity for MSI tumours to remain localized. As discussed in the prognosis chapter, variation in inclusion criteria (particularly stage) may therefore explain why our findings differ from studies that found MSI cancer had a better prognosis than MSS, with or without chemo.

We found both MSI and MSS cancer groups derive survival benefit from 5FU-based chemotherapy but that the effect is not greater in one or the other. There may have been a trend toward a differentially better response in MSI but this could not be confirmed. Significance was not reached on specific testing for an interaction, though MSI status was closer than the other examined factors.

Unfortunately the study numbers for this analysis were less than anticipated in the power calculations. Fewer cases than expected were available for inclusion. This was partly due to registries‟ limitations but also because data from a fourth hospital was

unavailable, despite clinician best intentions, because of ethical privacy issues. This, combined with a lower than expected rate of MSI (as stage C only), contributed to the shortfall. Thus, despite having over 800 cases, lack of power in the MSI analysis may have contributed to a type 2 error and prevented significance being reached. For this reason, it was felt the observed trend was worthy of note.

Our findings vary from the main two previous (opposing) works that demonstrate an influence of MSI on chemotherapy effect. Elsaleh et al. found that MSI predicted a marked improvement in 5-year survival with chemotherapy (37% without treatment vs 90% with chemotherapy) whereas the MSS group did not benefit (32% vs 35%) [21]. While there were methodological deficiencies (the analysis was unadjusted and groups not matched, as discussed in the site and gender section), the difference is so great it is difficult to imagine that confounding factors could have been sufficiently disproportionate to account for these findings, especially given the marked discrepancy between the two groups (the majority of patients derived no benefit from chemotherapy; essentially all the responders were in the MSI group).

In the Elsaleh et al. study, the number in the MSI group that received chemotherapy was small and in this group only one event was recorded (only one patient died of cancer). This may have contributed to the discrepancy in the findings. Using 5-year survival as the endpoint may also have produced misleading results. When we looked at the unadjusted 5-year survivals, the difference between MSI and MSS did appear more dramatic (chemo vs non-chemo in the MSI group was 65% vs 26%, MSS 56% vs 42%). However we know from logrank testing across the survival curves that this is not significant, highlighting the lack of reliability of using a point in time for analysis. Furthermore, the survival curves only confirm the better survival of the MSI

cases compared to the MSS in those given chemotherapy (similar to the difference observed in the whole group) not that the chemotherapy effect was greater in the MSI cohort.

Thus, the trend observed in our results is similar in direction to the findings of the Elsaleh et al. study and whilst it is possible that MSI cases may have a greater benefit from adjuvant chemotherapy, we disagree that they are the only group to benefit and we cannot support a lack of benefit in MSS cases.

Ribic et al. on the other hand conclude that MSI cases do worse with chemotherapy whereas MSS cases still benefit [48]. Their five-year unadjusted survivals (of stage B and C cases) show MSI without treatment was 75.5% while with treatment was 70.7%. MSS survival improved from 68.4% to 88% with chemotherapy. However, this difference between the chemotherapy group and non-chemotherapy group was not significant, despite the apparent worse outcome in MSI cases treated with chemotherapy. Again this part of their analysis is unadjusted as well as a point in time measure and may be deceptive.

The hazard ratio of dying was calculated for chemotherapy effect in the MSI and MSS group. No survival advantage with adjuvant therapy was observed across the whole patient group, which is most likely due to inclusion of stage B as well as stage C cases. On subgrouping, the HR within the MSI group given chemotherapy showed a trend towards a worse outcome but was not significant (HR dying 2.14 95% CI 0.83-5.49 ns), suggesting that chemotherapy may be detrimental in MSI cases. The above trend is observed in a breakdown based on stage (stage B and C subgroups) but again neither was significant and the confidence intervals were very wide.

