Non-digestible Carbohydrates, the WNT Signalling Pathway and Bowel Cancer Risk

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In Fulfilment of the Requirements for the Degree

Doctor of Philosophy

September 2014

Abstract

Epidemiological and experimental evidence suggests that non-digestible carbohydrates (NDCs) e.g. resistant starch (RS) are protective against colorectal cancer (CRC), resulting primarily from the production of the short-chain fatty acid (SCFA) butyrate. The WNT signalling pathway is central to the maintenance of homeostasis within the large bowel through regulation of physiological processes and is frequently aberrantly activated in CRCs. Butyrate has been shown to positively modulate WNT signalling, affecting functional outcomes such as apoptosis and proliferation and, therefore, protecting against CRC.

To investigate the molecular mechanisms through which NDCs may reduce CRC risk in humans, we undertook a double-blind, randomised, controlled trial (The DISC Study) in which 75 healthy participants were supplemented with two NDCs: RS (Hi-maize® 260) and polydextrose (PD) (Litesse® *Ultra*™) for 50 days using a 2x2 factorial design. Colorectal mucosal biopsies were collected before and at the end of intervention and used to quantify expression of WNT pathway-related genes and of *BAX* and *BCL-2* (two regulators of apoptosis) and to assess colonic crypt cell proliferation. WNT signalling pathway genes were also investigated in an additional 38 participants at higher risk of CRC because of quiescent ulcerative colitis or a history of adenomatous polyps.

RS supplementation significantly reduced expression of *CTNNB1* (p=0.045), encoding β-catenin (a key component of the WNT pathway), and *c-MYC* (p=0.037), suggesting a reduction in WNT signalling in these participants. RS and/or PD significantly reduced expression of two antagonists of WNT signalling, *SFRP1* and *SFRP2*, suggesting an increase in WNT activity. RS and PD did not affect *SFRP1* methylation or expression of miRNAs that may target this gene. RS increased total colonic crypt cell proliferation (p=0.030) but did not alter the proportion of mitotic cells in the top half of the crypt (a marker of crypt health).

Participants at higher risk of CRC had increased expression of *c-JUN* (p=0.046) and *WNT11* (p=0.040) and increased *SFRP1* methylation. The *BAX* to *BCL-2* ratio was lower in higher-risk participants, suggesting a reduction in BCL-2

family-mediated apoptosis. Unexpectedly, crypt cell proliferation was also reduced in higher-risk participants (p=0.009).

These findings suggest that the increased cell proliferation with RS supplementation may have resulted from induction of the WNT pathway. However, these effects were not mediated via alterations in *SFRP1* methylation or expression of miRNAs. This study has also shown that WNT signalling is aberrantly active in the macroscopically-normal mucosa of people at higher risk of CRC.

Dedication

This thesis is dedicated to my wonderful parents, Tom and Elita Malcomson.

Acknowledgements

First and foremost, I am indebted to my principal supervisor Prof. John Mathers for his expert guidance and constant support throughout these past four years, first for my Master's and then my Ph.D. I really appreciate his genuine interest in my personal development and future career prospects, for example by encouraging me to attend both national and international courses and conferences. His knowledge and expertise have been invaluable and for this my thanks are immeasurable.

I would also like to record my grateful thanks to my secondary supervisor, Mr. Seamus Kelly, for his contributions to this Ph.D. project. I am also very thankful to my progression panel, Prof. John Hesketh and Prof. Dianne Ford, for their help and guidance throughout my Ph.D. studies.

I am very grateful to The DISC Study Team: Naomi Willis, Iain McCallum and Long Xie, for without the DISC Study and all the team's hard work recruiting participants and collecting samples this Ph.D. project would not have been possible. In particular, Naomi was pivotal to the completion of the DISC study and I thoroughly appreciate her willingness to help and guide me during this Ph.D. I also wish to acknowledge the DISC Study participants for taking part and the staff at Wansbeck and North Tyneside Hospitals for the important role they played in this study. I would also like to extend my appreciation to the DISC Study collaborators, particularly Prof. Ian Johnson and Dr. Nigel Belshaw at the IFR. I am very thankful to Nigel Belshaw and Wing Leung for contribution of their designed primer sequences for gene expression quantification. I am most grateful to the BBSRC DRINC for funding this Ph.D. studentship and for allowing me to further develop my skills as a Ph.D. student and network with other early-career researchers at regular dissemination events.

I would also like to thank other members of the Mathers group whom I have met over the last four years and I consider myself very fortunate to have joined such an excellent research group. In particular, a special thanks to Jo Gorniak, Frances Wong, Jill McKay, Sofia Lisanti and Sabine Langie for their help with laboratory techniques and Carlos Celis for his statistical expertise. Thank you very much also to Julie Coaker who patiently taught me how to perform the

analyses on the colonic crypt cell proliferative state by whole crypt microdissection, which formed a vital part of this thesis. I am also very grateful to Idoia Ibero, who arrived as an Erasmus student from the University of Navarra but left Newcastle University as a new friend, for performing the quantification of the expression of apoptotic genes and *WNT11* methylation.

The undertaking of this Ph.D. also allowed me to make a number of special friends, particularly Caroline Shaw and Lynn Barron who embarked on this journey with me when we started the MRes together in September 2010. I thank you all and could not have asked for better friends and colleagues to accompany me throughout these four years.

I would like to say a special thank you amazing boyfriend, Adam Trueman, who has supported me consistently over the last six years. Throughout my studies, Adam has continued to encourage me and has never failed to put a smile on my face during stressful times. I know that my Ph.D. experience would not have gone as smoothly without his daily support and motivation.

I am extremely thankful to my wonderful twin sister, Shona, who has always supported me and been there for me unconditionally. Shona has always been ready to help me in whatever way possible. During my Ph.D. studies, she has listened to countless presentation practices and has been especially supportive in the last few months, accompanying and encouraging me during the writing of this thesis and I do not know how I would have managed without her.

Lastly, but most importantly, I would like to thank my marvellous parents who have always encouraged me throughout my studies and without whose loving support I would not be where I am today. I owe all of my achievements to their valuable guidance and inspiration to help me reach my goals.

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Abbreviations

5mC	5-methylcytosine
8-oxo	8-hydroxy-2-deoxyguanosine
ACF	Aberrant crypt foci
AGO	Argonaute
AICR	American Institute for Cancer Research
ANOVA	Analysis of variance
AOM	Azoxymethane
APC	Adenomatous polyposis coli
AR	Argentinian corn maize variety
ATP	Adenosine triphosphate
BAX	Bcl-2-associated X
BCFA	Branched-chain fatty acid
BCL-2	B-cell lymphoma 2
BM DNA	Bisulphite-modified DNA
BMI	Body mass index
βTrCP	β-transducin repeat-containing protein
CAC	Codex Alimentarius Committee
CAPP	Colorectal Adenoma/carcinoma Prevention Programme
CCPS	Crypt cell proliferative state
cDNA	Complementary DNA
CI	Confidence interval
CK1	Casein kinase I

CK1δ	Casein kinase I delta
COBRA	Combined bisulphite restriction analysis
COX	Cyclooxygenase
CRC	Colorectal cancer
CRP	C-reactive protein
CRUK	Cancer Research UK
Ct	Cycle threshold
CtBP	C-terminal binding protein
DKK1	Dickkopf-related protein 1
DMH	1,2-dimethylhydrazine dihydrochloride
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
dNTP	Deoxynucleotide triphosphate
dsDNA	Double-stranded DNA
dsRBD	Double-stranded RNA-binding domain
DVL	Dishevelled
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPIC	European Prospective Investigation into Cancer and Nutrition
FAP	Familial adenomatous polyposis
FFPE	Formalin-fixed paraffin-embedded
FFQ	Food frequency questionnaire
gDNA	Genomic DNA
GI	Gastrointestinal

GLM	General linear model
GSK3β	Glycogen synthase kinase 3β
GUAT	Guatemalan corn maize variety
HAMS	High-amylose maize starch
HAMSB	Butyrylated HAMS
HAT	Histone acetyl transferase
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HNPCC	Hereditary non-polyposis colorectal cancer
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
HRM	High red meat
hsCRP	High-sensitivity CRP
IBD	Inflammatory bowel disease
ICAT	Inhibitor of β-catenin
IFR	Institute of Food Research
IHC	Immunohistochemistry
IL	Interleukin
KCALs	Kilocalories
LEF	Lymphoid enhancer factor
LINE-1	Long interspersed nuclear element-1
LOH	Loss of heterozigosity
LRP	Low-density lipoprotein receptor-related protein
LSM	Least squares mean

miRNA	microRNA
miRTC	miRNA RT control
MMR	Mismatch repair
mRNA	Messenger RNA
NDC	Non-digestible carbohydrate
NFκB	Nuclear transcription factor kappa B
NSAID	Non-steroidal anti-inflammatory drug
NTC	No template control
O^6-MeG	O^6-methyl-2-deoxyguanosine
PCP	Planar cell polarity
PCR	Polymerase chain reaction
PD	Polydextrose
PLCSB	Polysaccharide of Larimichthys crocea swim bladder
PPAR	Peroxisome proliferator-activated receptor
PPC	Positive PCR control
PPi	Pyrophosphate
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
QC	Quality control
qPCR	Quantitative PCR
R^2	Coefficient of determination
Rb	Retinoblastoma
RCT	Randomised controlled trial
RISC	RNA-induced silencing complex

RNA	Ribonucleic acid
ROX	Carboxy-X-rhodamine
RS	Resistant starch
RTC	Reverse transcriptase control
RT-qPCR	Reverse-transcription qPCR
SAM	*S*-adenosyl methionine
SCCAI	Short Clinical Colitis Activity Index
SCFA	Short-chain fatty acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SELENBP1	Selenium-binding protein 1
SEM	Standard error of the mean
SFRP	Secreted Frizzled-related protein
snRNA	Small nuclear RNA
snoRNA	Small nucleolar RNA
ssRNA	Single-stranded RNA
TBE	Tris-borate-EDTA
TCF	T-cell factor
TE	Tris EDTA
Tm	Melting temperature
TP53	Tumour protein 53
TRBP	Transactivating response RNA-binding protein
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labelling
UC	Ulcerative colitis

UD	Undetectable
UK	United Kingdom
WCRF	World Cancer Research Fund

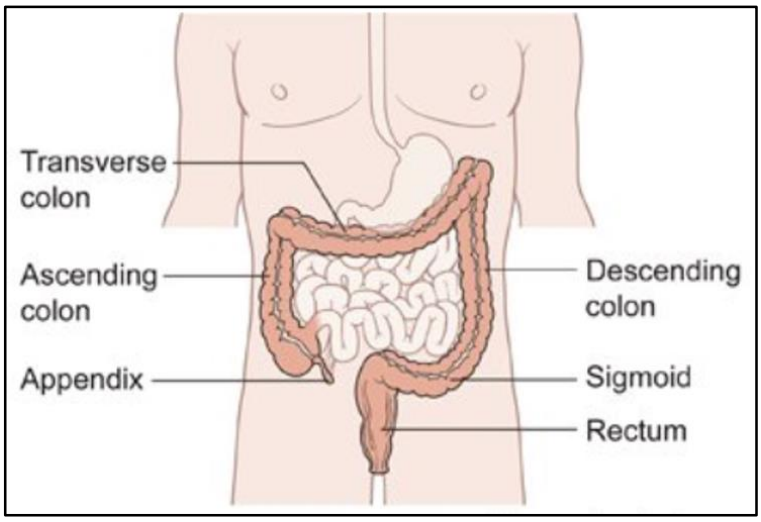
Chapter 1 General Introduction

1.1 The human large bowel

The large bowel, or large intestine, is found in the lower section of the gastrointestinal (GI) tract and comprises the caecum, colon and rectum. The colon can be divided into the right side (proximal colon), comprising the ascending colon and proximal transverse colon, and left side (distal colon), which includes the distal transverse colon, descending colon, sigmoid colon and rectum (Figure 1.1).

The colorectal epithelium, which forms a barrier separating the gut lumen from the rest of the body, consists of a single layer of columnar epithelial cells (Humphries and Wright, 2008). Although the intestinal luminal surface of the large bowel epithelium is flat, there are numerous invaginations, named crypts of Lieberkuhn, which house the stem cells responsible for populating this tissue.

Figure 1.1 The large bowel.



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1.1.1 Function of the large bowel

The primary function of the large bowel is the fermentation of undigested carbohydrates, such as resistant starch (RS), by commensal colonic bacteria to produce energy with short-chain fatty acids (SCFAs) as major end-products. These SCFAs, including butyrate, are absorbed by the colonic epithelium and

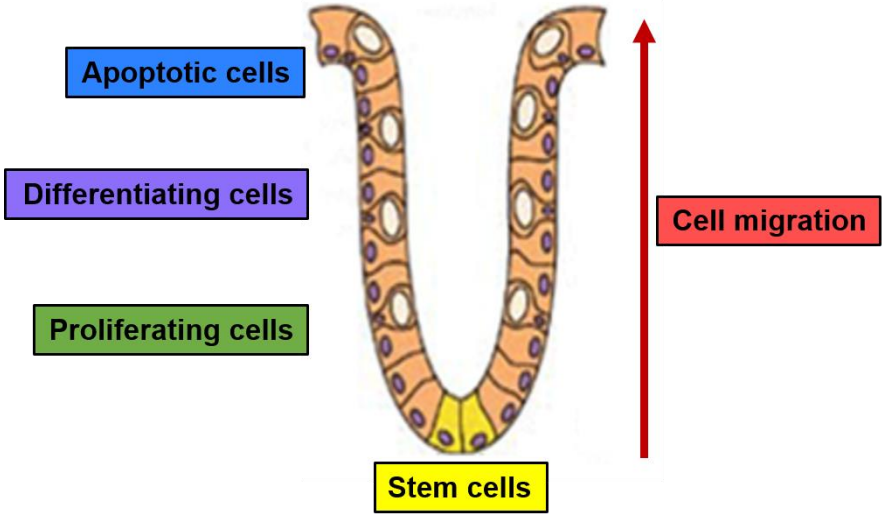
contribute to host energy supply as well as the health of the mucosa (Lanham-New *et al.*, 2011). In addition, colonic bacteria synthesise amino acids and vitamins, including vitamin B12 and vitamin K. Another important function of the large bowel is the absorption of electrolytes and water, which aids the formation of semi-solid faeces (Lanham-New *et al.*, 2011).

1.1.2 The colonic crypt

Colonic crypts are invaginations formed by the epithelium that penetrate into the connective tissue (Humphries and Wright, 2008). There are approximately ten million crypts within the human large bowel (Zhao and Michor, 2013). The average crypt is 80 cells in depth and 40 cells in circumference and comprises approximately 2,000 cells, including an average of five stem cells (Potten *et al.*, 1992b). Stem cells are constantly dividing and producing proliferating progenitor cells which then migrate towards the top of the crypts and differentiate into epithelial cells (Clevers, 2013). Four different cell types are found in the large bowel: colonocytes, enteroendocrine cells, goblet cells and Paneth cells (Kinzler *et al.*, 1991; Schneikert and Behrens, 2007).

The intestinal epithelium is continuously renewing and is completely renewed every two to seven days (Brittan and Wright, 2004). This is achieved through the constant upward migration of proliferating progenitor cells which, once differentiated into epithelial cells, cease proliferating, undergo apoptosis and are shed (Schneikert and Behrens, 2007) (Figure 1.2). Both the migration of progenitor cells and proliferation are controlled by WNT signalling (Clevers and Batlle, 2006).

Figure 1.2 The colonic crypt.



Within the colonic crypt, stem cells, which reside at the base of the crypt, produce cells that proliferate in the proliferative compartment (basal half of the crypt) and differentiate as they migrate upwards towards the mouth of the crypt. It is here that cells undergo apoptosis and are shed. The continuity of this process allows constant renewal of the colonic epithelium.

1.1.2.1 The colonic crypt cell proliferative state

Cell proliferation describes the growth and replication of cells. In the healthy colonic crypt, cell proliferation is a tightly regulated process that is confined to the lower compartments of the crypt, known as the proliferative compartment, towards the base of the crypt where the stem cells are found (Potten *et al.*, 1992a). It has been calculated that approximately 10% of all cells within the crypt are undergoing proliferation at one time (Potten *et al.*, 1992b).

Hyperproliferation is a characteristic of CRC and, importantly, an altered pattern of proliferating cells within the crypt, characterised by expansion of the proliferative compartment towards the luminal surface, is one of the earliest detectable alterations in the normal mucosa during colorectal carcinogenesis (Risio *et al.*, 1991).

1.1.2.2 Apoptosis in the colonic crypt

Kerr, Wylie and Currie first described apoptosis in 1972 as *"a distinctive process […], which plays a complementary but opposite role to mitosis in the regulation of animal cell populations"* (Kerr *et al.*, 1972). Not only is apoptosis important in

the removal of aged cells through programmed cell death, but it also removes any cells with genetic damage, providing a defence mechanism against tumourigenesis.

In the healthy colonic crypt, apoptosis is particularly important at the top of the crypt, towards the luminal surface, where there is a constant cell turnover and shedding of apoptotic cells from the surface of the colonic epithelium which maintains the homeostatic state. As in the case of cell proliferation, apoptosis is tightly controlled within the colonic epithelium. An imbalance between apoptosis and proliferation, favouring a hyperproliferative state, may lead to tumour development. This can result from an imbalance in pro- and anti-apoptotic genes, which has been associated with colorectal carcinogenesis (Evertsson *et al.*, 1999). Any apoptosis in the lower compartments of the crypt, where proliferation predominates, is believed to be a defensive response to genetic damage in proliferating cells (Strater *et al.*, 1995; Koornstra *et al.*, 2003).

A systematic review of studies investigating changes in apoptosis from normal mucosa to adenomas and ultimately carcinomas was completed by Koornstra and colleagues (Koornstra *et al.*, 2003). The majority of studies reviewed found an increase in apoptosis when comparing adenomas with normal mucosa, and carcinomas with adenomas. When assessing changes in apoptosis in adenomas, a correlation between the level of dysplasia and an increase in apoptosis was observed. In adenomatous polyps, a reduction in apoptosis at the mouth of the crypts, where apoptotic cells are predominantly found in healthy crypts, was accompanied by an increase in apoptosis at the base of the crypts (Moss *et al.*, 1996a; Sinicrope *et al.*, 1996).

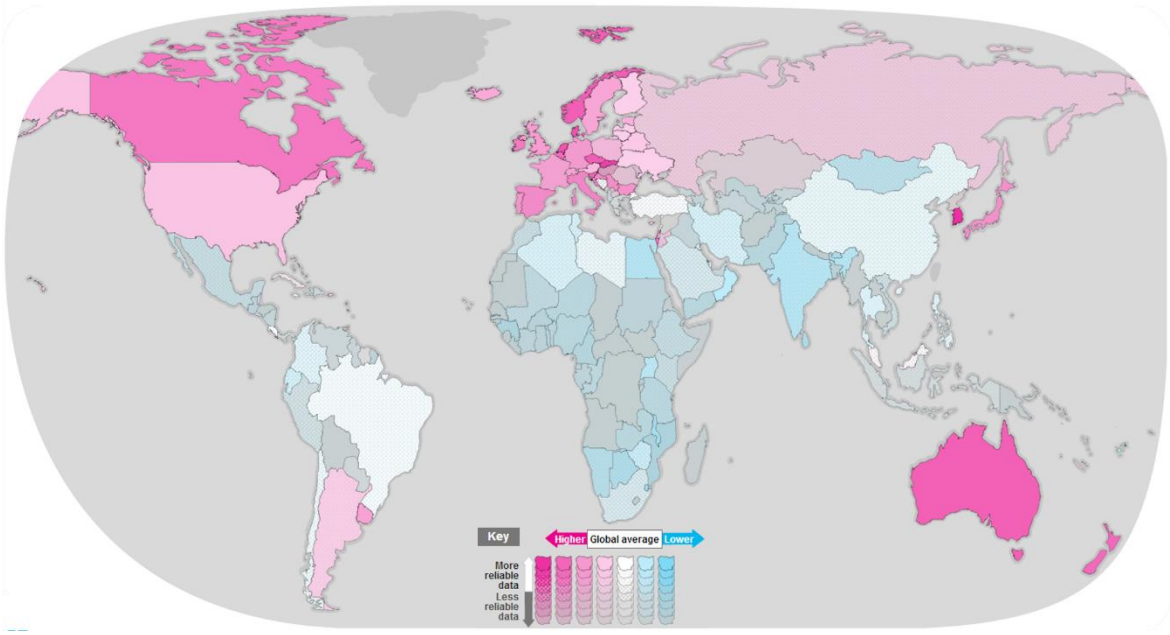
1.2 Colorectal cancer

Colorectal cancer (CRC), or bowel cancer, is a common cause of morbidity and mortality in the United Kingdom (UK) and worldwide. It is therefore important that the molecular mechanisms responsible for the pathogenesis of this cancer are understood to predict predisposing factors and to develop suitable risk biomarkers which would facilitate CRC prevention and/or treatment. As discussed in section 1.2.3 below, CRC risk is significantly altered by environmental factors such as diet, suggesting that lifestyle modifications may prevent a large proportion of CRC cases.

1.2.1 Colorectal cancer incidence

CRC is the third most common cancer worldwide and second most common cancer in Europe (CRUK, 2014a). However, as can be observed in Figure 1.3, incidence varies greatly between different regions in the world. In 2012, CRC incidence rates were greatest in developed regions, such as Australia, New Zealand and Western Europe, and lowest in developing regions, particularly Africa and South-Central Asia (Ferlay *et al.*, 2013).

Figure 1.3 Worldwide colorectal cancer incidence (2012 estimates).



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In the UK, CRC is the fourth most common cancer, with close to 110 new CRC diagnoses each day (CRUK, 2014b). In 2010, it was calculated that males in the UK had a 1 in 14 lifetime risk of developing CRC and that this is slightly lower in females, who have a 1 in 19 lifetime risk (CRUK, 2014a). In the UK, CRC incidence has increased since the mid-1970s (CRUK, 2014a) and has risen by 6% in the last decade (CRUK, 2014b). In 2011 there were over 40,000 new CRC diagnoses in the UK (CRUK, 2014b).

1.2.2 Colorectal cancer mortality and survival

Worldwide, CRC is the fourth most common cause of cancer-related death and is the second-most common cause of cancer-related death in Europe. In the UK, it is also the second most common cause of cancer-related death, which translates to approximately 10% of all cancer deaths or over 15,000 deaths in 2011 (CRUK, 2014c). Although the incidence of CRC in the UK has risen since the 1970s, mortality rates have decreased by 63% from the early 1970s to between 2009 and 2011 (CRUK, 2014c). Survival rates have also improved, with one-year survival rates increasing from approximately 40% in the early 1970s to over 70% between 2005 and 2009 (CRUK, 2012).

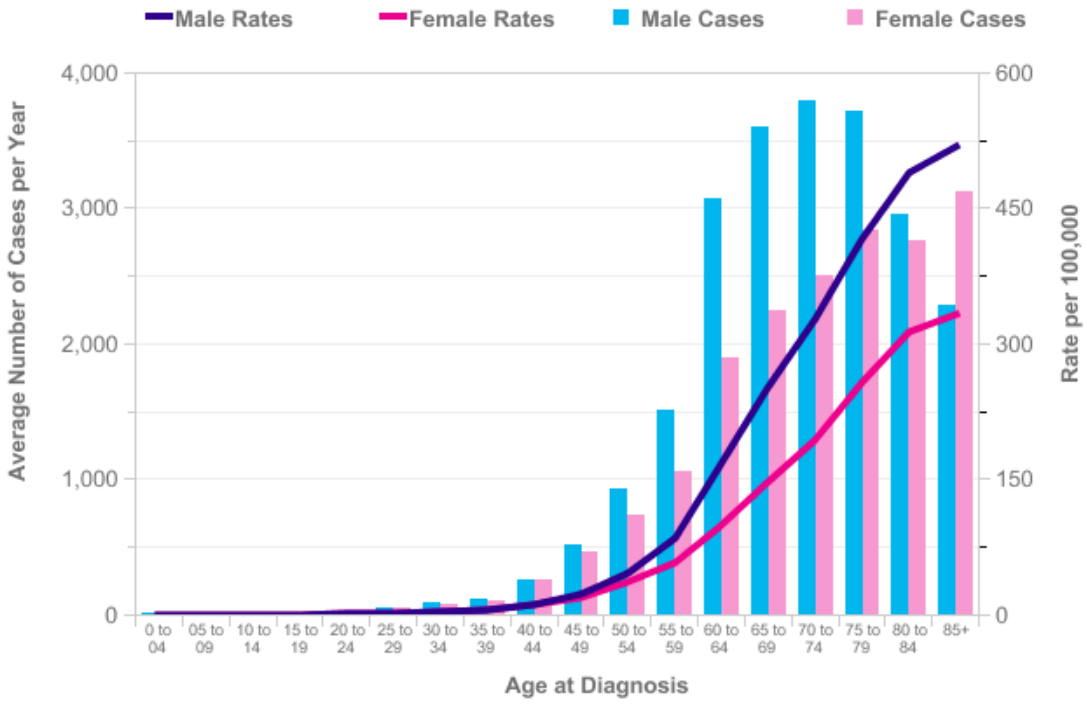
1.2.3 Risk factors for colorectal cancer

A number of factors have been demonstrated to alter CRC risk such as age, other diseases of the large bowel, genetic factors and lifestyle factors including diet, physical activity and smoking.

1.2.3.1 Age

There is a strong positive correlation between age and CRC incidence, particularly after the age of 50. Between 2009 and 2011, 95% of CRC cases diagnosed in the UK were observed in people aged 50 and over (CRUK, 2014a) (Figure 1.4). This correlation is particularly strong in males who, up to the age of 80 years, have greater CRC incidence compared with females.

Figure 1.4 CRC incidence rates and average number of cases by year by age group, UK, 2009-2011.



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1.2.3.2 Diet and alcohol

The American Institute for Cancer Research (AICR)/World Cancer Research Fund (WCRF) Continuous Update Project Report: 'Food, Nutrition, Physical Activity and the Prevention of Colorectal Cancer' released in 2011 concluded that a reduction in CRC risk could be achieved with higher consumption of dietary fibre (convincing evidence) (discussed in more depth in section 1.5), garlic, milk and calcium (probable evidence) (WCRF/AICR, 2011). On the contrary, higher intakes of red meat and processed meat (convincing evidence) and alcohol (convincing evidence for males, probable for females) were associated with increased CRC risk (WCRF/AICR, 2011). Limited/suggestive evidence was found for a protective effect of non-starchy vegetables, fruits and foods containing vitamin D, and for a detrimental effect of animal fats, cheese, sugars and foods containing iron (WCRF/AICR, 2011). A summary of the conclusions from this report are described in Table 1.1.

Table 1.1 Summary of the conclusions from the WCRF/AICR Continuous Update Report (2011): 'Food, Nutrition, Physical Activity and the Prevention of Colorectal Cancer'.

Evidence	Decreases risk	Increases risk
Convincing	• Physical activity of all types (occupational, household, transport and recreational) • Foods containing dietary fibre[1]	• Red meat (beef, pork, lamb and goat) • Processed meat (preserved by smoking, curing, salting or addition of chemical preservatives) • Alcoholic drinks (men) • Body fatness • Abdominal fatness • Adult attained height
Probable	• Garlic • Cow milk • Calcium	• Alcoholic drinks (women)
Limited-suggestive	• Non-starchy vegetables • Fruits • Foods containing vitamin D[1]	• Foods containing iron[1] • Cheese • Foods containing animal fats[1] • Foods containing non-milk extrinsic sugars
Limited- no conclusion	• Fish • Glycaemic index • Folate • Vitamin C • Vitamin E • Selenium • Low fat • Dietary pattern	

[1]Includes both foods naturally containing the constituent or with the constituent added

1.2.3.3 Body mass index and abdominal adiposity

There is a positive correlation between both body mass index (BMI) and abdominal adiposity and CRC incidence. A recent systematic review concluded that obese people (BMI ≥ 30kg/m^2) have a 33% greater CRC risk compared with those with a normal BMI (Ma *et al.*, 2013). It has been estimated that 13% of the new CRC cases diagnosed in the UK in 2010 were attributable to overweight or obesity (Parkin and Boyd, 2011). Abdominal adiposity is also associated with an increased risk of CRC; those with the largest waist circumference have almost double the CRC risk observed in those with the smallest waist circumference (Ma *et al.*, 2013).

1.2.3.4 Physical activity

The WCRF/AICR Continuous Update Report also concluded that convincing evidence exists for a protective effect of total physical activity against CRC and that greatest levels of overall physical activity were associated with the lowest risk of CRC (WCRF/AICR, 2011) (see Table 1.1).

In a meta-analysis of three cohort studies conducted as part of the Continuous Update Project, the authors concluded that for every 5 metabolic equivalent of task (MET) per day of total physical activity there was a 3% reduction in CRC risk (WCRF/AICR, 2011). For recreational physical activity, a dose-response meta-analysis conducted in three studies revealed a significant inverse correlation between recreational physical activity and colorectal cancer risk, with an 11% reduction in CRC risk per 30 minutes of recreational physical activity per day (WCRF/AICR, 2011). Physical activity may protect against colorectal carcinogenesis through improving metabolic efficiency and capacity as a consequence of the increased metabolic rates and oxygen uptake associated with moderate levels of physical activity (Slattery, 2004). Physical activity is also associated with anti-inflammatory effects, a reduction in insulin and in insulin resistance. Another mechanism through which physical activity may protect against CRC is by reducing body fatness, which, as described in section 1.2.3.3, is associated with an increased risk of CRC.

1.2.3.5 Smoking

There is a strong association between tobacco smoking and CRC incidence. In the UK, 8.1% of CRCs in 2010 were attributable to tobacco smoking (Parkin *et al.*, 2011). A meta-analysis which examined 36 prospective studies reported a 17% and 25% greater risk of CRC in smokers and former smokers respectively compared with non-smokers (Liang *et al.*, 2009). Furthermore, there is a dose response relationship between the daily number of cigarettes and CRC risk, with 20 cigarettes per day being associated with 17.5% greater CRC incidence which increased to 38.5% with 40 cigarettes per day (Liang *et al.*, 2009).

1.2.3.6 Inflammatory bowel disease

Inflammatory bowel disease (IBD) encompasses diseases characterised by inflammation of the GI tract, primarily Crohn's disease and ulcerative colitis (UC). Patients with IBD are at greater risk of developing CRC and account for approximately 2% of new CRC diagnoses per year (Triantafillidis *et al.*, 2009; Lutgens *et al.*, 2013).

1.2.3.6.1 Ulcerative colitis

UC is characterised by inflammation in the mucosal layer of the colorectum (Ford *et al.*, 2013; Rogler, 2014). Symptoms of UC include rectal bleeding, tenesmus and diarrhoea (Ford *et al.*, 2013). UC normally develops in early adulthood between 20 and 30 years of age (Rogler, 2014) and is believed to result from an abnormal immunological response to an alteration in the colonic environment in genetically susceptible people (Ford *et al.*, 2013).

In a study of 600 patients with UC, CRC risk was 1.5-fold greater compared with healthy participants (Rutter *et al.*, 2006). A meta-analysis investigated the prevalence of UC-associated CRCs in 116 studies and reported that CRC prevalence in UC patients was 3.7% (Eaden *et al.*, 2001). Studies have reported a correlation between CRC risk and disease duration, with UC patients with a disease duration of 20 years having a 7% risk, doubling to up to 14% at 25 years and may increase to up to 30% after 35 years (Rogler, 2014).

Eaden *et al.* proposed that increased CRC risk is apparent approximately eight to ten years following the onset of UC-related inflammation (Eaden *et al.*, 2001), demonstrating that the associated risk results from inflammation in these patients. CRCs that develop as a result of UC, also known as colitis-associated cancers, are a consequence of the chronic inflammatory state associated with this disease (Rogler, 2014). This is confirmed by the observation that UC patients treated with anti-inflammatory agents, such as thiopurines, have reduced CRC risk (Eaden, 2003; Seidelin *et al.*, 2013; Nieminen *et al.*, 2014).

Colitis-associated CRCs tend to develop from the progression of low-grade to high-grade dysplasia (Ullman and Itzkowitz, 2011). Although colitis-associated CRCs do not follow the adenoma-carcinoma sequence observed in sporadic cases, they do share similarities in pathogenetic alterations such as chromosome instability and DNA hypermethylation (Rogler, 2014). An early event in colitis-associated CRC is the loss of the p53 tumour suppressor, occurring in up to 85% of cases (Burmer *et al.*, 1992), loss of heterozygosity (LOH) or loss of function (Hussain *et al.*, 2000; Rosman-Urbach *et al.*, 2004). Another shared event by sporadic and colitis-associated CRCs is the loss of the adenomatous polyposis coli (*APC*) tumour suppressor, a component of the WNT signalling pathway. However, in colitis-associated cancers this is a much rarer event and occurs at later stages of the progression to CRC (Tarmin *et al.*, 1995).

Colitis-associated CRCs also have altered epigenetic mechanisms, particularly DNA methylation. Similarly to that in sporadic CRC, genes such as the hMLH1, a mismatch repair gene, are frequently hypermethylated and consequently downregulated in colitis-associated CRCs (Fleisher *et al.*, 2000). The methylation state of several members of the WNT signalling pathway, which is frequently aberrantly activated in CRC, have also been reported in colitis-associated CRC. WNT components such as *SFRP1, SFRP4, DKK1* and *WIF1* showed greater methylation both in UC compared with normal tissue and in colitis-associated neoplasia compared with UC tissue (Dhir *et al.*, 2008).

1.2.3.6.2 Crohn's disease

Crohn's disease is the other common type of IBD and is an autoimmune disease that can develop in any part of the GI tract (Bandzar *et al.*, 2013). Symptoms of Crohn's disease include inflammation, loss of appetite and abdominal pain (Bandzar *et al.*, 2013). Microscopically, up to two-thirds of patients with Crohn's disease develop granulomas which are masses of inflamed tissue (Ramzan *et al.*, 2002). Three disease subtypes have been described viz. inflammatory, obstructive and fistulating, however these may co-exist (Maglinte *et al.*, 2003).

Although both UC and Crohn's disease increase CRC risk, this relationship is greater, and has been explored more extensively, in UC (Andersen and Jess, 2013). A meta-analysis of 20 clinical studies investigated the risk of cancers, including CRC, in patients with Crohn's disease. Results from this study revealed that, in age-matched patients, CRC risk was two to three-fold greater in patients with Crohn's disease compared with healthy people (Laukoetter *et al.*, 2011). Results from another meta-analysis using six population-based studies suggested that overall risk estimates for patients with Crohn's disease were almost two-fold greater compared with healthy people (Jess *et al.*, 2005).

1.2.3.7 Colorectal polyps

Polyps have been defined as *"any mass protruding into the lumen of a hollow viscus"* (Shussman and Wexner, 2014). Macroscopically, colorectal polyps may be classified as sessile polyps or pendunculated polyps.

Some polyps, such as hyperplastic polyps, do not appear to progress to carcinoma and several types of non-neoplastic polyps have been identified (Shussman and Wexner, 2014):

- Hyperplastic: the most common colorectal polyp. Hyperplastic polyps are predominantly small in size and located in the distal colon and rectum.
- Hamartomatous: smooth, pendunculated polyps that are predominantly made up of connective tissue.

- Inflammatory: masses of inflammatory infiltrations. These are commonly found in UC patients.

Neoplastic polyps are known as adenomatous polyps. Although adenomatous polyps are benign, these could ultimately develop into carcinomas and therefore their removal is warranted because the majority of CRCs result from the progression of adenomatous polyps (Shussman and Wexner, 2014). Polyp size influences the risk of progression into carcinomas. Higher grades of neoplasia were observed with increasing polyp size from diminutive (<5mm) to small (6-9mm) and ultimately large (≥10mm) polyps (Sakamoto *et al.*, 2013).

Histological analysis has been used to differentiate subtypes of adenomatous polyps (Shussman and Wexner, 2014):

- Tubular: the most common subtype, found in up to 80% of polyps which are characterised by branched tubular glands.
- Villous: constitute up to 10% of neoplastic polyps and have finger-like projections.
- Tubule-villous: make up to 25% of polyps, and share combined characteristics of tubular and villous polyps.

1.2.4 Inherited risk of colorectal cancer

Although the majority of CRC cases develop sporadically, up to 10% occur due to hereditary defects such as familial adenomatous polyposis (FAP) syndrome or hereditary non-polyposis colorectal cancer (HNPCC), which are inherited in an autosomal dominant pattern (Lynch and de la Chapelle, 2003). Up to 80-100% of those with FAP or HNPCC may develop CRC (Rustgi, 2007). In addition, up to 25% of CRCs may develop due to a strong family history (Bogaert and Prenen, 2014).

1.2.4.1 Familial colorectal cancer

Familial CRC describes a cancer which develops following an increased risk associated with the presence of CRC in more than two first- or second-degree relatives (Lynch and de la Chapelle, 2003). A systematic review and meta-analysis revealed that people with one first-degree relative suffering from CRC

have more than double the risk of CRC compared with those without a family history (Johns and Houlston, 2001). This risk was more than four-fold greater in those with more than one relative with CRC (Johns and Houlston, 2001).

1.2.4.2 Familial adenomatous polyposis syndrome

FAP accounts for an estimated 1% of all CRCs and is characterised by the development of hundreds or thousands of adenomatous polyps in the colorectum of young adults. FAP develops due to a germline mutation in the *APC* tumour suppressor gene on chromosome 5q22 (Galiatsatos and Foulkes, 2006). If left untreated, it is very likely that one or more of the polyps in those with FAP will progress into carcinomas by the age of 40 (Bisgaard *et al.*, 1994). Although polyps are benign, the majority of CRC cases are a consequence of progression of an adenoma to a malignant carcinoma. Furthermore, FAP patients are also prone to other malignancies including desmoid tumours, hepatoblastoma and pancreatic cancer (Galiatsatos and Foulkes, 2006).

1.2.4.3 Hereditary non-polyposis colorectal cancer

HNPCC, also known as Lynch Syndrome, is responsible for approximately 4% of CRC cases (Rustgi, 2007). The lifetime risk of developing CRC is 80% in patients with HNPCC (Rustgi, 2007) and HNPCC patients are also prone to other cancers including gastric cancer, ovarian cancer and skin cancer (Umar *et al.*, 2004).

HNPCC results from an inherited defect in DNA mismatch-repair (MMR) genes, most commonly *hMSH-2* or *hMLH-1* (Chung and Rustgi, 2003; Umar *et al.*, 2004). MMRs encode proteins that correct mismatched DNA bases and small insertions or deletions and consequently lead to errors in DNA replication (Rustgi, 2007).

1.2.5 Colorectal cancer pathogenesis

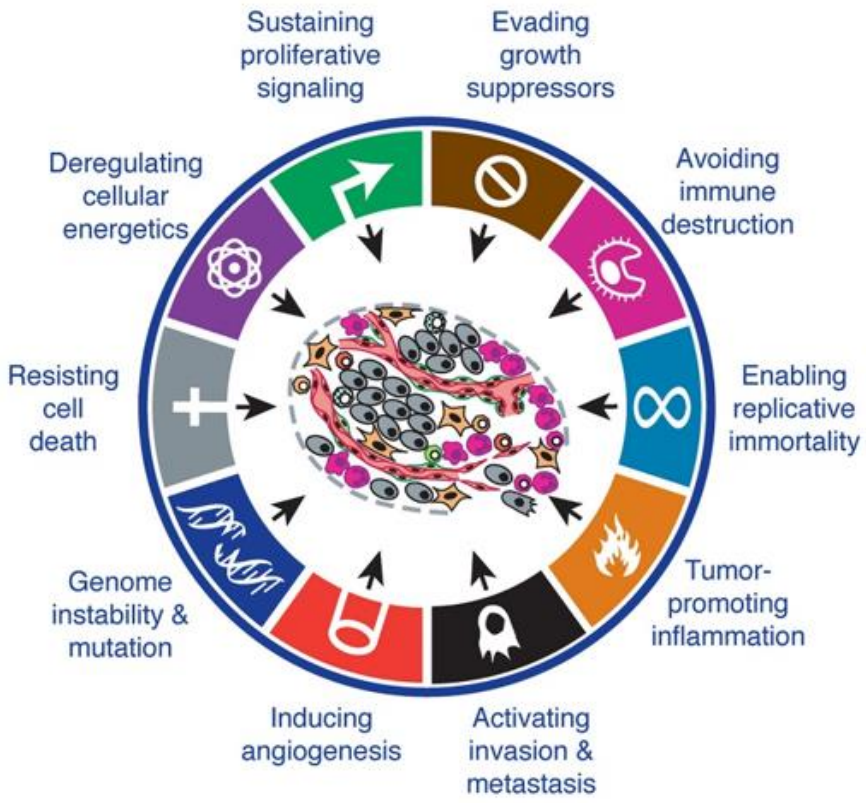
Cancers develop from a multi-step process, comprising genomic and epigenomic alterations, whereby normal cells progressively transform into neoplastic cells. These alterations give tumour cells, and cells in pre-neoplastic

lesions such as polyps, novel properties, or hallmarks, that are not present in healthy cells. In 2000, Hanahan and Weinberg proposed six hallmarks of cancer that are acquired during carcinogenesis and give cells their tumorigenic and ultimately malignant properties and allow cancer cells to survive, proliferate and metastasise (Hanahan and Weinberg, 2000). These are:

- Chronic proliferation
- Evading tumour suppressors that regulate cell growth, such as tumour protein p53 (TP53) and retinoblastoma (Rb)
- Resisting cell death
- Immortalisation (unlimited replicative potential)
- Angiogenesis
- Invasion and metastasis

In 2011, Hanahan and Weinberg updated their proposed hallmarks of cancer following the emergence of two novel hallmarks in the last decade (Hanahan and Weinberg, 2011). These were (i) the modification of cellular metabolism to fuel cell proliferation and (ii) evasion of immunological destruction. In addition, the authors proposed a further two characteristics that enable cancer cells to acquire these hallmarks. These were (i) genomic instability and mutability and (ii) a tumour-promoting inflammatory response. The ten updated hallmarks and enabling characteristics of cancer proposed by Hanahan and Weinberg in 2011 are shown in Figure 1.5.

Figure 1.5 Hallmarks and enabling characteristics of cancer proposed by Hanahan and Weinberg (2011).



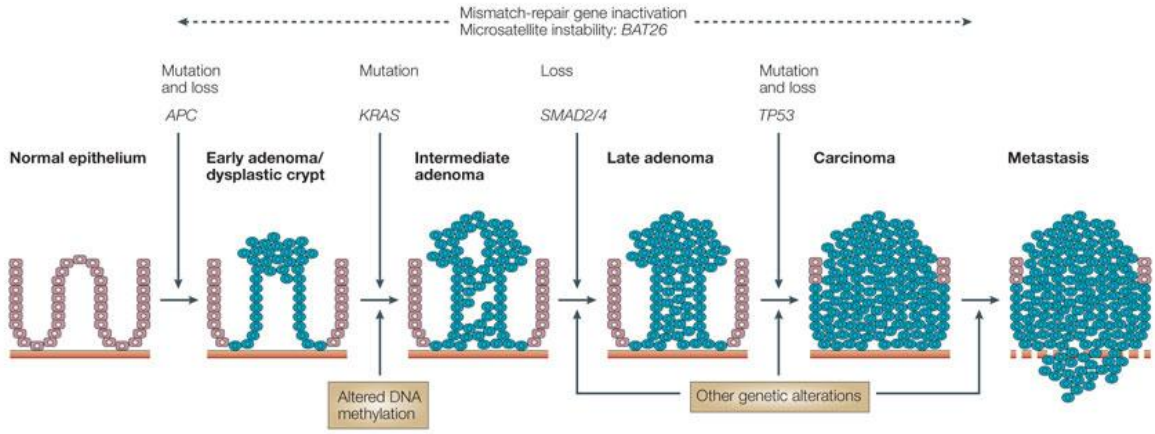
Adapted with permission from Elsevier: Cell (Hanahan and Weinberg, 2011), Copyright (2011).

The majority of colorectal carcinomas develop following the adenoma-carcinoma sequence, which is a multi-step process describing the progression from a normal epithelium to carcinoma (Figure 1.6). The sequence comprises several independent genetic and epigenetic alterations that result in aberrant expression of tumour-suppressor genes, including *APC* and *TP53,* proto-oncogenes such as *KRAS* and *c-MYC*, and DNA MMR genes, for example *MLH1* and *MSH2* (Leslie *et al.*, 2002; Shussman and Wexner, 2014). Consequently, the balance of proliferation and apoptosis is disturbed, favouring a hyperproliferative state which leads to tumour growth, carcinoma and ultimately metastasis into other tissues. Macroscopically, the different pathological stages have been well characterised, with progression from small adenomas increasing to large adenomas and ultimately to carcinoma.

Eighty-five percent of sporadic CRCs are believed to result from defects in the 'gatekeeper' pathway, that is genes involved in the control of cell proliferation

and tumour growth (Kinzler and Vogelstein, 1997), of which *APC* mutations are the most common. Patients with FAP inherit one defective copy of the *APC* gene and, following loss of function of the second copy, develop a large number of polyps during early-adulthood. Both copies of *APC* can be mutated somatically in sporadic CRC. The remaining 15% of sporadic CRCs develop following disruption of the 'caretaker' pathway, involved in maintaining genomic integrity and stability (Kinzler and Vogelstein, 1997).

Figure 1.6 Colorectal adenoma-carcinoma sequence.



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1.2.6 Colorectal cancer epigenetics

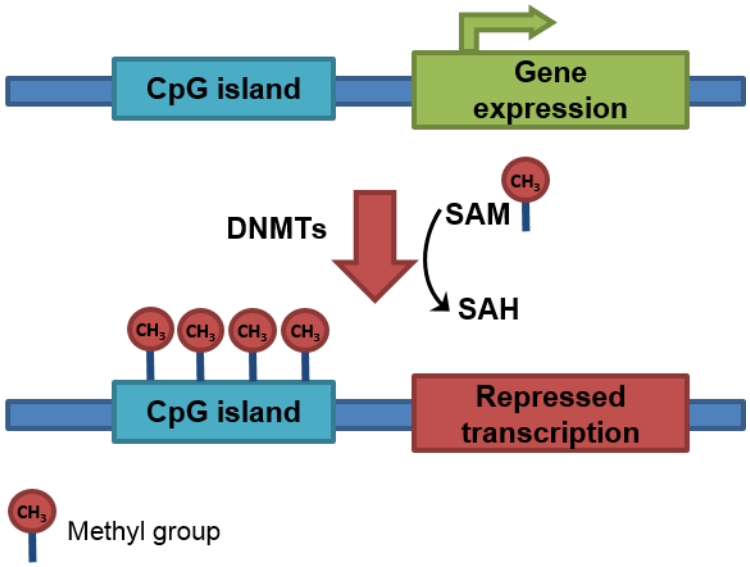
Epigenetics describes heritable changes in chromatin without alterations in the DNA sequence (Dupont *et al.*, 2009). Three epigenetic mechanisms have been described: DNA methylation, histone modification and the expression of micro riboxynucleic acids (RNAs) (miRNAs).

1.2.6.1 DNA methylation

DNA methylation is the addition of a methyl group to the carbon five position within a cytosine residue that is followed by a guanine, known as CpG sites, to produce 5-methylcytosine (5mC) (Bird, 2002) (Figure 1.7). The addition of methyl groups, which are donated by *S*-adenosyl methionine (SAM), is catalysed by DNA methyltransferases (DNMTs), primarily DNMT1, DNMT3a and DNMT3b (Robertson, 2001). Regions that are rich in CpG sites are

described as CpG islands and are not frequently methylated (Robertson, 2005). Hypermethylation, particularly of CpG sites close to, or within, the promoter region of a gene, is associated with reduced gene expression. This results from failure of transcription factor binding directly, because of the presence of 5mC, and also indirectly, because of blockage by methyl-CpG-binding proteins (Baylin and Herman, 2000; Robertson, 2005).

Figure 1.7 DNA methylation.



Low levels of methylation of CpG sites within CpG islands close to or within gene promoters are associated with active transcription and gene expression (top). The addition of a methyl group (DNA methylation) by DNA methyltransferases (DNMTs), donated by *S*-adenosyl methionine (SAM) to produce *S*-adenosyl homocysteine, is associated with repressed transcription (bottom).

In cancers, such as CRC, hypermethylation and therefore reduced expression of tumour suppressor genes are observed (Toyota *et al.*, 1999). Interestingly, an association between age and gene hypermethylation has been reported extensively for a number of genes (Ahuja *et al.*, 1998), suggesting that this may be one of the mechanisms that contributes to the increase in CRC risk and incidence associated with ageing. Genes that are hypermethylated in CRC include members of the WNT signalling pathway e.g. *SFRP1* (Suzuki *et al.*, 2004; Aguilera *et al.*, 2006; Farkas *et al.*, 2014; Zhang *et al.*, 2014), genes involved in the regulation of transcription e.g. *GATA4* (Akiyama *et al.*, 2003; Herbst *et al.*, 2009) and DNA repair genes e.g. *MLH1* (Kane *et al.*, 1997; Lahtz and Pfeifer, 2011; van Engeland *et al.*, 2011).

Coincident with the hypermethylation of tumour suppressor genes, hypomethylation and consequently elevated expression of oncogenes occur in CRC. For example, hypomethylation of *CDH3*, encoding P-cadherin which is involved in the regulation of cell adhesion and differentiation via interactions with the WNT pathway (see section 1.3 below), and consequent upregulated expression have been found in the early stages of colorectal carcinogenesis (Milicic *et al.*, 2008). The oncogene *c-MYC* is also frequently hypomethylated in CRC. Sharrard and colleagues observed a reduction in the DNA methylation of *c-MYC* in the progression from the normal mucosa to dysplasia, adenomas, carcinomas and ultimately metastases (Sharrard *et al.*, 1992). Importantly, the enhanced expression of *c-MYC* resulting from hypomethylation during colorectal carcinogenesis has been associated with an increase in cell proliferation (Sharrard *et al.*, 1992).

Furthermore, cancers have also been observed to show global demethylation (Baylin and Herman, 2000). Global hypomethylation is an early event during colorectal carcinogenesis and has been linked to functional alterations such as chromosome instability and loss of imprinting (Cui *et al.*, 2002; Eden *et al.*, 2003). Global DNA hypomethylation appears to increase with age (Suzuki *et al.*, 2006). A variety of methods exist to quantify levels of global DNA methylation, including the quantification of methyl cytosine by high-performance liquid chromatography (HPLC) and polymerase chain reaction (PCR)-based methods, which quantify methylation of specific genomic repeat elements such as long interspersed nuclear element-1 (LINE-1) (Lisanti *et al.*, 2013).

Studies have observed a reduction in global DNA methylation in adenomas and carcinomas compared with healthy tissue (Goelz *et al.*, 1985; Feinberg *et al.*, 1988; Hernandez-Blazquez *et al.*, 2000). In adenoma and cancer tissues, Feinberg *et al.* have reported an 8% and 10% decrease in global DNA methylation, assessed by HPLC, respectively when compared with matched normal tissue (Feinberg *et al.*, 1988). This approach of comparing cancerous and adenoma tissue in CRC patients with tissue retrieved from healthy subjects has been used in several studies. Using a global DNA hypomethylation assay, Bariol *et al.* observed greater global demethylation in hyperplastic polyps, adenomas and carcinomas compared with both normal tissue from healthy subjects and normal tissue from cancer patients (Bariol *et al.*, 2003). These

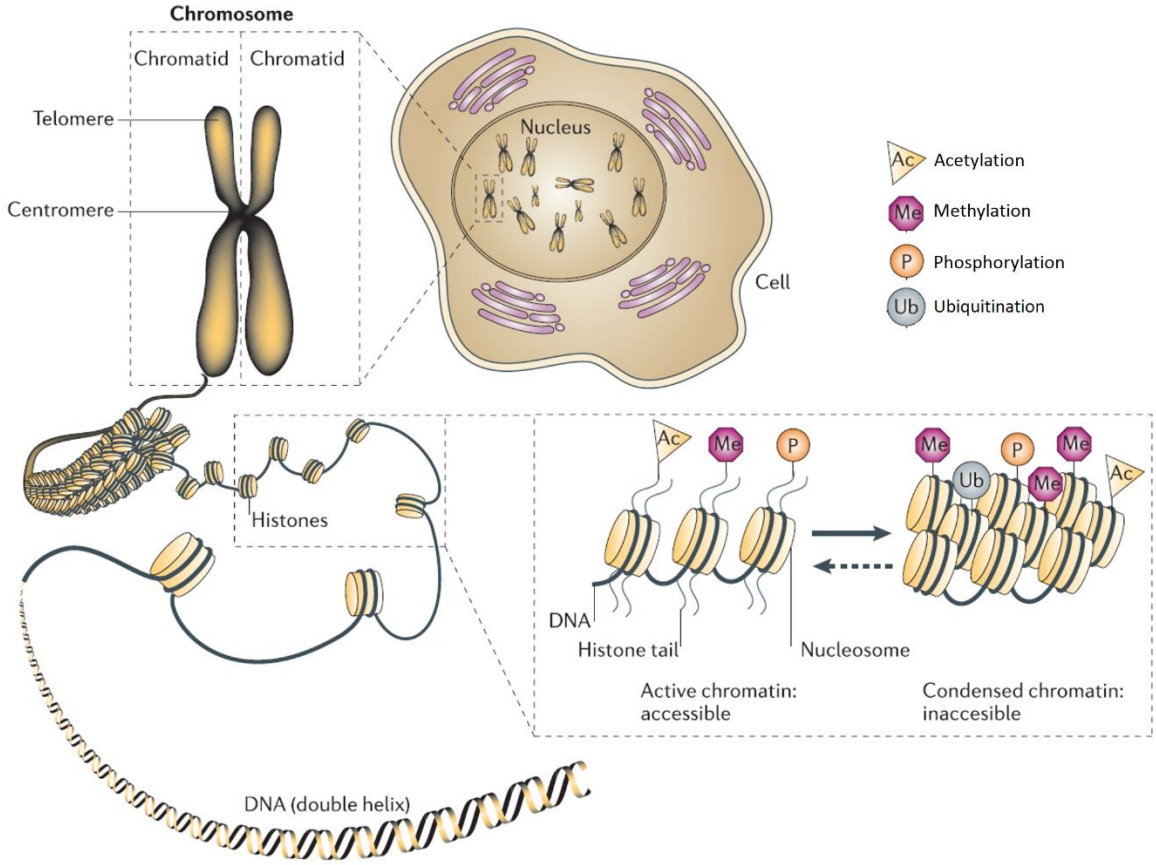
findings demonstrate that global hypomethylation is apparent in pre-neoplastic lesions such as hyperplastic polyps, and therefore is an early event in the adenoma-carcinoma sequence.

When comparing different samples taken from within patients, LINE-1 methylation has been observed to be significantly lower in cancerous tissue compared with adjacent normal tissue (Iacopetta *et al.*, 2007). Chalitchagorn *et al.* have also reported significantly greater LINE-1 hypomethylation in cancerous tissue compared with normal colonic tissue, analysed by combined bisulphite restriction analysis (COBRA) PCR (Chalitchagorn *et al.*, 2004). In addition, they observed a positive correlation between malignant progression and LINE-1 hypomethylation, suggesting that this occurs early in colorectal carcinogenesis and is associated with cancer progression.

1.2.6.2 Histone modifications

Histones are proteins that form the core of the wound structure of DNA into chromatin. Histones may undergo post-translational modifications such as acetylation, methylation and phosphorylation, which regulate expression of DNA (Bannister and Kouzarides, 2011) (Figure 1.8). Certain modifications are associated with active transcription due to the accessibility of the chromatin, such as acetylation of lysine 14 on histone H3 or lysine 4 trimethylation on histone H3 (Sparmann and van Lohuizen, 2006). On the contrary, other modifications, including trimethylation of lysines 9 and 27 on histone 3, are associated with condensed chromatin and ultimately repressed transcription (Sparmann and van Lohuizen, 2006).

Figure 1.8 Histone modifications.



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For the purpose of this thesis, I will focus on histone acetylation as butyrate, a product of dietary fibre fermentation with anti-neoplastic effects in the large bowel (discussed in section 1.5.4), represses histone deacetylation through the inhibition of histone deacetylase. Histone acetylation describes the addition of acetyl groups by histone acetyltransferase (HAT) to the lysine residues within the tail regions of histones (Struhl, 1998). Alternatively, histone deacetylation is the removal of such acetyl groups by histone deacetylase (HDAC). The lysine residues are positively charged, thus contributing to the compact packaging of the DNA due to an attraction with negatively-charged DNA. The addition of a negatively-charged acetyl group results in a neutrally-charged histone, which causes relaxation of the DNA. Consequently, acetylated DNA, named euchromatin, is associated with greater gene expression as it is more easily accessed by transcription factors. On the contrary, deacetylation restores the

tight interaction between histones and DNA and is associated with a more condensed DNA structure known as heterochromatin. Histone deacetylation results in repressed transcription.

Histone acetylation, in particular acetylation of histones H3 and H4, has been associated with cancer development and progression (Esteller, 2007). Karczmarski *et al.* found a total of 96 modifications, principally acetylation, to histones including H2A, H2B, H3 and H4 in CRC tissue compared with matched normal tissue (Karczmarski *et al.*, 2014). The investigators reported the novel finding of the acetylation of lysine 27 within histone H3 in CRC compared with normal tissue. Altered acetylation may also result from abnormal expression of HATs and HDACs. For example, an increase in the expression of HDAC2, as a consequence of activated WNT signalling, was associated with inhibition of apoptosis and has been reported in colon cancer cells (Zhu *et al.*, 2004).

