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SFRP4 as an Epigenetic Biomarker of Colorectal Cancer Risk

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DECLARATION

This thesis has not been previously submitted for a degree to any other university and represents entirely my own original work.

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Preface

There is a lack of robust biomarkers of CRC risk. Epigenetic changes in the WNT-related *SFRP4*, a gene whose expression is down-regulated early in CRC development, may be a potential CRC risk biomarker. If *SFRP4* promoter methylation proved to be a useful biomarker of CRC risk, it would have potential implications for CRC screening. In addition, it could be used as a surrogate endpoint for investigations of CRC risk modifying interventions.

SFRP4 methylation at several CpG sites was quantified in macroscopically normal rectal mucosal biopsies from volunteers at a relatively lower and higher CRC risk in two studies viz. the BORICC Study and the DISC Study. In the BORICC Study, the mean *SFRP4* methylation of the 5 CpG sites investigated was significantly (p=0.036) higher in those in the higher risk group than in healthy controls. In the DISC Study, *SFRP4* methylation was also higher at all CpG sites in the higher risk groups than in healthy controls but the differences were not statistically significant.

In the BORICC Study *SFRP4* methylation was also quantified in buccal cells matched to the rectal biopsies for the volunteers at a relatively lower and higher CRC risk. In contrast with the findings from the rectal mucosa, *SFRP4* methylation was significantly (p<0.001) lower at all CpG sites in those in the higher risk group than in healthy controls. At CpG sites 1 and 4 only, *SFRP4* methylation in the rectal biopsies and buccal cells was correlated significantly (p=0.001 and p=0.041 respectively).

The healthy controls in the DISC Study were entered into a 50 day dietary intervention study and randomised to two potential chemoprevention agents; resistant starch and polydextrose in a 2×2 factorial design. *SFRP4* methylation levels were quantified before and after the dietary intervention. Individually, resistant starch and polydextrose had no detectable effect on *SFRP4* methylation levels. However, there was evidence of an interaction between the two intervention agents which was qualitatively similar at all CpG sites investigated. This interaction was statistically significant (p=0.008) at CpG site 2. The biological interpretation of this interaction cannot be determined until the study is unblinded.

This study has provided preliminary evidence that *SFRP4* methylation may be a novel epigenetic biomarker of CRC risk and that measurement in DNA from buccal cells may be a useful surrogate for invasive measurements on rectal mucosa.

To Dad, Mum, Jenny and Peter

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Abbreviations

1,25(OH)2D3	1α,25-hydroxyvitamin D3
25(OH)D3	25 hydroxyvitamin D3
ACF	aberrant crypt foci
ANOVA	analysis of variance
APC	adenomatous polyposis coli
AUC	area under curve
BMI	body mass index
BORICC	Biomarkers of Risk in Colorectal Cancer
CAPP	Concerted Action Polyp Prevention
CIMP	CpG island methylation phenotype
CRC	colorectal cancer
DAPK	death associated protein kinase
DBP	vitamin D binding protein
DISC	dietary intervention, stem cells and colorectal cancer
DNA	deoxyribonucleic acid
DVL	Dishevelled family protein
EC cell	embryonal carcinoma cell
EDTA	ethylenediaminetetracetic acid
EPIC	European Prospective Investigation into Cancer and Nutrition
ES cell	embryonic stem cell
FAP	familial adenomatous polyposis

FDA	Federal Drug Administration
FOBT	faecal occult blood testing
FZ	frizzled membrane receptor
GSK	glycogen synthase kinase
GTP	guanosine 5-triphosphate
HAT	histone acetyltransferase
HDAC	histone deacetylase
HNPCC	hereditary non-polyposis colorectal cancer
K-RAS	Kirsten rat sarcoma viral oncogene homolog
LRP5	low density lipoprotein receptor-related protein 5
LRP6	low density lipoprotein receptor-related protein 6
MLH1	MutL homolog 1
MMR	mismatch repair
MSH2	MutS homolog 2
MSH6	MutS homolog 6
MSI	microsatellite instability
MSI-H	microsatellite instability high
MSI-L	microsatellite instability low
MSP	methylation specific polymerase chain reaction
MSS	microsatellite stable
NHS	National Health Service
NSAID	non-steroidal anti-inflammatory drug
OSCC	oral squamous cell carcinoma
P53	protein 53

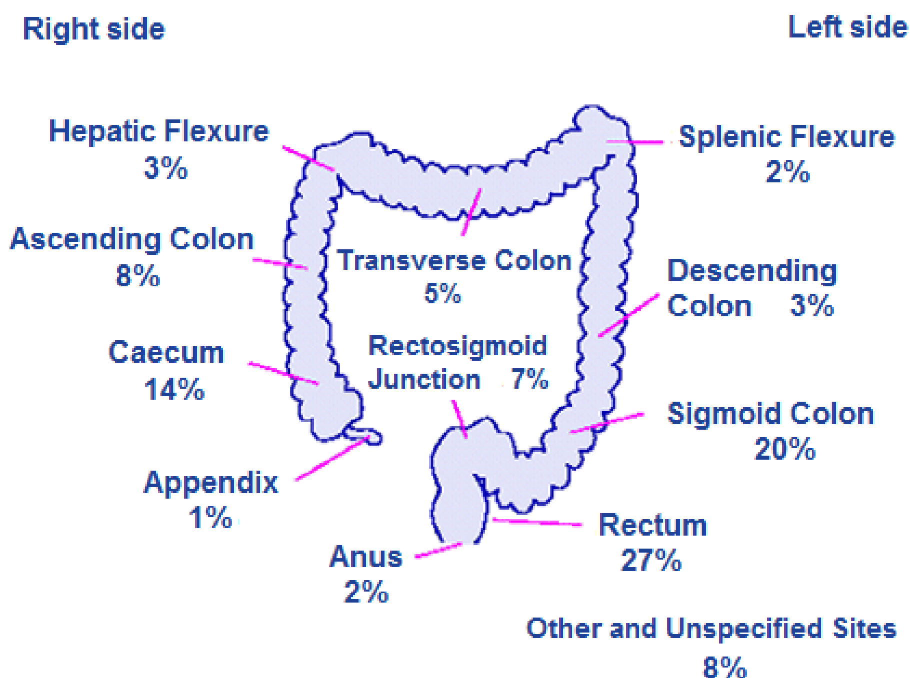
PCR	polymerase chain reaction
RNA	ribonucleic acid
ROC	receiver operator curve
RS	resistant starch
RT-QMSP	real time quantitative methylation specific PCR
SCFA	short chain fatty acid
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SET-SDS	SET-sodium dodecyl sulphate
SFRP	secreted frizzled related protein
TBE	Tris, boric acid, EDTA
TCF	T-cell factor
TGF	transforming growth factor
UC	ulcerative colitis
UK	United Kingdom
UV	ultraviolet
WCRF	World Cancer Research Fund

1 Introduction

1.1 Colorectal cancer

Colorectal cancer (CRC) arises from uncontrolled cell growth in the columnar epithelium most commonly in the rectum but this cancer can occur anywhere in the large intestine (Figure 1.1). It is the 3rd most common cancer (males and females combined) in the United Kingdom with approximately 110 new cases diagnosed daily (CRUK 2013). The lifetime risk of developing CRC in the UK is 1 in 16 for men and 1 in 20 for females (CRUK 2013).

Figure 1.1Distribution of cancer within the colon, UK (2007-2009) (CRUK 2013)



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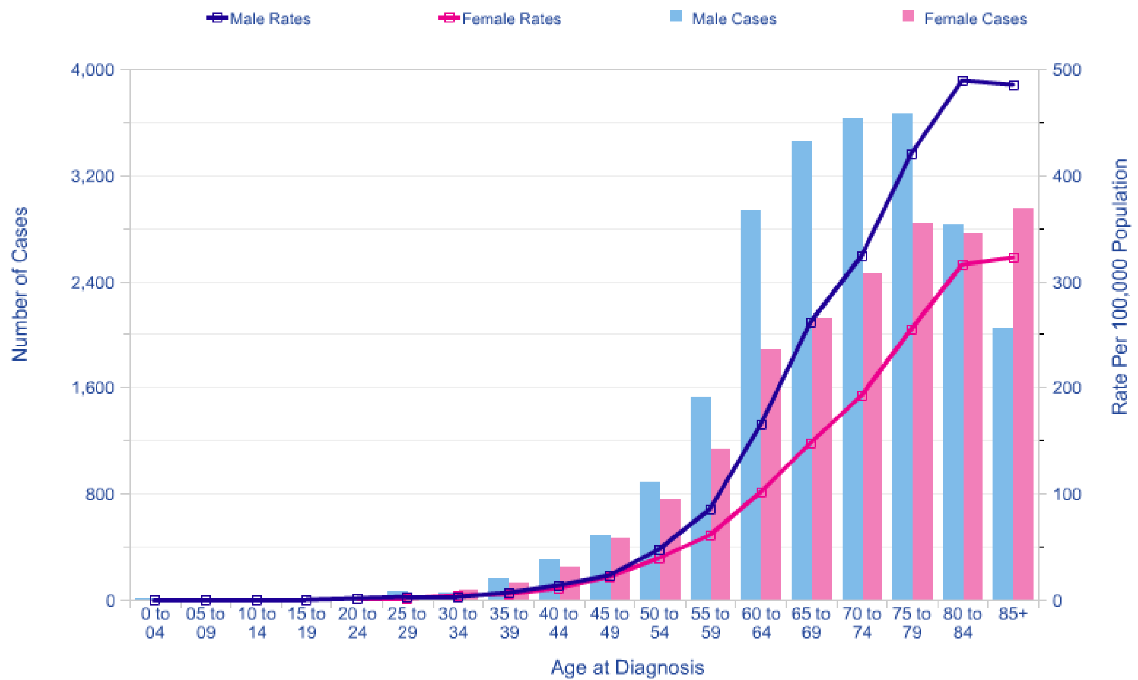
CRC risk increases progressively with age with 86% of cases arising in those who are over 60 years old (CRUK 2013). Below the age of 50 years, there are similar rates of CRC in both sexes, but after this age, CRC becomes more common in males (CRUK 2013) (Figure 1.2).

Figure 1.2 Number of new CRC cases per year and age-specific incidence rates, UK (2007-2009) (CRUK 2013)



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1.2 CRC mortality

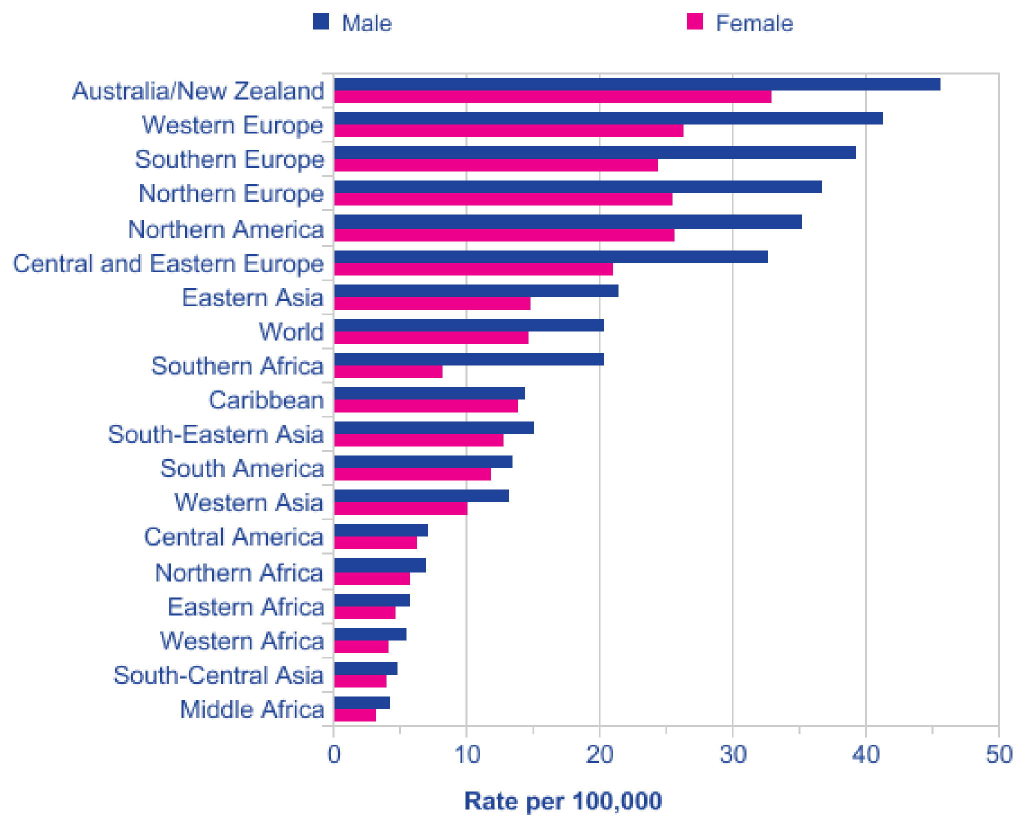
In 2008, there were 608,000 deaths from CRC and 1,234,000 new cases of CRC worldwide (Ferlay et al. 2010). Current trends in CRC mortality statistics from many developed countries are encouraging but only 50% of those diagnosed with CRC survive more than 5 years from diagnosis (CRUK 2013).

1.3 CRC epidemiology

The incidence of CRC is not uniform across the world. CRC is predominantly a disease of developed countries with over 63% of cases occurring in the developed world (Hagger & Boushev 2009). Identifying the reasons responsible for the inequality in CRC distribution globally would provide a starting point in reducing incidence and increasing survival from CRC. However, it is relevant to note that increasing age is a known risk factor for CRC (Section 1.4.1); and life expectancy is significantly greater in developed countries (where there is a

higher incidence of CRC) than in developing countries. However, this alone is unlikely to account for the ten-fold difference between the countries with the highest (Australia and New Zealand) and lowest (Africa) CRC incidence (Jemal et al. 2010, CRUK 2013) (Figure 1.3).

Figure 1.3 Worldwide incidence rates of CRC, 2008 (CRUK 2013)



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1.4 <u>CRC aetiology</u>

There are several risk factors associated with the development of CRC which can be categorized into modifiable and non-modifiable risk factors.

1.4.1 Non-modifiable CRC risk factors

Non-modifiable risk factors include increasing age and hereditary factors. CRC increases with age, with more than 90% of CRC occurring in people over the age of 50 years (CRUK 2013, Howlander et al. 2013) (Figure 1.2). CRC incidence is greatest in those over the age of 80 years, where CRC incidence peaks (CRUK 2013). Therefore with an ageing population, it is expected that the incidence of CRC will rise. Up to the age of 80 years, CRC incidence is greater in males. Above the age of 45 years, the incidence is significantly higher in males than in females (CRUK 2013).

Five percent of CRCs are hereditary and arise in individuals who have a genetic defect which predisposes them to the early development of CRC (Burt 2000). A genetic defect that predisposes to CRC is suspected in individuals who have a family history of CRC (Burt 2000, Fearnhead et al. 2002); though it is possible that a family history of CRC may be the result of family members being exposed to the same environmental factors that may impact upon the risk of CRC.

The most common inherited CRC-related conditions are Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC) which both demonstrate an accelerated progression through the pathways that lead to CRC (Burt 2000, Fearnhead et al. 2002, Lynch & de la Chapelle 2003). Some of the genes responsible for these forms of inherited CRC have been identified.

1.4.1.1 Hereditary non-polyposis colorectal cancer

Up to 6% of CRC is as a result of HNPCC (WCRF 2007). HNPCC is an autosomal dominant condition associated with mutations in genes involved in the DNA mismatch repair (MMR) pathway. The lifetime risk of developing CRC in an individual with an inherited MMR mutation is as high as 80%, with CRC usually presenting before the age of 50 years (Fearnhead et al. 2002, Lynch & de la Chapelle 2003, Burt 2007, Steinke et al. 2013). HNPCC patients are also

at an increased risk of cancer at other anatomical sites including the endometrium, ovary, stomach, small intestine, hepatobiliary system and brain (Lynch & de la Chapelle 2003, Burt 2007, Steinke et al. 2013).

HNPCC is caused by inherited mutations in one of five DNA MMR genes: *MSH2, MLH1, PMS1, PMS2, MSH6*; which can be identified phenotypically by microsatellite instability (MSI), a hallmark feature of HNPCC. Ninety percent of HNPCC is a result of an inherited mutation in the *MSH2* and *MLH1* DNA MMR genes (Lynch & de la Chapelle 2003).

Microsatellites are segments of the DNA sequence containing tandem repeats in the nucleotide sequence which are vulnerable to errors during DNA replications (Chung & Rustgi 1995, Boland et al. 1998, Boland & Goel 2010). This vulnerability or (microsatellite) instability results in changes in the length of the nucleotide repeats, which can be of varying lengths. All of the 4 nucleotide bases may be involved, though nucleotide repeats of cytosine and adenine are the most common (Boland & Goel 2010) (Section 1.6.1.6).

1.4.1.2 Familial adenomatous polyposis

FAP is an autosomal dominant condition which accounts for less than 1% of CRC cases and is caused by mutations in the tumour suppressor gene adenomatous polyposis coli (*APC*) which is located at chromosome 5q21-q22 (Chung 2000, Galiatsatos & Foulkes 2006) (Section1.6.1.1). Individuals suffering from FAP characteristically develop multiple adenomatous polyps at a relatively young age (often in adolescence) and malignant transformation of at least one of these adenomatous polyps occurs on average by the age of 35 years (mean age of CRC diagnosis) (Galiatsatos & Foulkes 2006, Hagger & Boushev 2009).

If untreated, the incidence of malignancy in patients with FAP approaches 100%; with almost 100% of malignant transformation occurring by the time the patient is 40 years old (Fearnhead et al 2002, Davies et al. 2005, Burt 2007). Therefore, in individuals with FAP, CRC is almost inevitable and preventative measures must be taken to prevent the occurrence of CRC. This is achieved with surgery to remove the colon (Fearnhead et al. 2002, WCRF 2007). However, despite prophylactic colectomy, individuals with FAP remain at a high risk of other cancers (stomach, duodenum) and regular endoscopic surveillance to screen for these cancers is still required (Burt 2007).

1.4.1.3 Colorectal polyps

Colorectal polyps or adenomas are benign growths occurring on the lining of the colon or rectum (Zuber & Harder 2001). They arise from the epithelial lining of the colorectum and the 2 most common types are hyperplastic polyps and adenomatous polyps. Other types of colorectal polyps which are less common are juvenile polyps and hamartomatous polyps (Zuber & Harder 2001).

1.4.1.4 Hyperplastic polyps

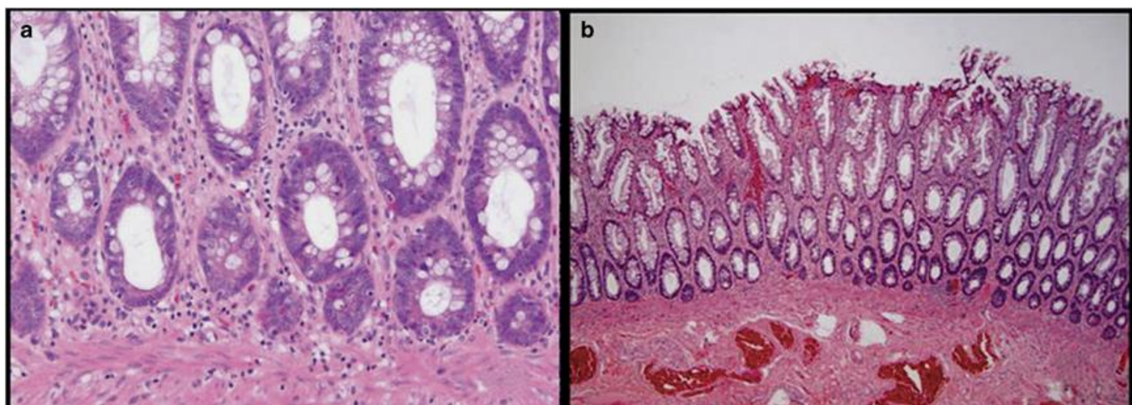
Hyperplastic polyps account for the majority (90%) of colorectal polyps (Hyman et al 2004). Histologically, hyperplastic polyps contain larger numbers of glandular cells with less cytoplasmic mucus. They lack nuclear hyperchromatism and atypia (Jass 2007, Guarinos et al. 2012) (Figure 1.4). Hyperplastic polyps are benign growths but do have malignant potential if they occur because of a hyperplastic polyposis syndrome (Hyman et al. 2004). Hyperplastic polyposis syndromes result when there are multiple colorectal serrated polyps (Guarinos et al. 2012). Risk factors for increased CRC risk in hyperplastic polyps include: polyp size greater than 1cm in diameter; a focus of adenoma within the hyperplastic polyp (a serrated adenoma); more than 20 hyperplastic polyps in the colon; a family history of hyperplastic polyposis or CRC (Jass & Burt 2000, Guarinos et al. 2012).

A serrated adenoma is a pre-cancerous lesion of the large bowel. Serrated adenomas develop into CRC via the serrated pathway which differs from the majority of CRCs which arise from adenomatous polyps via the adenoma-carcinoma sequence (Guarinos et al. 2012) (Figure 1.18).

The serrated adenoma pathway accounts for approximately 20% of sporadic CRC (Jass 2007). In this pathway, colorectal cells acquire genetic mutations in the *BRAF* oncogene and/or the *K-RAS* oncogene (Yang et al. 2004, Stefanius et al. 2011). The *BRAF* oncogene is associated with high MSI (as a result of hypermethylation of the promoter region of *MLH1*) (Section 1.6.1.6) and high CpG island methylation phenotype (CIMP) (Section 1.10.1) (Kambara et al. 2004). *K-RAS* mutations result in low levels of methylation and MSI (Stefanius et al. 2011).

Figure 1.4 Histology of hyperplastic polyp (Owens et al 2008)

Hyperplastic polyps have small basal crypts largely lacking in cytoplasmic mucus production (a and b)



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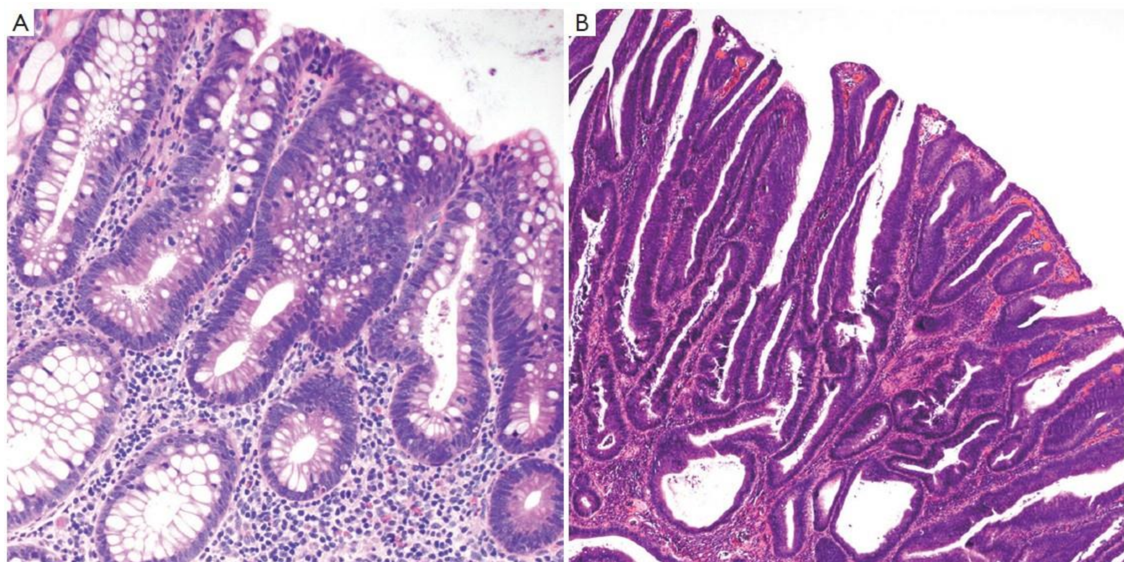
1.4.1.5 Adenomatous polyps

Adenomatous colorectal polyps have malignant potential and are of particular interest to this research project. The malignant potential of adenomatous polyps is demonstrated by i) evidence that the prevalence of adenomatous polyps peaks approximately 5 years earlier than that of CRC (Muto et al. 1975);

ii) the prevalence of adenomas in different geographical areas correlates with that of CRC incidence in those areas (Clark et al. 1985); iii) approximately one third of bowel resections for CRC will also contain one or more adenomatous polyps (Leslie et al. 2002); and iv) the risk of CRC increases significantly with increasing number of adenomatous polyps (Leslie et al. 2002).

Adenomatous polyps contain nuclei that are large, hyperchromatic and crowded together (Figure 1.5). Adenomatous polyps can be classified on histological grounds into 3 types viz. tubular, villous and tubulovillous (a combination of tubular and villous). Tubular adenomas are composed of branched tubules. Villous adenomas are composed of digitiform villi. Tubulovillous adenomas contain a mixture of branched tubules and digitiform villi (Cappell 2005).

Figure 1.5 Histology of tubular adenoma (A) and tubulovillous adenoma (B) (Fleming et al. 2012)



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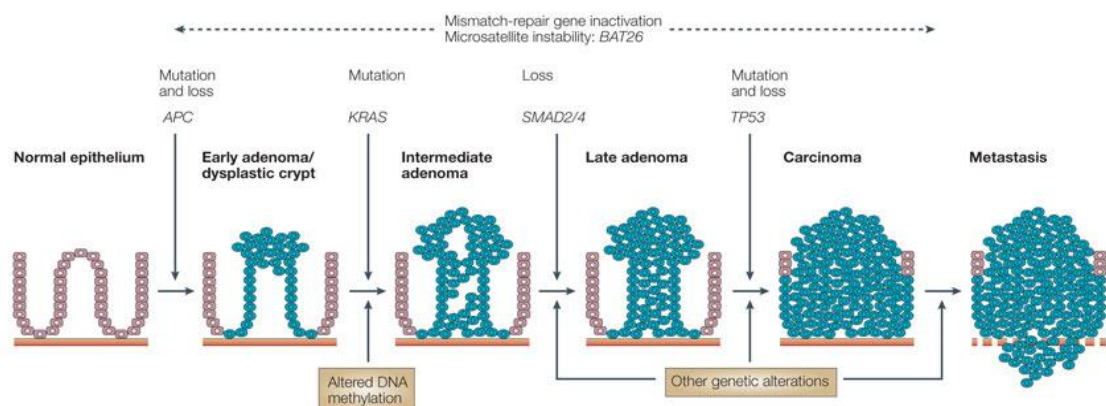
Approximately 5-10% of adenomatous polyps develop into CRC and the risk of an adenomatous polyp progressing to CRC is related to the type of adenomatous polyp and its size and shape (Muto et al. 1975). Those polyps

which are larger than 1cm, with a villous component greater than 25% and are sessile in shape (as opposed to pedunculated) are at an increased risk of progressing to CRC (Bond 2000).

Adenomatous polyps of the large intestine are precursor lesions of CRC. The development from adenomatous polyp to sporadic CRC is demonstrated by Fearon and Vogelstein's (1990) adenoma-carcinoma sequence (Figure 1.6). Fearon and Vogelstein (1990) proposed a genetic model for CRC development and suggested that a combination of oncogene activation and tumour suppressor gene inactivation results in the stepwise progression from adenomatous polyp to sporadic CRC development (Figure 1.6). This genetic model is based on the extensive data that suggest that the majority of CRCs arise from pre-existing benign adenomatous polyps. The total accumulation of these genetic changes was more important than the order in which they occurred with a minimum of mutations in 4–5 genes being required for CRC development (Fearon & Vogelstein 1990). Therefore individuals with a history of this type of polyp are at an increased risk of developing CRC and removal of the polyp before malignant transformation will reduce CRC risk (Winawer et al. 1993).

Figure 1.6 Fearon and Vogelstein's (1990) adenoma-carcinoma model of CRC (Davies et al. 2005)



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The transformation from adenomatous polyp to CRC requires a long latency period (Hagger & Boushev 2009). Therefore regular surveillance of the colon with endoscopy (colonoscopy or flexible sigmoidoscopy) to identify and remove the polyps will reduce the risk of the development of CRC (Atkin et al. 1992, Selby et al. 1992, Muller & Sonnenberg 1995, Thiis-Evensen et al. 1999). This is supported by the findings of the National Polyp Study which reported that CRC incidence was reduced by endoscopic removal of adenomatous polyps (Winawer et al. 1993). More recent evidence from a study by Zauber et al. (2012) corroborates the findings of the National Polyp Study (Winawer et al. 1993). Zauber et al. (2012) found that patients with adenomatous polyps did not have an increased incidence of CRC when compared with the patients from the non-adenomatous polyps group and the general population following endoscopic removal of their adenomatous polyp. Atkin et al. (2010) have suggested that regular endoscopic surveillance may not be needed and that only one flexible sigmoidoscopy screening test between the ages of 55 and 64 years is sufficient to reduce sporadic distal CRC incidence and mortality. The rationale for this is that if distal CRC was to develop, an adenomatous polyp would be expected by 60 years of age.

1.4.1.6 Ulcerative Colitis

Ulcerative colitis (UC) is an idiopathic, chronic relapsing inflammatory disease of the bowel where there is inflammation of the mucosa of the colon and rectum and long-standing, poorly-controlled UC increases the risk of CRC development (Fish & Kugathasan 2004). The incidence of UC is increasing, and is higher in westernized countries (Cosnes et al. 2011). Currently the worldwide incidence ranges from 1.2 to 20.3 cases per 100,000 people per year (Danese & Fiocchi 2011). Although UC accounts for only 1% of CRC, cancer is a serious consequence of UC occurring in up to 20% of patients (Eaden et al. 2001, Matter et al. 2011). The relative risk of CRC in all patients with UC has been estimated to be 4 to 20 fold (Gillen et al. 1994).

The environment, genetics and immunological factors play a role in UC development. Smoking is the only environmental factor reproducibly shown to

impact on UC. It is associated with milder disease, demonstrated by trials showing that nicotine can induce remission in active UC (McGrath et al. 2004); whereas smoking cessation is associated with UC relapse resulting in more hospital admissions and operations (McGrath et al. 2004, Danese & Fiocchi 2011, Cabre & Domenech 2012).

Many environmental factors have been investigated, including diet but the findings are inconsistent (Molodecky & Kaplam 2010). In the European Prospective Investigation into Cancer and Nutrition (EPIC) Study, the only significant dietary agent implicated in UC was linoleic acid (Hart 2008). In this study 126 out of 200,000 participants developed UC and analysis of their dietary intake showed that a higher intake of linoleic acid was associated with more than double risk of UC (odds ratio 2.49) (Hart 2008).

Lower faecal short chain fatty acid (SCFA) levels have been found in UC patients (Takaishi et al. 2008, Huda-Faujan et al. 2010). The SCFA level correlates with severity of inflammation; with lower SCFA levels found with increasing severity of inflammation (Topping & Clifton 2001). SCFA are end-products of anaerobic fermentation of dietary fibre within the large intestine. SCFA play a role in maintaining colonic health and is the principle energy source for colonocytes (Topping & Clifton 2001) (Section 1.4.2.4). In the colon, butyrate is the most important SCFA because it is the principle energy source for colonocytes (Wong et al. 2006). Impaired oxidation of butyrate has been demonstrated in patients with both active and inactive UC (Cabre & Domenech 2012).

Genome-wide association studies have suggested there are many gene variants associated with UC. Currently, there are 99 confirmed associations but at present most of the associated variants do not have any known function and many implicate regions within multiple genes (Khor et al. 2011). The various genes implicated in the development of UC include those affecting: (i) the

function of the epithelial lining of the colon; (ii) transcriptional regulation; (iii) the interleukin-23 signalling pathway; and (iv) cell death (Khor et al. 2011).

A genetic component is further suggested by the strong familial aggregation demonstrated in twin studies; with a sibling risk of 9-fold greater for UC compared to the general population, and an overall UC concordance rate in non-selected twin studies of 15% in monozygotic and 4% in dizygotic twins (Khor et al. 2011).

1.4.2 Modifiable CRC risk factors

Modifiable risk factors are environmental factors which include social and lifestyle factors such as diet, physical activity and smoking.

1.4.2.1 Dietary factors and CRC

Dietary factors account for 30% of all cancers in developed countries (WCRF 2007). 60% of CRC occurs in developed countries, with particularly high CRC rates in North America and Western Europe (CRUK 2013) (Figure 1.3).

Dietary products are one of the most common luminal antigens in the intestine that may influence intestinal health. Mechanisms of action include: a direct antigenic effect, alteration of gene expression, modulation of inflammatory mediators, changes in the composition of intestinal flora, and altered gut permeability (Cabre & Domenech 2012).

Studies have shown that CRC incidence increases among migrants when they move from a low to a high risk country and that CRC rates for second generation migrants can be double that of the first (Boyle & Langman 2000, Cappell 2005, Center et al. 2009, CRUK 2013). This suggests that environmental factors may play an important role in the development of CRC.

One possible environmental factor is the “westernisation” of dietary intake that occurs on moving from a low to a high risk country (Boyle & Langman 2000, Key et al. 2004, CRUK 2013).

The World Cancer Research Fund (2007) reported that CRC risk was significantly affected by various lifestyle factors (Table 1.1). These same lifestyle factors have been found to affect the development of CRC by Lee et al. (2011) and Parkin et al. (2011). In general, as found by the World Cancer Research Fund (2007), a high intake of red and processed meat is associated with a higher CRC risk and a high intake of fruit and vegetables and calcium may lower the risk of CRC. There is evidence that not smoking, avoiding excess alcohol consumption and avoiding weight gain by maintaining regular physical activity can reduce the risk of CRC (WCRF 2007, Parkin et al. 2011).

In comparison with the developing world, the “Western diet” has a higher intake of red meat, animal fat and alcohol and a lower intake of dietary fibre, fruit and vegetables (Cordain et al. 2005). Therefore the “Western diet” may contribute to the higher incidence of CRC in the Western world.

1.4.2.2 Dietary fibre and CRC

The intake of dietary fibre differs between high and low CRC risk countries and this may be partly accountable for the differences in CRC rates in Africa (low risk) and Westernised (high risk) countries (Boyle & Langman 2000, Key et al. 2004, Cordain et al. 2005, Center et al. 2009, CRUK 2013).

The EPIC Study reported a linear decrease in the risk of CRC with increasing dietary fibre intake (Bingham et al. 2003). However, the results of multiple epidemiological studies investigating the association between CRC risk and dietary fibre intake have been inconsistent (COMA 1998, Fuchs et al. 1999, Pietinen et al. 1999, Alberts et al. 2000, Bonithon-Kopp et al. 2000, Schatzkin et al. 2000, Terry et al. 2001, WCRF 2007). This is because of differences in the

study designs and dietary data collected using food frequency questionnaires; and the possibility of confounding by other dietary components (Day et al. 2001, Kipnis et al. 2001). In 2011, Aune et al. (2011) published a meta-analysis of the outcomes from prospective cohort studies on the relationship between dietary fibre on CRC risk. The meta-analysis consisted of 21 prospective studies and included over 1.7 million participants and 12,000 CRC cases, providing sufficient statistical power to show there is an inverse association between dietary fibre intake and CRC risk. Aune et al. (2011) concluded that there was a 10% reduction in CRC risk for each 10g/day intake of dietary fibre.

Table 1.1 Summary of World Cancer Research Fund (2007) findings of relationships between dietary and anthropometric factors and CRC risk

Strength of Evidence	**Decreases CRC risk**	**Increases CRC risk**
Convincing	Physical activity of all types (occupational, household, recreational)	Red meat Processed meat Alcohol (men) Body fatness Abdominal fatness
Probable	Dietary fibre Garlic Milk Calcium	Alcohol (women)
Suggestive	Non-starchy vegetables Fruits Folate Selenium Fish Vitamin D	Iron Cheese Animal fats Sugars
Inconclusive	Cereals, potato, poultry, seafood, other dairy products, total fat, fatty acid composition, cholesterol, caffeine, total carbohydrate, starch, vitamin A, C, E retinol, meal frequency, energy intake	
Substantial effect on risk unlikely	None identified	

The Polyp Prevention Trial investigated the effect of a low fat, high fibre and high fruit/vegetable diet on the recurrence of resected adenomatous polyps in the colon (Lanza et al. 2007). Just over 2000 participants with recently excised polyps were randomized into 2 groups: (1) Participants underwent intensive counselling to adopt a low fat, high fibre and high fruit/vegetable diet; and (2) participants were given a brochure on healthy eating (control group). After 4 years, there was no difference in the rate of polyp recurrence in both groups (Lanza et al. 2007). However, it is possible that dietary fibre may influence cellular events that result in the prevention of adenomatous polyp formation, but has no effect upon reducing adenomatous polyp and CRC risk once the initial insult to the colorectal tissue has occurred (Kim & Milner 2007).

The Wheat Bran Fibre Study investigated the effect of a wheat bran fibre supplement on the recurrence of resected adenomatous polyps in the colon (Earnest et al. 1999). Just fewer than 1,500 participants with recently excised polyps were randomized into 2 groups: (1) Daily high wheat bran fibre cereal supplement; and (2) low wheat bran fibre cereal supplement. After 3 years, there was no difference in the rate of polyp recurrence between the 2 groups (Earnest et al. 1999).