Specific interaction testing did reach significance (p=0.02) indicating that there was a compounding effect of MSI and chemotherapy on survival and given the above trend, this would imply the MSI cases have less survival benefit compared to MSS cases. However, it cannot be concluded that there is no benefit or indeed a deleterious effect of treatment, despite their stated conclusions.

Essentially, both this study and Ribic et al."s work find that MSI status does not significantly influence chemotherapy effect. However, both produced results with a discernable trend but in opposite directions. The studies are similar in size and methodology, with robust procedure and analyses. There are however several differences that may have influenced these divergent conclusions.

Ribic et al. included stage B and C cases whereas we included only stage C. This may account for their finding of significant prognostic effect of MSI without treatment against our finding of worse outcome in MSI. There is no obvious reason why this would have skewed an interaction between MSI and chemotherapy, especially as their findings persisted following adjustment for stage.

The inclusion criteria were otherwise similar though the patient group varied slightly between the studies. Patient demography is likely to be similar as both studies drew from a largely Caucasian population. The Ribic et al. patient group was markedly younger (median age 59.8 years against our 71.1 years). This may be explained by case selection from other trials, although most did not have entry age criteria. Adjustment was not made for age in their analysis, although the MSI and MSS group were matched in median age. Chemotherapy age matching was not stated but the study was randomised so should have been comparable. Our analysis was age

adjusted and used cancer-specific survival as an endpoint, which is less age dependent. For these reasons, while age may have varied, it is unlikely to account for the variation in findings. Whether the influence in young stage B cases varies from older stage C patients sufficiently to explain findings is debatable.

The Ribic et al. study group used previously randomised patients whereas our study was retrospective and non-randomised and therefore had potential selection bias. As highlighted previously, our group matching shows variation between the chemotherapy and non-chemotherapy cohorts in regards to gender, age and histology. However these factors were adjusted for in analysis and as such, any discrepancies should have been corrected. Cancer-specific survival was used as an endpoint, which minimizes the influence of age and co-morbidities (factors considered in selection for chemotherapy). We did not include other potential prognostic indicators due to concern regarding accuracy of retrospective collation (i.e. surgeon, co-morbidities) or because of lack of recording (apical node). However, these factors have not been found to have a large influence on outcome. It is conceivable there are yet to be identified influential factors we have not considered.

The Ribic et al. analysis only adjusted for stage and grade and the matching of other prognostic variables across the groups is not stated. It is possible the MSI cohort had disproportionate rates of some influential factors. However this would not explain the different prognostic direction in the non-chemotherapy and chemotherapy groups (if there were more poor prognostic features, both groups would have done worse).

There were some methodological differences in particular regarding the endpoints used and follow-up. Ribic et al. examined overall survival compared to our cancer-

specific survival. Without adjustment for other factors (such as age and gender) this may have skewed their results. It is unclear how perioperative deaths are dealt with. Our median follow-up time is shorter, reflecting the increased number of deaths in our study (as all were more advanced stage). Median follow-up of the alive-patients is closer to Ribic‟s median follow-up. There is no mention of how pathology was assessed or if there was independent evaluation. As is clear from our work, pathology reporting and registry data can be unreliable. While it is assumed that all cases were curative given the usual trial entry criteria, this is not stated. These factors could have influenced the results.

Thus, while there are differences in the studies, none adequately explains the variation in our findings and it cannot be concluded that one is necessarily more valid than the other. We feel that the current study, while having some deficiencies (power and retrospective) is a thorough examination of the issue, with appropriate adjustment and robust statistics and that we can be confident in the results.

The noting of a trend towards better chemotherapy effect in MSI cases is not an attempt to find meaning where there is not significance but to provide a fertile direction for future research. From our results, we do not believe that MSI is the molecular indicator of response to 5FU chemotherapy but it is possible that a factor associated with MSI will provide the answer.