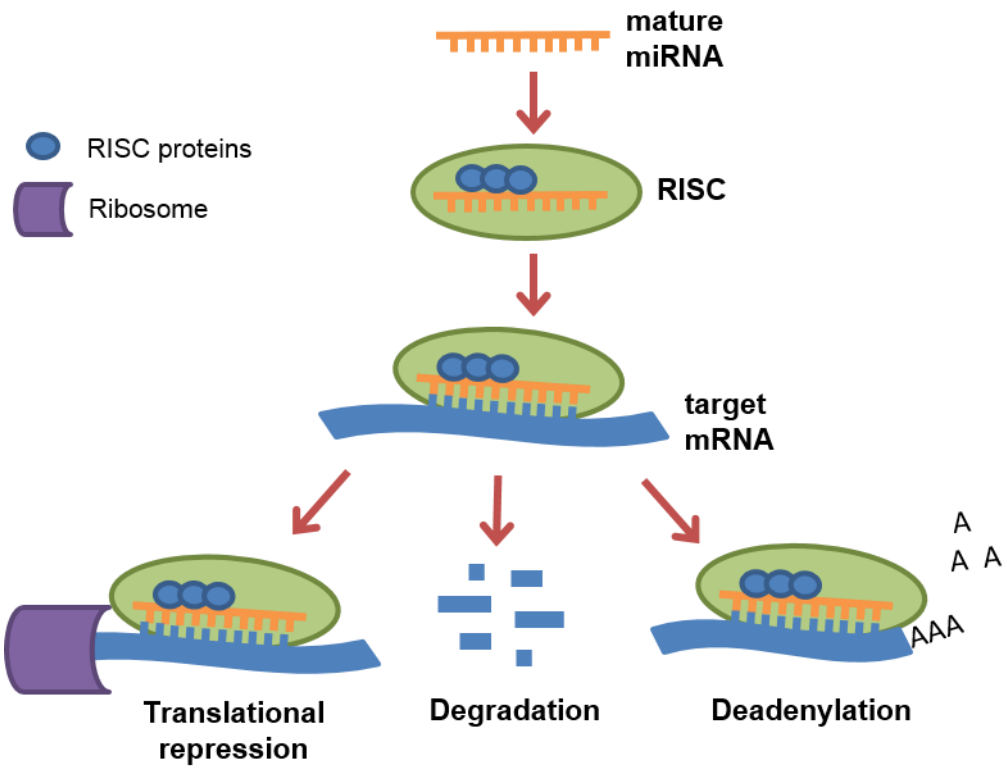
1.2.6.3 MicroRNA expression

Approximately 1,000 miRNAs have been identified in humans (Bentwich *et al.*, 2005), some of which are implicated in colorectal carcinogenesis. miRNAs are short, non-coding RNAs typically 22 nucleotides long that regulate expression of their target genes. A single miRNA may have numerous target genes and, likewise, a single gene may be targeted by multiple miRNAs (Lewis *et al.*, 2005).

Within the nucleus, miRNA genes are transcribed to primary miRNAs (pri-miRNAs) by RNA polymerase II, which are then cleaved by Drosha (a RNase III endonuclease) and DGCR8/Pasha (a double-stranded RNA-binding domain (dsRBD) protein) to produce precursor miRNAs (pre-miRNAs) that are approximately 70 nucleotides long (Bushati and Cohen, 2007). The pre-miRNAs are then transported to the cytoplasm by Exportin-5 and are again cleaved by Dicer (RNase III endonuclease) and TRBP/Loquacious (dsRBD protein) to form a duplex comprising the mature miRNA and its complementary strand (miRNA*), each approximately 22 nucleotides long (He and Hannon, 2004). In most cases, the miRNA* strand is subsequently degraded and is normally found at a 100-times lower concentration of that of the functional miRNA. However, in some cases, the miRNA* strand may also be functional (Ruby *et al.*, 2006).

The mature miRNA forms a complex, named the RNA-induced silencing complex (RISC), with proteins including dicer, Argonautes (AGO), such as Ago2, and the transactivating response RNA-binding protein (TRBP) (Bushati and Cohen, 2007) (please see Figure 1.9). RISC binds untranslated 3' regions of target mRNAs through complementary seed regions (6-8 nucleotides long) within the miRNA (Bushati and Cohen, 2007). If these regions are perfectly complementary to those of the target mRNA, the latter is cleaved by Ago2, which has endonuclease activity, and the mRNA is degraded (Bushati and Cohen, 2007). Otherwise, the translation of the mRNA into protein by the ribosome is inhibited. miRNAs may also result in destabilisation of the mRNA through deadenylation leading to degradation (Djuranovic *et al.*, 2012).

Figure 1.9 Regulation of target mRNAs by miRNAs.



Mature miRNAs form RISC with proteins such as Ago2 and TRBP and bind to complementary sequences within their target mRNAs and lead to post-transcriptional repression through the inhibition of translation, mRNA degradation or mRNA deadenylation and consequent destabilisation.

miRNAs regulate most cell functions including cell proliferation and differentiation, and aberrant miRNA expression may contribute to colorectal carcinogenesis. To date, irregular expression of more than 400 miRNAs have

been reported in CRC (Hogan *et al.*, 2012). Furthermore, the proposed targets of miRNAs dysregulated in CRC include members of the WNT pathway, such as *APC, CTNNBIP1* and *c-MYC* (Hogan *et al.*, 2012).

A systematic review found that six miRNAs were aberrantly expressed in each of seven studies viz. *miR-125a, miR-133a, miR-145, miR-30a-3p* and *mir-139* which were downregulated and *miR-106a* which was upregulated (Ma *et al.*, 2012). In addition, miRNAs from the miR-17-92 cluster (*miR-17, miR-18a, miR-19a, miR-19b*, *miR-20a* and *miR-92a*), also known as oncomir-1, are commonly dysregulated in cancers including CRC. All six mature miRNAs are overexpressed in colorectal carcinomas and are associated with cancer progression (Diosdado *et al.*, 2009). Additionally, the expression of certain dysregulated miRNAs have been associated with clinicopathological features. For example, downregulation of *miR-143* and *miR-145* has been reported early during the adenoma-carcinoma sequence (Michael *et al.*, 2003). On the contrary, upregulation of *miR-21* correlated with clinical stage and was associated with lymph node involvement and poor survival in CRC patients (Slaby *et al.*, 2007).

Abnormal miRNA expression in CRC is not restricted to colorectal tissue but has also been observed in plasma and faeces of CRC patients (Link *et al.*, 2010; Wu *et al.*, 2012a) (Kalimutho *et al.*, 2011; Ahmed *et al.*, 2013). Luo and colleagues observed that nine miRNAs were differentially expressed in the plasma of CRC patients compared with healthy controls (Luo *et al.*, 2013). These were *miR-18a, miR-20a, miR-21, miR-29a, miR-92a, miR-106b, miR-133a, miR-143* and *miR-145*. In stool samples, Ahmed *et al.* identified 12 miRNAs that were overexpressed in patients with colorectal adenomas and cancers (*miR-7, miR-17, miR-20a, miR-21, miR-92a, miR-96, miR-106, miR-134, miR-183, miR-196a, miR-199a-3p* and *miR-214*) (Ahmed *et al.*, 2013). Altogether, these findings suggest that these differentially-expressed miRNAs have potential as non-invasive biomarkers of CRC.

1.3 The WNT signalling pathway

The WNT signalling pathway is involved in normal developmental and physiological processes including cell proliferation, apoptosis, tissue self-renewal and cell migration (Bienz and Clevers, 2000). WNT signalling occurs in several tissue and cell-types, particularly in self-renewing tissues where it is active throughout life (Clevers, 2006). Three different WNT pathways have been discovered: the canonical WNT/beta-catenin (β-catenin) pathway, the non-canonical planar cell polarity (PCP) pathway and the WNT/calcium pathway. The canonical pathway is the best understood and most relevant in disease (especially cancer) pathophysiology.

1.3.1 The canonical WNT signalling pathway

The canonical WNT signalling cascade regulates functions such as proliferation, migration and apoptosis following activation of transcription of WNT target genes within the nucleus. A key player in this pathway is β-catenin which is regulated by the tumour suppressor APC (Groden *et al.*, 1991; Kinzler *et al.*, 1991). APC forms a multiprotein complex named the β-catenin destruction complex which comprises APC, AXIN, glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1) (Schneikert and Behrens, 2007). AXIN is a scaffolding protein which is bound to each component of the complex and facilitates the phosphorylation of β-catenin by GSK3β (Bienz and Clevers, 2000) and may be the limiting factor in the formation and disassembly of the destruction complex (Lee *et al.*, 2003).

1.3.1.1 Inactive state

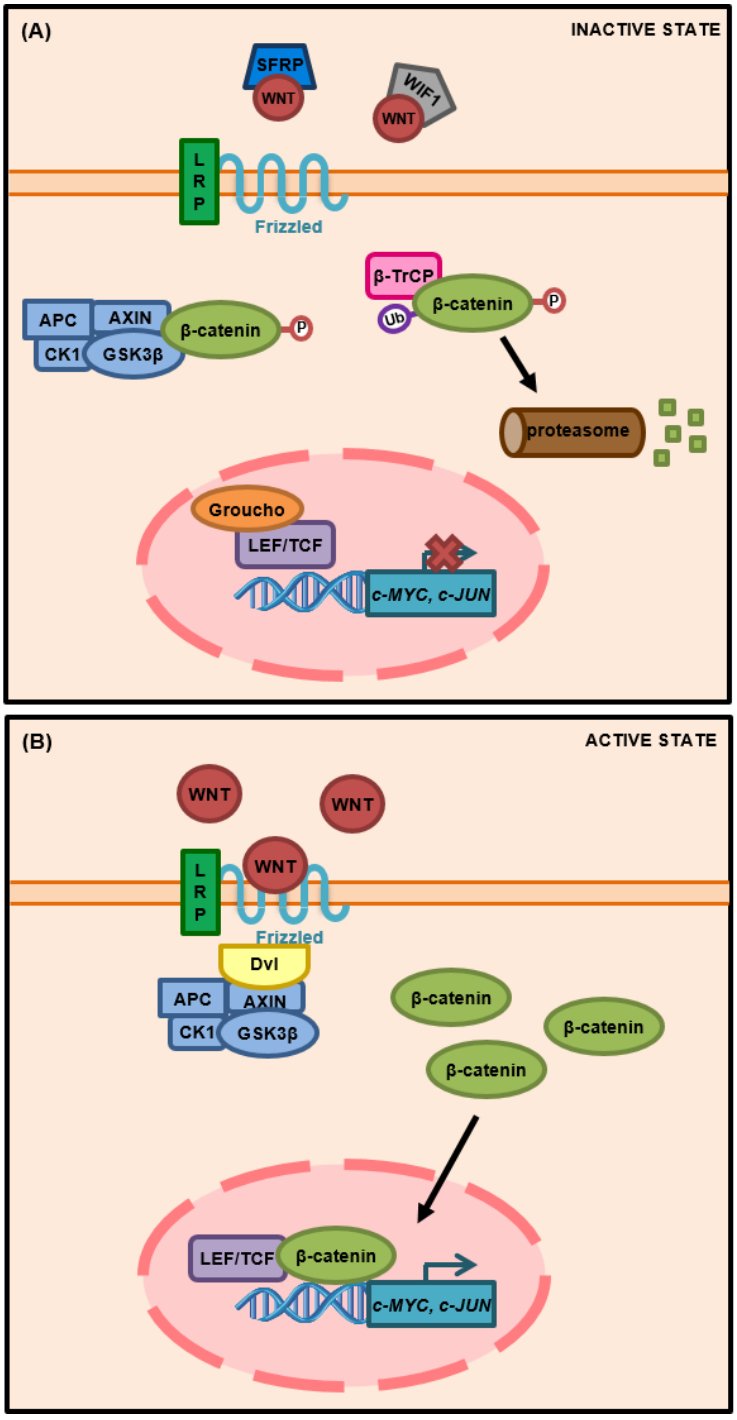
When there is no binding of a WNT ligand to its transmembrane receptor Frizzled, or when WNT antagonists such as *SFRP1* and *WIF1* inhibit WNT pathway activation (Schneikert and Behrens, 2007), β-catenin is phosphorylated by the β-catenin destruction complex (see Figure 1.10 (A)). CK1 phosphorylates β-catenin at serine 45 which then enables GSK3β to phosphorylate β-catenin at serine/threonine residues 41, 37 and 33. The β-transducin repeat-containing protein (βTrCP) targets phosphorylated β-catenin for ubiquitination by the E3 ubiquitin ligase complex (Schneikert and Behrens, 2007), which subsequently

labels β-catenin for degradation by the proteasome (Clevers, 2006). Inside the nucleus, Groucho and TLE co-repressors bind to and repress T cell factor (TCF) and lymphoid enhancer factor-1 (LEF) DNA-binding proteins, which inhibits transcription of WNT target genes (Clevers, 2006).

1.3.1.2 Active state

Activation of the WNT pathway by WNT ligands (see Figure 1.10 (B)), such as WNT1, binding to the Frizzled receptor leads to the interaction with the co-receptors, low-density lipoprotein receptor-related proteins LRP5 and LRP6, and the initiation of signal transduction (Clevers, 2006). The cytoplasmic tail of LRP is phosphorylated by CK1 and GSK3β (Schneikert and Behrens, 2007). Dishevelled (Dvl), a cytoplasmic protein, translocates to the membrane where it binds to Frizzled. AXIN is recruited to the membrane where it is bound to and inhibited by Dvl (Bienz and Clevers, 2000). As the destruction complex is inactive, β-catenin is no longer phosphorylated nor degraded, so cytoplasmic levels of this protein rise. Stabilised cytoplasmic β-catenin translocates into the nucleus where it displaces Groucho/TLE and interacts with TCF/LEF transcription factors, leading to the transcription of target genes. TCF forms a complex with β-catenin and other components such as histone acetylases, C-terminal-binding proteins (CtBP) and other proteins. These other interacting components are involved in the positive and negative regulation of the TCF/β-catenin complex and, ultimately, transcription (Sierra *et al.*, 2006). In addition, nuclear β-catenin activity can be repressed by the inhibitor Chibby and the inhibitor of β-catenin (ICAT).

Figure 1.10 The canonical WNT signalling pathway.



(A) Inactive state: in absence of activation by a WNT ligand binding to the Frizzled receptor, β-catenin is phosphorylated by the β-catenin destruction complex (APC, AXIN, CK1 and GSK3β), which destabilises β-catenin and targets it for ubiquitination and subsequent proteasomal degradation. Within the nucleus, LEF/TCF transcription factors are bound to and repressed by co-repressors such as Groucho. **(B)** Upon activation of the WNT pathway by WNT ligands e.g. WNT1, the Frizzled receptor forms a complex with LRP5/6 to initiate signal transduction. Dvl is activated and inhibits the β-catenin destruction complex. Levels of stabilised β-catenin accumulate in the cytoplasm, and β-catenin translocates into the nucleus where it binds to TCF/LEF to activate transcription of WNT target genes such as *c-MYC* and *c-JUN*.

1.3.2 The role of WNT signalling in the normal colonic mucosa

WNT proteins are expressed in intestinal crypt cells where the WNT signalling pathway is essential for maintaining homeostasis within the large bowel (Gregorieff *et al.*, 2005). Pericryptal myofibroblasts, which surround the stem cells at the base of the crypt, appear to be responsible for the production of WNT ligands, which then activate WNT signalling in the adjacent stem cells (Fevr *et al.*, 2007). Towards the base of the crypt, where the stem cell and proliferating progenitor cell populations are found, there are increased levels of nuclear β-catenin and active TCF/LEF, indicative of a stimulated, active WNT cascade (Clevers and Batlle, 2006).

WNT signalling is involved in the control of the continuous renewal of the colonic epithelium. Stimulation of the canonical WNT cascade results in activation of target genes that ultimately lead to the control of epithelial cell proliferation, organisation and fate (Schneikert and Behrens, 2007). Several *in vivo* studies have investigated the role of WNT signalling in the GI tract and have observed that loss or perturbed WNT signalling leads to an abnormal colonic phenotype (Korinek *et al.*, 1998; Wong *et al.*, 2002; Pinto *et al.*, 2003; Ireland *et al.*, 2004; Kuhnert *et al.*, 2004). Studies in mice have shown that WNT signalling is essential in maintaining the correct intestinal epithelium structure and that permanent stimulation of this pathway is required for precursor cell proliferation (Schneikert and Behrens, 2007).

Deletion of β-catenin in mice has been reported to result in crypt ablation and in alteration of the normal population of cell types in the epithelium, including a reduction in secretory cells and Paneth cells (Ireland *et al.*, 2004). Similarly, Fevr *et al.* used mutant mice (villin-creERT2-β-catenin$^{-/lox}$) to investigate the effects of complete ablation of β-catenin and found that these mice experienced complete loss of all colonocytes and the majority of crypts (Fevr *et al.*, 2007). This was paralleled by cessation of proliferation and reduced expression of *c-MYC,* (He *et al.*, 1998), a target gene of the WNT pathway that is involved in the regulation of cell proliferation.

Two other studies investigated the effect of overexpressing a WNT inhibitor, Dickkopf-related protein 1 (Dkk1), to reduce WNT pathway activity. Pinto *et al.* found a loss of proliferation and villi in the small intestine (Pinto *et al.*, 2003). A

later study by Kuhnert *et al.* used adenoviral expression of Dkk1 and found a rapid cessation of WNT signalling and ultimately proliferation in the small intestine, caecum and colon (Kuhnert *et al.*, 2004). They also observed structural disintegration with a progressive loss of crypts and villi, leading to ulceration and apoptosis.

Together, these findings suggest that WNT signalling is essential for maintaining the normal continuous proliferative state found in the adult small and large intestine. Dysregulation of this normal colonic epithelium maintenance, for example following mutations in WNT pathway genes and leading to aberrant activation of WNT signalling, can result in colorectal carcinogenesis.

1.4 The role of WNT signalling in CRC

Aberrant WNT signalling is implicated in the pathogenesis of several diseases including Alzheimer's disease (Inestrosa and Toledo, 2008), Dupuytren's skin disease (Dolmans *et al.*, 2011) and renal fibrosis (Pulkkinen *et al.*, 2008), as well as a number of cancers including CRC (Luo *et al.*, 2007). The canonical WNT pathway is frequently altered in CRC and is a key player in the pathogenesis of both inherited and sporadic forms of this cancer. Constitutively active WNT signalling is present in approximately 90% of sporadic CRCs (Klaus and Birchmeier, 2008). In particular, loss of function of the APC tumour suppressor, resulting from defects in *APC*, is found in approximately 85% of all CRCs (Kinzler and Vogelstein, 1996), including up to 70% of sporadic cases (Powell *et al.*, 1992). In the remaining 15% of CRC cases, other alterations to components of the WNT signalling pathway, such as mutations in the gene encoding β-catenin, *CTNNB1*, and epigenetic modifications of WNT inhibitors, have been identified.

1.4.1 Mutations in WNT genes

Most alterations to the WNT signalling pathway result from mutations in WNT genes. The principal mutations associated with CRC are those in *APC*. Germline mutations in *APC*, which are causal for the FAP syndrome, are found in 1% of all CRC cases (Segditsas and Tomlinson, 2006). In addition, mutations in *APC* are also found in over two-thirds of sporadic CRC cases (Segditsas and Tomlinson, 2006). Loss of *APC*, a member of the β-catenin destruction complex, leads to increased levels of dephosphorylated, active β-catenin (Bienz and Clevers, 2000).

Gain of function mutations in WNT genes associated with increased pathway activity, such as those encoding β-catenin and WNT ligands, are also common. Activating mutations in β-catenin, which prevent its recognition by GSK3β and therefore degradation, occur in up to 5% of CRCs (Ilyas *et al.*, 1997; Morin *et al.*, 1997). Truncating mutations in *AXIN* and *AXIN2* have also been discovered which, like loss of *APC*, lead to stabilisation of β-catenin (Liu *et al.*, 2000).

1.4.2 Abnormal expression of WNT pathway-related genes in colorectal cancer

Nguyen *et al.* investigated the expression of WNT pathway-related genes in the progression from normal colonic mucosa to tubular adenomas and ultimately to villous adenomas (Nguyen *et al.*, 2010). They found significant differences in the pattern of expression of 34 WNT pathway-related genes among normal, tubular adenoma and villous adenoma tissues. For 15 WNT pathway-related genes, there were larger significant ($p<0.01$) differences in expression between villous adenoma tissue and normal tissue and seven of these also showed significant differences in expression between tubular and villous adenoma tissues. Expression of *CTNNB1*, the gene encoding β-catenin, was almost three times greater in tubular adenomas compared with normal tissue and over six-fold higher in villous compared with tubular adenomas (Nguyen *et al.*, 2010). These observations led the authors to conclude that elevated WNT signalling is involved in the progression of adenomas.

Hlubek and colleagues demonstrated differential expression of several WNT target genes when comparing tumour with normal tissue and also in intra-tumorous comparison of cells at the invasive front with those at the tumour centre (Hlubek *et al.*, 2007). Twenty-nine WNT pathway-related genes, including *WISP1, CCND1, LEF1* and *TCF7,* were overexpressed in the colorectal carcinoma tissue compared with normal tissue (Hlubek *et al.*, 2007).

1.4.3 Abnormal methylation of WNT pathway-related genes in colorectal cancer

As discussed in Section 1.2.6.1, hypermethylation of specific genes has been associated with CRC. These genes include members of the WNT signalling pathway such as *APC* and WNT inhibitors e.g. *SFRP1.*

In a study conducted in Iran, the methylation state of *APC, AXIN1, AXIN2* and *GSK3β* was quantified by methylation-specific PCR and aberrant methylation was observed for *APC* and *AXIN2* in CRC patients (Naghibalhossaini *et al.*, 2012). A Taiwanese study also utilised methylation-specific PCR to explore the methylation state of six genes including *APC, AXIN1* and *GSK3β* in cancerous and normal tissue from sporadic CRC patients (Lin *et al.*, 2004). The

investigators reported significantly greater methylation of *APC* in cancerous compared with matched healthy tissue. Hypermethylation of the promoter region of *APC*, resulting in transcriptional silencing, has also been reported in serrated adenomas (Fu *et al.*, 2009) and was greater in colorectal carcinomas compared with adenomas, suggesting that it is an early event in colorectal carcinogenesis (Esteller *et al.*, 2000).

Promoter hypermethylation of WNT antagonists such as *DKK1* (Aguilera *et al.*, 2006), *WIF1* (Lee *et al.*, 2009) and *SFRP1* has also been reported (Qi *et al.*, 2007). Hypermethylation of WNT antagonists and consequent reduced expression would be expected to reduce the inhibition of WNT signalling and, therefore, permit increased pathway activity.

Secreted Frizzled-related proteins (SFRPs) are an important family of WNT antagonists and are frequently downregulated in CRC. Hypermethylation of *SFRP1, SFRP2, SFRP4* and *SFRP5* has been reported in both colorectal adenomas and carcinomas compared with normal and adjacent tissues (Qi *et al.*, 2006). Importantly, hypermethylation of these SFRPs was associated with downregulation or gene silencing. *SFRP1* hypermethylation has been reported *in vitro* in CRC cells and also *in vivo* in primary colorectal carcinoma tissues (Suzuki *et al.*, 2002). Furthermore, a recently-published meta-analysis, which included eight cohort studies with 942 participants, concluded that *SFRP1* promoter methylation was significantly increased in cancerous tissue compared with adjacent, normal and benign tissues (Chen *et al.*, 2014b). Significantly greater *SFRP1* methylation has also been reported in faecal samples from CRC patients compared with healthy subjects (Salehi *et al.*, 2012). Higher degrees of hypermethylation of *SFRP2* and *WIF1* have also been observed in faecal samples from patients with hyperplastic polyps, adenomas and carcinomas compared with normal subjects (Zhang *et al.*, 2014). Furthermore, *SFRP2* hypermethylation has also been reported in faecal, serum and tissue samples in CRC and polyp patients (Tang *et al.*, 2011).

1.5 Diet and colorectal cancer

The majority of CRC cases develop sporadically and various epidemiological studies, including migrant studies (Kolonel, 1980; Flood *et al.*, 2000) and secular trend studies (Kono, 2004), have provided evidence for an important role of environmental factors, principally diet and physical activity, in modulating the risk of developing this cancer. The panel from the WCRF/AICR concluded that "*food and nutrition have a highly important role in the prevention and causation of cancers of the colon and rectum"* (WCRF/AICR, 2007).

As described in Section 1.2.3.2, numerous dietary components have been associated with either a reduction or increase in CRC risk. Several studies, primarily migration studies, have investigated this impact of environmental factors on the risk of CRC and have found an increase in CRC risk when migrating from lower to higher-risk areas, such as from Asia to the western United States (Flood *et al.*, 2000). Investigations into the role of diet in CRC have focused on dietary risk factors such as obesity and the consumption of red meat, fat and alcohol (WCRF/AICR, 2007). However, of particular relevance to the present project, in the early 1970s, Dr. Denis Burkitt used his observation of low CRC incidence in a Western African population consuming high intakes of dietary fibre intake to propose a protective role of dietary fibre against CRC (Burkitt, 1971).

1.5.1 Dietary Fibre

Dietary fibre has been defined by the Codex Alimentarius Committee (CAC) as *"carbohydrate polymers with ten or more monomeric units which are not hydrolysed by the endogenous enzymes in the small intestine of humans"* (CAC, 2009). It encompasses non-digestible carbohydrates (NDCs), which are not absorbed in the small bowel and therefore reach the large bowel undigested (Ha *et al.*, 2000; DeVries, 2003). In the large bowel, NDCs are fermented by commensal bacteria which produce short chain fatty acids (SCFAs). The WCRF/AICR Continuous Update Project Report released in 2011 concluded that there is now convincing evidence for a protective role of dietary fibre against CRC (WCRF/AICR, 2011).

The WCFR/AICR Continuous Update Project Report analysed the effect of dietary fibre in 28 cohort studies (WCRF/AICR, 2011). In the majority of the studies, an increase in dietary fibre intake reduced the risk of developing CRC. A meta-analysis of the data from the cohort studies revealed a 10% reduction in CRC risk for every 10g per day of dietary fibre (WCRF/AICR, 2011).

The European Prospective Investigation into Cancer and Nutrition (EPIC) study in 2003 examined the association between dietary fibre intake and CRC incidence in over 500,000 individuals (Bingham *et al.*, 2003). An inverse correlation between dietary fibre consumption and CRC incidence [0.75 RR, 95% CI 0.59-0.95] was observed. The investigators concluded that a 40% reduction in CRC cases could be achieved by doubling dietary fibre intake. Importantly, the greatest protective effect of dietary fibre was observed in the left-side of the bowel, which is where the majority of CRC cases arise. A more recent analysis of EPIC data, with a mean follow-up of 11 years and a three-fold increase in the number of CRC cases, showed a 17% reduction in CRC risk per 10g of dietary fibre daily (Murphy *et al.*, 2012).

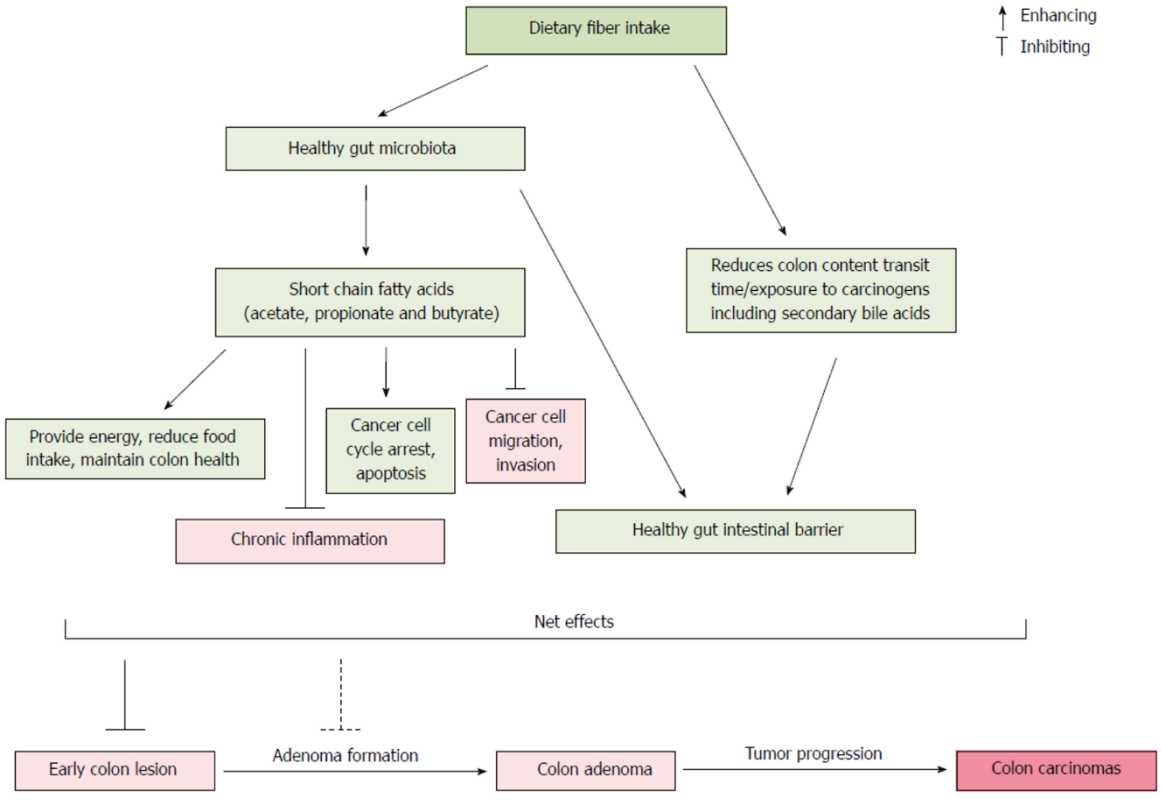
A systematic review and meta-analysis published in 2011 found inverse associations between intakes of dietary fibre and of wholegrains and CRC risk using data from 25 prospective studies (Aune *et al.*, 2011). This supports results from a previous meta-analysis of case-control studies that also observed this association (Jacobs *et al.*, 1998). However, in such observational studies it is impossible to attribute causality, and the authors discussed the possibility that some of the observed association between dietary fibre intake and CRC risk could result from other dietary or lifestyle confounding factors such as increased calcium and folate status, healthier weight and/or lower intakes of red meat, which could also reduce CRC risk (Aune *et al.*, 2011).

A more recently-published meta-analysis focussed on the protective effects of dietary fibre against colorectal adenomas, the precursor lesions of carcinomas, in 20 case-control and cohort studies with a total of just under 11,000 patients (Ben *et al.*, 2014). The results from this study also supported a protective role for dietary fibre against colorectal adenoma development. Analyses of twenty studies, which compared low versus high dietary fibre intake, showed a 28% reduction in adenoma risk per daily 10g of dietary fibre. However, a dose-

response model performed in 14 studies suggested a 9% decrease in adenoma risk per daily 10g of dietary fibre.

The mechanisms by which dietary fibre exerts its apparently protective effects are not completely understood and were originally proposed to result from a decrease in intestinal transit time, increase in stool bulk and the dilution of faecal content (Hylla *et al.*, 1998), therefore reducing exposure to carcinogens (Lipkin *et al.*, 1999). However, dietary fibre's beneficial effects have been shown to result primarily from effects of its major fermentation products i.e. SCFAs (Lipkin *et al.*, 1999). Although the molecular mechanisms by which dietary fibre produces these protective effects are not fully understood, there is compelling evidence of anti-neoplastic effects of butyrate, one of the major SCFAs. A summary of the effects of dietary fibre involved in the modulation of CRC risk, which will be discussed in more detail in the following sections of this chapter, as proposed by Zeng *et al.* (2014) are shown in Figure 1.11.

Figure 1.11 Effects of dietary fibre involved in the modulation of CRC risk as proposed by Zeng *et al.* (2014).



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1.5.2 Resistant starch

RS has been defined by Cummings and Stephen as *"the sum of starch and products of starch digestion that are not absorbed in the small bowel"* (Cummings and Stephen, 2007). RS therefore reaches the large bowel undigested, where it undergoes fermentation by colonic bacteria.

1.5.2.1 Resistant starch sources and intake

RS can be found naturally in different foods including cereals, vegetables and green (unripe) bananas (Niba, 2002). Particularly high sources of RS may be found in barley (16g/100g), chickpeas (6.6g/100g) and raw bananas (6.4g/100g) (Roberts *et al.*, 2004).

Four subtypes of RS have been described (Englyst *et al.*, 1992; Topping and Clifton, 2001):

- Type 1: physically inaccessible RS found in seeds;
- Type 2: resistant granules found in uncooked potato and green bananas;
- Type 3: retrograded RS which is present in bread and cooked, cooled potato;
- Type 4: chemically modified RS used widely for technical reasons in processed foods.

Baghurst and colleagues have recommended a RS intake of 20g per day, which may be up to four-times greater than that which is consumed daily in the Western diet (Baghurst *et al.*, 1996). RS intakes of up to 45g per day are well-tolerated in adults (Grabitske and Slavin, 2009).

Hylla *et al.* have investigated the effects of a diet high in RS in the colon of healthy human volunteers (Hylla *et al.*, 1998). The study demonstrated the feasibility of supplementing foods with RS without causing any unwanted gastrointestinal side effects. The supplementation of food and beverage products with RS may be a more effective means of increasing RS intake than recommending greater consumption of RS-containing foods as the average daily RS intake in the Western diet is very low.

1.5.2.2 Effects of resistant starch in the large bowel

In the large bowel, RS promotes faecal bulking which consequently shortens faecal transit time, promotes laxation and relieves constipation (Topping and Clifton, 2001). RS, including both types 2 and 3, increases faecal mass significantly so that RS has a mild laxative effect (Cummings *et al.*, 1996). RS also lowers pH (Noakes *et al.*, 1996), ammonia, phenols and secondary bile acid concentrations in the colon (van Munster *et al.*, 1994; Birkett *et al.*, 1996; Hylla *et al.*, 1998).

RS may also be protective by enhancing the maintenance of the colonic mucosal barrier (Toden *et al.*, 2005; Toden *et al.*, 2006) which acts as the first-line of defence in the colon (Gaudier *et al.*, 2004). Nofrarías *et al.* found that long-term intake of raw potato starch, a source of RS, improved mucosal integrity in the colon of pigs (Nofrarias *et al.*, 2007). This was indicated by increased mucin sulphation, reduced magnesium concentrations in the lumen and decreased epithelial proliferation. Improved mucosal integrity may help to protect against CRC as diseases which predispose to this cancer, such as IBD, often show a breakdown of this mucosal barrier (Itzkowitz and Yio, 2004). Furthermore, decreased mucin sulphation has been observed in patients with UC (Van Klinken *et al.*, 1999).

The principal physiological effect of RS consumption is the increase in levels of SCFAs, particularly butyrate (Scheppach *et al.*, 1988; Cummings *et al.*, 1996; Noakes *et al.*, 1996). Mathers *et al.* demonstrated substantial increases in large bowel butyrate concentrations in Wistar rats fed raw potato starch as a source of RS (Mathers *et al.*, 1997). Caecal butyrate concentrations were 70% greater and caecal butyrate pools were four-fold greater in rats fed a raw potato starch diet (80g/kg of diet) compared with the control diet (maize starch). It is thought that the consequential chemoprotective effects of RS consumption primarily result from an increase in butyrate levels, as well as other SCFAs, following RS fermentation.

1.5.2.3 Resistant starch as a prebiotic

Dietary prebiotics are described as *"selectively fermented ingredients that result in specific changes, in the composition and/or activity of the gastrointestinal*

microbiota, thus conferring benefit(s) upon host health" (Gibson, 2010). RS is a substrate for fermentation by colonic bacteria and all four types of RS have been reported to increase levels of *Bifidobacteria in vivo.*

One study randomised rats to a control diet, a granular potato starch diet (RS type 1) or a modified potato starch diet (RS type 2) for five months and observed modification of intestinal microflora with both types of RS (Kleessen *et al.*, 1997). Rats on both RS diets had significantly greater caecal *Bifidobacteria* counts than rats on the control diet. Differences in caecal bacteria counts between the two types of RS were also apparent. RS type 2 significantly increased caecal bacteria of the *Lactobacilli*, *Streptococci* and *Enterobacteria* genera compared with both the control and RS type 1 diets. This was also reflected in the faecal analyses. In a porcine model, increased faecal *Bifidobacteria* have been observed in animals given a probiotic supplemented with RS compared with those without RS (Brown *et al.*, 1997).

Studies investigating the role of RS as a prebiotic have also been conducted in humans. In a double-blind, randomised, placebo-controlled study, 200 healthy participants were randomised to a placebo group or one of four NDCs, including retrograded tapioca maltodextrin, a RS type 3 (Bouhnik *et al.*, 2004). Feeding 10g of retrograded tapioca maltodextrin per day for seven days increased faecal concentrations of *Bifidobacteria* significantly compared with placebo. This was the first study to show the prebiotic effects of a type 3 RS in humans. A double-blind, placebo-controlled crossover trial investigated the effects of RS types 2 and 4 on faecal microbiota in ten healthy subjects (Martinez *et al.*, 2010). This study revealed that only RS type 2 increased bacteria from the phylum Firmicutes compared with placebo. Bacteria from the Ruminococcaceae family and the *R. bromii* and *E. rectale* species were also significantly greater with RS type 2. On the other hand, RS type 4 increased faecal concentrations of the phyla Bacteroidetes and Actinobacteria and reduced Firmicutes compared with placebo. At the species level, significantly greater concentrations of *B. adolescentis, P. distasonis, C. clostridioforme* and significantly lower concentrations of *F. prausnitzii* and *D. formicigenerans* compared with placebo were observed.

1.5.2.4 Anti-neoplastic effects of resistant starch

Studies that have investigated the chemoprotective effects of RS against colorectal carcinogenesis have yielded inconsistent findings. Although a reduction in colorectal lesions have been reported with RS, other studies have not observed a protective effect of RS and some have even reported a detrimental effect of RS.

The pre-neoplastic lesions, aberrant crypt foci (ACF), can be used as an early marker of colorectal carcinogenesis in animal studies. Using the formation of ACF as an endpoint, Liu *et al.* investigated the effects of raw potato starch (RS type 2) in Wistar rats treated with the carcinogen azoxymethane (AOM) (Liu and Xu, 2008). They observed a significant reduction in ACF formation in rats that were given the RS-containing diets for three weeks post-carcinogen treatment compared with the control diet and there was a dose-dependent effect of RS in reducing ACF formation. Conversely, RS enhanced ACF formation when modelling the pre-initiation stage of carcinogenesis by administering the RS before exposure to AOM. These findings suggest that RS exerts its chemoprotective effects at the promotion stage of colorectal carcinogenesis but may have adverse effects at the pre-initiation stage, before the induction of carcinogenesis. It must be noted, however, that supplementation with RS at the pre-initiation stage increased faecal bulk and decreased faecal pH significantly which are regarded as positive markers of bowel health (Liu and Xu, 2008).

Two additional studies have also shown protective effects of RS at the promotion stage of colorectal carcinogenesis following carcinogen treatment. Thorup *et al.* reported significantly lower ACF formation in AOM-treated rats fed a raw potato starch diet compared with rats on corn starch and control diets (Thorup *et al.*, 1995). There was significantly reduced ACF formation in RS-fed rats (whether supplemented alone or synergistically with vitamin A for 12 weeks) and then treated with the carcinogen, 1,2-dimethylhydrazine dihydrochloride (DMH) (Cassand *et al.*, 1997). The ability of RS to reduce colorectal tumour formation has also been reported. Bauer-Marinovic *et al.*, reported a significantly greater incidence of colonic tumours in DMH-treated Sprague-Dawley rats fed a standard diet compared with those given Novelose 330, a type 3 RS, where there were no tumour-bearing animals (Bauer-

Marinovic *et al.*, 2006). A type 2 RS, high-amylose maize starch (HAMS), fed to rats for four weeks prior to injection with AOM also reduced the incidence and number of colonic adenocarcinomas (Le Leu *et al.*, 2007).

The Le Leu *et al.* group have also investigated the effects of symbiotic treatment of RS and *Bifidobacterium lactis* for four weeks on colorectal cancer development in AOM-treated Sprague-Dawley rats (Le Leu *et al.*, 2010). When rats were given both RS and *B. lactis* in combination, the incidence and number of colonic tumours was reduced and, although there was a trend for a protective effect for RS administered alone, this was not statistically significant.

Winter *et al.* investigated the effects of RS intake in mice fed red meat (which increases CRC risk) by measuring levels of a known mutagenic lesion implicated in colorectal carcinogenesis, O^6-methyl-2-deoxyguanosine (O^6-MeG) DNA adduct formation (Winter *et al.*, 2011). Faecal analysis showed that RS supplementation significantly increased concentrations of SCFAs, including butyrate and total SCFAs, and lowered faecal pH, ammonia and phenol concentrations. Furthermore, the addition of RS to the diet significantly reduced DNA adduct formation that resulted from the consumption of red meat. This suggests that RS may abrogate some of the mutagenic effects of red meat consumption by providing extra carbohydrate for bacterial fermentation and, therefore, increasing butyrate concentrations (Winter *et al.*, 2011). In a more recent study, RS, in the form of HAMS, was fed to rats which consumed both short-term (one month) and long-term (18 months) heme iron, believed to be the oncogenic component in red meat (Winter *et al.*, 2014). As in their previous study, O^6-MeG adduct formation as an indicator of colorectal carcinogenesis was one of the study endpoints. In addition, they quantified the formation of another pro-mutagenic adduct, 8-hydroxy-2-deoxyguanosine (8-oxo). After both short- and long-term feeding, there were no between-diet differences in O^6-MeG or 8-oxo adduct formation but there was a trend for lower colonic tumour incidence in rats fed RS compared with those not given RS.

In contrast with these positive effects of RS, some animal studies have reported no effect or adverse effects of RS on CRC risk. Despite increased butyrate concentrations following RS (3% or 10%) consumption, there were no effects on the development of colorectal tumours in DMH-treated rats compared with

those fed a control diet or cellulose (Sakamoto *et al.*, 1996). Similarly, amylose maize, a type 3 RS, did not protect against the formation of the pre-neoplastic lesions, ACF, in DMH-treated rats (Maziere *et al.*, 1998). In addition, ACF density and colonic tumour formation were increased in DMH-treated rats that were fed potato starch for 31 weeks (Young *et al.*, 1996). In a study from the Mathers laboratory, a mouse model of sporadic CRC, where mice carry a mutation in *APC* causing them to develop intestinal tumours, raw potato starch and Hylon VII (both type 2 RS) were fed to mice for up to five months and resulted in an increase in tumour formation in the small intestine (Williamson *et al.*, 1999). Interestingly, the co-administration of aspirin, a non-steroidal anti-inflammatory drug (NSAID), prevented this adverse effect.

In humans, the effects of RS (and of aspirin) on large bowel neoplasia have been investigated in a series of large-scale, multi-centre intervention studies led by Professors John Burn, Tim Bishop and John Mathers using individuals with familial bowel cancer. The first of these, known as the Colorectal Adenoma/carcinoma Prevention Programme (CAPP) 1, was a randomised, placebo-controlled study, which used a 2x2 factorial design to investigate the effects of RS (provided as a 1:1 mix of raw potato starch and Hylon VII) and/ or of aspirin on colorectal carcinogenesis in FAP patients (Burn *et al.*, 2011a). The primary endpoint of CAPP1 was polyp formation with additional endpoints including assessment of the size of the largest polyp and colonic crypt cell proliferation. Following a median intervention of 17 months, results from this study suggested that neither RS (nor aspirin) had protective effects on the number of polyps formed in young FAP patients. A protective effect of aspirin, but not RS, was observed on the size of the largest polyp. However, the authors reported an increase in crypt cell proliferation by 28% in patients supplemented with RS compared with placebo.

Using a similar randomised, double-blind placebo-controlled study with a 2x2 factorial design in Lynch Syndrome patients, the CAPP2 study, the team observed no effects of either aspirin or RS on neoplasia at the end of the intervention (up to four years) (Burn *et al.*, 2008). However, with longer follow up, those who had been randomised to aspirin has substantially reduced incidence of CRC and other Lynch Syndrome cancers (Burn *et al.*, 2011b) but there was no protective, or adverse, effect in those randomised to RS (Mathers

et al., 2012). The authors concluded that RS did not emulate the apparently protective effects of diets rich in dietary fibre (Mathers *et al.*, 2012). However, the findings from these studies, where a reduction in polyp size in young FAP patients and in CRC incidence in Lynch Syndrome patients with aspirin supplementation were observed, provided *prima facie* evidence that neoplasia of the colorectum are driven by inflammation and, therefore, reducing inflammation may reduce CRC risk (Burn *et al.*, 2011a; Burn *et al.*, 2011b).

1.5.2.5 Additional beneficial effects of resistant starch

Given that inflammation drives tumourigenesis in the large bowel (and in other tissues (Elinav *et al.*, 2013)) RS may have anti-neoplastic actions through its anti-inflammatory effects. A rat model of inflammation, with symptoms similar to those observed in UC patients, has been used to investigate the effects of RS intake on inflammation (Jacobasch *et al.*, 1999). The rats fed RS had increased concentrations of SCFAs and an accelerated regenerative response to induced colitis, including the development of new crypts in the epithelia.

Anti-inflammatory effects have also been reported in mice fed RS, as well as other dietary fibres. Mice deficient in the anti-inflammatory cytokine interleukin-10 (IL-$10^{-/-}$ mice) were used to investigate the potential prevention of inflammatory diseases by RS. Significant improvements in markers of disease, including reduced rectal bleeding and diarrhoea, as well as in the presence of inflammatory lesions, were observed in mice given RS compared with the control group (Bassaganya-Riera *et al.*, 2011). These findings provide evidence for a reduction of gastrointestinal inflammation following RS consumption and therefore a possible prevention of the development of inflammatory diseases. The anti-inflammatory effects of RS may be mediated via changes in the microbiota (as described in section 1.5.2.3), as intestinal dysbiosis (microbial imbalance), such as an increase in the ratio of *Firmicutes* to *Bacteroidetes* (Lee and Hase, 2014), and pathogenic microbial colonisation may induce an inflammatory response and have been associated with CRC (Elinav *et al.*, 2013).

Higgins *et al.* have investigated the effects of the ingestion of different carbohydrates on insulin sensitivity in rats (Higgins *et al.*, 1996). Amylose, a

starch resistant to digestion, or amylopectin, a carbohydrate readily digested in the small intestine, were fed to rats and the insulinaemic response to glucose was measured. Results from the study showed the development of insulin resistance in amylose-fed rats at 26 weeks of feeding, but no further increase in resistance was observed after this (Higgins *et al.*, 1996). In contrast, the group fed amylopectin showed increasing insulin resistance up to 26 weeks of feeding. The improved insulin sensitivity in rats fed RS compared to non-resistant starch could suggest protection against the development of diabetes. A human study also investigating the effects of RS consumption on insulin sensitivity found that subjects supplemented with 60g of RS had significantly lower postprandial plasma glucose and insulin levels as well as better insulin sensitivity compared with controls (Robertson *et al.*, 2003).

RS has also been shown to improve satiety. In a randomised, double-blind, crossover study, Willis *et al.* investigated the effects of different dietary fibres, including RS, incorporated into five different muffins consumed in the fasted state on short-term satiety (Willis *et al.*, 2009). The results from this study demonstrated that RS, as well as corn bran, improved satiety as assessed through indicators of hunger, prospective food intake and satisfaction. Furthermore, the duration of the satiety effect by RS was longer than the other tested fibres. Similar findings have been reported with the addition of RS to the evening meal of healthy subjects, which resulted in reduced consumption at breakfast compared with a low fibre meal (Nilsson *et al.*, 2008). Moreover, a randomised, single-blinded, crossover study has reported a reduction in total energy intake in healthy subjects fed 48g of RS compared with a control diet (Bodinham *et al.*, 2010).

Higgins *et al.* have also demonstrated in human volunteers that RS can significantly increase lipid oxidation and therefore reduce accumulation as adipose tissue (Higgins *et al.*, 2004). As increased adiposity can enhance the risk of several diseases including cancers and type 2 diabetes, this establishes another protective effect of RS.

1.5.3 Polydextrose

Polydextrose (PD) is a synthetic NDC originally developed by Pfizer as a low-calorie sweetener with bulking properties (Stowell, 2009b). It is a soluble fibre and a glucose polymer with sorbitol end groups (Stowell, 2009b). It has been proposed by many that PD can be classified as a dietary fibre and therefore is integrated into several foods to add its beneficial properties. Furthermore, several other health effects have been observed both *in vitro* and *in vivo* and a number of these have been attributed to the production of butyrate by PD fermentation.

1.5.3.1 Polydextrose as a food ingredient

PD has been incorporated as an ingredient in a variety of food and drink products including dairy products, confectionery and pasta (Stowell, 2009b). This is facilitated by the fact that PD is heat- and acid-stable and is compatible with the majority of food and drink matrices. Moreover, it can provide products with beneficial properties such as improving their moisture management due to its humectant properties and also as a thickening agent. PD has less energy content than that found in carbohydrates and fats, making it an ideal candidate to be added into products with low/lower energy content, low/lower glycaemic index, high fibre content and/or prebiotic properties (Stowell, 2009b).

As GI side effects, including abdominal cramps and bloating (Sandler *et al.*, 2000), have been reported following increased dietary fibre consumption, Boler *et al.* investigated the physiological effects of PD and soluble maize fibre consumption (Boler *et al.*, 2011). The results from tolerance tests indicated minor discomfort, including increased flatulence and distension. However, volunteers were consuming up to 21g of PD per day, which is more than that given in previous studies, indicating that this dose can be administered with minimal GI discomfort (Boler *et al.*, 2011).

1.5.3.2 Effects of polydextrose in the large bowel

A number of human studies have reported an improvement in large bowel function following PD consumption, such as a dose-dependent improvement in defaecation, augmented faecal weight and reduced faecal pH in healthy human

participants (Jie *et al.*, 2000). A placebo-controlled, randomised intervention supplemented 45 healthy participants with 8g of PD per day (Hengst *et al.*, 2009). PD supplementation in yoghurts was associated with a significant reduction in faecal PH, excretion of cholesterol degradation products, excretion of total bile acids and orofaecal transit time in addition to an improvement in ease of defaecation. A reduction in abdominal discomfort accompanied by an improvement in bowel habits has also been reported with PD consumption (Costabile *et al.*, 2011). The former may be explained by reduced gas production, reported *in vitro*, resulting from PD fermentation compared with other dietary prebiotics (Hernot *et al.*, 2009).

A chronic state of inflammation in the large bowel has been associated with increasing the risk of CRC. Increased levels of the mucosal cyclooxygenase (COX) COX-2, which is associated with increased inflammation, have been reported in tumourigenesis and CRC development (Wendum *et al.*, 2004), with at least 80% of colorectal carcinomas having raised COX-2 levels (Eberhart *et al.*, 1994). Furthermore, COX-2 antagonists are able to reduce CRC risk or even reverse carcinogenesis (Krause and DuBois, 2001). *In vitro* and *in vivo* studies have demonstrated that PD reduces COX-2 expression in the large bowel in a dose-dependent manner (Makivuokko *et al.*, 2005; Fava *et al.*, 2007), suggesting another protective effect of PD against CRC.

In addition, a reduction in levels of branched-chain fatty acids (BCFAs), markers of protein decomposition, have been observed in the large bowel following supplementation with PD (Smith and Macfarlane, 1997). This constitutes a protective effect against CRC as certain metabolites produced during this process are carcinogenic. More recently, Boler *et al.* investigated the digestive physiological effects of PD and soluble maize fibre, including gastrointestinal tolerance and SCFA concentrations, in healthy male volunteers (Boler *et al.*, 2011). This study also found a reduction in putrefactive compounds, including BCFAs and ammonia, which are produced by protein fermentation.

1.5.3.3 Polydextrose as a prebiotic

Adhering to the definition proposed by The WHO/FAO Codex Alimentarius Committee on Nutrition and Foods for Special Dietary Uses in 2005, PD falls

under the category of a dietary fibre (Stowell, 2009b). Furthermore, several studies have demonstrated comparable physiological effects to those of dietary fibre produced by PD.

In 1981, Figdor and Rennhard determined the disposition of PD in the rat to be approximately 35% (Figdor and Rennhard, 1981). This was later confirmed in humans by Figdor and Bianchine, where it was discovered that between 30% and 50% of PD is fermented in the intestine (Figdor and Bianchine, 1983). The effect of PD supplementation on the intestinal microbiota as well as on the immune function was investigated by Fava *et al.* in a porcine model (Fava *et al.*, 2007). The investigators observed the fermentation of PD in the distal colon, suggesting that this process occurs throughout the large bowel. This had previously been demonstrated *in vitro* by Makivuokko *et al.* in a human model of the large bowel (Makivuokko *et al.*, 2005). These observations are particularly important as they imply that PD can exert its protective effects even in the distal colon, where the majority of colorectal tumours develop.

In humans, a double-blind, crossover intervention study explored the prebiotic effects of a daily dose of 8g of PD, particularly focusing on its modulation of faecal microbiota (Costabile *et al.*, 2011). The study demonstrated an effect of PD on colonic microbiota, including an increase in the microbiota responsible for the production of butyrate such as *Faecalibacterium prausnitzii*. Despite this, there were no significant changes observed in faecal butyrate content. A more recent randomised, double-blind, placebo-controlled trial supplemented 20 healthy males with 21g of PD, placebo or soluble corn fibre for 21 days and assessed microbiota levels in faecal samples by pyrosequencing (Hooda *et al.*, 2012). Although the investigators did not observe an effect of either treatment on overall faecal bacteria diversity following PD supplementation, participants fed PD had greater faecal Clostridiaceae, Veillonellaceae and Verrucomicrobiaceae and reduced Lachnospiraceae, Eubacteriaceae, Lactobacillales, Bifidobacteriaceae, Coriobacteriaceae and Hyphomicrobiacea compared with the no fibre diet. PD added to chocolate as a sweetener and fed to 40 healthy participants for fourteen days has been reported to significantly increase faecal *Bifidobacteria* and *Lactobacilli,* and this remained apparent six weeks post-consumption (Beards *et al.*, 2010). Interestingly, the investigators

also reported a significant increase in faecal butyrate and propionate concentrations.

PD has been shown to increase levels of SCFAs, as well as decrease levels of BCFAs, both *in vitro* and *in vivo* in human studies. *In vitro,* Makivuokko *et al.* observed that total SCFAs increased progressively along the colon following PD fermentation (Makivuokko *et al.*, 2005). *In vivo,* a rise in total SCFAs in the colon has also been observed. Jie *et al.* investigated PD supplementation in a randomised controlled study in Chinese volunteers and measured a number of physiological factors including SCFA production, faecal microflora and colonic mucosal cell proliferation (Jie *et al.*, 2000). Following PD intake, the authors observed significantly increased levels of *Lactobacilli* and *Bifidobacteria* as well reduced levels of *Bacteroides*. Furthermore, a significant increase in butyrate concentrations was found in those volunteers given 8g or 12g of PD, suggesting a PD supplementation of more than 8g per day can yield the beneficial effects produced by butyrate in the colon (Jie *et al.*, 2000). The augmented levels of butyrate observed were confirmed by measuring cell proliferation in colonic crypts, an indicator of SCFA production, where increased proliferation, particularly in the basal compartments, was observed for all PD doses.

1.5.3.4 Anti-neoplastic effects of polydextrose

Although not as extensively-studied as RS, several studies have reported an anti-carcinogenic activity of PD. However, these have either been *in vitro* or *in vivo* animal studies and human studies have not been conducted to date.

In vitro, Putaala *et al.* used the Enteromix® colon stimulator to model the fermentation of PD and subsequently treated Caco-2 colon adenocarcinoma cells with the produced fermentation metabolome (Putaala *et al.*, 2011). Treatment of Caco-2 cells with PD fermentation products resulted in an induction of apoptosis and inhibition of proliferation. Furthermore, PD positively modulated the expression of genes reported to be dysregulated in CRC, such as the peroxisome proliferator-activated receptors (PPAR)-responsive genes, which have been reported to be downregulated in colon cancer tissue (Martinasso *et al.*, 2007).

In a study using ACF formation as the primary endpoint, 35 male Wistar/ST rats were randomised to one of five groups fed a control fibre-free diet or PD at 7 days prior to DMH injection or on days 0, 1 and 7 post-injection (Ishizuka *et al.*, 2003). All rats continued consuming their respective diets until five weeks post-injection. Rats fed PD prior to DMH injection had a significantly lower number of aberrant crypts and ACF in the colorectum compared with the fibre-free diet. A similar finding was observed for all of the PD diets, however they were not statistically significant. Interestingly, the majority of PD's effects were visible in the rectum, where a significantly lower number of aberrant crypts and ACF were counted in rats from all of the PD diet groups bar the one consumed seven days post-DMH injections compared with the fibre-free diet. Although PD appeared to be protective at all time-points of administration, this was only statistically significant when consumed prior to carcinogen exposure. This suggests that the timing of intervention is important and that PD may be particularly effective at the pre-promotion stage of colorectal carcinogenesis.

Another study in DMH-treated rats investigated the effects of the replacement of sucrose with PD and varying concentrations of lactulose sweeteners in a guava preserve on ACF formation (Menezes *et al.*, 2012). However, there were no protective effects of PD and lactulose observed in carcinogen-treated rats, which all developed ACF. Consistent with these findings, another study in DMH-treated mice did not report an effect of PD on colon tumour formation (Kumemura *et al.*, 1998).

1.5.3.5 Other beneficial effects of polydextrose

McMahon has demonstrated a glycaemic and insulinaemic response in volunteers with type 2 diabetes who administered a single 50g dose of PD or glucose (McMahon, 1974). Positive effects on other metabolic parameters in patients with type 2 diabetes, including total cholesterol and plasma triglycerides, as well as a reduction in body weight and BMI have also been observed in a dietary intervention study supplementing female volunteers with a combination of PD and oligofructose for six weeks (Cicek, 2009). Furthermore, an attenuation of the postprandial rise in plasma glucose and insulin response has been observed in response to PD (Stowell, 2009a).