It is possible that the participants of both the Polyp Prevention Trial and the Wheat Bran Fibre Study were not followed up for an adequate length of time or the intervention was not given for long enough. The development of CRC takes many years and interventions lasting 4 years and 3 years respectively may not have been long enough to have a significant impact. The participants of the Polyp Prevention Trial were followed up for a further 4 years after completion of the initial study and even after 8 years, participants taking a low fat, high fibre and high fruit/vegetable diet did not have a reduced polyp recurrence rate when compared to the group following a standard healthy diet (Lanza et al. 2007).

1.4.2.3 Dietary fibre

Dietary fibre is a collective term for a group of dietary carbohydrates with a diverse range of physical, physiological and chemical properties (Cummings & Stephen 2007). Dietary fibre refers to a physiological concept – the proportion of food derived from the cellular wall of plants which is resistant to digestion by human digestive enzymes. More specifically, dietary fibre consists of intrinsic plant cell wall polysaccharides (Cummings & Stephen 2007).

Two categories of dietary fibre have been described: Insoluble dietary fibre and soluble dietary fibre. In general, insoluble dietary fibres are those that are slowly and incompletely fermented, allowing them to have a bulking action and thus more pronounced effects on bowel habit (Cummings & Stephen 2007). Soluble dietary fibres are viscous and fermentable in the large intestine. They

delay gastric emptying and slow the transit of food material through the small intestine and have effects on glucose and lipid absorption in the small intestine (Cummings & Stephen 2007). However, the separation of dietary fibre into these two categories is pH dependent, thus making associations with specific distinct physiological properties uncertain (Cummings & Stephen 2007).

Dietary fibre has multiple beneficial effects on the gastrointestinal tract related to its inability to be digested in the small intestine (Cummings & Stephen 2007). The two most important actions with regards to colonic health are: (i) it protects against CRC by absorbing or diluting potential carcinogens within the large intestine (Fuchs et al. 1999, Lipkin et al.1999); and (ii) it acts as a substrate for fermentation by anaerobic bacteria within the large intestine, where it may be hydrolysed and metabolised to products such as SCFA. Butyrate, acetate and propionate are the 3 major types of SCFA which are produced as end-products of fermentation of dietary fibre in the colon. Of these, butyrate is the most important with regards to colonic health because it is the principle energy source for colonocytes (Cook & Sellin 1998, Nugent 2005).

1.4.2.4 Butyrate

Butyrate promotes cell differentiation and induces apoptosis in CRC cells; both actions are protective against CRC (Scheppach et al. 1992, Hague et al. 1995). Butyrate is a natural histone deacetylase inhibitor (Section1.9), and has anti-carcinogenic effects on colonic epithelial cells such as down-regulation of expression of oncogenes (Davis 2003, Rada-Iglesias et al. 2007). Evidence exists that butyrate reduces the number and size of aberrant crypt foci (ACF) (the earliest detectable change to the normal colorectal cell that suggests it may progress to CRC), and ultimately the development of CRC (Kim & Milner 2007). It also stimulates colonic blood flow and fluid and electrolyte uptake (Scheppach et al. 1994, Topping & Clifton 2001). Butyrate also reduces the conversion of primary bile acids to secondary bile acids. Secondary bile acids have been linked to an increased risk of CRC (Topping & Clifton 2001, Costarelli et al. 2002).

The mechanism responsible for the antineoplastic effects of butyrate is not known, but butyrate influences several processes important in tumourigenesis, including cell proliferation, apoptosis, angiogenesis, immunosurveillance and inflammation (Scheppach et al. 1992, Perrin et al. 1994, Hague et al. 1995, Davis 2003, Mathers 2003, Rada-Iglesias 2007). These effects of butyrate have been attributed to changes in gene expression, possibly as a consequence of epigenetic processes including hyperacetylation of histones (Boffa et al. 1978, Parker et al. 1986, Boffa et al. 1994, Davis 2003, Williams et al. 2003, Rada-Iglesias et al. 2007).

1.4.2.5 Resistant Starch

Starch is a glucose homopolymer found in two forms: amylose and amylopectin. Starch is present as crystalline granules in raw food. Resistant starches are all the starch and starch degradation products that resist small intestinal digestion and are therefore available to enter the large intestine where they act as substrates for fermentation into SCFA such as butyrate (Englyst et al. 1996). They are now included within the broad classification of dietary fibre. There are 4 types of resistant starch, classified by their (botanical) source and food processing effects which result in resistance to pancreatic α-amylase (Cummings & Stephen 2007) (Table 1.2).

Resistant starch has an energy value of 2kcal/g which is half that of digestible starch (Narina et al. 2012). It is found in various foods including rye bread, bananas and cooked potatoes which have been left to cool, and can be commercially manufactured (Hi-maize) and used in the production of many foods such as cereal bars, baked products and nutrition bars (Nugent 2005).

Table 1.2 Types of resistant starch (Nugent 2005)

RS1	Physically inaccessible starch – starches are trapped within the plant cell wall which resists digestion because of a physical barrier
RS2	Starches consisting of poorly gelatinized granules. Starch is packed tightly in a radial pattern and relatively dehydrated. This compact structure limits the accessibility of digestive enzymes and are hydrolysed slowly and resistant to digestion
RS3	Retrograded starches (after food processing) – the most resistant starch fraction. Mainly retrograded amylose formed during the cooling and crystallization of gelatinized starch – the process of heating and then cooling has rendered the starch resistant to digestion
RS4	Chemically modified starches as a result of novel chemical bonds

The Concerted Action Polyp Prevention (CAPP) 2 Study published by Mathers et al. (2012) investigated the effect of resistant starch with and without aspirin taken daily for up to 4 years in a randomised control trial using a 2×2 factorial design on 937 participants with HNPCC. The CAPP2 Study found no significant effect of resistant starch on the development of CRC. There was also no significant effect of aspirin on colorectal neoplasia during the study (Burn et al. 2008). However, the CAPP2 Study did find that 600mg aspirin daily provided protection against CRC development in long-term follow up (Burn 2011). Furthermore, the CAPP1 Study also investigated the effect of resistant starch and/or aspirin given daily for at least a year in a randomised control trial on participants with FAP. Despite showing that resistant starch led to reduced cell proliferation, there was no significant difference in the adenomatous polyp count in the large intestine of the participants who had taken resistant starch compared to those who had not (Burn et al. 2011).

The CAPP1 Study and CAPP2 Study have not found any clinical effect of resistant starch on the development of CRC (Burn et al. 2011, Mathers et al. 2012). However, there are multiple observational studies that do show a protective effect of dietary fibre such as resistant starch (Trock et al. 1990,

Howe et al. 1992). Aune et al. (2011) reported that dietary fibre significantly reduced CRC development in a meta-analysis of 21 prospective cohort and nested case-control studies. It is possible that resistant starch affects individuals genetically predisposed to CRC development (e.g. HNPCC, FAP) differently to the general population.

1.4.2.6 Polydextrose

Polydextrose is a synthetic polysaccharide which is largely non-digestible in the small intestine and only partially fermentable in the large intestine. It has a low energy value (1 kcal/g) (Cummings & Stephen 2007). It is a low molecular weight randomly bonded polysaccharide and is prepared by the bulk melt polycondensation of glucose and sorbitol with small amounts of food grade acid in vacuo (Flood et al. 2004). It is used widely as a low calorie sugar replacement (Flood et al. 2004). Extensive studies supported by the Food and Drug Administration (FDA) has shown polydextrose to be safe for dietary consumption (FDA 2013).

Polydextrose has physiological actions similar to that of dietary fibres as a result of its poor digestibility in the small intestine and incomplete fermentation in the large intestine (Topping & Clifton 2001). In individuals with a higher intake of polydextrose, the production of butyrate has been observed to increase (Jie et al. 2000). Polydextrose also promotes growth of colonocytes, with growth occurring mainly at the base of the colonic crypts where the stem cells are found (Topping & Clifton 2001) (Section 1.5).

1.4.2.7 Physical activity, body mass index and CRC

Observational evidence has suggested that body mass index (BMI) and levels of physical activity (occupational, household, recreational activity) play a role in the development of CRC. Higher body fatness and abdominal fatness increase CRC risk (WCRF 2007, Vrieling & Kampman 2010). The mechanistic link between raised adiposity and CRC remains uncertain but increased BMI is

associated with insulin resistance which results in hyperinsulinaemia and hyperinsulinaemia may induce cell division (Godsland 2010). An increased BMI is also associated with chronic low grade inflammation as a result of the release of proinflammatory cytokines by adipose tissue which may have a carcinogenic effect in the colon (van Kruijsdijk et al. 2009). Ben et al. (2012) performed a meta-analysis to investigate the effect of BMI on CRC risk and demonstrated that a 5 unit increase in BMI was associated with a 19% increased risk of CRC.

Higher levels of physical activity are associated with reduced risk of CRC and may be protective against CRC. There is evidence to suggest a dose-response effect, with frequency and intensity of physical activity inversely associated with CRC risk (Boyle & Langman 2000, WCRF 2007, Hagger & Boushev 2009).

1.4.2.8 Smoking and CRC

Cigarette smoking is a risk factor for CRC development and is the most important avoidable risk factor for cancer in the UK (Zisman et al. 2006, Tsong et al. 2007, Hagger & Boushev 2009). Cigarette smoking may be causally linked to CRC by exposure of the colonic epithelium to carcinogenic compounds in the cigarette smoke via the systemic circulation or from direct exposure from their ingestion (Chan & Giovannucci 2010). There is a: (i) 38% increase in CRC risk for an increment of 40 cigarettes/day; (ii) 20% increase in CRC risk for an increment of 40 years duration; (iii) 51% increase in CRC risk for an increment of 60 pack years; and (iv) 4% decrease in CRC risk for a delay of 10 years in smoking initiation (Chan & Giovannucci 2010).

Cigarette smoking is important in both the formation and growth rate of adenomatous polyps which is demonstrated by an earlier average age of onset of CRC and the presence of larger polyps in smokers (Zisman et al. 2006, Tsong et al. 2007, Botteri et al. 2008). There is a time lag of approximately 30-40 years between the exposure to carcinogenic compounds from cigarette smoking and the development of CRC (Giovannucci et al. 1994). The incidence

of CRC is also higher than normal in previous cigarette smokers suggesting that the carcinogenic compounds in cigarette smoke may be important in the initiation of CRC (Snowden 2009).

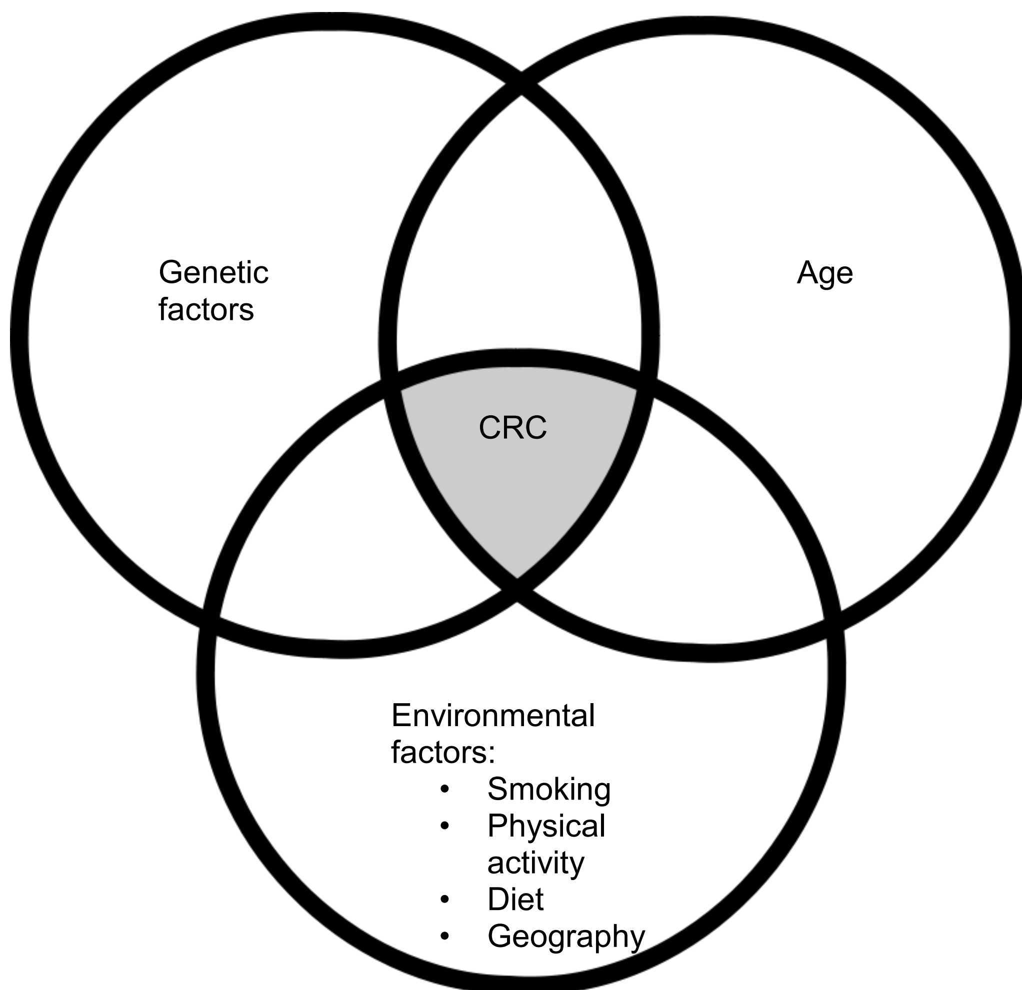
Interestingly, smoking protects against the acute exacerbation of UC (Molodecky & Kaplam 2010, Cosnes et al. 2011, Danese & Fiocchi 2011). The mechanism(s) by which this occurs requires further investigation. It has been suggested that cigarette smoke compounds may: alter gut permeability; modify gut motility; alter mucosal blood flow; increase the production of colonic mucus; and/or change the levels of circulating cytokines (Birrenback & Bocker 2004). Individuals with more severe forms of UC or with frequent exacerbations of the disease are at an increased risk of CRC (Kewenter et al. 1978, Hendriksen et al. 1985, Gillen et al. 1994). Therefore it seems contradictory that cigarette smoking is also a significant factor in the development of CRC. This would suggest that multiple factors play a role in the development and risk of CRC.

1.4.3 Aetiology of CRC: Conclusions

CRC aetiology is complicated because many of the aetiological factors are correlated. Multiple epidemiological studies have investigated the effects of dietary choices, BMI and physical activity on the development of CRC. These studies are subject to confounding e.g. one dietary choice may be associated with another or various dietary components may interact with one another. It is also possible that an individual opting for a healthier diet may also have a healthier lifestyle with more physical activity, decreased alcohol intake and is less likely to smoke cigarettes.

Several diverse factors contribute to the increased risk and development of CRC including the environment, age, genetics, cigarette smoking, physical activity and diet (Figure 1.7). With the exception of hereditary CRC, there does not appear to be any one factor that is causative for CRC. Therefore there are likely to be different pathways that results in development of CRC.

Figure 1.7 Factors that play a role in the development of sporadic colorectal cancer



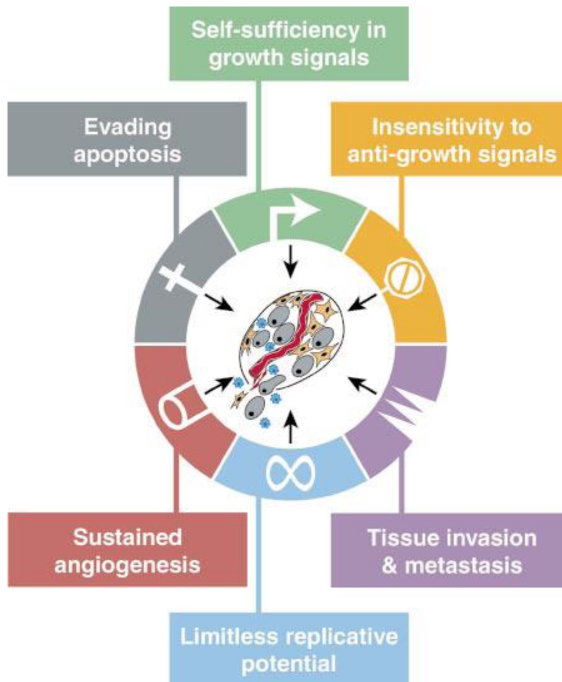
1.5 CRC development

Cancer is a disease involving dynamic changes in the genome (Vogelstein & Kinzler 1993). Cancer is a multistep process in which a normal human cell is transformed progressively into a malignant cell by multiple genetic and epigenetic alterations that disrupt the regulatory controls on cell proliferation, homeostasis and cell death (Vogelstein & Kinzler 1993). Disruption of normal cell proliferation and/or homeostasis and/or cell death may occur by one or more alterations allowing the cell to acquire unique molecular, biochemical and cellular traits including self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan & Weinberg 2011) (Figure 1.8). Each of these changes represents the successful overriding of a normal cell's anti-cancer defence mechanisms.

CRC arises from cells within the single layer of columnar epithelial cells which forms the barrier between the gut lumen and the inside of the body (van der Flier & Clevers 2009). Within this layer of epithelial cells, colorectal stem cells are found at the base of colonic crypts (invaginations of the colorectal epithelium). CRC stem cells proliferate and give rise to the cells that line the colonic crypt (Figure 1.9 (A)). Damage to these colorectal stem cells gives rise to CRC (Humphries & Wright 2008).

Pathological studies have demonstrated that a normal cell transforms into invasive CRC via a series of pre-malignant states which become more frequent with age (Vogelstein & Kinzler 1993). This suggests there are multiple genetic and/or epigenetic changes that occur in the development of CRC with each genetic and/or epigenetic adaptation conferring a growth advantage that allows the progressive change from a normal cell to a cancerous cell (Vogelstein et al. 1988). The fact that there are multiple intermediate steps in the pathway from a normal cell to cancer provides multiple possible steps that could potentially be "intercepted" and prevented or reversed to halt the progression of a normal cell to cancer.

Figure 1.8 Acquired capabilities of cancer (Hanahan & Weinberg 2011)



Self-sufficiency in growth signals: Cancer cells alter exogenous growth signals and the way in which exogenous growth signals are transferred across cell membranes.

Insensitivity to anti-growth signals: Multiple anti-growth signals maintain cellular quiescence and tissue homeostasis in normal tissue. Uncontrolled proliferation results from the insensitivity of cancer cells to growth-inhibitory signals.

Evading apoptosis: Acquired resistance against programmed cell death is a hallmark of cancer. An apoptotic program is present in a latent form in all cell types. Once triggered, it results in programmed cell death. Cancer cells are able to "switch off" the activation of programmed cell death.

Limitless replicative potential: Cancerous cells are immortalized, having acquired limitless replicative potential during cancer development.

Sustained angiogenesis: Cell function and survival is dependent upon oxygen and nutrient supply via blood vessels.

Tissue invasion and metastasis: The capability for invasion and metastasis enables cancer cells to colonize sites distant from its primary location. Successful invasion and metastasis depend upon all five acquired hallmark capabilities of cancer cells already described.

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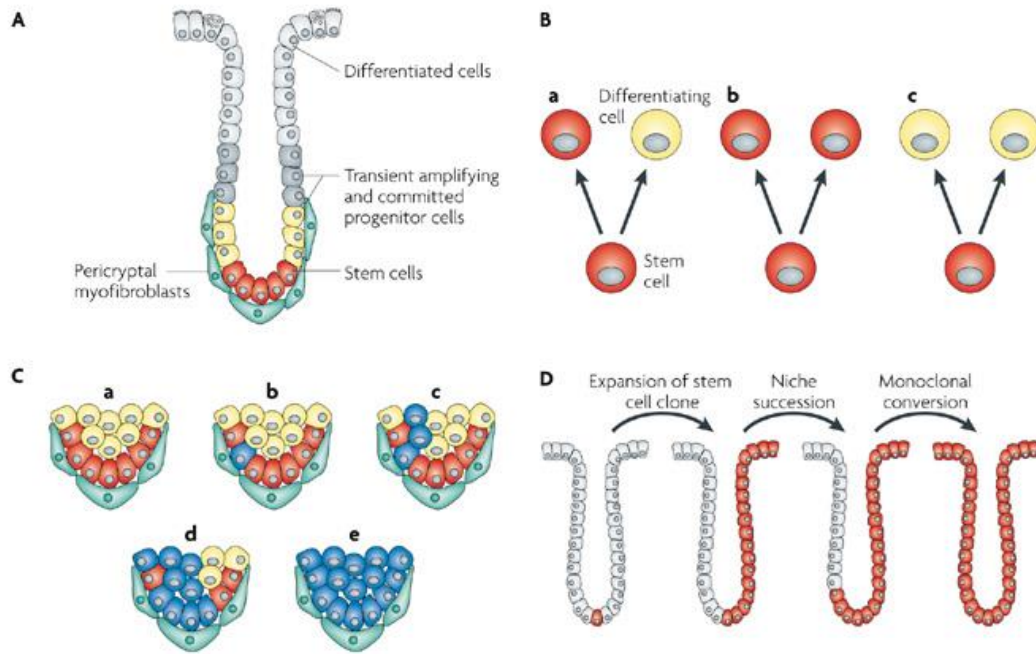
Figure 1.9 The colonic crypt (Humphries & Wright 2008)



(A) Stem cells reside at the base of colonic crypts where they communicate with the cells within the colonic niche.

(B) Types of stem cell division: (a) asymmetric stem cell division results in the production of one differentiated progenitor cell and one identical stem cell; (b) "lineage expansion" – symmetric stem cell division results in the production of two identical stem cells; (c) "lineage extinction" – symmetric stem cell division results in the production of two differentiated cells.

(C) Development of CRC: (a) colonic crypt niche houses the stem cells; (b) a stem cell (blue) develops a mutation; (c) asymmetric division results in the production of clones of the mutated cell amongst the non-stem cells lining the colonic crypt; (d) the niche is colonized as further clones of the mutant stem cell are produced; (e) the mutant stem cell dominates the niche.

(D) Clonal conversion: the progeny of a mutant stem cell replaces all other cells in the crypt.

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1.5.1 Stem cells and CRC

Colorectal stem cells are characterized by their ability to self-renew throughout the lifetime of the individual (Radtke & Clevers 2005, Boman et al. 2007). The accumulation of mutations and other genomic damage, including epigenetic

changes, within colorectal stem cells causes CRC (Humphries & Wright 2008, Salama & Platell 2009) (Figure 1.9 (C) and (D)).

Colorectal stem cells within the base of the colonic crypt divide to produce transit amplifying cells which, after a small number of rounds of proliferation, undergo differentiation to provide a continuous supply of the cell lineages required for normal crypt function including colonocytes, goblet cells and enteroendocrine cells (Humphries & Wright 2008). These cell lineages migrate towards the surface of the colonic crypt where they die or are sloughed into the colonic lumen within 4-8 days (Stappenbeck et al. 1998, Radtke & Clevers 2005). The stem cell niche is the microenvironment that houses the stem cell and controls its functions (Spradling et al. 2001, Humphries & Wright 2008).

There are two types of stem cell division: (i) symmetrical stem cell division and (ii) asymmetrical stem cell division (Humphries & Wright 2008, Salama & Platell 2009) (Figure 1.9 (B)). Symmetric stem cell division is the division of a stem cell to produce either two stem cells or two differentiated cells. If two stem cells are produced, symmetric stem cell division results in “lineage expansion” (Figure 1.9 (B:b)) whereas if two differentiated cells are produced, the result is “lineage extinction” (Figure 1.9 (B:c)). Symmetric division of CRC stem cells is essential in achieving exponential numbers of tumour cells (Boman et al. 2007).

Asymmetric stem cell division results in the production of one progenitor cell and a stem cell identical to the parental cell which allows for both the maintenance of the stem cell population and the production of a progenitor cell that may further divide into differentiated cells to allow a constant regeneration of organs and tissues (Humphries & Wright 2008) (Figure 1.9 (B:a)). The new stem cell retains the original strand of DNA to minimize DNA replication errors; the “immortal strand hypothesis” (McDonald et al. 2006).

1.6 Genetics and CRC

Fearon and Vogelstein (1990) described the adenoma-carcinoma sequence, a genetic model for CRC, where multiple genetic alterations are responsible for the stepwise progression from a “normal” cell to dysplastic epithelium to carcinoma (Figure 1.6). The accumulative effect of each genetic alteration may confer a significant growth and/or survival advantage over the “normal” cell; which may subsequently lead to the further development of dysplasia and ultimately CRC (Fearon & Vogelstein 1990). These genetic alterations occur at the stem cell level and 3 classes of genes play a central role in the genetic alterations which result in tumour development i.e. oncogenes, tumour suppressor genes and DNA repair genes (Vogelstein et al. 2000).

Oncogenes have the potential to cause cancer. Under normal conditions, oncogenes promote cell growth by encouraging mitosis (Land et al. 1983, Knudson 1985). Cancer may be the result of abnormal oncogene activation which may occur by a variety of mechanisms including (i) oncogene mutation resulting in increased activity or a loss of the usual control mechanisms; (ii) defective protein expression leading to increased concentration of the protein encoded by the oncogene and (iii) defective gene expression resulting from chromosomal translocation (Hanahan & Weinberg 2000). Aberrantly activated oncogenes result in exaggerated growth signals, causing survival of malignant cells and their resistance to anti-growth signals (Hanahan & Weinberg 2000, Vogelstein et al. 2000).

Tumour suppressor genes act as “stop” signals halting the progression of a cell through the cell cycle or promoting apoptosis. When the expression of tumour suppressor genes is abnormal, normal inhibitory control is lost and the cell may progress to cancer (Vogelstein et al. 2000).

Cells cannot function if DNA damage corrupts the integrity and accessibility of essential information in the genome. The majority of DNA damage affects the

primary structure of the DNA double helix (Lodish et al. 2004). Damaged DNA may be “repaired” by DNA repair genes and cells use unmodified complementary strands of DNA to recover its original information. DNA repair genes then act by restoring the original spatial configuration of the DNA helix by binding to the site of damage and inducing other molecules to bind and form a complex that enables repair to occur (Watson et al. 2004). DNA repair genes are only able to “repair” DNA that has been structurally damaged (Dinant et al. 2008).

Mutations to DNA repair genes render them unable to recognise and repair damaged DNA and as a result, the damaged cells may (i) enter a state of dormancy; (ii) die by apoptosis or (iii) divide in an unregulated fashion. Unregulated cell division may lead to the development of cancer (Hanahan & Weinberg 2000).

1.6.1 Genetic Alterations and CRC

The earliest detectable macroscopic change to the normal colorectal cell that suggests it may progress to CRC is the ACF. ACF are clusters of mucosal cells with an enlarged or thicker layer of epithelia that surround the normal crypt cells (Bird 1987, Alrawi et al. 2006). Further genetic alterations in ACF are needed for progression to adenomatous polyps and CRC (Alrawi et al. 2006).

1.6.1.1 Adenomatous polyposis coli

APC is a tumour suppressor gene located on chromosome 5q21-q22 (Bodmer et al. 1987). *APC* mutations or allelic losses of 5q are present in 40-80% of CRC (Vogelstein et al. 1988, Miyoshi et al. 1992, Powell et al. 1992, Miyaki et al. 1994), occur early in the adenoma-carcinoma sequence and are the first step in the initiation of CRC (Bodmer et al. 1987). This results in the progression from a normal colon cell to ACF (Figure 1.6). The *APC* gene is responsible for CRC resulting from FAP (Bodmer et al. 1987). *APC* mutation is a very important initiation step that sets a normal colonocyte onto a pathway that may potentially

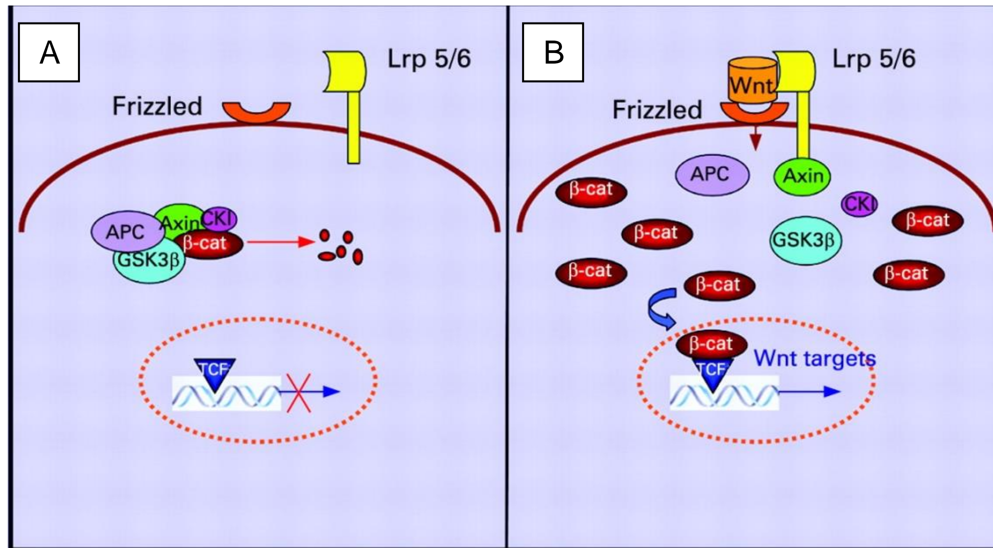
lead to adenomatous polyp or CRC development. Therefore, individuals with FAP, who inherit a mutant form of the *APC* gene, are predisposed to the accelerated development of adenomatous polyps and CRC (Burt 2000, Fearnhead et al. 2002).

The *APC* gene produces a large multifunctional protein that interacts with many proteins, most importantly β-catenin and glycogen synthase kinase (GSK) 3β (Rubinfeld et al. 1996, Kaplan et al. 2001) to form a complex which regulate intracellular β-catenin concentrations through the phosphorylation of β-catenin which signals β-catenin degradation (Munemitsu et al. 1995) (Figure 1.10 (A)).

Mutated *APC* may result in no APC protein being formed or may code for a protein that is unable to bind β-catenin or GSK 3β. In such cases, β-catenin is no longer degraded, but instead accumulates within the cytoplasm (Munemitsu et al. 1995, Ricci-Vitiani et al. 2008) and translocates to the nucleus where it binds to the T-cell factor (TCF) family of transcription factors and activates gene transcription (Molenaar et al. 1996) (Figure 1.10 (B)).

The effects of mutant *APC* can be observed in colonic stem cells residing at the base of colonic crypts. When a stem cell divides, its daughter cells proliferate further and migrate up the crypt towards the surface. The APC protein usually acts from the mid-crypt level upwards resulting in β-catenin degradation and suppression of cell proliferation. Mutated *APC* results in cells that are independent of physiological signals controlling β-catenin activity (insensitivity to growth inhibitory signals (Figure 1.8)). These aberrant cells continue to migrate to the crypt surface where ACF results (Bach et al. 2000, Sansom et al. 2004).

Figure 1.10 The actions of APC within the WNT signalling pathway (Ricci-Vitiani et al. 2008)



(A) In the normal colon epithelial cell, the APC-β-catenin-GSK 3β complex results in the binding of β-catenin; causing β-catenin degradation.

(B) Mutant APC is unable to bind β-catenin or GSK 3β; β-catenin is no longer degraded, instead it accumulates and translocates to the nucleus where it is able to activate gene transcription.

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1.6.1.2 *K-RAS*

K-RAS is an oncogene located on chromosome 12p12.1 (Shuangshoti 2011). *K-RAS* mutations occur early in the adenoma-carcinoma sequence and are present in 35-42% of CRC (Bos et al. 1987, Forrester et al. 1987, Vogelstein et al. 1988, Andreyev et al. 1998). *K-RAS* mutations are responsible for the progression of ACF to adenoma and for the further development of an adenoma to become more dysplastic (Fearon & Vogelstein 1990) (Figure 1.6).

The *K-RAS* oncogene produces a protein involved in the signal transduction of regulatory pathways. In its non-mutated form, *K-RAS* ensures that normal cell proliferation and differentiation occurs. In its active state, K-RAS is bound to guanosine 5'triphosphate (GTP) protein in the cell membrane. K-RAS becomes

inactive when GTP is hydrolysed by GTPase to guanosine 5'diphosphate. GTPase activity is reduced when K-RAS becomes mutated, leading to constitutively active K-RAS protein; abnormal cell proliferation and differentiation follows (Bos et al. 1989, McCormick 1989, Bourne et al. 1990).

1.6.1.3 Chromosome 18q loss

Chromosome 18q is lost in 70% of CRC (Vogelstein et al. 1988, Boland & Goel 1995). The tumour suppressor genes *SMAD2* and *SMAD4* are carried on chromosome 18 and play a role in the development of CRC; in particular, loss of *SMAD2* and *SMAD4* increases the degree of dysplasia within an adenoma (Leslie 2002) (Figure 1.6). *SMAD2* and *SMAD4* are intracellular mediators of the inhibitory transforming growth factor (TGF) β signalling pathway which regulates cell growth, differentiation and cell death (Heldin et al. 1997, Duff & Clarke 1998).

1.6.1.4 *P53*

P53 is a tumour suppressor gene which is located on the short arm of chromosome 17 (Vogelstein et al. 1988). 17p allelic loss or *P53* mutation occurs in 50-75% of CRC (Vogelstein et al. 1988, Kaklamanis et al. 1993, Darmon et al. 1994, Boland et al. 1995, Hardingham et al. 1998, Kaserer et al. 2000). *P53* mutation is implicated in the progression of an adenoma and its malignant transformation to adenocarcinoma (Vogelstein et al. 1988, Fearon & Vogelstein 1990, Boland et al. 1995) (Figure 1.6).

When there is DNA damage, *P53* normally blocks cell proliferation and stimulates DNA repair. If this DNA repair is unsuccessful, *P53* promotes cell death. Mutated *P53* causes defective cell proliferation and the accumulation of damaged DNA as a result of the defective cell's ability to evade apoptosis (Lane 1992, Carder et al. 1995) (programmed cell death (Figure 1.8)).

1.6.1.5 Chromosome instability

The genetic alterations described: *APC* mutations, *K-RAS* mutations and chromosome 18q loss, are each implicated in CRC development via the chromosome instability pathway. The chromosomal instability pathway, which accounts for 85% of sporadic CRC, (Grady & Markowitz 2000, Worthley & Leggett 2010) (Figure 1.11) occurs when there are increased rates of chromosome mis-segregation during mitosis. This leads to an inability to maintain the correct chromosomal complement via one of two mechanisms: (i) Mis-segregation which leads to aneuploidy as a result of loss or gain of whole chromosomes and (ii) unbalanced structural rearrangements which result in the loss of, and/or gain of, chromosomal regions (Pino & Chung 2010). Extensive loss of heterozygosity is a hallmark of chromosome instability and is the loss of either the maternal or paternal allele (Lengaur et al. 1998).

1.6.1.6 Microsatellite instability (section 1.4.1.1)

The MSI pathway accounts for 10-15% of sporadic CRC (Aaltonen et al. 1998, Salovaara et al. 2000, Samowitz et al. 2001, Hampel et al. 2008, de la Chapelle & Hampel 2010) (Figure 1.11). Defective MMR resulting in MSI may occur at any point along the adenoma-carcinoma sequence (Figure 1.6). MSI is a measure of defective MMR. Normally, the DNA MMR system eliminates mismatch of nucleotide insertions and deletions resulting from strand slippage occurring during DNA synthesis. MMR proteins recognise these errors and correct them. The mismatched nucleotide bases are removed leaving a gap in the DNA double helix and DNA repair polymerase fills in the gap by adding new correct bases. When defective MMR function is present, the nucleotide repeat sequences are at risk of replication errors (Chung & Rustgi 1995).