8 CONCLUSION

8.1 Summary of Findings

8.1.1 Prognostic influences

8.1.1.1 Aim 1 and 2 - Gender and Site

The first two prognostic aims of the study were to determine the influence of gender and tumour site on survival following curative resection for stage C CRC, hypothesising that both female gender and proximal site would be associated with an improved outcome. We found, however, that neither had prognostic significance in this group of patients. Combinations of these clinical factors also failed to predict outcome.

8.1.1.2 Aim 3 - Tumour Histology

The third aim of the study was to determine which histological features of CRC had prognostic significance in stage C CRC, in particular whether recently described parameters were prognostically useful

The pathological factors found to be significantly associated with a worse prognosis were higher nodal stage, perforation, extramural vascular invasion, perineural invasion and an infiltrating margin. Tumour grade and type had a minor influence. Factors not prognostically useful included tumour size, degree of bowel wall invasion and obstruction. The recently described factors budding, stroma type, TILs and Crohn‟s- like lymphocytes did not influence outcome in this group. The role of mural vascular invasion and peritumoral lymphocytes was unclear.

8.1.1.3 Aim 4 - MSI

The fourth aim was to determine the prognostic influence of MSI on survival from stage C CRC, predicting patients with MSI tumours would have improved outcome. We did not find this to be the case; MSI status of the tumour did not affect outcome in this group of stage C CRCs.

8.1.2 Predictors of Chemotherapy Response

8.1.2.1 Aim 1 and 2 - Gender and Site

The first aim of this section was to determine whether gender and site influenced the magnitude of the survival benefit from 5FU-based adjuvant chemotherapy in stage C CRC, predicting that women with proximal cancers may have the most to gain. We found, however, that neither gender nor site (or combination subgroups) significantly influenced response to chemotherapy. No one group gained greater benefit and all subgroups showed benefit.

8.1.2.2 Aim 3 - Tumour Histology

The third chemotherapy aim was to determine if histological variables (or combinations) identified a responsive target group for adjuvant chemotherapy. We found that no factor predicted a greater survival response from adjuvant treatment and, in particular, that patients with poor prognostic tumours did not have an increased benefit over others.

8.1.2.3 Aim 4 - MSI

The final chemotherapy aim was to determine if MSI status influenced response to adjuvant chemotherapy in stage C CRC. We found that both the MSI and MSS cancer groups derived a significant survival benefit and that the magnitude of the benefit was not significantly different. There was a trend towards a better response in the MSI cohort that did not reach significance.

8.1.2.4 Chemotherapy Conclusion

From this work, we conclude that a target group for adjuvant 5FU-based chemotherapy for stage C curatively resected colorectal cancer cases cannot be predicted according to gender, tumour site, tumour histology or MSI status. Thus, continuing the current practice of offering adjuvant therapy to all stage C cases is appropriate and importantly no subgroup should be excluded based on the above factors.

8.1.3 MSI Associations

The secondary aim of this thesis was to investigate the clinical features and tumour histology associated with MSI colorectal cancers. We found that MSI CRCs were significantly associated with female gender and location in the proximal colon, with very few distal cancers being MSI positive. The association was even stronger in women with proximal lesions with 30% being microsatellite unstable. Proximal tumour site combined with indicative histological features was also useful to predict MSI status. Female gender in combination with histology was less so. There was no significant difference in age distribution between MSI and MSS cases.

Histological factors that were significantly associated with MSI tumours were poor differentiation, mucinous component, tumour infiltrating lymphocytes and pushing margin (or lack of infiltrating margin). While an uncommon finding, the presence of TILs was highly suggestive of a MSI tumour. The presence of TILs and mucin in a poorly differentiated stage C CRC almost certainly indicates a MSI cancer.

8.2 Study Limitations

8.2.1 Retrospective

The research study was retrospective and thus potentially subject to selection bias and inaccuracies. To minimize selection bias, as many cases as possible from the years preceding standard adjuvant chemotherapy were included. For the later cases, selection bias in administration of chemotherapy was partially countered by adjusting for age. It was not possible to accurately include other factors such as comorbidities. However, we used cancer-specific outcome as the endpoint to minimize the influence of these factors on the survival rate.