PD may also be an appetite-suppressant as it has been reported that the addition of PD to yoghurt significantly increased post-prandial satiety and reduced energy intake in healthy human participants (King *et al.*, 2005). A reduction in *ad libitum* energy intake has been confirmed by Astbury *et al.*, who compared the effects of varying concentrations of PD administered in a liquidised form in a randomised, cross-over design of 21 healthy subjects (Astbury *et al.*, 2013). All three doses of PD (6.3, 12.5 and 25g) resulted in a significantly lower energy intake during the test meal compared with the control. A significantly lower total daily energy intake was also observed with PD, however this was only statistically significant for the two highest doses.

Additional health-benefitting effects of PD are its abilities to modulate serum cholesterol and triglyceride levels. Pronczuk *et al.* have reported a hypocholesterolemic effect of PD in gerbils and humans (Pronczuk and Hayes, 2006). In the animal studies, gerbils were either given a 0.15% cholesterol diet with or without 6% PD for four weeks or a cholesterol-free diet for three weeks. Supplementation of both diets with 6% PD, equivalent to approximately 30g per day in humans, resulted in significantly reduced plasma and liver cholesterol concentrations compared with the control diet. In the human study, 15g and 30g of PD per day were given to 12 hypercholesterolemic participants for four weeks. Consequently, a significant reduction in low-density lipoprotein cholesterol and a trend for lower total cholesterol was observed in five responding participants given the 30g per day dose of PD.

1.5.4 Butyrate

Butyrate, one of the SCFAs generated by the fermentation of dietary fibre, is one of the principal SCFAs produced from RS (Noakes *et al.*, 1996). Butyrate is primarily produced by bacteria of *Clostridia, Roseburia* and *Eubacteria* (Pryde *et al.*, 2002; Louis *et al.*, 2007). In the large bowel, butyrate plays an important role in the maintenance of large bowel function, particularly by providing energy to colonic epithelial cells (Hamer *et al.*, 2008). Butyrate has also been proposed to be responsible for the majority of dietary fibre's protective effects against CRC through the regulation of processes such as apoptosis and proliferation. A large array of *in vitro* and *in vivo* studies have investigated the effects of butyrate in the large bowel. However, the majority of these have utilised cancer cells or

tissues and findings from healthy compared with cancerous models have been conflicting.

1.5.4.1 Effects of butyrate in the large bowel

Butyrate is the preferred source of fuel for colonocytes, providing the majority of the energy requirement for these cells (Hamer *et al.*, 2008). Butyrate provides between 60% and 70% of the total energy required by colonocytes (Roediger, 1980). It also regulates many cellular processes such as proliferation, differentiation and apoptosis in both normal and cancerous tissues (Nofrarias *et al.*, 2007).

In the large bowel, the effects of butyrate are primarily on cell proliferation. Studies in the healthy crypt have observed an induction of proliferation by butyrate that maintains adequate levels necessary to preserve homeostasis (Kripke *et al.*, 1989). The effects of butyrate on colonic crypt cell proliferation are discussed in more detail in section 4.5.1.4. On the other hand, levels of apoptosis in the healthy crypt have been shown to be reduced by butyrate (Wachtershauser and Stein, 2000).

As SCFAs are acidic, another consequence of increased SCFA production following, for example, dietary fibre intake is a reduction in colonic pH (Topping and Clifton, 2001). This is another beneficial effect of butyrate in the large bowel as the lower pH may prevent the colonisation or over-growth of certain pathogenic bacteria.

Butyrate has also been shown to have anti-inflammatory properties within the large bowel, particularly via the modulation of regulators of inflammation including Interleukins -8 (IL-8), -10 (IL-10), -12 (IL-12) and -32 alpha (IL-32α) (Huang *et al.*, 1997; Kobori *et al.*, 2010), suppression of the nuclear transcription factor kappa B (NFκB) (Russo *et al.*, 2012) and downregulation of nitric oxide synthase (Stempelj *et al.*, 2007). Furthermore, butyrate has been demonstrated to induce apoptosis of T cells and prevent the induction of inflammation by Interferon-gamma, which is implicated in the development of inflammation-associated CRCs (Zimmerman *et al.*, 2012). Recently, Singh *et al.* have also reported an induction of anti-inflammatory signals via the activation of the GPR109A receptor by butyrate, promoting the differentiation of T cells and

consequently the production of the anti-inflammatory cytokine IL-10 (Singh *et al.*, 2014).

1.5.4.2 Anti-neoplastic effects of butyrate

Following the observation of a lower CRC risk in native Africans by Burkitt (Burkitt, 1971), where native Africans have a <1:100,000 CRC risk compared with 65:100,000 in African Americans (O'Keefe *et al.*, 2007), O'Keefe *et al.* have proposed a critical role of colonic microbiota in large bowel health (O'Keefe, 2008). In further investigations of the observed differences in CRC risk, they hypothesised that this was at least partially mediated by differences in colonic bacterial metabolism (O'Keefe *et al.*, 2009). Indeed, their results showed that colonic butyrate concentrations, as well as that of other major SCFAs, were significantly higher in native Africans, who have the lowest CRC risk, compared with African Americans. In addition, it was proposed that the increased levels of SCFAs observed in native Africans resulted from a higher intake of RS in their diet, such as from their maize meals. These findings suggested that the protective effects of dietary fibres such as RS resulted, at least in part, from the production of SCFAs and it was later revealed that butyrate in particular has anti-neoplastic properties, such as its role as a histone deacetylase inhibitor (HDACi) and through regulation of proliferation and apoptosis. The anti-neoplastic effects of butyrate in normal and cancer cells are summarised in Figure 1.12.

McIntyre *et al.* found that the protection against CRC observed in a rat model was associated with the dietary fibre type administered that resulted in the highest butyrate levels in the distal colon (McIntyre *et al.*, 1993). Additionally, Weaver *et al.* observed lower butyrate levels in enema samples from polyp and CRC subjects compared with normal controls, suggesting that these reduced levels, possibly associated with reduced colonic fermentation, may be linked to polyp and CRC development (Weaver *et al.*, 1988). A similar finding was discovered by Bradburn *et al.* who reported lower butyrate concentrations in faecal samples from patients at higher risk of developing CRC (FAP gene carriers) compared with the control group (Bradburn *et al.*, 1993). Altogether, these findings suggest that butyrate is the key fermentation product associated

with anti-tumourigenic properties and that, furthermore, a reduction in butyrate may be associated with an increased risk of CRC.

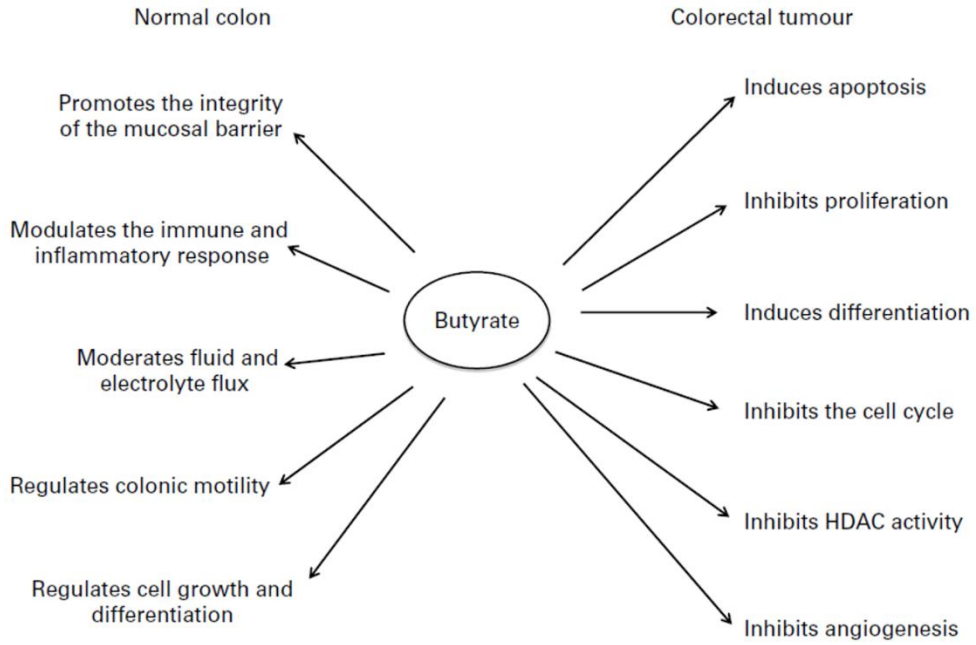
In vivo studies that have investigated the effects of butyrate on colorectal carcinogenesis have primarily been in rodents. Kameue *et al.* investigated the effects of dietary sodium gluconate, which increases the production of butyrate in the large bowel, on tumour development in rats (Kameue *et al.*, 2004). Rats were treated with AOM and deoxycholic acid to initiate and promote colorectal carcinogenesis. The rats which ingested the sodium gluconate had a significantly lower incidence of colorectal tumours and a lower average number of tumours compared with the controls. These results were attributed to the production of butyrate as dietary sodium gluconate does not undergo significant digestion or absorption before reaching the large bowel, where it is fermented by bacteria to produce butyrate. The authors investigated the levels of butyrate and reported a 60% increase in caecal butyrate concentrations in rats given the sodium gluconate. The effects of butyrate delivered directly to the caecum via intra-caecal administration in DMH-treated rats have also been explored and a reduction in colorectal malignancies and carcinomas was observed in rats which were administered caecal butyrate (Medina *et al.*, 1998).

The formation of ACF has also been utilised as a study endpoint for investigations of butyrate on colorectal carcinogenesis. A significant inverse correlation between caecal butyrate concentrations and colonic ACF formation has been reported (Coleman *et al.*, 2002). Rats fed a diet rich in α-cellulose, which resulted in higher butyrate levels, combined with fish oil conferred the greatest protective effect against ACF formation compared with RS or soy fibre diets, suggesting that the anti-neoplastic effects observed most likely resulted from butyrate production.

The anti-neoplastic effects of butyrate result primarily from the modulation of colonic crypt cell proliferation and apoptosis. In CRC cells or tissues, where a state of hyperproliferation is apparent, butyrate has been shown to reduce cell proliferation and induce apoptosis (Hodin *et al.*, 1996; Mortensen *et al.*, 1999; Comalada *et al.*, 2006; Clarke *et al.*, 2012). The anti-neoplastic effects of butyrate on cell proliferation are discussed in more detail in section 4.5.1.5. The

observation that butyrate's effects differ between healthy and cancerous cells suggest that it is highly selective (Fung *et al.*, 2012).

Figure 1.12 The effects of butyrate in the normal colon and in colorectal tumour cells.



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1.5.4.3 Butyrate as a histone deacetylase inhibitor

The effects of butyrate on histone acetylation have been studied extensively and its role as a HDACi is well established (Berni Canani *et al.*, 2012). By inhibiting histone deacetylation, which is associated with reduced gene expression, butyrate restores the expression of genes and consequently levels of processes including cell proliferation and differentiation. Furthermore, it has been suggested that the induction of apoptosis in cancerous cells by butyrate results primarily from the inhibition of HDACs (Fung *et al.*, 2012). In addition, butyrate has also been reported to modulate the acetylation of non-histone proteins, such as the transcription factors Sp1 and Sp3 (White *et al.*, 2006; Waby *et al.*, 2010).

For example, modulation of the acetylation state of *WNT5A,* a WNT ligand whose expression is reduced in CRC, by butyrate is associated with restored *WNT5A* expression in SW620 metastatic colon cancer cells (Li and Chen,

2012). The effects of butyrate as a HDACi were confirmed using the chromatin immunoprecipitation assay, whereby the acetylation of histone 3 at the promoter region was significantly greater following treatment with 5mmol/L butyrate for 48 hours. Furthermore, the investigators observed a significant increase in the expression of β-catenin at the protein level, suggesting that the restoration of *WNT5A* expression by butyrate through inhibition of histone deacetylation resulted in normal activation of the WNT signalling pathway.

Recently, Wang and colleagues have reported the restored expression of selenium-binding protein 1 (SELENBP1), whose expression was increased in CRC compared with normal tissue and was associated with the regulation of differentiation, with 2mM sodium butyrate treatment of colon cancer cell lines (Wang *et al.*, 2014). Similar findings were observed following treatment with another HDACi, trichostatin A, but not with an inhibitor of DNA methylation, suggesting that butyrate's effects on *SELENBP1* expression are mediated via modifications to histones.

1.5.4.4 Effects of butyrate on the WNT signalling pathway

More recently, the modulation of the WNT signalling pathway by butyrate has been investigated and has been the main focus of the group at The Commonwealth Medical College led by Dr. Michael Bordonaro, who primarily conduct *in vitro* studies to investigate the effects of butyrate on processes such as apoptosis through the regulation of WNT signalling. It has been proposed that the positive modulation of the WNT pathway, which is frequently aberrantly activated in CRC, is one of the mechanisms by which butyrate exerts its anti-neoplastic effects and regulates processes such as cell proliferation and apoptosis. The effects of butyrate on the expression and epigenetic regulation of WNT pathway components are described in more detail in section 4.2.1.2.

One of the Bordonaro group's earliest investigations explored the effects of butyrate on apoptosis and to what extent this was a consequence of its effects on WNT signalling. In SW620 colon carcinoma cells, apoptosis was induced following treatment with 5mM butyrate for 24 hours (Bordonaro *et al.*, 1999). Unexpectedly, this was associated with a stimulation of WNT pathway activity as indicated by enhanced activity of the TCF transcription factor and increased

formation of β-catenin-TCF complexes. Although WNT signalling regulated apoptosis, the observation that butyrate increased WNT pathway activity was surprising as this pathway is frequently hyperactive in CRC and previous studies had suggested that the induction of apoptosis was associated with reduced WNT signalling in CRC cells (Bordonaro *et al.*, 2008a). The findings from this study were later confirmed in ten CRC cell lines, where the greatest apoptotic response was associated with the highest levels of WNT activity (Lazarova *et al.*, 2004). Furthermore, an induction of WNT pathway activity by butyrate, demonstrated by an increase in active, dephosphorylated β-catenin, was reported in eight CRC cells (Bordonaro *et al.*, 2007).

Other studies have investigated the effects of butyrate on WNT pathway-mediated cell differentiation. In LIM2537 colon cancer cells, Vincan *et al.* observed an induction of cell differentiation with butyrate treatment (Vincan *et al.*, 2000). This was associated with an increase in WNT activity, particularly resulting from a reduction in the activity of GSK3β and, consequently, greater levels of stable β-catenin. Furthermore, an inverse correlation between the expression of GSK3β and differentiation was perceived.

Despite an indication of an increase in WNT pathway activity, Vincan *et al.* did not observe an increase in the expression of *CCND1* or *c-MYC,* two target genes of WNT signalling (Vincan *et al.*, 2000). Interestingly, butyrate has been reported to reduce expression of four WNT target genes which have been found to be upregulated in CRC (*CCND1, c-MYC, FOSL1* and *FST*) in CC531 rat colon carcinoma cells (Germann *et al.*, 2003). More recently, Bordonaro and colleagues identified a total of 1587 direct and indirect targets of the WNT pathway, following analyses by microarray, including those involved in the regulation of processes such as cell differentiation, migration and DNA replication, whose expression was modulated by 5mM butyrate in HCT-116 cells (Lazarova *et al.*, 2014b).

Bordonaro *et al.* have ultimately proposed the idea that a gradient of canonical WNT pathway activity occurs in the large bowel, whereby inactive WNT signalling is associated with cell differentiation and apoptosis, low levels of activity result in tissue self-renewal, moderate activity leads to uncontrolled proliferation and high levels result in apoptosis (Bordonaro *et al.*, 2008a;

Bordonaro *et al.*, 2008b). This proposition would explain previous findings where particular cells, which exhibited an induction of WNT signalling in response to butyrate treatment, consequently showed greater levels of apoptosis compared with those that did not show stimulation of the WNT pathway (Lazarova *et al.*, 2004). Furthermore, Lazarova *et al.* have suggested that early-stage neoplasms may be more responsive to butyrate following the observation that LT97 cells, a microadenoma cell line representative of an early stage of colorectal carcinogenesis, exhibited a greater induction of WNT activity and consequently stimulated apoptosis (Lazarova *et al.*, 2014a).

1.6 Hypotheses, Aims & Objectives of this Ph.D. project

1.6.1 Introduction to the DISC Study

This Ph.D. project was part of the DISC Study, a large, double-blind, placebo-controlled, randomised dietary intervention that supplemented 75 healthy participants with two NDCs, RS and PD, for seven weeks, and investigated the effects of the NDCs on biomarkers of colonic health. Furthermore, additional participants at differential risk of CRC, with quiescent UC and a history of adenomatous polyps, were recruited and did not take part in the dietary intervention.

The DISC Study was not subject to a formal power calculation as it aimed to test the impact of NDCs, known to be beneficial to GI health, on a panel of novel and well-established biomarkers. A target of 75 participants, allowing for a 10% dropout rate, was set. This number represented an achievable target, given the timeframe for recruitment and sample collection, predicted to identify effects of NDCs based on a previous study which detected significant effects of supplementing 65 CRC patients with RS for up to four weeks on colonic crypt cell kinetics and gene expression (Dronamraju *et al.*, 2009).

Samples, more specifically rectal mucosal biopsies, collected for the DISC Study were used for the investigations described in this Ph.D. thesis and to test the hypotheses defined below. The DISC Study is described in more detail in section 2.1.

1.6.2 Hypotheses

- Expression of WNT pathway-related genes is altered in the macroscopically-normal mucosa of participants at higher risk of CRC, with quiescent UC or prior history of polyps.
- The altered expression of WNT pathway-related genes in the macroscopically-normal mucosa of participants at higher risk results from epigenetic mechanisms such as DNA methylation and abnormal expression of miRNAs.

- Participants at higher risk of CRC have altered levels of colonic crypt cell proliferation and apoptosis.
- Dietary intervention in participants at normal risk of CRC with RS and/or PD will positively modulate the expression of WNT pathway-related genes.
- Dietary intervention in participants at normal risk of CRC with RS and/or PD will regulate the expression of WNT pathway-related genes via epigenetic mechanisms such as DNA methylation and the expression of miRNAs.
- Dietary intervention in participants at normal risk of CRC with RS and/or PD will positively modulate levels of colonic crypt cell proliferation and apoptosis.

1.6.3 Aims

This Ph.D. project aimed to test these hypotheses by investigating differences in the WNT signalling pathway and two of its functional outcomes, cell proliferation and apoptosis, in the macroscopically-normal mucosa of people at higher risk of CRC, with quiescent UC and a prior history of adenomatous polyps. Furthermore, it aimed to explore the effects of supplementing healthy human participants with two NDCs, RS and PD, on the WNT signalling pathway and its functional effects in the large bowel.

1.6.4 Objectives

- To identify a subset of WNT pathway-related genes which are differentially expressed in the macroscopically-normal colonic mucosa of participants with a prior history of polyps who are at higher risk of CRC;
- To compare the expression of the selected WNT pathway-related genes in the macroscopically-normal mucosa of participants at normal and higher risk of CRC i.e. those with quiescent UC or a prior history of adenomatous polyps;

- To investigate the roles of two epigenetic mechanisms (DNA methylation and miRNA expression) in the altered expression of the selected WNT pathway-related genes in the macroscopically-normal mucosa of participants at higher risk of CRC;
- To examine differences in two functional outcomes of WNT signalling that are dysregulated in CRC i.e. colonic crypt cell proliferation and apoptosis in the macroscopically-normal mucosa of participants at higher risk of CRC;
- To quantify the expression of the selected WNT pathway-related genes prior to, and following, a dietary intervention supplementing healthy participants with NDCs (the DISC Study);
- To investigate the effects of the dietary intervention on two epigenetic mechanisms (DNA methylation and miRNA expression) which may regulate the expression of the selected WNT pathway-related genes;
- To investigate the effects of the dietary intervention on two functional outcomes of WNT signalling that are dysregulated in CRC i.e. colonic crypt cell proliferation and apoptosis.

Chapter 2 Methods

2.1 Participant Recruitment and Sample Collection

This project is part of the DISC Study - a double-blind, randomised, placebo-controlled dietary intervention study using healthy human participants. The study is a randomised controlled trial (RCT) in which participants underwent a dietary intervention for 7 weeks during which they were asked to consume supplements of RS (Hi-maize® 260) and/or PD (Litesse®*Ultra*™) or placebo (a carbohydrate that is digested completely in the small bowel) in a 2*2 factorial design. Both RS and PD NDCs were selected because of their positive effects within the large bowel (and rest of the gut) including their ability to reduce/prevent inflammation. This was a significant factor in the choice of these intervention agents because of the evidence that chronic inflammation can predispose to CRC.

2.1.1 Study Approval

Ethical approval for this project was provided as part of a larger application for the DISC Study by the Newcastle and North Tyneside Research Ethics Committee from which a favourable ethical opinion was received on 10th December 2009 (REC No. 09/H0907/77) (Appendix A). Caldicott approval for the storage of data was given by the Northumbria NHS Foundation Trust (C1792). The clinical trial is registered with ClinicalTrials.gov (ClinicalTrials.gov Identifier NCT01075893).

2.1.2 Participant Recruitment

The participants contributing to this project were recruited by the DISC Study Team (Dr. Naomi Willis, Mr. Iain McCallum and Dr. Long Xie) prior to the commencement of this Ph.D. as part of the DISC Study. Endoscopy patient lists were used to identify potentially suitable participants from the gastroenterology out-patients departments at North Tyneside General Hospital, North Shields, UK and Wansbeck General Hospital, Ashington, UK. After excluding a large number of patients who did not meet the inclusion criteria

(Section 2.1.3) by revising hospital databases and previous endoscopy reports, potential participants were sent a letter with detailed information about the study prior to their endoscopy appointment at the hospital. The letter sent to potential intervention participants describing the DISC Study can be found in Appendix B. Similar letters were sent to potential 'UC' and 'Polyp' group participants but did not describe the dietary intervention arm of the study. At endoscopy, potential study participants were screened for exclusion criteria (Appendix C).

2.1.3 Exclusion Criteria

2.1.3.1 Intervention study exclusion criteria

- Aged <16 or >85 years
- Being a prisoner at the time of endoscopy
- Pregnancy or planning to become pregnant
- Diabetes mellitus
- Familial adenomatous polyposis syndrome
- Lynch Syndrome (HNPCC)
- Known colorectal tumour or prior CRC
- Prior colorectal resection
- Active colonic inflammation at endoscopy
- Iatrogenic perforation at endoscopy
- Incomplete left-sided colonic examination
- Colorectal carcinoma discovered at endoscopy
- CRC on histology
- Chemotherapy in the last 6 months
- Administering NSAIDs e.g. aspirin

- Administering other immunosuppressive medication e.g. methotrexate, steroids
- Administering warfarin or other anticoagulants

2.1.3.2 'Polyp' group exclusion criteria

- As described in Section 2.1.3

2.1.3.3 'UC' group exclusion criteria

- As described in Section 2.1.3
- Active colitis defined as a score >5 for Short Clinical Colitis Activity Index (SCCAI)

Eligible patients willing to participate were given a leaflet with information about the DISC Study and contact details of the DISC Team and were offered the opportunity to ask further questions about the study before providing informed written consent (Appendix D).

Four groups of participants were recruited as part of the DISC Study:

- Intervention group ('Normal' participants): patients with macroscopically normal colons willing to participate in the dietary intervention arm of the study.
- UC group: patients with quiescent UC, determined using the SCCAI, were recruited to this group.
- Polyp group: patients with previous non-malignant colorectal neoplasia viz. tubular adenoma, tubulovillous adenoma or villous adenoma that had been removed were recruited to join the 'Polyp' arm of the study and are referred to as the 'Polyp' group.
- Stem cell study group: 11 "normal" patients who underwent a full colonoscopy were recruited to this group. This group was not used in the present Ph.D. project and will not be described further in this thesis.

Participants from the first three groups then formed two groups of participants at differential risk of CRC that were compared in the second section of this Ph.D. project:

- 'Normal' group: those at normal risk of CRC, comprising baseline (pre-intervention) samples from participants taking part in the dietary intervention as well as any dropout participants.
- 'Higher-risk' group: those at higher risk of CRC, comprising participants from the 'UC' and 'Polyp' groups.

2.1.4 Human sample collection

Pre-intervention and post-intervention samples were collected from the dietary intervention participants. Samples from 'UC' and 'Polyp' participants were collected at the initial endoscopy appointment. Rectal mucosal biopsies were taken 10 cm from the anal margin (mid-rectum) from apparently-normal, healthy tissue during colonoscopy or flexible sigmoidoscopy pre-intervention and during rigid sigmoidoscopy post-intervention. In addition, participants provided blood, urine, stool and buccal cell samples. Anthropometric measurements were taken pre- and post-intervention and participants were asked to complete a Food Frequency Questionnaire (FFQ) (Appendix E) and Lifestyle Questionnaire (Appendix F).

2.1.4.1 Rectal mucosal biopsy collection

Rectal mucosal biopsies were collected by the DISC Study Team using biopsy forceps (Medical Innovations Biobite Biopsy forceps, diameter 2.3mm, length 230cm with spike, coated, REF BF23230-S/C, Lot 20110218). Nine biopsies were taken from the mid-rectum of each participant and were processed and stored as described below:

- Five biopsies were embedded in Tissue-Tek® OCT™ Compound (Sakura Finitek Europe), a cryoprotectant, snap-frozen in liquid nitrogen-cooled isopentane and stored at -80°C.

- Two biopsies were formalin-fixed paraffin-embedded (FFPE). These were fixed in 10% formalin and paraffin-embedded and blocked in the pathology laboratories at the Northumbria Healthcare NHS Foundation Trust.
- One biopsy was placed in RNAlater® (Ambion, USA) and stored at -80°C.
- One biopsy was preserved in 1ml of Carnoy's solution (70% ethanol, 30% acetic acid) overnight at 4°C and then stored in 70% ethanol at 4°C.

2.1.4.1.1 Rectal mucosal biopsy samples from intervention participants ('Normal')

Nine rectal biopsies were taken from each healthy participant before ('baseline'/'pre-intervention' samples) and after ('post-intervention' samples) intervention. Pre-intervention samples were collected during sigmoidoscopy or colonoscopy as described in Section 2.1.4.1. Post-intervention biopsies were taken from the mid-rectum, 10cm from the ano-rectal verge, during a rigid sigmoidoscopy using biopsy forceps (4mm Sarratt, Stericom, UK).

2.1.4.1.2 Rectal mucosal biopsy samples from the 'higher-risk' participants

Nine biopsies were collected from each 'UC' and 'Polyp' participant as described in Section 2.1.4.1. All samples from both groups were taken during their endoscopy appointment. Mid-rectal biopsies were taken 10cm from the ano-rectal verge.

2.1.4.2 Blood samples

Blood samples were collected from the study participants in seven 4ml BD Vacutainer® K_3EDTA (K_3E) tubes (Becton Dickinson, Oxford, UK) and one 5ml BD Vacutainer® SST™ II Advance tube with gold hemogard closure (Becton Dickinson, Oxford, UK). At baseline, participants having a colonoscopy would have been fasting for 24 hours and consequently their blood samples, but not the samples of those having a flexible sigmoidoscopy, were fasted. Post-intervention blood samples, collected at the time of the rigid sigmoidoscopy procedure, were un-fasted.

High-sensitivity C-reactive protein (hsCRP) levels in serum and vitamin B_{12} and red cell folate concentrations in plasma were analysed at the Department of Blood Sciences laboratories at the Freeman Hospital, Newcastle upon Tyne. Plasma selenium levels were quantified at the University of Liverpool.

2.1.5 Dietary intervention

The primary aim of the DISC Study dietary intervention was to investigate the response of a panel of established and novel biomarkers of CRC risk to each of the intervention agents and to assess the effectiveness of these outcomes as biomarkers of gut health.

2.1.5.1 Participant randomisation

The healthy participants ('Normals') took part in the dietary intervention study. Following their colonoscopy or flexible sigmoidoscopy at the endoscopy unit, participants were given at least a week to recover from the procedure to minimise any possible effects of the bowel preparation on the gut microflora and the colorectal epithelium. At this first visit, participants were randomised by choosing one of four sealed opaque envelopes labelled A, B, C and D. Participant randomisation was stratified by pre-intervention endoscopy procedure to avoid a confounding effect of the different bowel preparation methods associated with each procedure. Un-blinding was not performed until completion of all data collection and statistical analyses.

2.1.5.2 Interventions

Two active intervention agents, RS and PD, and their respective placebos were administered in a 2x2 factorial design. The intervention agents were provided in foil sachets labelled with a code which contained the agents in the form of a white powder.

The participants were asked to consume 35g of intervention supplement per day, divided into four sachets (two sachets of each agent). These were given in boxes containing a week's worth of sachets. The supplements were added to cold foods or liquids, such as yoghurt and orange juice, or mixed with cold

water. Participants took the supplements for a total of 50 days. They were asked to keep all of their sachets, both those that were consumed and those that were not, for the assessment of compliance at the end of the intervention.

2.1.5.2.1 Resistant starch

The RS supplied was Hi-Maize® 260 (Ingredion™, formerly National Starch, Food Innovation, USA), a type 2 RS that is isolated from high-amylose corn hybrids and occurs in the natural granular form (Ingredion, 2014). The RS content of Hi-Maize® 260 is approximately 60%, with the remaining 40% comprising digestible starch. Hi-Maize® 260 has an energy content of 1.4 kilocalories (kcal) per gram.

2.1.5.2.2 Polydextrose

PD was given in the form of Litesse® *Ultra*™ (DuPont™ Danisco®, Finland). Litesse® *Ultra*™ is a low-calorie (1kcal per gram), sugar-free soluble fibre developed as a sweetener (Danisco, 2011). It is a glucose polymer produced from sorbitol, dextrose and citric acid, which are derived naturally from corn.

2.1.5.2.3 Placebos

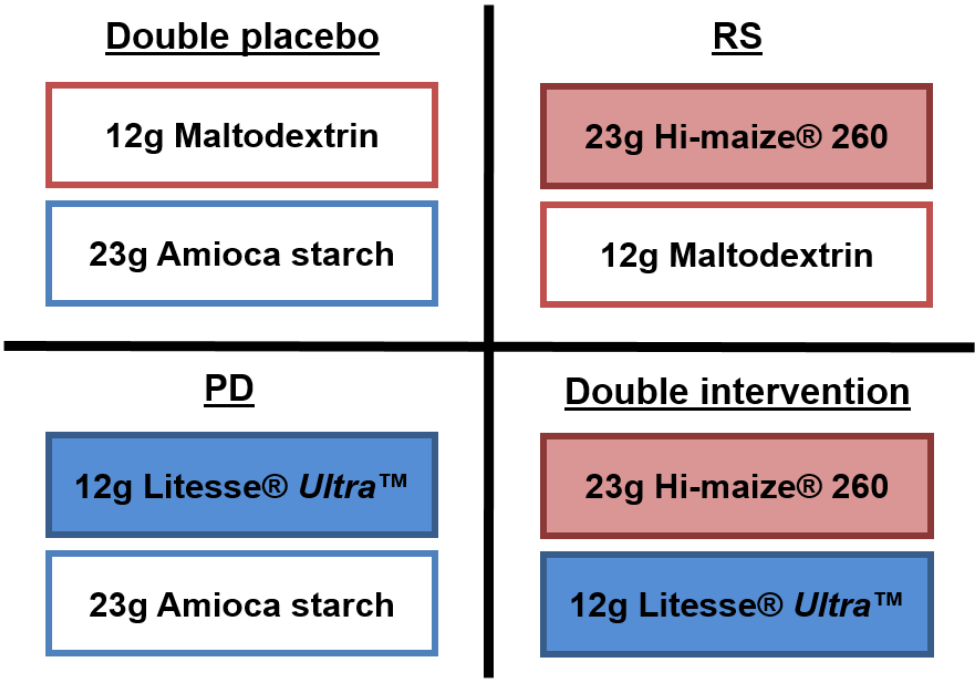
The placebos utilised for RS and PD were Maltodextrin and Amioca starch respectively. Maltodextrin is a polysaccharide produced from the partial hydrolysis of starches that can be derived from corn or wheat. Amioca starch is a low-amylose, waxy starch comprising amylopectin. Neither maltodextrin nor amioca starch contain RS and are easily digested and consequently absorbed in the small intestine (Kennedy *et al.*, 1995). Therefore, they do not have any effects in the large bowel.

2.1.5.3 Intervention groups

The four intervention groups were the double placebo group, the RS group, the PD group and the double intervention group (see Figure 2.1).

- Double placebo group: 12g of maltodextrin and 23g of amioca starch
- RS group: 23g of Hi-maize® 260 and 12g of maltodextrin
- Polydextrose group: 12g of Litesse® *Ultra*™ and 23g of amioca starch
- Double intervention group: 23g of Hi-maize® 260 and 12g of Litesse® *Ultra*™

Figure 2.1 Four intervention groups of human dietary intervention.



2.2 Laboratory Methods

2.2.1 Gene expression analyses

Quantitative PCR (qPCR) amplifies specific DNA sequences for the detection and quantification of a target in real time. Synthesis of complementary DNA (cDNA) followed by reverse-transcription qPCR (RT-qPCR) is now regarded as the optimal approach for quantifying messenger ribonucleic acid (mRNA) abundance (Bustin, 2000).

Primers are used to bind to a specific sequence of the template DNA to be amplified in the presence of DNA polymerase. Then a thermal cycler is used to produce cycles, customarily 40, of the following stages:

1. Denaturation: denaturation of the double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA) at a high temperature, typically 95°C.
2. Annealing: Hybridisation of primers (and probes) to complementary template sequences, at approximately 60°C.
3. Extension: Primer extension by DNA polymerase, typically 70-72°C where polymerase activity is optimal.

Quantification by qPCR is based on the fluorescence intensity detected at the end of each cycle and this increases as the PCR product is amplified. This reaches 100% efficiency during the exponential amplification phase where the quantity of PCR product is doubled in each cycle (Edwards *et al.*, 2004). A passive reference dye, such as carboxy-X-rhodamine (ROX), is also included in the reaction to normalise the fluorescent signals and to adjust for any differences between wells (Edwards *et al.*, 2004).

Two detection methodologies exist: primer-dye based assays and probe-based assays. The first uses a dye, commonly SYBR Green, that binds dsDNA, i.e. the PCR product, and fluoresces (Dorak, 2006). A downfall of the use of such dyes is that they are non-specific and are able to bind to any dsDNA, which can lead to inaccurate quantification of the target of interest. Melting curve, or dissociation, analyses are run directly after the PCR reaction to confirm that the PCR has generated a single PCR product and identify whether primer-dimers have been detected. This is particularly important when running primer-dye

based assays where the dye binds non-specifically to dsDNA. Each target PCR product has a unique melting temperature (Tm), where half of the template is ssDNA (Dorak, 2006), and a single fluorescence peak should be visualised at, or close to, the Tm of the PCR product on the dissociation curve (Dorak, 2006).

This issue of specificity associated with dye methods is overcome by using probe-based assays, labelled with a fluorescent reporter dye (5' end) and a quencher (3' end). When the probe is not bound and the reporter and quencher are in close proximity, the reporter signals are absorbed by the quencher by fluorescence resonance energy transfer (Dorak, 2006). During the extension stage of the reaction, the probe is degraded by exonuclease activity present in the polymerase. This releases and separates the reporter dye and quencher, allowing the reporter to emit fluorescent signals which are detected by the qPCR machine. Once the reaction has terminated, a threshold fluorescence value is set in the exponential phase for each gene and the threshold cycle (Ct value), which is the number of cycles taken for each sample to reach this fluorescence value, is determined (Edwards *et al.*, 2004). The Ct value is inversely proportional to the amount of the target gene in the template sample.

Two different methods may be used for gene expression quantification: quantification relative to the expression of reference genes or absolute quantification. In the present study, the relative quantification method was used where one or more reference, or housekeeping, genes that show stable expression in the tissue of interest and are not altered by treatment groups or condition are selected (Fink *et al.*, 1998). The expression of the reference genes is quantified alongside that of the target genes, allowing for normalisation of gene expression which overcomes issues such as differences in template cDNA volumes and in quality of the extracted RNA.

To investigate differences in gene expression, both between groups following the dietary intervention and between different CRC risk groups ('Normal', 'UC' and 'Polyp'), two-step RT-qPCR was used to quantify and compare the expression of the selected genes to be analysed. The dye detection method using SYBR Green was utilised, followed by relative quantification by normalisation with *18S* and *β2M* reference genes.

2.2.1.1 Extraction of RNA from rectal mucosal biopsies

Total RNA was extracted using Qiagen's RNeasy Mini Kit (Qiagen, UK) following the manufacturer's instructions. OCT-embedded samples were removed from storage at -80°C and the biopsy was removed from the excess OCT using sterile pipette tips. Whole biopsies were cut in half using a new, sterile scalpel blade for each sample and RNA was extracted from half a biopsy. The remaining half biopsy not used was immediately placed back into its tube and returned to storage at -80°C. The tissue was scraped into a RNase-free tube containing 500µl RNA*later*® and vortex mixed.

Tissue disruption and homogenisation were performed using 3mm glass beads (VWR, UK) followed by QiaShredders (Qiagen, UK). Five glass beads were dispensed into 2ml tubes and 150µl of Buffer RLT were added. The tissue was transferred to the 2ml tube containing the beads and shaken for 1 minute using an amalgamator. An additional 450µl of Buffer RLT were added to the lysate, yielding a total volume of 600µl of Buffer RLT. The lysate and beads were poured into the QiaShredder column and centrifuged for 2 minutes at maximum speed. The lysate was then centrifuged for 3 minutes at maximum speed and the supernatant was removed and transferred into a new microcentrifuge tube. One volume of 70% nuclease-free ethanol (≥99.8%, Fluka Analytical, Ukraine) was added to the lysate and mixed immediately by pipetting. The sample (700µl) was loaded onto an RNeasy spin column placed in a 2ml collection tube and centrifuged for 15 seconds at 10,000rpm.

The spin column membrane was washed with 700µl Buffer RW1 and centrifuged for 15 seconds at 10,000rpm. Two further wash steps were performed with Buffer RPE to prevent the carryover of ethanol during RNA elution. A final centrifugation step was performed in a new 2ml collection tube at maximum speed for 1 minute to eliminate any possible carryover of Buffer RPE.

The RNA was eluted in 30µl of RNase-free water and only one elution step was performed in an attempt to maximise the total RNA concentration. All centrifugation steps were performed at room temperature.

2.2.1.1.1 RNA quantification

The RNA concentration and purity, indicated by the A260/A280 ratio, were quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific) and the NanoDrop 1000 Software version 3.7.1. The mean RNA concentrations and A260/280 ratios for each participant group are described in Table 2.1. These were of sufficient concentration for subsequent analyses and of adequate purity as indicated by an A260/280 ratio between 2.0 and 2.1.

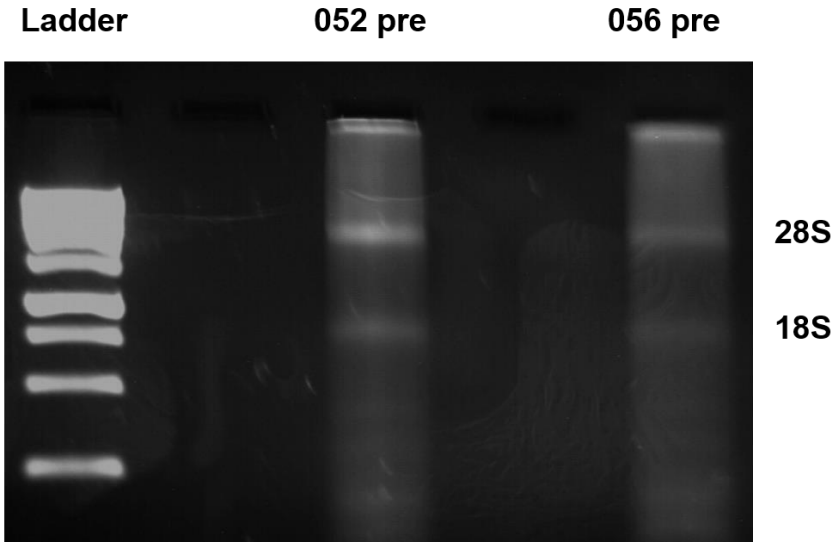
The quality and integrity of the RNA were assessed using agarose gel electrophoresis and the presence and intensity of 18S and 28S ribosomal RNA bands were analysed. A 1% agarose gel was prepared using agarose powder (Fermentas) and 1x TBE buffer (gibco®) and heated until the agarose had dissolved. The gel was stained with 3µl of GelRed™ dye (10,000x) (Biotium, UK) and the cooled mixture was poured into the electrophoresis tray and left to set for 30 minutes. A volume of 4µl of extracted RNA mixed with 3µl of 6x loading dye (Fermentas) was loaded into each well alongside a DNA molecular weight marker ladder (Fermentas). The gel was run at 120V for approximately 30 minutes using the Flowgen Bioscience CS-250V. UV light was used to visualise the RNA using the AlphaImage® (Alpha Innotech) gel documentation system and AlphaEase® FC Software Version 4.1.0. An example image is shown in Figure 2.2.

Table 2.1 RNA concentrations and A260/280 ratios for Intervention, UC and Polyp samples.

Measurement	Intervention	UC	Polyp
RNA concentration (ng/µl) mean (SEM)	121.2 (5.5)	153.6 (25.3)	148.9 (12.2)
A260/A280 ratio mean (SEM)	2.04 (0.01)	2.03 (0.02)	2.04 (0.01)

Data are presented as means and standard error of the mean (SEM).

Figure 2.2 RNA agarose gel electrophoresis image showing 28S and 18S rRNA bands.



A volume of 4µl of each RNA sample (equating to 0.665µg of 052 pre and 0.452µg of 056 pre) were loaded onto a 1% agarose gel and electrophoresed for 30 minutes at 120V.

2.2.1.2 cDNA synthesis

cDNA was synthesised by reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen, UK). A total amount of 1µg of RNA was reverse transcribed as described in the manufacturer's manual.

Firstly, a genomic DNA (gDNA) elimination step was performed by incubating the gDNA elimination reaction components (see Table 2.2) with the RNA for 2 minutes at 42°C and then placing immediately on ice.

Table 2.2 gDNA elimination reaction components.

Component	Volume per reaction (µl)
gDNA Wipeout Buffer (7x)	2
Template RNA	Variable (1µg)
RNase-free water	Variable (make up to 14µl)

The reverse transcription master mix was prepared as summarised in Table 2.3. This mix (6µl) was added to the template RNA (14µl from the gDNA elimination reaction) to produce a total volume of 20µl of cDNA.

Table 2.3 Reverse transcription reaction components.

Component	Volume per reaction (µl)
Quantiscript Reverse Transcriptase	1
Quantiscript RT Buffer (5x)	4
RT Primer Mix	1

Alongside the samples, two negative controls were included to determine the presence of any contaminants. These were:

- A no template control (NTC) containing all the reaction components except the template RNA, which was replaced with RNase-free water.
- A no reverse transcriptase control (RTC) containing all the reaction components except the reverse transcriptase enzyme, which was replaced with 1µl of water.

The tubes were mixed and incubated for 30 minutes at 42°C followed by 3 minutes at 95°C to inactivate the Quantiscript Reverse Transcriptase. All incubation steps were performed using the Sensoquest lab cycler (Göttingen, Germany). The cDNA was stored at -20°C. Prior to running the qPCR analyses, the cDNA was diluted 10x with RNase-free water to a total volume of 200µl.

2.2.1.3 Gene expression analysis

The expression of WNT pathway-related genes was quantified by qPCR using the Applied Biosystems® StepOnePlus™ System. All samples, including RTC and NTC controls were run in duplicate on a 96-well plate.

Assay validation was performed for the designed primers prior to analysing the samples by producing standard curves from serial dilution experiments and determining the amplification efficiency (Figure 2.3). For all qPCR reactions described in this thesis, data collection was performed during the extension stage of the 3-step cycling and melting curve analysis of the PCR products was performed following the cycling program to verify the specificity of the reactions (Figure 2.4).

A total of 12 WNT pathway-related genes were selected to be quantified by qPCR in the intervention study and in participants at differential risk of CRC. The process used for the selection of these genes is described in section 3.3.

Figure 2.3 qPCR standard curve analysis of *CTNNB1.*

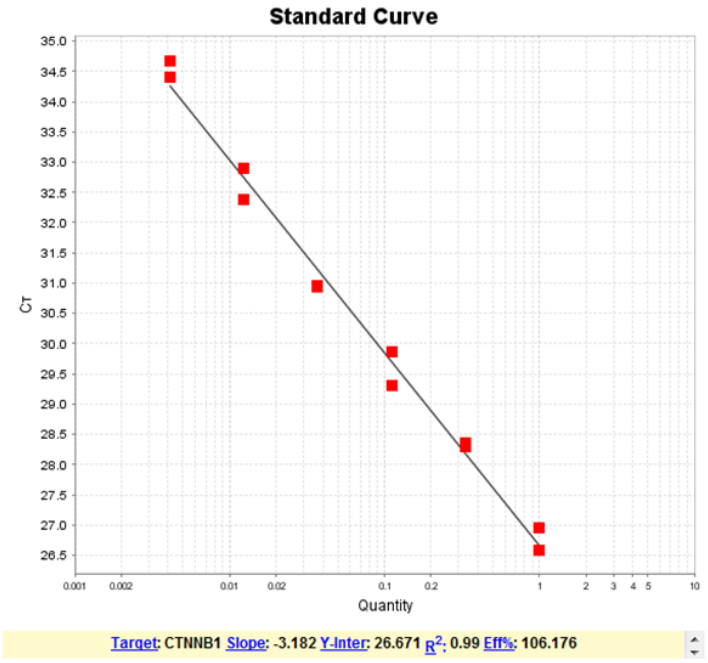
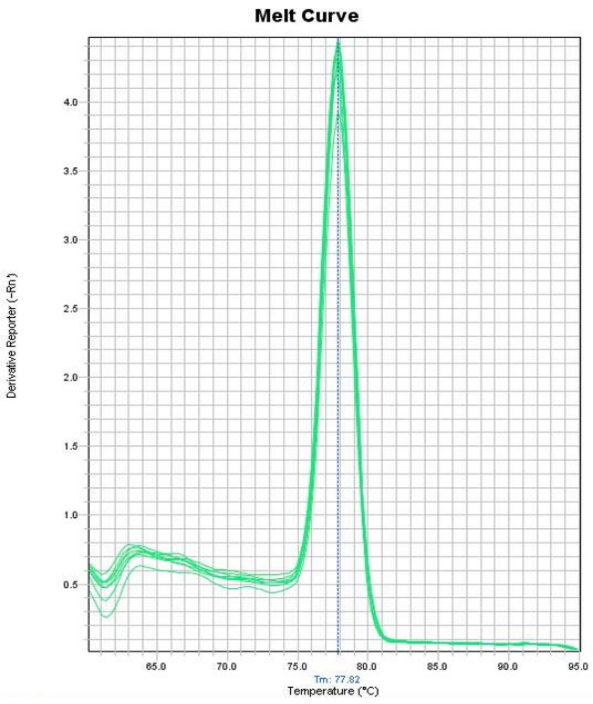


Figure 2.4 qPCR melt curve analysis of *CTNNB1.*



2.2.1.3.1 Quantification of *CCND1, c-MYC* and *SFRP1*

Quantification of *CCND1, c-MYC* and *SFRP1,* alongside the two reference genes *18S* and *B2M*, was performed using primers designed and optimised by Dr. Nigel Belshaw and Dr. Wing Leung (IFR, Norwich) (Table 2.4).

Table 2.4 Primer sequences for quantification of *CCND1, c-MYC* and *SFRP1* by qPCR.

Gene	Forward primer sequence	Reverse primer sequence
18S	GGCTCATTAAATCAGTTATGGTTCCT	GTATTAGCTCTAGAATTACCACAGTTATCC
B2M	AAAGATGAGTATGCCTGCCGT	ACTTAACTATCTTGGGCTGTGACAA
CCND1	TTGTACCTGTAGGACTCTCATTCG	ACAGCACTGTGAGCTGGCT
c-MYC	AGATCCGGAGCGAATAGGG	GTCCTTGCTCGGGTGTTGTA
SFRP1	TGGTGTGGATCTATTGGCTG	TCACTTTCTGGGCTTGACCT

A qPCR master mix was prepared as defined in Table 2.5. A 7µl volume of master mix was dispensed into each well of a 96-well plate followed by 3µl of the respective cDNA to produce a 10µl total reaction volume. The plates were sealed, centrifuged at 1,000rpm for 1 minute and then placed in the real-time cycler to commence the cycling program as described in Table 2.6.

Table 2.5 qPCR master mix components for quantification of *CCND1, c-MYC* and *SFRP1.*

Component	Volume per reaction (µl)
ImmoMix™ (2x) (Bioline, UK)	5.00
$MgCl_2$ (50mm) (Bioline, UK)	0.10
BSA (10mg/ml) (Ambion, UK)	1.00
ROX Reference Dye (50x) (Invitrogen, UK)	0.20
SYBR Green (100x) (Invitrogen, UK)	0.06
RNase-free water	0.60
Forward primer (100µM)	0.02
Reverse primer (100µM)	0.02

Table 2.6 Cycling programme used for quantification of *CCND1, c-MYC* and *SFRP1* using the Applied Biosystems® StepOnePlus™ System.

Step	Time	Temperature	Number of cycles
PCR initial activation step	10 minutes	95°C	1
3-step cycling: Denaturation Annealing Extension	 30 seconds 30 seconds 30 seconds	 95°C 60°C 72°C	40 Data collection

2.2.1.3.2 Quantification of *APC, AXIN2, CTNNB1, FOSL1, GSK3β, c-JUN, SFRP2, WNT5A* and *WNT11*

Quantification of *APC, AXIN2, CTNNB1, FOSL1, GSK3β, c-JUN, SFRP2, WNT5A* and *WNT11* alongside the two reference genes *18S* and *β2M* was performed using the QuantiTect® SYBR® Green PCR Kit (Qiagen, UK) and the QuantiTect® Primer Assays (please see Table 2.7). Lyophilised QuantiTect® Primer Assays were reconstituted in 1.1ml Tris EDTA (TE), pH 8.0 and kept at -20°C.

Table 2.7 QuantiTect® Primer Assays for quantification of WNT pathway-related gene expression by qPCR.

Gene	QuantiTect® Primer Assay name
18S	Hs_RRN18S_1_SG
β2M	Hs_B2M_1_SG
APC	Hs_APC_2_SG
AXIN2	Hs_AXIN2_1_SG
CTNNB1	Hs_CTNNB1_1_SG
FOSL1	Hs_FOSL1_1_SF
GSK3β	Hs_GSK3B_1_SG
c-JUN	Hs_JUN_1_SG
SFRP2	Hs_SFRP2_1_SG
WNT5A	Hs_WNT5A_1_SG
WNT11	Hs_WNT11_1_SG

A qPCR master mix was prepared as defined in Table 2.8 and 15µl of master mix were dispensed into each well of a 96-well plate followed by 5µl of the respective cDNA to produce a 20µl total reaction volume. The plates were sealed, centrifuged at 1,000rpm for 1 minute and then placed in the real-time cycler to commence the cycling program as described in Table 2.9.

Table 2.8 Reaction components for gene expression analyses using QuantiTect primer assays by qPCR.

Component	Volume per reaction (µl)
QuantiTect SYBR Green PCR Master Mix (2x)	10
QuantiTect Primer Assay (10x)	4
RNase-free water	1

Table 2.9 Cycling conditions for gene expression analyses using QuantiTect primer assays by qPCR.

Step	Time	Temperature	Number of cycles
PCR initial activation step	15 minutes	95°C	1
3-step cycling: Denaturation Annealing Extension	 15 seconds 30 seconds 30 seconds	 94°C 55°C 72°C	40 Data collection

2.2.1.4 Gene expression data processing

Prior to analysis of the qPCR data, the melt curves were checked for a single peak as multiple peaks can be indicators of lack of specificity or the formation of primer-dimers. A constant threshold value was set for each gene separately for all samples as described in Table 2.10. This was calculated by taking the average of the set thresholds for each gene for every plate.

Table 2.10 Set thresholds for gene expression analyses.

Gene	Set Threshold
18S	1.451
β2M	1.384
APC	1.336
AXIN2	1.804
CCND1	2.066
c-MYC	1.800
CTNNB1	1.706
FOSL1	0.960
GSK3β	1.820
c-JUN	1.748
SFRP1	2.060
SFRP2	1.481
WNT5A	1.805
WNT11	1.272

Two reference genes were quantified on each plate alongside the target genes for each sample. The geometric mean of *18S* and *β2M* housekeeping genes was calculated for each sample and used for the normalisation of the expression of each gene as described by Vandesompele *et al.* (Vandesompele *et al.*, 2002).

The delta Ct (ΔCt) value was calculated for each gene for each sample by subtracting the mean Ct value of the geometric mean of the reference genes from the mean Ct value of duplicates for the gene of interest:

ΔCt = Ct (target gene) – Ct (geometric mean of *18S* and *β2M*)

The relative copies for each gene were then calculated using the formula:

Relative copies = $2^{-\Delta Ct}$

The adjusted copies were calculated by multiplying the relative copies by a constant factor, in this case 10,000.

Adjusted copies = relative copies x 10,000

The standard deviation (SD) was calculated for each gene for each sample and any samples with SD >0.5 were repeated or excluded from statistical analysis.

2.2.2 MicroRNA expression analyses

2.2.2.1 Total RNA including miRNA extraction from rectal mucosal biopsies

Total RNA was extracted using Qiagen's miRNeasy Mini Kit (Qiagen, UK) following the manufacturer's instructions. OCT-embedded samples were removed from storage at -80°C. Whole biopsies were cut in half using a new, sterile scalpel blade for each sample and RNA was extracted from half a biopsy. The remaining half not used was immediately placed back into its tube and returned to storage at -80°C. Tissue disruption and homogenisation were performed using glass beads (VWR, UK) followed by QiaShredders (Qiagen, UK). Tissue was disrupted in 2ml tubes containing five 3mm glass beads and 700µl QIAzol Lysis Reagent and shaken for 1 minute using an amalgamator.

The lysate and beads were poured into the QiaShredder column and centrifuged for 2 minutes at maximum speed. The homogenate was left at room temperature for 5 minutes to promote dissociation of nucleoprotein complexes. A 140µl volume of chloroform (Sigma-Aldrich) was added to the homogenate and shaken vigorously for 15 seconds using the amalgamator. After incubation at room temperature for 2 minutes, the sample was centrifuged for 15 minutes at 12,000*g* at 4°C. The upper aqueous phase, approximately 330µl in volume, was transferred to a new 2ml collection tube and 495µl of 100% RNase-free ethanol were added and mixed thoroughly. Up to 700µl of the sample were pipetted into a RNeasy Mini spin column in a 2ml collection tube and centrifuged at 10,000rpm for 15 seconds at room temperature. The flow-through was discarded and the step was repeated with the remainder of the sample.

The spin column membrane was washed with 700µl of Buffer RWT and centrifuged at 10,000rpm for 15 seconds. The flow-through was discarded and 500µl Buffer RPE were added to the column. The tube was centrifuged at 10,000rpm for 15 seconds at room temperature to wash the column and the flow-through was discarded. Another 500µl of Buffer RPE was added and centrifuged at 10,000 rpm for 2 minutes at room temperature to dry the column membrane and ensure that no ethanol was carried over during the elution step. A final centrifugation step was performed in a new 2ml collection tube at maximum speed for 1 minute to eliminate any possible carryover of Buffer RPE.

The RNeasy Mini spin column was then transferred to a new 1.5ml tube and 30µl of RNase-free water were pipetted directly onto the column membrane. The tube was centrifuged at 10,000rpm for 1 minute at room temperature to elute the RNA.

2.2.2.2 cDNA synthesis

cDNA was synthesised from 0.8µg of RNA using the miScript II RT Kit (Qiagen) as described in the manufacturer's manual. The miScript HiSpec Buffer (5x) was used during reverse transcription to enable subsequent quantification of mature miRNAs.

The reverse transcription master mix was prepared as described in Table 2.11 and template RNA (variable volumes, equal to 0.8µg) was added to the respective tubes.

Table 2.11 miScript II RT Kit reverse transcription master mix.

Component	Volume per reaction (µl)
miScript HiSpec Buffer (5x)	4
miScript Nucleics Mix (10x)	2
miScript Reverse Transcriptase Mix	2
RNase-free water	Variable

Alongside the samples, two negative controls were included to determine the presence of any contaminants. These were:

1. A NTC containing all the reaction components except the template RNA, which was replaced with RNase-free water (12µl).
2. A RTC containing all the reaction components except the reverse transcriptase enzyme, which was replaced with 2µl of RNase-free water.

The tubes were mixed, centrifuged briefly and incubated for 60 minutes at 37°C followed by 5 minutes at 95°C to inactivate the miScript Reverse Transcriptase Mix. All incubation steps were performed using the Sensoquest lab cycler (Göttingen, Germany). The cDNA was stored at -20°C. Prior to running the

qPCRs, the cDNA samples were diluted to a total volume of 200µl with RNase-free water.

2.2.2.3 cDNA quality control prior to profiling mature miRNAs

The miScript miRNA Quality Control (QC) PCR Array (Qiagen) was run to assess the quality of a random selection of eight cDNA samples obtained from all three participant groups prior to the analyses of the selected miRNAs by qPCR.

QuantiTect SYBR Green PCR Master Mix, miScript Universal Primer, template cDNA and RNase-free water were thawed at room temperature and mixed. A PCR master mix was prepared as described in Table 2.12 for each of the eight cDNA samples.

Table 2.12 qPCR master mix for miScript miRNA QC PCR Array.

Component	Volume (µl)
QuantiTect SYBR Green PCR Master Mix (2x)	175
RNase-free water	135
miScript Universal Primer (10x)	35
Template cDNA	5

The miScript miRNA QC PCR Array was carefully removed from storage at -80°C and 25µl of master mix were dispensed into the respective wells. The plate was sealed and centrifuged for 1 minute at 1,000rpm at room temperature. The real-time cycler was programmed and run as described in Table 2.13.