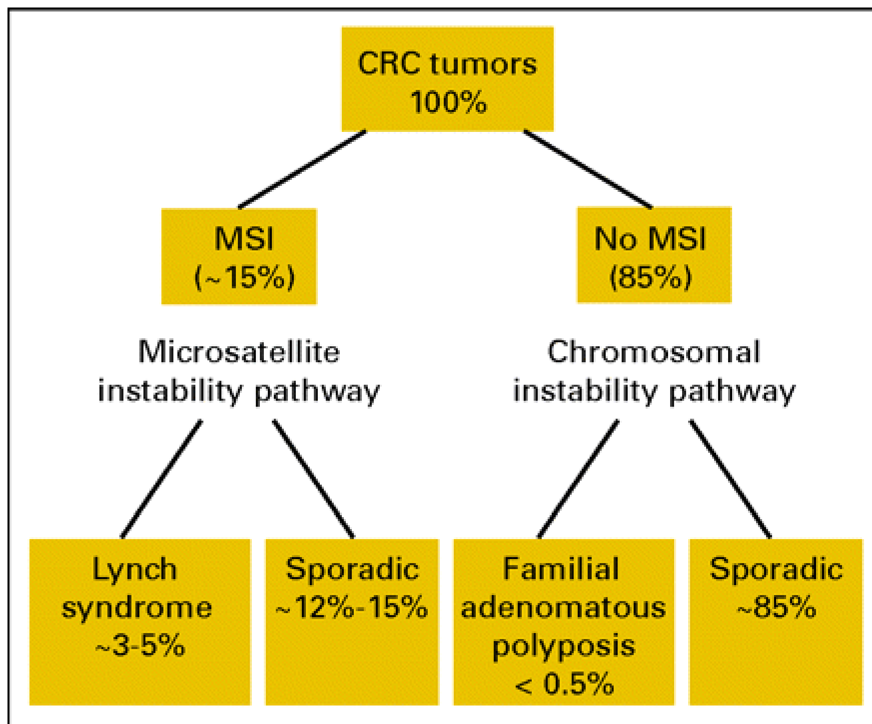
CRC with MSI have distinct features. They tend to arise in the proximal colon, have a poorly differentiated, mucinous or signet ring appearance and have a worse prognosis in comparison with CRC without MSI (Boland & Goel 2010). To classify whether or not a CRC expresses MSI, a panel of five specific microsatellite markers have been recommended for MSI evaluation. This was the outcome of the National Cancer Institute Workshop on MSI held in

Bethesda in 1996 to promote consistency amongst studies evaluating MSI (Boland et al. 1998). The five microsatellite markers are three dinucleotide repeats (D2S123, D5S346, D17S250) and two mononucleotide repeats (BAT26, BAT25). MSI is deemed to be present if 40% or more of the markers tested are unstable (at least 2 of the 5 marker panel) and these CRCs are termed MSI-high (MSI-H). The remainder of CRCs with no MSI and are described as microsatellite stable (MSS). The minority of CRCs that display low levels of MSI (0-40% unstable markers) are MSI-low (MSI-L) (Boland et al. 1998) (Table 1.3).

Table 1.3 Classification of MSI (de la Chapelle & Hampel 2010)

MSI classification	**Proportion of unstable markers**	**Positive markers in the 5 marker panel**
MSI high	≥40%	≥2/5
MSI low	<40%	1/5
Microsatellite stable	0%	0/5

Figure 1.11 The classification of colorectal cancers (de la Chapelle & Hampel 2010)



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1.7 WNT signalling pathway

WNT proteins are a family of 19 glycoproteins that activate WNT signalling pathways and play a role in tissue homeostasis, cell growth and differentiation. WNT signalling pathways can be grouped as “canonical” and “non-canonical” pathways (Katoh & Katoh 2007, Bovolenta et al. 2008).

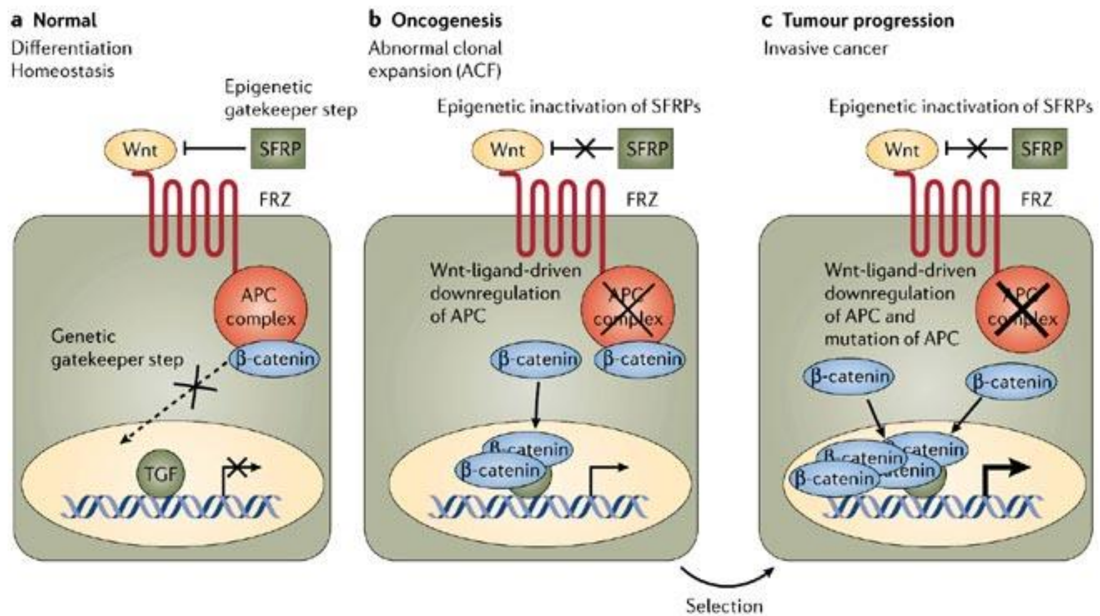
The canonical pathway is involved in cell fate determination (Katoh & Katoh 2007). This results from canonical WNT signals that are transduced to the β-catenin signalling cascade via binding of WNT proteins to frizzled membranes receptors (FZ) and LRP5/LRP6 co-receptors (Bhanot et al. 1996, Katoh & Katoh 2007). LRP5 and LRP6 are low density lipoprotein related protein receptors that assist FZ in binding WNT proteins (Bovolenta et al. 2008). In the normal cell, WNT proteins bind to cell surface (frizzled) receptors which activates the Dishevelled (DVL) family proteins. The DVL family proteins are signal

transduction proteins. The DVL family protein-WNT receptor complex inhibits APC. As discussed above, APC regulates intracellular β-catenin levels by promoting its degradation (Munemitsu et al. 1995) (Figure 1.12(A)). Inhibition of APC allows β-catenin to accumulate within the cell and to enter the nucleus, where it interacts with the TCF family of transcription factors to promote gene transcription and thus modify cell behaviour (Baylin & Ohm 2006) (Figure 1.12 (B)) (Section 1.6.1.1). The non-canonical pathway plays a role in the control of cell movement and tissue polarity. The non-canonical WNT signals are also transduced through FZ to affect cytoskeletal reorganisation during metastasis (Katoh & Katoh 2007).

1.8 Secreted frizzled related proteins

Secreted frizzled related proteins (SFRPs) are a family of 5 secreted glycoproteins structurally related to the cysteine rich domain of FZ of the WNT protein (Bovolenta et al. 2008). They are tumour suppressor genes that play a role in controlling cell growth and differentiation (Suzuki et al. 2004). *SFRPs* mediate cell communication pathways involving the canonical WNT signalling pathway (Figure 1.12) through binding both WNT ligands and FZ. They inhibit the WNT signalling pathway by competition with FZ for WNT ligands or by direct formation of non-signalling complexes with FZ (Baylin & Ohm 2006, Bovolenta et al. 2008).

Figure 1.12 WNT signalling pathway and the actions of *SFRP* (Baylin & Ohm 2006)



(A) In the normal colon epithelial cell, SFRP competes with WNT proteins for binding to the FZ. When WNT signalling is inactive, the APC complex degrades β-catenin.

(B) When SFRP expression is lost, WNT signalling is activated. This inactivates the APC complex, which allows β-catenin to accumulate in the cytoplasm and nucleus, where it activates gene transcription.

(C) Persistent activation of the WNT signalling pathway. These aberrant cells are selected because of their survival and proliferative advantages.

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Aberrant WNT pathway signalling is an early event in CRC development (Fodde et al. 2001, Suzuki et al. 2004). In the normal colon epithelial cell, *SFRP* competes with WNT proteins for binding to FZ. When WNT signalling is inactive, the APC complex degrades β-catenin (Figure 1.12(A)). When SFRP expression is lost, WNT signalling is activated which inactivates the APC complex allowing β-catenin to accumulate in the cytoplasm and then to translocate to the nucleus, where it activates transcription (Baylin & Ohm 2006) (Figure 1.12(B)).

There is relatively little literature on the various members of the *SFRP* family. It is known that *SFRP3* has a different structure to the other four members (Hoang et al. 1996, Bovolenta et al. 2008). *SFRP1, 2, 4* and *5* are similar in structure and are thought to play a role in CRC development by negatively regulating the WNT signalling pathway (Rattner et al. 1997, Suzuki et al. 2004, Bovolenta et al. 2008). Huang et al. (2010) showed that there was no difference in the expression of *SFRP2* between CRC and normal tissue. In the same study, Huang et al. (2010) also showed that *SFRP4* was overexpressed in CRC in comparison with normal tissue. The difference in study results for the different members of the *SFRP* family suggests that each member of the *SFRP* family may work in different ways. Further studies are needed in order to clarify this.

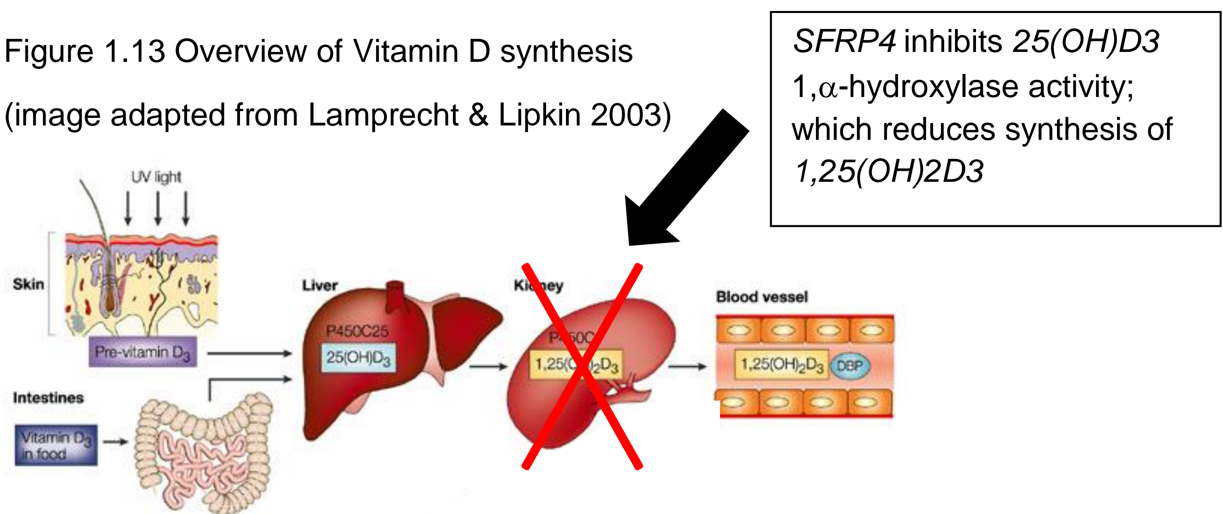
Promoter hypermethylation of *SFRP1* and *SFRP2* have been observed from the earliest stages of CRC development. However, *Sfrp* null mice do not show increased incidence of spontaneous CRC formation which implies that additional genetic alterations may be needed for CRC development (Esteve & Bovolenta 2010). This supports Fearon and Vogelstein's (1990) hypothesis that multiple genetic mutations are required in order for CRC to occur.

Hypermethylation of the genes coding for SFRP1 and SFRP2 may lead to suppression of *SFRP1* and *SFRP2* gene expression respectively, which may silence their tumour suppressor actions, leading to constitutively active WNT signalling and CRC formation (Suzuki et al. 2004). This has been reported in colorectal adenomas and premalignant ACF, the earliest lesion in the pathway from normal colon mucosa to CRC. The frequency of hypermethylation of *SFRP1* and *SFRP2* is increased from normal mucosa to hyperplastic polyp to adenoma (Wang & Tang 2007).

1.8.1 *SFRP4*

The *SFRP4* gene is located on chromosome 7 (NCBI 2013). SFRP4 plays an important role in phosphorus homeostasis by inhibiting the synthesis of vitamin D, and thus intestinal absorption of inorganic phosphate (Berndt et al. 2003, Berndt & Kumar 2009). SFRP4 specifically inhibits the activity of 25-hydroxyvitamin D3 (*25(OH)D3)* 1,α-hydroxylase which thus reduces the synthesis of the active form of the vitamin i.e. 1α,25-hydroxyvitamin D3 (*1,25(OH)2D3)* (Berndt et al. 2003) (Figure 1.13). The mechanisms by which it does this require further research. SFRP4 also has an important role in soft tissue homeostasis because inorganic phosphate is required for basic cell processes including nucleic acid synthesis and energy metabolism (Berndt & Kumar 2009).

Figure 1.13 Overview of Vitamin D synthesis (image adapted from Lamprecht & Lipkin 2003)



The active form of vitamin D, 1,25(OH)2D3 is formed in a multistep pathway: Following cutaneous production or intestinal absorption from dietary sources, inactive vitamin D3 is transported to the liver, where it undergoes hydroxylation to produce 25-hydroxyvitamin D3 (25(OH)D3). Further hydroxylation by 25(OH)D3 1,α-hydroxylase in the kidneys produces 1,25(OH)2D3 (the active form) which is lipophilic and is transported in the blood to target cells bound to vitamin D binding protein (DBP).

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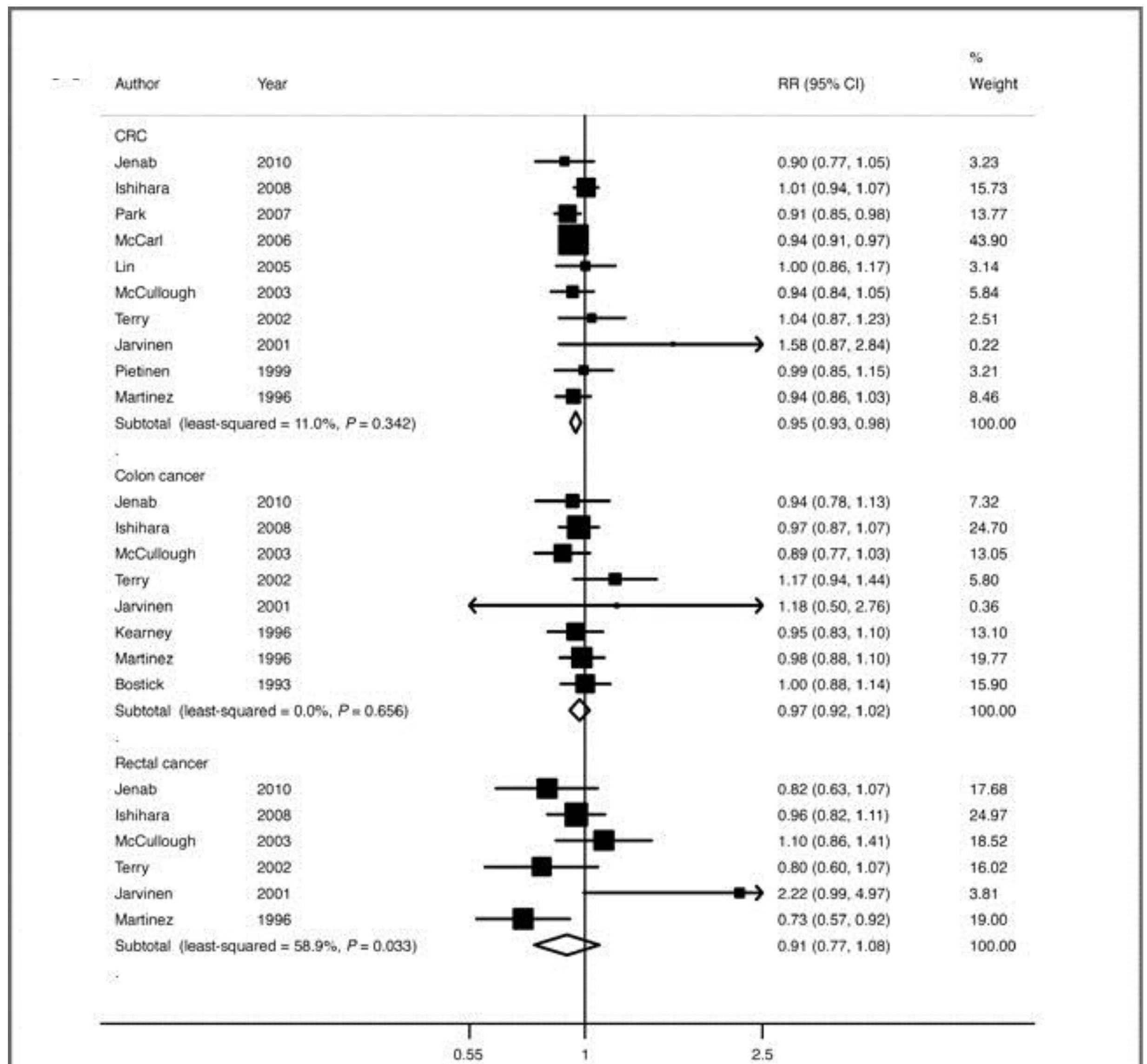
Interestingly, low levels of vitamin D intake, and low vitamin D status (usually measured as circulating concentrations of 25-hydroxyvitamin D3), have been associated with increased risk of CRC (Table 1.1). Touvier et al. (2011) performed a systematic review on vitamin D intake, 25-hydroxyvitamin D3 status, vitamin D receptor polymorphisms and CRC risk. They found a statistically significant inverse association between dietary vitamin D and CRC risk for an increase of 100IU/day based on data from 10 independent studies (Figure 1.14). Vitamin D is a major regulator of gene expression and may lower CRC risk by reducing cell proliferation and inducing apoptosis in colon cancer cells (Lamprecht & Lipkin 2003, Palmer et al. 2003, Larriba et al 2008, Krishnan & Feldman 2011). The active form of vitamin D3 inhibits the accumulation of β-catenin by facilitating its degradation (Kim & Milner 2007).

Figure 1.14 Dose-response meta-analysis of dietary and total vitamin D intake, circulating 25-hydroxyvitamin D3 and CRC risk (for an increase of 100IU/day) (Touvier et al. 2011)



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Since abnormal expression of *SFRP1* and *SFRP2* genes is implicated in CRC development, abnormal *SFRP4* gene expression may also be implicated in CRC development (Suzuki et al. 2004, Wang & Tang 2007, Esteve & Bovolenta 2010, Huang et al. 2010). Whilst the underlying mechanism for this proposed link is uncertain, it is possible that this may involve vitamin D. Abnormal *SFRP4* gene expression prevents the production of the active form of vitamin D3 and as summarised above, low vitamin D intake and low vitamin D status are

associated with increased CRC risk (Berndt et al. 2003, Berndt & Kumar 2009, Touvier et al. 2011). Further research is needed to ascertain whether defective *SFRP4* gene expression causes an increased risk of CRC by preventing the production of the active form of vitamin D.

1.9 Epigenetics

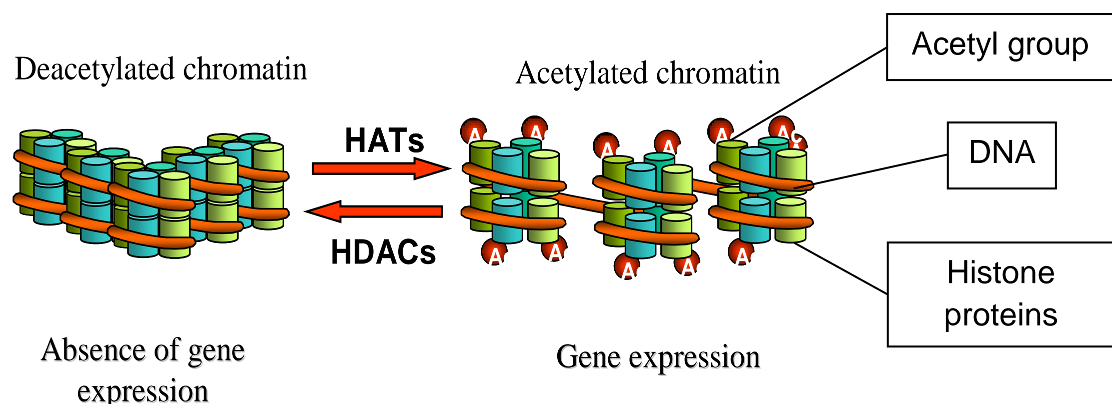
Epigenetic alterations leading to aberrant expression of tumour suppressor genes and oncogenes play a role in the multistep pathway that initiates changes in the normal cell and causes its progression to CRC (Jones & Laird 1999, Jones & Baylin 2002, Herman & Baylin 2003). Epigenetics is the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being (Waddington 1942) . Epigenetic patterns are biomarkers that can be used to identify pathways to disease, such as CRC; and therefore possible treatments (Berg & Soreide 2011). Epigenetic epidemiology can illuminate the mechanisms by which genes are regulated and the consequences of changes in epigenetic signals to be identified (Jones & Baylin 2002, Ushijima 2005, Baylin & Ohm 2006).

Epigenetic inheritance is defined as cellular information, other than the DNA sequence, that is heritable from one cell to its daughter cell during cell division. Importantly, epigenetic marks and molecules regulate gene expression (Feinberg & Tycho 2004). There are three ways in which epigenetic marks can be inherited viz. DNA methylation, genomic imprinting and histone modification (Feinberg & Tycho 2004).

Genomic imprinting refers to those genes that are silent when maternally inherited but expressed with paternally inherited, and vice versa (Ubeda 2008). Genomic imprinting is an epigenetic process that involves DNA methylation and histone modifications which results in monoallelic gene expression without altering the genetic sequence (Bartolomei & Ferguson-Smith 2011).

Histones are proteins found within cell nuclei that package the DNA into nucleosomes. Histones play a role in gene regulation via a complex post-translational modification of the N-terminus tails including covalent addition (or removal) of acetyl, methyl, phosphate and ubiquitin groups (Lugor 2001, Lachner et al. 2003, Wong et al. 2007, Bannister & Kouzarides 2011). The patterns of histone modifications alters their interaction with DNA and nuclear proteins which alters cell function e.g. through regulating gene expression and through allowing access for the DNA repair machinery. The balance of the opposing actions of histone acetyltransferase (HAT) (which acetylates histones) and histone deacetylases (HDAC) (which remove acetyl groups from histones) regulates gene expression via chromatin modification (Figure 1.15). Removal of acetyl groups from histones results in chromatin condensation and transcriptional inactivation of the associated DNA which can contribute to the suppression of tumour suppressor gene expression and enhanced tumorigenesis. Histone acetylation allows transcription by weakening the association of the histone with DNA and permits binding by transcription factors and other components of the transcription machinery (Fukeda et al. 2006, Sharma et al. 2010, Bannister & Kouzarides 2011).

Figure 1.15 Acetylation of histones and gene expression (Gillet et al. 2007)



Histone acetyl transferase (HAT) adds acetyl groups to histones and histone deacetylase (HDAC) removes acetyl groups from histones.

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1.10 DNA methylation

DNA methylation is an important epigenetic mark and is the addition of a methyl group at the 5' position on a cytosine residue located 5' to a guanosine residue in a CpG dinucleotide. Within the human genome, there are CpG islands which are CpG rich regions of DNA that are often associated with the transcription start sites of genes. Here CpG islands are normally unmethylated (Bird 1986) (Figure 1.16).

The transcription start site of a gene, and therefore the CpG islands, are at the proximal promoter region of a gene (Vavouri & Lehner 2012). CpG islands are normally unmethylated in expressed genes (Bird 1985, Wong et al. 2007, Illingworth et al. 2010) and hypermethylation of the CpG islands within these proximal promoter regions causes transcriptional silencing of genes by interfering with transcription initiation (Bird 2002, Wong et al. 2007).

Epigenetic regulation of gene expression is essential for the normal function of cells and epigenetic marks and molecules are often abnormal in cancerous cells resulting in aberrant gene expression (Jones & Laird 1999, Jones & Baylin 2002, Feinberg & Tycho 2004). Promoter hypermethylation is a mechanism that can suppress gene activity (Figure 1.16) and there appears to be a reciprocal relationship between the density of methylated cytosine residues in promoter regions and the transcriptional activity of the corresponding gene (Wong et al. 2007). Therefore abnormal promoter CpG island hypermethylation may result in transcriptional silencing (Jones & Baylin 2002) (Figure 1.17). Such epigenetic changes may precede genetic changes in premalignant cells or may facilitate the accumulation of further genetic and/or epigenetic changes.

The process of methylation involves methionine, the substrate for s–adenosylmethionine (SAM), which is the methyl group donor for DNA methylation. Dietary folate is converted to the circulatory form 5-methyltetrahydrofolate (5-methylTHF) and this metabolite plays an important role by providing methyl groups for the re-synthesis of methionine from

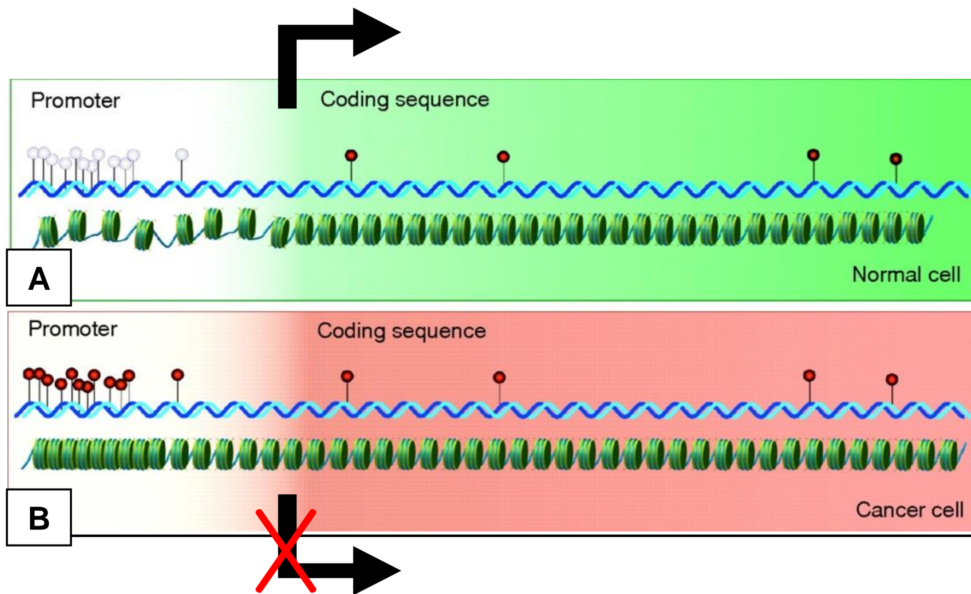
homocysteine. The enzyme methylenetetrahydrofolate reductase (MTHFR) catalyzes the conversion of 5,10-methylenetetrahydrofolate (5,10-methylene THF) to 5-methyl THFwhich is the primary methyl donor for the remethylation of homocysteine to methionine (Krumen & Fowler 2014, Tang et al. 2014). Deficiencies in folate would result in a lack methionine and, therefore of SAM, and this can lead to mis-methylation of DNA (Nijhout & Reed 2014). In addition, alcohol appears to antagonise the processes that allow the production of SAM. Therefore, the availability of methyl groups for SAM production may be affected adversely by high alcohol intake and inadequate intake of dietary folate (Krumen & Fowler 2014).

1.10.1 DNA methylation and CRC

The first epigenetic abnormality identified in cancer was the loss of DNA methylation (hypomethylation) (Feinberg & Tycho 2004). DNA hypomethylation can lead to oncogene activation and chromosome instability (Ehrlich 2002, Gaudet et al. 2003). Epigenetic abnormalities can also lead to hypermethylation which can result in gene silencing and silencing of tumour suppressor genes by promoter hypermethylation will increase CRC risk (Jones & Baylin 2002).

There are at least four types of genetic abnormalities described in CRC: Chromosome instability; MSI; CIMP (DNA hypermethylation of gene promoters containing CpG islands); and global DNA hypomethylation (Pancione et al. 2012) (Figure 1.18).

Figure 1.16 CpG methylation in normal and cancer cells (Wong et al. 2007)



(A) (Green panel): In the normal cell, CpG islands remain unmethylated (white pins); whereas scattered cytosine residues elsewhere are methylated (red pins). In the absence of methylation of this CpG island, DNA in the promoter region is accessible to transcription factors and the gene may be expressed.

(B) (Red panel): A cancer cell shows characteristic CpG island methylation, causing silencing of gene expression.

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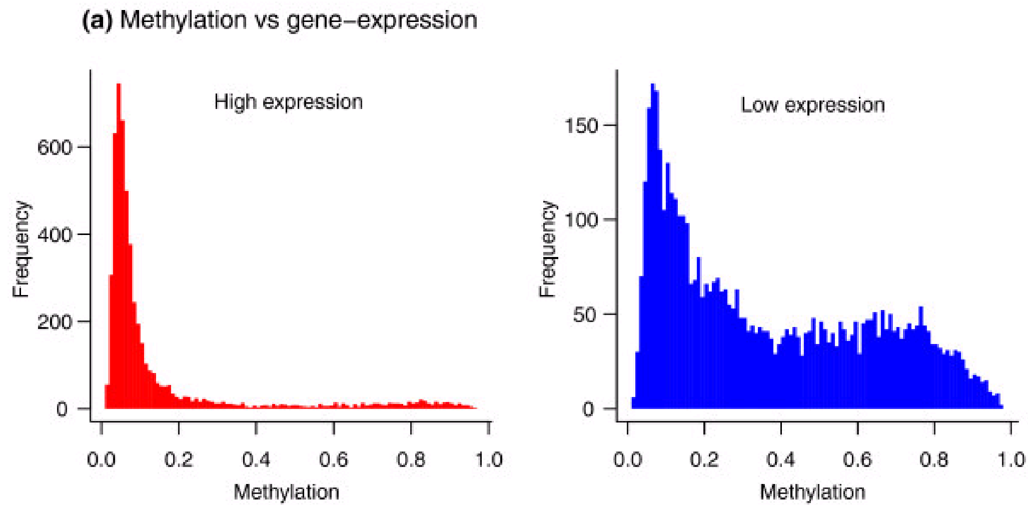
Figure 1.17 The relationship between DNA methylation and gene expression (Bell et al. 2011)



DNA methylation is negatively correlated with gene expression. Methylation levels are low in the top quartile of highly expressed genes (left); and high in the bottom quartile of lowly expressed genes (right); data from 12,670 autosomal genes examined in 77 lymphoblastic cell lines. Gene expression was measured using RNA sequencing.

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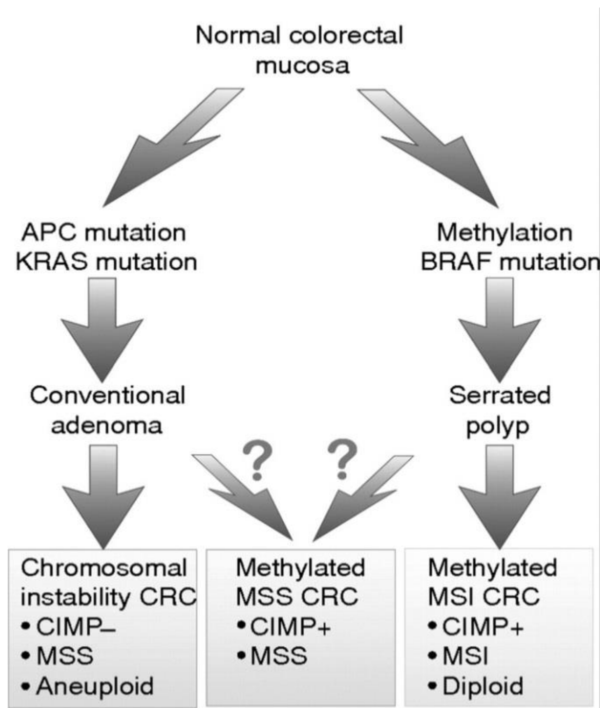
Figure 1.18 Pathways for CRC development (Wong et al. 2007)



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CpG islands within the promoter regions of genes may be methylated which leads to gene silencing (Wong et al. 2007). CIMP defines a group of cancers with a 3-5 fold elevated frequency of aberrant gene methylation. These cancers represent a clinically and aetiologically distinct group of cancers that are characterised by epigenetic instability (Issa 2004).

Cancer associated DNA hypomethylation is as prevalent as cancer-linked hypermethylation (Ehrlich 2002). These two types of epigenetic abnormalities affect different parts of the DNA sequence. DNA hypermethylation tends to occur within CpG islands within the promoter region of genes whereas hypomethylation is observed frequently in the main gene body in cancer (Bird 2002, Ehrlich 2002, Jones & Baylin 2002, Feinberg & Tycho 2004, Wong et al. 2007, Pancione 2012). Global DNA hypomethylation is thought to result in chromosomal instability (Ehrlich 2002, Gaudet et al. 2003).

1.10.2 Reversibility of DNA methylation

Both epigenetic marks and germ-line mutations are heritable from one cell to its daughter; however, unlike germ-line mutations, epigenetic processes such as DNA methylation are potentially reversible. It is possible for gene expression to be re-established by the demethylation of hypermethylated promoter regions offering the potential for therapeutic treatments to be designed to treat cancer or environmental adaptation, for example through diet, to reduce the risk of cancer development (Yoo & Jones 2006, Huang et al. 2010, Ushijima 2010).

Proof of principle of the reversibility of DNA methylation marks can be seen from the action of 5-aza-2-deoxycytidine which inhibits DNA methyltransferase, the enzyme responsible for DNA methylation. 5-aza-2-deoxycytidine is a powerful inhibitor of DNA methylation because it is incorporated into the nucleic acids of dividing cells, where it is able to act as a mechanism-based inhibitor of DNA methyltransferases. Reduction of DNA methyltransferase activity by 5-aza-2-deoxcytidine reduces colorectal adenoma formation (Robertson & Jones

2000, Das & Singal 2004). However, re-expression of the silenced gene in response to 5-aza-2-deoxycytidine is transient and the gene is re-silenced as a result of DNA hypermethylation upon removal of 5-aza-2-deoxycytidine (Bender et al. 1998, Egger et al. 2007). Also, the use of DNA methylation inhibitors may cause inappropriate activation of genes within normal healthy cells because it is not known whether they will be selective for just the hypermethylated cancerous cell.

1.11 The Human Epigenome Project

DNA methylation is an important epigenetic modification that is known to affect various cellular processes and is implicated in the development of various human diseases. The Human Epigenome Project aims to identify, catalogue and interpret genome-wide DNA methylation patterns of all human genes in all major tissues. Methylation combined with genetics and the environment, plays a role in disease aetiology. Differences in gene methylation gives rise to distinct patterns that are believed to be specific for different disease states. Therefore, the degree of DNA methylation is a potential epigenetic marker and this has implications in the diagnosis, treatment and prevention of disease (HEP 2013).

1.12 Epigenetic biomarkers of CRC risk

Hypermethylation of CpG islands is an early event in CRC development and is detectable in the macroscopically normal rectal epithelium of individuals who are at an increased risk of CRC or who have already developed CRC (Baylin & Ohm 2006, Belshaw et al. 2010). Hypermethylation can be measured with a high degree of sensitivity and, therefore, quantification of DNA methylation levels may allow the identification of individuals with early CRC or who may go onto develop CRC (Jones & Baylin 2002). The literature describes differential levels of DNA methylation in those with and without CRC in DNA derived from a variety of biological sources (including rectal tissue and stool samples). The quantification of DNA methylation as an epigenetic biomarker of CRC risk shows promise.

Adult cancers may derive from stem or early progenitor cells and epigenetic regulation of gene expression is essential for the normal functioning of these early cells. Ohm et al. (2007) studied 29 tumour suppressor genes known to be frequently hypermethylated in various cancer cell lines and in primary tumour samples (evidence from a review of the literature and from their previous studies). The epigenetic status (levels of DNA methylation) in both normal embryonic stem (ES) cells and their malignant counterparts (embryonal carcinoma (EC) cells) of these 29 selected genes were compared. The genes which exhibited promoter CpG island DNA hypermethylation in adult human cancer cells tended to remain unmethylated in both ES and EC cells. 13 of the 29 genes studied were hypermethylated in one adult colon cancer line (HCT-116). None of these were hypermethylated in ES cells and only a small fraction was completely methylated in the EC cells (Ohm et al. 2007).

It is suggested that aberrant DNA promoter hypermethylation and subsequent gene silencing in adult cancers is a result of transient silencing of important growth regulatory genes in stem or progenitor cells. Ohm et al. (2007) have shown that genes with frequently hypermethylated DNA in adult cancers usually lack such DNA methylation in normal and neoplastic embryonic cells. Of the 29 genes studied by Ohm et al. (2007), *SFRP1*, *SFRP*2, *SFRP4*, *DAPK*, *GATA*-4 and *GATA*-5 were i) hypermethylated in the adult colon cancer cell line HCT-116, ii) unmethylated in both embryonic stem cells and embryonal carcinoma cells and iii) had notable methylation described in the literature. Therefore *SFRP1*, *SFRP*2, *SFRP4*, *DAPK*, *GATA-4* and *GATA-5* could be potential epigenetic biomarkers of CRC risk. This research project focuses on *SFRP4* as an epigenetic biomarker of CRC.

1.13 Prevention of CRC

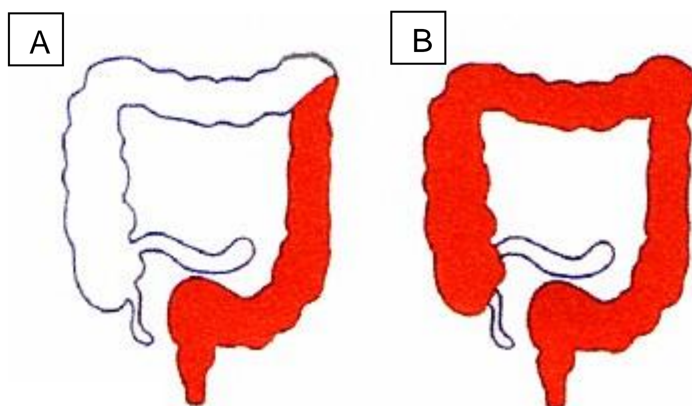
CRC screening programmes are an opportunity to alter the survival of patients with precancerous lesions or early CRC (Moiel & Thompson 2011). The common screening tests for CRC and advanced adenoma are faecal occult blood testing (FOBT) and visualisation of the bowel. Several expert groups,

including the NHS Bowel Cancer Screening Programme (2013) recommend that those who are at average risk should undergo CRC screening from the age of 60 years.