Retrospective review of data is fraught with inaccuracy. Data for this study was gathered from several prospective databases, which were subject to comparison. South Australia has an advantage when performing this type of study. The government-run cancer registry is dedicated to the accurate and thorough collation of cancer data. Information concerning death is meticulously gathered and while there is some state migration, these cases are detected through frequent national checks. Hospital databases were established early and while they have not always been maintained with vigour they have improved in recent years. The numerous exclusions

were mostly accounted for by inaccuracies at the hospital level and reflect the need to be vigilant when using databases especially those staffed by non-medical personnel.

The numerous cross-checks performed and exclusion of cases with non-verifiable information or inconsistencies, meant we could be confident the patient information accrued for this study is accurate. The numerous exclusions are unlikely to be informative. It is possible that the need to exclude some synchronous and metachronous cases and those with other cancers may have decreased the number of familial cases. Ideally, familial cases would have been excluded. However it is unlikely from available information that they were clinically recognised as is probably the case in many of the population studies.

8.2.2 Prognostic Factors

It is possible that not all prognostic influences were included or accounted for in this analysis. Recognised factors such as surgeon grade and emergency surgery could not be reliably ascertained from casenotes but are unlikely to have significantly skewed or changed the findings. Other non-recognised factors may affect retrospective results that would not occur with randomisation.

8.2.3 Pathology

All histology was thoroughly re-evaluated, however the pathological assessment had some potential inaccuracies. Slide deterioration made some parameters (especially stroma) difficult to assess for some cases. Secondly, limited sampling of the original specimen meant some cases had few blocks available for assessment and some parameters may have been under-appreciation. Furthermore, tumours tend to be

heterogeneous and features may vary depending on sampling. Fortunately, most parameters are measured at the leading edge, which is usually included in sampling.

Lymph node yields or sampled yields in the past were not always the number recommended today. In this study the average number of total nodes identified was 10.5 for the years 1980 and 1991 compared to 12.7 between 1992 and 2003. This may have led to an under-estimation of involved nodes. Further examination of lymph nodes did not always occur once positive nodes were identified. Therefore, while the stage may be accurate, the number of involved nodes may not. It is also probable that some stage B cases have been under-staged and thus should have been in our cohort. Apical node could not be included due to inconsistent reporting.

8.2.4 Power

Despite having a large number of cases, power calculations determined we would require even more cases to detect subtle differences. This was not possible from the databases that were available. For this reason, trends may be worthy of consideration - not to draw conclusions but to guide future research.

8.2.5 MSI Determination

We did not perform the complete NCI panel and thus may be criticized. The accuracy of BAT26 for MSI, however, is sufficiently high that few cases would have been missed. We aimed to find a tool to target therapy that was simple, reproducible and accessible. Result interpretation relies on visual assessment of allele length variation however, with only 4 equivocal cases from 729 in this study, interpretation should not be a significant hurdle to its usefulness.

8.2.6 5FU-Based Therapy

We determined the response to standard 5FU-based therapy. Today regimens are often varied in dosage, administration and combinations, with frequent inclusion of oxaliplatin and thus the relevance of this work to current practice may be challenged. While there is variance, the basis of adjuvant therapy is still 5FU and thus these findings relevant.

8.2.7 Rectal Cancers

Rectal cancers are increasingly being recognised as distinct from colon cancer in terms of (neo) adjuvant therapy, importance of surgical technique, nature of recurrence and survival. Given the variance, separating rectal cases for analysis may have led to more specifically relevant results. Yet the smaller number in this subgroup would have meant less robust conclusions if analysed separately. It may be advisable to separate colonic and rectal cases in future research.