Table 2.13 Cycling programme used to quantify miRNAs using the Applied Biosystems® StepOnePlus™ System.

Step	Time	Temperature	Number of cycles
PCR initial activation step	15 minutes	95°C	1
3-step cycling:			40
Denaturation	15 seconds	94°C	
Annealing	30 seconds	55°C	
Extension	30 seconds	70°C	Data collection

2.2.2.4 miRNA quantification by qPCR

Quantification of the selected miRNAs and two reference controls, the small nucleolar RNA (snoRNA) *SNORD68* and the small nuclear RNA (snRNA) *RNU6*, were performed using the miScript SYBR® Green PCR Kit (Qiagen, UK) and the miScript Primer Assays (please see Table 2.14 and Table 2.15). Lyophilised miScript Primer Assays were reconstituted in 550µl TE Buffer, pH 8.0 and kept at -20°C.

Table 2.14 Primer assays used for quantification of selected miRNAs for Intervention participants.

miRNA	miScript Primer Assay	Mature miRNA sequence
SNORD68 control	Hs_SNORD68_11	
RNU6 control	Hs_RNU6-2_11	
miR-17	Hs_miR-17_2	5' CAAAGUGCUUACAGUGCAGGUAG
miR-19a	Hs_miR-19a_1	5' UGUGCAAAUCUAUGCAAAACUGA
miR-19b	Hs_miR-19b_2	5' UGUGCAAAUCCAUGCAAAACUGA
miR-20a	Hs_miR-20a_1	5' UAAAGUGCUUAUAGUGCAGGUAG
miR-25	Hs_miR-25_1	5' CAUUGCACUUGUCUCGGUCUGA
miR-93	Hs_miR-93_1	5' CAAAGUGCUGUUCGUGCAGGUAG
miR-106b	Hs_miR-106b_1	5' UAAAGUGCUGACAGUGCAGAU
miR-424	Hs_miR-424_1	5' CAGCAGCAAUUCAUGUUUUGAA

Table 2.15 Primer assays used for quantification of selected miRNAs in participants at differential risk of CRC.

miRNA	miScript Primer Assay	Mature miRNA sequence
miR-101	Hs_miR-101_3	5' UACAGUACUGUGAUAACUGAA
miR-122a	Hs_miR-122a_1	5' UGGAGUGUGACAAUGGUGUUUG
miR-135b	Hs_miR-135b_1	5' UAUGGCUUUUCAUUCCUAUGUGA
miR-145	Hs_miR-145_1	5' GUCCAGUUUUCCCAGGAAUCCCU
miR-335	Hs_miR-335_1	5' UCAAGAGCAAUAACGAAAAAUGU

A qPCR master mix was prepared as defined in Table 2.16 and 18µl of master mix were dispensed into each well of a 96-well plate followed by 2µl of the respective cDNA to produce a 20µl total reaction volume. The plates were sealed, centrifuged at 1,000 rpm for 1 minute at room temperature and then placed in the real-time cycler to commence the cycling program as described in Table 2.13.

Table 2.16 Reaction components for miRNA expression analyses by qPCR.

Component	Volume per reaction (µl)
QuantiTect SYBR Green PCR Master Mix (2x)	10
RNase-free water	4
miScript Universal Primer (10x)	2
miScript Primer Assay (10x)	2

2.2.2.5 miRNA expression data processing

Prior to analysis of the qPCR data, the melt curves were checked for a single peak as multiple peaks can be indicators of lack of specificity or the formation of primer dimers (Figure 2.5). A constant threshold value was set for each miRNA separately for all of the samples as described in Table 2.17 (for the miRNAs selected for intervention analyses) and in Table 2.18 (for the miRNAs selected for analysis in participants at differential risk of CRC). This was calculated by taking the average of the set thresholds for each miRNA for every plate.

Figure 2.5 qPCR melt curve analysis of *miR-25.*

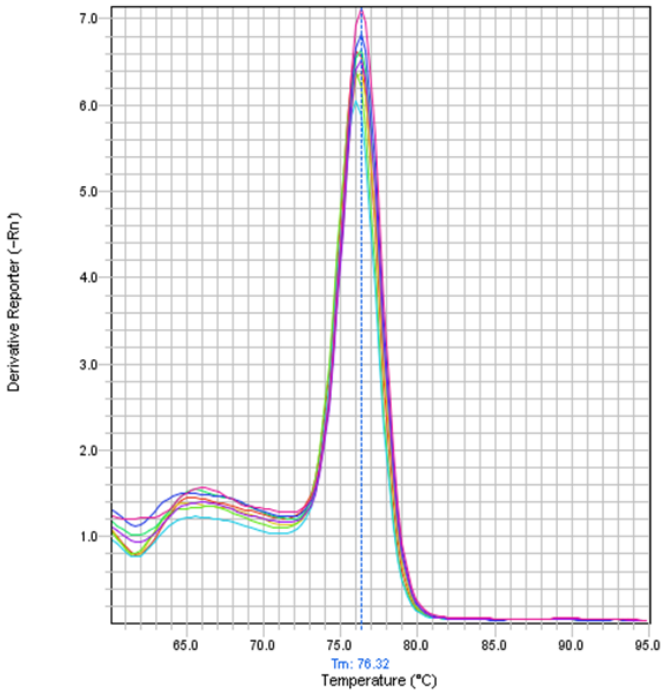


Table 2.17 Set thresholds for analyses of miRNAs selected for quantification in intervention participants.

miRNA	Set Threshold
RNU6	1.72
SNORD68	1.11
miR-17	1.74
miR-19a	1.23
miR-19b	1.31
miR-20a	1.50
miR-25	1.83
miR-93	1.81
miR-106b	1.64
miR-424	1.91

Table 2.18 Set thresholds for analyses of miRNAs selected for quantification in participants at differential risk of CRC.

miRNA	Set Threshold
RNU6	1.72
SNORD68	1.11
miR-101	2.11
miR-122a	1.40
miR-135	1.19
miR-145	2.05
miR-335	1.26

Two control RNAs, *RNU6* and *SNORD68*, were quantified for each sample alongside the target miRNAs. The geometric means of *RNU6* and *SNORD68* controls were calculated and used for the normalisation of the expression of each miRNA.

The delta Ct (ΔCt) value was calculated for each miRNA for each sample by subtracting the geometric mean of the control RNAs from the mean Ct value of duplicates for the target miRNA:

ΔCt = Ct (target miRNA) – Ct (geometric mean of *RNU6* and *SNORD68*)

The relative copies for each miRNA were then calculated using the formula:

Relative copies = $2^{-\Delta Ct}$

The adjusted copies were calculated by multiplying the relative copies by a factor, in this case 1,000.

Adjusted copies = relative copies x 1,000

The standard deviation for the duplicates was calculated and any samples with a standard deviation >0.5 were repeated or excluded from statistical analysis.

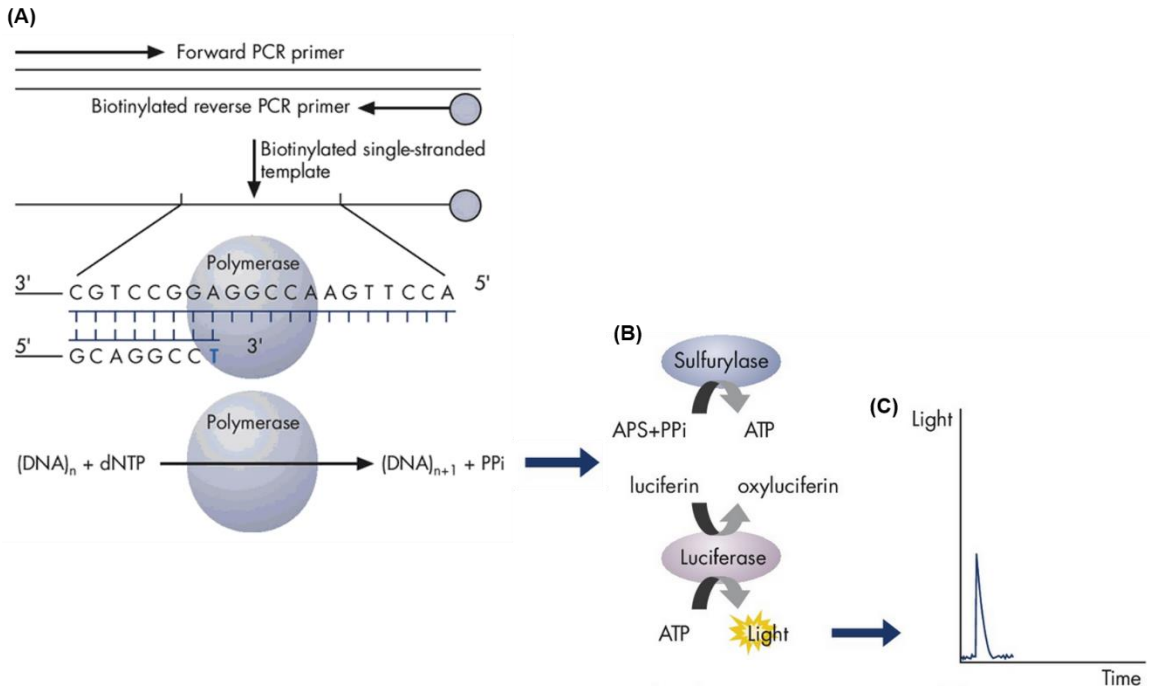
2.2.3 Methylation analyses

DNA methylation analyses were performed using the bisulphite modification protocol developed by Frommer and colleagues (Frommer *et al.*, 1992). This method involves bisulphite modification, which is the conversion of unmethylated, but not methylated (5-methylcytosine), cytosine residues to uracil by deamination. This provides a robust method which allows for the distinction between methylated and unmethylated DNA. Sequencing primers for a region of interest, commonly CpG islands known to contain several CpG sites, are designed and used to amplify the region of interest by PCR of the bisulphite-modified (BM) DNA.

Following amplification of the BM DNA, DNA methylation was quantified at specific CpG sites by pyrosequencing, a 'sequencing by synthesis' technique regarded as the gold standard (Migheli *et al.*, 2013). Pyrosequencing involves the quantification of methylation at individual cytosine residues within a specific region of interest, expressed as percentage methylation. Methylation analyses were performed using commercially-available, optimised Qiagen assays, which include both the PCR primer and sequencing primer. Details about the sequences analysed and primers can be found in sections 3.5 and 5.4.3).

Firstly, the denatured single-stranded PCR product sequence is hybridised by the sequencing primer and is elongated by DNA polymerase with the addition and incorporation of nucleotides. When a deoxynucleotide triphosphate (dNTP) is added, a molecule of pyrophosphate (PPi) is released (Figure 2.6 (A)). This pyrophosphate is then converted into adenosine triphosphate (ATP) by sulfurylase. Luciferin is oxidised by luciferase using the generated ATP (Figure 2.6 (B)), and this produces a light signal that is detected by the pyrosequencer and expressed as a peak for the incorporated nucleotide on the Pyrogram® (Figure 2.6 (C)). If the nucleotide was not incorporated, it is degraded by apyrase and there is no subsequent generation of ATP or resultant peak. The heights of the resultant peaks for each dNTP on the Pyrogram are proportional to the number of nucleotides added.

Figure 2.6 Steps involved in the quantification of DNA methylation by pyrosequencing.



Adapted with permission from Qiagen (Qiagen, 2014).

2.2.3.1 DNA extraction

DNA was extracted from half a rectal mucosal biopsy using a phenol-chloroform protocol. OCT-embedded samples were removed from storage at -80°C and excess OCT compound was removed. Whole biopsies were cut in half using a new, sterile scalpel blade for each sample and the remaining half not used was immediately placed back into its tube and returned to storage at -80°C.

Samples were homogenised on a shaker set to 900rpm for 8 hours at 55°C in 500µl of SET-sodium dodecyl sulphate solution (25ml Tris (50mM), 12.5Mm EDTA, 0.5% sodium dodecyl sulphate (SDS)) with the addition of 16µl of Proteinase K (Fermentas). The homogenised samples were centrifuged at 13,000rpm for 3 minutes. DNA was precipitated using 600µl of chloroform:isoamyl alcohol 24:1 (Fermentas). Phase lock gel (5 Prime, Hamburg, Germany) was utilised to separate the DNA by centrifuging at 10,000rpm for 5 minutes at room temperature. The upper phase was poured into a new tube and 16µl of RNase A/T1 (Fermentas) were added. After incubating at 37°C for 30 minutes, 42µl of sodium acetate solution (3M, pH 5.2) were added. The tubes were inverted several times and 400µl of isopropanol

and 2.5µl of glycogen (Fermentas) were added. The tubes were inverted several times and then centrifuged at 13,000rpm for 5 minutes at room temperature. The supernatant was discarded and the DNA pellet was washed twice by adding 500µl of 70% ethanol and centrifuging at 13,000rpm for 2 minutes at room temperature, discarding the ethanol in-between washes. The DNA pellet was left to air dry for an hour, re-suspended in 50µl of 2mM Tris and left to dissolve overnight at 4°C on the shaker.

The DNA purity and concentration were measured using the NanoDrop 1000 spectrophotometer, similarly to that described for RNA in Section 2.2.1.1.1.

DNA extraction from the Intervention, 'UC' and 'Polyp' biopsy samples was performed by Mr. Iain McCallum, Dr. Naomi Willis and Dr. Long Xie.

2.2.3.2 Bisulphite modification of DNA

Bisulphite modification of DNA was performed using the EZ DNA Methylation-Gold™ Kit (Zymo Research). A total of 250ng of DNA made up to a volume of 20µl with water was prepared and 130µl of CT Conversion Reagent (900µl water, 300µl M-Dilution Buffer and 50µl M-Dissolving Buffer) were added. The samples were mixed by pipetting up and down, centrifuged and placed in the Sensoquest lab cycler programmed to perform the following steps for DNA denaturation followed by CT-conversion reaction:

1. 98°C for 10 minutes
2. 64°C for 2.5 hours
3. Hold at 4°C for storage

DNA samples were loaded into Zymo-Spin™ IC Columns containing 600µl of M-Binding Buffer placed in a collection tube. These were centrifuged at maximum speed and the flow-through discarded. Samples were washed by adding 100µl of M-Wash Buffer and centrifuging at maximum speed for 30 seconds. The samples were then incubated at room temperature with 200µl of M-Desulphonation Buffer for 20 minutes. After the incubation, samples were centrifuged at maximum speed for 30 seconds. Two further wash steps were performed by adding 200µl of M-Wash Buffer, centrifuging at maximum speed for 30 seconds and repeating. The columns were placed into 1.5ml

microcentrifuge tubes, and to elute the BM DNA 10µl of M-Elution Buffer were added followed by a centrifugation step for 30 seconds at maximum speed.

DNA bisulphite modification reactions of the Intervention samples were performed Dr. Naomi Willis and Dr. Long Xie as part of the DISC Study.

2.2.3.3 Amplification of bisulphite-modified DNA

A PCR master mix was prepared as described in Table 2.19 and 24µl were dispensed into 0.5ml tubes. BM DNA (1µl) was added to each tube, with each sample performed in duplicate, with the exception of the negative controls which included 1µl of Epitect human control DNA (Qiagen) or 1µl of water. All PCR reactions were performed on the S1000™ (Bio-Rad) PCR machine under the conditions described in Table 2.20.

Table 2.19 Pyrosequencing PCR reaction components.

Component	Volume per reaction (µl)
HotStarTaq Master Mix (2x)	12.5
H_2O	9.5
PCR primer	2

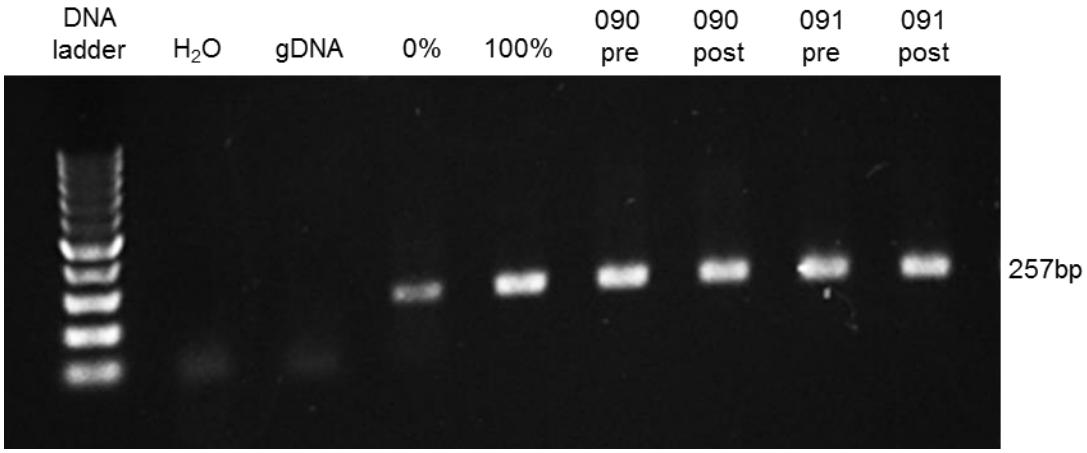
Table 2.20 Cycling conditions for pyrosequencing PCR.

Step	Time	Temperature	Number of cycles
Initialization	15 minutes	95°C	1
3-step cycling:			40
Denaturation	20 seconds	95°C	
Annealing	40 seconds	55°C	
Extension	20 seconds	72°C	
Final elongation	5 minutes	72°C	1

2.2.3.3.1 Agarose gel electrophoresis of PCR products for methylation analyses

PCR products and negative controls were run on a 1% agarose gel stained with SafeView (NBS Biologicals Ltd, Cambridgeshire, UK) by loading 4µl of PCR products with 1µl of loading dye alongside 3µl of 100bp DNA ladder. Samples were electrophoresed at 120mV, 300mA for 35 minutes to check that the correct sequence was amplified and that there was no contamination in the negative controls (H_2O and gDNA) (Figure 2.7).

Figure 2.7 Agarose gel electrophoresis image of *SFRP1* assay PCR product.



Agarose gel electrophoresis was used to check the PCR products prior to analysis by pyrosequencing. There were no bands present in the negative controls (H_2O and gDNA) indicating that there was no contamination. Bands of the correct product size (257bp) were observed for all sample and control PCR products.

2.2.3.4 Pyrosequencing

Pyrosequencing was performed using the Pyromark Q96 ID (Qiagen) Pyrosequencer and Pyromark Gold Q96 reagents (Qiagen). A master mix for the 96-well PCR plate was prepared as described in Table 2.21, and 70µl of master mix were added to each well followed by 5µl of PCR product. The PCR plate was sealed and shaken for 10 minutes at 15rpm to prevent sedimentation of the beads.

Table 2.21 Pyrosequencing master mix for PCR plate.

Component	Volume per reaction (µl)
Binding Buffer (Qiagen)	38
Nuclease-free water (Qiagen)	30
Streptavidin Sepharose High Performance Beads (GE Healthcare, Uppsala, Sweden)	2

In a pyrosequencing plate (Qiagen), 9µl of annealing buffer (Qiagen) and 3µl of sequencing primer (Qiagen) were added to each well. The PyroMark Q96 Vacuum Workstation (Qiagen) was used to aspirate the samples from the PCR plate using the vacuum. The vacuum probes were then washed in 70% ethanol for 5 seconds, denatured in denaturing buffer (0.8% NaOH solution) for 5 seconds and washed in Pyromark wash buffer (Qiagen) for 5 seconds. The vacuum was switched off and the samples were transferred to the pyrosequencing plate and heated at 80°C for at least 2 minutes to bind the sequencing primer. The enzyme, substrate mixtures and nucleotides were dispensed into their respective reagent and nucleotide tips as determined by the PyroMark CpG Software 1.0.11. The cartridge and plate were inserted and, after successful tip dispensation testing, run on the pyrosequencer.

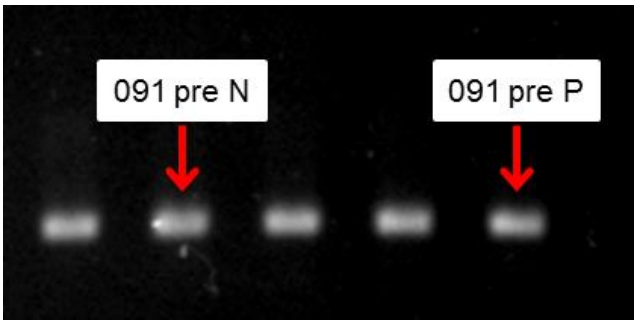
All samples were run in duplicate using duplicates from two individual PCR amplification reactions. Participant sample order was randomised so that comparable numbers of each gender and endoscopy procedure, and intervention group as appropriate, were present on each pyrosequencing run plate. Pre- and post-intervention samples were run on the same pyrosequencing plate for the intervention samples. On each pyrosequencing plate, two negative controls to check for any contamination and 0% and 100% methylation controls were run alongside the samples.

2.2.3.5 Bisulphite modification quality control

Quantification of methylation by pyrosequencing included an internal control that confirmed full bisulphite conversion i.e. that all templates show conversion to thymine where unmethylated cytosines are not followed by guanine. Any samples indicating an incomplete bisulphite conversion were flagged by the Pyromark software (highlighted in yellow) at the analysis stage and would be consequently excluded from subsequent analyses. I did not encounter this problem during my analyses.

As, in a minority of cases, previously bisulphite-modified and stored DNA samples were utilised for quantification of methylation by pyrosequencing, methylation was quantified and compared in newly bisulphite-modified and previously bisulphite-modified samples. The results from this analysis showed there were no differences in the amplification of BM DNA by PCR and that methylation levels of *SFRP1* at each CpG site were highly comparable between both newly- and previously- bisulphite-modified samples (see Table 2.22).

Figure 2.8 Agarose gel electrophoresis image for comparison of *SFRP1* assay PCR products amplified from 091 pre-intervention newly bisulphite-modified samples and previously bisulphite-modified samples.



Agarose gel electrophoresis was used to check the PCR products prior to analysis by pyrosequencing. In this image, PCR products from newly bisulphite-modified (N) and previously bisulphite-modified (P) DNA from sample 091 pre-intervention are compared. Bands of the correct product size (257bp) were observed for both samples and were of comparable intensity.

Table 2.22 Comparison of 091 pre-intervention *SFRP1* methylation levels using newly bisulphite-modified samples and previously bisulphite-modified samples.

	***SFRP1* methylation (%)** Mean (SD)	
CpG site	**091 pre N**	**091 pre P**
1	27.3 (3.2)	26.2 (0.6)
2	21.6 (1.8)	21.5 (2.3)
3	22.0 (2.9)	18.9 (0.6)
4	29.2 (0.2)	24.8 (4.3)
5	19.4 (1.3)	19.4 (1.4)
6	26.1 (3.2)	24.5 (2.4)
7	25.6 (2.4)	24.9 (1.7)
Mean	24.4 (3.5)	22.9 (2.9)

SFRP1 methylation levels (%) at each CpG site and the mean methylation across all seven CpG sites expressed as mean and standard deviation for newly bisulphite-modified (N) and previously bisulphite-modified (P) samples for 091 pre-intervention.

2.2.3.6 Methylation data processing

The means of the duplicates of the pyrosequencing data, expressed as percentage methylation (%), were taken and used for statistical analyses. Duplicate data were checked for a difference of no greater than 5%. Samples with a discordance between duplicates of >5% were repeated, and subsequently excluded from analyses if a difference of ≤5% was not achieved.

2.2.4 Assessment of colonic crypt cell kinetics

Colonic crypt cell kinetics, particularly the colonic crypt cell proliferative state (CCPS), were assessed by counting the number of mitotic figures following histochemical staining and whole crypt microdissection of rectal biopsies.

Schiff reagent was utilised for staining using the DNA-specific Feulgen technique, or nucleal reaction, which was first described in 1914 by Robert Feulgen (Feulgen, 1914). The DNA is first hydrolysed in warm, weak hydrochloric acid (HCl), resulting in removal of the purine bases and formation of aldehyde groups on deoxyribose sugars (Ross and Pawlina, 2011). Schiff reagent then reacts with the formed aldehyde residues via a condensation reaction to produce a magenta colour (Mescher, 2010), which is visible under a light microscope.

The whole crypt microdissection method was first validated in 1991 by Goodlad *et al.* who compared mitoses counts and crypt cell mass data derived from the microdissection technique with an autoradiography-based technique in a study in dogs (Goodlad *et al.*, 1991). The authors concluded that the microdissection technique had resulted in comparable data but was significantly less time-consuming.

Whole crypt microdissection is now the preferred method for the assessment of GI cell proliferation in human studies compared with *in vitro* methods, such as immunohistochemical staining, which may not yield as reliable data and are more time-consuming and labour intensive (Wong and Wright, 1999). Compared with autoradiography techniques that label DNA synthesising cells, an advantage of using microdissected crypts is that, although the numbers of mitotic figures counted are significantly lower, data are produced for entire crypts (Wong and Wright, 1999).

Mills *et al.* have established the reliability and reproducibility of mitosis counting using Schiff reagent-stained microdissected crypts taken from healthy participants and participants with adenoma, carcinoma or FAP for the assessment of human colonic crypt cell proliferation (Mills *et al.*, 2001). They found that, within an individual, mitotic counts were consistent in three biopsies

from the same site (the rectum), suggesting that data counted in a single biopsy may be used to represent mitosis at that site.

2.2.4.1 Tissue hydrolysis and staining

Carnoy's-fixed biopsies were removed from storage and halved using a sterile scalpel blade. Half a biopsy was transferred into new tubes containing 1ml of 50% ethanol, and the remaining half was returned to storage. Samples were left to hydrate for 10 minutes at room temperature followed by another 10 minute hydration step in 25% ethanol. The biopsies were transferred into new tubes containing 1ml of 1M HCl for 10 minutes at 60°C in a water bath to be hydrolysed. The samples were then stained in Schiff reagent (Surgipath™) for 1 hour at room temperature. The Schiff reagent was removed and replaced with 1ml of 45% acetic acid. Stained samples were microdissected immediately.

2.2.4.2 Whole crypt microdissection

The stained crypts were microdissected using the Olympus SZ40 dissecting microscope and Leica CLS 150X light source. A clean microscope slide (Surgipath™, Leica Microsystems, UK) was placed on the base of the dissecting microscope and a drop of 45% acetic acid was added to the centre of the slide. The stained biopsy was placed in the acetic acid and arranged so that the bases of the crypts were facing upwards. Using 25G x 5/8" fine gauge hypodermic needles (Terumo®, Belgium) attached to 2ml syringes (Terumo®, Belgium), rows of crypts were carefully teased apart by separating the serosa and muscularis mucosa and spreading over the acetic acid. Any connective tissue or Peyer's patches were removed. The crypts were covered with a cover slip (Surgipath® Premier Cover glass 22 x 22 #1.5 Leica, UK) and slight pressure was applied using the rubber-end of a pencil to spread out the crypts. Sufficient pressure was applied so that the crypts were in focus but not enough to burst the cells. Prepared samples were counted immediately to prevent the samples from drying out.

2.2.4.3 Crypt dimension measurement and mitosis counting

A light microscope was used to examine the microdissected crypts under 40x magnification to allow visualisation of individual cells in single crypts. Ten intact crypts to be analysed were selected randomly. The length of the crypt was measured by placing the graticule (reticle eyepiece) at the middle of the base of the crypt in the middle plane of focus. The width of the crypt was measured by placing the graticule at the widest part of the crypt in the middle plane of focus and measuring from one edge to the opposite edge. Using the length measurement, the crypt was divided into ten equal compartments i.e. if the crypt length is 140 graticule marks, the compartments were 1-14, 15-28, 29-42, etc. Graticule marks were converted into micrometres (µm) by multiplying the number of graticule marks by 2.47 as calculated following calibration of the graticule with a stage micrometre.

To analyse colonic crypt cell mitosis and apoptosis, the number of cells in mitosis or apoptosis in each compartment were counted by focusing up and down through the crypt, commencing with the first compartment at the base of the crypt. Cells in prophase, metaphase, anaphase or telophase were characterised as mitotic. The ten compartments were counted per crypt, and a total of ten crypts were analysed per tissue sample.

2.2.5 Quantification of BAX and BCL-2 expression as markers of apoptosis

Although the assessment of colonic crypt cell kinetics following whole crypt microdissection allowed for the successful quantification of proliferating cells, it was uncommon to count an apoptotic cell within the crypts. Because of this, the expression of the pro-apoptotic gene Bcl-2 associated X protein (*BAX*) and the anti-apoptotic gene B-cell lymphoma 2 (*BCL-2*) were quantified by qPCR as markers of apoptosis within the crypts.

The RNA utilised for this analysis was the total RNA extracted for the miRNA analyses as described in Section 2.2.2.1. cDNA was synthesised from 0.5µg of RNA by reverse transcription using the QuantiTect Reverse Transcription Kit as described in Section 2.2.1.2. cDNA was diluted 10x with RNase-free water to yield a total volume of 200µl.

Quantification of *BAX* and *BCL-2* alongside *18S* and *β2M* was carried out utilising the QuantiFast SYBR® Green PCR Kit (Qiagen, UK) and the QuantiTect® Primer Assays (please see Table 2.23).

Table 2.23 QuantiTect® primer assays for quantification *BAX* and *BCL-2.*

Gene	QuantiTect® Primer Assay
18S	Hs_RRN18S_1_SG
β2M	Hs_B2M_1_SG
BAX	Hs_BAX_1_SG
BCL-2	Hs_BCL2_1_SG

A qPCR master mix was prepared as described in Table 2.24 and 12µl were dispensed into the appropriate wells of a 96-well PCR plate followed by 8µl (20ng) of the respective cDNA samples. The plate was sealed and centrifuged at 1,000rpm for 1 minute.

Table 2.24 Reaction components for *BAX* and *BCL-2* gene expression analyses by qPCR.

Component	Volume per reaction (µl)
QuantiFast SYBR Green PCR Master Mix (2x)	10
QuantiTect Primer Assay (10x)	2

Samples, alongside negative controls, were run in duplicate using the fast protocol on the Applied Biosystems® StepOnePlus™ System as described in Table 2.25.

Table 2.25 Fast cycling programme used to quantify *BAX* and *BCL-2* mRNA using the Applied Biosystems® StepOnePlus™ System.

Step	Time	Temperature	Number of cycles
PCR initial activation step	5 minutes	95°C	1
2-step cycling: Denaturation Annealing	 10 seconds 30 seconds	 95°C 60°C	40 Data collection

qPCR data were processed as previously described in Section 2.2.1.3. Thresholds were calculated as described in Section 2.2.1.4 and are described in Table 2.26.

Table 2.26 Set thresholds for analyses of *BAX* and *BCL-2.*

Gene	Set Threshold
18S housekeeping gene	1.43
β2M housekeeping gene	1.54
BAX	1.35
BCL-2	1.42

2.2.6 Statistical analyses

Prior to statistical analyses, the data were checked for normal distribution using the Kolmogorov-Smirnov normality test and producing a normality plot. Data not normally distributed, where $p<0.05$, were transformed using an appropriate transformation (usually $\log_{10}$ or taking the square root). All statistical analyses were conducted on Minitab® 16.1.0 and graphs were produced using the SigmaPlot™ 12.5 software.

2.2.6.1 Statistical analyses of intervention participants

For normally distributed data, the analysis of variance (ANOVA) general linear model (GLM) was used to investigate post-intervention differences in the analysed outcomes (gene expression, miRNA expression, methylation and colonic crypt cell kinetics) between the intervention groups by using the pre-intervention measurement as a covariate. The ANOVA GLM uses a least squares regression approach and analyses the relationship between factors, as well as covariates, and the response outcome. Other covariates included in the ANOVA GLM were age, gender, endoscopy procedure, BMI and smoking status. Where data were not normally distributed despite transformation, the Kruskal-Wallis one-way analysis of variance test was used. Kruskal-Wallis is the non-parametric equivalent of the ANOVA GLM.

To investigate the effects of each dietary agent (RS or PD) individually and a potential interaction effect, the ANOVA GLM was used to examine the effects of “Treatment 1” and “Treatment 2” (referring to the RS and PS) on the analysed outcomes post-intervention, with the pre-intervention measurement, age, gender, endoscopy procedure, BMI and smoking status included as covariates. Where data were not normally distributed, the Kruskal-Wallis non-parametric test was used, inserting the analysed outcome as the response and “Treatment 1” and “Treatment 2” as the factors (individually). In this case, pre-intervention differences in the outcome measurement (gene expression, methylation or proliferation) between the intervention groups were investigated to check that there were no differences at baseline that could have effects on the post-intervention analysis outcomes. The Bonferroni method was applied to correct

for multiple comparisons. An example of the analyses outcomes using the ANOVA GLM can be found in Appendix G.

2.2.6.2 Statistical analyses of participants at differential risk of CRC

To compare differences in the analysed outcomes between the 'Normal' and higher-risk ('UC' and 'Polyp') groups, the ANOVA GLM was used with age, gender, endoscopy procedure, BMI and smoking status including as covariates. Dunnett's test was applied to correct for multiple comparisons and the Bonferroni method was used for pairwise comparisons. Where data were not normally distributed, the Kruskal-Wallis non-parametric test was used.

Chapter 3 Selection of WNT pathway-related genes and microRNAs for expression and methylation analyses

3.1 Introduction to Chapter 3

The aim of this chapter is to describe the processes behind the selection of the genes and miRNAs that were quantified and the methylation assays that were used for analyses in this Ph.D. The primary outcome measurement of this Ph.D. was the quantification of expression of WNT pathway-related genes as an indicator of WNT pathway activity. A total of 12 genes were selected by reviewing the literature to find genes that are altered in CRC and/or modified by butyrate and by conducting a pilot study to investigate differences in expression of WNT pathway-related genes in people at higher risk of CRC, with a prior history of adenomatous polyps. The selected 12 WNT pathway-related genes were quantified both in intervention participants (section 4.2) and in people at differential risk of CRC ('Normal', 'UC' and 'Polyp' participants) (section 5.2).

A similar selection process was applied for the selection of miRNAs to be quantified in the intervention participants (section 3.4.1). miRNAs reported to be aberrantly expressed in CRC and modulated by butyrate were identified from the literature. In addition, as effects of RS and PD were observed on expression of *SFRP1*, miRNAs predicted to target this gene were included. The eight selected miRNAs were also quantified in people at differential risk of CRC with the addition of five miRNAs reported to be abnormally expressed in CRC and involved in the regulation of the WNT signalling pathway (section 3.4.2).

Lastly, this chapter describes the selection of the assay used for the analysis of *SFRP1* methylation in both intervention participants (section 4.4) and in people at differential risk of CRC (section 5.5) by pyrosequencing.

3.2 Pilot Study: WNT pathway-related gene expression in the macroscopically-normal colorectal epithelium of people at higher risk of CRC

3.2.1 Introduction

A pilot study was performed to investigate differences in expression of a panel of WNT pathway-related genes in the macroscopically-normal mucosa of people at normal and higher risk of developing CRC. Analysis of the 'Normal' participants used pre-intervention samples from the DISC Study intervention arm and the higher-risk group comprised participants with a prior history of adenomatous polyps.

3.2.2 Hypotheses, Aims and Objectives

3.2.2.1 Hypotheses

The hypothesis for this pilot study was that participants at higher risk of CRC, i.e. those with a prior history of adenomatous polyps, would have differential expression of WNT pathway-related genes compared with 'Normal' participants. In particular, it was hypothesised that 'Polyp' participants would have increased expression of genes that indicate an increase in WNT activity, such as WNT ligands and target genes, and reduced expression of genes that would suggest a decrease in WNT activity, such as WNT inhibitors, compared with 'Normal' participants.

3.2.2.2 Aims

This pilot study aimed to test this hypothesis by quantifying expression of a panel of WNT-pathway related genes in the macroscopically-normal mucosa of people at differential CRC risk i.e. those at normal risk of developing CRC and those with a prior history of adenomatous polyps, at higher risk of developing CRC. The ultimate aim of this pilot study was to identify a subset of WNT genes which are differentially expressed in the macroscopically-normal mucosa of people at higher CRC risk that could be used as possible markers of increased CRC risk to test the effects of the dietary intervention supplementing healthy participants with RS and PD.

3.2.2.3 Objectives

- To obtain colorectal biopsies from the macroscopically-normal mucosa of two groups of DISC Study participants at differential risk of CRC i.e. 'Normal' participants and 'Polyp' participants;
- To identify matched pairs of 'Normal' and 'Polyp' participants in the DISC Study;
- To optimise isolation of RNA from OCT-embedded rectal mucosal biopsies;
- To extract an adequate amount of high-quality RNA from OCT-embedded rectal mucosal biopsies;
- To synthesise cDNA by reverse transcription;
- To quantify expression of a panel of 70 WNT pathway-related genes by qPCR;
- To compare gene expression in the 'Normal' and 'Polyp' participants.

3.2.3 Methods

3.2.3.1 Selected participants

Eleven 'Normal' and nine 'Polyp' participants were selected. 'Normal' participants were selected from the dietary intervention arm of the DISC Study and baseline (pre-intervention) samples were used for analyses. The 'Normal' and 'Polyp' participants were age and gender-matched where possible. There were similar numbers of male and female participants in each risk group, with the exception of the 'Polyp' group which consisted of five males and four females. Additionally, it was ensured that mean ages for the normal and higher-risk participants were comparable within a gender. The selected participants were all non-smokers to avoid smoking habit as a potential confounder. The BMI was also determined for each participant and the mean BMIs for each gender were compared between risk groups.

3.2.3.2 Total RNA extraction from OCT-embedded rectal mucosal biopsies

Total RNA was extracted using Qiagen's RNeasy Mini Kit (Qiagen, UK) following the manufacturer's instructions. This was performed as described in the methods section 2.2.1.1, with the exception of tissue disruption, which was achieved by grinding the tissue in lysis buffer (600µL Buffer RLT) using a sterile plastic pestle (Starlab). Tissue disruption was further optimised after completion of this pilot study to that described in the methods chapter.

3.2.3.2.1 RNA Quantification

The total RNA yield and purity (A260/280 ratio) were quantified using the NanoDrop 1000 spectrophotometer (Thermo Scientific) and the NanoDrop 1000 software version 3.7.1.

The quality and integrity of the RNA were assessed by agarose gel electrophoresis. A 1% agarose gel was prepared using agarose powder (Fermentas) and 1x Tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE) buffer and heated until the agarose had dissolved. The gel was stained with 2µL ethidium bromide (Invitrogen™) and the cooled mixture was poured into the electrophoresis tray and left to set for 30 minutes. A 3µl volume of 6x loading dye (Fermentas) was added to a 4µL aliquot of each RNA sample and made up to 12µl with nuclease-free water. The samples and a DNA molecular weight marker ladder (Fermentas) were loaded onto the gel and run at 120V for approximately 30 minutes. RNA was visualised under UV light using the Uvitec Gel Doc and the UVIPhotoMW Software version 11.01.

3.2.3.3 cDNA synthesis

cDNA was synthesised from 1µg of template RNA by reverse transcription using the QuantiTect Reverse Transcription Kit (Qiagen, UK) as described in the methods section 2.2.1.2.

3.2.3.4 Quantification of WNT pathway-related gene expression

The expression of 70 WNT pathway-related genes (see Table 3.1) were quantified by qPCR using primers developed by Dr. Nigel Belshaw and Dr.

Wing Leung at the Institute of Food research (IFR), Norwich. Expression levels (described as highly, moderately or weakly expressed in Table 3.1) had been previously determined at the IFR following quantification by qPCR. In addition, expression of two housekeeping genes, 18S rRNA (*18S*) and beta-2-microglobulin (*β2M*), was quantified. Samples were analysed on a gene by gene basis by running all of the samples in duplicate on a 96-well plate. In addition, each plate contained a no template control (NTC) and a reverse transcriptase negative control (RTC) to detect any possible contamination.

The 20µl of cDNA was first diluted to a total volume of 500µl using elution buffer. A PCR master mix was prepared in excess as described in the methods section 2.2.1.3.1, and kept on ice. A volume of 3µl of cDNA was added to 7µl of PCR master mix to yield a total reaction volume of 10µl. A 10 minute start step was performed at 95°C followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 60°C for 30 seconds and extending at 72°C for 30 seconds using the ABI 7300 real-time PCR machine (Applied Biosystems). For quantification of weakly expressed genes (see Table 3.1), the number of cycles was increased to 50 cycles. Data were expressed as deltaCT (ΔCt) relative to the geometric mean of *18S* and *β2M* and the adjusted copies were calculated using the formula adjusted copies = $2^{-\Delta Ct}$ x 1,000.

Table 3.1 Pilot study WNT pathway-related genes analysed by qPCR.

Highly expressed	Moderately expressed	Weakly expressed
AES	*AXIN1*	*APC*
CCND1	*BCL9*	*CSNK1G1*
CCND2	*CTNNB1P1*	*DKK1*
CCND3	*DIXDC1*	*FOXN1*
CSNK1A1	*FOSL1*	*FZD4*
CSNK1D	*FRAT1*	*LEF1*
CSNK2A1	*FZD1*	*LGR5*
CTBP1	*FZD2*	*NKD1*
CTBP2	*FZD6*	*PITX2*
CTNNB1	*FZD7*	*PYGO1*
DAAM1	*FZD8*	*SFRP4*
DVL1	*PORCN*	*SOX17*
EP300	*PPP2CA*	*TCF7L1*
FBXW2	*SFRP1*	*WIF1*
FBXW4	*TCF7*	*WISP1*
FBXW11	*WNT2B*	*WNT1*
FRZB	*WNT3*	*WNT2*
FZD5	*WNT5B*	*WNT5A*
GSK3A	*WNT6*	
GSK3B	*WNT11*	
c-JUN		
KREMEN		
LRP5		
LRP6		
MYC		
NLK		
PPP2R1A		
RHOU		
SENP2		
SLC9A3R1		
TLE1		
TLE2		

3.2.3.5 Statistical analyses

Statistical analyses were performed using the Minitab 16 Statistical Software. Prior to analyses, the distribution of each dataset was examined and, where necessary, data were transformed to achieve normally-distributed data. The ANOVA GLM was used to examine differences in gene expression between the two risk groups on a gene-by-gene basis. All analyses were adjusted for the effect of age and gender as covariates and the Bonferroni method was used to correct for multiple testing. A p value of <0.05 was considered statistically significant.

3.2.4 Results

3.2.4.1 Participant characteristics

A total of 20 volunteers (11 ‘Normal’ and 9 ‘Polyp’ participants) were used in this pilot study. Participants were age- and gender-matched, where possible, and mean ages for ‘Normal’ and ‘Polyp’ participants were comparable within a gender (Table 3.2). All participants were Caucasian and non-smokers to avoid these as possible confounders.

Table 3.2 Characteristics of selected 'Normal' and 'Polyp' participants for the pilot study.

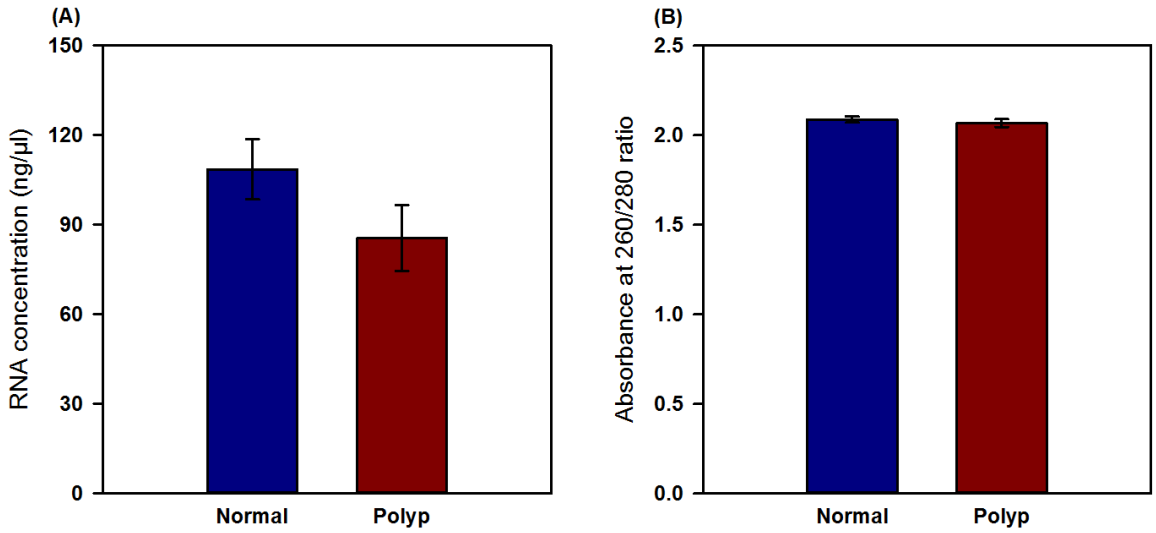
Characteristics	'Normal' group	'Polyp' group
Number of participants	11	9
Age (mean years) Range (years)	62.6 47 – 78	61.6 48 – 79
Gender Male (%) Female (%)	 5 (46%) 6 (54%)	 5 (56%) 4 (44%)
BMI (kg/m^2) (mean) Range (kg/m^2)	30.5 22.9 – 41.7	28.4 20.6 – 40.3

3.2.4.2 Total RNA quantity and quality

Total RNA was isolated from OCT-embedded rectal mucosal biopsies using the RNeasy Kit (Qiagen) as described in Section 3.2.3.2. The RNA quantity and quality were analysed to determine the RNA yield and purity (indicated by the absorbance at 260/280 ratio).

The average concentration of the extracted RNA was 98ng/µl (109ng/µl and 86ng/µl for the 'Normal' and 'Polyp' samples respectively) (Figure 3.1 (A)). However, this yield ranged from 55ng/µl to 182ng/µl due to the large inter-participant variability in biopsy size and quality. The average 260/280 ratio was 2.08 (2.09 and 2.07 for 'Normal' and 'Polyp' samples respectively) (Figure 3.1 (B)), indicating good purity of the RNA.

Figure 3.1 RNA concentrations and absorbance at 260/280 ratios for 'Normal' and 'Polyp' participants in the pilot study.



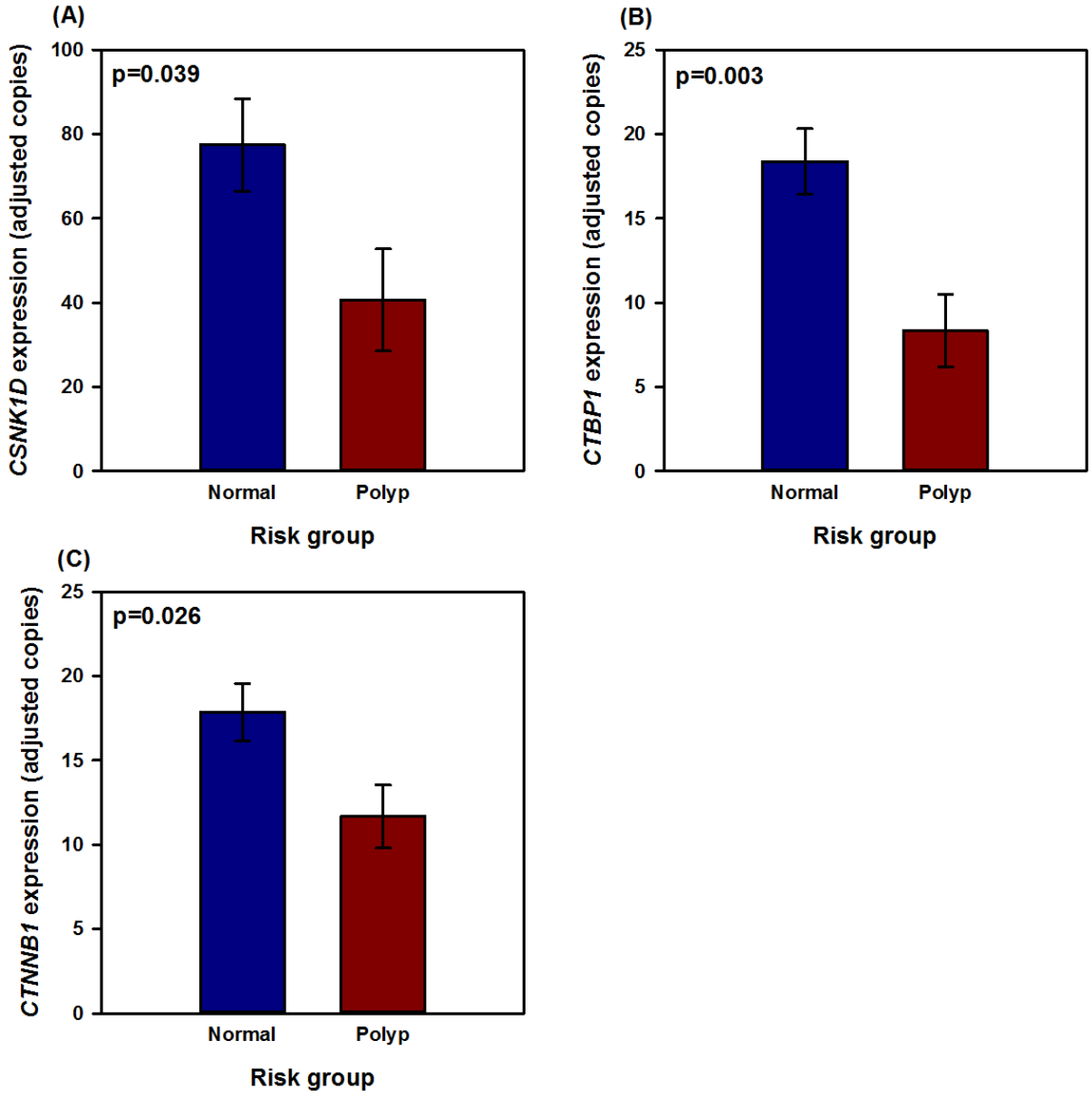
Data are presented as means and error bars represent SEM. N=20

3.2.4.3 WNT pathway-related gene expression in people at higher risk of CRC

Expression of a total of 70 WNT pathway-related genes was quantified in this pilot study. Significant differences in expression of three WNT pathway-related genes were observed, with lower expression of *CSNK1D, CTBP1* and *CTNNB1* in 'Polyp' participants compared with 'Normal' participants (Figure 3.2).

Expression of *CSNK1D* was almost two-fold lower in 'Polyp' compared with 'Normal' participants (Figure 3.2 (A)). Similarly, *CTPB1* expression was greater than two-fold lower in the 'Polyp' group (Figure 3.2 (B)). Expression of β-catenin, encoded by *CTNNB1,* was reduced by one-and-a-half times in participants with a prior history of polyps compared with 'Normal' participants (Figure 3.2 (C)).

Figure 3.2 Expression of (A) *CSNK1D* (B) *CTPB1* and (C) *CTNNB1* in the macroscopically-normal rectal mucosa of 'Normal' and 'Polyp' participants in the pilot study.



Data are expressed as adjusted copies ($2^{-\Delta Ct}$ x 1,000 relative to the geometric mean of *18S* and *β2M*) and presented as least squares means (LSMs). Error bars represent SEMs. N= 20.

The gene expression data were also expressed as fold difference ('Polyp' compared with 'Normal' participants) and the 15 genes showing the largest positive difference (Table 3.3) and 15 genes showing the largest negative difference (Table 3.4) were determined, however the majority of these differences were not statistically significant.

Table 3.3 WNT pathway-related genes showing the 15 largest positive fold differences when comparing 'Polyp' with 'Normal' participants in the pilot study.

Gene	Fold difference	P value
TCF7	1.68	0.254
SFRP4	1.54	0.408
NKD1	1.37	0.337
c-JUN	1.36	0.295
AES	1.21	0.444
WNT5A	1.20	0.530
WNT3	1.16	0.751
PORCN	1.15	0.507
SOX17	1.14	0.701
SLC9AR1	1.14	0.555
KREMEN	1.13	0.703
TCF7L1	1.11	0.630
WNT11	1.10	0.689
CCND2	1.08	0.803
FBXW4	1.07	0.769

Table 3.4 WNT pathway-related genes showing the 15 largest negative fold differences when comparing 'Polyp' with 'Normal' participants in the pilot study.

Gene	Fold change	P value
FOXN1	-18.03	0.056
WNT6	-3.18	0.625
WNT2	-2.86	0.166
TLE1	-2.30	0.057
CTBP1	-2.21	**0.003**
WISP1	-2.02	0.060
CSNK1D	-1.91	**0.039**
LGR5	-1.76	0.385
GSK3A	-1.61	0.095
RHOU	-1.56	0.258
CTNNB1	-1.53	**0.026**
CCND3	-1.51	0.166
AXIN1	-1.49	0.129
FOSL1	-1.47	0.549
CSNK1A1	-1.46	0.180

Statistically significant p-values are shown in bold.

3.2.5 Discussion

This pilot study aimed to quantify expression of a panel of 70 WNT pathway-related genes in the macroscopically-normal mucosa of people at normal and at higher risk (with a prior history of adenomatous polyps) of CRC. The results from this study demonstrated that expression of *CSNK1D, CTBP1* and *CTNNB1* was significantly reduced in 'Polyp' compared with 'Normal' participants.

CSNK1D encodes casein kinase 1 delta (CK1δ), a member of the casein kinase 1 family (McKay *et al.*, 2001). CK1δ is a positive regulator of the canonical WNT signalling pathway (Peters *et al.*, 1999), therefore a reduction in *CSNK1D* expression in 'Polyp' participants suggests a decrease in WNT activity. To my knowledge, altered *CSNK1D* expression has not been reported previously in

CRC. A germline missense mutation in *CSNK1D*, *CSNK1D*(R324H), was discovered in a patient with multiple adenomatous polyps and with a high familial incidence rate of CRC (Tsai *et al.*, 2007). This individual developed larger and a greater number of polyps at a much earlier age than both his affected siblings, suggesting that the mutation may affect polyp formation and therefore the risk of developing CRC. The mutant form was more active and promoted colony formation in a colon cancer cell line, however these effects were found to be independent of WNT signalling as WNT pathway activity did not increase when stimulated by the mutant compared with the wildtype *CSNK1D*.

CTBP1 encodes C-terminal binding protein 1, a nuclear protein that activates WNT signalling via a positive feedback loop following stimulation of the WNT pathway by WNT ligands. However, in the absence of WNT pathway activation by WNT ligands, CTBP1 acts as a repressor. Phelps *et al.* have suggested that CTBP1 plays a role in tumour initiation following the loss of *APC* and observed upregulation of CTBP1 at the protein level in both adenoma samples from FAP patients and adenocarcinoma samples (Phelps *et al.*, 2009). *CTBP1* has previously been found to be overexpressed in tissue samples taken from all four different stages of CRC compared with normal tissue (Gujral and MacBeath, 2010). In addition, greater expression of *CTBP1* was noted in colon cancer cells with activated WNT signalling compared with normal cells and that CTBP1 expression levels correlated positively with cell proliferation and migration. These findings were confirmed at the protein level, where increased CTBP1 expression was observed in tumour compared with normal tissue. Based on these findings, the authors concluded that *CTBP1* may be a potential marker of CRC progression with *CTBP1* upregulation following WNT pathway activation occurring at all four stages of CRC. The findings from both studies suggest a role for CTBP1 at the stage of adenoma formation, progression and early events in colorectal carcinogenesis.

A reduction in expression of *CTNNB1*, encoding β-catenin, a key component of canonical WNT signalling that induces the transcription of WNT target genes following activation of this pathway, in people with a prior history of adenomatous polyps, at higher risk of developing CRC, suggests that WNT signalling pathway activity is reduced in these participants. This observation is

contrary to previous studies that have reported increased β-catenin expression, predominantly at the protein level, in tissue from adenomatous polyps compared with normal tissue. Iwamoto *et al.* quantified β-catenin protein levels in the normal mucosa, hyperplastic polyps, adenomas and carcinomas and observed an increase in β-catenin, particularly within the nucleus, in adenomatous polyps and carcinomas compared with normal tissue and hyperplastic polyps (Iwamoto *et al.*, 2000). In a case control study, Ahearn *et al.* also observed 3% greater β-catenin protein levels in sporadic colorectal adenoma cases compared with controls, however this was not statistically significant. In a recent study, β-catenin expression, again at the protein level, was assessed specifically in hyperplastic polyps, sessile serrated adenomas, traditional serrated adenomas, CRCs and in the normal colon (Fang *et al.*, 2014). A correlation between the abnormal expression of β-catenin and increasing severity of the colorectal lesion was observed, with all 14 CRCs analysed showing abnormal β-catenin expression. As the present study used macroscopically-normal tissue from patients with a prior history of polyps, this could explain the differences in the findings from my study compared with the literature, which used samples from the polyp tissues themselves.

In conclusion, expression of three genes (*CSNK1D, CTBP1* and *CTNNB1*) differed significantly between normal and higher-risk participants. In all cases, participants with a prior history of polyps had reduced expression of these genes. A total of 30 WNT pathway-related genes (15 with the largest positive fold-difference and 15 with the largest negative fold-difference) were taken forward to be used for the selection of genes to be quantified in the intervention participants and in the larger study of participants at differential risk of CRC, as described in section 3.3. These included the three genes, *CSNK1D, CTBP1* and *CTNNB1* that differed significantly between the two participant groups.