CRC screening aims to prevent deaths from CRC by identifying CRC at a pre-cancerous or early stage to maximise the chance of curative treatment. In addition, it has a role in the secondary prevention of CRC. There are three levels of prevention: (i) Primary prevention employs health promotion activities to prevent the occurrence of CRC (for example, promotion of a healthy diet and more physically active lifestyle); (ii) secondary prevention promotes screening in those at high risk for CRC (for example, regular colonoscopies in those with a history of adenomatous polyps or a familial predisposition for CRC); and (iii) tertiary prevention is directed towards preventing the reoccurrence of CRC once treated or the reduction of CRC related complications (for example, blood transfusions for anaemia) (WHO 2010).

Visualisation of the bowel can be by colonoscopy, sigmoidoscopy or computed tomography colonography (Figure 1.19). Colonoscopy and sigmoidoscopy have the benefit of allowing simultaneous biopsy of the bowel mucosa and removal of premalignant lesions. Colonoscopy is the most effective means of detecting CRC (Strul & Arber 2007).

Figure 1.19 Flexible sigmoidoscopy versus colonoscopy



(A) Flexible sigmoidoscopy visualises the left side of the large bowel.

(B) Colonoscopy visualised the entire bowel.

FOBT is advantageous because it is safe, non-invasive, cheaper than endoscopy and can be undertaken at home without the need for a healthcare professional. FOBT relies on the stool sample coming into contact with blood shed into the colon from a CRC. A limitation of FOBT is that any bleeding into the colon can result in a positive test and therefore all positive FOBT results should be followed up with colonoscopy (Strul & Arber 2007).

DNA-related biomarkers can also be measured in faecal-based assays (Strul & Arber 2007). These assays rely on the mutant DNA, for example *K-RAS, APC,* and *P53,* present in cancerous lesions in the bowel being shed into the gastrointestinal tract and excreted in stool. It has been claimed that such faecal-based DNA assays have a higher sensitivity and no reduction in specificity when compared with FOBT (Alquist et al. 2000, Calistri et al. 2003, Tagore et al. 2003, Imperiale et al. 2004, Strul & Arber 2007) but, at present, there is a lack of robust biomarkers that can predict CRC risk (Song et al. 2004). Currently, tissue biopsies are often taken at endoscopy and processed in the laboratory to look for features of cancer. Theoretically, these tissue biopsies could also be processed to identify the presence of any DNA-related biomarkers of CRC risk but there is insufficient reproducible scientific evidence to support

the regular use of DNA assays for the diagnosis of CRC (Song et al. 2004, Strul & Arber 2007).

1.14 The Alimentary Canal

The alimentary tract runs from the mouth to the anus. Therefore cells from the alimentary tract can be obtained from both the rectum and the mouth, as well as other locations such as the oesophagus, stomach and small intestine. The literature describes a phenomenon known as “field changes”. Field changes are defined as abnormalities in epithelial gene expression affecting the mucosal surface, rendering it vulnerable to neoplasia (Polley et al. 2006, Belshaw et al. 2008). Field abnormalities of the colon may encompass the entire mucosal field, and DNA extracted from any point along the colon has been shown to demonstrate abnormal gene promoter methylation at sites distant to the area of pathology (Polley et al. 2006). Field changes have only been described within the colon for CRC. There is no literature investigating whether the phenomenon of field changes extends to the entire alimentary tract in respect of CRC risk. If such field changes extended throughout the whole GI tract, this would raise the possibility that any abnormality in gene promoter methylation in rectal biopsies might be reflected in buccal cells obtained from the mouth.

The potential for use of buccal cells as a surrogate for rectal mucosal biopsies has not been investigated previously. Buccal cells are easily obtained by taking a swab of the buccal mucosa. This contrasts with rectal biopsies which require the individual to undergo either a rigid or flexible sigmoidoscopy or colonoscopy in order to obtain a rectal tissue sample. It is possible to extract DNA from both buccal cells and rectal biopsies and the DNA can then be processed in the same way.

1.15 Buccal cells and *SFRP4*

There is little literature regarding *SFRP4* in buccal cells. Pannone et al. (2010) observed increased levels of *SFRP4* methylation in DNA extracted from oral

squamous cell carcinoma (OSCC) tissue in comparison with oral mucosal biopsies obtained from matched individuals without OSCC.

1.16 Formulation of research hypotheses

Of the five members of the *SFRP* family, *SFRP3* is unusual because it lacks CpG islands in its promoter region. The other 4 members of the *SFRP* family (*SFRP1, SFRP2, SFRP4, SFRP5*) do contain CpG islands in their promoter regions and are thought to have similar modes of action (Hoang et al. 1996, Bovolenta et al. 2008). There is good evidence that *SFRP1* and *SFRP2* are differentially methylated in those with and without CRC (Wang & Tang 2007). As yet there is limited information about the methylation status of *SFRP4* in respect of CRC and it was hypothesised that *SFRP4* would be differentially methylated in those at higher CRC risk.

This research project aimed to test the hypothesis that *SFRP4* was differentially methylated in those at higher and lower risk for CRC. For this purpose, the higher risk group were patients with a history of adenomatous polyps or of UC, and the lower risk group were healthy volunteers. A difference in *SFRP4* methylation between those at a higher and lower risk for CRC seemed plausible since *SFRP1* and *SFRP2* methylation levels increased with increasing CRC risk from normal volunteers to adenomatous polyp patients to CRC patients (Wang & Tang 2007).

The use of buccal DNA as a possible surrogate for DNA from mucosal rectal biopsies is of interest because it may provide a more acceptable (to patients) method of collecting DNA for CRC screening programmes and for the investigation of modifiers of CRC risk. If the “field effect” phenomenon (whereby an abnormality in epithelial gene expression renders the whole mucosa vulnerable to CRC development) (Polley et al. 2006), extended throughout the alimentary track, such vulnerability might be detectable in the mouth as well as in the colorectum.

If there is a difference in *SFRP4* methylation between those at a higher and lower CRC risk, the reversibility of *SFRP4* hypermethylation may provide a useful biomarker to assess the effect of potential CRC risk modifiers, e.g. resistant starch and polydextrose.

1.17 Project hypotheses, aims and objectives

Hypothesis 1:

The *SFRP4* promoter is differentially methylated in rectal DNA in those at a lower and higher risk for CRC.

Hypothesis 2:

Buccal cells will show the same patterns of *SFRP4* promoter methylation as rectal biopsies in subjects at a lower and higher risk of CRC and will act as a surrogate tissue for CRC biomarker assay.

Hypothesis 3:

SFRP4 promoter hypermethylation in rectal biopsies is reversible by dietary supplements of resistant starch and polydextrose (both alone and in combination).

Aims and Objectives 1:

To test Hypothesis 1 by using pyrosequencing to quantify *SFRP4* promoter methylation in rectal DNA obtained from volunteers at a lower (normal healthy volunteers) and higher (patients with a history of adenomatous polyps or a history of non-active UC) risk for CRC using rectal mucosal biopsies obtained in the BORICC Study (Mathers et al. 2010) and the Dietary Intervention, Stem Cells and Colorectal Cancer (DISC) Study (2011).

Aims and Objectives 2:

To test Hypothesis 2 by using pyrosequencing to quantify *SFRP4* promoter methylation in matched samples of rectal DNA and buccal DNA from volunteers at a lower (normal healthy volunteers) and higher (patients with a history of adenomatous polyps) risk for CRC using rectal mucosal biopsies and buccal cells obtained in the BORICC Study (Mathers et al. 2010).

To investigate possible correlations between *SFRP4* promoter methylation levels in matched samples of rectal DNA and buccal DNA.

Aims and Objectives 3:

To test Hypothesis 3 by using pyrosequencing to quantify *SFRP4* promoter methylation levels in rectal DNA obtained from healthy volunteers in the DISC Study (2011) before and after a randomised controlled trial of effects of dietary supplementation with resistant starch and polydextrose in a 2×2 factorial design.

2 General methods

2.1 Introduction

Mucosal rectal biopsies and buccal cell samples collected in previously conducted, or ongoing, studies at Newcastle University were used in this research project. These biological samples have been collected from healthy volunteers in the BORICC1 Study (Mathers et al. 2010) (relatively lower CRC risk) and from volunteers with adenomatous polyps in the BORICC2 Study (Mathers et al. 2010) (relatively higher risk of CRC). The DISC Study (2011) provided equivalent biological samples from healthy volunteers, from patients with adenomatous polyps and also from patients with inactive UC (both relatively higher CRC risk). The healthy volunteers in the DISC study (2011) participated in a dietary intervention study, providing two sets of biological samples; one before and one after the dietary intervention. Table 2.1 summarises the participant groups within this research project.

2.2 Ethical approval

The biological samples used in this research project are taken from the biobank established by the BORICC Studies (Mathers et al. 2010) and the DISC study (2011). Ethical approval was gained from the Northumberland Local Research Ethics Committee for both studies (BORICC Study: Project reference 04/Q0902/6/, 2004; DISC Study: Project reference 09/H0907/77).

Table 2.1 Overview of studies and of participant groups

Study	Participant group	Population represented
BORICC1 Study	Healthy volunteers	Relatively lower risk of CRC[1]
BORICC2 Study	Patients with a history of adenomatous polyps	Relatively higher risk of CRC[2]
DISC Study	Healthy volunteers	Relatively lower risk of CRC[1 3]
DISC Study	Patients with a history of adenomatous polyps	Relatively higher risk of CRC[2]
DISC Study	Patients with inactive UC	Relatively higher risk of CRC

[1]These two groups are thought to be comparable

[2]These two groups are thought to be comparable

[3]This group also participated in a dietary intervention study

2.3 The Biomarkers of Risk in Colorectal Cancer (BORICC) Study

The BORICC study (Mathers et al. 2010) was designed to identify and to validate novel biomarkers of CRC risk and to investigate their relationships with dietary intake and with nutritional status. The biological samples (rectal mucosal biopsies, buccal cell swabs, urine, stool and blood) were collected in 2004/05. In the BORICC1 Study (Mathers et al. 2010), the biological samples were collected from healthy volunteers whereas in the BORICC2 Study (Mathers et al. 2010), the biological samples were collected from participants with a current or recent history of colorectal adenomatous polyps. One set of biological samples was obtained from each volunteer and, in both studies, the rectal mucosal biopsies were collected from the macroscopically normal bowel.

2.4 The Dietary Intervention, Stem cells and Colorectal cancer (DISC) Study

The DISC Study (2011) is a dietary intervention study in which healthy volunteers were randomised to two potential chemoprevention agents, resistant starch and polydextrose, in a 2×2 factorial design. Two sets of biological

samples were collected from each volunteer; one before the intervention (baseline), and one after. A single set of biological samples was also collected from patients with a current or recent history of colorectal adenomatous polyps and patients with non-active UC.

2.5 Participant recruitment

All volunteers for both the BORICC Study (Mathers et al. 2010) and the DISC Study (2011) were recruited via endoscopy lists within Northumbria Healthcare NHS Foundation Trust. Patient details were reviewed at least 5 days before the scheduled endoscopy appointment and those without any exclusion criteria (section 3.2.1) were posted information about the study prior to their attendance for endoscopy of the large bowel (flexible sigmoidoscopy or colonoscopy) (see appendix) and inviting them to participate. For those indicating willingness to volunteer, at their scheduled endoscopy appointment, the information about the study was reiterated, potential recruits were offered the opportunity to ask questions and written consent was obtained.

2.6 Biological samples

An extensive panel of biological samples was collected from each participant in both the BORICC Study (Mathers et al. 2010) and the DISC Study (2011) for immediate use in each study and to create a “biobank” for future studies. The biological samples of interest to this research project are the rectal mucosal biopsies and buccal cell samples.

2.7 Demographic data

Age, sex, smoking status and anthropometric measurements were also obtained for each study participant. In both studies, the anthropometric measurements included height and weight (for calculation of BMI) and waist

and hip circumferences. In the DISC Study (2011) only, upper thigh circumference was also measured.

2.8 Data Recording

Data from laboratory analyses were collected and transferred to a Microsoft excel 2010 spreadsheet for storage. To ensure the correct data had been transferred against the correct sample identifier, the data were checked on a second occasion and then on a third occasion if, upon statistical analysis, Minitab (version 16) statistical software had identified particular data-points as being unexpected.

2.9 Statistical analyses

All statistical analyses were performed using Minitab (version 16) statistical software.

3 *SFRP4* promoter methylation in rectal DNA: Lower Vs higher CRC risk

3.1 Introduction

This chapter aims to investigate whether the *SFRP4* promoter is differentially methylated in rectal DNA in those at a higher and lower risk for CRC.

3.2 Methods

Participants were recruited to the BORICC Studies (Mathers et al. 2010) and the DISC Study (2011) from endoscopy lists within Northumbria Healthcare NHS Foundation Trust as outlined in section 2.5. Those without any exclusion criteria were invited to participate.

3.2.1 Exclusion criteria

3.2.1.1 The BORICC Study and the DISC Study participant exclusion criteria

Table 3.1 lists the exclusion criteria for volunteers who wanted to participate in the BORICC Study (Mathers et al. 2010) and DISC Study (2011).

Table 3.1 Exclusion criteria for healthy volunteers participating in the BORICC Study and the DISC Study

General exclusion criteria
Age <16 or >85 years
Familial adenomatous polyposis (FAP)
Hereditary non-polyposis colorectal cancer (HNPCC)
Known CRC
Previous colorectal surgery
Pregnancy
Chemotherapy within the past 6 months
Aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs)
Immunosuppressive medication/therapy
Active colonic inflammation at endoscopy
Incomplete left sided examination
CRC found at endoscopy or on histology
Iatrogenic perforation at endoscopy

There were two main types of exclusion criteria: (1) Exclusion criteria for practicality reasons (e.g. age, pregnancy); and (2) exclusion of participants who may have an altered risk of CRC (e.g. those with a personal or family history of CRC and those taking non-steroidal anti-inflammatory drugs (NSAIDs). A normal endoscopic examination to the splenic flexure (left-sided examination) using a flexible sigmoidoscope or colonoscope was required to deem a participant at a lower risk of CRC (providing other exclusion criteria did not exist). This is also provided the examination of the rectum and sigmoid colon by flexible sigmoidoscopy is normal and the patient has no iron deficiency anaemia or abdominal mass (Thompson et al. 2008). Colorectal specialists triage patients referred for large bowel endoscopy to flexible sigmoidoscopy or colonoscopy based upon their symptoms and signs. Therefore, in this study, it

is assumed the appropriate endoscopy test has been performed and that those who have a normal flexible sigmoidoscopy are low risk for CRC.

Individuals with FAP and HNPCC are at a higher risk of CRC (Burt 2000, Fearnhead et al. 2002, Lynch & de la Chapelle 2003) It is possible that some individuals may not be aware of a family history of FAP or HNPCC and therefore in screening potential study participants, the Amsterdam II criteria 3-2-1 rule was used (Vasen et al. 1991) (Table 3.2). Any potential volunteers with any of the Amsterdam II criteria were excluded from the study.

The CAPP2 Study demonstrated that 600mg aspirin given daily for 2 years significantly reduces the risk of CRC in patients with HNPCC (Mathers et al. 2012). Therefore, aspirin, and other NSAIDs may protect against CRC and regular use of these drugs by potential participants resulted in their exclusion from the study. An individual's immune response is suppressed by chemotherapy and other immunosuppressive therapies leading to an increased risk of CRC (CRUK 2013). Therefore potential participants undergoing these treatments were excluded.

Table 3.2 Clinical criteria for identifying persons at risk of HNPCC. The Amsterdam II criteria: The 3-2-1 rule (Vasen et al. 1991)

Amsterdam II criteria
At least 3 relatives with HNPCC associated cancer (colorectal, endometrium, ovarian, gastric, small intestine, hepatobiliary, urinary, brain, skin) 2 generations affected by HNPCC associated cancer 1 HNPCC cancer diagnosed before the age of 50 years

3.2.1.2 Exclusion criteria for those with ulcerative colitis (UC)

The DISC Study (2011) recruited patients with UC as a higher CRC risk group. Individuals with a simple clinical colitis activity index score greater than 5 were excluded from the study (Table 3.3). This validated tool is able to predict active colitis with 92% sensitivity and 91% specificity (when the score is 5 and above) (Walmsley et al. 1998). Active colitis was excluded in this study as the interpretation of samples and the data generated from such participants may be difficult. Saito et al. (2011) found there was increased DNA methylation in active inflamed colon mucosa compared with quiescent mucosa in UC patients when they investigated 6 genes in 28 surgically resected UC patients.

Table 3.3 Clinical scoring system for simple clinical colitis activity index (Walmsley et al. 1998)

Symptom	Score
Bowel frequency (day)	
- 1-3	- 0
- 4-6	- 1
- 7-9	- 2
- >9	- 3
Bowel frequency (night)	
- 1-3	- 1
- 4-6	- 2
Urgency of defecation	
- Hurry	- 1
- Immediately	- 2
- Incontinence	- 2
Blood in stool	
- Trace	- 1
- Occasionally frank	- 2
- Usually frank	- 3
General well being	
- Very well	- 0
- Slightly below par	- 1
- Poor	- 2
- Very poor	- 3
- Terrible	- 4
Extracolonic features	1 per manifestation

3.2.2 Biological samples

Rectal mucosal samples were collected by biopsy from each participant in both the BORICC Study (Mathers et al. 2010) and the DISC Study (2011) for immediate use in each study and to create a “biobank” for future studies. Blood samples collected by venepuncture were also taken from the participants in the BORICC1 Study (Mathers et al. 2010). Samples were analysed by the biochemistry laboratory at the Royal Victoria Infirmary, Newcastle to quantify the plasma concentration of 25(OH)D3.

3.2.2.1 Rectal biopsies

Each participant in both the BORICC Study (Mathers et al. 2010) and the DISC study (2011) donated multiple colorectal mucosal samples, which were taken by "pinch" biopsy from the rectum 10cm from the anal verge at the time of endoscopic examination using a flexible sigmoidoscopy or colonoscopy. 2.3mm spiked flexible endoscopy forceps were used to obtain the biopsy (Biobite forceps, Medical Innovations, Essex, UK). The biopsy samples used in this research project had been immediately snap-frozen in liquid nitrogen and, upon return to the laboratory, transferred to a −80°C freezer.

3.2.3 Laboratory methods

The following laboratory methods were used in this research project: DNA extraction, bisulphite modification, polymerase chain reaction (PCR), gel electrophoresis and pyrosequencing. The final methods chosen for the project were based upon: Available resources, cost effectiveness, experience and personal preference within the laboratory of the research group, time constraints and the production of high quality, reproducible results.

3.2.3.1 DNA extraction: Mucosal rectal biopsies

Mucosal rectal biopsies were removed from -80°C storage and thawed at room temperature. 500µl of SET-sodium dodecyl sulphate (SET-SDS) made from 25µl 50mM Tris, 12.5mM ethylenediaminetetracetic acid (EDTA) and 0.5% sodium dodecyl sulphate (SDS), and 16µl proteinase K (Fermantas) was used to homogenize the rectal biopsy at 55°C, shaking at 900rpm for 8 hours. The homogenized solution was centrifuged at 13,000rpm for 3 minutes at room temperature and 600µl 24:1 chloroform:isoamyl alcohol (Fermentas) was added to precipitate the DNA. 2ml heavy phase lock gel (5-Prime) centrifuged at 10,000rpm for 5 minutes separated the DNA from interphase proteins. The DNA was incubated with 16µl RNAase/T1 (Fermentas) for 37°C for 30 minutes, and 42µl 3M sodium acetate, 400µl isopropanolol and 2.5µl (20mg/ml solution) glycogen (Fermentas) was added. The DNA pellet after centrifugation was

washed twice with 500µl 70% ethanol and centrifuged at 13,000rpm for 2 minutes at room temperature. The DNA pellet was air dried for 1 hour and re-suspended in 50µl 2mM Tris. The DNA purity and concentration were then measured using a spectrophotometer (NanoDrop 1000).

3.2.3.2 Bisulphite modification

This was performed using EZ DNA Methylation Gold™ Kit (Zymo research). A 20µl solution of DNA in water [(500 ÷ DNA concentration as determined by the NanoDrop 1000 spectrophotometer) microlitres DNA and volume of water to make a 20µl solution] was prepared and added to 130µl CT conversion reagent (900µl water, 300µl M dilution buffer and 50µl M dissolving buffer). This was placed in a thermal cycler and programmed to run at 98°C for 10 minutes; 64°C for 2.5 hours; and then to hold at 4°C. Six hundred microlitres M binding buffer was added to a Zymo-Spin™ IC Column, to which the DNA sample was added. This was then centrifuged at full speed for 30 seconds and the flow-through discarded. One hundred microlitres of M wash buffer was then added to the column and the mix centrifuged at full speed for 30 seconds. Two hundred microlitres M desulphonation buffer was then added to the column and the mix left to incubate at room temperature for 20 minutes. After incubation, the mix was centrifuged at full speed for 30 seconds. Two hundred microlitres of M wash buffer was added to the column and the mix centrifuged at full speed for 30 seconds. A further 200µl M wash buffer was added to the column, and centrifuged at full speed for an additional 30 seconds. The column was then placed into a microcentrifuge tube and 10µl M elution buffer added to the column. The mix within the column and the microcentrifuge tube it was in was then centrifuged at full speed for 30 seconds to elute the DNA.

3.2.3.3 *SFRP4* assay design

Genomatix software was used to identify *SFRP4* promoter sequences. The sequences generated with a cytosine and guanine content greater than 50% within a 200 base pair sequence with a cytosine-guanine ratio greater than 0.6 were selected. The selected CpG rich *SFRP4* promoter sequences were

bisulphite modified, and inputted into Pyrosequencing Assay Design software to allow *SFRP4* primers to be generated. The set of *SFRP4* primers with the highest suitability score (as determined by the Pyrosequencing Assay Design software) was selected for use in this research project.

The *SFRP4* primers chosen for this research project are located within 411 to 657 base pairs downstream from the transcription start site (Genomatix) (Figure 3.1-Figure 3.3). This primer which is located downstream of the transcription start site was chosen in preference of other primers, some of which were upstream of the transcription start site, because it had a high cytosine-guanine ratio, scored a high suitability score on the Pyrosequencing Assay Design software and was the only primer that had a correlation coefficient greater than 0.95 at each CpG site in the valiation process (section 3.2.3.4).

There is limited literature on *SFRP4* methylation and its association with CRC risk. Only 2 studies have quanitified *SFRP4* methylation in rectal mucosal biopsies, (Belshaw et al. 2008, Qi et al. 2006) both of which used different methods of *SFRP4* methylation quantification from that used in this research project. Therefore, there are no published data that are directly comparable with the data obtained in this research project. The lack of published literature regarding *SFRP4* gene expression and its possible involvement in the pathways that lead to CRC limits the interpretation of data on *SFRP4* methylation at whichever DNA site it is measured.

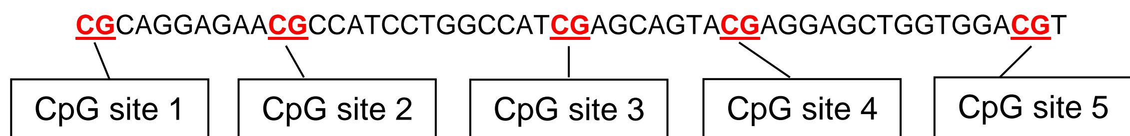
Figure 3.1 *SFRP4* DNA sequence 411 to 657 base pairs downstream from the transcription start site (Genomatix)

CTGTGCCTGTGGCTGCACCTGGCGCTGGGCGTGCGCGGCGCGCCCTGCGAGGCGGTGC
GCATCCCTATGTGCCGGCACATGCCCTGGAACATCACGCGGATGCCCAACCACCTGCACC
ACAGCA**CG**CAGGAGAA**CG**CCATCCTGGCCAT**CG**AGCAGTA**CG**AGGAGCTGGTGGA**CG**TG
AACTGCAGCGCCGTGCTGCGCTTCTTCCTCTGTGCCATGTACGCGCCCATTTGCACCCTG
GAGTTCCTGC

Figure 3.2 *SFRP4* DNA sequence 411 to 657 base pairs downstream from the transcription start site following predicted bisulphite modification and showing locations of primer sequences generated by Pyrosequencing Assay Design Software

TTGTGTTTGTGGTTGTATTTGGC/TGTTGGGC/TGTGC/TGC/TGGC/TGC/TGTTTTGC/TGAG
GC/TGGTGC/TGTATTTTTATGTGTC/TGGTATATGTTTTGGAATATTAC/TGC/TGGA**TGTTTA**
ATTATTTGTATTATAGTA**C/T**GTAGGAGAA**C/T**GTTATTTTGGTTAT**C/T**GAGTAGTA**C/T**GAGG
AGTTGGTGGA**C/T**GTGAATTGTAGC/TGTC/TGTGTTGC/TGTTTTTTTTTTTGTGTTATGTAC/T
GC/TGTTTATTT**GTATTTTGGAGTTTTTGT**

Figure 3.3 Location of CpG sites in *SFRP4* DNA sequence 532 to 584 base pairs downstream from the transcription start site



Nuclease-free water was added to the designed forward, reverse and sequence *SFRP4* primers (MWQ Eurofins) to make a concentration of 10pmol/µl. PCR (section 3.2.3.5) using a temperature gradient was used to establish the optimal annealing temperature which was 47°C. The PCR product size and purity were assessed using agarose gel electrophoresis (section 3.2.3.6).

3.2.3.4 Assay validation

PCR (section 3.2.3.5) and pyrosequencing (section 3.2.3.7) were used to validate the *SFRP4* gene assay. For pyrosequencing, the reverse primer (MWQ Eurofins) was biotin-labelled at the 5' end. Epitect Control DNA (Qiagen) allowed PCR and pyrosequencing with known percentages of methylation using 0% methylated and 100% methylated DNA to make up the different methylation levels (0%, 25%, 50%, 75%, 100%). This was performed using pre- and post-PCR dilutions and the observed methylation levels were then correlated with the known methylation levels and plotted using Microsoft Excel 2010. The correlation coefficient was determined and if this was greater than 0.95 at each CpG site, the assay was validated.

3.2.3.5 Polymerase Chain Reaction

Two microlitres of bisulphite modified DNA was added to a mix of 12.5µl HotStarTaq DNA polymerase (Qiagen), 1µl 10pmol/µl forward *SFRP4* primer (MWQ Eurofins), 1µl 10pmol/µl biotin labelled reverse *SFRP4* primer (MWQ Eurofins) and 9.5µl water and then placed in a thermal cycler and following an initiation step of 95°C for 15 minutes, the thermal cycler was programmed to perform 50 cycles of: (i) denaturation step 95°C for 30 seconds; (ii) annealing step 47°C for 90 seconds; (iii) elongation step 72°C for 45 seconds. PCR was completed with the final elongation step (72°C for 5 minutes).

3.2.3.6 Agarose Gel Electophoresis

Agarose gel electrophoresis was used to determine whether PCR has generated the anticipated DNA fragment. The agarose gel was made using 0.5g 1% agarose powder in 60ml of 1×TBE (Tris, boric acid and EDTA) buffer. 3μl SafeView nucleic acid stain (NBS Biological) was added before the gel was left to set. Once the agarose gel had set, 1×TBE buffer was added and 5μl PCR product was mixed with 2μl bromophenol blue loading buffer (Fermentas) and placed into the agarose gel wells. Gel electrophoresis was run at 70V for 40 minutes before immediate viewing under ultraviolet (UV) light to compare the size of the PCR product against a DNA ladder (Fermentas).

3.2.3.7 Quantification of DNA Methylation: Pyrosequencing

DNA methylation was quantified by Pyrosequencing using a PyroMark MD™ (biotage) platform; using PyroMark Gold Q96 reagents (Qiagen). Ten microlitres of PCR product was added to the PCR mix (2μl streptavidin beads, 38μl binding buffer, 30μl nuclease free water) and shaken at 15rpm for 10 minutes. The plate containing the PCR mix was loaded onto the vacuum prep worktable, the vacuum turned on, and the vacuum prep tool used to capture the streptavidin beads. The vacuum prep tool was then transferred to a trough containing 70% ethanol (wash step) for 5 seconds, a trough containing 0.2M sodium hydroxide solution (denaturation step) for 5 seconds, and a trough containing wash buffer (10mM Tris acetate buffer (Qiagen)) (wash step) for 5 seconds on the vacuum prep worktable. The vacuum was turned off and the streptavidin beads transferred into the pyrosequencing plate containing the pyrosequencing mix (11.5μl annealing buffer (Qiagen), 0.5μl *SFRP4* sequencing primer (MWQ Eurofins)). The pyrosequencing plate was heated at 80°C for 2 minutes. The pyrosequencing plate and cartridge containing the enzyme, substrate and nucleotides (Qiagen) (volumes dictated by the PyroMark CpG Software 1.0.11) were loaded into the PyroMark MD Genetic Analysis System and analysed to quantify DNA methylation.

3.2.4 Reproducibility of results

All analyses of *SFRP4* DNA promoter methylation levels were run in duplicate. The results for a given sample were deemed reproducible if the values for the repeat samples were within 5% of each other. If the initial results were not reproducible, the pyrosequencing assay was repeated. If after further pyrosequencing runs, the values for *SFRP4* methylation were not reproducible, the values for this sample were excluded from analysis. When comparing study participant groups, mean values for each sample were used for statistical analysis.

3.2.5 Comparability of BORICC and DISC data

The analysis of samples for the BORICC Study was undertaken first followed by analysis of samples from the DISC Study. Therefore there is a hypothetical risk of systematic bias associated with the temporal difference in analysing samples from the 2 studies. To investigate the possibility of systematic bias between analyses of samples from the 2 studies, samples of BORICC and DISC DNA were selected and *SFRP4* methylation was quantified for these selected samples under exactly the same conditions. The samples were run simultaneously on the same plates using the same reagents in the same machines under the same conditions (Figure 3.4). Each DNA sample was analysed in duplicate within the same plate and to further assess reproducibility, the experiment was repeated using the same DNA samples and an identical arrangement on the pyrosequencer plate. This quantified *SFRP4* promoter methylation 4 more times for each of the selected DNA samples.

The DNA chosen for this quality control assessment study were obtained from 4 groups 1) baseline “before intervention” samples from healthy participants in the DISC Study, 2) BORICC1 participants, 3) polyp patients from the DISC Study and 4) BORICC2 participants. DNA samples that had the lowest and highest *SFRP4* promoter methylation levels when analysed originally and a random selection of samples with intermediary methylation levels were selected for

each of the study groups. The samples selected in each group were matched for participant characteristics.

Figure 3.4 Assessment of the reproducibility of *SFRP4* methylation in rectal DNA obtained from the BORICC Study and the DISC Study

Diagram representing the set-up of the pyrosequencing plate. Two identical plates were analysed on two separate occasions.

B	B	0	0	100	100	222	222	214	214	165	165
126	126	260	260	004	004	067	067	207	207	108	108
046	046	101	101	039	039	100	100	096	096	083	083
041	041	074	074	057	057	044	044	095	095	11	11
79	79	17	17	01	01	12	12	43	43	62	62
89	89	43	43	06	06	05	05	07	07	11	11
16	16	14	14	12	12	24	24				

BORICC 1 *BORICC 2* *DISC NORMAL* *DISC POLYP*

B = blank/control *0 = 0% control* *100 = 100% control*

The numbers in each box represent the ID of the study participant who donated the colorectal biopsy from which the DNA sample was isolated.

3.2.6 Measurement of serum vitamin D concentration

Serum vitamin D concentration measurements were obtained in the BORICC1 Study only. Circulating concentrations of vitamin D were measured as serum 25(OH)D3. This was determined using a commercial enzyme immunoassay kit (Immunodiagnostics Systems Limited, Tyne & Wear, UK).

3.2.7 Statistical analyses

3.2.7.1 Comparison of characteristics of BORICC1 and BORICC2 participants

Characteristics of the participants of the BORICC1 Study and BORICC2 Study were compared using: Unpaired t-test to identify any significant differences in the ages and BMI of the two groups; χ^2 test to identify any significant difference in the smoking status of the two groups; and Fisher's exact test to identify any significant difference in the male:female ratio between the two study groups. $p<0.05$ was considered to be significant.

3.2.7.2 Comparison of participant characteristics in the DISC Study

Characteristics of the participants from the 3 risk groups (healthy volunteers, patients with adenomatous polyp(s) and patients with UC) were compared using: Analysis of variance (ANOVA) in a general linear model to identify any significant differences in the ages and BMI between the groups; χ^2 test to identify any significant difference in the smoking status and the male:female ratio between the different groups. $p<0.05$ was considered to be significant.

3.2.7.3 Comparison of characteristics of the BORICC Study and the DISC Study participants

Characteristics of the participants in the BORICC Study and the DISC Study were compared using: Unpaired t-test to identify any significant differences in the ages and BMI between the two groups; χ^2 test to identify any significant difference in the smoking status between the two groups; and Fisher's exact test to identify any significant difference in the male:female ratio between the two study groups. In each case, the characteristics of the participants of the BORICC1 Study were compared with the healthy volunteers from the DISC Study and the characteristics of the participants of the BORICC2 Study were compared with the patients with adenomatous polyps in the DISC Study. $p<0.05$ was considered to be significant.

3.2.7.4 *SFRP4* methylation in rectal DNA: Comparison between low and high CRC risk groups

Differences between the levels of *SFRP4* promoter DNA methylation between volunteers of low and higher CRC risk were analysed using ANOVA in a general linear model. Age, sex, smoking status and BMI were used as covariates. Results were expressed as least square means ± standard error of the mean (SEM). $p<0.05$ was considered to be significant.

3.2.7.5 Comparability of the BORICC Study and DISC Study data

Further data generated to assess the comparability of the BORICC Study and DISC Study data were compared and analysed using ANOVA in a general linear model. Results were expressed as least square means ± SEM. $p<0.05$ was considered to be significant.

3.2.7.6 Correlation between *SFRP4* methylation and vitamin D concentration

Regression analysis was used to investigate associations between *SFRP4* promoter methylation in rectal DNA and circulating concentrations of vitamin D measured as 1,25(OH)2D3 in serum. This analysis was performed for the BORICC 1 Study data only because this was the only study in which serum vitamin D concentrations were measured. $p<0.05$ was considered to represent a significant correlation.

3.2.7.7 Sensitivity and specificity of *SFRP4* as a biomarker of CRC risk

Minitab (version 16) statistical software was used to perform binary logistic regression to assess potential variation in *SFRP4* promoter methylation levels between those at lower and higher risk for CRC. This allowed the sensitivity and specificity of *SFRP4* promoter methylation to identify those at a higher or lower risk of CRC to be calculated. Receiver operating curves (ROCs) were plotted to represent the results of the binary logistic regression and the area under the curve (AUC) represented how well *SFRP4* methylation can

distinguish between those at a higher and lower risk for CRC and therefore its potential to be used as a diagnostic test. Table 3.4 shows how the AUC is used to determine the accuracy of the diagnostic test.

Table 3.4 How the area under the receiver operating curve is used to determine the accuracy of a diagnostic test (Hanley & McNeil 1982)

Area under curve (AUC)	**Accuracy of diagnostic test**
0.90-1	Excellent
0.80-0.89	Good
0.70-0.79	Fair
0.60-0.69	Poor
<0.60	Fail

3.3 Results

3.3.1 Patient demographics

3.3.1.1 Biomarkers of Risk in Colon Cancer (BORICC) Study: Rectal Biopsies

SFRP4 methylation was quantified in DNA extracted from biopsies of macroscopically normal rectal mucosa obtained from 253 healthy individuals (relatively lower CRC risk) who were participants in the BORICC1 Study. *SFRP4* methylation was also quantified in DNA extracted from biopsies from a further 96 patients with adenomatous polyps (relatively higher CRC risk) who were participants in the BORICC2 Study. DNA was available from 268 biopsies of macroscopically normal rectal mucosa from healthy individuals and 101 biopsies of macroscopically normal rectal mucosa from patients with adenomatous polyps. However 15 normal samples and 5 polyp samples were excluded from this analysis because of an inability to obtain reproducible values for *SFRP4* methylation. The demographics of study participants are shown in Table 3.5. The BORICC2 Study (higher risk group) participants were significantly ($p<0.001$, unpaired t-test) older than the BORICC1 Study participants. BORICC2 patients were older as would be expected by the increasing incidence of polyps with age (Grahn & Varma 2008). There were no significant differences in BMI ($p=0.3010$, unpaired t-test) or smoking status ($p=0.99$, χ^2 test) between the participants of the BORICC1 Study and the BORICC2 Study. The male:female ratio was significantly different ($p<0.001$, Fisher's exact test) between the 2 groups (see Table 3.5). The rate of CRC is similar in males and females up to the age of 50 years, but after this age, CRC becomes more common in males (CRUK 2013). The difference in the male:female ratio between the participants in the BORICC1 Study and in the BORICC2 Study was addressed by including sex as a covariate in the analyses.