8.2.8 Select Stage

We included only stage C cases and as such conclusions can only be drawn for this stage of disease. The prognostic usefulness of some factors may be of greater relevance in either earlier or more advanced cases. It would not be unreasonable to extend the trends we have identified in chemotherapy response to further research in earlier stage disease. Identification of biomarkers to guide adjuvant therapy is even more relevant to stage B CRC because it is difficult to justify administering deleterious treatment to all cases when the number needed to treat for a survival gain is so high.

8.3 Study Strengths

The strength of this study lies in its size, the purity of the dataset, the accuracy of the pathology, the comprehensiveness of adjusted analysis and appropriately directed statistical methodology. The number of cases included was larger than comparative studies. Accuracy of information was assured by the numerous crosschecks, in particular that the stage was correct and that the surgery was curative. Using databases without checking was shown to be unsatisfactory by the number of cases subsequently excluded. By re-evaluating all histological slides, pathological assessment was thorough, consistent and accurate. Modern examination protocols were applied allowing assessment of more recently recognised factors.

The list of prognostic factors examined was comprehensive. The inclusion of these for adjusted analysis therefore better predicted those with true independent influence. Specific analysis was used to test for an interaction between the studied factors and chemotherapy. This was important to ensure a compounding effect was tested for not just a prognostic influence.

8.4 Future Research

Despite MSI not being a clinically useful factor with which to target chemotherapy, the trend identified warrants further attention. It is possible that a factor related to MSI may provide the answer and that methylation is the key. As MMR gene methylation may occur as part of more extensive methylation (CIMP or otherwise), it is possible that associated methylation of another gene, possible one relevant to the folate pathway and thus the mechanism of action of 5FU, will prove to be a useful biomarker. It is hoped future work will unravel these relationships.

Given recent advances and current emphasis, there is no doubt that pharmacogenomics (targeting therapy according to genotype) will be the future in colorectal cancer treatment.

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10 APPENDIX

Database Fields

- Demographic information and death data
    - Automated study identification number
    - Hospital number
    - Surname
    - First name
    - Date of birth
    - Postcode
    - Died
    - Date of death
    - Cause of death

- Operative data and adjuvant chemotherapy (applicable to each operation)
    - Automated operation identifying number
    - Procedure number (if multiple)
    - Date of operation
    - Procedure type
    - Chemotherapy
        - If received
        - Date commenced
    - Radiotherapy
        - If received
        - Date commenced

- Histology for each tumour
    - Automated histology identifying number
    - Tumour number (if multiple)
    - Size
    - TNM stage (individual)
    - Proximal/distal
    - Subsite
    - Differentiation
    - Type
    - Signet ring component
    - Lymphocytes
        - Peritumoral
        - Crohn‟s-like
        - Tumour infiltrating (TILs)
    - Stroma type
    - Obstruction
    - Perforation
    - Infiltrating margin
    - Budding
    - Margin involvement
        - Proximal
        - Distal
        - Radial
    - Neurovascular invasion
        - Mural vascular

        - Extramural vascular
        - Perineural
    - Nodal status
        - Apical
        - Total number examined
        - Number positive
        - Micrometasases
    - Polyps
        - Residual
        - Other adjacent polyps

- MSI information
    - Overall status
    - BAT 26 result
    - BAT 40 result
    - Tumour block used and identifying number on DNA database
    - Normal block used and identifying number on DNA database

- Check boxes
    - HNPCC recognised
    - FAP recognised
    - Synchronous tumours
        - Synchronous across colon
    - Metachronous tumours

        - Metachronous across colon
    - Note to be checked
    - Notes checked
    - Check with registry
    - Operation elsewhere
    - No pathology (no slides or blocks available or laboratory number unable to be located)
    - Analysis exclusions
        - Not for site analysis (see multiple cancer section)
        - Not for pathology analysis (see multiple cancers section)
        - Not for MSI analysis (blocks not available but pathology able to be reviewed