3.3 Selection of WNT pathway-related genes for expression analyses in intervention and higher-risk participants

One of the aims of this Ph.D. study was to investigate changes in WNT pathway-related gene expression following supplementation with NDCs (RS and PD) and to compare expression in people at differential risk of CRC ('Normal', 'UC' and 'Polyp' participants). Therefore, the following criteria were applied in the process of WNT pathway-related gene selection:

a) Genes whose expression is altered in patients with CRC

b) Genes whose expression is modified by butyrate treatment (butyrate is an anti-neoplastic SCFA produced when NDCs are fermented in the large bowel)

c) Genes that showed a significant difference and/or the largest fold difference in expression when comparing normal-risk participants with those at higher risk of CRC ('Polyp' participants in the pilot study) (Section 3.2)

A total of 12 WNT pathway-related genes were selected for quantification by qPCR. The WNT gene selection process is illustrated in a Venn diagram in Figure 3.3. Firstly, the literature was reviewed to investigate WNT genes that were a) implicated in colorectal carcinogenesis (selection criterion 1) and b) whose expression is modified by butyrate (selection criterion 2). The literature search was conducted on PubMed and the date on which the last search was performed was March 2013. The keywords used in this literature search are detailed in Table 3.5.

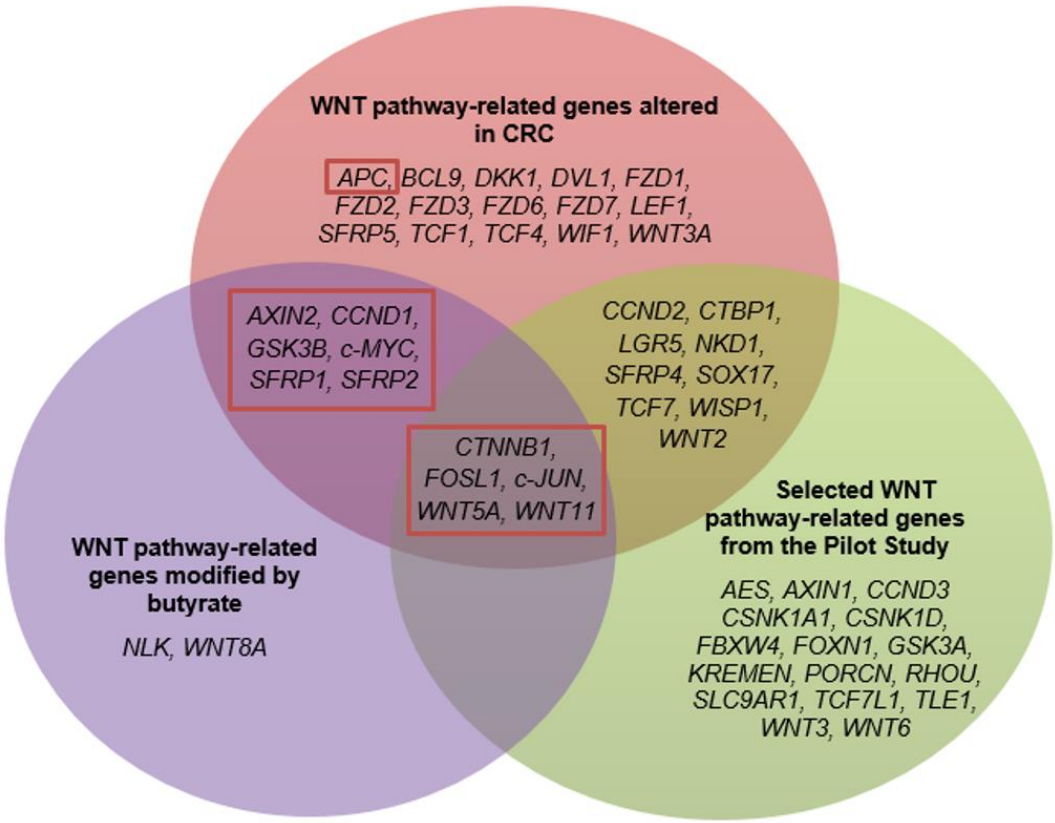
Table 3.5 Keywords used for the literature search on PubMed for WNT pathway-related gene selection.

Search	Keywords
Expression change in CRC	*'WNT','WNT signalling', 'WNT pathway', 'colon', 'large bowel', 'colon cancer', 'colorectal cancer', 'rectal cancer'*
Effect of butyrate	*'WNT','WNT signalling', 'WNT pathway', 'butyrate', 'short-chain fatty acids', 'SCFA',*

A pilot study was also conducted to investigate c) WNT genes with altered expression in participants with a prior history of adenomatous polyps and, therefore, at higher risk of CRC (section 3.2). The genes showing the largest differences in expression (15 with the largest positive fold-difference and 15 with the largest negative fold-difference) between normal and higher-risk participants were included in the selection criterion 3 set.

The WNT pathway-related genes that belonged in the intersection of all three selection criteria were prioritised for gene expression analyses, followed by those whose expression is altered in CRC and modified by butyrate (Table 3.6). This yielded a total of eleven genes that were (i) reported to be altered in CRC and in response to butyrate and showed the largest differences in expression between normal and higher-risk participants in the polyp study or that were (ii) altered in CRC and modulated by butyrate. In addition, *APC* (found in the 'WNT genes altered in CRC' set) was selected due to its key role in the WNT signalling pathway and in CRC.

Figure 3.3 Venn diagram illustrating the criteria for the selection of WNT pathway-related genes to be quantified in intervention participants and in participants at differential risk of CRC.



The three Venn diagram sets show the selection criteria used for the selection of WNT pathway-related genes to be quantified by qPCR: (i) altered expression in CRC (results from the literature search), (ii) modified by butyrate (results from the literature search) and (iii) differentially expressed in the macroscopically-normal mucosa of people at higher risk of CRC with a prior history of adenomatous polyps (results from the pilot study). The 12 selected genes are shown in red boxes and either belonged in all three sets or were reported to be altered in CRC and modified by butyrate. In addition, *APC* was also selected due to its role in both sporadic and inherited forms of CRC.

Table 3.6 Evidence from the literature on changes in expression of WNT pathway-related genes in CRC and in response to butyrate treatment.

Gene	Selection criterion 1: Expression change in CRC	References	Selection criterion 2: Effect of butyrate on expression	References
AXIN2	Increased	(Wu *et al.*, 2012b), (Metcalfe *et al.*, 2010), (Lustig *et al.*, 2002)	Increased	(Daly *et al.*, 2005)
CCND1	Increased	(Nakashima *et al.*, 2002)	Decreased	(Daroqui and Augenlicht, 2010), (Maier *et al.*, 2009), (Germann *et al.*, 2003), (Lallemand *et al.*, 1996)
CTNNB1	Increased	(Wang *et al.*, 2011a)	Decreased	(Kucerova *et al.*, 1999)
FOSL1	Increased	(Zhang *et al.*, 2005), (Tice *et al.*, 2002)	Decreased	(Germann *et al.*, 2003)
GSK3β	Increased	(Wang *et al.*, 2011a)	Decreased	(Vincan *et al.*, 2000)
c-JUN	Increased	(Wang *et al.*, 2000), (Zhang *et al.*, 2005)	Increased Increased	(Tsai *et al.*, 2000), (Velazquez *et al.*, 1996)

Gene	Selection criterion 1: Expression change in CRC	References	Selection criterion 2: Effect of butyrate on expression	References
c-MYC	Increased	(Sikora *et al.*, 1987), (Smith *et al.*, 1993)	Decreased	(Daroqui and Augenlicht, 2010), (Tsai *et al.*, 2000), (Souleimani and Asselin, 1993)
SFRP1	Decreased	(Caldwell *et al.*, 2006), (Qi *et al.*, 2006), (Caldwell *et al.*, 2004)	Increased	(Shin *et al.*, 2012)
SFRP2	Decreased	(Qi *et al.*, 2006)	Increased	(Shin *et al.*, 2012)
WNT5A	Decreased	(Cao *et al.*, 2012), (Li and Chen, 2012), (Ying *et al.*, 2008)	Increased	(Li and Chen, 2012), (Bordonaro *et al.*, 2011)
WNT11	Increased	(Nishioka *et al.*, 2013)	Increased	(Bordonaro *et al.*, 2011)

3.4 Selection of microRNAs for expression analyses in intervention and in higher-risk participants

Another aim of this Ph.D. study was to investigate changes in expression of miRNAs, to test the hypothesis that gene expression is regulated by epigenetic mechanisms, such as expression of miRNAs, following supplementation with NDCs (RS and PD) and to compare miRNA expression in people at differential risk of CRC ('Normal', 'UC' and 'Polyp' participants).

Therefore, eight miRNAs that fit the following selection criteria were selected for quantification in intervention participants:

- miRNAs whose expression is altered in patients with CRC
- miRNAs whose expression is modified by butyrate treatment
- miRNAs that are predicted to target *SFRP1*, that was differentially expressed following supplementation with RS and PD

In addition, five miRNAs whose expression is altered in patients with CRC and that are implicated in the regulation of the WNT signalling pathway were selected to be quantified in people at differential risk of CRC ('Normal', 'UC' and 'Polyp' participants). The eight miRNAs selected as part of the intervention study were also quantified in these participants.

3.4.1 Selection of microRNAs for quantification in intervention participants

In preparation for investigation of the effects of supplementation with NDCs (RS and PD) on miRNA expression in biopsies from the intervention study participants, a rigorous miRNA selection process was applied and is outlined in Figure 3.4. This involved a literature search conducted on PubMed. The last search was performed in December 2013.

The first step of the miRNA selection process was to review the literature for miRNAs whose expression was reported to be altered by butyrate treatment either in healthy or cancerous colorectal tissue or cells. The keywords used in this literature search were: *'microRNA', 'microRNAs', 'miRNA', 'miRNAs', 'butyrate', 'short-chain fatty acids' and 'SCFAs'*. This first investigation yielded a

total of 45 miRNAs previously reported to be altered by butyrate treatment (please refer to Appendix H).

The next step was to investigate whether members of this subset of miRNAs were reported to be abnormally expressed in colon, rectal or colorectal cancer. The keywords used in this literature search were: *'microRNA', 'microRNAs', 'miRNA', 'miRNAs', 'colon', 'large bowel', 'colorectal cancer', 'colon cancer'* and '*rectal cancer'*. In addition each of the 45 miRNAs were searched for individually with the addition of the keywords *'colon', 'large bowel', 'colorectal cancer', 'colon cancer'* and '*rectal cancer'*. Of the 45 miRNAs altered by butyrate treatment, 31 have been previously observed to be abnormally expressed in CRCs (please refer to Appendix H).

SFRP1 was selected as a potentially important target gene because it showed statistically significant differences in expression between intervention groups post-intervention (section 4.2.4.2). In the next stage of the miRNA selection process, I reviewed the literature to identify those miRNAs that may regulate *SFRP1* expression. The keywords used in this literature search were: *'microRNA', 'microRNAs', 'miRNA', 'miRNAs', 'secreted Frizzled-related protein'* and *'SFPR1'.*

Fu and colleagues observed an inverse correlation between 26 CRC-specific miRNAs and *SFRP1* following analysis by microarray (Fu *et al.*, 2012). Of these 26 miRNAs, the authors found that only one (*miR-27a*) was predicted to target *SFRP1* by two miRNA databases (TargetSCAN Human and miRanda). However, despite not being predicted to target *SFRP1* by both the TargetSCAN Human and miRanda miRNA databases, the inverse correlations between both *miR-21* and *miR-29a* and *SFRP1* observed during microarray analyses were confirmed by qPCR. Of the 31 miRNAs abnormally expressed in CRC and altered by butyrate treatment, 11 were reported to show a significant inverse correlation with *SFRP1* and therefore have been predicted to target *SFRP1* (Fu *et al.*, 2012).

The remaining 11 miRNAs were examined and those for which expression was reported to be affected by butyrate treatment on both papers by Hu *et al.* and Humphreys *et al.* were shortlisted (Hu *et al.*, 2011; Humphreys *et al.*, 2013). These were *miR-17, miR-19a, miR-19b* and *miR-20a.*

A further four miRNAs for which altered expression following butyrate treatment was confirmed by qPCR analysis *(miR-25, miR-93, miR-106b* and *miR-424)* in the study by Hu *et al.* (2011) were also selected, yielding a total of eight miRNAs for quantification by qPCR (Table 3.7).

Figure 3.4 Selection process used to identify eight miRNAs that are differentially expressed in CRC, responsive to butyrate treatment and predicted (or known) to target *SFRP1* for analysis in participants in the intervention study.

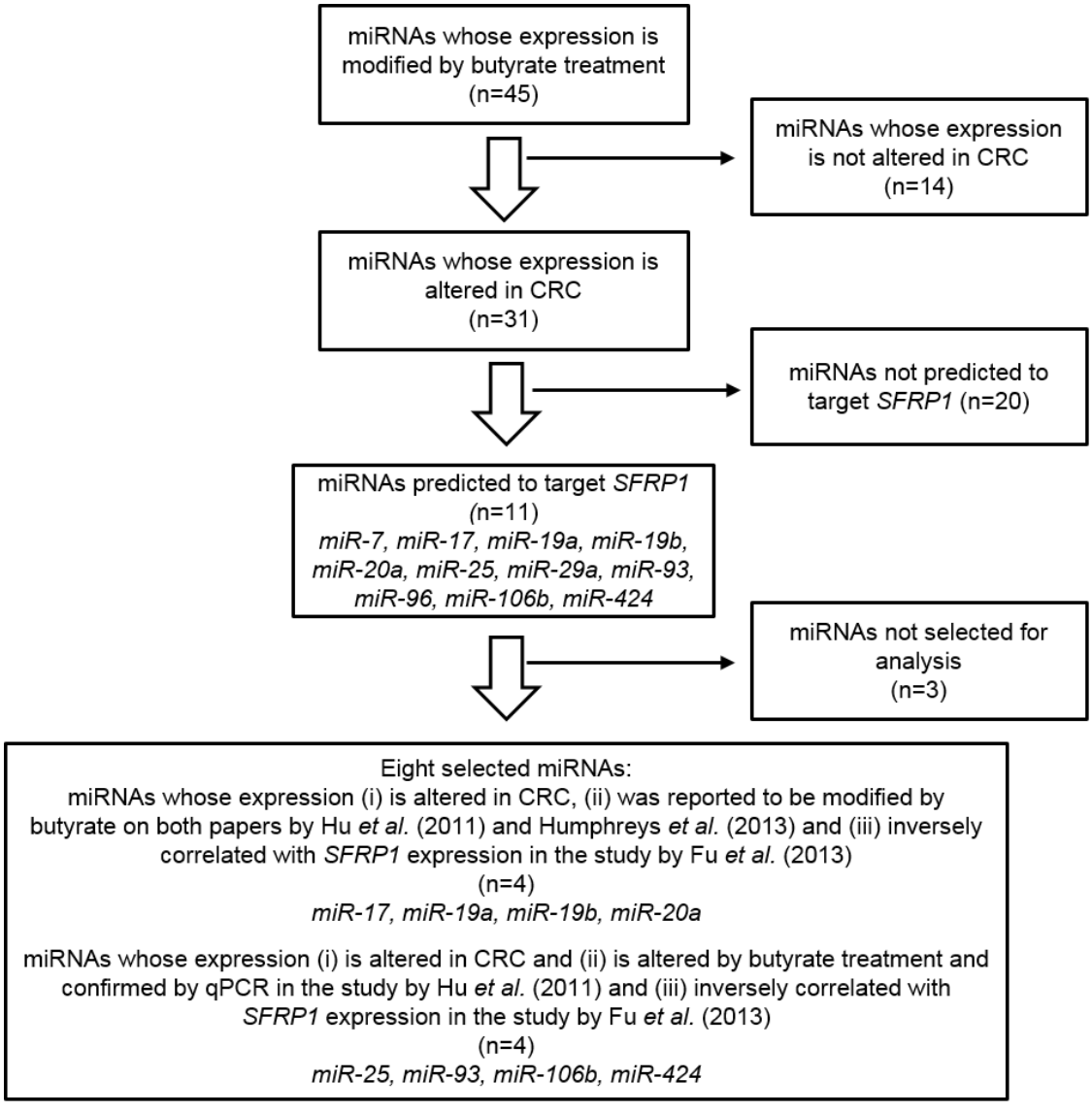


Table 3.7 Eight selected miRNAs for analysis by qPCR in intervention participants - relationship with *SFRP1* and effects of butyrate treatment.

Study	Hu *et al.* (2011)		Humphreys *et al.* (2013)	Fu *et al.* (2012)	
miRNA	Effect of butyrate (microarray data) (fold change)	Effect of butyrate (qPCR data) (fold change)	Effect of butyrate (microarray analysis, confirmed by qPCR)	CRC vs. normal tissue (fold change)	Correlation with *SFRP1* (Pearson's R)
miR-17	-1.25	-1.50	↓	+2.59	-0.781
miR-19a	-1.25	X	↓	+2.66	-0.579
miR-19b	-1.20	-1.50	↓	+2.57	-0.675
miR-20a	-1.25	-2.00	↓	+2.58	-0.785
miR-25	-1.20	-1.90		+1.59	-0.776
miR-93	-1.20	-1.70		+1.76	-0.827
miR-106b	-1.20	-2.00		+2.16	-0.703
miR-424	+1.50	+1.50		+4.60	-0.758

3.4.2 Selection of microRNAs for analysis in people at differential risk of colorectal cancer

In addition to the eight miRNAs selected for analysis in the intervention participants, that were also quantified in participants at differential risk of CRC ('Normal', 'UC' and 'Polyp'), five miRNAs were selected to be analysed specifically in 'Normal', 'UC' and 'Polyp' participants.

As this Ph.D. investigated differences in the WNT signalling pathway in participants at differential risk of CRC, the following criteria were applied for the selection of miRNAs to be analysed in 'Normal', 'UC' and 'Polyp' participants:

a) miRNAs whose expression is altered in CRC

b) miRNAs involved in the regulation of the WNT signalling pathway

A similar literature search-based process was used as in the selection of miRNAs for quantification in the intervention participants. The literature search

was performed on PubMed and the last search was performed in December 2013. The keywords used in this literature search are described in Table 3.8.

Table 3.8 Keywords used for the literature search on PubMed for the selection of miRNAs to be quantified in people at differential risk of CRC.

Search	Keywords
Expression change in CRC	*'microRNA', 'microRNAs', 'miRNA', 'miRNAs', 'colon', 'large bowel', 'colon cancer', 'colorectal cancer', 'rectal cancer'*
Regulation of the WNT signalling pathway	*'microRNA', 'microRNAs', 'miRNA', 'miRNAs', 'WNT', 'WNT signalling', 'WNT pathway'*

A total of five miRNAs were selected for quantification by qPCR that fulfilled the two criteria above (see Table 3.9). These were *miR-101, miR-122a, miR-135b, miR-145* and *miR-335*.

Table 3.9 Evidence from the literature identifying five miRNAs whose expression is altered in CRC and involved in the regulation of the WNT signalling pathway.

miRNA	Implicated in CRC	References	Involvement in the regulation of the WNT signalling pathway	References
miR-101	Downregulated	(Schee *et al.*, 2012)	- WNT activation induces *miR-101* repression - *miR-101* re-expression impairs β-catenin nuclear translocation and subsequent transcription	*(*Strillacci *et al.,* 2013*)*
miR-122a	Downregulated	(Wang *et al.*, 2009b)	Aberrant WNT activation mediates *miR-122a* downregulation	(Wang *et al.*, 2009b)
miR-135b	Upregulated	(Wang *et al.*, 2010) (Bandres *et al.*, 2006)	Suppresses APC expression and induces WNT activity	(Nagel *et al.*, 2008)
miR-145	Downregulated	(Schee *et al.*, 2012) (Wang *et al.*, 2009a) (Bandres *et al.*, 2006) (Michael *et al.*, 2003)	Impairs β-catenin nuclear translocation and subsequent transcription	(Yamada *et al.*, 2013b)
miR-335	Upregulated	(Wang *et al.*, 2010)	Activates WNT signalling and downregulates *DDK1*	(Yan *et al.*, 2012) (Zhang *et al.*, 2011)

3.5 Pyrosequencing bioinformatic analysis and *SFRP1* assay selection

Since expression of *SFRP1* differed between the intervention groups (section 4.2.4.2), it was hypothesised that this may have been due to epigenetic factors such as DNA methylation. Therefore, the methylation status of key regulatory domains within the promoter region of the *SFRP1* gene was analysed to determine whether these differences in gene expression could be attributed to differences in DNA methylation.

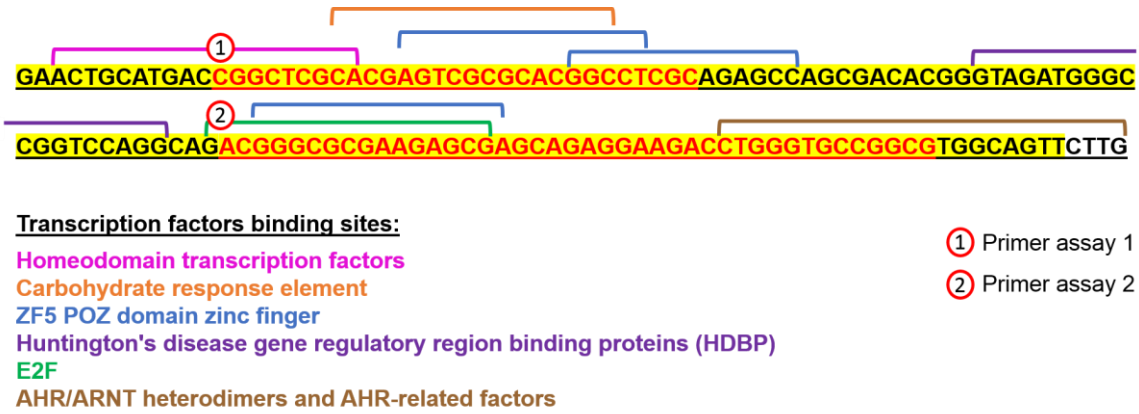
Promoter sequences of *SFRP1* were identified using the Genomatix Software. Two promoter sequences were identified at 41166172 – 41167556 and 41164408 – 41165009. The NCBI Nucleotide database was used to obtain the nucleotide sequence for a region encompassing both promoter sequences, i.e. between 41164300 – 41167700. MethPrimer (Li and Dahiya, 2002) was then used to find CpG islands within this region employing the following criteria: island size >100 bases, GC ratio >0.5 and observed to expected ratio >0.6. The sequences of the two available PyroMark CpG assays (Qiagen) for *SFRP1* were identified within the DNA sequence. Both promoter sequences, CpG islands and the two possible assay sequences are illustrated in Figure 3.5. Both assay sequences, in red, are located within the second promoter region and in the first CpG island identified.

Figure 3.5 *SFRP1* nucleotide sequence illustrating promoter sequences and CpG islands.

Promoter sequences are in bold and underlined, CpG islands are highlighted in yellow and sequences identified as potential targets for methylation quantification using the two available PyroMark CpG assays (Qiagen) are in red.

The Genomatix Software Suite v3.2 (Genomatix, 2014) was used to identify transcription factor binding sites identified in cells from the digestive system tissue within the *SFRP1* promoter sequence. The transcription factor binding sites (shown in brackets) within close proximity of the two *SFRP1* PyroMark CpG assays were identified (Figure 3.6).

Figure 3.6 Transcription factor binding sites within, or close to, domains in the two PyroMark CpG assay sequences within the second promoter of *SFRP1*.



CpG islands are highlighted in yellow. Coloured brackets represent transcription factor binding sites. Primer assays 1 and 2 are shown by circled numbers.

Primer assay 1 was selected to be used for analysis of *SFRP1* methylation because it a) contained more CpG sites (seven CpG sites compared with six) and b) contained or was close to more transcription factor binding sites than primer assay 2 (four transcription factor bindings sites compared with three). The seven CpG sites which were analysed are highlighted in yellow in the sequence below:

Primer assay 1: **CG**GCT**CG**CA**CG**AGT**CGCG**CA**CG**GCCT**CG**

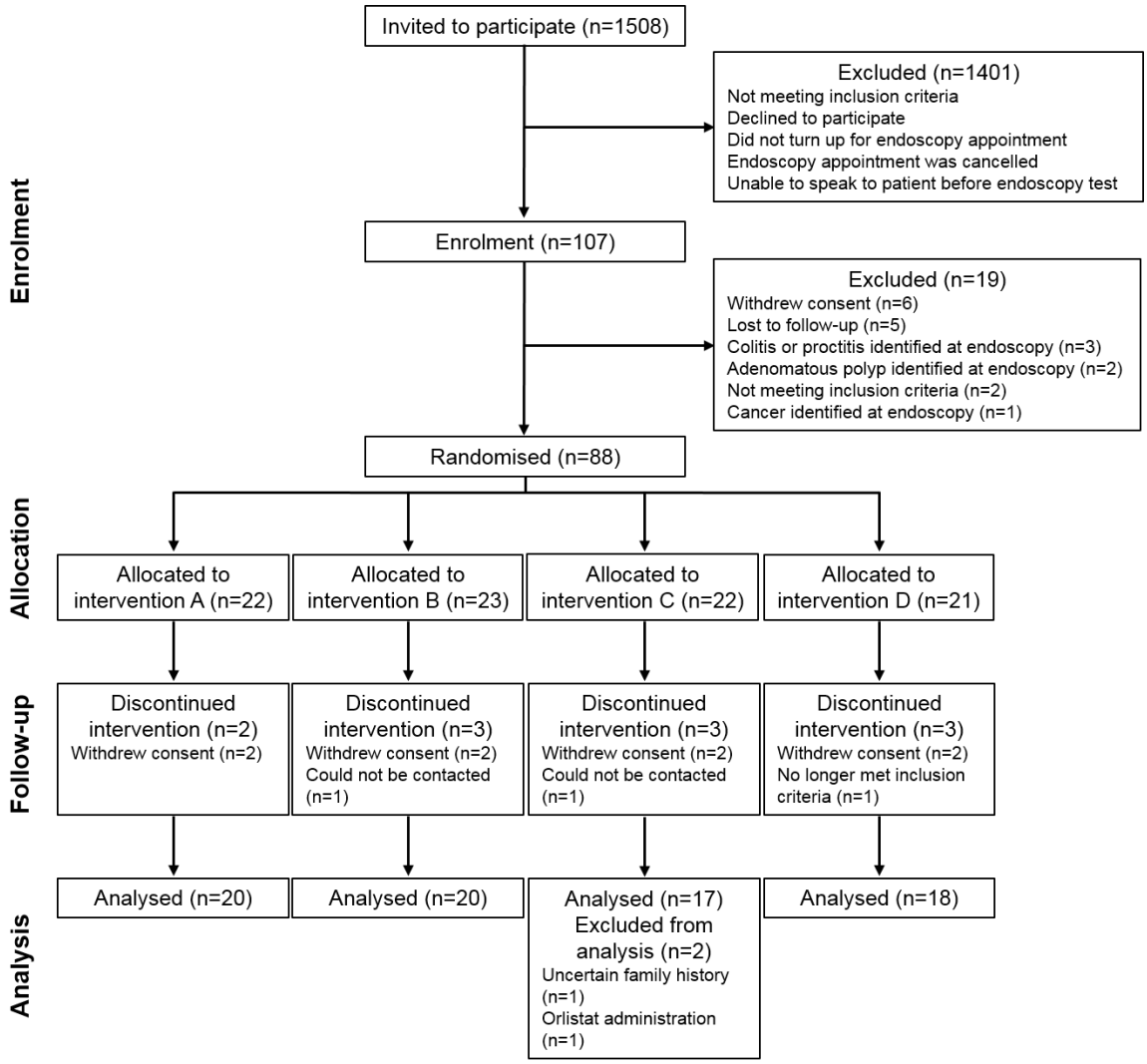
Chapter 4 The effects of non-digestible carbohydrates on the WNT signalling pathway and its functional outcomes

4.1 The DISC Study intervention participants

Over 1,500 potential participants were invited to take part in the DISC Study, of whom 1,401 were excluded, primarily because they did not meet the inclusion criteria or were not willing to participate. Eighty-eight healthy participants were recruited by the DISC Team and randomised for the intervention arm of the study. Following randomisation, 11 participants (12.5%), equally distributed amongst the four intervention groups, were lost to follow up. Eight participants withdrew consent, two could no longer be contacted and one no longer met the inclusion criteria.

A total of 77 participants completed the dietary intervention successfully, however two participants from intervention group C were excluded at the analysis stage. One of these was excluded as their family history was uncertain and they could not be contacted for this to be verified. The other participant was administering Orlistat, an inhibitor of fat digestion, which could be associated with effects on the microbiota as dietary fat has been shown to alter the microflora. The flow of participants throughout the DISC study is presented in a CONSORT diagram (Figure 4.1).

Figure 4.1 CONSORT diagram: flow of participants throughout the DISC Study RCT.



4.1.1 Baseline characteristics of intervention participants

A total of 75 participants completed the DISC Study dietary intervention arm and were included in the analyses. Table 4.1 describes the baseline (pre-intervention) characteristics for all of the intervention participants by intervention group. The ANOVA GLM was used to analyse mean age and mean BMI (kg/m^2) and Chi-square tests were used for the analysis of intervention group differences in other participant characteristics at baseline.

The majority of the participants were Caucasian. All variables, with the exception of age, were comparable between the four intervention groups. Participants in group B (mean age 58.3 years) were significantly older than participants in the other groups (p=0.041). The mean participant age across all

groups was 52.4 years, with the youngest and oldest participants being 30 and 84 years old respectively. A large proportion of participants were overweight (36%) or obese (47%) with only 17% of the intervention participants having a normal BMI (18.5-25kg/m^2). Furthermore, 12 participants had a BMI greater than 35kg/m^2. To avoid any confounding effects of intervention group differences in age, gender, endoscopy procedure, BMI or smoking status on the measured outcomes, these variables were included as covariates during statistical analyses using the ANOVA GLM.

Table 4.1 Baseline characteristics of intervention participants in the DISC Study.

Intervention group	All	A	B	C	D	P value
	RS	**-**	**-**	**+**	**+**	
	PD	**-**	**+**	**-**	**+**	
Number of participants	75	20	20	17	18	
Ethnicity Caucasian (%) Black African (%) Mixed race (%)	 73 (97) 1 (1.33) 1 (1.33)	 19 (95) 1 (5) 0 (0)	 19 (95) 0 (0) 1 (5)	 17 (100)	 18 (100)	
Gender Female (%) Male (%)	 40 (53) 35 (47)	 10 (50) 10 (50)	 11 (55) 9 (45)	 13 (76) 4 (24)	 6 (33) 12 (67)	0.084
Age Mean (years) Range	 52.4 30-80	 48.0 30-70	 58.3 33-80	 53.4 42-67	 50.1 36-74	**0.041**
Smoking status Never (%) Former (%) Current (%)	 38 (51) 21 (28) 16 (21)	 12 (60) 4 (20) 4 (20)	 12 (60) 5 (25) 3 (15)	 8 (47) 6 (35) 3 (18)	 6 (33) 6 (33) 6 (33)	0.604
BMI Mean (kg/m^2) Range	 30.0 23.0 – 49.3	 29.8 23.2 - 37.1	 29.7 23.2 - 49.3	 30.9 23.0 - 43.1	 29.9 23.4 - 41.8	0.901
Procedure Colonoscopy (%) Flexible Sigmoidoscopy (%)	 23 (31) 52 (69)	 6 (30) 14 (70)	 6 (30) 14 (70)	 6 (35) 11 (65)	 5 (28) 13 (72)	0.969

4.2 The effects of supplementation with RS and PD on WNT pathway-related gene expression in the human colorectal mucosa

4.2.1 Introduction

The WNT signalling pathway is an important regulator of physiological processes, including cell proliferation, differentiation and apoptosis, that maintain a homeostatic environment within the colorectal epithelium. However, aberrant WNT signalling is implicated in colorectal carcinogenesis, including both sporadic and inherited forms of this cancer, resulting in inappropriate activation of the WNT pathway. Due to its role in CRC, and because of speculations that NDCs and butyrate may be protective against CRC via positive modulation of this pathway, the effects of the dietary intervention on WNT signalling were investigated. Here, I report the results of quantification of the expression of WNT pathway-related genes in biopsies of colorectal mucosa as an indicator of WNT pathway activity.

4.2.1.1 Effects of non-digestible carbohydrates on WNT pathway-related gene expression

To date, there is only one study which has investigated the effects of RS on WNT pathway-related gene expression in the colon and no studies on the effects of PD. In addition, the single published study is based on work in rats (see below) so that, to my knowledge, my study is the first to have been carried out in humans.

The effects of RS on the expression of eight WNT pathway-related genes have been investigated in both normal (untreated) and carcinogen-treated rats (Cray, 2013). Three corn maize varieties were tested: Argentinian (AR), Guatemalan (GUAT) and the AR x GUAT hybrid and fed for ten weeks. The highest RS content was found in the GUAT diet (18.6%) and the lowest in the AR diet (5.4%). In normal rats, there was a significant increase in β-catenin expression in rats fed the GUAT diet compared with the hybrid diet. However, in carcinogen-treated rats, significantly lower β-catenin expression was observed in rats fed the hybrid diet compared with the AR diet, which had a lower RS content. *AXIN2* expression was significantly lower in rats fed the higher RS GUAT diet compared with the lower RS AR diet in both normal and carcinogen-

treated rats. Expression of *WISP1,* an inhibitor of WNT signalling, was also significantly lower in rats fed the two diets with highest RS content compared with the AR diet. In carcinogen-treated rats, the investigators also observed significantly higher *SFRP4* expression in the GUAT diet compared with the two diets of lower RS content.

4.2.1.2 Effects of butyrate on WNT pathway-related gene expression

The effects of butyrate on the WNT signalling pathway were first investigated *in vitro* by Bordonaro and colleagues in 1999 (Bordonaro *et al.*, 1999). Prior to that study, the induction of cell cycle arrest, differentiation and apoptosis in colonic carcinoma cell lines by butyrate treatment was already well established (Williams *et al.*, 2003) but the pathways involved in the regulation of these processes in the colorectal epithelium were relatively unknown. Bordonaro *et al.* (1999) aimed to investigate the effects of butyrate on the canonical WNT signalling pathway, due to its role in colon cancer aetiology, in colonic epithelial cells. Treatment of SW620 colonic carcinoma cells with 5mM butyrate (a physiologically relevant concentration) resulted in a five-fold increase in TCF activity at 24 hours. TCF is a transcription factor that activates transcription of WNT target genes when bound to β-catenin following β-catenin translocation to the nucleus. Butyrate treatment also increased the formation of β-catenin/TCF complexes. Furthermore, during the 24 hours after exposure to butyrate, apoptosis was induced as indicated by several markers including G_0-G_1 cell cycle arrest, caspase-3 activation and DNA fragmentation. The authors concluded that the induction of apoptosis by butyrate was linked to the increase in TCF activity (Bordonaro *et al.*, 1999). Following this finding, the authors were interested in investigating the mechanism by which butyrate is able to modulate the WNT pathway in different colon carcinoma cell lines. It was observed that the modulation of WNT signalling by butyrate was both promoter- and cell-type dependent (Bordonaro *et al.*, 2002).

A recent study by the Bordonaro group continued their investigations into the modulation of the WNT pathway by butyrate using a total human genome microarray (Lazarova *et al.*, 2014b). These authors treated HCT-116 CRC cells with a physiologically-relevant concentration of butyrate (5mM) and observed significantly altered expression of 1587 genes, including both direct and indirect

targets of WNT signalling ($p<0.05$). These included upregulation of the expression of *AXIN2, DKK1, WNT5B* and c-*JUN* and downregulation of *FOSL1* and *c-MYC.* A similar earlier study by the group compared the effects of 5mM butyrate treatment on LT97 colon microadenoma and on SW620 colorectal adenocarcinoma cell lines. Their results showed a significant effect of butyrate on the expression of genes including downregulation of *c-MYC* in both cell lines (Lazarova *et al.*, 2014a).

Another group used oligonucleotide microarrays to investigate the effects of butyrate treatment on the expression of 12 WNT target genes in the CC531 rat colon carcinoma cell line (Germann *et al.*, 2003). In response to butyrate, the expression of four target genes normally induced by activated WNT pathway viz. *CCND1*, *c-MYC*, *FOSL1* and *FST* all decreased. Importantly, *c-MYC*, *CCND1* and *FOSL1* have been reported to be overexpressed in colorectal carcinomas (Wong and Pignatelli, 2002) .

4.2.2 Hypotheses, Aims and Objectives

4.2.2.1 Hypotheses

The hypothesis for this study was that NDCs modulate the WNT pathway positively by altering the expression of WNT signalling genes. Specifically, it was hypothesised that the expression of genes that resulted in reduced WNT pathway activity, such as WNT inhibitors, would be increased, and that genes associated with enhanced WNT pathway activity, such as WNT ligands and target genes, would be decreased.

4.2.2.2 Aims

This study aimed to test this hypothesis by investigating the effects of supplementing healthy human participants with NDCs on the expression of 12 selected WNT pathway-related genes.

4.2.2.3 Objectives

- To select a subset of WNT pathway-related genes for analysis;
- To obtain colorectal mucosal biopsies from DISC Study participants before and after 50 days supplementation with NDCs;
- To extract an adequate amount of high-quality RNA from OCT-embedded rectal mucosal biopsies;
- To synthesise cDNA by reverse transcription of RNA;
- To quantify the expression of the selected WNT genes by qPCR;
- To investigate the effects of the supplementation with NDCs on the expression of the selected WNT genes;
- To explore the effects of key epidemiological factors including age and gender on WNT pathway-related gene expression.

4.2.3 Methods

The methods utilised for this study can be found in section 2.2.1. The analyses were performed using RNA extracted from OCT-embedded rectal biopsies, which was then reverse transcribed to cDNA. The synthesised cDNA was used for the quantification of the 12 selected WNT pathway-related genes by qPCR.

The effects of the interventions were determined by analysis of post-intervention expression data using the ANOVA GLM, with the corresponding pre-intervention value as a covariate. Other covariates in the analyses were age, gender, endoscopy procedure, BMI and smoking status. The Bonferroni correction was used to adjust for multiple comparisons.

4.2.4 Results

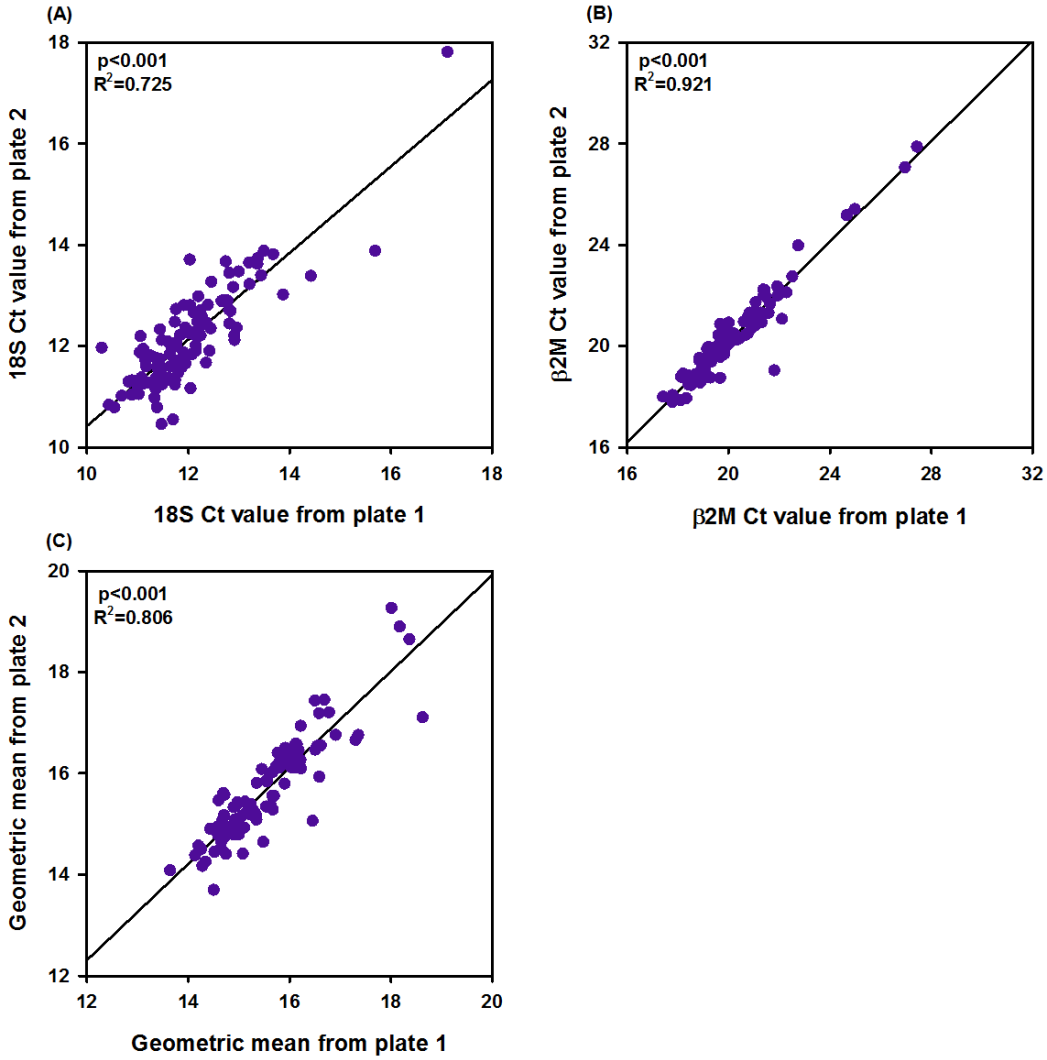
4.2.4.1 Reproducibility analysis

As quantification of the selected genes for each participant was performed using two separate PCR plates, the housekeeping genes *18S* and *β2M* were

analysed twice (once on each PCR plate). The data from both qPCR runs were utilised for a reproducibility analysis.

To examine the correlation between the two qPCR runs, Spearman's rank correlation was used to analyse the Ct values for *18S* and *β2M* as well as the geometric mean of the housekeeping genes using SigmaPlot v.12.5. Strong, positive correlations between qPCR runs were observed for *18S*, *β2M* and the geometric mean of the two (Figure 4.2). The Spearman correlation coefficients were 0.813 and 0.931 for *18S* and *β2M* respectively ($p<0.001$) (Figure 4.2 (A) and (B)). Consequently, a strong correlation between the geometric means of the two housekeeping genes was also achieved, with a Spearman correlation coefficient of 0.887 ($p<0.001$) (Figure 4.2 (C)).

Figure 4.2 Positive correlation of (A) *18S* expression, (B) *β2M* expression and (C) geometric mean data between plate 1 and plate 2.



Data are expressed as Ct values. (A) N=113, (B) N=108 and (C) N=100.

4.2.4.2 Effects of RS and PD on WNT pathway-related gene expression

Expression of each of the 12 WNT pathway-related genes in the rectal mucosa of healthy participants in the DISC Study was quantified by qPCR. Expression levels for each of the 12 genes in the rectal mucosa were characteristically different, with the most abundantly expressed gene being *CCND1,* and the gene with lowest expression being *SFPR2.* From the 12 WNT pathway-related genes quantified, significant effects of the intervention agents on the expression of *CTNNB1, c-MYC, SFRP1* and *SFRP2* were observed (Table 4.2).

Expression of *CTNNB1* (which encodes β-catenin) was significantly lower (reduced by 25%) in participants supplemented with RS (p=0.045).There was no effect of PD supplementation on *CTNNB1* expression.

Participants supplemented with RS also had significantly lower *c-MYC* expression (p=0.037) but, again, there was no effect of PD. *c-MYC* expression following RS supplementation was approximately half that observed in the placebo group.

Supplementation with each of the intervention agents lowered *SFRP1* expression, which was reduced by approximately two thirds following RS (p=0.005) and PD (p=0.053) supplementation. PD supplementation had a similar effect on *SFRP2* expression (p=0.01), but there were no detectable effects of RS on expression of this gene.

When applying Bonferroni's correction for multiple testing, the set p-value is lowered to 0.004 and so the statistically significant effects of RS and PD on expression of *CTNNB1, c-MYC, SFRP1* and *SFRP2* are no longer present.

Whilst there was no evidence of significant effects of RS and PD, there appeared to be interactions between RS and PD on expression of both *FOSL1* (p=0.055) and *c-JUN* (p=0.08). Supplementation with RS increased expression of *FOSL1* when given with the PD placebo, but reduced *FOSL1* expression when given with PD (Figure 4.3 (A)). On the contrary, expression of *c-JUN* reduced when RS was given with the PD placebo, but increased when administered with PD (Figure 4.3 (B)).

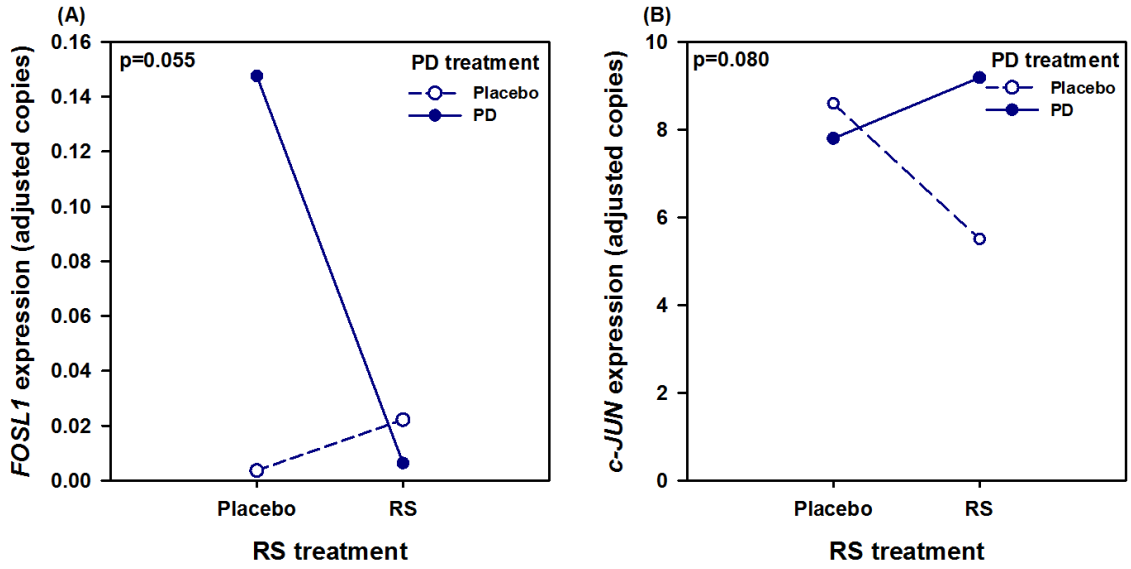
Table 4.2 Effects of supplementation with RS and PD on expression of 12 WNT pathway-related genes in the human rectal mucosa.

	RS allocation LSM (95% CI)			**PD allocation** LSM (95% CI)			**Interaction between RS and PD**
Gene	**Placebo (Amioca)**	**RS (Hi-Maize® 260)**	**P value**	**Placebo (Maltodextrin)**	**PD (Litesse® Ultra™)**	**P value**	**P value**
APC N=35	1.83 (1.48 – 2.22)	1.49 (1.17 – 1.85)	0.227	1.55 (1.24 – 1.89)	1.77 (1.43 – 2.17)	0.410	0.873
AXIN2 N=66	2.15 (1.81 – 2.50)	2.22 (1.89 – 2.55)	0.230	2.31 (1.98 – 2.65)	2.42 (2.07 – 2.77)	0.682	0.188
CCND1 N=35	66.5 (45.9 – 96.3)	45.2 (30.6 – 66.7)	0.171	50.9 (33.4 – 77.6)	59.0 (37.7 – 92.4)	0.678	0.352
CTNNB1 N=63	14.9 (12.3 – 17.7)	11.1 (9.0 – 13.5)	**0.045**	12.5 (10.3 – 15.1)	13.3 (10.8 – 16.0)	0.679	0.484
FOSL1 N=16	0.02312 (0.00534 – 0.10010)	0.01172 (0.00536 – 0.02565)	0.474	0.00887 (0.00384 – 0.02050)	0.03055 (0.00565 – 0.16521)	0.303	0.055

Gene	Placebo (Amioca)	RS (Hi-Maize® 260)	P value	Placebo (Maltodextrin)	PD (Litesse® Ultra™)	P value	P value
GSK3β N=64	5.31 (4.56 – 6.07)	4.08 (4.08 – 5.53)	0.351	4.92 (4.18 – 5.65)	5.20 (4.44 – 5.96)	0.615	0.843
c-JUN N=64	8.20 (6.54 – 10.04)	7.23 (5.73 – 8.91)	0.436	6.96 (5.48 – 8.63)	8.48 (6.78 – 10.37)	0.230	0.080
c-MYC N=37	10.18 (6.97 – 14.85)	5.41 (3.58 – 8.19)	**0.037**	8.06 (5.01 – 12.59)	6.84 (4.37 – 10.73)	0.668	0.925
SFRP1 N=39	6.19 (3.63 – 10.57)	1.78 (1.00 – 3.18)	**0.005**	5.49 (2.97 – 10.14)	2.01 (1.07 – 3.76)	0.053	0.320
SFRP2 N=49	0.00695 (0.00397 – 0.01216)	0.00685 (0.00378 – 0.01243)	0.980	0.01242 (0.00705 – 0.02188)	0.00383 (0.00210 – 0.00699)	**0.010**	0.233
WNT5A N=63	0.133 (0.097 – 0.183)	0.103 (0.076 – 0.140)	0.267	0.104 (0.075 – 0.144)	0.132 (0.095 – 0.184)	0.332	0.843
WNT11 N=42	0.1174 (0.0918 – 0.1463)	0.0889 (0.0666 – 0.1143)	0.145	0.1038 (0.0785 – 0.1327)	0.1015 (0.0777 – 0.1285)	0.910	0.684

Data are expressed as Least Square Means (LSMs) and 95% confidence intervals for adjusted values ($2^{-\Delta Ct}$ x 10,000) relative to the *18S* and *β2M* housekeeping genes

Figure 4.3 Interaction between RS and PD supplementation on *FOSL1* (A) and *c-JUN* (B) expression.



Data are expressed as adjusted copies and presented as LSMs. (A) N=16 and (B) N=64.

4.2.4.3 Exploration of additional factors influencing WNT pathway-related gene expression

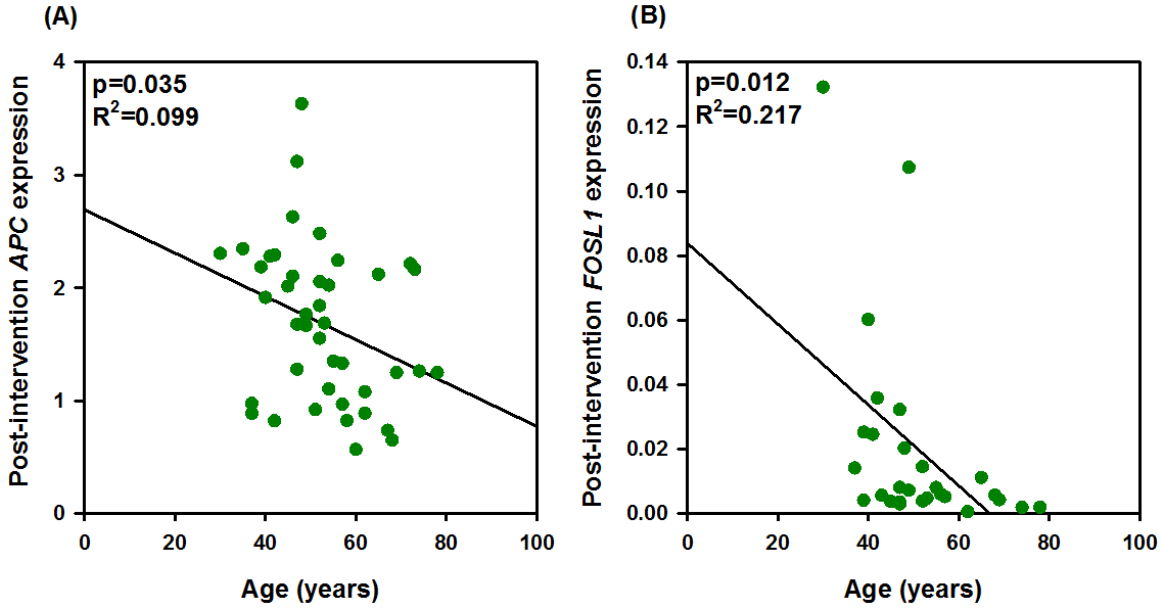
Expression of the gene of interest plus the variables age, gender, endoscopy procedure, BMI and smoking status were included as covariates in statistical analyses. Gender, endoscopy procedure, BMI and smoking status did not affect the expression of any of the 12 genes quantified. However, there were significant positive effects of pre-intervention expression levels on post-intervention expression for six of the genes (Table 4.3). Age was also a determinant of post-intervention expression levels for *APC* (p=0.035) and *FOSL1* (p=0.012).

Table 4.3 P values for the effects of pre-intervention expression, age, gender, endoscopy procedure, BMI and smoking status on post-intervention WNT pathway-related gene expression.

Gene	Pre-intervention expression	Age	Gender	Endoscopy procedure	BMI	Smoking status
APC	0.607	**0.035**	0.743	0.671	0.119	0.860
AXIN2	**0.001**	0.990	0.896	0.716	0.294	0.503
CCND1	**0.001**	0.885	0.207	0.235	0.225	0.619
CTNNB1	**0.005**	0.589	0.448	0.795	0.203	0.310
FOSL1	0.287	**0.012**	0.230	0.175	0.142	0.418
GSK3β	**0.001**	0.724	0.601	0.782	0.962	0.061
c-JUN	0.284	0.861	0.969	0.805	0.144	0.409
c-MYC	**<0.001**	0.955	0.751	0.264	0.603	0.365
SFRP1	0.828	0.883	0.625	0.441	0.700	0.460
SFRP2	0.399	0.866	0.258	0.388	0.676	0.056
WNT5A	**<0.001**	0.186	0.766	0.706	0.943	0.178
WNT11	0.104	0.317	0.334	0.620	0.664	0.635

Spearman correlation analyses revealed inverse correlations between age and post-intervention expression of both *APC* (Spearman correlation coefficient -0.371, p=0.016) (Figure 4.4 (A)) and *FOSL1* (Spearman correlation coefficient -0.513, p=0.005) (Figure 4.4 (B)).

Figure 4.4 Inverse correlations between age and post-intervention expression of (A) *APC* and (B) *FOSL1* in the human rectal mucosa.



Expression data are expressed as adjusted copies. (A) N=35 and (B) N=16.

4.2.5 Discussion

To investigate the effects of supplementation with RS and PD on WNT activity in the human colorectal epithelium, 12 WNT pathway-related genes were selected to be quantified by qPCR. These were selected by reviewing the literature to identify WNT pathway-related genes implicated in colorectal carcinogenesis and whose expression had been reported to be modulated by butyrate as described in section 3.3. A pilot study (section 3.2) was also conducted to compare the expression of a panel of 70 WNT pathway genes in the macroscopically-normal mucosa of people at higher risk of CRC, with a prior history of adenomatous polyps.

Supplementation with RS lowered *CTNNB1* expression by approximately 25% (p=0.045) but PD supplementation had no effect on *CTNNB1* expression (Figure 4.5 (A)). *CTNNB1* encodes β-catenin, a central component of the canonical WNT signalling pathway which, when WNT signalling is activated, accumulates in the cytoplasm and translocates to the nucleus. In the nucleus, β-catenin acts as a co-activator and is recruited by the transcription factor family TCF/LEF which binds to DNA in enhancer elements of genes and switches on expression of oncogenes. A reduction in *CTNNB1* expression at the mRNA

level may result in a decrease in the β-catenin protein and is suggestive of reduced WNT pathway activity. To my knowledge, there are no similar studies of RS or PD on *CTNNB1* expression in the human colorectum. However, in AOM-treated rats, Cray *et al.* did not observe significant differences in β-catenin expression in the colonic epithelium when the rats were fed a low or a high RS diet for eight weeks (Cray, 2013).

Previously, Bordonaro and colleagues did not observe any effect of sodium butyrate on total β-catenin levels in colon carcinoma cells (Bordonaro *et al.*, 2002). However, further investigation of the effects of 5mM sodium butyrate exposure for up to 24 hours produced significantly increased levels of active, dephosphorylated β-catenin, measured by Western Blot, in eight of ten CRC cell lines (Bordonaro *et al.*, 2007). In addition, significantly increased concentrations of dephosphorylated β-catenin in the nucleus were confirmed in SW620 and HCT-116 cell lines.

RS, but not PD, also lowered *c-MYC* expression significantly ($p=0.037$) so that post-intervention *c-MYC* expression in participants given RS was approximately half of that in participants given placebo (Figure 4.5 (B)). *c-MYC* is a target of the WNT pathway, whose expression is increased in response to WNT pathway activation (van de Wetering *et al.*, 2002) (He *et al.*, 1998). *c-MYC* is an oncogene considered to be a “master regulator” which controls many aspects of both cellular growth regulation and cellular metabolism (Miller *et al.*, 2012). It is frequently overexpressed in CRCs (Stewart *et al.*, 1986).

The findings from my study are consistent with results from a very recent study by the Bordonaro group, where they reported a 50% reduction in *c-MYC* expression ($p<0.05$) following treatment with 5mM butyrate (Lazarova *et al.*, 2014b). An earlier study by this group found that 5mM butyrate treatment significantly reduced the expression of *c-MYC* in both LT97 and SW620 cell lines, with butyrate:control ratios for *c-MYC* being 0.04 and 0.052 respectively (Lazarova *et al.*, 2014a). The authors argued that butyrate treatment would lead to an increase in WNT activity and that, although this would lead to an increase in *c-MYC* transcription, butyrate also inhibits transcriptional elongation (Wilson *et al.*, 2002), consequently leading to a reduction in *c-MYC* expression overall.