Table 3.5 Demographics of participants in the BORICC1 and BORICC2 Studies

	Rectal biopsies	
	Normal participants	**Patients with polyps**
Number of participants (male:female)	253 (115:138) (45%:55%)	96 (63:33) (66%:34%)
Mean age (standard deviation)	50 years (13.48)	60 years (11.64)
Mean BMI (standard deviation)	28.44 (5.63)	29.15 (5.82)
Smoking status (non/ex/current)	109/69/59 (43%/28%/23%)	43/27/24 (45%/28%/25%)

3.3.1.2 Dietary Intervention and Stem Cells (DISC) Study: Lower vs. higher CRC risk

SFRP4 methylation was quantified in DNA extracted from biopsies of macroscopically normal rectal mucosa obtained from 88 healthy volunteers, 26 patients with adenomatous polyps, and 12 patients with non-active UC. A further 12 biopsies of macroscopically normal rectal mucosa were available from healthy volunteers. However, these were excluded from the study because of an inability to obtain reproducible values for *SFRP4* methylaton. The demographics of the study participants are shown in Table 3.6. There were no significant differences in BMI (p=0.401, ANOVA) or smoking status (p=0.0551, χ^2 test) between the 3 different groups of participants in the DISC Study (healthy volunteers, patients with a history of adenomatous polyp, patients with a history of UC). Age (p=0.003, ANOVA) and the male:female ratio (p=0.004, Fisher's exact test) were both significantly different between the different groups of participants. On average, patients with polyps were 9 and 6 years older than normal participants and patients with UC respectively.

Table 3.6 Demographics of participants in the DISC Study by risk group

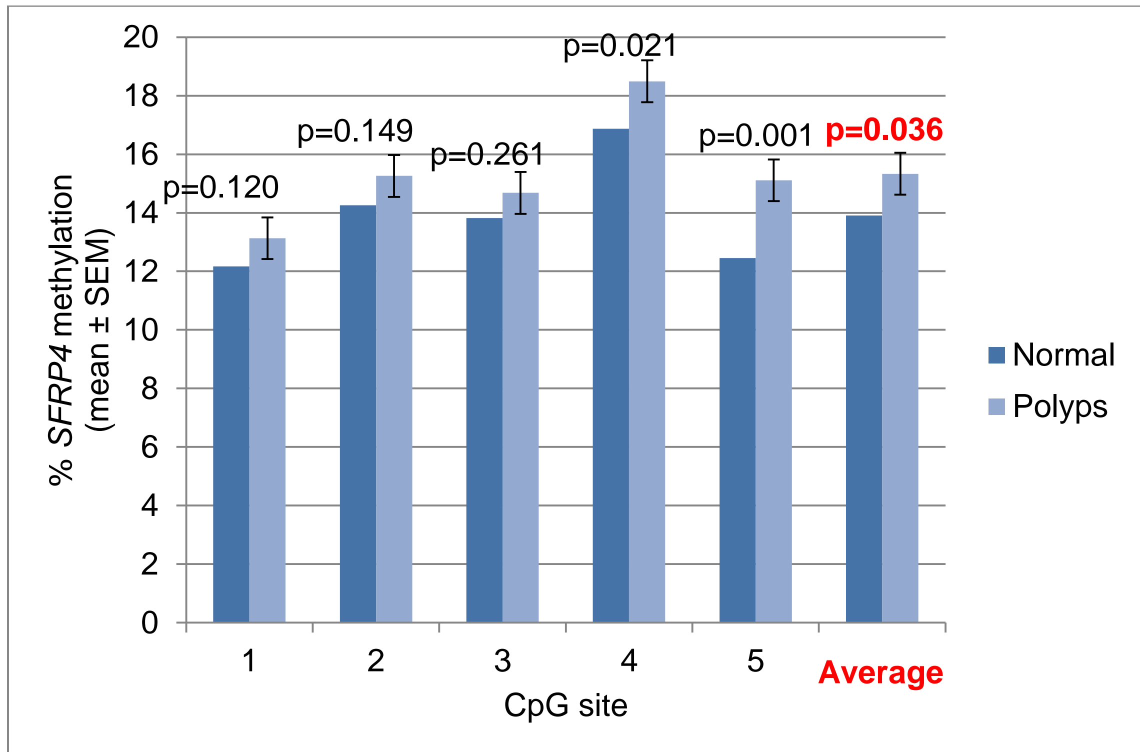
	Normal participants	**Patients with polyps**	**Patients with UC**
Number of participants (male:female)	88 (39:49) (44%:56%)	26 (20:6) (77%:23%)	12 (9:3) (75%:25%)
Mean age **(standard deviation)**	53 years (12.62)	62 years (9.53)	56 years (11.52)
Mean BMI **(standard deviation)**	30.09 (5.34)	28.82 (4.96)	28.39 (4.59)
Smoking status **(non/ex/current)**	44/22/17 (50%/25%/19%) [5 (6%) unknown]	8/10/5 (31%/38%/19%) [3 (12%) unknown]	3/8/1 (25%/67%/8%)

3.3.2 *SFRP4* methylation: Comparison of those at higher *v*. lower risk for CRC

3.3.2.1 BORICC Study

In the BORICC Study, *SFRP4* promoter methylation was quantified in DNA extracted from 253 volunteers at a relatively lower CRC risk (normal healthy volunteers) and 96 volunteers at a relatively higher CRC risk (patients with adenomatous polyps). Methylation of *SFRP4* was quantified at 5 CpG sites within the promoter. *SFRP4* methylation was highest at CpG site 4 and lowest at CpG site 1 with similar methylation at the other 3 sites. Across all 5 sites, methylation ranged from approximately 12-19% (Figure 3.5). At all 5 CpG sites, *SFRP4* methylation was higher in the higher CRC risk group. This difference was significant at CpG site 4 (p=0.021), CpG site 5 (p=0.001) and the mean of the 5 CpG sites (p=0.036) investigated. Averaged across all 5 CpG sites, methylation was 1.42% higher in polyp patients than in controls (15.33% and 13.91% methylation respectively; p=0.036) (Figure 3.5).

Figure 3.5 *SFRP4* methylation in DNA from rectal mucosa from participants in the BORICC Study (analysis has been adjusted for age)



Age was the only covariate which affected *SFRP4* promoter methylation significantly ($p<0.004$) in rectal DNA at all CpG sites individually and overall. At all 5 CpG sites, increasing age was significantly associated with higher *SFRP4* promoter methylation levels ($p<0.004$) and, therefore, all statistical analyses have been adjusted for age (Table 3.7).

At CpG sites 1 and 3, smoking was associated with significantly higher levels of *SFRP4* promoter methylation ($p=0.024$ and $p=0.032$ respectively). Smoking status was also associated with raised *SFRP4* methylation at the average of CpG sites 1-5 and at CpG site 2 but these effects were not statistically significant ($p=0.056$ and $p=0.071$ respectively) (Table 3.7).

There were no significant ($p>0.441$) associations between BMI and *SFRP4* methylation levels in rectal DNA for participants in the BORICC Study (Table

3.7). At CpG site 4, *SFRP4* methylation was significantly higher (p=0.046) in females than in males, but there were no significant differences between males and females in *SFRP4* methylation at the other CpG sites investigated (Table 3.7).

Table 3.7 Associations between age, sex, smoking and BMI and *SFRP4* methylation in DNA from rectal mucosa from participants in the BORICC Study (statistical analysis using ANOVA in Minitab (version 16) with age, sex, smoking and BMI as covariates)

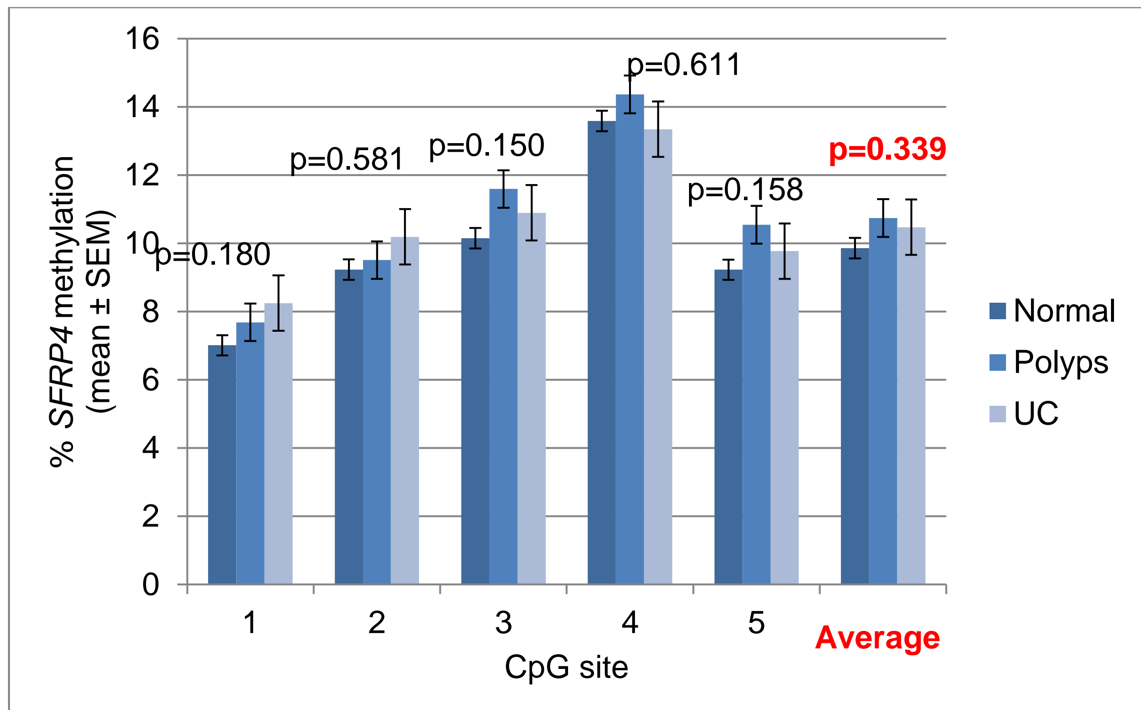
Factor	**CpG site 1**	**CpG site 2**	**CpG site 3**	**CpG site 4**	**CpG site 5**	**Average**
Age (p-value)	0.001	0.004	<0.001	<0.001	0.001	<0.001
Sex (p-value)	0.758	0.242	0.483	0.046	0.167	0.229
Smoking (p-value)	0.024	0.071	0.032	0.136	0.203	0.056
BMI (p-value)	0.441	0.674	0.907	0.886	0.975	0.798

3.3.2.2 *SFRP4* methylation in healthy volunteers and in patients with polyps and patients with UC in the DISC Study

Details of subjects in the DISC study are shown in Table 3.6. Methylation of *SFRP4* was quantified at 5 CpG sites within the promoter. *SFRP4* methylation was highest at CpG site 4 and lowest at CpG site 1 with similar methylation at the other 3 sites. Across all 5 sites, methylation ranged from approximately 7-14% (Figure 3.6). There was no detectable difference between patient groups (p>0.150) in percentage of *SFRP4* methylation at any CpG site. However, levels of *SFRP4* methylation tended to be lower at all CpG sites in those in the

lower CRC risk group (healthy volunteers) when compared with those in the higher CRC risk groups (patients with adenomatous polyps or UC) (Figure 3.6).

Figure 3.6 *SFRP4* methylation in DNA from rectal mucosa from participants in the DISC Study (analysis has been adjusted for age)



As with *SFRP4* promoter methylation in rectal DNA from participants in the BORICC Study, age was a significant ($p<0.010$) determinant of *SFRP4* methylation in rectal DNA from participants in the DISC Study with significant positive associations with age at CpG sites 1, 3, and 4 and at CpG sites 1-5 combined. Increasing age was associated with increased levels of *SFRP4* promoter methylation. For participants in the DISC Study, BMI was associated positively and significantly ($p<0.011$) with *SFRP4* promoter methylation at all CpG sites investigated in rectal DNA. Neither sex nor smoking status was a significant covariate (Table 3.8).

Table 3.8 Associations between age, sex, smoking and BMI and *SFRP4* methylation in DNA from rectal mucosa from participants in the DISC Study (statistical analysis using ANOVA in Minitab (version 16) with age, sex, smoking and BMI as covariates)

Factor	**CpG site 1**	**CpG site 2**	**CpG site 3**	**CpG site 4**	**CpG site 5**	**Average**
Age (p-value)	0.004	0.106	0.010	0.008	0.060	0.007
Sex (p-value)	0.884	0.923	0.989	0.299	0.798	0.742
Smoking (p-value)	0.467	0.519	0.490	0.836	0.607	0.676
BMI (p-value)	0.005	0.033	0.027	0.021	0.051	0.011

3.3.3 Comparison of *SFRP4* methylation in rectal DNA obtained from the participants in the BORICC Study and in the DISC Study

The BORICC Study and DISC Study are two independent studies that share several similarities including the medical history and geographical origin of the study participants and the protocols used for participant recruitment and for collection, storage and processing of biological samples. Both studies used similar inclusion and exclusion criteria to recruit adult volunteers at a relatively lower and higher risk for CRC from referrals to gastrointestinal outpatient clinics in Northumberland in the North East of England. Although the biological samples for the BORICC Study were collected in 2004/2005, and the biological samples for the DISC Study were collected in 2010/2011, it was expected that the lower CRC risk groups (healthy volunteers) would have similar levels of *SFRP4* promoter methylation between the 2 studies as would the higher CRC risk groups (patients with adenomatous polyps).

There were greater number of participants in the BORICC Study (253 healthy volunteers and 96 patients with adenomatous polyps) compared to the DISC Study (88 healthy volunteers and 26 patients with adenomatous polyps). Despite this, there was no significant difference between the male:female ratios of the lower risk group in both studies(p=0.9013, Fisher's exact test). Similarly, there was no significant age difference between the lower risk group participants in both of the studies(p=0.1809, unpaired t-test): The mean age of the BORICC Study and DISC Study lower risk groups was 50 years and 53 years respectively. In the BORICC2 Study the ratio was 66% male:34% female whereas in the DISC Study it was 77% male:23% female. These differences were not significantly different (p=0.3466, Fisher's exact test). There was no significant difference between the ages (p=0.4899, unpaired t-test) or the BMI (p=0.7971, unpaired t-test) of the higher risk groups in the BORICC Study and the DISC Study. There were no significant differences in the smoking status between the lower risk groups (p=0.5273, χ^2 test) in the BORICC Study and the DISC Study; or the higher risk groups (p=0.3887, χ^2 test) in the BORICC Study and the DISC Study (Table 3.5;Table 3.6). Based on the characteristic data available (age, sex, BMI, smoking status) for the two studies, the higher and lower CRC risk groups in the BORICC Study are comparable to the higher and lower CRC risk groups in the DISC Study.

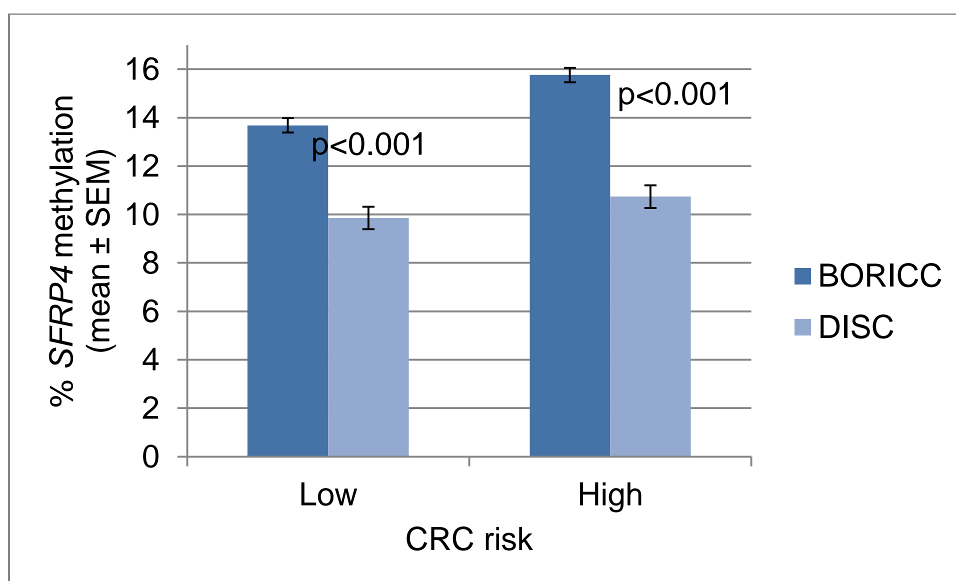
Figure 3.5 and Figure 3.6 show that for the healthy volunteers, *SFRP4* promoter methylation ranged from 12-17% in the rectal DNA obtained in the BORICC Study whereas in the DISC Study, *SFRP4* promoter methylation levels were consistently lower ranging from 7-14%. The *SFRP4* promoter methylation levels also differed in the two groups of adenomatous polyp patients with *SFRP4* promoter methylation ranging between: 13% and 19% in the BORICC Study rectal DNA; and 7% and 14% in the DISC Study rectal DNA.

Figure 3.7 shows *SFRP4* promoter methylation for the average of all CpG sites for both the BORICC Study and the DISC Study, grouped into the lower risk group (healthy volunteers) and higher risk group (patients with adenomatous polyps). There is a significant (p<0.001) difference observed between the

percentage *SFRP4* promoter methylation quantified in the BORICC Study and the DISC Study for both participant groups. This between study difference was also significant ($p<0.001$) at all CpG sites individually (data not shown).

Figure 3.7 *SFRP4* methylation in DNA from rectal mucosa from participants in the BORICC Study and in the DISC Study

(Averages for CpG sites 1-5 shown)



3.3.3.1 <u>Analysis of comparability of *SFRP4* methylation in rectal DNA obtained from the BORICC Study and the DISC Study</u>

Section 3.2.5 details a set of experiments designed to assess the comparability of the BORICC Study and DISC Study data and to investigate the possibility of systematic bias between analyses of samples from the two different studies. Selected samples from the BORICC Studies and the DISC Study were processed under exactly the same conditions and methylation of *SFRP4* was quantified by Pyrosequencing in both sample sets simultaneously on the same plate. Each DNA sample was analysed in duplicate, both within the same plate, and in a further (second) identical experiment.

Figure 3.8 shows *SFRP4* promoter methylation for the average of all CpG sites for the samples of DNA from the BORICC Studies and the DISC Study chosen to assess study comparability as described in section 3.2.5. *SFRP4* promoter methylation has been grouped into the lower risk group (healthy volunteers) and higher risk group (patients with adenomatous polyps). There is a significant difference observed between the percentage *SFRP4* promoter methylation quantified in the BORICC Study and the DISC Study for both participant groups (lower CRC risk p=0.007; higher CRC risk p=0.018). As observed in Figure 3.7, Figure 3.8 shows that the levels of *SFRP4* promoter methylation in the DISC Study are significantly lower for both participant groups than the BORICC Studies. There was a significant (p<0.001, R=0.961-R=0.99) positive correlation between the duplicate selected BORICC Study and DISC Study rectal DNA samples between the first and second analysis at all CpG sites. Figure 3.9 shows the line of regression for *SFRP4* methylation at CpG site 4.

Figure 3.8 *SFRP4* methylation in DNA from rectal mucosa from a sample of participants in the BORICC Study and in the DISC Study (section 3.2.5)

(Averages for CpG sites 1-5 shown)

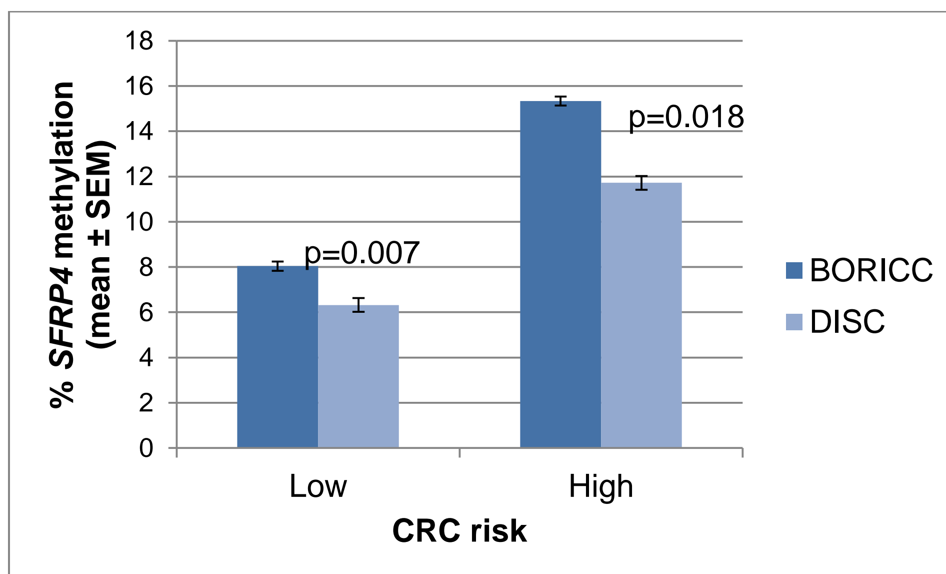
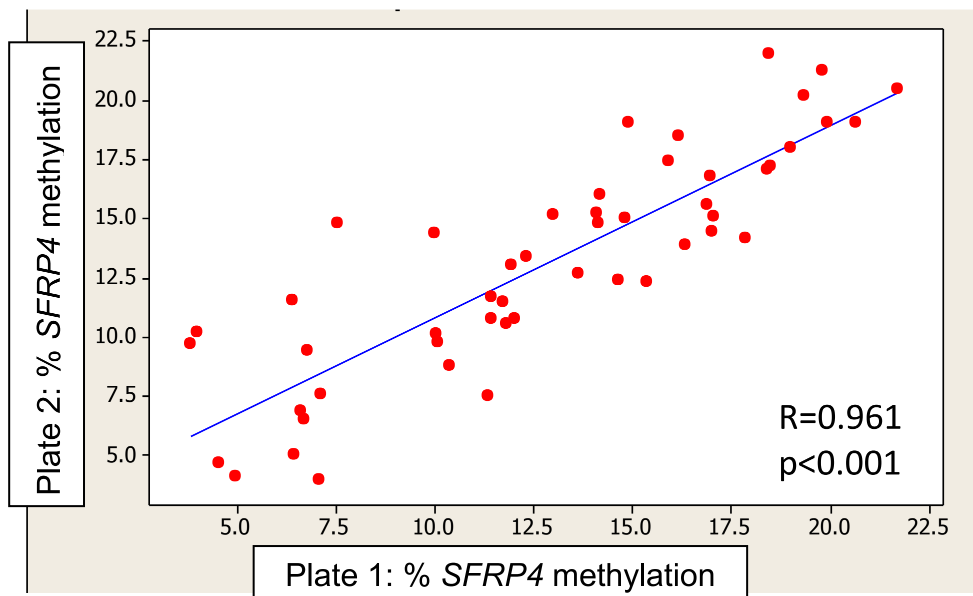


Figure 3.9 *SFRP4* methylation at CpG site 4 in the rectal DNA samples obtained from a sample of BORICC Study and DISC Study participants as detailed in section 3.2.5: Plate 1 Vs. Plate 2



3.3.4 *SFRP4 methylation and serum vitamin D concentration*

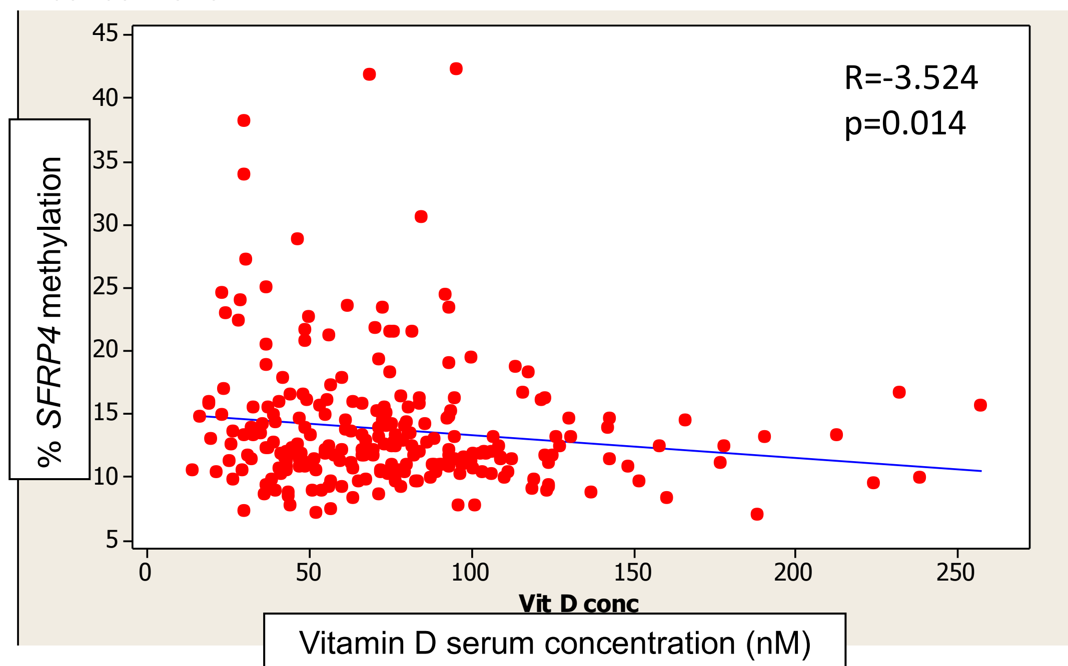
SFRP4 promoter methylation data from the BORICC1 samples were regressed against the participant's serum vitamin D concentration. Serum vitamin D concentration measurements were not made in any of the other studies.

Table 3.9 tabulates the outcomes of the regression analysis of vitamin D serum concentration versus percentage *SFRP4* promoter methylation. There were negative correlations between the 2 variables at all CpG sites investigated and these were significantly negative at CpG site 4 (p=0.014, R=-0.16), CpG site 5 (p=0.009, R=-0.17) and at all CpG sites combined (p=0.023, R=-0.14). Figure 3.10 shows the line of regression for *SFRP4* promoter methylation in rectal DNA from participants in the BORICC 1 Study versus serum vitamin D concentration at all CpG sites combined.

Table 3.9 Regression analysis of serum vitamin D concentration versus percentage *SFRP4* methylation in rectal DNA from participants in the BORICC Study

	CpG site 1	CpG site 2	CpG site 3	CpG site 4	CpG site 5	Average
R coefficient	-0.12	-0.11	-0.13	-0.16	-0.17	-0.14
p-value	0.065	0.085	0.052	0.014	0.009	0.023

Figure 3.10 Average *SFRP4* methylation across all CpG sites Vs. serum vitamin D concentration



3.3.5 *SFRP4* methylation as a screening test for risk of CRC

Figure 3.5 and Figure 3.6 show that there were differences in *SFRP4* promoter methylation in rectal DNA between those at higher and lower risk for CRC. These differences could be clinically important if *SFRP4* promoter methylation could be used to identify those at an increased CRC risk with a high sensitivity and specificity and therefore have the potential for use as a diagnostic test.

Logistic regression was used to investigate differences in *SFRP4* promoter methylation levels in those at lower and at higher risk for CRC with age as a covariate. The BORICC Study and DISC Study data were each analysed separately (section 3.2.7.7).

3.3.5.1 *SFRP4* methylation as a screening test for risk of CRC: BORICC Study data

SFRP4 promoter methylation in rectal DNA obtained from the BORICC Studies were highly specific at identifying those at a lower risk of CRC (specificity > 0.91 for all CpG sites individually and combined) (Table 3.10). However sensitivity was relatively poor. The highest sensitivity was 0.28 (all CpG sites combined) (Table 3.10).

ROCs demonstrated that *SFRP4* methylation in rectal DNA from the BORICC Studies would be a “fair” test to differentiate between those at a higher and lower risk for CRC (AUC 0.70-0.78) (Table 3.4; Table 3.10; Table 3.11) (Hanley & McNeil 1982).

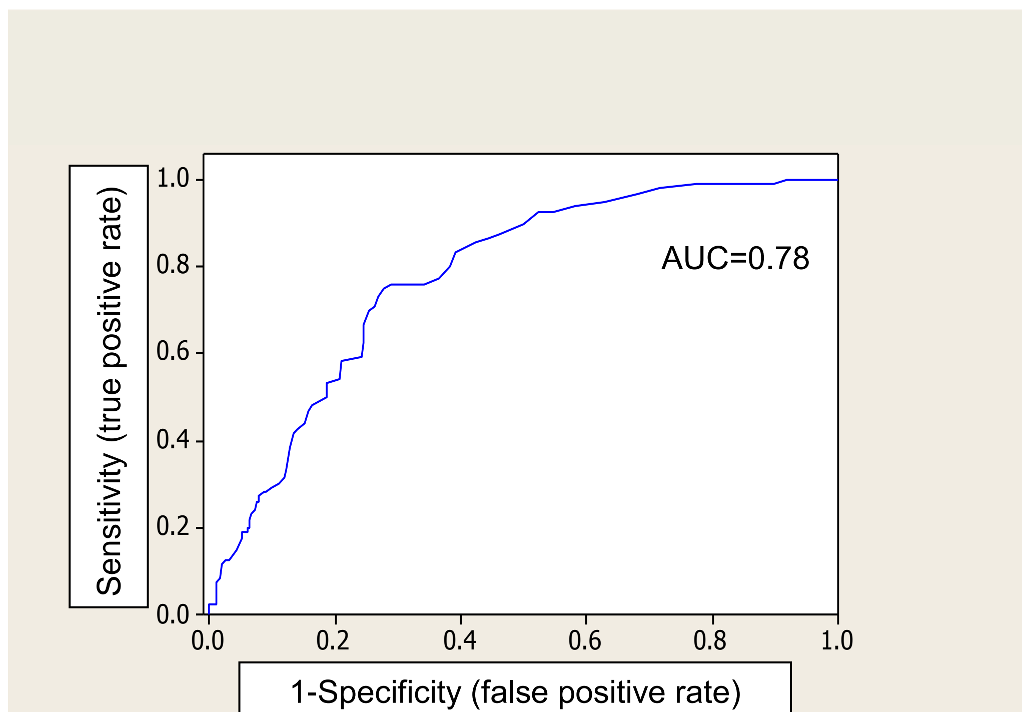
Table 3.10 *SFRP4* methylation in rectal DNA obtained from participants in the BORICC Study: Sensitivity and specificity for differentiating between those at a lower and higher risk for CRC

	Specificity	**Sensitivity**	**AUC***
CpG site 1	0.94	0.19	0.71
CpG site 2	0.95	0.18	0.71
CpG site 3	0.95	0.17	0.70
CpG site 4	0.94	0.22	0.71
CpG site 5	0.9	0.21	0.71
CpG sites averaged	0.94	0.19	0.71
CpG sites combined	0.91	0.28	0.78

**AUC refers to the area under the ROC curve.*

These calculations have been adjusted for age.

Figure 3.11 ROC curve: *SFRP4* methylation in rectal DNA obtained from the BORICC Study: All CpG sites combined



3.3.5.2 *SFRP4* methylation as a screening test for risk of CRC: DISC Study data

SFRP4 promoter methylation in rectal DNA obtained from the DISC Study was highly specific at identifying those at a lower risk of CRC (specificity > 0.84 for all CpG sites individually and combined) (Table 3.11). However sensitivity was very poor. The highest sensitivity was 0.077 (all CpG sites combined) (Table 3.10). The ROCs demonstrated that *SFRP4* methylation in rectal DNA would “fail” as a test to differentiate between those at a higher and lower risk for CRC (AUC 0.49-0.57) (Table 3.4)

Table 3.11 *SFRP4* methylation in rectal DNA obtained from participants in the DISC Study: Sensitivity and specificity for differentiating between those at a lower and higher risk for CRC

	Specificity	**Sensitivity**	**AUC***
CpG site 1	1.00	0	0.55
CpG site 2	1.00	0	0.49
CpG site 3	0.86	0	0.54
CpG site 4	1.00	0	0.53
CpG site 5	0.84	0.038	0.54
CpG sites averaged	1.00	0	0.55
CpG sites combined	0.85	0.077	0.57

**AUC refers to the area under the ROC curve.*

These calculations have been adjusted for age.

3.4 Discussion

3.4.1 *SFRP4* methylation: Comparison of those at higher *v*. lower risk for CRC

In the BORICC Study, the mean percentage *SFRP4* promoter methylation of the 5 CpG sites investigated was significantly (p=0.036) higher in the higher CRC risk group. In the DISC Study, there was no significant difference between patient groups (p>0.150) in *SFRP4* promoter methylation at any of the 5 CpG sites investigated. However, levels of *SFRP4* methylation tended to be lower at all CpG sites in those in the lower CRC risk group when compared to those in the higher CRC risk groups (section 3.3.2). This trend (higher *SFRP4* methylation in the higher risk groups) may not have reached a significance level of p<0.05 because of the small sample size (88 healthy volunteers vs. 26 polyp patients vs. 12 UC patients). A retrospective power analysis showed that for the differences in *SFRP4* methylation observed between the three different groups of study participants in the DISC Study, to achieve a power of 80%, 1569 study participants would be needed. This demonstrates that the sample size for the DISC Study was too small. The data range of *SFRP4* methylation in the BORICC Study differed from the DISC Study. If it is assumed that the BORICC Study, with a larger number of study participants (253 healthy individuals and 96 patients with adenomatous polyps), where a difference in *SFRP4* methylation was identified, is a better representation of the range of *SFRP4* methylation levels in rectal DNA, using the BORICC Study *SFRP4* methylation data, a power analysis shows that 228 study participants would have been needed in the DISC Study to identify a significant difference in *SFRP4* methylation.

Although, no strong conclusions regarding *SFRP4* methylation in those at a higher risk of CRC can be made from this research project, the trend is that *SFRP4* methylation is higher in the higher CRC risk groups. This adds to the limited *SFRP4* literature available, but still, it is difficult to make general conclusions.

Qi et al. (2006) examined the methylation and expression of *SFRP* genes in colorectal tumours, comparing methylation levels of *SFRP1, SFRP2, SFRP4* and *SFRP5* in DNA extracted from CRC, colorectal adenoma, ACF and normal mucosa. Qi et al. (2006) found that there were significant differences in *SFRP4* methylation between: (i) CRC and normal mucosa (p=0.002), (ii) adenoma and normal mucosa (p<0.0462) and (iii) ACF and normal mucosa (p=0.017), where there was no detectable *SFRP4* methylation in the normal mucosa. However, there were no significant differences in *SFRP4* methylation between: (i) CRC and adenoma (p=0.228), and (ii) ACF and adenoma (p=1.00). This suggests that hypermethylation of *SFRP4* occurs as an early event in Fearon and Vogelstein's (1990) adenoma-carcinoma sequence; and a lack of difference in *SFRP4* methylation levels between ACF and adenoma, and adenoma and CRC, suggests that unlike *SFRP1* and *SFRP2, SFRP4* promoter hypermethylation does not increase from normal mucosa to hyperplastic polyp to adenoma to CRC (Wang & Tang 2007).

Section 1.10 describes how promoter hypermethylation is a mechanism that can suppress gene expression and there appears to be a reciprocal relationship between the density of methylated cytosine residues in promoter regions and the transcriptional activity of the corresponding gene (Wong et al. 2007). Qi et al.'s (2006) findings are in keeping with this – reduced expression of *SFRP4* was significantly associated with hypermethylation of the *SFRP4* gene. *SFRP4* expression was down-regulated in CRC, though unlike the reduced expression of *SFRP1, SFRP2* and *SFRP5,* this was not to a level of significance (*SFRP4* p=0.438; *SFRP1, SFRP2, SFRP5* p<0.001). Therefore Qi et al (2006) came to the conclusion that *SFRP1, SFRP2* and *SFRP5* were more specific to CRC than *SFRP4* and would be more suitable as biomarkers for CRC. It is plausible that *SFRP4* may differ in its suitability as a biomarker for CRC in comparison to *SFRP1, SFRP2* and *SFRP5* because it shows less homology with the other members of the *SFRP* family (Bovolenta et al. 2008).

Qi et al.'s (2006) study suggests that hypermethylation of *SFRP4* occurs as an early event in the development of CRC. The patients with polyps in this

research project are at a relatively increased risk of CRC and higher levels of *SFRP4* methylation were found in DNA extracted from the rectal mucosa of these patients. Therefore the findings of this research project are in keeping with the findings of the Qi et al.'s (2006) study.

In contrast, Huang et al. (2010) found that *SFRP4* was overexpressed in CRC. *SFRP4* gene expression was investigated in DNA from 95 CRC, 51 colorectal adenomas and 38 normal colon epithelial samples. This included 20 paired CRC and adjacent normal mucosa. Huang et al. (2010) found that *SFRP4* protein and RNA expression was significantly increased (RNA expression p=0.001, protein expression p<0.0001) in CRC compared to non-cancerous tissue. In the same study, Huang et al. (2010) demonstrated that *SFRP1* and *SFRP5* gene expression were down-regulated in CRC, and there was no difference in the levels of *SFRP2* gene expression amongst cancerous and non-cancerous tissue.