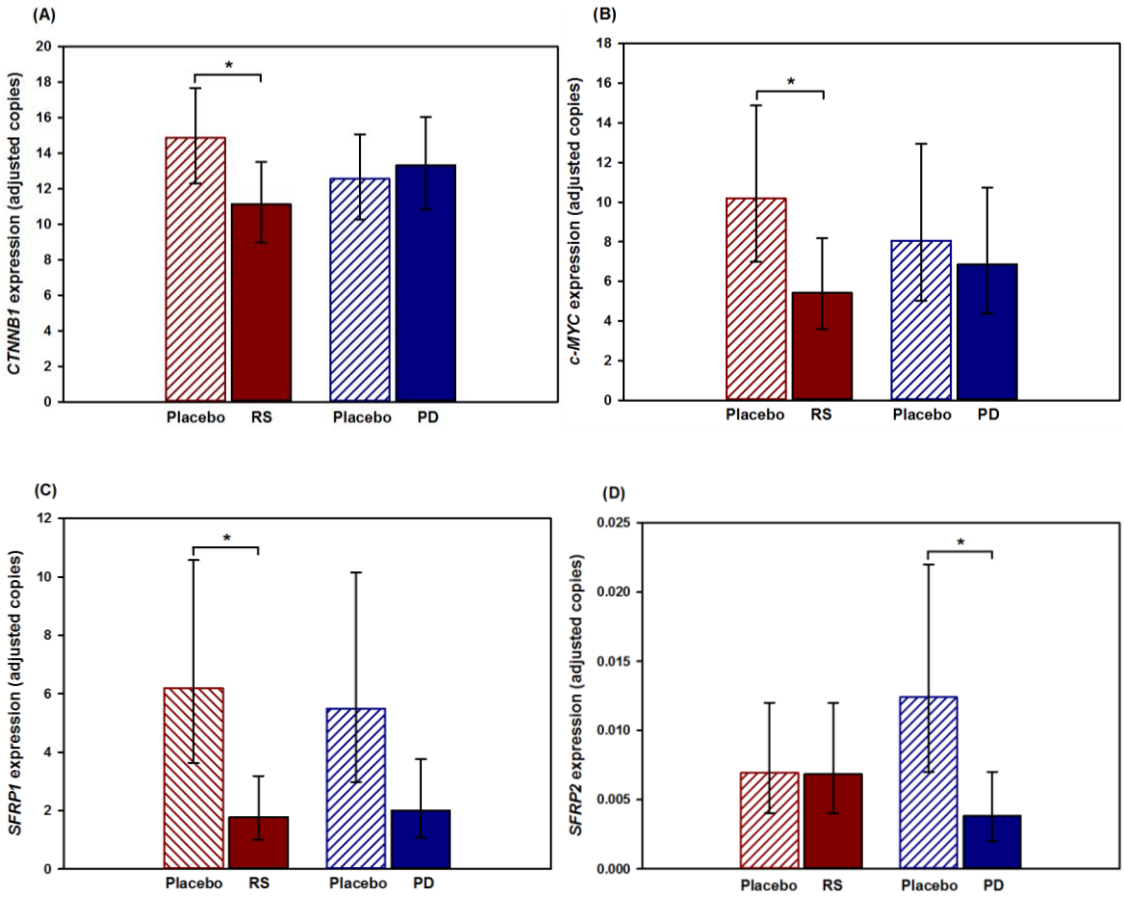
The reduction of *c-MYC* expression by butyrate was first observed by Herold and Rothberg in 1988. They found that treatment of SW837 rectal carcinoma cells with butyric acid, with maximal reduction observed at a 1mM concentration, decreased mRNA levels of *c-MYC* in a dose-dependent manner (Herold and Rothberg, 1988). The Rothberg group further explored the attenuation of *c-MYC* transcription by butyrate. They observed a reduction in *c-MYC* expression following treatment of SW837 with 2mM butyrate. A follow-up study by this group later identified that this reduction in expression resulted from prevention of transcriptional elongation as opposed to a reduction in the initiation of transcription or induction of RNA degradation (Heruth *et al.*, 1993).

The effects of butyrate on protein levels of c-MYC have been investigated in six human colon tumour cell lines and the investigators found that a similar reduction in c-MYC was achieved by butyrate in all six cell lines (Taylor *et al.*, 1992).

Both RS and PD supplementation reduced expression of *SFRP1* (Figure 4.5 (C)). This was statistically significant for RS ($p=0.005$) and close to reaching statistical significance for PD ($p=0.053$), with expression levels which were only one third or less of expression levels compared with their placebos. In addition, PD, but not RS, significantly reduced *SFRP2* expression ($p=0.01$) to a similar degree (Figure 4.5 (D)). *SFRP1* and *SFRP2* encode proteins which are members of the WNT antagonist family of SFRPs. SFRPs have a N-terminal cysteine-rich domain similar to that of Frizzled receptors and inhibit the WNT pathway competitively by competing with the Frizzled receptors for WNT ligand binding (Bafico *et al.*, 1999).

To my knowledge, this is the first study which has investigated the effects of NDCs or of butyrate on *SFRP1* or *SFPR2* expression in the colon. However, work in two gastric cancer cell lines, AGS and MKN45, showed that treatment with 2µM sodium butyrate restored SFRP1 and SFRP2 protein levels, which are diminished in cancer (Shin *et al.*, 2012).

Figure 4.5 Significant effects of supplementation with RS and PD on expression of (A) *CTNNB1,* (B) *c-MYC,* (C) *SFRP1* and (D) *SFRP2* in the human rectal mucosa.



Data are expressed as LSMs for adjusted copies relative to the geometric mean of *18S* and *β2M* housekeeping genes. Error bars represent 95% CIs. *p<0.05 for effect of active agent versus placebo.(A) N=63, (B) N=37, (C) N=39 and (D) N=49.

As shown in Figure 4.4, there were significant inverse relationships between age and expression of both *APC* (p=0.035) and *FOSL1* (p=0.012). This is the first report of correlations between age and *APC* and *FOSL1* expression in the colon. However, using bone marrow mesenchymal stem cells from cardiac patients, Brunt and colleagues have previously reported positive correlations between age and *FOSL1* expression (Brunt *et al.*, 2012).

APC is a key component of the WNT signalling pathway and loss of APC through mutation or epigenetic silencing results in failure to degrade β-catenin and its accumulation in the cytoplasm. A reduction in *APC* expression with age suggests a decrease in APC protein levels, which would lead to reduced β-catenin degradation by the destruction complex (which includes APC and GSK3β) and therefore to an increase in WNT activity. This could be one of the

possible mechanisms that contribute to the increasing risk of CRC with age. Conversely, *FOSL1* is a target gene of the WNT pathway and therefore a reduction in *FOSL1* expression with age could be an indication of reduced WNT pathway activation.

In conclusion, RS and/or PD significantly modulated the expression of four of the 12 selected WNT pathway-related genes. RS significantly reduced the post-intervention expressions of *CTNNB1* and *c-MYC* compared with placebo. *CTNNB1* encodes β-catenin, perhaps the key component of the canonical WNT pathway, that drives transcription of WNT target genes when the pathway is activated, and *c-MYC* is a target of the WNT pathway. A decrease in their expression would therefore be suggestive of a reduction in WNT signalling.

On the contrary, both RS and PD reduced the expression of *SFPR1* and PD reduced the expression of *SFRP2* post-intervention. *SFRP1* and *SFRP2* are both inhibitors of the WNT signalling pathway and therefore a decrease in their expression, if paralleled at the protein level, would lead to reduced inhibition of the WNT pathway and subsequently an increase in WNT activity.

4.3 The effects of supplementation with RS and PD on microRNA expression in the human colorectal mucosa

4.3.1 Introduction

miRNAs are small, non-coding RNAs that are able to regulate gene expression epigenetically. As described in section 1.2.6.3, several miRNAs are aberrantly expressed in CRC, particularly members of the miR-17-92 cluster, leading to altered expression of their target genes.

As an effect of both RS and PD was observed on *SFRP1* expression, the effects of the intervention on the expression of miRNAs that may target *SFRP1* were investigated. A total of eight miRNAs were selected as described in section 3.4.1. These comprised miRNAs reported to be altered in colorectal carcinogenesis, modified by butyrate and predicted to target *SFRP1*.

4.3.1.1 Effects of non-digestible carbohydrates on miRNA expression in the colon

Very few studies have investigated the effects of NDCs or butyrate on expression of miRNAs that target the WNT signalling pathway in the colon.

A very recent paper published by Humphreys *et al.* was the first study to investigate the effects of RS, and butyrate, on miRNA expression in colorectal cells *in vivo* (Humphreys *et al.*, 2014). Healthy participants consumed a high red meat (HRM) diet, which is associated with increasing CRC risk, and a HRM diet plus butyrylated RS for four weeks in a cross-over design. The HRM diet increased expression of miR-17-92 cluster miRNAs. However, the authors observed lower expression of miR-17-92 cluster miRNAs following the HRM plus butyrylated RS diet compared with the HRM diet alone, suggesting that RS was able to reverse the detrimental effects of HRM on miRNA expression. This was statistically significant for *miR-17, miR-19a, miR-19b, miR-20a* and *miR-92.*

4.3.1.2 Effects of butyrate on miRNA expression in the colon

Hu *et al.* explored the effects of butyrate treatment of HCT-116 colon cancer cells on expression of miRNAs using a miRNA microarray (Hu *et al.*, 2011). They found that butyrate significantly altered expression of 44 miRNAs,

including reduced expression of members of the miR-17-92 cluster. Interestingly, the authors found that *miR-17, miR-20a, miR-20b, miR-93, mi-106a* and *miR-106b*, that were downregulated in butyrate-treated cells, were significantly overexpressed in colon tumour tissue compared with normal tissue. This suggested that one of the mechanisms by which butyrate is protective against CRC may be through the positive modulation of miRNAs that are abnormally overexpressed in CRC.

As miRNAs from the miR-17-92 cluster have been reported to be overexpressed in CRC, Humphreys *et al.* investigated the effects of butyrate treatment on miRNA expression, particularly from this cluster, in HT-29 colorectal adenocarcinoma and HCT-116 colorectal carcinoma cells (Humphreys *et al.*, 2013). They assessed the effects of 48-hour treatment of HT-29 cells with 5mM butyrate on miRNA expression by microarray analysis and found that butyrate reduced and increased expression of 30 and 39 miRNAs respectively. To validate the observed effects, miRNA expression in both HT-29 and HCT-116 cells treated with 1, 5 and 10mM butyrate were quantified by qPCR. Treatment with 5mM butyrate significantly downregulated miRNAs belonging to the miR-17-92 cluster (*miR-18a, miR-19a, miR-19b, miR-20a* and *miR-92a)* in both cell lines. Furthermore, the authors observed decreased proliferation with 48-hour butyrate treatment of HT-29 and HT-116 cells, and this was statistically significant with the physiologically-relevant concentration of 5mM.

4.3.2 Hypotheses, Aims and Objectives

4.3.2.1 Hypotheses

The hypothesis for this study was that changes in the expression of *SFRP1* observed following supplementation with RS and PD resulted from altered expression of miRNAs predicted to target *SFRP1*.

4.3.2.2 Aims

This study aimed to test this hypothesis by investigating the effects of supplementing healthy human participants with NDCs on the expression of eight selected miRNAs that may target *SFRP1*.

4.3.2.3 Objectives

- To select a subset of miRNAs for analysis;
- To extract an adequate amount of high-quality total RNA, including small RNAs such as miRNAs, from OCT-embedded rectal mucosal biopsies;
- To synthesise cDNA by reverse transcription of RNA;
- To conduct a quality control PCR array experiment;
- To quantify the expression of the selected miRNAs by qPCR;
- To investigate the effects of supplementation with NDCs on expression of the selected miRNAs;
- To explore the effects of key epidemiological factors including age and gender on expression of the selected miRNAs.

4.3.3 Methods

The methods for this study can be found in section 2.2.2. Total RNA, including small RNAs such as miRNAs, was extracted from OCT-embedded rectal biopsies and reverse transcribed to cDNA. A quality control (QC) PCR array was run prior to quantification of the selected miRNAs by qPCR.

The effects of the interventions were determined by analysis of post-intervention expression data using the ANOVA GLM, with the corresponding pre-intervention value as a covariate. Other covariates in the analyses were age, gender, endoscopy procedure, BMI and smoking status. For data that were not normally-distributed, the Kruskal-Wallis non-parametric test was utilised.

4.3.4 Results

Expression of the eight selected miRNAs was quantified to investigate the effects of the dietary intervention. Prior to quantification, a QC PCR array was run to ensure that cDNA quality was suitable for subsequent analyses by qPCR.

4.3.4.1 cDNA quality control prior to profiling mature miRNAs

Prior to profiling the expression of the selected miRNAs, a cDNA QC PCR Array was run as described in section 2.2.2.3. This array included primers for the detection of *C. elegans miR-39*, three ubiquitously expressed miRNAs (*miR-16, miR-21* and *miR-191*), three snoRNA controls (SNORD61, SNORD95 and SNORD96A) and two controls (PPC and RTC).

The positive PCR control (PPC) assessed PCR performance and detected any inhibition of the PCR reaction. Ct values 19 ± 2 are indicative of high quality RNA. This was achieved for all samples run on the array, with a mean PPC Ct value of 18.7 for the eight cDNA samples. Likewise, the miRNA reverse transcription control (miRTC) assessed the reverse transcription reaction performance and detected any inhibition of reverse transcription by quantifying the template synthesised from a control RNA integrated in the miScript II RT Kit. The ΔCt was calculated from the PPC and miRTC duplicate mean values and should be less than 7. As shown in Table 4.4, the ΔCt values for all eight samples were less than 7, with a mean value of 2.52, indicating that there was no inhibition of reverse transcription.

Table 4.4 miScript QC PCR Array PPC and miRTC data.

Sample	PPC Ct mean	miRTC Ct mean	ΔCt
011 pre	18.9	20.8	0.42
032 pre	18.6	23.9	3.82
066 pre	18.7	26.3	6.13
079 post	18.7	22.5	2.34
082 pre	18.6	20.6	0.52
093 post	18.7	22.9	2.71
109	18.7	20.4	0.21
211	18.8	24.3	4.01

Data are presented as duplicate mean Ct values for PPC and miRTC. $\Delta Ct = Ct^{miRTC} - Ct^{PPC} - 1.5$. N=8.

The *C. elegans miR-39* should only be detectable when the Syn-cel-miR-39 miScript miRNA Mimic has been spiked into the samples. All eight cDNA samples correctly resulted in undetectable (UD) levels of *C. elegans miR-39* as shown in Table 4.5.

Three miRNAs that are ubiquitously expressed across a variety of cell, tissue and fluid types, *miR-16, miR-21* and *miR-191*, were detected and showed adequate Ct values for all eight samples analysed (Table 4.5).

Table 4.5 miScript QC PCR Array *C. elegans miR-39, miR-16, miR-21* and *miR-191* data.

Sample	*C. elegans miR-39*	*miR-16*	*miR-21*	*miR-191*
011 pre	UD	16.7	14.0	18.5
032 pre	UD	19.2	14.7	20.3
066 pre	UD	20.9	15.0	21.1
079 post	UD	17.6	14.2	18.9
082 pre	UD	16.4	13.8	18.7
093 post	UD	18.0	13.7	19.4
109	UD	16.0	13.3	18.2
211	UD	19.4	14.5	20.4

Data are presented as Ct values. N=8

Three snoRNAs, SNORD61, SNORD95 and SNORD96A, that may be utilised as endogenous controls were quantified. The expression of these controls has been shown to be relatively stable across different tissue and cell types. This is reflected in the QC array data from the analysed samples (Table 4.6).

Table 4.6 miScript QC PCR Array control snoRNA data for SNORD61, SNORD95 and SNORD96A.

Sample	SNORD61	SNORD95	SNORD96A
011 pre	19.0	19.7	19.1
032 pre	21.8	22.4	22.4
066 pre	23.4	22.9	22.1
079 post	20.2	20.2	20.0
082 pre	19.7	19.5	19.6
093 post	20.7	19.9	19.9
109	18.0	18.7	18.7
211	22.3	21.6	21.6

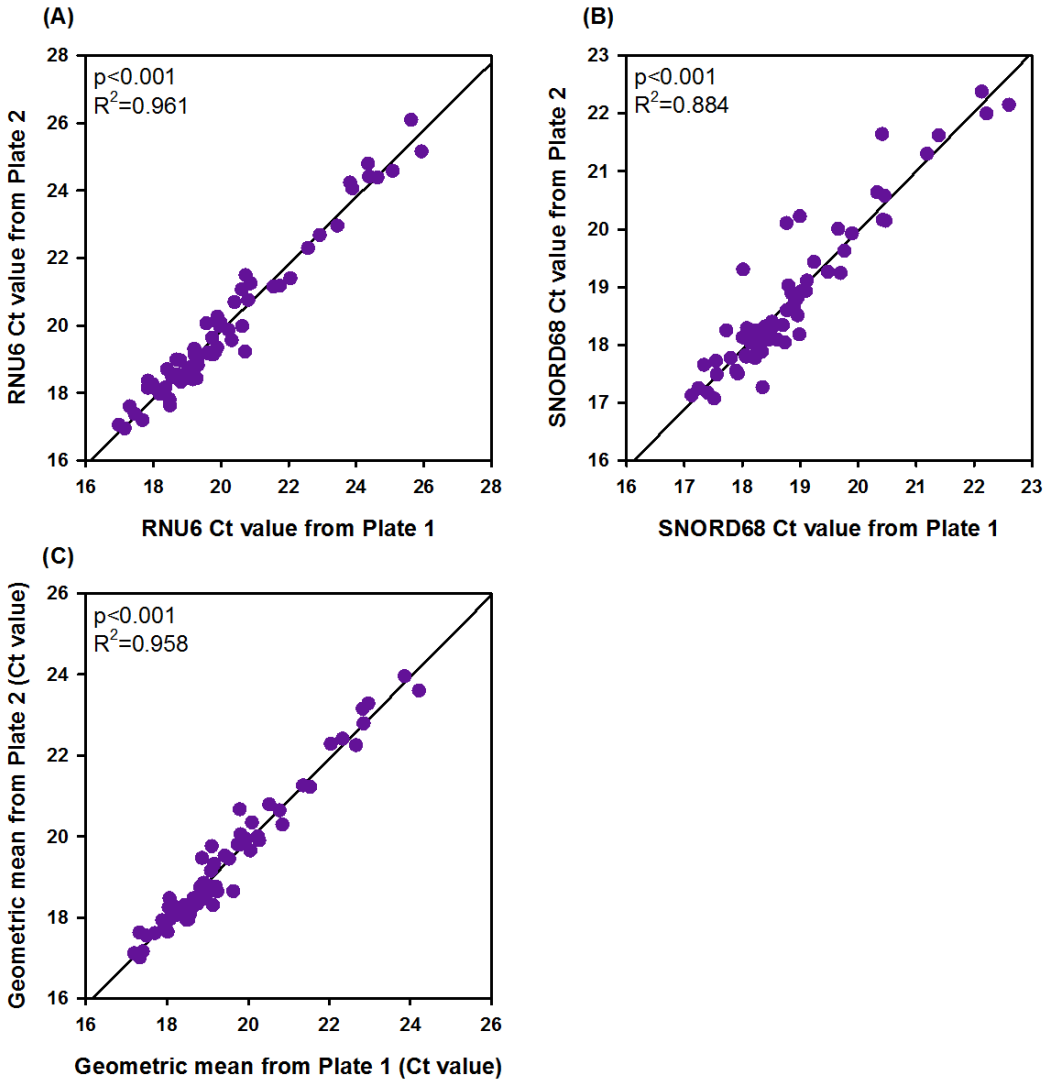
Data are presented as Ct values. N=8

4.3.4.2 Reproducibility analysis

As for 25 of the 'Normal' (pre-intervention) participants and the 'Polyp' and 'UC' groups the control RNAs RNU6 and SNORD68 were analysed twice (once on the plate analysing the miRNAs selected for the intervention and once on the plate analysing the miRNAs selected analysis of participants at differential risk of CRC), the data from both qPCR runs were utilised for a reproducibility analysis.

To examine the correlation between the two qPCR runs, Spearman's rank order correlation test was run on the Ct values for RNU6 and SNORD68 as well as the geometric mean of the two control RNAs using SigmaPlot v.12.5. Strong, positive correlations between qPCR runs were observed for both RNU6 and SNORD68. For RNU6, Spearman's correlation coefficient was 0.968 ($p<0.001$) and the linear regression graph had a R^2 value of 0.961 (Figure 4.6 (A)). For SNORD68, Spearman's correlation coefficient was 0.899 ($p<0.001$) with an R^2 value of 0.884 (Figure 4.6 (B)). Consequently, a strong correlation between the geometric means was also achieved, with a Spearman's correlation coefficient of 0.963 ($p<0.001$) and an R^2 value of 0.958 (Figure 4.6 (C)).

Figure 4.6 Positive correlation of (A) RNU6 expression, (B) SNORD68 expression and (C) geometric mean data between plate 1 and plate 2.



Data are expressed as Ct values. N=63.

4.3.4.3 Effects of the non-digestible carbohydrates on miRNA expression

For three of the selected miRNAs, *miR-17*, *miR-19b* and *miR-424*, the ANOVA GLM was used to analyse for an effect of RS and PD. The effects of the intervention agents on the expression of the remaining five miRNAs were analysed using the non-parametric test Kruskal-Wallis as it was not possible to achieve normally-distributed data for these miRNAs despite transformation. RS and PD did not have an effect on expression of the eight miRNAs quantified. A series of trends, however, were observed, with participants supplemented with RS appearing to have reduced expression of *miR-17, miR-19a, miR-19b, miR-20a* and *miR-25* (Table 4.7).

Table 4.7 Effects of supplementation with RS and PD on *miR-17*, *miR-19b* and *miR-424* expression in the human rectal mucosa.

	RS allocation LSM (95% CI)			**PD allocation** LSM (95% CI)			**Interaction between RS and PD**
miRNA	**Placebo (Amioca)**	**RS (Hi-Maize® 260)**	**P value**	**Placebo (Maltodextrin)**	**PD (Litesse® Ultra™)**	**P value**	**P value**
miR-17	153 (133 – 178)	136 (119 – 157)	0.279	140 (122 – 163)	148 (128 – 173)	0.631	0.261
miR-19b	187 (128 – 280)	139 (97 – 203)	0.290	150 (105 – 219)	172 (118 – 258)	0.625	0.307
miR-424	27.3 (22.0 – 34.7)	24.9 (20.3 – 31.3)	0.581	23.4 (19.1 – 29.3)	29.2 (23.1 – 38.0)	0.205	0.202

Data are expressed LSMs and 95% confidence intervals for adjusted values ($2^{-\Delta Ct}$ x 1,000) relative to the reference RNAs RNU6 and SNORD68 (ANOVA GLM). N=56.

Table 4.8 Effects of supplementation with RS and PD on expression of *miR-19a, miR-20a, miR-25, miR-93* and *miR-106b* in the human rectal mucosa.

	RS allocation Median (lower and upper quartiles)			**PD allocation** Median (lower and upper quartiles)		
miRNA	**Amioca (placebo)**	**Hi-Maize® 260 (RS)**	**P value**	**Maltodextrin (placebo)**	**Litesse® Ultra™ (PD)**	**P value**
miR-19a	283 (82 – 532)	211 (102 – 429)	0.870	245 (93 – 473)	273 (98 – 548)	0.762
miR-20a	366 (295 – 474)	338 (294 – 456)	0.987	357 (287 – 470)	355 (316 – 419)	0.889
miR-25	75.5 (60.4 – 200.7)	70.6 (50.0 – 127.0)	0.394	70.1 (55.8 – 178.2)	74.3 (58.0 – 132.0)	0.762
miR-93	69.2 (58.5 – 109.2)	76.8 (59.7 – 87.6)	0.935	72.5 (57.4 – 104.5)	71.8 (59.9 – 92.4)	0.700
miR-106b	172 (158 – 255)	173 (141 - 213)	0.724	174 (148 - 211)	171 (158 - 255)	0.853

Data are expressed median and lower and upper quartiles for adjusted values ($2^{-\Delta Ct}$ x 1,000) relative to the reference RNAs RNU6 and SNORD68 (Kruskal-Wallis test). N= 56, with the exception of *miR-106* where N=55.

4.3.4.4 Exploration of additional factors influencing miRNA expression

Expression of the eight selected miRNAs plus the variables age, gender, endoscopy procedure, BMI and smoking status were included as covariates in statistical analyses (Table 4.9). A significant effect of pre-intervention expression was observed on post-intervention *miR-19b* ($p<0.001$) and *miR-424* ($p<0.001$) expression. Post-intervention *miR-19b* expression was significantly affected by age ($p=0.012$), and smoking status was a determinant of post-intervention *miR-93* expression ($p=0.017$) (Figure 4.7).

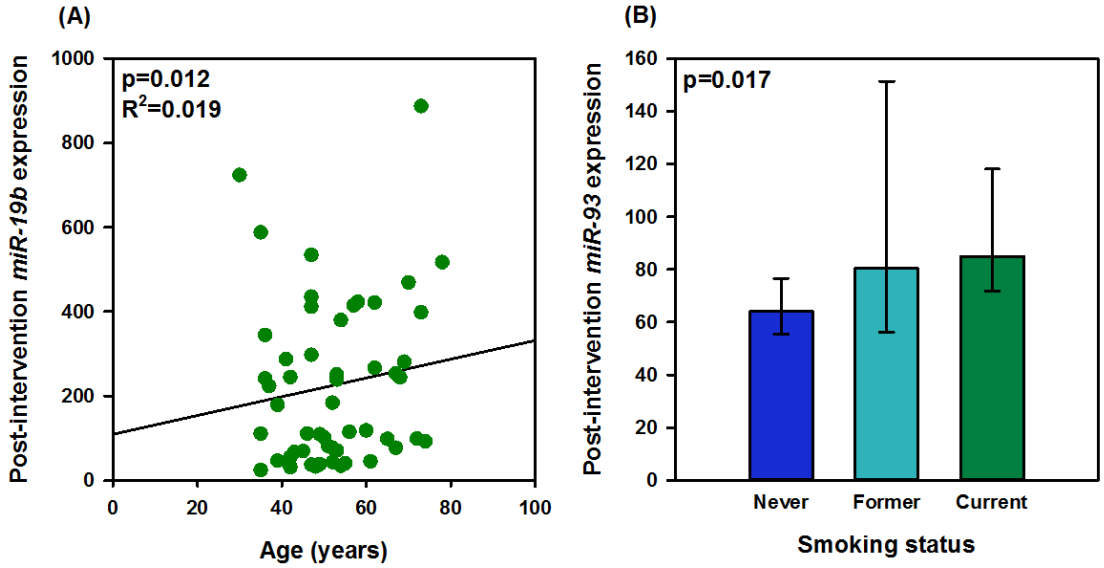
Table 4.9 P values for the effects of pre-intervention expression, age, gender, endoscopy procedure, BMI and smoking status on post-intervention miRNA expression.

miRNA	Pre-intervention expression	Age	Gender	Endoscopy procedure	BMI	Smoking status
***miR-17*[1]**	0.325	0.255	0.170	0.959	0.066	0.202
***miR-19a*[2]**	0.475	0.617	0.543	0.256	0.475	0.136
***miR-19b*[1]**	**<0.001**	**0.012**	0.355	0.703	0.144	0.055
***miR-20a*[2]**	0.475	0.738	0.430	0.233	0.475	0.091
***miR-25*[2]**	0.475	0.330	0.384	0.691	0.475	0.231
***miR-93*[2]**	0.475	0.717	0.543	0.621	0.475	**0.017**
***miR-106b*[2]**	0.474	0.510	0.866	0.118	0.474	0.070
***miR-424*[1]**	**<0.001**	0.406	0.513	0.074	0.837	0.649

Spearman correlation analyses revealed a positive correlation between age and post-intervention *miR-19b* expression (Spearman correlation coefficient of 0.179). Figure 4.7 (B) shows the correlation between the two following the removal of three outliers with very high post-intervention *miR-19b* expression. The Spearman correlation coefficient following the removal of the three outliers was 0.156.

Smokers, comprising both current and former smokers, had higher post-intervention *miR-93* expression by approximately 25% compared with participants who had never smoked Figure 4.7 (B).

Figure 4.7 Significant effects of age and smoking status on *miR-19b* and *miR-93* expression.



(A) Positive correlation between age and *miR-19b* expression. (B) *miR-93* expression by smoking status. Expression data are expressed as adjusted copies. Data are presented as median values following analysis using the Kruskal-Wallis test. Error bars represent lower and upper quartiles. N=56.

4.3.5 Discussion

miRNAs are small, non-coding RNAs that regulate gene expression at a post-transcriptional level, by resulting in either RNA degradation or by blocking translation into proteins. The expression of eight selected miRNAs was quantified by qPCR to investigate the effects of supplementation with RS and PD and to test the hypothesis that changes in gene expression may result from epigenetic regulation, such as by miRNAs. As *SFRP1* expression was downregulated by both RS and PD, miRNAs predicted to target *SFRP1* were selected.

Neither RS nor PD had effects on expression of the selected eight miRNAs. However, a trend for reduced expression of *miR-19a* and *miR-19b* by approximately 25% in participants supplemented with RS was observed. RS also appeared to reduce *miR-17* expression by approximately 11% compared with placebo.

Humphreys *et al.* have previously observed downregulation of miRNAs from the miR-17-92 cluster with butyrate treatment, including *miR-19a* and *miR-19b* that

were upregulated in HT-29 and HCT-116 CRC cells (Humphreys *et al.*, 2013). An *in vivo* study by this group also observed a significant reduction in *miR-17, miR-19a* and *miR-19b* by approximately 20% with a HRM diet supplemented with butyrylated RS compared with the HRM diet alone (Humphreys *et al.*, 2014).

In contrast, the opposite effect was observed for PD, with a trend for an increase in *miR-424* and *miR-19b* expression by approximately 20% and 13% respectively compared with placebo. Both *miR-19b (*Tsuchida *et al.,* 2011*)* and *miR-424* (Wang *et al.*, 2010) have been reported to be upregulated in colonic cancer tissue compared with adjacent normal tissue.

A significant effect of age was observed on the post-intervention expression of *miR-19b* in the rectal mucosa. This finding is consistent with results from a study by Hackl and colleagues where a reduction in *miR*-19b expression was observed in six cell ageing models (Hackl *et al.*, 2010). The post-intervention expression of *miR-93* was significantly lower in smokers compared with participants who had never smoked. This has not been reported previously.

4.4 The effects of supplementation with RS and PD on *SFRP1* DNA methylation in the human colorectal mucosa

4.4.1 Introduction

Another epigenetic mechanism that regulates gene expression is DNA methylation. DNA methylation is the addition of a methyl group to the carbon-5 position within a cytosine residue (Smith and Meissner, 2013). A hypermethylated state is associated with inhibition of transcription and consequently gene silencing. As both RS and PD reduced expression of *SFRP1*, the DNA methylation state of *SFRP1* was assessed to test the hypothesis that changes in gene expression may have resulted from alterations in *SFRP1* methylation. Furthermore, *SFRP1* is frequently downregulated in CRC due to DNA hypermethylation.

4.4.1.1 SFRP1

SFRP1, encoding secreted-Frizzled-related protein 1, is one of five members of the SFRP family of WNT pathway antagonists (Mao *et al.*, 2010). *SFRP1* is located at the human chromosome 8, p12-11.1 and encodes the 35.4kDA protein (Mao *et al.*, 2010). It inhibits WNT pathway activation by competing with the Frizzled receptor for binding to WNT ligands (Bafico *et al.*, 1999). In addition, SFRP1 can also directly bind to Frizzled receptors, thus inhibiting ligands from binding and preventing WNT pathway activation (Mao *et al.*, 2010).

4.4.1.2 SFRP1 methylation state in colorectal carcinogenesis

SFRP1 expression has been observed to be reduced in CRC and *SFRP1* has been identified as a tumour suppressor (Caldwell *et al.*, 2004). A reduction in *SFRP1* expression has correlated with, and is believed to result from, DNA hypermethylation (Rawson *et al.*, 2011). Increased *SFRP1* methylation has been observed in ACF, colorectal adenomas and carcinomas compared with normal tissue, resulting in reduced *SFRP1* expression in these samples (Qi *et al.*, 2006). Epigenetic silencing of *SFRP1* results in increased WNT activity and has been proposed to be a contributing phenomenon in early colorectal tumourigenesis (Suzuki *et al.*, 2004).

A very recent meta-analysis investigated the role of *SFRP1* promoter methylation in colorectal carcinogenesis using eight cohort studies comprising a total of 942 CRC patients (Chen *et al.*, 2014b). Results from this meta-analysis revealed that *SFRP1* promoter methylation was significantly higher in cancer tissue compared with both adjacent and normal tissues.

SFRP1 hypermethylation has also been observed in the DNA isolated from stool samples of CRC patients (Salehi *et al.*, 2012). Consequently, Salehi *et al*. have proposed the use of *SFRP1* promoter methylation state as an epigenetic biomarker for the diagnosis of CRC.

A recent study by Lyu *et al.* in China investigated methylation levels of the *SFRP1* promoter region in serum samples collected from patients with sporadic CRC by methylation-specific PCR (Lyu *et al.*, 2014). Similar to findings from studies performed in colorectal tissue and stool samples, *SFRP1* methylation was significantly higher, by 30.5%, in the sera from cancer patients compared with healthy participants.

4.4.1.3 Effect of NDCs and butyrate on SFRP1 methylation

To date, only one study has reported an effect of butyrate on the methylation state of *SFRP1.* This study was conducted by Shin *et al.* and investigated the epigenetic mechanisms by which sodium butyrate altered *SFRP1* expression in human gastric cancer cells (Shin *et al.*, 2012). Expression of *SFRP1,* and *SFRP2*, was quantified in nine gastric cancer cell lines by qPCR. None of the nine cell lines showed *SFRP1* expression, however, treatment of the cells with an inhibitor of DNA methyltransferase, 5'-Aza-dC, resulted in restored *SFRP1* expression, suggesting that *SFRP1* silencing resulted from hypermethylation. The authors then investigated the effects of treatment with 2μM sodium butyrate in two gastric cancer cell lines: AGS and MKN45. They observed *SFRP1* promoter demethylation following sodium butyrate treatment and this correlated with restored *SFRP1* expression.

In humans, a double-blind, placebo-controlled, crossover trial by Worthley *et al.* published in 2009 did not report an effect of a four-week RS diet, or of probiotic or symbiotic supplementation, on *SFRP1* methylation in rectal mucosal biopsies (Worthley *et al.*, 2009).

4.4.2 Hypotheses, Aims and Objectives

4.4.2.1 Hypothesis

The hypothesis for this study was that reduced expression of *SFRP1* in participants supplemented with RS and with PD may be explained by alterations in *SFRP1* DNA methylation. More specifically, reduced expression of *SFRP1* in participants supplemented with RS and with PD may have resulted from *SFRP1* hypermethylation.

4.4.2.2 Aims

This study aimed to test this hypothesis by investigating the effects of supplementing healthy human participants with NDCs on the methylation state of *SFRP1* in the colorectum.

4.4.2.3 Objectives

- To select a primer assay, within the promoter region and encompassing as many CpG sites as possible, to be used for the assessment of *SFRP1* methylation by pyrosequencing;
- To bisulphite-modify rectal mucosal DNA previously extracted by members of the DISC Study team;
- To amplify the specific region to be sequenced by PCR;
- To perform a validation pyrosequencing experiment using standard curves to test the quality of the primer;
- To quantify the methylation of *SFRP1* at seven CpG sites by pyrosequencing;
- To investigate the effects of the supplementation with NDCs on *SFRP1* methylation at each CpG site and on the mean methylation across all sites;
- To explore the effects of key epidemiological factors including age and gender on *SFRP1* methylation.

4.4.3 Methods

The methods used for the quantification of *SFRP1* methylation can be found in section 2.2.3. DNA, extracted by members of the DISC Study team, was first bisulphite modified. PCR was utilised to amplify the specific region to be analysed by PCR. *SFRP1* methylation was quantified at each CpG site by pyrosequencing.

The effects of the interventions were determined by analysis of post-intervention methylation data using the ANOVA GLM, with the corresponding pre-intervention value as a covariate. Other covariates included in the analyses were age, gender, endoscopy procedure, BMI and smoking status.

4.4.4 Results

Quantification of *SFRP1* methylation was performed at seven CpG sites within the promoter region by pyrosequencing. Prior to this, an assay validation experiment was performed using standard curves.

4.4.4.1 Assay Validation

SFRP1 assay validation was performed using both pre- and post-PCR dilutions. SigmaPlot 11.0 was used to plot the results with a line of best fit and to determine the coefficient of determination (R^2). The R^2 values for all of the regression analyses comparing expected and observed methylation values were >0.98 (Figure 4.8 and Figure 4.9).

Figure 4.8 Pre-PCR standard curves for the *SFRP1* pyrosequencing assay validation at all seven CpG sites.

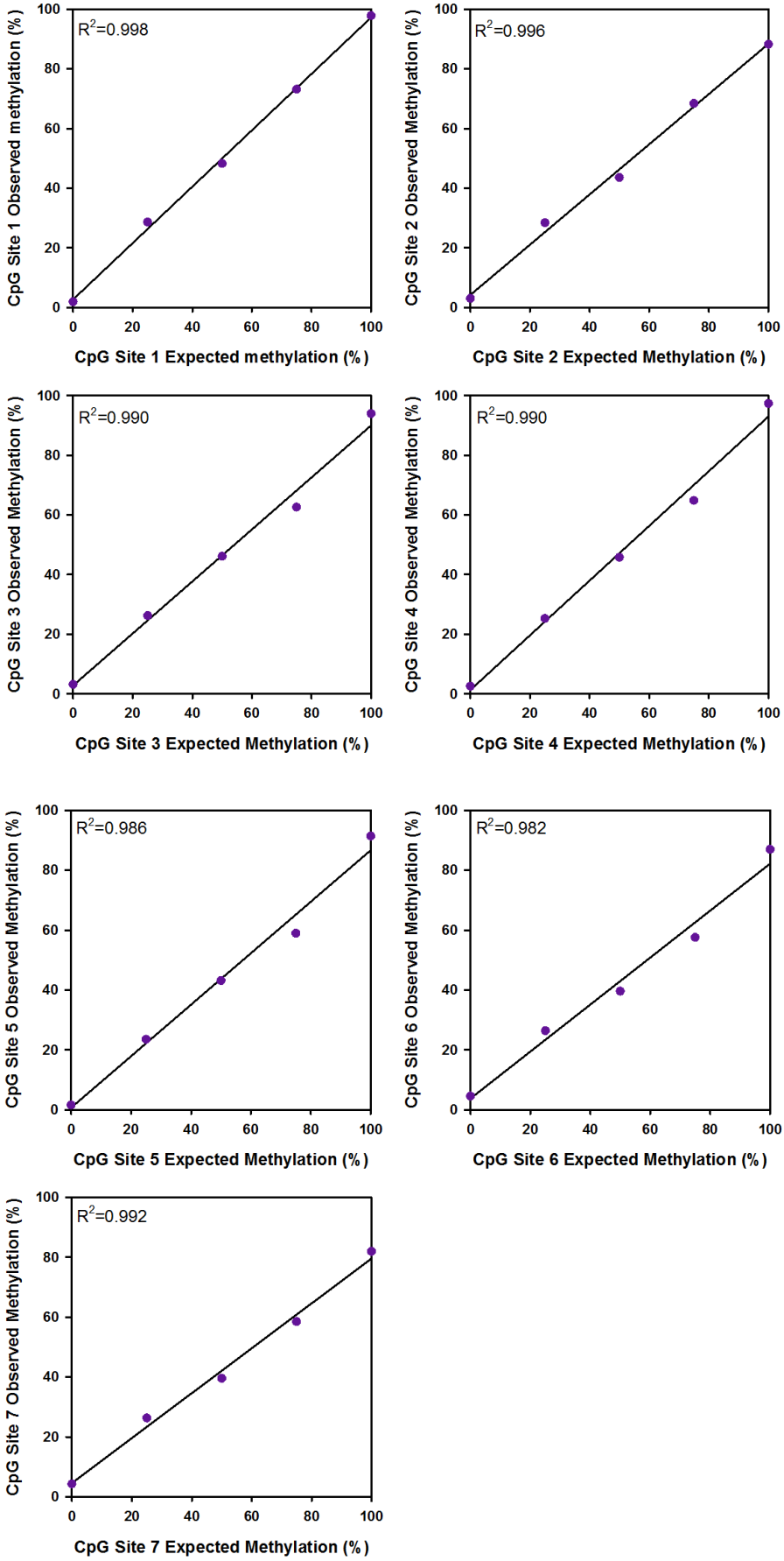
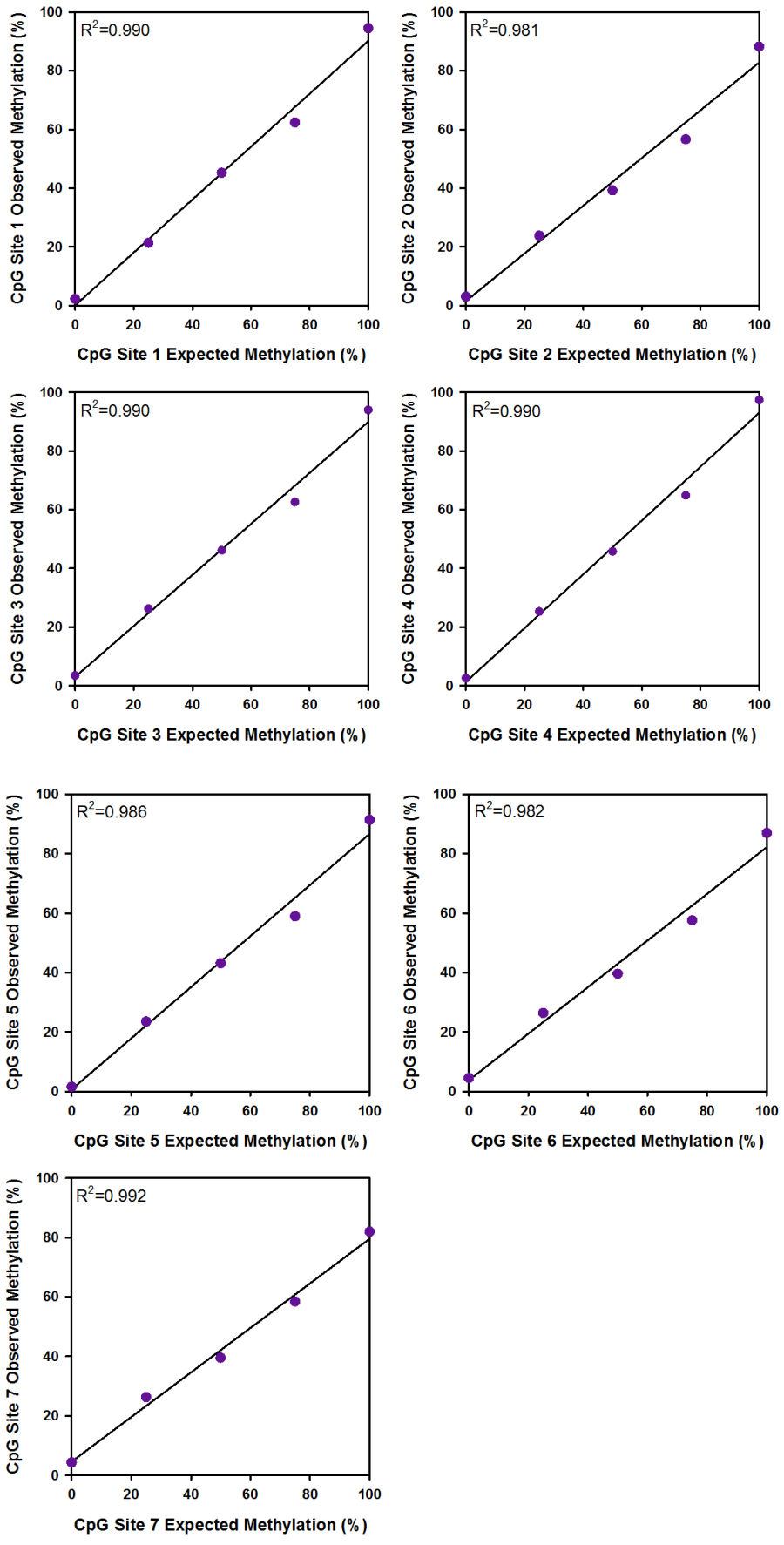


Figure 4.9 Post-PCR standard curves for the *SFRP1* pyrosequencing assay validation at all seven CpG sites.



4.4.4.2 Effects of non-digestible carbohydrates on SFRP1 Methylation

SFRP1 DNA methylation, quantified at the seven CpG sites within the promoter region, was moderate, with a mean pre and post-methylation of 26.3% and 29.6% respectively across all groups. Mean pre-intervention methylation across all groups was lowest at CpG site 1 (29.8%) and highest at site 7 (29.9%). Mean post-intervention methylation across all groups was lowest at CpG site 5 (25.6%) and highest at CpG site 2 (33.3%). Across all seven CpG sites and all four intervention groups, pre-intervention methylation ranged from 13.6% to 44.9% and post-intervention methylation ranged from 13.2% to 56.1%.

There were no statistically significant effects of RS or PD on *SFRP1* methylation at any of the CpG sites or on the mean methylation (Table 4.10). Participants supplemented with PD appeared to have reduced *SFRP1* methylation at CpG sites 4, 5, 6 and 7 and a close to significant effect of PD was observed on the mean methylation across all CpG sites (p=0.055).

Table 4.10 Effects of supplementation with RS and PD on *SFRP1* methylation in the human rectal mucosa.

	RS allocation LSM (95% CIs)			**PD allocation** LSM (95% CIs)			**Interaction between RS and PD**
CpG site	**Amioca (placebo)**	**Hi-Maize® 260 (RS)**	**P value**	**Maltodextrin (placebo)**	**Litesse® Ultra™ (PD)**	**P value**	**P value**
1 N=66	31.7 (30.1 – 33.5)	32.6 (30.7 – 34.6	0.526	33.4 (31.5 – 35.4)	31.0 (29.4 – 32.7)	0.075	0.811
2 N=66	25.2 (23.9 – 26.5)	25.9 (24.6 – 27.3)	0.456	26.0 (24.7 – 27.4)	25.1 (23.8 – 26.4)	0.348	0.601
3 N=66	23.9 (22.4 – 25.7)	24.4 (22.8 – 26.3)	0.705	25.2 (23.5 – 27.0)	23.2 (21.7 – 25.0)	0.129	0.646
4 N=67	31.9 (30.3 – 33.6)	33.0 (19.5 – 57.0)	0.417	33.3 (31.6 – 35.1)	31.5 (29.8 – 33.2)	0.149	0.520
5 N=63	23.8 (22.4 – 25.4)	24.7 (23.0 – 26.4)	0.500	25.1 (23.4 – 26.9)	23.5 (22.0 – 25.1)	0.181	0.514
6 N=62	30.1 (28.5 – 31.7)	30.5 (28.8 – 32.2)	0.714	31.3 (29.7 – 33.1)	29.3 (27.7 – 31.0)	0.097	0.453

	RS allocation LSM (95% CIs)			PD allocation LSM (95% CIs)			Interaction between RS and PD
CpG site	**Amioca (placebo)**	**Hi-Maize® 260 (RS)**	**P value**	**Maltodextrin (placebo)**	**Litesse® Ultra™ (PD)**	**P value**	**P value**
7 N=58	31.1 (29.1 – 33.1)	32.4 (30.4 – 34.4)	0.404	32.8 (30.8 – 34.8)	30.7 (28.6 – 32.7)	0.152	0.605
Mean N=63	29.0 (27.3 – 30.7)	28.8 (27.1 – 30.7)	0.954	30.2 (28.5 – 32.0)	27.7 (26.0 – 29.4)	0.055	0.777

Data are expressed LSMs and 95% CIs for percentage methylation (%) at each CpG site and the mean across all seven sites.

4.4.4.3 Exploration of additional factors influencing SFRP1 methylation

The effect of pre-intervention *SFRP1* methylation, age, gender, endoscopy procedure, BMI and smoking status on post-intervention methylation as covariates were analysed for each CpG site and mean *SFRP1* methylation using the ANOVA GLM. Gender, endoscopy procedure, BMI and smoking status did not affect post-intervention *SFRP1* methylation.

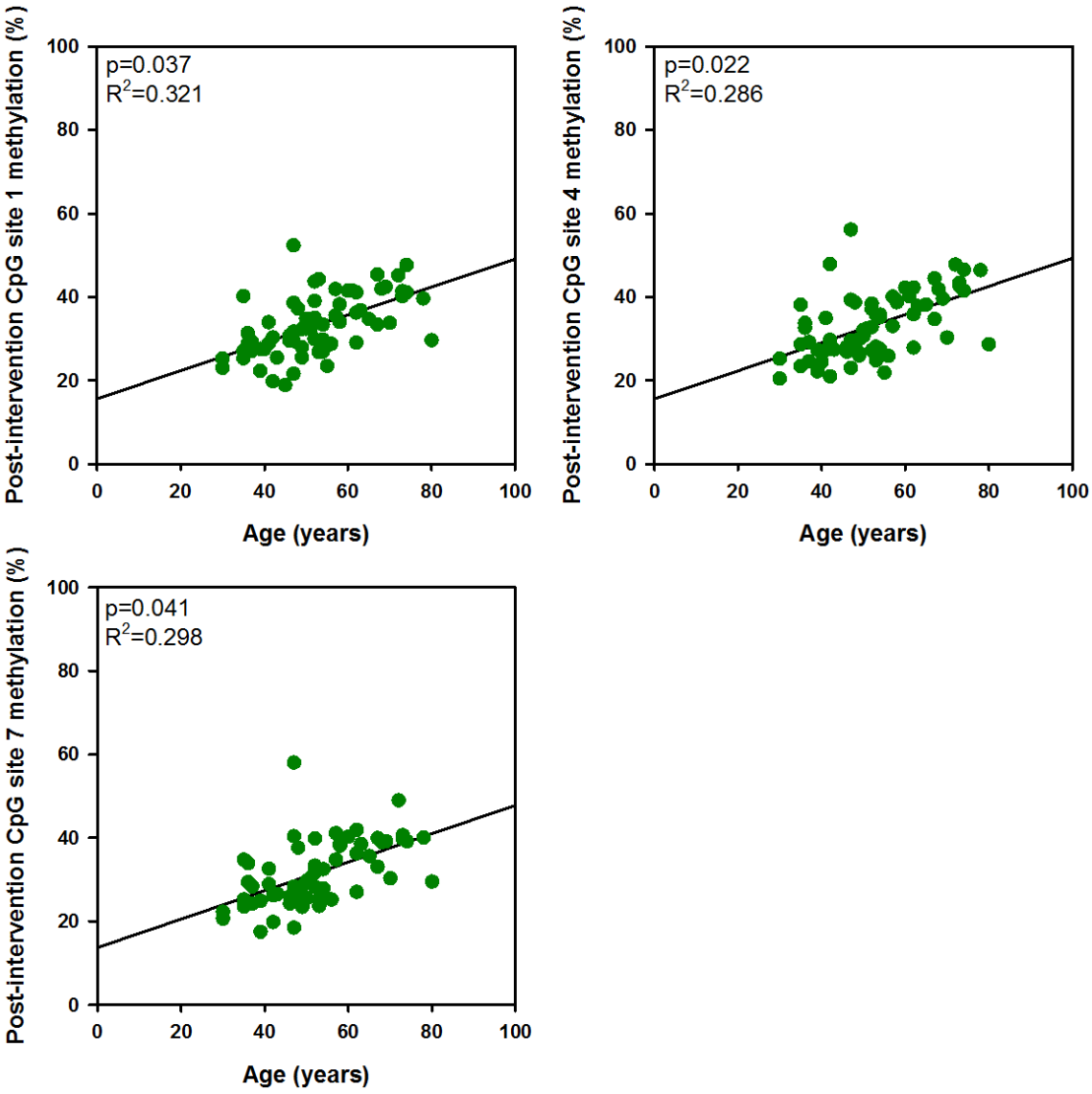
Post-intervention *SFRP1* methylation was significantly affected by pre-intervention levels at all sites and for the mean methylation across all sites ($p<0.001$) (Table 4.11). Age was also a determinant of post-intervention methylation at CpG sites 1 ($p=0.037$), 4 ($p=0.022$) and 7 ($p=0.041$) but did not affect the mean methylation.

Table 4.11 P values for the effects of pre-intervention methylation, age, gender, endoscopy procedure, BMI and smoking status on post-intervention *SFRP1* methylation.

CpG Site	Pre-intervention methylation	Age	Gender	Endoscopy procedure	BMI	Smoking status
1	**<0.001**	**0.037**	0.447	0.116	0.557	0.300
2	**<0.001**	0.110	0.341	0.310	0.251	0.170
3	**<0.001**	0.352	0.745	0.354	0.275	0.341
4	**<0.001**	**0.022**	0.709	0.171	0.179	0.949
5	**<0.001**	0.062	0.597	0.523	0.194	0.208
6	**<0.001**	0.070	0.499	0.225	0.146	0.542
7	**<0.001**	**0.041**	0.535	0.084	0.336	0.879
Mean	**<0.001**	0.119	0.602	0.553	0.155	0.535

Spearman correlation analyses revealed positive correlations between age and post-intervention *SFRP1* methylation at CpG sites 1, 4 and 7, with Spearman correlation coefficients of 0.596 ($p<0.001$), 0.555 ($p<0.001$) and 0.606 ($p<0.001$) respectively (Figure 4.10).

Figure 4.10 Positive correlations between age and *SFRP1* methylation for CpG sites 1, 4 and 7.

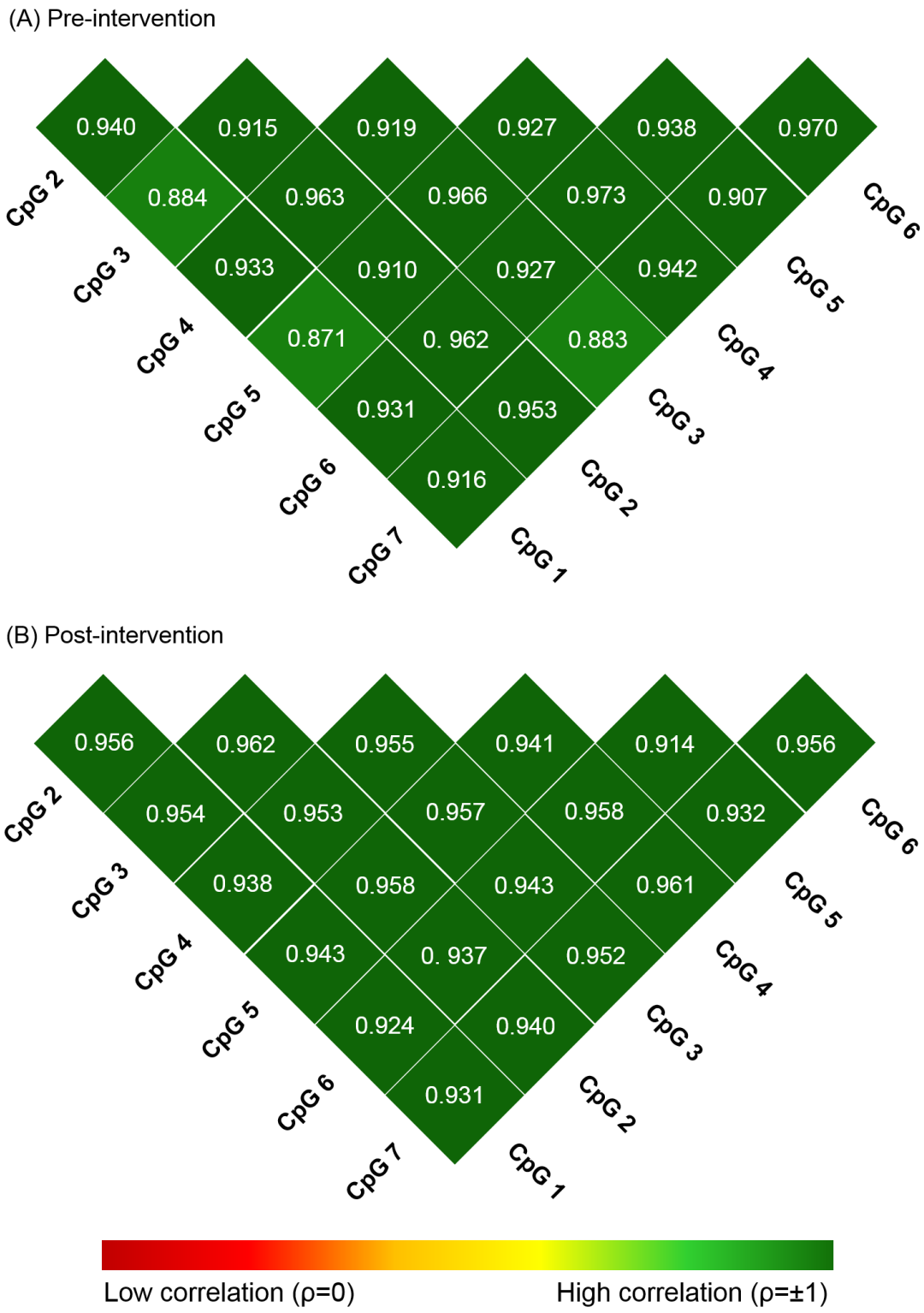


Correlation between age and post-intervention methylation. (A) N=66, (B) N=67 and (C) N=58.

4.4.4.4 Correlation of SFRP1 methylation between CpG sites

Spearman correlation analyses were used to investigate the correlation between levels of *SFRP1* methylation between the seven CpG sites. Both pre- and post-intervention, *SFRP1* methylation was strongly correlated between the seven CpG sites (Figure 4.11).

Figure 4.11 Correlation of *SFRP1* methylation between CpG sites.



Values represent Spearman correlation coefficients (ρ) pre- (A) (N=69) and post-intervention (B) (N=64). P<0.001.

4.4.4.5 Correlation between SFRP1 methylation and expression

Spearman correlation analyses were used to investigate whether levels of DNA methylation correlated with *SFRP1* expression. Inverse correlations between pre-intervention *SFRP1* methylation and expression were observed at each CpG site and the overall mean (Table 4.12). Although this was not statistically significant, a close to significant p value was observed at CpG sites 4 and 5.