A study by Feng Han et al. (2006) also reported findings which differed from those of Qi et al. (2006). Feng Han et al. (2006) evaluated the expression of *SFRP4* in 1044 CRC samples to investigate possible associations with clinical pathological features and prognosis. No significant associations were found. Although in their study there was no comparison with normal colorectal mucosa, Feng Han et al. (2006) state that CRC expressed higher levels of *SFRP4* compared with adjacent normal mucosa. This is the opposite of what Qi et al. (2006) found in their study: *SFRP4* promoter methylation was significantly higher (p<0.001) in DNA from CRC compared to DNA from the adjacent normal mucosa. As it is expected that promoter hypermethylation results in transcriptional silencing, increased *SFRP4* methylation would translate into a lower level of *SFRP4* expression.

Belshaw et al. (2008) investigated the methylation levels of 18 genes, including *SFRP4* in rectal DNA from 20 morphologically normal colonic mucosa of neoplasia-free subjects, 13 adenomatous polyp patients and 19 CRC patients.

There was no significant difference (p=0.511) in *SFRP4* methylation between normal mucosa and CRC. The median (range) percentage *SFRP4* methylation for DNA extracted from normal mucosa and from morphologically normal mucosa from patients with polyps was 0.77% (0.3-3.9%) and 1.99% (0.5-4.6%) respectively but this comparison was not tested statistically. However, these data suggest that there was higher *SFRP4* methylation levels in the higher risk group (polyp patients) and this is similar to findings of the present research project and Qi et al.'s (2006) study.

Although there was only a significant difference in *SFRP4* methylation between the different CRC risk groups in the BORICC Study, the findings of this study appear to be in keeping with studies published by Qi et al. (2006) and Belshaw et al. (2008).

3.4.1.1 Difference in *SFRP4* methylation between the BORICC Study and the DISC Study

This project quantified *SFRP4* promoter methylation in DNA obtained from rectal biopsies from participants in two independent studies: The BORICC Study and the DISC Study. The laboratory methods used to quantify *SFRP4* promoter methylation was the same for both studies and the promoter region of interest was identical (section 3.2.3). The values obtained for *SFRP4* methylation were significantly (p<0.001) different between the two studies (Figure 3.7). Levels of *SFRP4* methylation from participants in the BORICC Study were significantly greater than those from participants in the DISC Study (Figure 3.5; Figure 3.6).

Further experiments to assess the comparability of *SFRP4* methylation in rectal DNA obtained from the BORICC Study and the DISC Study were conducted to investigate the possibility of systematic bias between analyses of samples from the 2 different studies (Section 3.2.5). In this second set of experiments, there are also significant differences (lower CRC risk p=0.007; higher CRC risk p=0.018) observed in the levels of *SFRP4* methylation quantified in the

BORICC Study and DISC Study (Figure 3.9), where *SFRP4* methylation in the DISC Study are lower for both participants groups than the BORICC Studies. The difference in the studies is unexpected so it is important to examine possible explanations for these inter-study differences.

3.4.1.1.1 <u>Participant characteristics</u>

Section 3.3.1 describes the characteristics of the study participants for both the BORICC Studies and the DISC Study (Table 3.5; Table 3.6). Section 3.3.3 compares the characteristics of the participants of the BORICC Studies with the DISC Study. Although there were a greater number of participants in the BORICC Study (253 healthy volunteers and 96 patients with adenomatous polyps) compared to the DISC Study (88 healthy volunteers and 26 patients with adenomatous polyps), there was no statistically significant difference in any of the characteristics (age, sex, BMI, smoking status) recorded for each of the study participants in either risk group. Therefore the higher and lower CRC risk groups in the BORICC Studies are comparable to the higher and lower CRC risk groups in the DISC Study.

3.4.1.1.2 <u>Methods and analysis</u>

The rectal biopsies for both studies were obtained from the same endoscopy departments in the North of England. Both studies used the same participant recruitment methods and targeted similar patient groups and the same techniques were used to obtain the rectal biopsies and process them. The rectal biopsies in the BORICC Studies were collected in 2004/05, whereas the rectal biopsies in the DISC Study were collected in 2010/11. It is possible to hypothesize that methylation marks may be affected over time. However there is limited literature investigating stability of methylation marks over time in biopsy samples stored at -80 degrees.

DNA methylation refers to the modification of DNA and is thought to be the most stable, heritable and well conserved epigenetic change, and persists even in the absence of the conditions that established them (Bird 2002). Byun et al. (2012)

suggest that DNA methylation can exhibit different temporal behaviours, varying between stability and instability of the DNA sequence. There is no human data available that allows distinction between stable and non-stable methylation marks.

Byun et al. (2012) and Talens et al. (2010) conducted two independent studies analysing a panel of DNA methylation markers to assess variability in methylation marks over time. Neither included the *SFRP4* gene in their study. Byun et al. (2012) investigated short term variability in blood DNA methylation of 12 genes in 63 healthy individuals, where 2 sets of blood samples were obtained; one on day 1, and the following on day 4. This study found that DNA methylation of different genes in blood DNA have different degrees of short-term variability (Byun et al. 2012). Though whether these results can be extended to other cell types requires further investigation.

Talens et al. (2010) investigated whether pre-existing stored DNA would be suitable for epigenetic epidemiological studies. Thirty-four individuals were selected to allow an assessment of the stability of DNA methylation in a panel of 16 genes over time. DNA samples from blood and buccal cells were obtained in these individuals and processed to allow comparison with previously collected DNA samples from the same individuals 11-20 years (blood) and 2-8 years (buccal cells) previously. Overall, DNA methylation was similar at the two time points. The study by Talens et al. (2010) is more applicable to this research project because the time frame is in years as opposed to days as in the Byun et al. (2012) study. It shows that methylation levels do appear to be stable over time, and that therefore the difference in timing of collection of the rectal biopsies in the BORICC study and DISC study is unlikely to account for the significant differences in *SFRP4* methylation levels.

3.4.1.2 Difference in *SFRP4* methylation between the BORICC Study, the DISC Study and the published literature

The levels of *SFRP4* promoter methylation quantified in this research project differ from the values published by Qi et al. (2006) and Belshaw et al. (2008). This may be the result of different research methods or different sites within the *SFRP4* promoter that the various research groups have focussed upon. The studies by Feng Han et al. (2006) and Huang et al. (2010) did not measure levels of *SFRP4* promoter methylation.

Qi et al.'s (2006) method for measuring *SFRP4* methylation was methylation-specific PCR (MSP) whereas Belshaw et al. (2008) used real-time quantitative methylation specific PCR (RT-QMSP). The data in Qi et al.'s (2006) study is presented as the number of samples in which *SFRP4* methylation could be detected (24.2% of DNA samples from patients with adenomatous polyps compared to 0% of DNA samples from healthy volunteers). This differs from the data collected in Belshaw et al.'s (2008) study and that of this research project, where the data are presented as a percentage methylation of each CpG site within each DNA sample. Therefore, the results of this research project are not directly comparable to the results of the study by Qi et al. (2006).

Both this research project and Belshaw et al.'s (2008) study generated data on the percentage *SFRP4* promoter methylation at various CpG sites of interest. Furthermore, Belshaw et al. (2008) recruited the participants of their study from the same population as the BORICC Studies and the DISC Study. The rectal biopsies obtained in Belshaw et al.'s (2008) study were collected from the same endoscopy departments using similar methods to the BORICC Studies and the DISC Study.

The CpG sites of interest in this research project differ from those of Belshaw et al.'s (2008). This may account for the differences in *SFRP4* methylation levels between the two studies. For the BORICC Study and the DISC Study, *SFRP4*

methylation was quantified at 5 CpG sites 411 to 657 base pairs downstream to the transcription start site, whereas Belshaw et al. (2008) quantified *SFRP4* methylation at 6 CpG sites 123 base pairs upstream to 128 base pairs downstream of the transcription start site (Figure 3.12)

Figure 3.12 *SFRP4* DNA sequence 123 base pairs upstream to 657 base pairs downstream of the transcription start site (adapted from Genomatix)

GAAGAAAAAAGACTGGCCAGACTAAAAAGGAGGGACTTTAGGGGGAAAGCAGGCTTCCAGCCCTGGGCTGCGG
CCCAGAGGGGGTGATGTCACCGCTTCTGCACCGACGGGCCTGGGGGTGGG**G**CGGCCGAGGGGGAGCCCGCGCC
GCGGCTGCAGCTGCCAAGGGAGCGTTCCGAGCCCACGTCAGGGGAGGTGTCGGGATAAATAGGGTCCCGCAATG
GCCGTGGCTGGCTGCGCTCCGAGCTGCGGAGTCCGGGACTGGAGCTGCCCGGGCGGGTTCGCGCCCCGAAGGCT
GAGAGCTGGCGCTGCTCGTGCCCTGTGTGCCAGACGGCGGAGCTCCGCGGCCGGACCCCGCGGCCCCGCTTTGCT
GCCGACTGGAGTTTGGGGGAAGAAACTCTCCTGCGCCCCAGAGGATTTCTTCCTCGGCGAAGGGACAGCGAAAG
ATGAGGGTGGCAGGAAGAGAAGGGCGCTTTCTGTCTGCCGGGGTCGCAGCGCGAGAGGGCAGTGCCATGTTCCT
CTCCATCCTAGTGGCGCTGTGCCTGTGGCTGCACCTGGCGCTGGGCGTGCGCGGCGCGCCCTGCGAGGCGGTGC
GCATCCCTATGTGCCGGCACATGCCCTGGAACATCACGCGGATGCCCAACCACCTGCACCACAGCACGCAGGAGA
ACGCCATCCTGGCCATCGAGCAGTACGAGGAGCTGGTGGACGTGAACTGCAGCGCCGTGCTGCGCTTCTTCCTCT
GTGCCATGTACGCGCCCATTTGCACCCTGGAGTTCCTGC

G Transcription start site

Locations of CpG sites in Belshaw et al.'s (2008) Study: 123 base pairs upstream to 128 base pairs downstream of the transcripton start site

Location of CpG sites in current research project (BORICC Study and DISC Study): 411 to 657 base pairs downstream to the transcription start site

3.4.1.3 The effect of age on *SFRP4* methylation in rectal DNA

Age was the only statistically significant (p<0.01) covariate affecting *SFRP4* promoter methylation in rectal DNA extracted from both the BORICC Study and the DISC Study participants although the age effect was not statistically significant at CpG site 2 (p=0.105) or CpG site 5 (p=0.06) in the DISC Study.

Importantly, in both studies and at all CpG sites investigated, *SFRP4* methylation levels increased with age.

CRC risk increases with age (CRUK 2013) and the age-related greater methylation of *SFRP4* observed in the present project was also reported in Belshaw et al.'s (2008) study. At present it is not known whether the age-related increase in *SFRP4* methylation is causal for CRC or an association. The hypothesis that the accumulation of epigenetic alterations such as *SFRP4* hypermethylation that may occur during the ageing process, may directly contribute to the formation of CRC needs further investigation.

The process of ageing comprises anatomical, physiological, biochemical and epigenetic changes during an individual's life time. Epigenetic factors are heritable at the cellular level and may be modulated by external factors such as the environment (Choi & Friso 2010). Interestingly, the epigenetic changes that occur during the ageing process are the same epigenetic changes associated with cancer i.e. promoter hypermethylation and genomic global DNA hypomethylation (Ehrlich 2002).

Cancer, ageing, environmental factors and specific epigenetic alterations such as DNA hypermethylation and global genomic hypomethylation, theoretically may all occur together; yet they all do not have to occur together – not everyone develops cancer as they age. Perhaps, the *SFRP4* methylation occurring during the ageing process may not directly cause the development of CRC in isolation, but, in association with modifiable environmental factors such as diet, may initiate the pathway of genetic and epigenetic alterations that accumulate and result in CRC formation.

3.4.1.4 *SFRP4* methylation in patients with ulcerative colitis

SFRP4 methylation at all CpG sites investigated tended to be higher in rectal DNA extracted from patients with non-active UC in comparison with the healthy volunteers, but this difference was not statistically significant (p=0.339). It is possible that the lack of statistical significance is affected by the relatively small number of UC patients investigated in the DISC Study.

This is the first study which has investigated methylation of the WNT antagonist *SFRP4,* or any other members of the *SFRP* family, in UC patients. However, van Dekken et al. (2007) demonstrated the involvement of the WNT signalling pathway in the development of CRC in patients with UC by evaluating the immunolabelling patterns of β-catenin and products of WNT-related genes (E-cadherin, cyclin D1 and c-myc) along the successive stages of inactive colitis, dysplasia and adenocarcinoma in UC using surgical resection specimens from 18 CRCs, 17 dysplastic lesions and 11 inactive colitis (normal control). The findings were up-regulation of β-catenin, cyclin D1 and c-myc in the preneoplastic state and down-regulation of E-cadherin, in keeping with an activated WNT signalling pathway. This study suggests that the WNT pathway is activated early in the malignant progression of UC (van Dekken et al. 2007). Since *SFRP4* is a WNT antagonist, this implies a possible role for SFRP4 gene expression in the development of CRC in patients with UC (Baylin & Ohm 2006, Bovolenta et al. 2008) and may account for the increased *SFRP4* methylation in patients with UC.

Sporadic CRC in patients without UC follows the adenoma-carcinoma sequence (Fearon & Vogelstein 1990). The mechanisms that cause the transition from UC to CRC are unclear but may involve the “inflammation-dysplasia-carcinoma progression sequence”. This sequence, similar to the adenoma-carcinoma sequence, may involve changes to the WNT signalling pathway (Harpaz & Polydorides 2010, Shenoy et al. 2012).

3.4.2 *SFRP4* methylation and serum vitamin D concentration

Circulating concentrations of Vitamin D were available for the participants in the BORICC1 Study only. In this study there were associations between percentage *SFRP4* methylation and serum vitamin D concentration (section 3.3.4). There were negative correlations between the 2 variables at all CpG sites investigated (Table 3.9) which were significantly negative at CpG site 4 ($p=0.014$, $R=-0.16$), CpG site 5 ($p=0.009$, $R=-0.17$) and at all CpG sites combined ($p=0.023$, $R=-0.14$). For the remainder of the CpG sites, the p-value approached significance ($p<0.064$). These findings support the proposed link between *SFRP4* promoter methylation and vitamin D concentration described in the literature (Section 1.8.1).

It is not known whether a low vitamin D intake and subsequent low vitamin D serum concentrations are causal for raised *SFRP4* promoter methylation levels, and whether this may be a mechanism by which low vitamin D status increases the risk of CRC. Alternatively, serum concentrations of vitamin D, CRC risk and their associations with *SFRP4* methylation may be mechanistically independent of each other and low vitamin D status may lead to increased risk of CRC via different mechanisms.

Cutaneous biosynthesis following skin exposure to ultraviolet light is the predominant source of vitamin D for most people (Parfitt et al. 1982) and seasonal differences in circulating concentrations of 25(OH)D3 are well documented (McKenna et al. 1985; Rapuri et al. 2002; Webb et al. 1988). In northern latitudes such as the UK, sunlight during winter is insufficient to stimulate cutaneous biosynthesis of vitamin D (van der Wielen et al. 1995). Serum vitamin D concentration data are available for the participants of the BORICC1 Study only which investigated novel biomarkers of CRC risk in a low CRC risk healthy population, and data collection spanned a year-long period over 2004-2005. At latitudes similar to the UK, there is good evidence for seasonal variation in circulating concentrations of 25(OH)D and future work should include estimation of season-specific 25(OH)D and use of these

adjusted values in investigation of relationships with *SFRP4* methylation (Shoben et al, 2005).

3.4.3 *SFRP4* methylation as a screening test for CRC

SFRP4 methylation is significantly different in the DNA extracted from the rectal mucosa of BORICC1 and BORICC2 Study participants. Although a similar difference between those at higher and lower risk for CRC in the DISC Study was also observed, this difference was not statistically significant. These differences observed would be clinically applicable if *SFRP4* methylation could identify those at an increased CRC risk with high sensitivity and specificity. Only 50% of those diagnosed with CRC survive more than 5 years from diagnosis (CRUK 2013). CRC incidence has not been affected by changes in treatment and survival although it has been shown to be influenced by improved diagnostic techniques and screening programmes (Hagger & Boushev 2009). Therefore, the emphasis should be on improving screening and diagnosis for CRC.

Although endoscopic examination of the colon is the gold standard investigation for screening for CRC, it is a time consuming procedure and requires skilled healthcare professionals to carry out the procedure. Other simpler investigations can be used to help prioritise which individuals within the population should undergo endoscopic evaluation of their bowel to assess risk of CRC. FOBT is a non-invasive screening method and involves the individual providing a stool sample for assessment which is then tested for any evidence of blood (Strul & Arber 2007). FOBT has decreased mortality from CRC by 15-33% due to the early detection of colorectal adenoma (Hardcastle et al. 1986, Mandel et al. 1993, Kronborg et al. 1996, Niv et al. 2002).

Since the development of FOBT, further non-invasive screening tests for CRC have been developed. Stool-based DNA tests involve the individual providing a stool sample, which is then processed to identify a panel of epigenetic and

genetic alterations that are thought to relate to CRC risk. Stool-based DNA tests are 4 times more effective than FOBT for detecting CRC (Salehi et al. 2012). Also, stool-based DNA tests specifically designed to test for CRC are theoretically more accurate than FOBT because the stool-based DNA test assesses the stool sample for evidence of known biomarkers of CRC risk whereas FOBT assesses the stool sample for blood. Blood in the stool is a potential sign of CRC, but there are other causes such as benign conditions such as haemorrhoids, or secondary to dietary red meat intake.

This research project has investigated the potential use of tissue biopsies in DNA tests using mucosal biopsies from the rectum and *SFRP4* as a biomarker of CRC risk. *SFRP4* promoter methylation in rectal DNA obtained from the BORICC Studies were highly specific at identifying those at a lower risk of CRC (specificity > 0.91 for all CpG sites individually and combined) (Table 3.10). However sensitivity was relatively poor. The highest sensitivity was 0.28 (all CpG sites combined) (Table 3.10). ROCs demonstrated that *SFRP4* methylation in rectal DNA from the BORICC Study would be a “fair” test to differentiate between those at a higher and lower risk for CRC (AUC 0.70-0.78) (Table 3.4; Table 3.10; Table 3.11) (Hanley & McNeil 1982) (section 3.3.5.1). However, ROCs demonstrated that *SFRP4* methylation in rectal DNA obtained from the DISC Study would “fail” as a test to differentiate between those at a higher and lower risk for CRC (AUC 0.49-0.57) (Table 3.4; Table 3.11). *SFRP4* promoter methylation in rectal DNA obtained from the DISC Study were highly specific at identifying those at a lower risk of CRC (specificity > 0.84 for all CpG sites individually and combined) (Table 3.11). However sensitivity was very poor. The highest sensitivity was 0.077 (all CpG sites combined) (Table 3.10) (section 3.3.5.2).

SFRP4 methylation in rectal DNA is not sensitive for identifying those at a higher risk of CRC; and would underestimate the true number of people at higher CRC risk. However, it should be noted that the data in this research project were generated from a case control study. In order to classify participants as high or low risk for CRC, a prospective study design is required

in which participants are followed over until it is determined whether or not they develop CRC. It is only then that a true sensitivity and specificity can be calculated.

In Belshaw et al.'s (2008) study, *SFRP4* was one of the 6 (out of 18) informative genes in classifying study samples into CRC, polyps and neoplasia free. Collectively, all 18 genes investigated in Belshaw et al's (2008) study were able to correctly classify 67.3% of study samples; and when the classifications were grouped into cancer and no cancer, the panel of 18 genes were able to correctly classify 87.9% of study samples, where *SFRP4* was one of the three most informative genes.

3.5 Conclusions

This research project has shown that *SFRP4* promoter methylation is greater in those at a higher CRC risk. Mean *SFRP4* methylation was significantly greater (p=0.036) in those at a higher risk for CRC in the BORICC Study. *SFRP4* methylation was also greater in those at a higher CRC risk in the DISC Study, though this difference was not significant (p>0.15). This direction of change is in keeping with studies published in the literature by Qi et al. (2006) and Belshaw et al. (2008).

Further studies investigating the differential methylation of *SFRP4* in those at a lower and higher risk for CRC should include investigating why there were differences in *SFRP4* methylation levels in rectal DNA from the BORICC and DISC Studies. Also, the patients in the BORICC Study and DISC Study could be followed to establish whether or not they develop CRC and to determine whether or not the *SFRP4* methylation level quantified in this study was predictive of CRC risk.

This study also found a negative correlation between percentage *SFRP4* methylation and serum vitamin D concentration at all CpG sites investigated, and this supports the literature which proposes that there is a link between *SFRP4* methylation and vitamin D concentration. Further investigation is required to assess whether low vitamin D concentrations are causal for a raised *SFRP4* methylation level, and whether this may be a possible mechanism by which low vitamin D status increases the risk of CRC.

This research project has found that *SFRP4* is not a suitable epigenetic biomarker for CRC risk. Although *SFRP4* methylation levels in rectal DNA obtained from the BORICC Studies and the DISC study were both highly specific in identifying those at a lower risk of CRC (specificity>0.84), *SFRP4* methylation levels in rectal DNA obtained from both studies had poor sensitivity levels (sensitivity<0.28). Both this project and the literature has suggested that

there are differences in *SFRP4* methylation in those at a higher and lower risk for CRC. Further investigations should focus on whether *SFRP4* could be used in combination with other genes as an epigenetic biomarker of CRC risk.

4 Buccal cells as a surrogate tissue for CRC biomarker assay using *SFRP4* promoter methylation as an exemplar

4.1 Introduction

Although colorectal mucosal biopsies are a good source of information about molecular events which may lead to CRC development, collecting such tissue biopsies is an invasive process. It requires specific medical skills and may involve some discomfort for the study participant. In contrast, samples of buccal cells can be collected safely and readily by the participant without the need for skilled assistance. The buccal mucosa is part of the gastrointestinal mucosa and is exposed to some of the same factors as the colorectum e.g. host genotype, endogenous factors such as obesity-related inflammation and dietary factors. As a consequence, it is hypothesised that the buccal mucosa might be a useful surrogate for measurement of biomarkers of CRC risk including *SFRP4* promoter methylation. Therefore, this chapter aims to investigate relationships between *SFRP4* promoter methylation in matched samples of DNA from rectal mucosal biopsies and from buccal cells from participants at lower (normal healthy volunteers) and higher (patients with a history of adenomatous polyps) risk for CRC using rectal mucosal biopsies and buccal cells obtained in the BORICC Study (Mathers et al. 2010).

4.2 Methods

Participants were recruited to the BORICC Study (Mathers et al. 2010) from endoscopy lists within Northumbria Healthcare NHS Foundation Trust as outlined in section 2.5. Those without any exclusion criteria as described in section 3.2.1.1 were invited to participate.

4.2.1 Biological samples

Rectal samples and matched buccal cell swabs were collected at the same time from each participant in the BORICC Study. Rectal biopsies were sampled and stored as described in section 3.2.2.1.

4.2.2 Buccal cell samples

Buccal cells were collected using Catch-All™ swabs (Epicentre Bio Technologies). Participants were asked to avoid food and drink for at least 2 hours before collection of the sample. To do so, participants were asked to rinse their mouth twice with water before a buccal swab was rolled against the inside of their cheek firmly 20 times on each side. The buccal swab was then placed back inside its original packaging and transported to the laboratory where it was stored at −80°C.

4.2.3 Laboratory methods

In addition to the laboratory methods described in section 3.2.3, the following technique for DNA extraction from the buccal cell swabs was used.

4.2.3.1 DNA extraction: Buccal cell samples

Buccal cell samples were removed from -80°C storage and thawed at room temperature. The BuccalAmp™ DNA Extraction Kit (Epicentre Bio Technologies) was used to extract DNA from the buccal cell samples. The swab end of the Catch-All™ sample collection swab (Epicentre Bio Technologies) was placed into a tube containing QuickExtract DNA extraction solution (Epicentre Bio Technologies) and vortexed for 10 minutes. The mixture was then incubated at 65°C for 1 minute followed by incubation at 98°C for 2 minutes. The DNA purity and concentration were then measured using a spectrophotometer (NanoDrop 1000)

4.2.4 Statistical analyses

4.2.4.1 Comparison of participant characteristics: BORICC1 Vs. BORICC2 participants

Characteristics of the participants of the BORICC1 Study (healthy volunteers) and BORICC2 Study (patients with polyps) were compared using: Unpaired t-test to identify any significant differences in the ages and BMI of the two groups; χ^2 test to identify any significant difference in the smoking status of the two groups; and Fisher's exact test to identify any significant difference in the male:female ratio between the two study groups. $p<0.05$ was considered to be significant.

4.2.4.2 *SFRP4* methylation in buccal DNA: Comparison between low and high CRC risk groups

Levels of *SFRP4* promoter DNA methylation in buccal DNA for participants at lower (BORICC1) and higher (BORICC2) CRC risk were analysed using ANOVA in a general linear model, as described in section 3.2.7.4. Age, sex, smoking status and BMI were used as covariates. Results were expressed as least square means $\pm$ SEM. $p<0.05$ was considered to be significant.

4.2.4.3 Relationships between *SFRP4* promoter methylation in DNA from Rectal Biopsies and from Buccal Cell Samples

Regression analysis using Minitab (version 16) statistical software was used to investigate potential linear relationships between *SFRP4* promoter methylation in DNA from the rectal biopsies and from the buccal cells. $p<0.05$ was considered to represent a significant correlation.

4.2.4.4 Sensitivity and specificity of *SFRP4* methylation in buccal DNA as a biomarker of CRC risk

Minitab (version 16) statistical software was used to perform binary logistic regression to examine variation in *SFRP4* promoter methylation levels in buccal DNA in those at a lower and higher risk for CRC (section 3.2.7.7).

4.3 Results

4.3.1 Patient demographics

SFRP4 methylation was quantified in DNA extracted from buccal cells from 233 healthy individuals who were at a relatively lower risk of CRC (BORICC1 Study) and from 89 patients with adenomatous polyps who were at a relatively higher risk of CRC (BORICC2 Study). DNA was available from 268 buccal cells obtained from healthy individuals and 99 buccal cells from patients with adenomatous polyps. However 35 samples from healthy individuals and 10 samples from patients with polyps were excluded because of an inability to obtain reproducible values for *SFRP4* methylaton. The demographics of study participants are shown in Table 4.1. There were no significant differences in BMI (p=0.3291, unpaired t-test) or smoking status (p=0.8437, χ^2 test) between the participants of the BORICC1 Study and the BORICC2 Study. On average, the participants with polyps group was approximately 10 years older (p<0.001, unpaired t-test) and had a higher proportion of males (p<0.001, Fisher's exact test) than the healthy group within the BORICC Study.

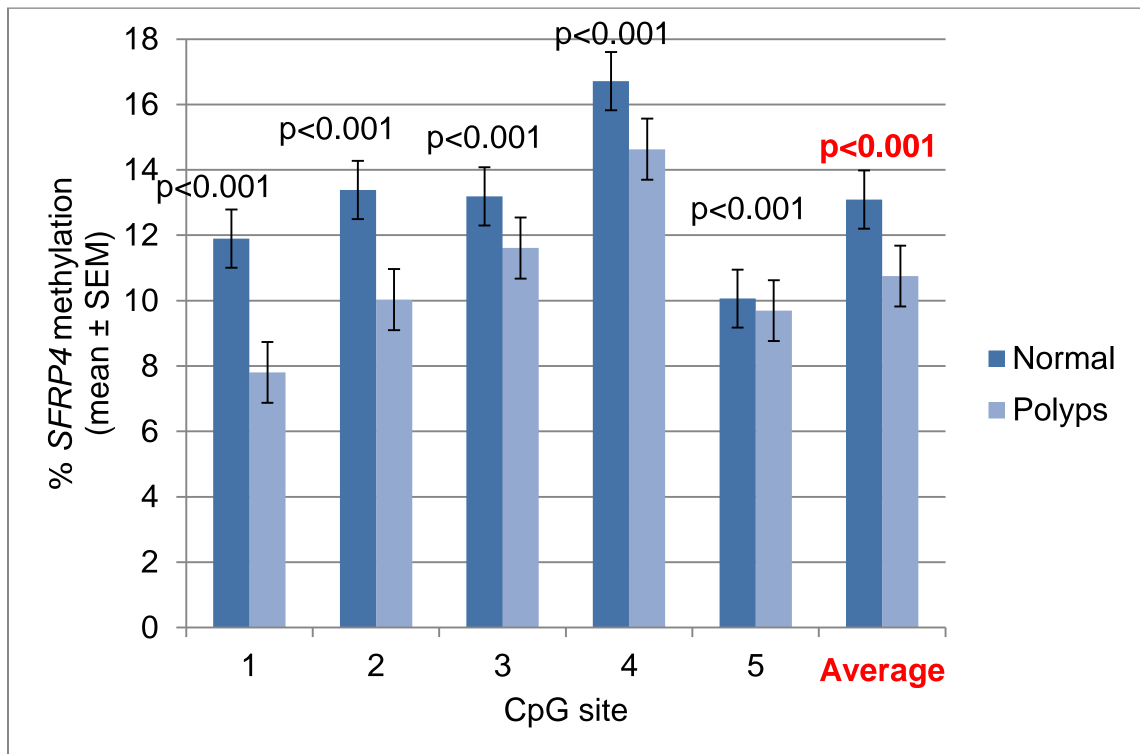
Table 4.1 Demographics of participants in the BORICC1 and BORICC2 Studies for whom buccal cell samples were available for analysis

	Normal participants	**Patients with polyps**
Number of participants (male:female)	233 (93:140) (40%:60%)	89 (63:26) (71%:29%)
Mean age (standard deviation)	50 years (13.50)	60 years (11.62)
Mean BMI (standard deviation)	28.37 (5.61)	29.04 (5.85)
Smoking status (non/ex/current)	104/70/57 (45%:30%:24%) [2 (1%) unknown]	42/24/23 (47%/27%/26%

4.3.2 Methylation of *SFRP4* in DNA from buccal cells: Comparison of those at lower vs. higher risk for CRC

Using DNA from buccal cells from the individuals detailed in Table 4.1, methylation was quantified at 5 CpG sites within the promoter of *SFRP4*. *SFRP4* methylation was highest at CpG site 4 and lowest at CpG site 1 with similar methylation at the other 3 sites. Across all 5 sites, methylation ranged from approximately 8-17% (Figure 4.1). At all 5 CpG sites, *SFRP4* promoter methylation was significantly ($p<0.001$) higher in the lower CRC risk group. Averaged across all 5 CpG sites, methylation was 2.34% lower in DNA from polyp patients than in controls (10.75% and 13.09% methylation respectively; $p<0.001$).

Figure 4.1 *SFRP4* methylation in DNA from buccal mucosa from participants in the BORICC Study (analysis has been adjusted for age)



Sex, smoking and BMI had no significant effects on *SFRP4* promoter methylation in the buccal DNA obtained from BORICC Study participants. However, age was a significant covariate for methylation at CpG site 3, CpG site 4 and all CpG sites combined, where increasing age was significantly ($p<0.02$) associated with a higher levels of *SFRP4* promoter methylation (Table 4.2).

Table 4.2 The effect of covariates upon *SFRP4* methylation in DNA from buccal cells obtained from participants in the BORICC Study

Factor	**CpG site 1**	**CpG site 2**	**CpG site 3**	**CpG site 4**	**CpG site 5**	**Average**
Age (p-value)	0.396	0.166	0.004	0.02	0.102	0.02
Sex (p-value)	0.262	0.405	0.456	0.532	0.619	0.542
Smoking (p-value)	0.807	0.293	0.404	0.274	0.427	0.289
BMI (p-value)	0.396	0.377	0.802	0.08	0.905	0.478

4.3.3 Correlation between *SFRP4* methylation in matched samples of buccal DNA and rectal DNA obtained from participants in the BORICC Study

Table 4.3 shows that there were relatively weak, but statistically significant, negative correlations between *SFRP4* promoter methylation in buccal cells and that in rectal mucosa at CpG site 1 (Figure 4.2) and CpG site 4 (Figure 4.3) only, (p=0.001, R=-0.184 and p=0.041 R=-0.114 respectively).

Table 4.3 Results of regression analysis of *SFRP4* promotor methylation in DNA from rectal biopsies and buccal cells (matched samples obtained from participants in the BORICC Study)

	CpG site 1	**CpG site 2**	**CpG site 3**	**CpG site 4**	**CpG site 5**	**Average**
R coefficient	-0.184	-0.032	0.044	-0.114	-0.063	-0.084
Slope	-0.285	-0.055	0.105	-0.245	-0.168	-0.199
SE of the slope	0.083	0.093	0.151	0.119	0.147	0.127
Intercept	15.45	15.19	12.71	21.34	15.06	16.76
SE of the intercept	0.94	1.19	1.96	1.95	1.61	1.61
p-value	0.001	0.554	0.487	0.041	0.255	0.117

Figure 4.2 Relationship between *SFRP4* methylation at CpG site 1 in DNA from buccal cells with that from rectal mucosa obtained from participants in the BORICC Study

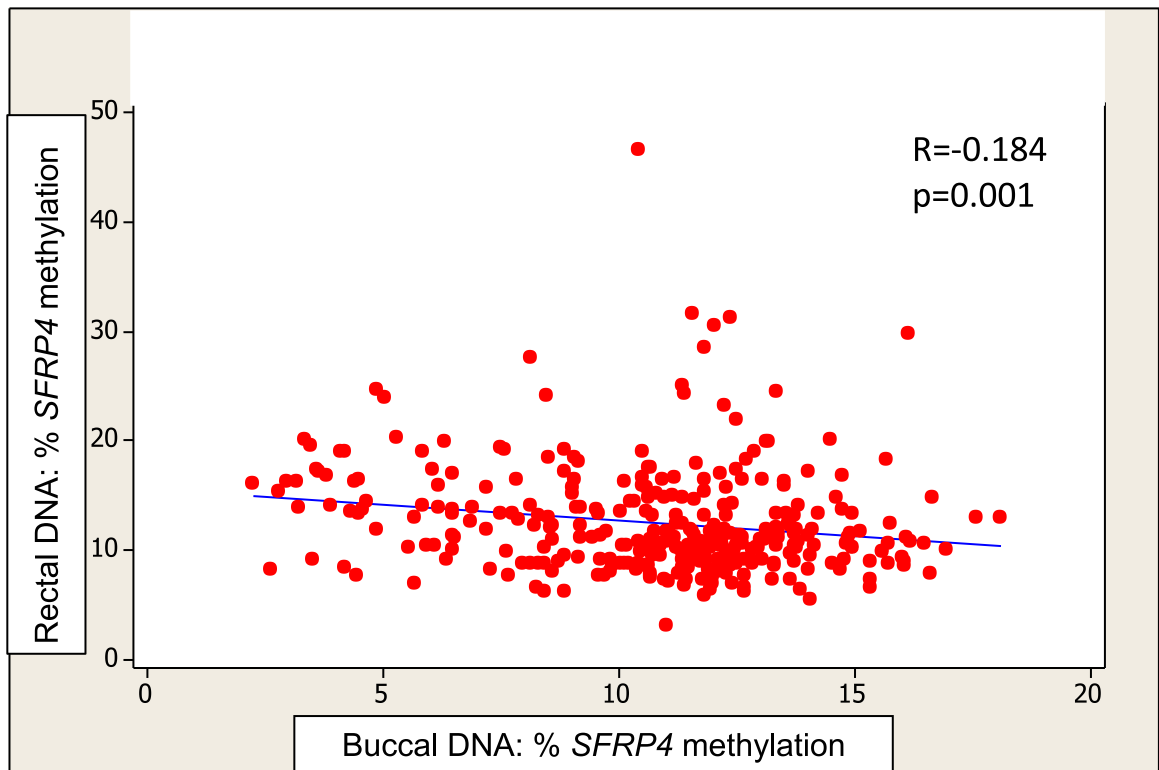
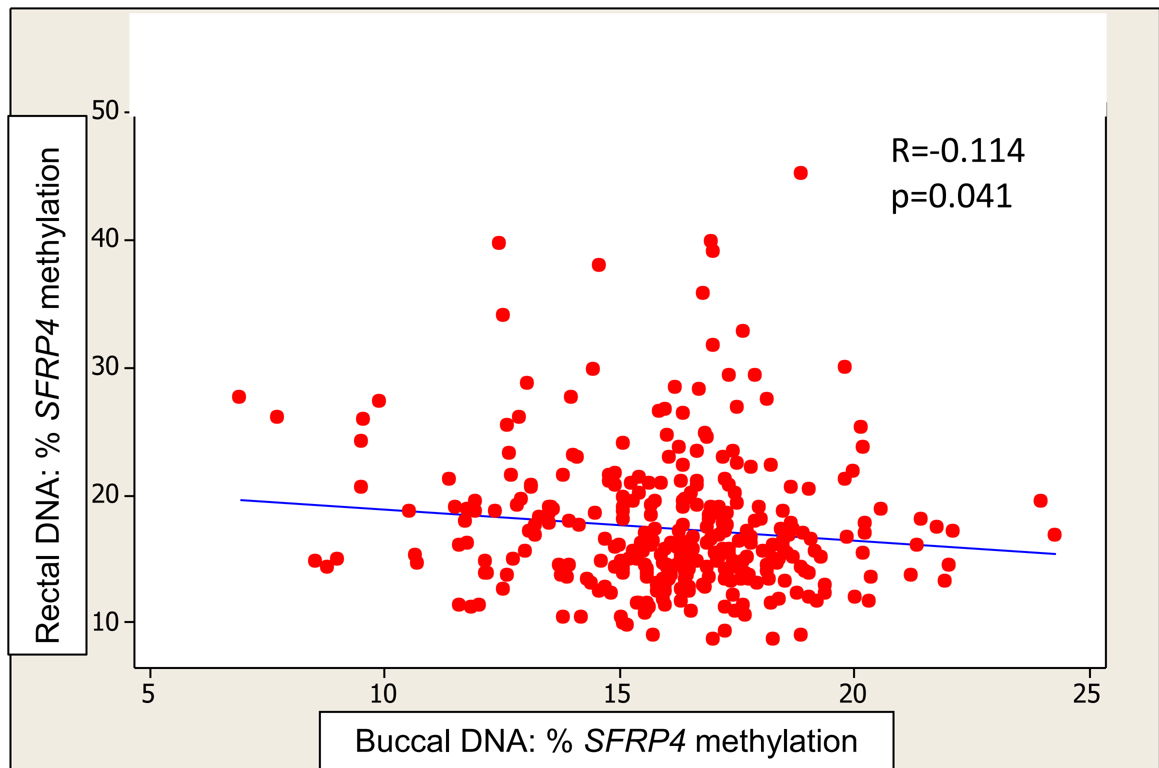


Figure 4.3 Relationship between *SFRP4* methylation at CpG site 4 in DNA from buccal cells and that from rectal mucosal biopsies obtained from participants in the BORICC Study



4.3.4 *SFRP4* methylation as a screening test for risk of CRC

Figure 4.1 shows that *SFRP4* promoter methylation in DNA from buccal cells was consistently greater in those at lower risk for CRC. This difference could be clinically important and might be the basis for development of a diagnostic test which uses *SFRP4* promoter methylation to identify those at an increased CRC risk. To investigate this potential, logistic regression was used to investigate differences in *SFRP4* promoter methylation levels in those at lower and at higher risk for CRC. Since age was significantly ($p<0.001$) lower in the "Normal" participants than in the patients with polyps, age was used as a factor in the analysis (section 4.3.1).