Interestingly, the opposite trend was observed for the post-intervention data, where DNA methylation correlated positively with *SFRP1* expression. This was statistically significant at CpG sites 1 (p=0.033), 3 (p=0.008), 6 (p=0.030) and 7 (p=0.030) (Table 4.12).

Table 4.12 Correlation between pre- and post-intervention *SFRP1* methylation and gene expression at each CpG site and for the mean across all sites.

	Pre-intervention		Post-intervention	
CpG site	**Spearman correlation coefficient**	**P value**	**Spearman correlation coefficient**	**P value**
1	-0.189	0.260	0.357	**0.033**
2	-0.294	0.073	0.309	0.071
3	-0.284	0.087	0.427	**0.008**
4	-0.304	0.063	0.300	0.071
5	-0.308	0.063	0.328	0.051
6	-0.282	0.095	0.367	**0.030**
7	-0.226	0.190	0.383	**0.030**
Mean	-0.200	0.240	0.313	0.067

Spearman correlation coefficients and corresponding p values for the correlation between *SFRP1* methylation and expression pre- and post-intervention. N=37.

4.4.5 Discussion

Gene expression may be regulated by epigenetic mechanisms such as DNA methylation. DNA hypermethylation is associated with reduced transcription and therefore a reduction in gene expression. To test the hypothesis that the observed effects of RS and PD on *SFRP1* expression may have resulted from effects on the methylation state of *SFPR1*, DNA methylation was quantified at seven CpG sites within the promoter region of *SFRP1*.

RS and PD did not have a significant effect on *SFRP1* methylation at any of the seven CpG sites or on the mean methylation across all sites. This suggests that changes in *SFRP1* gene expression resulted from mechanisms other than DNA methylation. However, a close to significant effect of PD was observed on the mean *SFRP1* methylation across all seven CpG sites (p=0.055). Mean *SFRP1* methylation appeared to be lower in participants supplemented with PD, which would be associated with an increase in gene expression. However, this is the opposite to findings from the analyses on *SFRP1* expression, where PD supplementation downregulated *SFRP1.*

Methylation at the seven CpG sites analysed correlated strongly, as investigated using the Spearman correlation analysis. Despite this, the effects of the intervention on *SFRP1* methylation were analysed at each CpG site as previous studies have shown that differences in methylation at specific, but not all, CpG sites may be observed between treatment groups. For example, Weaver *et al.* showed that nerve growth factor-inducible protein A methylation at CpG site 16 was increased in the offspring of high pup licking and grooming and arched-back nursing rat mothers whereas this is rarely methylated in rats with low levels of pup licking and grooming (Weaver *et al.*, 2004). In contrast, methylation at CpG site 17 does not differ with differences in maternal care. In addition, the biological relevance of methylation at each CpG site may vary due to differences in the proximity to transcription factor binding sites.

To further investigate the correlation between DNA methylation and expression levels, the Spearman correlation analysis was run on both pre and post-intervention data. A trend for an inverse correlation between *SFRP1* methylation and gene expression was observed for the pre-intervention data. However, the opposite was apparent for the post-intervention data, where a positive

correlation between post-intervention methylation at CpG sites 1, 3, 6 and 7 and *SFRP1* expression was observed. This is contrary to reports in the literature that show a reduction in gene expression with increased DNA methylation.

The effects of age, gender, endoscopy procedure, BMI and smoking status on post-intervention *SFRP1* methylation were also explored. Post-intervention methylation was significantly affected by pre-intervention methylation levels at each site and the mean methylation across all sites. In addition, positive correlations between age and post-intervention *SFRP1* methylation at CpG sites 1, 4, and 7 were observed. These findings are consistent with results from previous studies where *SFRP1* promoter methylation was observed to be more common in CRC patients aged over 50 years (Rawson *et al.*, 2011). A significant correlation between age and *SFRP1* methylation has also been reported in the distal, but not proximal, normal-appearing mucosa (An *et al.*, 2010).

4.5 The effects of supplementation with RS and PD on colonic crypt cell proliferative state in the human colorectal mucosa

4.5.1 Introduction

Regulation of cell proliferation within the crypt is imperative for the maintenance of homeostasis within the colon. Importantly, altered cell proliferation can lead to an imbalance in cell proliferation and apoptosis leading to a hyperproliferative state that may promote colorectal tumourigenesis. Here I report the results of the effects of supplementation with RS and PD on the colonic crypt cell proliferative state (CCPS) in microdissected, Schiff reagent-stained human rectal biopsies.

4.5.1.1 Effects of resistant starch on crypt cell proliferation in the healthy colon

To date, the majority of studies exploring the effects of supplementation with RS on cell proliferation in the healthy colon have reported an increase in cell proliferation or have not observed any effects. On the contrary, a very early study reported a reduction in cell proliferation in 14 healthy participants supplemented with 45g of native amylomaize, equivalent to 28g of type 2 RS, for two weeks (van Munster *et al.*, 1994).

Mentschel and colleagues have investigated the effects of supplementation with resistant potato starch, a type 2 RS, for 19 days in 12 male pigs (Mentschel and Claus, 2003). Faecal butyrate concentrations were significantly higher in pigs fed the RS diet compared with the control group. Although the crypt length was significantly longer with RS supplementation, there was no effect observed on overall total mitosis, determined by histocytochemical analysis of Ki-67. However, when dividing the crypt into thirds and determining levels of mitosis in each compartment, the investigators found that pigs fed RS had increased cell proliferation in the middle and luminal compartments. On the contrary, cell proliferation was reduced in the lower stem cell compartment in pigs fed RS.

In a small human study, 12 healthy participants consumed a control diet supplemented with corn starch (low RS) for four weeks, followed by a six-week washout period and another four weeks of control diet supplemented with up to

59.7g of highly resistant amylomaize starch per day (Wacker *et al.*, 2002). There were no significant differences in rectal colonic crypt cell proliferation levels, measured by bromodeoxyuridine labelling, observed between the low and high RS diet periods.

A randomised, double-blind, placebo-controlled, four-week crossover trial supplemented 20 healthy participants with RS, in the form of 25g/day of high-amylose maize starch (HAMS), and/or 5g/day of *Bifidobacterium lactis* (Worthley *et al.*, 2009). Again, the investigators did not observe an effect of either intervention on colonic crypt cell proliferation, assessed by immunohistochemical analysis of Ki-67.

4.5.1.2 Effects of resistant starch on crypt cell proliferation in the diseased colon

The majority of studies that have investigated the effects of RS on cell proliferation in the diseased colon used animal models of CRC, such as carcinogen-treated rats. Most of these studies have reported a reduction in crypt cell proliferation with RS administration.

An *in vivo* study investigated the effects of RS administration, in the form of HAMS, on AOM-induced colorectal carcinogenesis in rats (Le Leu *et al.*, 2007). Rats supplemented with both doses of RS (10% and 20%) showed significantly reduced proliferation in the distal colon, as indicated by a decrease in proliferating cell nuclear antigen labelling. SCFA analyses revealed that RS increased butyrate concentrations in the caecum, proximal colon, distal colon and in faeces and that this was statistically significant for the higher dose of RS (20% HAMS).

A more recent study, using the AOM-induced rat model of colorectal carcinogenesis, compared levels of colonic proliferation and apoptosis in rats fed a low RS diet, a low RS diet plus tributyrin, a HAMS diet or a 10% butyrylated HAMS (HAMSB) diet (Clarke *et al.*, 2012). The HAMSB diet yielded the highest colonic butyrate concentrations compared with the other three diet groups. Results from this study showed a trend for a reduction in cell proliferation, measured by immunohistochemical staining of Ki-67, in the distal colon of rats on the HAMS and HAMSB diets. Rats on the low RS diet

supplemented with tributyrin, a pro-drug of butyrate, also appeared to have lower levels of proliferation.

Another study investigated the effects of a RS type 3 in a chemically-induced rat model of colon carcinogenesis (Bauer-Marinovic *et al.*, 2006). Proliferation was assessed by immunohistochemical staining of Ki-67. The investigators observed a significant reduction in the number of proliferating cells, the size of the proliferative compartment and the crypt length in the distal, but not proximal, colon of rats fed the RS compared with standard diet.

In another model of colorectal tumourigenesis, Young *et al.* compared the effects of a control diet, a raw potato starch (type 2 RS) diet and a diet with both potato starch and wheat bran (a non-starch polysaccharide) in DMH-treated rats (Young *et al.*, 1996). Crypt depth was 15% longer in rats fed both potato starch diets compared with the control diet and this was associated with a significant increase in the number of cells entering metaphase during a three hour period, assessed histologically by haematoxylin and eosin staining.

Winter *et al.* investigated the effects of RS in a Western diet model of spontaneous CRC, where mice were given a control diet or a diet containing heme, RS or heme plus RS (Winter *et al.*, 2014). The diets containing RS significantly increased the faecal concentrations of butyrate, acetate and propionate both after four weeks and 18 months on the diet. Short-term data, after four weeks, indicated that mice fed the heme-rich diet had significantly increased cell proliferation in the colon. The investigators did not observe an effect of short-term supplementation with RS on cell proliferation in the colon. However, they found that RS significantly increased crypt height. Long-term analyses, following 18 months on the respective diets, showed that mice on the RS diets had both significantly increased colonic cell proliferation and increased crypt height.

A two part, randomised and blinded study in rats was designed by Perrin and colleagues to mimic a human prospective study (Perrin *et al.*, 2001). Mucosal proliferation was assessed after 44 days on the control diet or one of the following three high fibre diets: starch-free wheat bran, short-chain fructo-oligosaccharides or RS type 3. There were no differences in proliferation observed between the low and higher fibre diets, or between the high (RS and

short-chain fructo-oligosaccharides) and low (control and starch-free wheat bran) butyrate-producing diets.

Studies investigating the effects of RS in the diseased colon in humans with colorectal illnesses, however, have yielded conflicting results. Although a couple of studies have shown that RS consumption in humans resulted in reduced colonic crypt cell proliferation, many have not observed an effect of RS on proliferation.

In a RCT undertaken in 65 patients with CRC or symptoms of CRC, Dronamraju and colleagues investigated the effects of RS supplementation for up to four weeks on cell proliferation, assessed by whole-crypt microdissection of Schiff reagent-strained crypts (Dronamraju *et al.*, 2009). The trial comprised two separate studies, and there were no effects of RS observed on post-intervention total mitoses or crypt measurements in either study. However, supplementation with RS reduced the proportion of mitotic cells in the top half of the crypt, and this was statistically significant in Study 1 ($p=0.028$). When data from both studies were pooled, the authors observed a 44% reduction in the proportion of mitotic cells in the top half of the crypt in participants supplemented with RS compared with ordinary starch ($p=0.07$).

A more recent study by Burn *et al.* randomised 206 FAP patients from 12 international centres to one of the four following intervention groups: aspirin plus RS, aspirin plus RS placebo, aspirin placebo plus RS or aspirin placebo plus RS placebo (Burn *et al.*, 2011a). The investigators assessed proliferation by direct counting of mitotic cells in microdissected crypts and immunohistochemical analysis of Ki-67. A trend for an increased number of mitotic cells in participants given RS compared with the non-RS groups was observed ($p=0.12$), but this was not reflected in the data from the immunohistochemical analysis. There were no effects of RS on colonic crypt dimensions, although the authors did observe a significant reduction in mean crypt length over time in participants given RS compared with those given RS placebo ($p<0.001$).

A study by Grubben *et al.* investigated the effects of RS on cell proliferation in 23 patients with recently removed adenomas (Grubben *et al.*, 2001). Participants were stratified by gender and randomised to the control group or

the RS group, where they were supplemented with 45g of high amylomaize starch, equating to 28g of RS type 2, for four weeks. However, the investigators did not observe significant differences in cell proliferation between the two groups.

A similar finding was observed in a randomised, double-blind, placebo-controlled trial that recruited 111 patients with sporadic adenomas (van Gorkom *et al.*, 2002). Participants were randomised to the control group (two placebos), the calcium group (1g calcium plus RS placebo) or the RS group (30g amylomaize, equating to 19g of RS, plus calcium placebo) for two months. The investigators did not observe any differences in total or luminal proliferation, measured by bromodeoxyuridine incorporation, between the three intervention groups at any of the four sites (caecum, transverse colon, sigmoid colon or rectum).

4.5.1.3 Effects of polydextrose on colonic crypt cell proliferation in the colon

To date, studies investigating the effects of PD on colonic crypt cell proliferation are limited. *In vitro,* Putaala and colleagues used Enteromix®, an *in vitro* semi-continuous colon simulator comprising four vessels that mimic the human proximal, ascending, transverse and distal colon, to simulate the fermentation of PD and produce the PD metabolome (Putaala *et al.*, 2011). The latter was then used to treat Caco-2 human colonic adenocarcinoma cells to investigate the effects of the fermented PD, as well as 5mM butyrate and 100mM NaCl, on Caco-2 cells. After 24 hours, treatment with the fermentation metabolome from 2% PD retrieved from the proximal, ascending and distal colon vessels resulted in significantly reduced levels of proliferation compared with 0% PD.

In vivo, an animal study investigated the effects of PD in the large intestine of Sprague-Dawley rats (Yoshioka *et al.*, 1994). The first experiment divided 62 rats into six groups which administered three different doses of PD, cellulose or galatomannan. The second experiment further investigated the effects of different doses (5, 10 and 20%) of PD or a fibre-free diet in 35 rats. The authors used haematoxylin and eosin staining of caecal and colonic samples to assess crypt depth and muscular layer thickness but did not measure cell proliferation directly. Results from the first experiment did not report a significant difference

in caecal or colonic crypt lengths between the six groups. However, in the second experiment, the investigators observed significantly longer crypts in the caecum of rats given the highest PD dose (20%) compared with the lowest PD dose (5%) and the fibre-free diet.

A Chinese randomised, double-blind, placebo-controlled human study explored the effects of supplementation with three doses of PD (4g PD/day, 8g PD/day or 12g PD/day) or control for 28 days in 120 healthy participants (Jie *et al.*, 2000). Proliferation in the caecum was assessed using light microscopy following staining of samples with Schiff reagent. PD increased post-intervention caecal butyrate concentrations compared with pre-intervention levels and compared with the control group, and this was statistically significant for the two highest PD doses. All three PD doses resulted in significantly higher post-intervention total whole crypt proliferation compared with pre-intervention levels and the control group.

4.5.1.4 Effects of butyrate on crypt cell proliferation in the healthy colon

In the healthy colon, or in *in vitro* studies using normal cells, butyrate has either produced no effect on proliferation or has resulted in increased cell proliferation. *In vivo,* a study in piglets investigated the effects of colon-delivered butyrate on colonic cell proliferation (Kien *et al.*, 2007). Twenty-four piglets were randomised to either the control group, the caecal butyrate infusion group, the inulin supplementation group or the caecal butyrate infusion plus inulin supplementation group. Cell proliferation was assessed by bromodeoxyuridine labelling and crypt measurements were taken. The proliferative index in piglets in the caecal butyrate infusion group was significantly higher in the caecum and distal colon compared to the control group.

In humans, Mortensen *et al.* investigated the effects of butyrate administered directly to the rectum via Hartmann's procedure in eight patients (Mortensen *et al.*, 1999). Participants were given 100ml of placebo for 14 days followed by 100ml of SCFAs for 14 days in a blinded manner. Cell proliferation was determined by immunohistochemical analysis of Ki-67 in rectal biopsies. A significant increase in proliferation following Hartmann's procedure was observed in the rectal mucosa post-SCFA treatment compared with baseline

and post-placebo levels. Furthermore, the majority of the increase in proliferating cells was observed in the upper two thirds of the crypt.

Another study investigated the effects of three SCFAs at physiological concentrations utilising caecal biopsies taken from 45 healthy participants (Scheppach *et al.*, 1992). Tissues were incubated for three hours with acetate, propionate and butyrate, both in combination and individually. Caecal proliferation was assessed autoradiographically by thymidine incorporation analyses and was increased by all SCFAs both individually and in combination. Butyrate (10mmol/L) significantly increased cell proliferation by 89%, which was comparable to the increase achieved following combined incubation with all three SCFAs. When the crypts were divided into five compartments of equal size, the observed increase in proliferation was restricted to the basal three compartments.

4.5.1.5 Effects of butyrate on crypt cell proliferation in the diseased colon

In the diseased colon however, butyrate results in the opposite effect and has been reported to reduce cell proliferation. This has been named ‘the butyrate paradox’.

In a study by Hodin and colleagues, cell proliferation following butyrate treatment was assessed in HT-29 cells by both cell counts and thymidine incorporation analyses (Hodin *et al.*, 1996). Significantly reduced cell proliferation, as demonstrated by a 32% decrease in total cell number, was observed in HT-29 cells treated with 5mM butyrate for 48 hours compared with the control.

In vivo, Clarke *et al.* investigated the effects of increasing colonic butyrate concentrations through the delivery of butyrylated starch on colonic rates of apoptosis and proliferation in AOM-treated rats (Clarke *et al.*, 2012). The rats fed a 10% HAMSB diet had higher colonic digesta and plasma SCFA concentrations compared with the other three groups that were not given butyrate. Furthermore, the authors observed a correlation between luminal butyrate concentrations and an increase in apoptosis in the distal colon of the HAMSB rats. In addition, this increase in apoptotic rates occurred

predominantly in the lower, normally proliferative, crypt compartments. However, there were no effects of butyrate on the rates of proliferation.

To try and explain the contradictory outcomes observed by butyrate, Comalada *et al.* investigated the effects of butyrate treatment in foetal human normal colon cells and in colon adenocarcinoma HT-29 cells (Comalada *et al.*, 2006). An inhibitory effect of butyrate was observed on the proliferation of HT-29 cells. However, in the normal, healthy cells, the authors did not observe an effect of butyrate, or the other tested SCFAs, on cell proliferation at any of the tested concentrations.

4.5.2 Hypotheses, Aims and Objectives

4.5.2.1 Hypotheses

The hypothesis for this study was that NDCs would reduce CCPS and, in particular, decrease the proportion of mitotic cells in the top half of the crypt.

4.5.2.2 Aims

This study aimed to test this hypothesis by investigating the effects of supplementing healthy participants with NDCs on CCPS and crypt dimensions.

4.5.2.3 Objectives

- To stain, using Schiff reagent, and microdissect whole crypts from Carnoy's-fixed rectal biopsies;
- To count the number of mitotic cells in ten intact crypts from each participant;
- To record crypt measurements (length and width) from ten intact crypts from each participant;
- To calculate the proportion of mitotic cells in the top half of the crypts;
- To assess the effects of the intervention on CCPS and crypt dimensions;

- To explore the effects of key epidemiological factors including age and gender on CCPS and crypt dimensions.

4.5.3 Methods

The methods used for the assessment of CCPS can be found in section 2.2.4. Briefly, Carnoy's-fixed biopsies were hydrated, hydrolysed and stained in Schiff reagent prior to microdissection. CCPS was analysed in 38 participants, with ten intact crypts analysed per participant by light microscopy. The number of mitotic cells as well as crypt measurements were recorded.

4.5.4 Results

4.5.4.1 Baseline characteristics of intervention participants analysed for analysis of colonic crypt cell proliferation

Colonic crypt cell proliferation was analysed in a total of 38 participants comprising comparable numbers in each intervention group (Table 4.13). The characteristics of the participants in terms of age, gender, BMI, smoking status and endoscopy within each group were also representative of that observed in the complete set of 75 intervention participants.

Table 4.13 Baseline characteristics of intervention participants analysed for analysis of colonic crypt cell proliferation.

Intervention group	A	B	C	D
RS	**-**	**-**	**+**	**+**
PD	**-**	**+**	**-**	**+**
Number of participants	10	10	9	9
Ethnicity				
Caucasian (%)	9 (90)	9 (90)	9 (100)	9 (100)
Black African (%)	1 (10)	0 (0)	0 (0)	0 (0)
Mixed race (%)	0 (0)	1 (10)	0 (0)	0 (0)
Gender				
Female (%)	5 (50)	5 (50)	7 (78)	3 (33)
Male (%)	5 (50)	5 (50)	2 (22)	6 (67)
Age				
Mean (years)	47.6	62.3	53.6	52.6
Range	30 – 68	42 – 80	42 – 67	37 – 74
Smoking status				
Never (%)	8 (80)	6 (60)	6 (67)	4 (45)
Former (%)	1 (10)	2 (20)	2 (22)	3 (33)
Current (%)	1 (10)	2 (20)	1 (11)	2 (22)
BMI				
Mean (kg/m^2)	28.2	28.9	30.5	30.4
Range	23.2 – 36.2	23.2 – 38.4	26.3 – 34.6	23.4 – 41.8
Procedure				
Colonoscopy (%)	3 (30)	3 (30)	3 (33)	3 (33)
Flexible Sigmoidoscopy (%)	7 (70)	7 (70)	6 (67)	6 (67)

4.5.4.2 Effects of non-digestible carbohydrates on colonic crypt cell proliferative state

The colonic CCPS was analysed in ten crypts per participant using whole crypt microdissected Schiff reagent-stained rectal biopsies. Proliferation was assessed by counting the number of mitotic cells in ten equally-sized compartments per crypt and calculating the mean total number of mitotic cells per crypt and the percentage of mitotic cells in the top half of the crypts.

The data for the effects of supplementation with RS and PD on CCPS and crypt measurements are summarised in (Table 4.14). A significant effect of RS supplementation on the mean total number of mitotic cells per crypt ($p=0.03$) was observed. Participants supplemented with RS had significantly higher mean total mitoses counts, with a mean of 8 mitotic cells per crypt compared with 6 mitotic cells per crypt in participants given placebo. However, there was no significant effect of either RS or PD on the percentage of mitotic cells in the top half of the crypt.

When applying Bonferroni's correction for multiple testing, the set p-value is lowered to 0.01 and so the statistically significant effects of RS on the mean total number of mitotic cells per crypt ($p=0.03$) is lost.

In addition to mitotic counts, crypt measurements were also recorded in the form of crypt width and length, and the crypt volume was calculated. There were no effects of RS or PD observed on the crypt measurements.

Table 4.14 Effects of supplementation with RS and PD on CCPS and crypt dimensions. N=36.

	RS allocation LSM (95% CI)			**PD allocation** LSM (95% CI)			**Interaction between RS and PD**
	Placebo (Amioca)	**RS (Hi-Maize® 260)**	**P value**	**Placebo (Maltodextrin)**	**PD (Litesse® *Ultra*™)**	**P value**	**P value**
Total mitoses	6.20 (4.99 – 7.41)	8.33 (7.05 – 9.61)	**0.030**	7.37 (6.10 – 8.65)	7.15 (5.89 – 8.42)	0.821	0.685
Mitoses in top half of crypt (%)	4.44 (2.26 – 7.35)	4.54 (2.22 – 7.68)	0.960	4.29 (2.05 – 7.36)	4.69 (2.33 – 7.85)	0.848	0.270
Crypt length (µm)	540 (508 – 573)	532 (498 – 567)	0.765	538 (503 – 572)	535 (501 – 570)	0.930	0.612
Crypt width (µm)	117 (109 – 125)	120 (111 – 129)	0.648	115 (106 – 123)	122 (114 – 131)	0.268	0.958
Crypt volume (μm^3)	5.81×10^6 (4.85×10^6 – 6.86×10^6)	6.13×10^6 (5.09×10^6 – 7.27×10^6)	0.685	5.65×10^6 (4.66×10^6 – 6.74×10^6)	6.30×10^6 (5.25×10^6 – 7.45×10^6)	0.432	0.918

4.5.4.3 Exploration of additional factors influencing colonic crypt cell proliferative state

The effects of the pre-intervention measurement, age, gender, endoscopy procedure, BMI and smoking status were analysed as covariates using the ANOVA GLM.

There were no effects of any of the covariates observed on any of the outcome measurements of CCPS, with the exception of the total number of mitotic cells per crypt (Table 4.15). The post-intervention total number of mitotic cells per crypt was significantly affected by the pre-intervention measurement (p=0.006).

Table 4.15 P values for the effects of pre-intervention measurement, age, gender, endoscopy procedure, BMI and smoking status on CCPS measured outcomes.

	Pre-intervention measurement	Age	Gender	Endoscopy procedure	BMI	Smoking status
Total mitoses	**0.006**	0.147	0.847	0.537	0.930	0.726
Mitoses in top half of crypt (%)	0.085	0.721	0.531	0.570	0.244	0.125
Crypt length	0.053	0.697	0.309	0.263	0.784	0.902
Crypt width	0.139	0.238	0.543	0.965	0.323	0.895
Crypt volume	0.146	0.452	0.363	0.746	0.470	0.827

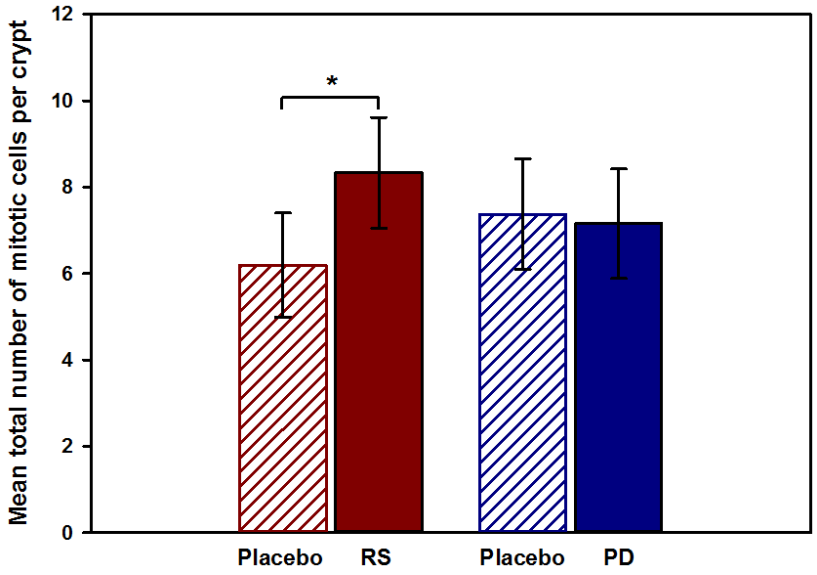
4.5.5 Discussion

Studies investigating the effects of RS, PD and butyrate on colonic crypt cell proliferation have yielded inconsistent results. In the normal colon, *in vitro* and *in vivo* studies have suggested that NDCs and butyrate may induce proliferation and others have reported no effect. This could be explained by the fact that butyrate is the main energy source for colonocytes and therefore may have trophic effects in the colon. However, in the diseased colon, the opposite has been observed, with a reduction in proliferation following NDC or butyrate administration *in vitro* in colorectal carcinoma cell lines and *in vivo,* both in animal models of CRC and in patients with CRC or other colonic diseases. This controversy is particularly true in studies investigating the effects of butyrate, where this phenomenon has been named the 'butyrate paradox'.

To test the effects of supplementation with RS and PD on the CCPS in the healthy colorectal mucosa, rectal biopsies were stained with Schiff reagent and microdissected. Ten intact crypts were analysed and divided into ten equal-sized compartments in which the number of proliferating cells were counted and crypt measurements were recorded.

An effect of RS, but not PD, was observed on the mean total number of mitotic cells per crypt (Figure 4.12). Total mitoses were significantly greater in participants supplemented with RS compared with placebo ($p=0.030$). As the distribution of mitotic cells may be more significant than the total number of proliferating cells themselves, with the majority of mitotic cells being localised to the basal half of the crypt, the proportion of mitotic cells in the lower and upper halves of the crypts were calculated. The percentage of mitotic cells in the top half of the crypt were comparable in all four intervention groups, suggesting that, although RS increased the total number of mitotic cells, RS did not have an adverse effect through alterations to the proportion of mitotic cells in the upper half of the crypts. There were no significant effects of either RS or PD on the additional measured outcomes.

Figure 4.12 Significant effect of supplementation with RS on the mean total number of mitotic cells per crypt.



Data are expressed as LSMs and error bars represent 95% CIs. *p<0.05 for effect of active agent versus placebo. N=36.

In conclusion, the findings from my study are consistent with findings from studies in the healthy colon where an increase in colonic crypt cell proliferation has been reported with RS or butyrate supplementation.

4.6 The effects of supplementation with RS and PD on expression of *BAX* and *BCL-2* apoptotic genes in the human colorectal mucosa

4.6.1 Introduction

The method utilised for the assessment of CCPS, whole crypt microdissection of Schiff reagent-stained biopsies, did not allow for the analysis of apoptosis as the recording of cells undergoing apoptosis was rare. Because of this, the effects of supplementation with RS and PD on apoptosis in the human colorectal mucosa were assessed by quantifying the expression of two apoptotic genes: Bcl-2-associated X (*BAX*), a pro-apoptotic gene, and B-cell lymphoma 2 (*BCL-2*), an anti-apoptotic gene.

4.6.1.1 BAX and BCL-2-mediated apoptosis

BAX and *BCL-2* are members of the Bcl-2 family of proteins that regulate apoptosis. BCL-2 was the first member of the family discovered and has been identified as a proto-oncogene (Gross *et al.*, 1999).

BAX is normally localised to the cytoplasm and, in the presence of a death stimulus, *BAX* forms a homodimer and translocates to the mitochondria where it acts as an integral membrane protein (Wolter *et al.*, 1997). On the other hand, BCL-2 is permanently located as an integral membrane protein in the mitochondria and, in the healthy colon, *BCL-2* is found at the base of the crypts (Biden *et al.*, 1999). In the presence of *BCL-2*, activation of *BAX* is inhibited and *BAX* and *BLC-2* are able to form heterodimers. More important than the expression of *BAX* or *BCL-2* individually in determining cell fate is the ratio of the two (Oltvai *et al.*, 1993).

4.6.1.2 The role of BAX and BCL-2 in colorectal carcinogenesis

Altered rates of apoptosis, leading to an imbalance in proliferation and apoptosis that favours tumour growth, have been associated with colorectal carcinogenesis. This can result from aberrant expression of genes involved in the regulation of apoptosis. Due to its role in inhibiting apoptosis and promoting cell survival, *BCL-2* has been defined as an oncogene (Hockenbery, 1992). Aberrant BCL-2 expression has been described as an early event in colorectal

carcinogenesis and is abnormally expressed in up to 75% of carcinomas (Biden *et al.*, 1999). This has also been reported in normal tissue adjacent to colorectal tumours (Bronner *et al.*, 1995) Expression of *BCL-2* may prevent the removal of cells containing DNA damage and can confer a growth advantage to cancerous cells.

On the contrary, expression of pro-apoptotic *BAX* has been reported to be reduced in CRC. *BAX* is also frequently mutated in CRC (Ouyang *et al.*, 1998), leading to gene inactivation. Progression of cancer stage has been associated with further reduction of *BAX* expression (Tsamandas *et al.*, 2007).

4.6.1.3 Effects of non-digestible carbohydrates on BAX and BCL-2 expression

A study investigated the protective effects of polysaccharide of *Larimichthys crocea* swimming bladder (PLCSB) in a mouse model of colon carcinogenesis (Chen *et al.*, 2014a). The authors observed a significant reduction in anti-apoptotic BCL-2 and an increase in pro-apoptotic BAX protein expression, determined by Western Blot, in the mice in the PLCSB group and found that this effect was enhanced in the group of mice given PLCSB plus RS type 3 microspheres.

To date, there have not been any previous studies investigating the effect of PD on *BAX* and *BCL-2* expression. To my knowledge, the present study is the first study to investigate the effects of supplementation with PD on expression of *BAX* and *BCL-2* in the healthy colorectal mucosa.

Furthermore, only one study has investigated the effects of PD on apoptosis in the colon. Putaala and colleagues used a semi-continuous colon simulator model, Enteromix®, to investigate the effects of 1% and 2% PD on apoptosis in the human adenocarcinoma cell line Caco-2 (Putaala *et al.*, 2011). The effects of PD on apoptosis were analysed by enzyme-linked immunosorbent assay (ELISA) and by analysis of caspase activation. The authors found a significant increase in apoptosis and in caspase-3 activation, suggesting that like RS, PD may also positively modulate the regulation of apoptosis in models of colorectal carcinogenesis by inducing apoptosis.

4.6.1.4 Effects of butyrate on BAX and BCL-2 expression

Similar to the butyrate paradox used to describe the conflicting effects of butyrate on cell proliferation, effects on apoptosis differ between healthy and diseased colon models. In the healthy colon, butyrate has been reported to reduce apoptosis, whereas in the diseased colon it has been observed to induce apoptosis.

In a model of the healthy colon, although Mentschel *et al.* did not observe an effect of butyrate on colonic crypt cell proliferation in pigs, they reported significantly lower levels of apoptosis, determined using the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay, in the pigs fed a raw potato starch diet, as a source of butyrate, compared with the control group (Mentschel and Claus, 2003). In addition, histocytochemical analyses revealed significantly higher expression of BCL-2 by two-fold in the butyrate group.

Incubation of guinea pig colon tissue sheets with 10mmol/L butyrate has been observed to result in a more sparse population of apoptotic cells compared with control, where a substantial amount of apoptotic cells were detected on the mucosal surface (Hass *et al.*, 1997). The effects of incubation with butyrate on BAX and BCL-2 protein levels were assessed by Western Blot. There were no differences observed in BCL-2 protein expression between control and butyrate-treated tissues. However, expression of BAX was three-fold higher in control tissue compared with tissue incubated with butyrate. These effects were confirmed by immunohistochemical analyses where more intense staining of BAX was observed in control tissue. Results from this study suggest that the reduction in apoptosis observed with butyrate may result from reduced expression of the pro-apoptotic protein *BAX.*

However, in models of colorectal carcinogenesis, studies have reported an induction of apoptosis by butyrate. An *in vitro* study investigated the effects of butyrate treatment of primary invasive human colonocytes on BAX and BCL-2 protein levels by immunofluorescent confocal microscopy (Emenaker *et al.*, 2001). Butyrate treatment (0.01mol/L) significantly increased BCL-2 expression by 187% ($p<0.001$) and reduced BAX expression by 15% ($p<0.001$).

Another *in vitro* study investigated the effects of the fermentation product of RS type 3 on proliferation and apoptosis in the human colon cancer cell line HCT-116 (Purwani *et al.*, 2012). Apoptosis was detected semi-quantitatively by fluorescence microscopy. Butyrate treatment at concentrations of 2.6mM and 5.3mM resulted in seven and nine-fold greater *BAX* expression respectively compared with untreated cells. However, there were no significant effects observed on *BCL-2* expression. These findings suggest that there was an increase in the BAX:BCL-2 ratio, with an increase in pro-apoptotic BAX, thus promoting apoptosis. Indeed, the authors also observed an increase in levels of caspase-3, an activator of apoptosis, assessed by ELISA, with butyrate treatment of HCT-116 cells.

A study by Ruemmele *et al.* reported that butyrate induced apoptosis by up to 79% in a time- and dose-dependent manner in Caco-2 cells, but did not observe any effects on the expression of the BCL-2 protein (Ruemmele *et al.*, 1999). A later study by the same group also did not observe effects of butyrate treatment, with concentrations up to 100mM, on the expression of BAX or BCL-2 proteins analysed by Western Blotting (Ruemmele *et al.*, 2003). However, the authors reported an effect of butyrate on the expression of another protein from the Bcl-2 family: BAK.

4.6.2 Hypotheses, Aims and Objectives

4.6.2.1 Hypotheses

The hypothesis for this study was that NDCs would induce apoptosis by increasing the expression of pro-apoptotic genes i.e. *BAX* and reducing the expression of anti-apoptotic genes i.e. *BCL-2*.

4.6.2.2 Aims

This study aimed to test this hypothesis by investigating the effects of supplementing healthy human participants with NDCs on the expression of two apoptosis-related genes: *BAX* (pro-apoptotic) and *BCL-2* (anti-apoptotic) in the colorectum.

4.6.2.3 Objectives

- To quantify expression of *BAX* and *BCL-2* by qPCR using cDNA already prepared for quantification of WNT pathway-related gene expression;
- To investigate the effects of the supplementation with NDCs on expression of *BAX* and *BCL-2* and on the ratio of *BAX* to *BCL-2*;
- To explore the effects of key epidemiological factors including age and gender on expression of *BAX* and *BCL-2* and on the ratio of *BAX* to *BCL-2.*

4.6.3 Methods

The methods for this study can be found in section 2.2.5. cDNA was synthesised from RNA previously extracted for the quantification of WNT pathway-related gene expression. The expression of *BAX* and *BLC-2,* as well as the two housekeeping genes *18S* and *β2M,* was quantified by qPCR. Reverse transcription and the quantification of *BAX* and *BCL-2* were undertaken under my supervision by Idoia Ibero, an Erasmus Project student, to whom I am very grateful.

4.6.4 Results

As assessment of CCPS following microdissection of Schiff reagent-stained rectal biopsies did not allow for the successful analysis of apoptosis, the effects of the intervention on the expression of two genes, *BAX* (pro-apoptotic) and *BCL-2* (anti-apoptotic), were investigated by qPCR.

4.6.4.1 Effects of non-digestible carbohydrates on the expression of BAX and BCL-2

Supplementation with RS and PD did not have an effect on expression of *BAX* or *BCL-2* (Table 4.16). As regulation of apoptosis results from a balance between pro- and anti-apoptotic genes, *BAX* and *BCL-2* expression data were expressed as a ratio (*BAX/BCL-2*). However, there were no significant effects of RS or PD on the ratio of *BAX* to *BCL-2* (Table 4.16).

Table 4.16 Effects of supplementation with RS and PD on expression of *BAX* and *BCL-2* in the human rectal mucosa.

	RS allocation			PD allocation			Interaction between RS and PD
Gene	**Placebo (Amioca)**	**RS (Hi-Maize® 260)**	**P value**	**Placebo (Maltodextrin)**	**PD (Litesse® Ultra™)**	**P value**	**P value**
***BAX*[1]**	2.61 (1.51 – 3.64)	1.97 (1.13 – 3.31)	0.390	2.10 (1.46 – 3.66)	2.61 (1.02 – 3.33)	0.888	-
***BCL-2*[1]**	0.266 (0.177 – 0.652)	0.231 (0.160 – 0.340)	0.538	0.266 (0.201 – 0.359)	0.220 (0.123 – 0.704)	0.375	-
***BAX/BCL-2*[2]**	0.808 (0.778 – 0.838)	0.826 (0.765 – 0.585)	0.425	0.819 (0.791 – 0.847)	0.816 (0.791 – 0.782)	0.871	0.524

[1]Data for *BAX* (N=49) and *BCL-2* (N=46) expression are expressed as median and lower and upper quartiles for adjusted values ($2^{-\Delta Ct}$ x 10,000) relative to *18S* and *β2M* housekeeping genes (Kruskal-Wallis test). [2]Data for the *BAX/BCL-2* ratios represent *BAX* ΔCt value to *BCL-2* ΔCt value, relative to *18S* and *β2M* housekeeping genes (N=43). Data are presented as LSMs and 95% CIs (ANOVA GLM).

4.6.4.2 Exploration of additional factors influencing BAX and BCL-2 expression

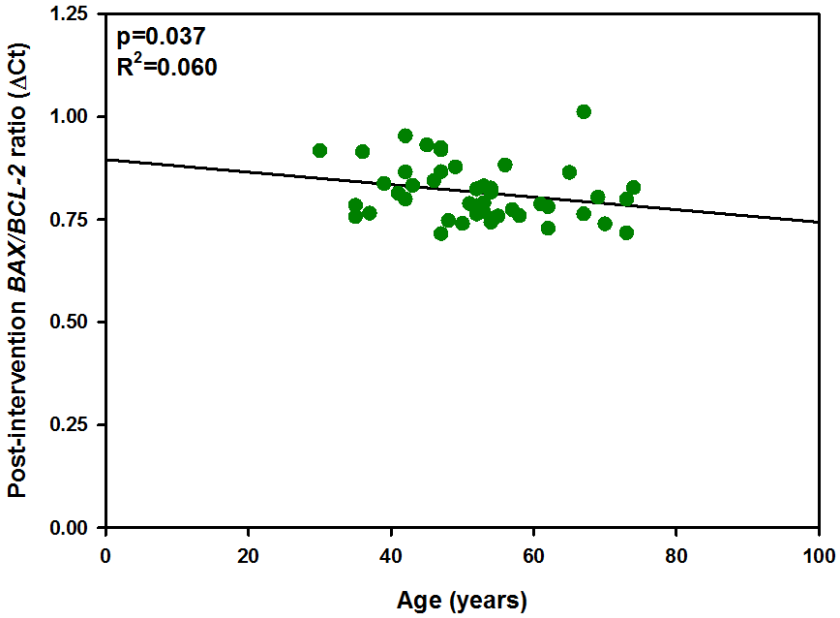
The effects of pre-intervention expression, age, gender, endoscopy procedure, BMI and smoking status on *BAX, BCL-2* and the ratio of *BAX/BCL-2* were assessed. There were no effects of any of the covariates observed for any of the outcomes with the exception of age. Age was a determinant of the post-intervention ratio of *BAX/BCL-2* (p=0.037).

Table 4.17 P values for the effects of pre-intervention expression, age, gender, endoscopy procedure, BMI and smoking status on post-intervention *BAX* and *BCL-2* expression and on the *BAX-BCL-2* ratio.

Gene	Pre-intervention expression	Age	Gender	Endoscopy procedure	BMI	Smoking status
BAX[2]	0.473	0.699	0.400	0.766	0.473	0.416
BCL-2[2]	0.471	0.331	0.272	0.608	0.472	0.524
BAX/BCL-2 (ΔCt)[1]	0.151	**0.037**	0.407	0.684	0.135	0.955

Analyses using Spearman's correlation test revealed an inverse correlation between age and the post-intervention *BAX/BCL-2* ratio (Spearman correlation coefficient of -0.300 (p=0.046)) (Figure 4.13).

Figure 4.13 Inverse correlation between age and post-intervention *BAX/BCL-2* ratio.



Data for the *BAX/BCL-2* ratio represent *BAX* ΔCt value to *BCL*-2 ΔCt value, relative to *18S* and *β2M* housekeeping genes. N=43.

4.6.5 Discussion

The effects of supplementation with RS and PD on apoptotic rates in the colonic crypt were first assessed by light microscopy following whole crypt microdissection of Schiff reagent-stained crypts. However, this was not an appropriate indicator of apoptosis as the numbers of apoptotic cells observed by this method were negligible. An alternative method was utilised as an indicator of apoptosis: the quantification of two genes involved in the regulation of apoptosis: *BAX,* a pro-apoptotic gene, and *BCL-2*, an anti-apoptotic gene.

Supplementation with RS or PD did not affect expression of *BAX* or *BCL-2.* As the regulation of apoptosis is achieved through a balance of pro- and anti-apoptotic genes, the ratio of *BAX* to *BCL-2* was calculated. Similar to that observed when the genes were expressed individually, there were no effects of RS or PD on *BAX*/*BCL-2.* However, an inverse correlation was observed between age and the post-intervention *BAX/BCL-2* ratio.

A small number of studies have specifically looked at the effects of NDCs on apoptosis via the quantification of *BAX* and *BCL-2* expression. One study reported a greater reduction in BCL-2 and an increase in BAX proteins with the

addition of RS type 3 to PLCSB in a mouse model of colon carcinogenesis (Chen *et al.*, 2014a). However, the results from this study showed no effect of either NDC on *BAX* or *BCL-2* expression or on the ratio of these regulators of apoptosis in the rectal mucosa of healthy participants.

Chapter 5 The WNT signalling pathway and its functional outcomes in the macroscopically-normal mucosa of people at differential risk of colorectal cancer

5.1 The DISC Study participants at differential risk of CRC

For this study, I used samples and data from a total of 91 normal-risk participants. This included baseline samples and data from the 75 participants who took part in the dietary intervention study within the DISC Study plus 16 dropout participants, i.e. those who did not complete the intervention or who were excluded for various reasons. The higher-risk groups consisted of two participant groups: the 'UC' group with 12 participants and the 'Polyp' group with 26 participants. The majority of participants in the 'Normal' group underwent endoscopic examination by flexible sigmoidoscopy, whereas almost all of the higher-risk participants had a colonoscopy.

The participant characteristics of each group are detailed in Table 5.1. The majority of the participants were Caucasian. Within the 'Normal' group, the proportion of females and males were comparable, but in both 'UC' and 'Polyp' groups the majority of participants (approximately 75%) were male. The participants in the 'Polyp' group were approximately 10 years older than those in the 'Normal' group ($p=0.001$), with the mean age of the 'UC' participants being midway between the other two groups.

There were no significant differences between groups in the mean BMI of the participants ($p=0.503$). The mean BMIs were high in all three participant groups. In the 'Normal' group, 13 participants had a normal BMI, 31 were classified as being overweight and 40 were classified as being obese. In the 'UC' group, three participants had a normal BMI, four were classified as being overweight and four were obese. In the 'Polyp' group, five participants had a normal BMI, eleven were overweight and ten were obese.

Table 5.1 Characteristics of participants in 'Normal', 'UC' and 'Polyp' groups.

Risk group	Normal	UC	Polyp	P value
Number of participants	91	12	26	
Ethnicity Caucasian (%) Black African (%) Mixed race (%)	 89 (98) 1 (1) 1 (1)	 12 (100) 0 (0) 0 (0)	 26 (100) 0 (0) 0 (0)	0.848
Gender Female (%) Male (%)	 50 (55) 41 (45)	 3 (25) 9 (75)	 6 (23) 20 (77)	**0.005**
Age Mean (years) Range	 51.8 26 – 80	 56.5 38 – 71	 61.9 44 – 79	**0.001**
Smoking status Never (%) Former (%) Current (%)	 43 (47) 22 (24) 17 (19)	 3 (25) 8 (67) 1 (8)	 8 (31) 11 (42) 5 (19)	0.051
BMI (kg/m^2) Mean Range	 30.1 23.0 – 49.3	 29.3 22.2 – 37.6	 28.8 19.8 – 40.3	0.503
Procedure Colonoscopy (%) Flexi Sigmoidoscopy (%)	 25 (27) 66 (73)	 12 (100) 0 (0)	 24 (92) 2 (8)	**<0.001**

Data on smoking status were not available for nine 'Normal' and two 'Polyp' participants and anthropometric data for seven 'Normal' and one 'UC' participants.

5.2 WNT pathway-related gene expression in the macroscopically-normal mucosa of people at differential risk of CRC

5.2.1 Introduction

As WNT signalling is frequently aberrantly activated in CRC, this study aimed to investigate the expression of a panel of WNT pathway-related genes in the macroscopically-normal mucosa of people at “normal” risk of CRC and in those at higher CRC risk because of quiescent UC or a prior history of adenomatous polyps.

5.2.1.1 Expression of WNT pathway components in the colorectum of people with ulcerative colitis

Due to its role in maintaining homeostasis and in regulating several processes including cell proliferation and apoptosis within the colon, several studies have been interested in investigating differences in WNT signalling in patients with UC. When analysing the expression of WNT components, the majority of studies have focussed on quantifying expression at the protein level.

A WNT array was used to investigate the expression of WNT pathway-related genes in uninflamed and inflamed tissue from six UC patients (You *et al.*, 2008). Seven WNT ligands (*WNT2B, WNT3A, WNT5B, WNT6, WNT7A, WNT9A* and *WNT11)* and two receptors from the Frizzled family (*FZD3* and *FZD4)* showed significantly higher expression in uninflamed tissue from UC patients compared with six healthy participants, which could be indicative of an increase in WNT pathway activity. However, expression of *FZD1* and *FZD5* was significantly reduced in UC patients. Within the UC patients, a significant increase in the expression of *COL1A1, DKK4, DVL2, SOX17* and a decrease in *CCND3, SFRP2, WISP3, WNT3A* was observed in inflamed compared with uninflamed mucosa, demonstrating differences in WNT activity between active and inactive disease. Increased expression of members of the Frizzled receptor family and *DVL* have also been observed in the inflamed mucosa of UC patients compared with healthy participants (Uthoff *et al.*, 2001).

Differences in the expression of WNT pathway components have also been observed in myofibroblasts isolated from colonic tissue from normal participants

and UC patients. These included a greater than three-fold reduction in the expression of *SFRP1*, an antagonist of WNT signalling, in UC (Hughes *et al.*, 2011). Similar to the findings by You *et al.* (2008), downregulation of *SFRP1* would consequently result in reduced inhibition of the WNT pathway and therefore increased WNT activity.

Van Dekken *et al.* investigated changes in the WNT signalling pathway in a cross-sectional study of the progression from dysplasia to carcinoma in 21 patients with UC (van Dekken *et al.*, 2007). The expression of four WNT pathway proteins, β-catenin, E-cadherin, cyclin D1 and c-MYC, was determined by immunohistochemical analysis in 18 adenocarcinomas and 17 dysplastic lesions. β-catenin abundance, particularly within the nucleus, was significantly increased in both dysplasia and adenocarcinoma compared with normal tissue. Significantly reduced E-cadherin expression was observed in cancerous compared with normal tissue. Nuclear cyclin D1 expression was significantly higher in cancerous tissue compared with normal tissue and there was a trend towards higher c-MYC expression in both dysplastic and cancerous tissues. Overall, the results from this study suggested that, in the progression to carcinoma in UC patients, WNT activity is increased, as demonstrated by enhanced β-catenin expression alongside an upregulation of cyclin D1 and c-MYC and reduced expression of E-cadherin.

More recently, Shenoy and colleagues also evaluated WNT pathway activity during the progression from colitis to cancer (Shenoy *et al.*, 2012). They showed significantly higher β-catenin expression in tissue from UC patients and sporadic CRC, increased by 2.5-fold and 4.5-fold respectively, compared with healthy controls. The authors concluded that the activity of WNT signalling in UC was intermediate between that in the healthy colon and in CRC.

The expression of cyclin D1, a target of WNT signalling which is involved in regulation of the cell cycle and is overexpressed in CRC (Maeda *et al.*, 1998) has been compared in patients with quiescent UC, active UC, dysplasia and UC-related cancer (Wong *et al.*, 2003). Whilst cyclin D1 expression in the mucosa from patients with quiescent UC was similar to that in healthy participants, significantly higher cyclin D1 expression was observed in patients with active UC or dysplasia, where expression levels were comparable. Based

on these results, Wong *et al.* (2003) suggested that cyclin D1 expression may be increased early during the progression from UC-associated inflammation to dysplasia and ultimately carcinoma. This is in contrast with Xie and Itzkowitz, who suggested that aberrant WNT signalling is observed at later stages in the progression from colitis to cancer (Xie and Itzkowitz, 2008).

A study by Aust *et al.* was the first to investigate the altered expression of WNT related proteins, β-catenin, E-cadherin and APC, in UC-related cancer using tissue from 33 UC-related cancer and 42 sporadic CRC patients (Aust *et al.*, 2001). Immunohistochemical analyses revealed that both cancer types were associated with an increase in cytoplasmic and nuclear β-catenin, as opposed to membrane-associated, whereas abnormal staining of nuclear β-catenin was significantly higher in sporadic cases. Reduced APC expression was observed in both cancer types compared with normal tissue, however there was no difference between the two cancers themselves. E-cadherin expression was also significantly lower in cancerous compared with normal tissue. Abnormal levels of membrane-associated E-cadherin were greater in UC-related cancers, however the opposite was found for nuclear E-cadherin. The authors reported a stronger association between abnormal levels of membrane-associated E-cadherin and abnormal levels of cytoplasmic β-catenin in UC-related cancers compared with sporadic cases. In sporadic cancers, they observed an association between abnormal APC levels and nuclear β-catenin expression. This demonstrated that, although abnormal expression of WNT proteins was observed in both UC-related and sporadic cancers of the colon, β-catenin expression in UC-related cancers was regulated by E-cadherin expression whereas APC had a more significant role in sporadic cancers.

5.2.1.2 Expression of WNT pathway components in the colorectum of people with polyps

Similar to studies examining the expression of WNT pathway components in the tissue of patients with UC, studies in patients with polyps have also primarily assessed protein levels. Furthermore, studies have also investigated differences in expression between different polyp types, such as hyperplastic polyps and tubular adenomas.

Dai *et al.* (2007) assessed levels of APC, β-catenin, c-MYC and cyclin D1 proteins in normal mucosa, adenomas and carcinomas (Dai *et al.*, 2007). APC expression was lower in colorectal carcinomas and malignant adenomas compared with non-malignant adenomas and the normal mucosa. Abnormal expression of β-catenin was observed significantly more in tissues from both non-malignant and malignant adenomas and carcinomas compared with the normal mucosa. In addition, an inverse correlation between the expression of APC and β-catenin was found. c-MYC and cyclin D1, two targets of WNT signalling, were also upregulated in carcinomas and both adenoma types compared with healthy tissue. Furthermore, c-MYC and cyclin D1 expression correlated positively with abnormal β-catenin expression. Altogether, the findings from this study demonstrate that abnormal expression of WNT proteins, resulting in reduced inhibition of β-catenin and increased WNT pathway activity, is not only observed in colorectal carcinomas but may be apparent early in the progression to CRC in non-malignant adenomas.

The sub-cellular localisation as well as the expression of the WNT pathway components APC, AXIN, AXIN2 and β-catenin have also been compared in healthy colonic crypts, adenomatous polyps and adenocarcinomas (Anderson *et al.*, 2002). An increase in nuclear β-catenin was found in adenocarcinoma tissue compared with healthy and polyp tissues, where the majority of β-catenin was localised to cell-cell junctions. In polyp tissue, levels of nuclear AXIN were reduced and levels of cytoplasmic AXIN were increased compared with healthy tissue. Levels of AXIN2 were comparable between the three tissues and were predominantly observed within the nuclei. In healthy tissue, expression of APC, AXIN and AXIN2 was representative of inhibition of WNT signalling and, consequently, a lack of nuclear β-catenin. However, in adenomatous polyps and adenocarcinomas, abnormal expression of APC, AXIN and β-catenin were observed.

β-catenin expression has also been investigated specifically in a variety of colorectal lesions including hyperplastic polyps, sessile serrated adenomas and traditional serrated adenomas (Fang *et al.*, 2014). Abnormal β-catenin was observed more frequently in all lesions compared with the normal mucosa. Furthermore, both sessile and traditional serrated adenomas showed an increased incidence of abnormal β-catenin expression compared with

hyperplastic polyps, and therefore the investigators concluded that the WNT pathway may play a more important role in the development of serrated lesions.

Iwamoto *et al.* investigated the expression of the two key components of the canonical WNT signalling pathway, APC and β-catenin, in the normal mucosa, hyperplastic polyps, adenomas and carcinomas (Iwamoto *et al.*, 2000). In the normal mucosa and in hyperplastic polyps, expression of APC and β-catenin was comparable. However, almost a third of the adenomas and more than three-quarters of the carcinomas analysed showed complete loss of APC. Furthermore, abnormal β-catenin expression, particularly that within the nucleus, was also observed in adenomas and carcinomas.

FAP is an inherited disease, usually resulting from a mutation in and loss of the *APC* gene, where patients characteristically develop hundreds of intestinal polyps (De Queiroz Rossanese *et al.*, 2013). Gaspar *et al.* have analysed gene expression in adenomatous polyps from patients with FAP and in polyps from a mouse model of FAP (Gaspar *et al.*, 2008). The investigators applied stringent criteria to select a total of 166 genes that were both differentially expressed in polyps from FAP patients compared with healthy mucosa and in polyps from C57BL6J $Apc^{+/1638N}$ compared with control mice. These genes included several WNT pathway-related genes such as *CCND1* and *CD44*, two target genes of WNT signalling that are upregulated early during colorectal carcinogenesis (Abbasi *et al.*, 1993).

Wang *et al.* also investigated expression of β-catenin and cyclin D1, encoded by *CCND1,* in the normal mucosa and in adenomas and carcinomas from FAP patients (Wang *et al.*, 2013a). Both β-catenin and cyclin D1 were significantly increased in low and high-grade adenomas and carcinomas compared with normal mucosa. Furthermore, nuclear β-catenin was significantly enhanced in adenomas and carcinomas. These findings demonstrate that abnormal β-catenin expression observed in colorectal adenomas and carcinomas is not only limited to sporadic cases but may also be observed in low-grade adenomas and later stages in patients with FAP.

An earlier study investigated the expression of two target genes of the WNT pathway, *c-MYC and NKD1,* and *SFRP1,* a WNT antagonist, at the mRNA level in patients with FAP and sporadic adenomas (Caldwell *et al.*, 2010). *SFRP1*

was downregulated in adenomas of both FAP and sporadic patients compared with the normal mucosa, however this was more pronounced in sporadic adenomas. An increase in *c-MYC* and *NKD1* expression was observed in adenomas from FAP patients and sporadic cases compared with the normal mucosa and, again, this was enhanced in sporadic cases. The results from this study demonstrate that similarities exist between adenomas occurring sporadically and those resulting from an inherited disease, FAP.

5.2.2 Hypotheses, aims and objectives

5.2.2.1 Hypotheses

The hypothesis for this study was that participants at higher risk of CRC, i.e. those with quiescent UC or a prior history of adenomatous polyps, would have aberrant expression of WNT pathway-related genes. In particular, it was hypothesised that higher-risk participants would have increased expression of genes that indicate an increase in WNT activity, such as WNT target genes, and reduced expression of those that suggest reduced activity, such as WNT inhibitors, compared with normal participants.

5.2.2.2 Aims

This study aimed to test the hypothesis by quantifying expression of the selected WNT-pathway related genes in the macroscopically-normal mucosa of people at differential CRC risk i.e. those with quiescent UC, those with a prior history of adenomatous polyps and those at normal risk of developing CRC.

5.2.2.3 Objectives

- To obtain colorectal biopsies from the macroscopically-normal mucosa of three groups of DISC Study participants at differential risk of CRC i.e. those with quiescent UC, those with a prior history of adenomatous polyps and “normal” participants;
- To extract an adequate amount of high-quality RNA from OCT-embedded rectal mucosal biopsies;

- To synthesise cDNA by reverse transcription;
- To quantify the expression of the 12 selected WNT genes by qPCR;
- To compare gene expression in the ‘Normal’ participants and in the two higher-risk groups (‘UC’ and ‘Polyp’ participants);
- To investigate the effects of key epidemiological factors including age and gender on WNT pathway-related gene expression.