SFRP4 promoter methylation in buccal DNA was highly specific at identifying those at a lower risk of CRC (specificity > 0.93) for all CpG sites individually and

combined) (Table 4.4). However sensitivity was relatively poor. The highest sensitivity for *SFRP4* promoter methylation in buccal DNA was 0.65 (all CpG sites combined) (Table 4.4).

Table 4.4 Outcomes of ROC analysis of *SFRP4* methylation in DNA from buccal cells obtained from participants in the BORICC Study: Sensitivity and specificity for differentiating between those at a lower and higher risk for CRC

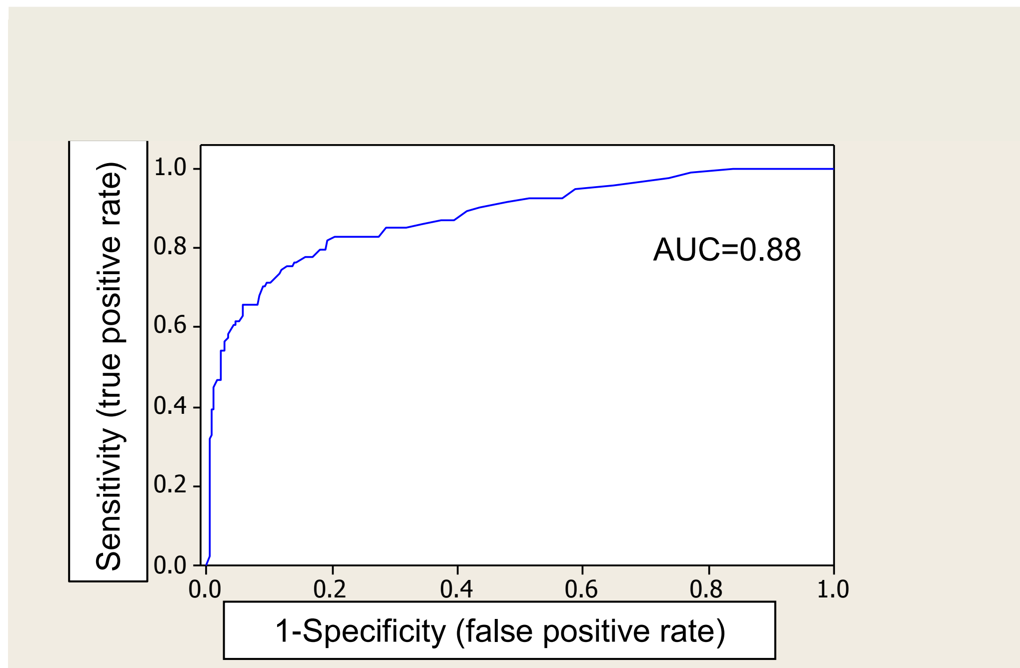
	Specificity	**Sensitivity**	**AUC***
CpG site 1	0.96	0.64	0.88
CpG site 2	0.96	0.53	0.84
CpG site 3	0.93	0.30	0.78
CpG site 4	0.93	0.41	0.80
CpG site 5	0.93	0.23	0.75
CpG sites averaged	0.93	0.53	0.84
CpG sites combined	0.94	0.65	0.88

**AUC refers to the area under the ROC curve.*

These calculations have been adjusted for age.

ROCs demonstrated that *SFRP4* methylation in DNA from buccal cells would be a “fair to good” test for differentiating between those at a higher and lower risk for CRC (AUC 0.75-0.88) (Table 3.4; Table 4.4; Figure 4.4) (Hanley & McNeil 1982).

Figure 4.4 ROC curve: *SFRP4* methylation in buccal DNA obtained from the BORICC Study: All CpG sites combined



Each point on the plotted line represents matched sensitivity and 1-specificity values. When specificity is high (0.0 on x-axis), sensitivity is low. Similarly, when sensitivity is high (0.9 on y-axis), specificity is low. There is a trade-off between maximising sensitivity and specificity. In screening tests, such as that proposed for the use of *SFRP4* methylation in buccal DNA, it is important to maximise sensitivity (to minimise false negatives). However this would mean settling for a lower specificity, which would result in higher false positives, causing people not at CRC risk to worry whilst undergoing further definitive investigations.

4.4 Discussion

4.4.1 *SFRP4* methylation in buccal DNA: Comparison of those at higher *v.* lower risk for CRC

The main finding from the work described in this chapter was that *SFRP4* promoter methylation in DNA from buccal cells from participants in the BORICC Study was significantly ($p<0.001$) higher in the lower CRC risk group at all 5 CpG sites investigated. This between risk group difference is the opposite to that observed for *SFRP4* promoter methylation in rectal DNA.

This is the first study which has investigated *SFRP4* promoter methylation in DNA from buccal cells from colorectal polyp patients. Indeed, there is limited literature on *SFRP4* promoter methylation in buccal cells under any circumstances. One study reported that *SFRP4* promoter methylation was increased in primary OSCC compared with matched normal oral mucosa (Pannone et al. 2010). Pannone et al. (2010) compared *SFRP4* methylation levels in DNA extracted from 37 OSCC and 37 controls of normal oral epithelium and found that the *SFRP4* promoter was significantly more ($p<0.001$) methylated in OSCC than in normal controls. None of the participants in the BORICC Study had OSCC. In addition, there is no literature investigating associations between CRC and OSCC which suggest that if an individual was at an increased risk of CRC, they would be at a decreased risk of OSCC and vice versa.

Since at the level of the individual genome (DNA molecule) methylation of a CpG site is binary i.e. the cytosine residue is either methylated or not methylated, it is expected that the percentage of *SFRP4* methylation in a tissue sample will reflect the proportion of cells within the buccal mucosa that are methylated (Mathers & Ford 2009). In other words, at the tissue level, a lower level of *SFRP4* promoter methylation suggests that there are fewer methylated cells within the buccal mucosa. It is also expected that *SFRP4* promoter methylation is correlated inversely with *SFRP4* gene expression. Therefore the percentage of *SFRP4* promoter methylation may allow the prediction of the

proportion of cells within the buccal mucosa in which the *SFRP4* gene is suppressed. This suggests that in buccal mucosa, the lower levels of *SFRP4* methylation in the higher risk group reflect a lower level of SFRP4 gene expression in the lower CRC risk group. This is the opposite to the findings in the rectal DNA and to what is expected given that SFRP4 is a tumour suppressor gene. This may be because:

- *SFRP4* promoter methylation in DNA from buccal cells is not different in those at a higher or lower risk for CRC and the results obtained in this research project have been due to chance. Whilst confounding by factors not considered in this study cannot be ruled out, the consistent difference in methylation between the Polyp group and the Normals in this study which was evident at all 5 CpG sites investigated suggests strongly that this is not a chance observation.

- The epigenetic processes that lead to *SFRP4* promoter methylation are influenced by the local environment and differ between mouth and rectum. For example, smoking and alcohol intake are both known risk factors for CRC and OSCC (CRUK 2013). However, they may affect DNA in cells in the mouth differently to DNA in cells from colonic tissue. This may be because of a more direct and concentrated effect in the mouth. Section 1.4.2.1 details how butyrate is a natural histone deacetylase inhibitor and is a product of dietary fibre fermentation in the colon. By preventing deacetylation, butyrate halts the process that leads to tumour gene expression suppression (Davis 2003, Rada-Iglesias et al. 2007). If butyrate plays a role in *SFRP4* gene expression, which may impact upon *SFRP4* methylation because of the inverse relationship between DNA methylation and gene expression, it may be partly accountable for the levels of *SFRP4* methylation quantified in rectal DNA. Butyrate is not present in the mouth and so if not available to produce the effects described for the large bowel. These possible differing effects of the local environment in the mouth and the large bowel will be addressed.

4.4.1.1 The effect of smoking on oral mucosa compared to the rectal mucosa

Cigarette smoke and its carcinogens come into direct contact with buccal mucosa and therefore may have more effect on buccal DNA than rectal DNA. The carcinogens in cigarette smoke may reach the colonic lumen as they are swallowed by the smoker. However, by the time these carcinogens reach the colon, their concentrations may be lower than when they were first ingested.

Naderi et al. (2012) used the micronucleus assay to assess the effect of smoking on DNA in buccal cells of smokers and non-smokers. Micronuclei result from chromosome fragments that are not included in the main daughter nuclei during nuclear division and provide a measure of chromosome breakage and chromosome loss, and is an indicator of DNA damage at the chromosome level (Fenech 1994). Naderi et al. (2012) assessed 500 buccal cells per study participant in 23 non-smokers, 14 participants who had smoked for up to 10 years and 26 participants who had smoked for more than 10 years. The mean percentage of micronuclei for each study group was quantified and found to be statistically different ($p<0.002$), where the lowest numbers of micronuclei were seen in the non-smokers. This suggests that smoking plays a role in buccal DNA damage. There are no studies assessing the same marker of DNA damage in both buccal and rectal mucosa which would allow a direct comparison.

Section 1.4.2.8 describes how smoking may be causally linked to CRC and polyp formation by exposure of the colonic epithelium to carcinogenic compounds in the cigarette smoke via the systemic circulation or from direct exposure from their ingestions (Chan & Giovannucci 2010). The mechanisms by which smoking may lead to polyp formation are not clear and require further investigation.

In this study, there was evidence that smoking behaviour influenced *SFRP4* methylation at CpG sites 1 and 3 in buccal DNA (Table 4.2). It is surprising that

not all CpG sites were influenced by smoking. This is because smoking has generally been associated with increased DNA promoter methylation (Zochbauer-Muller et al. 2003, Enokida et al. 2005, Marsit et al. 2007). However, as there is limited literature on *SFRP4* methylation, it is not known whether cigarette smoke has effects on methylation of specific genes such as *SFRP4*.

4.4.1.2 The effect of alcohol on oral mucosa compared to the rectal mucosa

Alcohol is an established risk factor for both CRC and OSCC (CRUK 2013) but the mechanisms through which alcohol affects CRC and OSCC development may differ. For example, alcohol may play a role in OSCC development as a result of direct contact of oral mucosa with alcohol. This is less likely in CRC development as the majority of alcohol is absorbed by the stomach and small intestine before it is able to reach the large intestine (Mumenthaler et al. 1999). The systemic effects of alcohol and its metabolism within the body are more likely to exert effects that play a role in CRC development. These same systemic mechanisms may also play a role in the development of OSCC.

Simanowski et al. (1995), Maier et al. (1994) and Seitz et al. (1998) have all investigated chronic alcohol consumption as a risk factor for various gastrointestinal cancers, including OSCC and CRC. Susceptibility to cancer development is the result of increased regeneration of gastrointestinal mucosa which has an increased susceptibility towards the action of carcinogens. Chronic alcohol use results in oral mucosal atrophy, which may play a role in OSCC development. Excess alcohol intake may also stimulate crypt cell production in the rectum, thus playing a role in the development of rectal cancer.

A more direct effect of alcohol on the oral mucosa was suggested by Zamora-Parez et al. (2013), who investigated the effects of alcohol-containing mouthwash on the induction of nuclear anomalies in buccal cells in 38 participants who used alcohol-containing mouthwash (26% ethanol

concentration) twice a day for 30 consecutive days, 36 participants who used a non-alcohol-containing mouthwash for the same time period, and 33 participants who did not use any mouthwash. There were significantly greater ($p<0.05$) nuclear anomalies in the group who used alcohol-containing mouthwash compared with both the control group and the non-alcohol mouthwash group.

4.4.1.3 The effect of diet on oral mucosa compared to the rectal mucosa

Dietary factors may affect buccal DNA and rectal DNA differently because food may be in one form in the mouth and another in the colon. Also, the different local environments may result in different effects upon buccal DNA and rectal DNA. To demonstrate this difference, an item of food may be followed from initial ingestion to the large intestine (should it reach that far along the alimentary canal). In the mouth, food will initially be in its undigested form. Digestion begins mechanically and the teeth break food into smaller pieces. Salivary glands release saliva to lubricate food to aid its digestion. Saliva also contains amylase and lipase which digest starches and fats respectively. The temperature of the food may be important. Food that is too cold or too hot may be irritant to the oral mucosa or it may cause the oral enzymes to work less efficiently because at extremes of temperatures, enzymes will denature and fail to function. Each of these aspects of the local oral mucosal environment may have an effect on buccal DNA and possibly its methylation.

Once swallowed, the food bolus passes down the oesophagus and into the stomach, where pepsinogen begins to digest proteins. In the small intestine, the now partially digested food comes into contact with amylases, trypsinogen and other proteases, nucleases and lipase secreted from the pancreas to further digest starches, proteins, nucleic acids and fats respectively the latter aided by bile from the gallbladder. The result is that most of the food is digested into simpler monomeric forms including sugars, amino acids, fatty acids, vitamins and minerals which are absorbed along the small intestine. In

summary, the majority of nutrients from the initial ingested food will not reach the large intestine.

In contrast, dietary fibre and resistant starches resist degradation and pass into the large intestine. Resistant starches and soluble dietary fibres are fermented in the colon to produce SCFA, such as butyrate, which has a local protective effect on colonic epithelium (Section 1.4.2.4). Butyrate is not present in the mouth on initially ingesting dietary fibres or resistant starch to have the same local effect on the oral mucosa.

4.4.1.4 Microflora of the oral mucosa compared with the rectal mucosa

The microflora of the oral mucosa and rectal mucosa differ, and this may account for some of the difference in *SFRP4* methylation at the two locations. Oral bacteria include streptococci, lactobacilli, staphylococci and anaerobes such bacteroides, whereas in the colon, whilst bacteroides are also present, coliforms are more prominent. The microflora in both the mouth and colon synthesises vitamins. Both are also able to stimulate the development and activity of immunological tissues, but the microflora of the colon has a greater ability to do this. Some of the bacteria found in the colon may be harmful, for example, bacteroides produce metabolites that are carcinogenic (Todar 2013).

4.4.2 Colorectal cells versus buccal cells

In evaluating the utility of buccal cells as surrogates for molecular events in the colorectal epithelium relevant to the development of CRC, it is useful to consider similarities and differences between the epithelia per se, and the nature of the collected samples, at the two ends of the GI tract. Buccal DNA was extracted from samples taken from the buccal mucosa. This is relatively thick non-keratinised epithelium that forms the lining of the mucous membrane in the mouth. The mucous membrane in the mouth consists of stratified squamous cells. In comparison, rectal DNA was extracted from mucosal biopsies obtained from the “simple” columnar lining of the rectum.

A rectal mucosal biopsy takes a piece of tissue from the rectum. This will contain cells from the single layer of epithelial cells that line the colon, and also other structures such as immune cells, epithelial cells of the crypt and blood vessels. In contrast, the buccal cells sampled when obtaining a buccal swab are the cells residing on the surface of the mucous membrane in the mouth, and this may not represent the level of *SFRP4* methylation in all layers of the stratified squamous epithelium. There is no literature available to suggest that *SFRP4* methylation or methylation of any other gene differ across these layers. However, in oral stratified squamous epithelium, cell division occurs at the deeper layers, and not in the cells available at the surface which are most likely to be sampled with a buccal swab. These cell differences could cause a difference in *SFRP4* methylation levels.

The alimentary canal starts at the mouth and ends at the anus, and involves both the mouth and rectum. It consists of continuous regions although some organs, for example the oesophagus, stomach, small and large bowel have distinct boundaries separating them. Field changes include abnormalities of epithelial gene expression affecting the mucosa rendering it vulnerable to neoplasia. This phenomenon has been described in the colon in which DNA extracted from the colon has shown changes in methylation levels when neoplasia is present at any site within that colon (Belshaw et al. 2008). As yet, there is no literature investigating whether field changes can be applied across the boundaries of different organs to different tissue types within the GI tract. For example, are changes in methylation levels in rectal DNA reflected in buccal DNA and vice versa? If so, there is the possibility that CRC screening tests could use buccal DNA – a test which may be more acceptable to patients than rigid sigmoidoscopies to obtain rectal biopsies for DNA extraction or measurement of other biomarkers.

The observation of significant negative correlations between *SFRP4* promoter methylation in rectal DNA and buccal DNA at CpG site 1 ($p=0.001$, $R=-0.184$) (Figure 4.2) and CpG site 4 ($p=0.041$, $R=-0.114$) (Figure 4.3) was a further novel finding from this study. This observation suggests that methylation of this

gene, at least at these 2 CpG sites, may be related in the 2 parts of the GI tract considered in this project. If similar factors influence *SFRP4* promoter methylation in these 2 gut regions, one might have expected that the correlations would have been positive. The finding of significant negative correlations is intriguing and implies that, if there is a mechanistic connection, then the mediating factor(s) operates in opposite directions in the mouth and the rectum. This is the first study investigating relationships between methylation levels of the *SFRP4* promoter in DNA from matched samples of rectal and buccal cells and further studies are needed to confirm, or to refute, these observations.

4.4.3 *SFRP4* methylation in buccal DNA as the basis for development of a screening test for CRC risk

This research project has investigated the use of buccal cells as a surrogate tissue for rectal biopsies when investigating potential biomarkers of risk of developing CRC. Collection of buccal cells has many practical advantages over obtaining biopsies from the rectum including i) the collection can be done anywhere and does not require that participants attend a clinic, ii) study participants can collect the buccal cells unaided i.e. without the requirement for specialised (expensive) medical staff, iii) sample collection is relatively non-invasive and essentially hazard-free e.g. there is no risk of significant bleeding or perforation which can occur with rectal biopsies and iv) the collected samples can be preserved immediately in tubes provided as part of the collection kit without recourse to specialised equipment (e.g. liquid nitrogen storage) and transferred to the laboratory by hand or by post. It is not known whether the field effect (changes in DNA methylation and gene expression which are indicative of vulnerability to CRC) that occurs in the colon extends to the mouth. However this study has shown that there are differences in *SFRP4* methylation in buccal DNA extracted from those at a higher and lower risk for CRC and this promising finding requires further investigation. If the hypothesis that buccal DNA can be used as a surrogate for rectal DNA is investigated rigorously and found to be true, DNA biomarkers specific to CRC may be measurable in buccal cells.

SFRP4 promoter methylation in buccal DNA obtained from the BORICC Studies were highly specific at identifying those at a lower risk of CRC (specificity > 0.93 for all CpG sites individually and combined) (Table 4.4). However sensitivity was relatively poor. The highest sensitivity was 0.65 (all CpG sites combined) (Table 4.4). ROCs demonstrated that *SFRP4* methylation in buccal DNA from the BORICC Study would be a "fair" to "good" test to differentiate between those at a higher and lower risk for CRC (AUC 0.75-0.88) (Table 3.4; Table 4.4; Figure 4.4) (Hanley & McNeil 1982) (section 4.2.4.4). Similar to *SFRP4* methylation in rectal DNA, *SFRP4* methylation in buccal DNA is not sensitive for identifying those at a higher risk of CRC (section 3.4.3).

It is unlikely that any test measuring DNA biomarkers to assess risk for CRC would rely on only 1 gene. All current DNA biomarker tests to assess risk for CRC use a panel of genes that are known to play a role in CRC development. This would be more reliable than measuring just 1 biomarker because CRC is a complex disease and aberrant expression of multiple tumour suppressor genes and oncogenes have been described. *SFRP4* promoter methylation in buccal DNA obtained from the BORICC Studies were highly specific at identifying those at a lower risk of CRC (specificity > 0.93). This needs further investigation to ensure the high level of specificity is reproducible, and if found to be, *SFRP4* methylation in buccal DNA could be used potentially as a biomarker for CRC risk. It is likely that such use would be as a component of a panel of genes assessing risk and that the other selected biomarkers had high sensitivity to compensate for the poor sensitivity of *SFRP4* at identifying CRC risk.

4.5 Conclusions

This research project has shown that *SFRP4* promoter methylation in buccal DNA is significantly greater ($p<0.001$) in those at a lower CRC risk, and this between risk group difference is the opposite to that observed for *SFRP4* methylation in rectal DNA.

Possible correlations between *SFRP4* promoter methylation levels in matched samples of rectal DNA and buccal DNA were investigated. Although the direction of change was opposite, at CpG site 1 and CpG site 4 only, there was a relatively weak, but statistically significant, negative correlation between *SFRP4* promoter methylation in buccal cells and that in rectal mucosa ($p=0.001$, $R=-0.184$, and $p=0.041$, $R=-0.114$ respectively).

This is the first study which has investigated *SFRP4* promoter methylation in DNA from buccal cells from colorectal polyp patients and further investigation is required to establish if these results are reproducible and to investigate causes for the unexpected difference in *SFRP4* methylation. In addition, further investigations should include the possible confounding effect of the local environment upon the differences in *SFRP4* methylation in buccal DNA and rectal DNA.

5 Effect of short-term supplementation with resistant starch and polydextrose on *SFRP4* methylation in the human colorectal mucosa.

5.1 Introduction

Chapter 3 of this thesis reports that methylation of the *SFRP4* promoter was consistently greater in patients with polyps i.e. at higher CRC risk than in "normal" volunteers without evidence of colorectal neoplasia. This suggests that *SFRP4* promoter methylation might be a useful biomarker of CRC risk. In a systematic review of 21 prospective cohort studies, including over 1.7 million participants and 12000 CRC cases, Aune et al. (2011) found that individuals with diets rich in dietary fibre have lower CRC risk. On this basis we hypothesised that dietary factors such as resistant starch and polydextrose which lower CRC risk might alter methylation of the *SFRP4* promoter. To test this hypothesis *SFRP4* promoter methylation levels were quantified in rectal DNA obtained from healthy volunteers who were participants in the DISC Study (2011). Rectal mucosal biopsies were collected before and after a randomised controlled trial of dietary supplementation with resistant starch and polydextrose in a 2×2 factorial design.

5.2 Methods

Participants were recruited to the DISC Study (2011) from endoscopy lists within Northumbria Healthcare NHS Foundation Trust as outlined in section 2.5. In addition to the exclusion criteria outlined in section 3.2.1.1., volunteers wishing to participate in the dietary intervention study could not have any of the exclusion criteria described in section 5.2.1.

5.2.1 Exclusion criteria for the healthy participants in dietary intervention component of the DISC Study

Potential participants recruited for the DISC Study (2011) dietary intervention study were excluded if they were taking anticoagulant medication such as warfarin or low molecular weight heparin. This exclusion criterion was included as a safety measure because participants taking such therapies would be at increased risk of bleeding when their second set of rectal biopsies was taken. Due to the relatively small carbohydrate doses used in the DISC Study (2011) RCT, it is unlikely that blood sugar control would be affected by the placebo dietary supplements used in the intervention study (amioca starch or maltodextrin). However, potential participants were also excluded if they were diabetic because diabetes is a risk factor for CRC. In a systematic review of 30 cohort studies on diabetes and CRC incidence, Jiang et al. (2011) demonstrated that the Relative Risk (RR) of CRC among diabetics was 1.27 (95% CI 1.21-1.34). By excluding diabetics, we were likely to reduce inter-individual heterogeneity in CRC risk and, possibly, in response to the interventions, among the study participants.

5.2.2 Randomisation to treatment within the DISC Study

The DISC Study (2011) was a randomised, placebo-controlled study. Recruited participants who had no exclusion criteria selected an opaque sealed envelope from a box which dictated which dietary supplements they would receive (section 5.2.5). Participants were also stratified into two separate groups dependent on which endoscopy procedure (flexible sigmoidoscopy or colonoscopy) they received at the time of collection of their initial biopsies. Randomisation was double blinded and, as this is an ongoing study, the investigators will not be “unblinded” as to which combination of dietary supplements study participants received until after the submission of this thesis.

5.2.3 Biological samples

Rectal mucosal biopsies were obtained from participants as described section 3.2.2.1.

5.2.4 The dietary intervention

Participants in the DISC study (2011) RCT took a dietary supplement for 50 days following collection of their initial rectal biopsies. This supplementation did not start immediately after their first biopsies as it was possible the bowel mucosa and colorectal microbiome would be affected by the bowel preparation given prior to their initial endoscopy investigation. Therefore a "washout" period of at least seven days was used before commencing the dietary supplements. A minimum of seven days "washout" period was deemed sufficient because colorectal stem cells divide and migrate from the base towards the surface of the colonic crypt, where they die or are sloughed off into the colonic lumen, in 4-8 days (Stappenbeck et al. 1998, Radtke & Clevers 2005). Upon completion of the 50 day supplementation, a repeat set of biological samples was collected.

5.2.5 The dietary supplements

The DISC Study used a 2×2 factorial design to test the impact of two "active" agents on colonic health The "active" agents were Hi-maize 260 (National Starch, USA) (resistant starch) and polydextrose (Danisco, Finland) and the corresponding placebo agents were amioca starch and maltodextrin respectively. This study design resulted in 4 intervention combinations:

1. Hi-maize 260 (23g/d) and polydextrose (12g/d)
2. Hi-maize 260 (23g/d) with maltodextrin (12g/d) (polydextrose placebo)
3. Polydextrose (12g/d) with amioca starch (23g/d) (Hi-maize 260 placebo)
4. Double placebo [amioca starch (23g/d) and maltodextrin (12g/d)].

All supplements were packaged into 8×8cm opaque silver sachets and coded according to their contents. The sachets were then packed into boxes to

contain a week's supply of supplements to make up each type of dietary intervention. The daily dose of each supplement was split equally between two sachets. Therefore each day, each participant was asked to consume 4 sachets; and each weekly box of supplements contained 28 sachets.

Participants were asked to consume the supplements on, or added to, cold food or mixed with cold water. They were asked to retain the sachets whether or not the supplement had been eaten to allow compliance to be assessed.

5.2.5.1 Hi-maize 260

Hi-maize 260 is a source of resistant starch which is isolated from a hybrid corn (maize) that is naturally high in amylose. It contains approximately 60% resistant starch and 40% digestible starch.

5.2.5.2 Polydextrose

Polydextrose is an indigestible synthetic polymer of glucose and sorbitol. It is very poorly digested in the small intestine and more than 95% of polydextrose available for fermentation in the colon.

5.2.5.3 Amioca starch

This starch consists mainly of amylopectin, an α[1-6]-branched polymer of glucose in which the glucose residues in the linear components are α[1-4]-linked It is completely digested in the small intestine and therefore none reaches the colon.

5.2.5.4 Maltodextrin

Maltodextrin is an oligosaccharide made by partial hydrolysis of starch. It is easily digestible and fully absorbed in the small intestine. None reaches the colon.

5.2.6 Laboratory methods

DNA was extracted from rectal biopsies and processed to quantify *SFRP4* promoter methylation as described in section 3.2.3.

5.2.7 Statistical analyses

5.2.7.1 Comparison of participant characteristics in the DISC Study

Characteristics of the participants in the four dietary intervention groups of the DISC Study were compared using ANOVA in a general linear model to identify any significant differences in the mean age and BMI between treatment groups; χ^2 test to identify any significant difference in the smoking status and the male:female ratio of the treatment groups. $p<0.05$ was considered significant.

5.2.7.2 Investigation of the effect of the dietary intervention within the DISC Study

The dietary intervention within the DISC Study was arranged as a 2×2 factorial design with the aim of testing the effects of resistant starch and polydextrose. This allowed investigation of the effects of each dietary agent individually and also any potential interaction between them. Data collected for this research project was analysed for the effects of “Treatment 1” and “Treatment 2”. It is unknown which of resistant starch or polydextrose “Treatment 1” and “Treatment 2” refer to. Minitab (version 16) statistical software was used to analyse the outcome data (after intervention) using ANOVA in a general linear model with the corresponding baseline data (before intervention) as a covariate. The direct effects of Treatment 1 and of Treatment 2 were assessed individually

and any potential interaction was also investigated. Results were expressed as least square means ± SEM. $p<0.05$ was considered to be significant.

5.3 Results

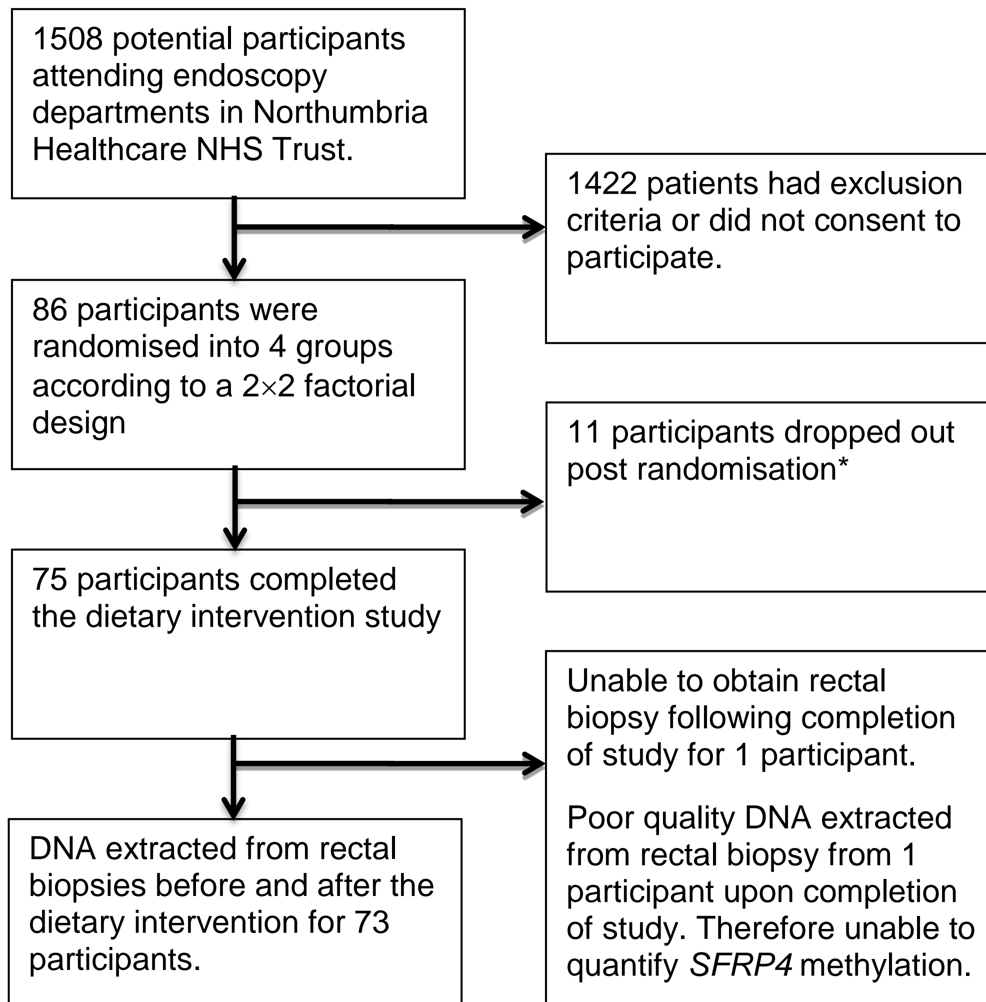
5.3.1 The DISC study

1508 potential participants attending the endoscopy departments in Northumbria Healthcare NHS Trust were invited to participate in the dietary intervention in the DISC study. Details are presented in the CONSORT diagram (Figure 5.1) Of these, 86 participants who did not have any exclusion criteria (section 5.2.1) and who consented to participate were recruited to the dietary intervention study and were randomised into 4 groups according to a 2×2 factorial design (section 5.2.2). Seventy five participants completed the intervention study (section 5.2.4). DNA was extracted from macroscopically normal rectal biopsies before and after the dietary intervention in 74 of the healthy volunteers. For one participant, it was not possible to obtain safely rectal biopsies after completion of the dietary intervention study because of inability to visualise the lining of the rectum because of large amounts of stool in the rectum. Therefore, for this individual, there was no DNA for quantification of *SFRP4* promoter methylation levels after completion of the dietary intervention. It was not possible to quantify *SFRP4* promoter methylation levels for DNA extracted from one other participant because the DNA extracted from the rectal biopsy obtained from this individual following completion of the dietary intervention was of insufficient quality to allow quantification of *SFRP4* promoter methylation (Figure 5.1).

5.3.2 Patient demographics

The demographics of the dietary intervention participants are shown in Table 5.1. There were no significant differences in age (p=0.062, ANOVA), the male:female ratio (p=0.1078, χ^2 test), BMI (p=0.566, ANOVA) or smoking status (p=0.6686, χ^2 test) between the four different dietary intervention groups.

Figure 5.1 CONSORT diagram summarising recruitment to, and participant flow through, the DISC Study (Schulz et al. 2011)



**Reasons for participant drop-out included: They did not like the intervention (n=2); they changed their mind (n=2); they were unable to participate because of time commitments (n=3); there were privacy issues with their family (n=1); they experienced bloating whilst taking the intervention (n=1); they became unwell during the intervention phase (not related to the intervention) (n=1); incorrect classification of endoscopy as "normal" by the study team (n=1).*

Table 5.1 Demographics of participants in the DISC Study by intervention group

	Group A*	**Group B***	**Group C***	**Group D***
Number of participants (male:female)	18 (9:9) (50%:50%)	21 (9:12) (43%:57%)	17 (5:12) (29%:71%)	17 (12:5) (71%:29%)
Mean age (standard deviation)	49 years (12.54 years)	58 years (14.96)	54 years (7.04)	50 years (10.18)
Mean BMI (standard deviation)	29.50 (4.84)	29.36 (6.10)	31.68 (5.00)	30.04 (5.60)
Smoking status (non/ex/current)	11/4/3 (61:22:17%)	12/6/2 (57:28:10%) [1 (5%) unknown]	8/5/4 (47:29:24%)	6/6/5 (35:35:30%)

**The four intervention groups are labelled groups "A-D" for purposes of illustration. It is not known what treatment combination each intervention group received.*

5.3.3 The dietary intervention

DNA was extracted from macroscopically normal rectal mucosal biopsies before and after the dietary intervention for 73 healthy volunteers.

5.3.4 The effects of Treatment 1 and Treatment 2 on *SFRP4* methylation in rectal DNA

Neither dietary intervention had any significant effect on *SFRP4* methylation in rectal DNA at any of the CpG sites investigated; see Figure 5.2 (Treatment 1) and Figure 5.3 (Treatment 2).

For those participants randomised to Treatment 1, With the exception of CpG site 1, *SFRP4* promoter methylation was higher in study participants before the treatment 1 (Figure 5.2). These differences were not statistically significant (p>0.498). *SFRP4* methylation ranged from approximately 8-14% and was highest at CpG site 4 and lowest at CpG site 1 in study participants who were given Treatment 1 (Figure 5.2).

Figure 5.2 *SFRP4* methylation in rectal DNA obtained from participants of the DISC Study: Treatment 1

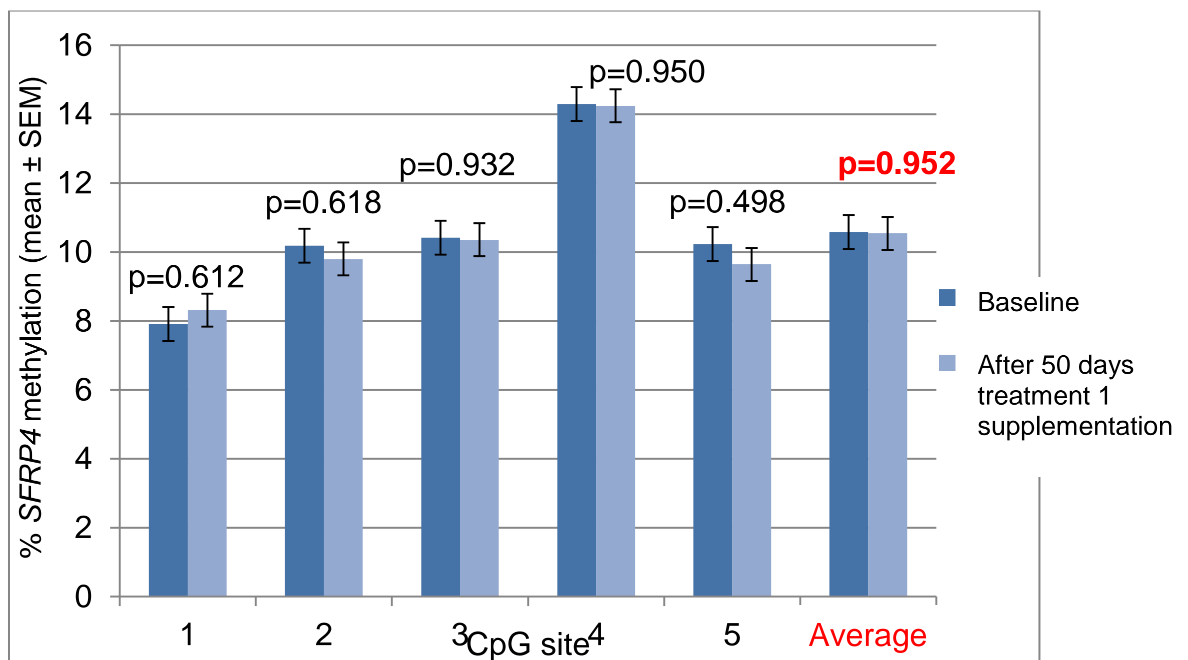
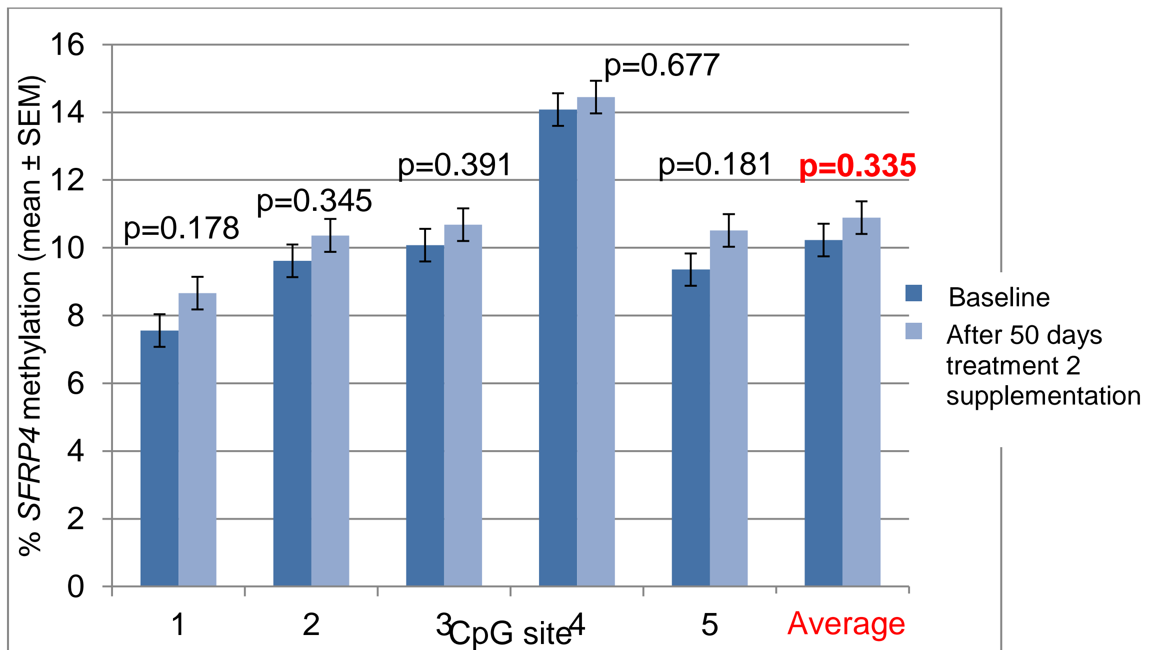


Figure 5.3 *SFRP4* methylation in rectal DNA obtained from participants in the DISC Study: Treatment 2

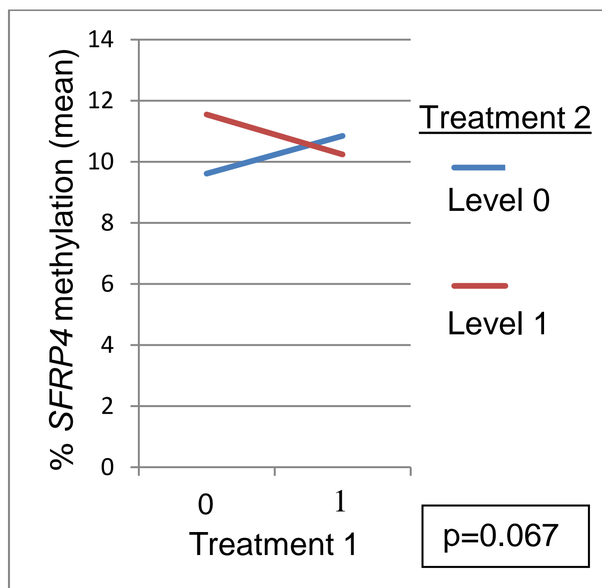


SFRP4 promoter methylation was higher in study participants after Treatment 2 (Figure 5.3) but these differences were not statistically significant (p>0.178). *SFRP4* methylation ranged from approximately 7-15% and was highest at CpG site 4 and lowest at CpG site 1 in study participants who were given Treatment 2.

5.3.5 *SFRP4* methylation in rectal DNA: Potential interaction between Treatment 1 and Treatment 2

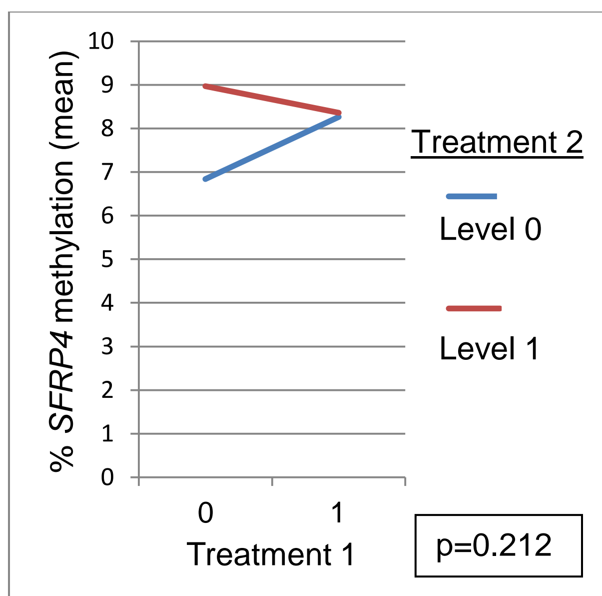
Although there was no evidence that either Treatment 1 or treatment 2 individually had any significant effect on *SFRP4* promoter methylation , the results shown in Figure 5.4-Figure 5.9 suggest that there may be interactions between the two dietary supplements on *SFRP4* promoter methylation ("0" and "1" indicate the two levels of treatment (placebo and active) but the identity of each remains blinded; the p-value refers to the interaction of Treatment 1 and Treatment 2).

Figure 5.4 Potential interaction between resistant starch and polydextrose on *SFRP4* methylation in rectal DNA obtained from the DISC Study – all CpG sites combined.



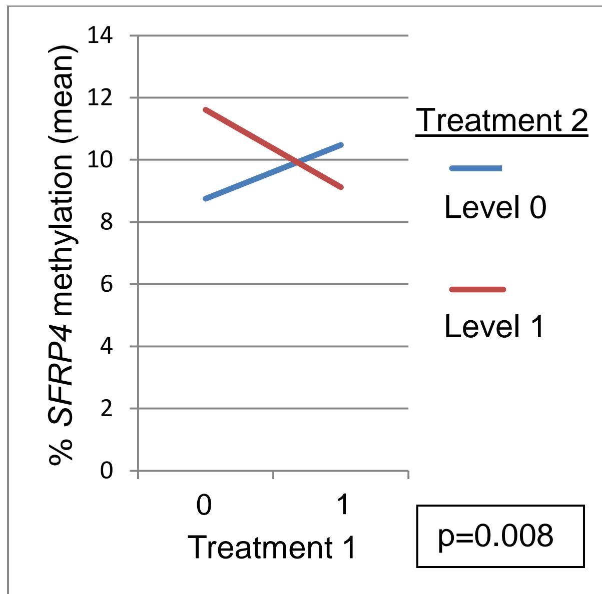
0 and 1 represent the two levels of treatment (active and placebo) but the identity of each remains blinded

Figure 5.5 Potential interaction between resistant starch and polydextrose on *SFRP4* methylation in rectal DNA obtained from the DISC Study – CpG site 1



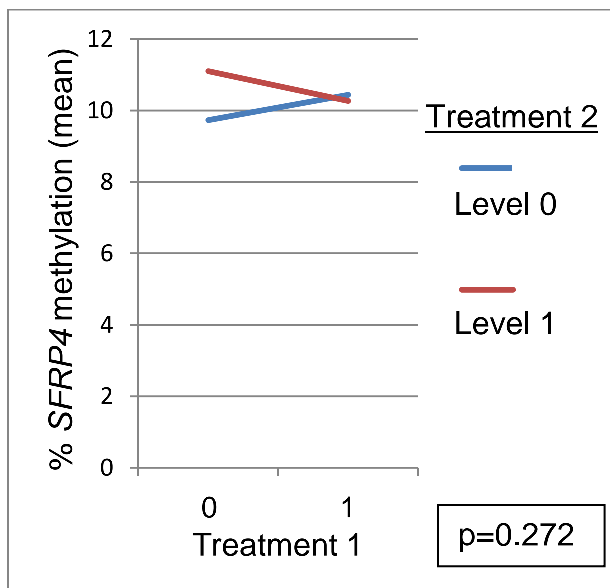
0 and 1 represent the two levels of treatment (active and placebo) but the identity of each remains blinded

Figure 5.6 Potential interaction between resistant starch and polydextrose on *SFRP4* methylation in rectal DNA obtained from the DISC Study – CpG site 2



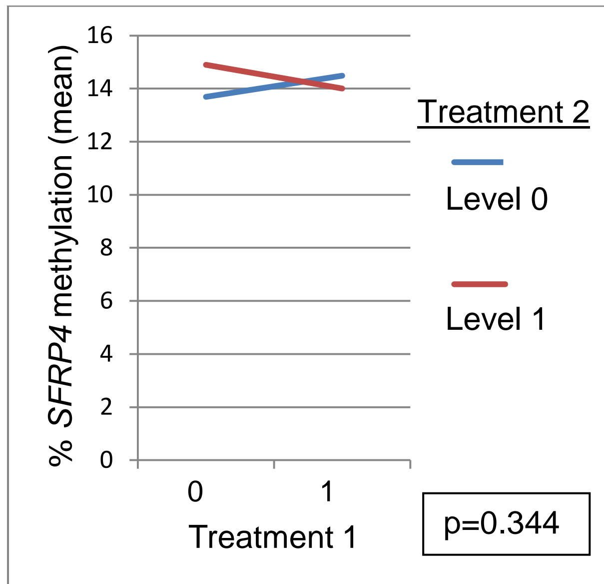
0 and 1 represent the two levels of treatment (active and placebo) but the identity of each remains blinded

Figure 5.7 Potential interaction between resistant starch and polydextrose on *SFRP4* methylation in rectal DNA obtained from the DISC Study – CpG site 3



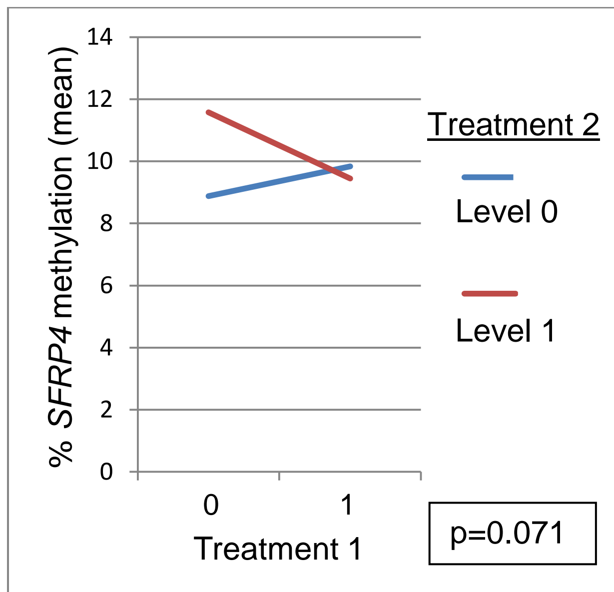
0 and 1 represent the two levels of treatment (active and placebo) but the identity of each remains blinded

Figure 5.8 Potential interaction between resistant starch and polydextrose on *SFRP4* methylation in rectal DNA obtained from the DISC Study – CpG site 4



0 and 1 represent the two levels of treatment (active and placebo) but the identity of each remains blinded

Figure 5.9 Potential interaction between resistant starch and polydextrose on *SFRP4* methylation in rectal DNA obtained from the DISC Study – CpG site 5



0 and 1 represent the two levels of treatment (active and placebo) but the identity of each remains blinded

At all individual CpG sites and for all CpG sites combined (Figure 5.4-Figure 5.9) there was evidence of an interaction between resistant starch and polydextrose on *SFRP4* methylation i.e. the effect of one treatment depends upon the presence (or absence) of the other. The nature of this interaction was

qualitatively similar at all CpG sites investigated. When Treatment 2 was given at Level 0, *SFRP4* methylation was higher when Treatment 1 was given at Level 1 than when given at Level 0. In contrast, when Treatment 2 was given at Level 1, *SFRP4* methylation was always lower when Treatment 1 was given at Level 1 than when given at Level 0. Although this interaction was apparent at all CpG sites, it is illustrated best at CpG site 2 (Figure 5.6) where the interaction was highly significant (p=0.008).

5.4 Discussion

5.4.1 Resistant starch and polydextrose given as chemoprevention agents independently

Resistant starch and polydextrose are so-called non-digestible carbohydrates i.e. they are not digestible in the small intestine and so flow to the large bowel where they are exposed to bacterial fermentation. As such, they may be classified as types of dietary fibre, and there have been multiple observational studies that show reduced CRC risk is associated with increased intake of dietary fibre (Bingham et al. 2003, Dahm et al. 2010, Aune et al. 2011). However, because of the close associations between dietary fibre and many other dietary components and, indeed, other lifestyle factors, it remains uncertain whether dietary fibre *per se* protects against the development of CRC or whether it is a marker of a lower risk diet/lifestyle. Chapter 3 shows that there are higher levels of *SFRP4* methylation in those at increased risk (patients with polyps compared with “normal” volunteers) which suggests that *SFRP4* is a possible biomarker of CRC risk. If resistant starch or polydextrose were chemoprotective, it was expected rectal DNA extracted from individuals taking resistant starch and/or polydextrose would have lower levels of *SFRP4* methylation. This difference was not seen. When given individually, neither dietary intervention had any significant effect on *SFRP4* methylation in rectal DNA at any of the CpG sites investigated.

The CAPP1 Study (Burn et al. 2011) and CAPP2 Study (Mathers et al. 2012) found no significant effect of resistant starch (with or without aspirin) on the development of CRC in individuals with FAP (CAPP1 Study) and HNPCC (CAPP2 Study), where colorectal adenoma (CAPP1 Study) and colorectal neoplasia (CAPP2 Study) was the primary outcome. In comparison, the study participants in this research project were healthy with no genetic predisposition to the development of CRC, and the outcome measure was the effect of resistant starch on *SFRP4* methylation. Therefore, the outcomes from the CAPP1 Study and CAPP2 Study are not directly comparable with those from this current research project. It is possible that resistant starch affects individuals genetically predisposed to CRC development (e.g. HNPCC, FAP)

differently to the general population. This is supported by Aune et al. (2011), who have reported that dietary fibre (e.g. resistant starch) significantly reduced CRC development in a meta-analysis.

The apparently protective effect of higher intakes of dietary fibre against CRC development which has been reported in observational studies may be the result of the health benefit of the whole dietary habits and/or lifestyles of the study participants rather than of the dietary fibre *per se*. This may explain why, in intervention studies, a protective effect of dietary fibre supplements alone has not been observed (Mathers et al. 2012).

In the current study, the effect of resistant starch and polydextrose was investigated in a group of individuals at relatively low CRC risk. As this group of study participants were already at lower CRC risk, it may not have been possible to reduce this risk further and this could account for why there was no significant drop in *SFRP4* methylation levels following intervention. It would have been interesting to investigate the effects of these dietary agents on those at a higher risk of CRC, who may have higher levels of *SFRP4* methylation, and to identify whether or not these dietary agents were able to reduce the levels of *SFRP4* methylation to those levels measured for the lower CRC risk group.

The lack of any significant effect of either of the dietary agents may be due to the small sample sizes of participants in the intervention study. A retrospective power analysis showed that for the differences in *SFRP4* methylation observed between the different treatment groups, to achieve a power of 80%, 201 study participants would be needed. This demonstrates that the sample size for the DISC Study was probably too small for this specific outcome measure.

In addition, or alternatively, the dose of each chemoprevention agent (12 grams/day of polydextrose and 23 grams/day of resistant starch) used in this study may have been too small. This is the first study to investigate the effects

of resistant starch and polydextrose on *SFRP4* methylation in colorectal DNA, and therefore there are no other studies for direct comparison. The latest data from the CAPP2 Study has shown that 30 grams of resistant starch given daily for a median of 24.4 months had no effect on CRC development after a median follow up time of 52.7 months (Mathers et al. 2012). Therefore, a dose of 23 grams/day of resistant starch given in this study given for only 50 days with no follow up may have been too small to exert any significant effect. However, contrasting to the results of the CAPP2 Study (Mathers et al. 2012), Aune et al. (2011) found that just 10g/day of dietary fibre was needed to reduce the risk of CRC risk by 10%. There is no literature regarding the effect of polydextrose on DNA methylation and what quantity is likely to have a therapeutic effect in the context of colorectal cancer risk and development.

The dietary agents may have been given for too small a time period for an effect to be observed. Cells in the colorectal mucosa have a life span of approximately 5 days during which they arise from the stem cell at the colonic base and migrate along the colonic crypt to the mucosal surface from where they are shed into the colonic lumen (Bach et al. 2000). Dronamraju et al. (2009) showed a significant reduction (p=0.028) in the proportion of mitotic cells in the top half of the colonic crypts in patients with CRC who were given resistant starch for just 2-4 weeks. There was also an increase in the expression of *CDK4* and *GADD45A* genes in CRC tissue following resistant starch intervention. These genes are associated with reduced cell proliferation (*CDK4*) and genomic stability (*GADD45A*). The participants in this research project (the DISC Study) took the intervention agent they were assigned for 50 days – almost twice as long as Dronamraju et al.'s (2009) study, and therefore it is likely that the dietary agents in this study were given for a long enough time period to exert a biological effect.

5.4.2 Resistant starch and polydextrose given as chemoprevention agents in combination

Resistant starch and polydextrose may interact together to affect *SFRP4* methylation. In this study, it appears that the effect of one treatment depended upon the presence (or absence) of the other. This is illustrated best for *SFRP4* methylation at CpG site 2 where the interaction was highly significant (p=0.008). However, the interaction was qualitatively similar at the 4 other CpG sites investigated which increases confidence that this may be a biologically important interaction. In each case, when Treatment 2 was present at Level 0, *SFRP4* methylation was higher when Treatment 1 was given at Level 1 than when given at Level 0. The opposite response occurred when Treatment 2 was given at Level 1 i.e. *SFRP4* methylation was reduced when Treatment 1 was given at Level 1 compared with that when given at Level 0.

At present this study remains blinded so that the identities of “Treatment 1” and “Treatment 2” are not known i.e. which is resistant starch and which is polydextrose, nor is it known which of Level 0 and Level 1 refers to “Placebo” and which to “Active agent”. As a consequence, the biological interpretation of the interaction summarised above cannot be determined until the DISC Study is unblinded. Of necessity, the following remarks are speculative and should be considered as ideas which will be pursued when the main study has been unblinded. Both resistant starch and polydextrose are substrates for colonic fermentation which produces butyrate and a range of other fermentation end-products including other SCFA. In addition, each individual carbohydrate may support the proliferation of a (subtly) different consortium of bacteria within the colon and it is likely that these bacteria or their metabolic end-products are responsible for effects in colonocytes within the epithelium including epigenetic marks such as DNA methylation. To my knowledge, there is no literature investigating whether resistant starch and polydextrose or other non-digestible carbohydrates interact in their effects on the gut microbiome, the metabolic end-products of this bacterial metabolism or down-stream effects on the colorectal mucosa. However, collaborators working within the DISC Study have collected data on several relevant factors including in faeces: SCFA concentrations,

bacterial species and concentrations of compounds with pro- and anti-inflammatory actions. In addition, the DISC team has undertaken extensive characterisation of cellular and molecular markers in the colorectal mucosal biopsies, including the methylation status of a larger panel of genes, which will be useful in determining i) whether interactions between polydextrose and resistant starch are apparent for other methylation of other genes involved in WNT signalling and ii) to help explain the biological basis for the observed interaction.

5.5 Conclusions

This research project has shown that following a 50 day dietary intervention study, 23 grams/day of resistant starch and 12 grams/day of polydextrose, individually and in combination had no significant effect on the *SFRP4* methylation in DNA extracted from colorectal cells obtained from healthy volunteers. Although the interaction effect was not statistically significant, this research project suggests that the direction of change exerted by the two dietary intervention agents on colon cell *SFRP4* methylation is opposite. The reasons for this are unknown and require further investigation.

Another area of future research is to assess whether resistant starch and/or polydextrose can reverse the higher levels of *SFRP4* methylation that have been observed in those at a higher risk of CRC in this research project.

6 Conclusions

6.1 Summary of findings

This research project aimed to test 3 hypotheses using pyrosequencing to quantify *SFRP4* methylation in various DNA samples obtained from rectal mucosal biopsies and buccal swabs taken from participants in the BORICC Study (Mathers et al. 2010) and the DISC study (2011).

The first hypothesis was that the *SFRP4* promoter is differentially methylated in rectal DNA obtained from volunteers at a lower (normal healthy volunteers) and higher (patients with a history of adenomatous polyps or a history of non-active UC) risk for CRC using rectal mucosal biopsies obtained in the BORICC Study and the DISC Study. There was strong evidence that *SFRP4* promoter methylation in DNA extracted from colorectal cells is greater in those at a higher CRC risk as demonstrated by the significantly ($p=0.036$) higher levels of *SFRP4* methylation in those at higher CRC risk in the BORICC Study. In addition, *SFRP4* methylation was also greater in those at a higher risk in the DISC Study, though this difference was not significant ($p>0.15$) in this much smaller study. This direction of change in *SFRP4* methylation is in keeping with studies published in the literature by Qi et al. (2006) and Belshaw et al. (2008).

This study also found a negative correlation between percentage *SFRP4* methylation and serum vitamin D concentration at all CpG sites investigated, and this supports the literature which proposes that there is a link between *SFRP4* methylation and vitamin D concentration.

This research project has found that *SFRP4* methylation alone is not a suitable epigenetic biomarker for CRC risk. Although *SFRP4* methylation levels in rectal DNA obtained from the BORICC Studies and the DISC study were both highly specific in identifying those at a lower risk of CRC (specificity>0.84), *SFRP4* methylation levels in rectal DNA obtained from both studies had poor sensitivity

levels (sensitivity<0.28). It remains to be discovered whether *SFRP4* methylation is a useful inclusion in a panel of markers of CRC risk.

The second hypothesis was that buccal DNA will show the same pattern of *SFRP4* promoter methylation as rectal biopsies in volunteers at a lower and higher risk of CRC and so will have potential as a surrogate tissue for CRC biomarker assay. *SFRP4* promoter methylation in matched samples of rectal DNA and buccal DNA from volunteers at a lower (normal healthy volunteers) and higher (patients with a history of adenomatous polyps) risk for CRC was quantified using rectal mucosal biopsies and buccal cells obtained in the BORICC study. Surprisingly, *SFRP4* promoter methylation in buccal DNA obtained from the BORICC Study was significantly ($p<0.001$) greater in those at a lower CRC risk. This between risk group difference is the opposite to that observed for *SFRP4* methylation in rectal DNA and deserves further investigation.

Possible correlations between *SFRP4* promoter methylation levels in matched samples of rectal DNA and buccal DNA were also investigated. At CpG site 1 and CpG site 4 only, there were statistically significant, but relatively weak, negative correlations between *SFRP4* promoter methylation in buccal cells and that in rectal mucosa ($p=0.001$, $R=-0.184$, and $p=0.041$, $R=-0.114$ respectively).

The third hypothesis was that *SFRP4* promoter hypermethylation in rectal biopsies is reversible by dietary supplements of resistant starch and polydextrose (both alone and in combination). Pyrosequencing was used to quantify *SFRP4* promoter methylation levels in rectal DNA obtained from healthy volunteers in the DISC Study (2011) before and after a randomised controlled trial of effects of dietary supplementation with 23 grams/day resistant starch and 12 grams/day polydextrose in a 2×2 factorial design. In this study, resistant starch and polydextrose had no significant effect individually or in combination on *SFRP4* methylation in colorectal DNA from healthy individuals after a 50 day trial. However, this study revealed evidence of possible

interactions between resistant starch and polydextrose on *SFRP4* methylation and this requires further investigation.

6.2 Suggestions for future research

Further studies investigating the differential methylation of *SFRP4* in those at a lower and higher risk for CRC should include investigating why there were differences in *SFRP4* methylation levels in rectal DNA from the BORICC and DISC Studies. In addition, it would be valuable to undertake longer term follow up of the patients in the BORICC Study and DISC Study to establish which of them develop CRC and to ascertain whether or not the *SFRP4* methylation level quantified at baseline in the present study predicted those who went on to develop CRC.

Further investigation is required to assess whether low vitamin D concentrations are causal for raised *SFRP4* methylation, and whether this may be a possible mechanism through which low vitamin D status increases the risk of CRC.

This is the first study which has investigated *SFRP4* promoter methylation in DNA from buccal cells from colorectal polyp patients and further investigation is required to establish if these results are reproducible and to investigate causes for the unexpected difference in *SFRP4* methylation. Further investigations should also include the possible confounding effect of the local environment upon the differences in *SFRP4* methylation in buccal DNA and rectal DNA.

Future research should determine whether or not resistant starch and/or polydextrose can reverse the higher levels of *SFRP4* methylation that have been observed in those at a higher risk of CRC in the present study. The apparent interaction between resistant starch and polydextrose on *SFRP4* methylation also requires further investigation.

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Appendix I

Letter to potential study participants

Dept of Surgery
North Tyneside General Hospital
Rake Lane
North Shields
NE29 8NH

Dear

We are writing to inform you about a study that we are conducting at North Tyneside General Hospital and Wansbeck General Hospital. We are writing to you because you have been booked for an endoscopy (a camera examination of the lower bowel).

Our research aims to examine how diet can influence cells in the bowel wall and the changes they sometimes undergo to become a cancer. To conduct this study we require samples taken from the bowel wall from normal volunteers without a cancer.

Please take your time reading the enclosed information. When you arrive for your endoscopy you will be seen by one of the research team who will be able to provide you with further information. If you decide to take part in the study you will have to sign a consent form. If you decide not to participate now, or at a later time within the study, it will not affect any other aspect of your treatment at the hospital.

Thank you for your cooperation which is very much appreciated.

Yours sincerely

Naomi Willis
Research Associate
Newcastle University

On behalf of the research team

John Mathers
Professor of Nutrition
Newcastle University

Iain McCallum
Research Fellow
North Tyneside Hospital

Naomi Willis
Research Associate
Newcastle University

Seamus Kelly
Consultant Surgeon
North Tyneside Hospital

Mike Bradburn
Consultant Surgeon
Wansbeck Hospital

Appendix II

Letter of invitation/research participant information sheet

Dear Sir/Madam,

You are being asked if you would be willing to participate in one of our research projects that is being conducted by Northumbria NHS Foundation Trust and Newcastle University.

Before you decide to participate, please read the details below. Take the time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear to you or if you would like more information. Your GP will be informed if you decide to participate in the study. Take time to decide whether or not you wish to take part. Deciding to take part or not to take part will not affect any other aspect of the care that you will receive.

Why have I been offered entry to the study?
You have been offered entry to the study because your doctor (GP or hospital doctor) has requested that we perform an endoscopy (camera examination of the lower bowel). For our research we require biopsy samples from patients' colons to examine particular cells. Depending on the findings at your endoscopy, you may be asked to participate in the study where a food supplement is taken for 50 days and a repeat camera test is done with new biopsies taken.

Why is this study being performed?
One part of the study is designed to examine colon stem cells *(stem cell study).* Stem cells are present in all tissues of the body and are responsible for renewing all cells in the body. Cancers are thought to originate from stem cells by a process of genetic alterations. If no abnormality is present on your endoscopy we would like to take nine biopsies (tiny tissue samples) so that we can count the number of stem cells present in the tissue. We will be able to compare your samples with other peoples' to see how the distribution of stem cells varies between people. We will also compare your samples with other people who have precancerous abnormalities to see how normal is different from them.

In the colon we know that a substance thought to be protective against cancer (non-digestible carbohydrates) can reverse some very early precancerous changes at a microscopic level in tissue from people who have colon cancer. Two non-digestible carbohydrates are resistant starch and polydextrose. Non-digestible carbohydrates occur naturally in the diet.

We believe that the changes in the cells in the bowel caused by non-digestible carbohydrates may be due to changes in the numbers of stem cells in the colon. To test this we would like you to take a food supplement which will be a type of non-digestible carbohydrate or a placebo (a substance that has no effect) for 50 days. We would then take further biopsy samples with a different type of telescope that only examines the last 15cm of the bowel to see if the number of stem cells has changed. This procedure doesn't require any medicines to cleanse the bowel beforehand.

The other part of the study *(marker study)* aims to help us understand the molecular changes that put some people at risk of colon cancer. We know that diet and lifestyle choices can affect the risk of developing colon cancer. However definite answers that would tell us how colon cancer could be prevented are hard to come by as we have to conduct experiments over very long time periods as we need to wait and see who develops a cancer and this is a very slow process (tens of years). We need to understand some of the changes that we can measure much earlier, particularly expression of certain molecules and genes. To know whether these molecules are sensitive enough to show the very early changes we are looking for we need to test their response to dietary supplementation. These tests would be carried out on the same samples that you provided for the stem cell part of the study.

What exactly would I have to do as a participant?

If you decide to participate and there are no abnormalities seen in your colon we will ask if you would carry on with the study. This would involve taking a food supplement twice a day for 50 days and then returning for a second endoscopy to look at the last 15cm of the bowel to collect further biopsies.

We would ask all patients to answer some questions at the start of the study about their lifestyle (smoking, dietary and exercise habits). We would take height, weight, waist, hip and thigh measurements at the start and end of the study. We would also like to collect a blood sample as well as a urine, stool and cheek cell sample at the start and the end of the study. To allow the effects of the bowel preparation to wear off so that our measurements are accurate we would ask that you wait for one week after your first endoscopy before starting the food supplement. Just before starting the food supplement we would ask you to give a urine and stool sample. We will give you more details on this if you decide to participate.

Deciding not to participate will not affect any other treatments or investigations that the hospital would provide for you.

How often do I need to visit the hospital during the study?

You would need to attend for the first endoscopy as you normally would. If you are asked to participate further the food supplement would be supplied to you and you would be asked to attend again for one further examination of the last 15cm of the bowel after 50 days of treatment. Before you start taking your supplement we would ask you to provide a urine and stool sample. This would be one week after the first

endoscopy and we will provide you with equipment and instructions to collect this at home. We would pick up this sample from your home at a time that suits you. You would then take your food supplement after this. Just before your second endoscopy we would ask you to provide a urine and stool sample in the same way which we would ask you to bring with you at the time of your second appointment.

There may of course be other clinic appointments or tests required due to the results of tests that are unrelated to this study. These would not alter although we would do our best to fit your one repeat endoscopy test around any other appointments that you had to minimise your inconvenience.

What food supplement would I be taking?
You will not be told and we would not know what supplement you were taking. You would either be taking resistant starch, polydextrose or a substance called a placebo which is something that will have no effect on your cells. Both the patient and the study staff not knowing what supplement anyone is taking makes the experiment fairer when it comes to looking at the results.

What do we know about non-digestible carbohydrates?
Non-digestible carbohydrate is a term for any starch molecule in the diet that is not broken down by the intestine until it reaches the colon. In the colon natural bacteria break down the resistant starch into active chemicals. We know that these chemicals have the ability to interact with genes in cells and are able to switch on anti-cancer genes. We have shown that treatment with resistant starch can alter very early pre-cancerous changes in mature cells. This study aims to find the effects on the stem cells in the colon and the effects on molecules that could be tested for to show the earliest signs of cancer development.

How is the supplement administered?
We will ask you to take four sachets of the supplement each day for 50 days. The powder in these sachets can be put on cold food or sometimes dissolved in juice and does not have any taste.

What side effects can I expect?
Non-digestible carbohydrates are part of the normal diet although we are providing a supplement to exaggerate any effect that they have on colon cells. In larger amounts non-digestible carbohydrates are known to sometimes cause: increased flatulence, bloating sensation, mild abdominal pain and mild laxative effects. These will all stop when the supplementation is stopped. No serious side-effects have ever been reported from non-digestible carbohydrates.

What are the risks of endoscopy examination and biopsies?
All operations and procedures carry a small risk and it is important that you understand this before deciding whether or not to participate. There is a very small chance of a perforation (a hole made in the bowel) of the bowel (1 in 15 000). A perforation will

almost always require an operation to fix it. Bleeding occurs more frequently (1 in 100-200) but is almost always minor and settles on its own. It is most common after removal of a polyp. Although you may require to have a polyp removed depending on what your consultant sees at your initial endoscopy this would not be a part of the study we are conducting. This risk of bleeding or perforation is obviously higher with each biopsy that is being taken. We plan to take nine biopsies at your first and second camera examinations.

What happens if anything goes wrong?
You are free to participate or not in the study and this will in no way affect your subsequent care in the hospital. There is no payment intended for patients or doctors. If you are harmed by taking part in this research project, there are no special compensation arrangements, but you will still be entitled to complain through your local NHS hospital procedure. If you are harmed due to someone's negligence or wrong doing, then you may have grounds for a legal action but you may have to pay for it. You may withdraw from the study at any time without explaining why, this will not affect any future care that you may receive.

Will the information be confidential?
Yes. Only those involved will be able to look at any information records. Specific details which identify you will only be available to the study doctors. Your own doctor (GP) will be informed that you are taking part in this study.

What will happen to the samples collected?
The samples that are collected will be examined at laboratories in Newcastle University. All samples will be stored securely. We will perform tests to look for the stem cells and tests to look at the activity of the various markers we are looking into. After the study has finished the samples will be stored in our laboratory freezers in accordance with government regulations. Your name and details will no longer be associated with the samples. We keep the samples so that if new techniques or markers are discovered we can do further testing without having to collect new samples from other volunteers.

What benefits may I get from the study?
We do not believe that there will be any direct benefit to the health of those who participate in the study. The research may well help us to understand the development of bowel cancer and develop prevention or treatment strategies.

We will give all the patients who take part in the intervention phase (i.e. taking the food supplement) a shopping voucher for £50 as a thank you for the extra time that they will have given up for the project. Travel expenses for the additional trip to hospital will also be provided.

Who is performing the research?

The research team consists of five members:

Mr Iain McCallum is a research fellow at Northumbria NHS Foundation Trust
Dr Naomi Willis is a research associate at Newcastle University
Professor John Mathers is the Professor of Human Nutrition at Newcastle University
Mr Seamus Kelly is a consultant surgeon at North Tyneside General Hospital and a senior lecturer at Newcastle University
Mr Mike Bradburn is a consultant surgeon at Wansbeck General Hospital

We hope that you agree to participate, if you have any questions please ask.

Study coordinator, Naomi Willis

Research Associate
Human Nutrition Research Centre
Newcastle University

Appendix III

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From: Supporter Services <supporter.services@cancer.org.uk>
Subject: Email Enquiry
Date: Thu, 22 May 2014 15:35:51 +0100
To: <helen.staley@doctors.org.uk>

Dear Helen

I can confirm that you can use the figures as long as Cancer Research UK is referenced.

If there's anything else I can help with please feel free to get back in contact by e-mail, by telephone or through our website.

Kind regards,

Jamie Cotton

Supporter Contact Advisor
Supporter Services & Operations

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Dear Dr. Staley,

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