5.2.3 Methods

A total of 12 WNT pathway-related genes were selected as described in section 3.3. Their expression was quantified by qPCR using cDNA synthesised from RNA extracted from rectal mucosal biopsies as detailed in section 2.2.1.

The ANOVA GLM was used to investigate differences in expression between the three participant groups, with age, gender, endoscopy procedure, BMI and smoking status incorporated as covariates. Dunnett’s test was used to correct for multiple comparison testing and pairwise comparisons were analysed using the Bonferroni method. When data were not normally-distributed, the non-parametric test Kruskal-Wallis was applied.

5.2.4 Results

The expression of the 12 selected WNT pathway-related genes was quantified in rectal biopsies from the macroscopically-normal mucosa of the three groups of participants at differential risk of CRC. Data for the expression of three of these genes is not included in this thesis (*CCND1, c-MYC* and *SFRP1*) because of technical difficulties with the assays, evident from the melt curves observed during qPCR, in the samples from ‘UC’ and ‘Polyp’ participants.

5.2.4.1 WNT pathway-related gene expression in the macroscopically-normal epithelium of ‘Normal’, ‘UC’ and ‘Polyp’ participants

Levels of expression differed considerably (by greater than three orders of magnitude) between each of the nine WNT pathway-related genes (Table 5.2). Across all three participant groups, the gene with the greatest expression in the rectal mucosa was *CTNNB1,* with a mean expression of 16.3 (adjusted copies)

whilst the gene with the lowest expression was *SFRP2*, with a mean expression of 0.06 (adjusted copies).

With the exception of *c-JUN* (p=0.046) and *WNT11* (p=0.040), expression of most of the genes was similar in all three groups. *c-JUN* expression was lowest in the 'Normal' group, a little higher in the 'UC' group and highest in the 'Polyp' group, where expression levels were 76% greater than those seen in the 'Normal' group. Similarly, *WNT11* expression was highest in the 'Polyp' group, where it was 56% higher than in the 'Normal' group.

When applying Bonferroni's correction for multiple testing, the set p-value is lowered to 0.004 and so the statistically significant differences in *c-JUN* (p=0.046) and *WNT11* (p=0.040) expression between the three participant groups are lost.

Although not statistically significant, there was evidence for apparent between group differences in expression for four other genes. Whilst mean expression of *FOSL1* was approximately five-fold greater in the 'Normal' group compared with that in the two higher-risk groups, this substantial difference was not statistically significant because of the large range in individual expression values within each group. In addition, expression of *APC*, *AXIN2* and *CTNNB1* appeared to be reduced in the 'UC' group compared with both the 'Normal' and 'Polyp' groups.

Table 5.2 Expression of WNT pathway-related genes in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.

Gene	Normal	UC	Polyp	P value
***APC*[1]** N=108	1.86 (1.33 – 2.44)	1.04 (0.73 – 2.37)	1.69 (1.09 – 2.13)	0.080
***AXIN2*[2]** N=108	2.41 (2.06 – 2.82)	1.76 (1.18 – 2.64)	2.51 (1.86 – 3.37)	0.293
***CTNNB1*[2]** N=108	14.6 (11.9 – 17.5)	11.0 (5.8 – 17.8)	15.9 (11.0 – 21.6)	0.432
***FOSL1*[1]** N=82	0.1647 (0.0228 – 0.4785)	0.0293 (0.0138 – 0.4003)	0.0309 (0.0144 – 0.2626)	0.325
***GSK3β*[2]** N=108	4.84 (4.06 – 5.69)	4.47 (2.73 – 6.65)	5.81 (4.31 – 7.54)	0.450
***c-JUN*[1]** N=116	6.28 (4.31 – 10.30)	7.39 (3.50 – 14.43)	11.05 (5.72 – 21.17)	**0.046**
***SFRP2*[2]** N=89	0.0231 (0.015 – 0.034)	0.0383 (0.013 – 0.109)	0.0322 (0.015 – 0.069)	0.679
***WTN5A*[2]** N=106	0.151 (0.116 – 0.198)	0.150 (0.078 – 0.291)	0.190 (0.117 – 0.306)	0.712
***WNT11*[2]** N=88	0.0905 (0.0706 – 0.1139)	0.0725 (0.0381 – 0.1230)	0.1454 (0.0999 – 0.2028)	**0.040**

Data are expressed as adjusted copies. [1]Data are presented as median (lower and upper quartiles). The non-parametric Kruskal-Wallis test was used to compare gene expression between the three groups. [2]Data are presented as LSMs (95% CIs). The ANOVA GLM was used to compare gene expression between the three groups and Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method was used for pairwise comparisons.

5.2.4.2 Exploration of additional factors influencing WNT pathway-related gene expression

Given the evidence from epidemiological studies that age, gender and lifestyle factors are major determinants of CRC risk, the effects of age, gender, BMI and smoking status on the expression of the selected gene panel were investigated. In addition, I examined the effect of the two endoscopy procedures (flexible

sigmoidoscopy and colonoscopy) used to collect colorectal biopsies since it was not known whether these procedures *per se* (or the different bowel preparations which preceded them) influenced gene expression in the colorectal mucosa. Potential effects of these factors were determined by their inclusion as covariates in the ANOVA GLM. However, as the data for *APC, FOSL1* and *c-JUN* expression were not normally-distributed despite transformation, the non-parametric Kruskal-Wallis test was utilised. The p values from the results of these analyses are shown in Table 5.3.

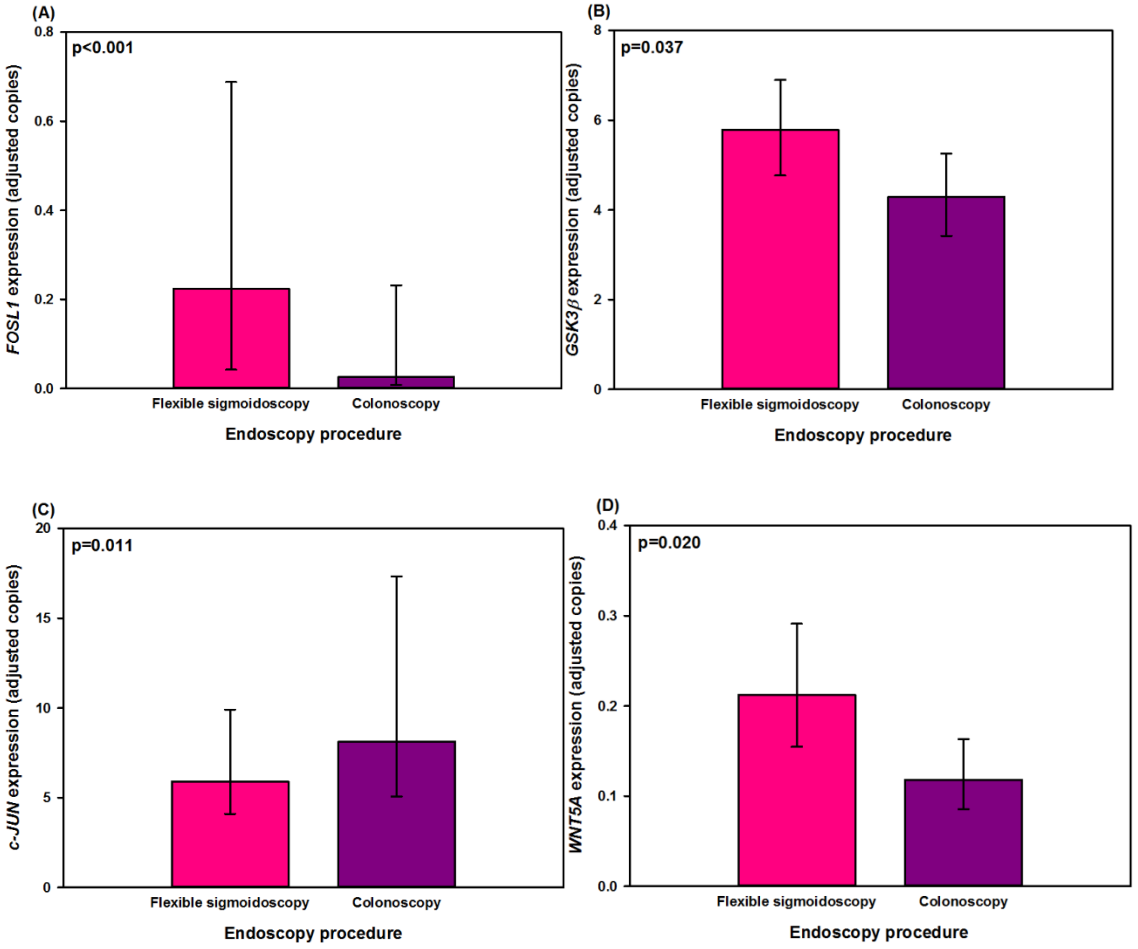
Table 5.3 P values for the effects of age, gender, endoscopy procedure, BMI and smoking status on WNT pathway-related gene expression.

Gene	Age	Gender	Endoscopy procedure	BMI	Smoking status
APC	0.296	0.069	0.142	0.413	0.079
AXIN2	0.463	0.711	0.057	0.340	0.148
CTNNB1	0.998	0.325	0.061	0.167	0.465
FOSL1	0.539	0.422	**<0.001**	0.446	**0.008**
GSK3β	0.254	0.763	**0.037**	0.441	0.768
c-JUN	0.149	0.893	**0.011**	0.505	0.104
SFRP2	0.572	0.103	0.823	0.244	0.295
WTN5A	0.283	0.390	**0.020**	0.436	0.134
WNT11	0.967	0.228	0.277	0.728	**0.024**

For these analyses, data from all three participants were pooled.

Despite the epidemiological evidence of their role in determining CRC risk, there was no evidence that age, gender or BMI were associated with expression of any of the selected WNT pathway-related genes. However, for four of the nine genes (*FOSL1, GSK3β, c-JUN* and *WNT5A*) there were statistically significant differences in expression between the two endoscopy procedures. Expressions of *FOSL1, GSK3β* and *WNT5A* were significantly lower in participants who had endoscopic examination by colonoscopy compared with flexible sigmoidoscopy (Figure 5.1 (A, B and D)) whereas the opposite effect was observed for *c-JUN* (Figure 5.1 (C)).

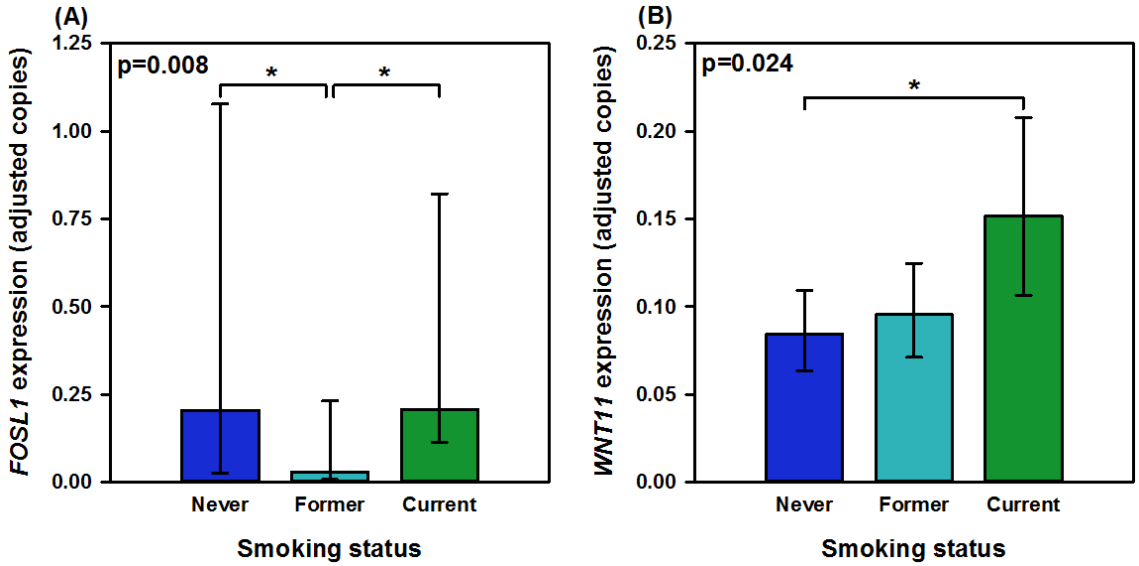
Figure 5.1 Effect of endoscopy procedure on expression of *FOSL1*, *GSK3β, c-JUN* and *WNT5A.*



Data are expressed as adjusted copies. Data for the expression of *FOSL1* (A) (N=82) and *c-JUN* (C) (N=116) are presented as medians and error bars represent lower and upper quartiles (Kruskal-Wallis). Data for the expression of *GSK3β* (B) (N=108) and *WNT5A* (D) (N=106) are presented as LSMs and error bars represent 95% CIs (ANOVA GLM).

The expression of *FOSL1*, as well as *WNT11*, was significantly affected by smoking status. Interestingly, *FOSL1* expression was significantly reduced in participants who were former smokers, compared with both those who had never smoked and those who were current smokers (Figure 5.2 (A)). In contrast, the expression of *WNT11* appeared to increase with cigarette exposure, and was significantly higher in current smokers than in those who never smoked (Figure 5.2 (B)).

Figure 5.2 Effect of smoking status on expression of *FOSL1* and *WNT11*.



Data are expressed as adjusted copies. Data for the expression of *FOSL1* (A) are presented as medians and error bars represent lower and upper quartiles (Kruskal-Wallis) (N=82). Data for the expression of *WNT11* (B) are presented as LSMs and error bars represent 95% CIs (ANOVA GLM) (N=88).

5.2.5 Discussion

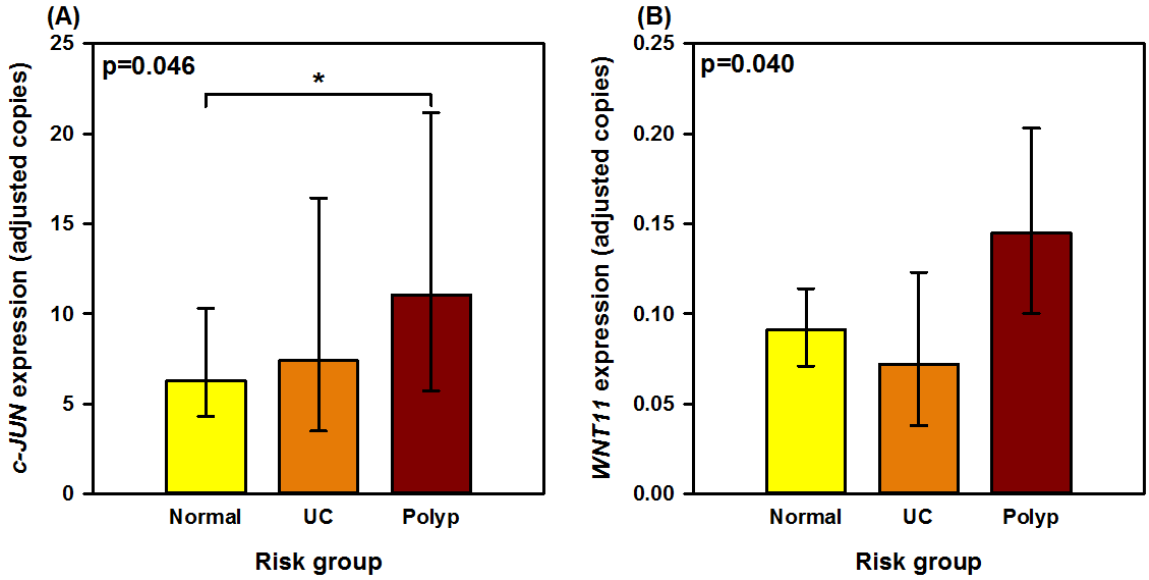
The expression of 12 WNT pathway-related genes that are implicated in CRC were quantified in the macroscopically-normal mucosa of participants with quiescent UC and a prior history of polyps by qPCR, however, for higher-risk participants, data for 9 of the genes were analysed. There were significant differences in the expression of *c-JUN* and *WNT11* between the three participant groups (Figure 5.3).

The expression of *c-JUN* increased with increasing CRC risk, and was significantly upregulated in the 'Polyp' compared with the 'Normal' group (Figure 5.3 (A)). *c-JUN* is a member of the JUN family of proto-oncogenes and is a stimulator of cell proliferation (Wang *et al.*, 2000). It is also a target gene of the WNT signalling pathway that is transcribed following the activation of the WNT pathway and consequent induction of transcription by β-catenin within the nucleus (Mann *et al.*, 1999). Therefore, an increase in *c-JUN* expression would suggest an increase in WNT signalling activity. Furthermore, at the protein level, *c-JUN* expression has been shown to be increased in colorectal carcinomas compared with the healthy mucosa (Pandey *et al.*, 1995) and to be higher in large tubulovillous adenomas, as well as in adenocarcinomas (Magrisso *et al.*,

1993). The present study is the first to observe increased expression of *c-JUN* in UC patients.

I also observed higher expression of *WNT11* in the macroscopically-normal mucosa of participants with a prior history of polyps compared with both 'Normal' and 'UC' participants (Figure 5.3 (B)). *WNT11* is a WNT ligand that, although originally believed to target only the non-canonical WNT signalling pathway (Rao and Kuhl, 2010), may also inhibit or activate the canonical WNT pathway (Maye *et al.*, 2004; Tao *et al.*, 2005). Increased expression of *WNT11* has been observed previously in colorectal adenocarcinomas (Kirikoshi *et al.*, 2001) and it is thought to contribute to cancer progression through the stimulation of proliferation, migration and invasion (Nishioka *et al.*, 2013). In a mouse model of Peutz-Jegher's syndrome (where patients develop polyps throughout the digestive tract), *WNT11* expression was approximately two-fold greater in gastric polyp tissue compared with healthy mucosa (Lai *et al.*, 2011). *WNT11* expression has been investigated in only one previous study where the authors reported significantly greater *WNT11* expression in the uninflamed mucosa of UC patients compared with healthy controls (You *et al.*, 2008). Their observation is contrary to my observation in the present study where *WNT11* expression was slightly lower in participants in the 'UC' group than in the 'Normal' group. The reason for this divergence between studies is not known but may relate to choice of "normal" (control) patients or other confounding factors.

Figure 5.3 Expression of *c-JUN* and *WNT11* in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.



Data are expressed as adjusted copies. Data for *c-JUN* expression are presented as medians and error bars represent lower and upper quartiles (Kruskal-Wallis) (N=116). Data for *WNT11* expression are presents as LSMs and error bars represent 95% CIs (ANOVA GLM) (N=88).

Interestingly, *WNT11* expression increased with increasing exposure to tobacco, and was significantly increased in participants who were current smokers compared with those who had never smoked. This effect has not been reported previously. Another novel finding from the present study was a significant effect of endoscopy procedure on expression of *FOSL1, GSK3β, c-JUN* and *WNT5A.*

5.3 microRNA expression in the macroscopically-normal mucosa of people at differential risk of CRC

5.3.1 Introduction

This study aimed to investigate the expression of miRNAs implicated in colorectal carcinogenesis and involved in the regulation of the WNT signalling pathway in the macroscopically-normal mucosa of people at “normal” risk of CRC and in those at higher CRC risk because of quiescent UC or a prior history of adenomatous polyps. The criteria for the selection of the miRNAs quantified in this study can be found in section 3.4.2. In addition, the miRNAs selected to be quantified in the intervention arm of this Ph.D. were also quantified in this study.

5.3.1.1 microRNAs

miRNAs are short (approximately 22 nucleotides long), non-coding, single-stranded RNAs that are able to regulate gene expression both by degrading mRNA or inhibiting transcription (Chen and Rajewsky, 2007). Each miRNA can target hundreds of genes (Thomas *et al.*, 2010) and therefore regulate a variety of cellular processes including apoptosis, differentiation and proliferation (Kloosterman and Plasterk, 2006).

5.3.1.2 microRNA expression in the colorectum of people ulcerative colitis

A number of studies investigating miRNA expression in UC exist, the majority of which are human studies. Wu and colleagues were the first to investigate differences in expression of miRNAs involved in the regulation of inflammation in the colonic mucosa of IBD patients (Wu *et al.*, 2008). The study recruited 15 patients with inactive UC and 15 with active UC as well as additional healthy participants and those with other colonic diseases. Microarray analyses revealed that 18 miRNAs, out of the 554 miRNAs quantified, were differentially expressed in tissue from active UC patients compared with normal participants. Of these, 12 were also differentially expressed in inactive UC samples compared with normal tissue. Analysis by qPCR confirmed the downregulation of three miRNAs (*miR-192, miR-375* and *miR-422b*) and upregulation of eight

miRNAs (*miR-16, miR-21, miR-23a, miR-24, miR-29a, miR-126, miR-195* and *Let-7f)* in active UC tissue compared with normal tissue.

A later study investigated differences in miRNA expression in the mucosa of patients with active UC and healthy controls by analysing a greater number of miRNAs (710) by microarray (Takagi *et al.*, 2010). Twenty-seven were differentially expressed in tissue from UC patients compared with healthy controls and two of these miRNAs, *miR-21* and *miR-150*, were confirmed to be significantly upregulated in active UC tissue by qPCR.

More recently, studies have also compared miRNA expression between inactive and active forms of UC. In a study which compared miRNA expression in patients with UC (active and quiescent), Crohn's disease or healthy participants, Coskun *et al.* identified five miRNAs that were upregulated in active UC and two miRNAs that were upregulated in quiescent UC compared with healthy controls (Coskun *et al.*, 2013). These findings demonstrate that miRNAs do not only have the potential to be used as biomarkers for the diagnosis of UC, but they could also be used to distinguish between disease activity.

Furthermore, studies have also coupled investigations into the expression of miRNAs in people with UC with explorations into their possible gene targets. For example, Pekow *et al.* showed a significant inverse correlation between *miR-143* and *miR-145,* that were downregulated in UC patients, with their targets *IRS-1, K-RAS, API5* and *MEK-2* (Pekow *et al.*, 2012). Koukos *et al.* also observed significantly increased *STAT3* expression in paediatric patients with UC and concluded that this may be explained by reduced *miR-124* expression that may regulate *STAT3* expression (Koukos *et al.*, 2013). TNF-α has also been described to be a target of a miRNA, *miR-19a,* found to be significantly downregulated in colon tissue from patients with UC (Chen *et al.*, 2013).

5.3.1.3 microRNA expression in the colorectum of people with polyps

Tsikitis *et al.* recently investigated expression of *miR-145, miR-192* and *miR-320a*, three miRNAs implicated in colorectal carcinogenesis, in 113 adenomas that varied histologically and in tumour grade (Tsikitis *et al.*, 2014). Analysis by microarray revealed that *miR-320* expression increased with histologic progression, from normal mucosa to tubular, sessile serrated and traditional

serrated adenomas and to, ultimately, high grade dysplasia. This finding disagrees with previous reports in the literature that have observed reduced *miR-320* expression in CRC (Sun *et al.*, 2012). Interestingly, one of the targets of *miR-320* is β-catenin, and it has previously been shown that *miR-320* inhibited β-catenin expression in CRC cells, resulting in reduced cell proliferation (Sun *et al.*, 2012). On the contrary, expression of both *miR-145* and *miR-192* decreased with histologic progression. *miR-192* expression has been reported to be downregulated in CRCs (Chiang *et al.*, 2012), and so findings from this study suggest that altered *miR-192* expression may be observed early during carcinogenesis. The results from this study, where a reduction in *miR-145* was observed with progression towards carcinoma, are in agreement with previous studies that have shown reduced *miR-145* expression in CRC and have identified it as a tumour suppressor (Slaby *et al.*, 2007).

Oberg and colleagues quantified expression of 735 miRNAs by microarray in normal colonic mucosa, tubulovillous adenomas and in adenocarcinomas in an attempt to further examine changes in miRNA expression in the progression from the normal mucosa to carcinoma (Oberg *et al.*, 2011). The differences in global miRNA expression patterns clearly distinguished the three tissue types and further analyses revealed a total of 31 miRNAs that were significantly up- or downregulated in adenomas compared with normal tissue. These included *miR-135b, miR137, miR-552* and *miR-99a.* Interestingly, Nagel *et al.* have previously shown that *miR-135b,* which was increased in both adenomas and carcinomas, targets *APC,* consequently resulting in an induction of the WNT signalling pathway (Nagel *et al.*, 2008).

A miRNA cluster that has been implicated in CRC, and more recently in pre-neoplastic lesions, is the miR-17-92 cluster, encoding *miR-17, miR-18a, miR-19a, miR-19b, miR-20a* and *miR-92*. Tsuchida *et al.* quantified expression of the six mature miRNAs encoded by this cluster in colorectal adenoma and carcinoma tissues by qPCR (Tsuchida *et al.*, 2011). All six miRNAs were upregulated in adenoma and carcinoma tissues compared with healthy controls, however this was only statistically significant for the carcinoma versus normal tissue comparison. Diosdado *et al.* conducted a similar study and also quantified expression of the six miRNAs encoded by the miR-17-92 cluster in healthy controls and patients with adenomas and adenocarcinomas (Diosdado

et al., 2009). The results from this study were consistent with the findings from the study by Tsuchida *et al.* (2011) and found that expression of all six miRNAs was increased in both adenoma and carcinoma tissues. In addition, they observed significantly higher expression of the miRNAs in carcinoma compared with adenoma tissues.

Studies that have investigated miRNA expression in polyps have not been limited to the colorectal mucosa. A study investigated differences in miRNA expression in the plasma of patients with adenomas with the aim of identifying potential biomarkers (Kanaan *et al.*, 2013). A total of 380 miRNAs were quantified and eight miRNAs (*miR-15b, miR-17, miR-142-3p, miR-195, miR-331, miR-532, miR-532-3p* and *miR*-652) were differentially expressed in the plasma of polyp patients compared with healthy controls. The authors concluded that, after further validation, the differentially expressed miRNAs could potentially be used as future biomarkers of colorectal adenomas and that their measurement in plasma provides a non-invasive and inexpensive method.

In stool, differences in miRNA expression in patients with polyps have also been detected. Wu and colleagues examined expression of *miR-21* and *miR-92a,* two miRNAs upregulated in the tissue (Schetter *et al.*, 2008) and plasma (Ng *et al.*, 2009) of CRC patients (Wu *et al.*, 2012a), and found significantly upregulated *miR-92a* expression in the stool of patients with polyps compared with healthy controls. However, there were no differences observed in expression of *miR-21.* These findings support the use of stool samples for the detection of miRNAs differentially expressed in pre-neoplastic lesions that may be used as biomarkers.

A later study by the group further investigated the use of miRNAs detected in stool samples as biomarkers of adenomas and carcinomas (Wu *et al.*, 2014). Expression of 667 miRNAs was quantified by microarray analysis in both stool and tissue samples collected from healthy participants and patients with advanced adenomas and carcinomas. *miR-31* and *miR-135b*, which were both upregulated, showed the largest difference between adenoma or carcinoma tissues and the normal mucosa. Similar analysis of stool samples agreed that *miR-135b,* but not *miR-31,* was significantly increased in patients with adenomas or carcinomas compared with healthy controls. The investigators

therefore identified another potential miRNA, *miR-135b,* which could be used as a biomarker of pre-cancerous lesions or carcinomas in stool samples.

5.3.2 Hypotheses, aims and objectives

5.3.2.1 Hypotheses

The hypothesis for this investigation was that participants at higher risk of CRC, i.e. those with quiescent UC or a prior history of adenomatous polyps, would exhibit differential expression of miRNAs implicated in CRC and involved in the regulation of the WNT signalling pathway.

5.3.2.2 Aims

This study aimed to test this hypothesis by investigating the differences in miRNA expression in the macroscopically-normal mucosa of people at differential CRC risk, i.e. those with quiescent UC, those with a prior history of adenomatous polyps and those at normal risk of developing CRC.

5.3.2.3 Objectives

- To obtain colorectal biopsies from the macroscopically-normal mucosa of three groups of DISC Study participants at differential rick of CRC i.e. those with quiescent UC, those with a prior history of adenomatous polyps and “normal” participants;
- To review the literature to select miRNAs to be quantified in participants at higher risk of CRC;
- To extract an adequate amount of high-quality RNA, including small RNAs such as miRNAs, from OCT-embedded rectal mucosal biopsies;
- To reverse transcribe RNA for the synthesis of cDNA;
- To utilise cDNA to quantify the expression of the selected miRNAs using qPCR;

- To compare miRNA expression in the ‘Normal’ participants and in the two higher-risk groups (‘UC’ and ‘Polyp’ participants);
- To investigate the effects of key epidemiological factors, including age and gender, and endoscopy procedure on miRNA expression.

5.3.3 Methods

The expression of miRNAs was quantified by qPCR as described in section 2.2.2. The ANOVA GLM was used to investigate differences in expression between the three participant groups, with age, gender, endoscopy procedure, BMI and smoking status incorporated as covariates. Dunnett’s test was used to correct for multiple comparison testing and pairwise comparisons were analysed using the Bonferroni method. Where data were not normally-distributed despite transformation, the non-parametric Kruskal-Wallis test was used.

5.3.4 Results

A total of five miRNAs were selected to be quantified in the ‘UC’ and ‘Polyp’ participants as part of the analyses in participants at differential CRC risk. These were *miR-101, miR-122a, miR-135b, miR-145* and *miR-335*. In addition, the eight miRNAs selected to be quantified in the dietary intervention arm of the study were also analysed in the higher-risk groups. The data for the intervention eight miRNAs from the higher-risk groups were compared with the pre-intervention data from the intervention study, which represented the ‘Normal’ group.

5.3.4.1 Expression of miR-101, miR-122a, miR-135, miR-145 and miR-335 in the macroscopically-normal mucosa of ‘Normal’, ‘UC’ and ‘Polyp’ participants

miR-101, miR-122a, miR-135b, miR-145 and *miR-335* were selected to be quantified in participants at higher risk of CRC. A total of 63 participants, comprising 25 ‘Normal’, 12 ‘UC’ and 26 ‘Polyp’ participants, were used in the analysis of the five selected miRNAs in participants at differential risk of CRC

(Table 5.4). These were age and gender-matched as best possible as well as attempting to match for BMI and smoking status.

Table 5.4 Participant characteristics for the quantification of miRNAs in participants at differential risk of CRC.

		Higher-risk	
Risk group	**Normal**	**UC**	**Polyp**
Number of participants	25	12	26
Ethnicity Caucasian (%)	25 (100)	12 (100)	26 (100)
Gender Female (%) Male (%)	 6 (24) 19 (76)	 3 (25) 9 (75)	 6 (23) 20 (77)
Age Mean (years) Range	 60.8 43 – 78	 56.5 38 – 71	 61.9 44 – 79
Smoking status Never (%) Former (%) Current (%)	 12 (48) 10 (40) 3 (12)	 3 (25) 8 (67) 1 (8)	 8 (31) 11 (42) 5 (19)
BMI Mean (kg/m^2) Range	 28.5 23.2 – 35.5	 29.3 22.2 – 37.6	 28.8 19.8 – 40.3
Procedure Colonoscopy (%) Flexi Sigmoidoscopy (%)	 5 (20) 20 (80)	 12 (100) 0 (0)	 24 (92) 2 (8)

Differences in expression of *miR-101, miR-122, miR-135b* and *miR-335* were analysed using the ANOVA GLM. Expression data for *miR-145* were not normally-distributed, despite transformation, and consequently the non-parametric test Kruskal-Wallis was utilised to investigate differences between the participant groups. There were no statistically significant differences in expression of the quantified miRNAs observed between the three participant

groups (Table 5.5). Differential expression of *miR-135b* between the three participant groups was close to reaching statistical significance (p=0.076), with expression being approximately two-fold higher in the 'UC' group compared with 'Normal' and 'Polyp' participants who had comparable expression levels.

Table 5.5 Expression of miRNAs selected for quantification in participants at differential risk of CRC in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.

miRNA	Normal	UC	Polyp	P value
***miR-101*[1]** N=60	38.8 (23.9 – 63.1)	26.1 (13.9 – 48.8)	36.5 (24.1 – 55.2)	0.588
***miR-122*[1]** N=54	0.0244 (0.0170 – 0.0349)	0.0163 (0.0101 – 0.0261)	0.0165 (0.0122 – 0.0223)	0.355
***miR-135b*[1]** N=60	5.37 (3.64 – 7.92)	10.06 (6.10 – 16.60)	5.38 (3.87 – 7.50)	0.076
***miR-145*[2]** N=63	1140 (880 - 1919)	767 (322 - 1545)	1040 (745 - 1479)	0.264
***miR-335*[1]** N=60	5.14 (3.72 – 7.08)	3.87 (2.56 – 5.85)	3.30 (4.35)	0.201

Data are presented as adjusted copies. [1]Data are presented as LSMs (95% CIs). The ANOVA GLM was used to compare miRNA expression between the three groups and Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method was used for pairwise comparisons. [2]Data are presented as median (lower and upper quartiles). The non-parametric Kruskal-Wallis test was used to compare miRNA expression between the three groups.

5.3.4.2 Expression of miR-17, miR-19a, miR-19b, miR-20a, miR-25, miR-93, miR-106b and miR-424 in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants

Differences in expression of *miR-17*, *miR-19b* and *miR-25* between participant groups were analysed using the ANOVA GLM. However, it was not possible to achieve normally-distributed data, despite transformation, for expression of *miR-19a, miR-20a, miR-93, miR-106b* and *miR-424* and, consequently, the non-parametric test Kruskal-Wallis was used.

Expression of *miR-424* differed significantly between the three participant groups (p<0.01) (Table 5.6). *miR-424* expression was greater than two-fold

higher in participants in the ‘UC’ group compared with the ‘Normal’ group and almost three-fold higher when compared with the ‘Polyp’ group. Furthermore, *miR-424* expression was significantly lower in ‘Polyp’ compared with ‘Normal’ participants.

When applying Bonferroni’s correction for multiple testing and adjusting the p-value threshold to 0.006, the differences in *miR-424* expression between the three participant groups remain statistically significant ($p<0.001$).

A close to significant effect of risk group was observed for *miR-20a* expression, with reduced *miR-20a* expression in both higher-risk groups compared with the ‘Normal’ group ($p=0.055$). There were no significant differences observed for any of the other miRNAs analysed. However, although not statistically significant, there was evidence for apparent between group differences in expression of *miR-19a* and *miR-19b. miR-19a* expression appeared to be 50% greater in participants in the ‘Polyp’ group compared with those in the ‘Normal’ and ‘UC’ groups. Participants in the higher-risk groups appeared to have increased *miR-19b* expression compared with the ‘Normal’ group by just under two-fold. Interestingly, *miR-19b* expression was highly comparable in the ‘UC’ and ‘Polyp’ participants.

Table 5.6 Expression of miRNAs selected for the intervention arm of the study in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.

miRNA	Normal	UC	Polyp	P value
***miR-17*[1]**	120 (109 – 132)	128 (110 – 151)	127 (109 – 149)	0.823
***miR-19a*[2]**	96.9 (36.8 – 268.8)	89.4 (64.1 – 356.3)	155.5 (61.3 – 373.0)	0.465
***miR-19b*[1]**	61.7 (46.7 – 83.2)	118.3 (61.0 – 262.2)	117.9 (72.0 – 206.7)	0.120
***miR-20a*[2]**	333 (258 – 424)	279 (238 – 311)	308 (251 – 374)	0.055
***miR-25*[1]**	98.4 (81.6 – 121.0)	86.2 (60.4 – 133.1)	66.3 (52.4 – 86.5)	0.097
***miR-93*[2]**	77.2 (59.0 – 97.5)	67.7 (60.7 – 78.3)	69.5 (62.2 – 75.2)	0.277
***miR-106b*[2]**	164 (138 - 222)	144 (119 – 174)	162 (120 – 193)	0.296
***miR-424*[2]**	32.5 (19.2 – 43.8)	68.6 (32.7 – 89.7)	23.9 (16.0 – 30.9)	**<0.001**

Data are expressed as adjusted copies. [1]Data are presented as LSMs (95% CIs). The ANOVA GLM was used to compare miRNA expression between the three groups and Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method was used for pairwise comparisons (N=91). [2]Data are presented as median (lower and upper quartiles). The non-parametric Kruskal-Wallis test was used to compare miRNA expression between the three groups (N=94).

5.3.4.3 Exploration of additional factors influencing microRNA expression

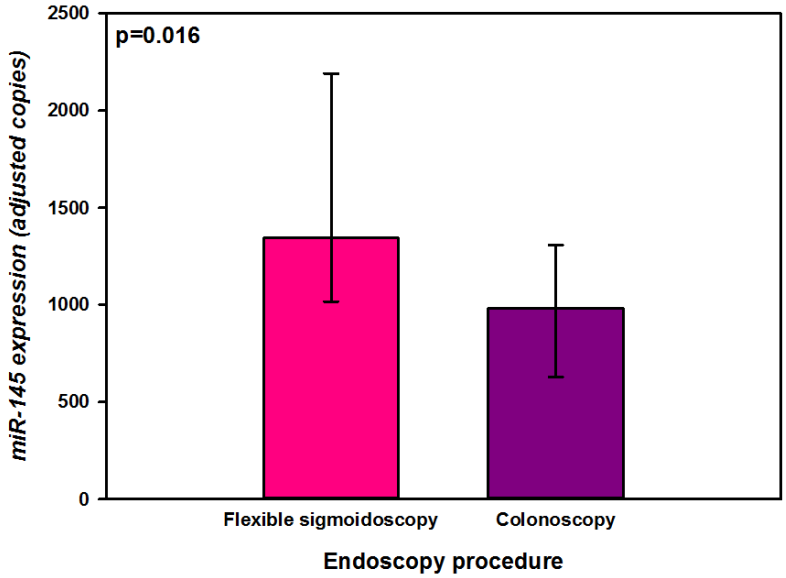
For the miRNAs selected to be analysed specifically in participants at differential risk of CRC, a significant effect of endoscopy procedure was observed on *miR-145* expression (p=0.016). There were no effects of any other the covariates observed on the other miRNAs (Table 5.7).

Table 5.7 P values for the effects of covariates on the expression of miRNAs selected for quantification in participants at differential risk of CRC.

miRNA	Age	Gender	Endoscopy procedure	BMI	Smoking status
miR-101	0.237	0.436	0.610	0.325	0.230
miR-122a	0.835	0.846	0.519	0.545	0.074
miR-135b	0.283	0.881	0.825	0.440	0.549
miR-145	0.739	0.699	**0.016**	0.416	0.546
miR-335	0.723	0.799	0.865	0.662	0.540

miR-145 expression was significantly higher, by greater than one and a half-fold, in participants who underwent examination by flexible sigmoidoscopy compared with those who had a colonoscopy (Figure 5.4).

Figure 5.4 Effect of endoscopy procedure on expression of *miR-145.*



Data are expressed as adjusted copies and presented as medians. Error bars represent low and upper quartiles (Kruskal-Wallis). N=63.

For the miRNAs selected for the intervention analyses, that were also quantified in participants at differential risk of CRC, a number of associations between the analysed additional factors and expression of the miRNAs were observed (Table 5.8). An effect of endoscopy procedure was observed on all but three of

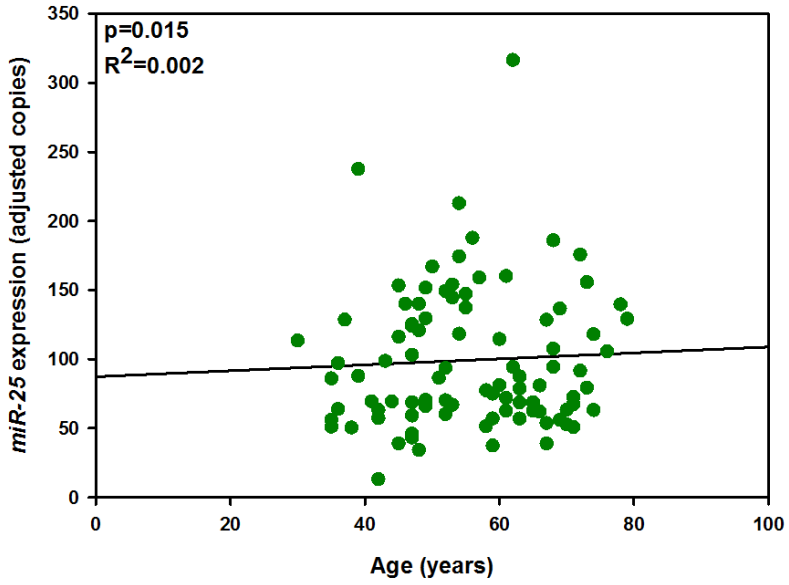
the miRNAs quantified. Smoking status was a determinant of *miR-19a* and *miR-19b* expression. Significant effects of BMI on expression of *miR-17* and *miR-19b* and of age on *miR-25* expression were also found.

Table 5.8 P values for the effects of covariates on the expression of miRNAs selected for the intervention arm of the study.

miRNA	Age	Gender	Endoscopy procedure	BMI	Smoking status
miR-17	0.159	0.050	**0.003**	**0.039**	0.081
miR-19a	0.517	0.799	0.753	0.438	**0.023**
miR-19b	0.969	0.294	**0.009**	**0.002**	**0.011**
miR-20a	0.600	0.239	**0.001**	0.432	0.068
miR-25	**0.015**	0.092	0.688	0.579	0.421
miR-93	0.233	0.060	**0.031**	0.422	0.207
miR-106b	0.365	0.113	**0.027**	0.424	0.165
miR-424	0.213	0.581	0.139	0.444	0.398

Analysis using the Spearman rank order correlation test revealed a positive, but weak, correlation between participant age and *miR-25* expression (Spearman correlation coefficient 0.062) (Figure 5.5).

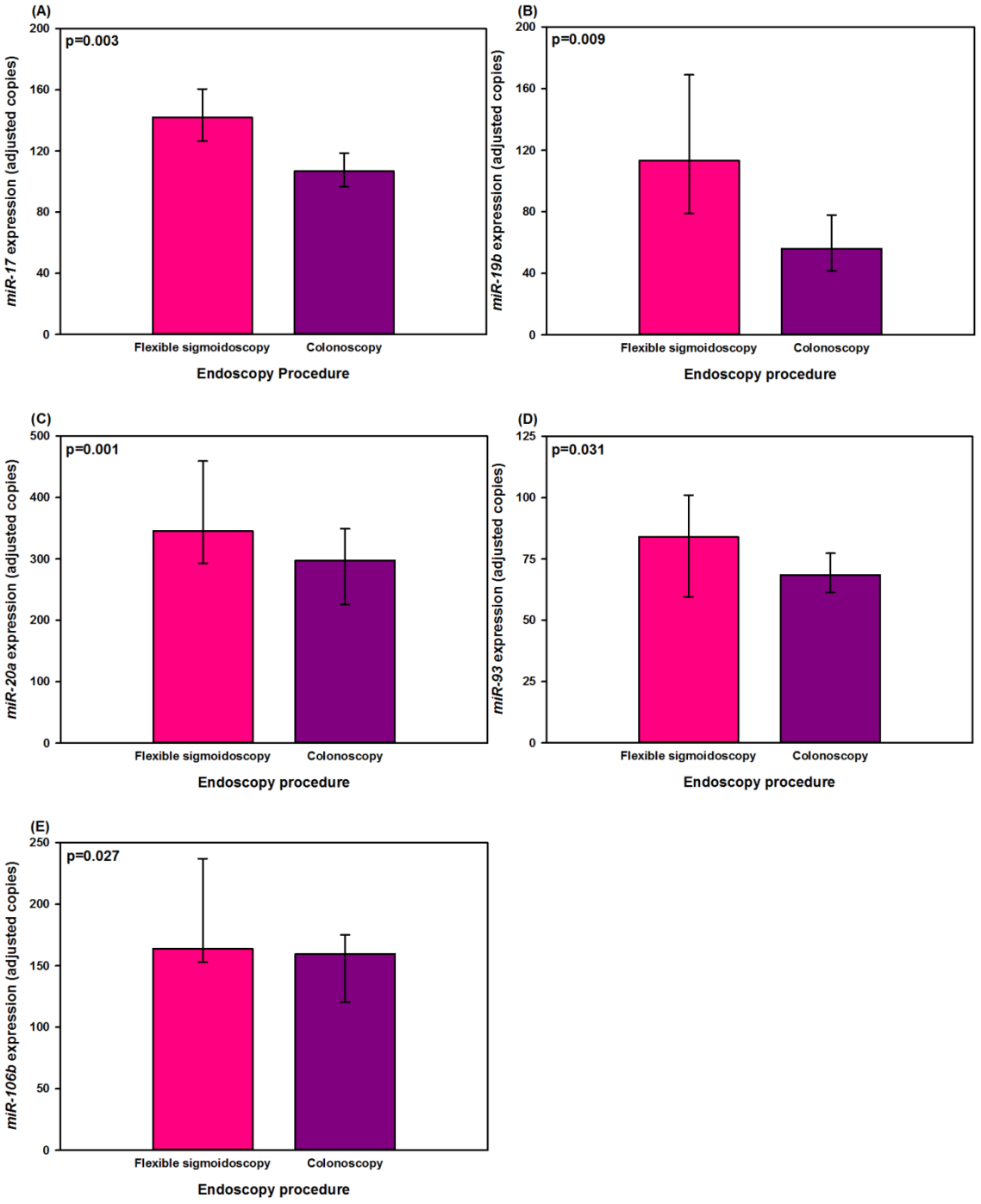
Figure 5.5 Positive correlation between age and *miR-25* expression.



N=91.

Interestingly, expression of *miR-17, miR-19b, miR-20a, miR-93* and *miR-106b* was significantly lower in participants who underwent endoscopic examination by colonoscopy compared with those who had a flexible sigmoidoscopy (Figure 5.6).

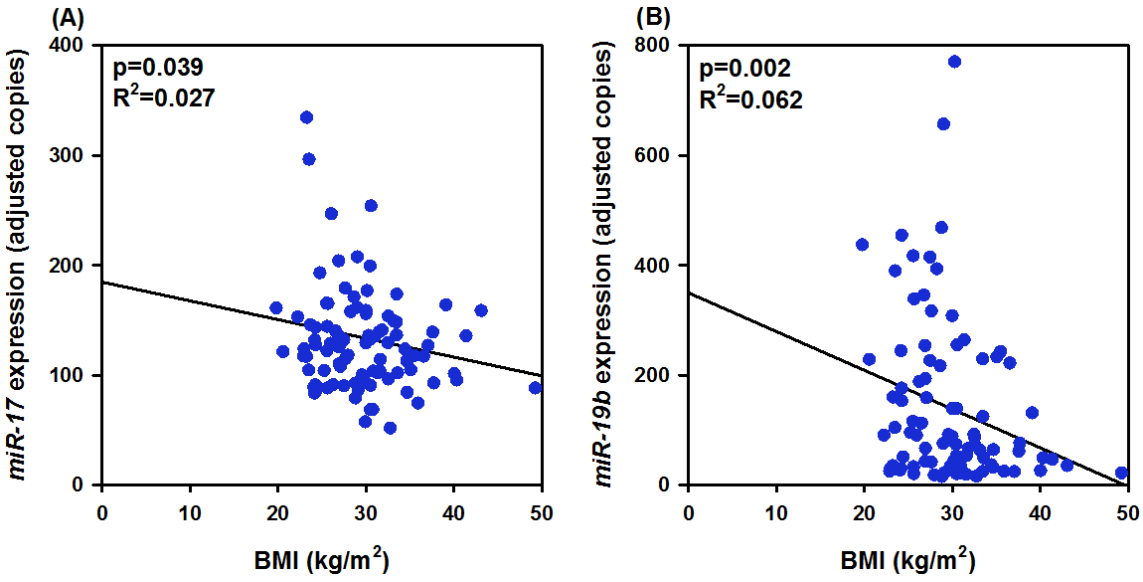
Figure 5.6 Effect of endoscopy procedure on miRNA expression.



Data are expressed as adjusted copies. Data for *miR-17* (A) and *miR-19b* (B) are presented as LSMs and error bars represent 95% CIs (ANOVA GLM) (N=91). Data for *miR-20a* (C), *miR-93* (D) and *miR-106b* (E) are presented as medians and error bars represent lower and upper quartiles (Kruskal-Wallis) (N=94).

In addition, expression of *miR-17* and *miR-19b* was also significantly affected by BMI. In both cases, an inverse correlation between BMI and miRNA expression was observed (Figure 5.7).

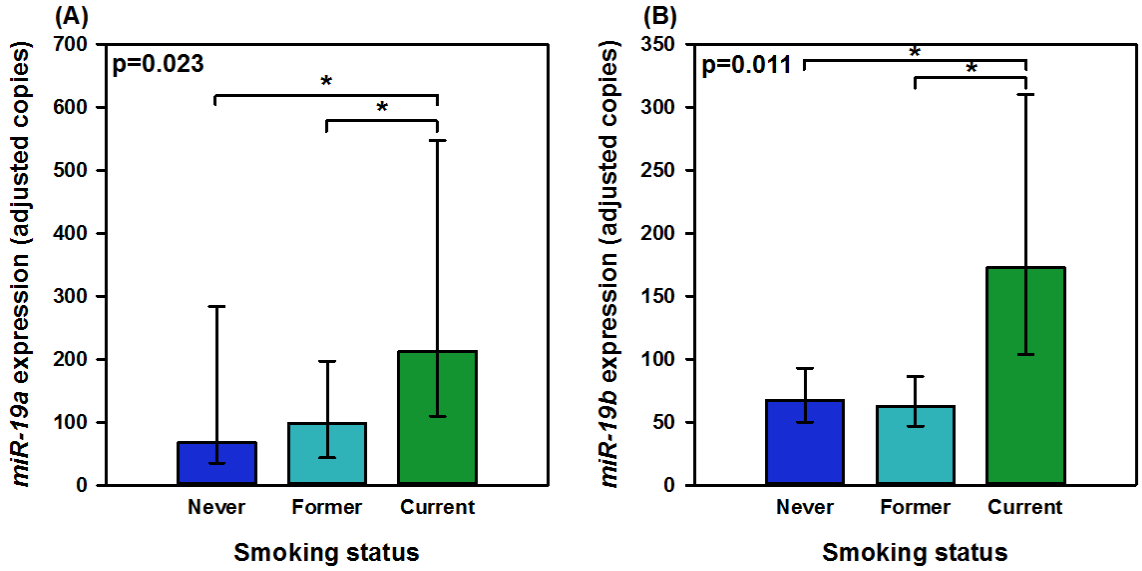
Figure 5.7 Inverse correlation between BMI and *miR-17* (A) and *miR-19b* (B) expression.



Data are expressed as adjusted copies and presented as LSMs (ANOVA GLM). N=91.

A significant association between smoking status and expression of *miR-19a* and *miR-19b* was found. Current smokers had significantly increased expression of both *miR-19a* and *miR-19b* compared with participants who had never smoked and former smokers (Figure 5.8). In both cases, miRNA expression was approximately two-fold greater in current smokers compared with non-smokers.

Figure 5.8 Effect of smoking status on *miR-19a* and *miR-19b* expression.



Data are expressed as adjusted copies. Data for *miR-19a* (A) are presented as medians and error bars represent the lower and upper quartiles (Kruskal-Wallis) (N=94). Data for *miR-19b* (B) are presented as LSMs and error bars represent 95% CIs (ANOVA GLM) (N=91).

5.3.5 Discussion

This study quantified miRNA expression in the macroscopically-normal mucosa of people at differential risk of CRC. The literature was reviewed to select miRNAs reported to be aberrantly expressed in CRC and involved in the regulation of the WNT signalling pathway.

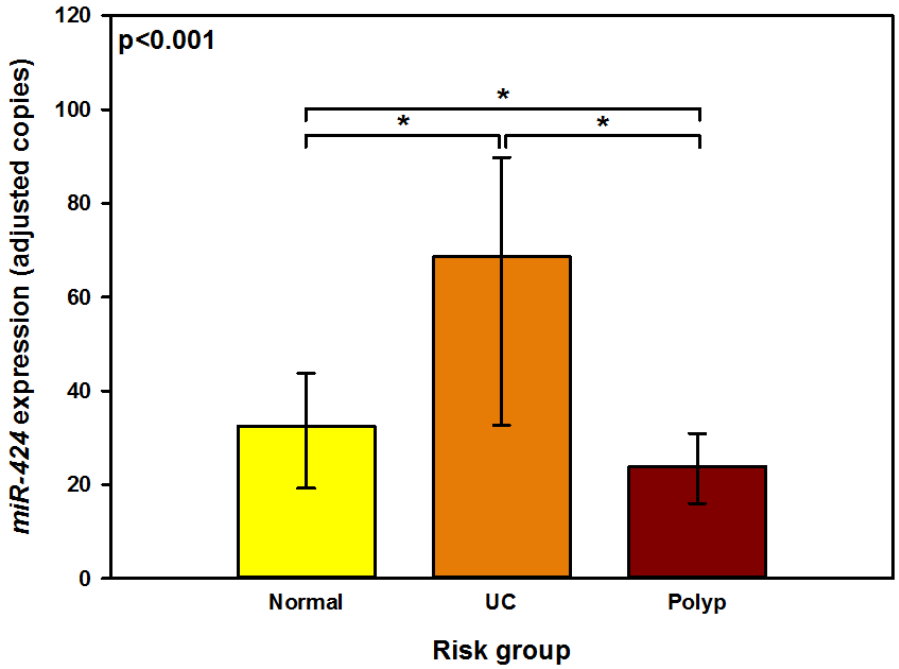
Previous studies have observed differential expression of miRNAs in the active and uninflamed mucosa of patients with UC compared with the normal mucosa (Wu *et al.*, 2008; Takagi *et al.*, 2010; Bian *et al.*, 2011; Coskun *et al.*, 2013). Differences in expression of miRNAs, particularly the miR-17-92 cluster (Diosdado *et al.*, 2009; Tsuchida *et al.*, 2011), have also been observed in the mucosa, plasma and stool of patients with polyps (Wu *et al.*, 2012a; Kanaan *et al.*, 2013). Furthermore, one study identified an increase in a miRNA that targets and inhibits β-catenin (Sun *et al.*, 2012), *miR-320,* in the histological progression from the normal mucosa to adenomas and ultimately high-grade dysplasia (Tsikitis *et al.*, 2014).

In the present study, there were no differences in expression of *miR-101, miR-122, miR-135b*, *miR-145* or *miR-335,* selected specifically for quantification in

the macroscopically-normal mucosa of participants at differential risk of CRC, between the three participant groups. Reduced *miR-145* expression has been reported in both the mucosa of patients with UC (Pekow *et al.*, 2012) and in the progression from normal mucosa to adenomas and dysplasia (Tsikitis *et al.*, 2014). On the other hand, *miR-135b* expression has previously been observed to be increased in the stool of patients with colorectal adenomas (Wu *et al.*, 2014). To date, altered expression of *miR-101, miR-122* or *miR-335* in UC or polyps have not been reported previously.

In addition, the eight miRNAs selected to be quantified in the intervention arm of the DISC Study, that are also implicated in colorectal carcinogenesis, were also quantified in the two higher-risk groups. From the eight miRNAs analysed, a significant difference between participant groups was discovered for the expression of *miR-424*. Participants with quiescent UC had greater *miR-424* expression compared with 'Normal' and 'Polyp' participants (Figure 5.9). This is consistent with findings from a study by Lin *et al.* where increased *miR-424* expression was observed in colonic tissue samples collected from IBD patients, comprising both UC and Crohn's disease patients (Lin *et al.*, 2014). Interestingly, an increase in expression of *miR-424* has also been previously observed in colonic cancer tissue compared with adjacent normal tissue in CRC without lymph node metastasis (Wang *et al.*, 2010). I also observed that *miR-424* expression was significantly lower in participants with a prior history of polyps compared with 'Normal' participants. This has not been reported previously.

Figure 5.9 Expression of *miR-424* in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.



Data are presented as adjusted copies and expressed as medians (Kruskal-Wallis). Error bars represent lower and upper quartiles. N=94.

Previous studies have reported an increase in *miR-17, miR-19a, miR-19b* and *miR-20a* expression in adenomas (Diosdado *et al.*, 2009; Tsuchida *et al.*, 2011). In the present study, although not statistically significant, an increase in *miR-19a* was observed in the 'Polyp' group and upregulated *miR-19b* was found in both higher-risk groups compared with the 'Normal' group.

5.4 *WNT11* DNA methylation in the macroscopically-normal mucosa of people at differential risk of CRC

5.4.1 Introduction

As differences in *WNT11* expression at the mRNA level were observed between the three participant groups, *WNT11* methylation was assessed to test the hypothesis that changes in gene expression may result from alterations in DNA methylation. To my knowledge, this is the first study to investigate *WNT11* methylation in the human colorectal mucosa.

5.4.1.1 The role of WNT11 in the WNT signalling pathway

WNT11 encodes a 45kD glycoprotein that acts as a ligand in the WNT signalling pathway and binds to Frizzled receptors, particularly Fzd7 (Uysal-Onganer and Kypta, 2012). WNT11 is a member of the WNT5A family of WNT proteins that comprises WNT4, WNT5A and WNT11 (Du *et al.*, 1995). Unlike the WNT1 family, the WNT5A family are not potent activators of the canonical WNT signalling pathway (Maye *et al.*, 2004).

Although WNT11 has been observed to act principally via the non-canonical WNT signalling pathway (Rao and Kuhl, 2010), it has also been suggested that WNT proteins may activate both canonical and non-canonical pathways (Maye *et al.*, 2004). In the canonical pathway, WNT11 has been shown to activate (Tao *et al.*, 2005) and inhibit WNT signalling (Maye *et al.*, 2004).

5.4.1.2 WNT11 in the healthy colon and in carcinogenesis

In the colon, Liu *et al.* have investigated the role of WNT11 in the inflammatory response to *Salmonella* infection (Liu *et al.*, 2011). An increased risk of IBD has been reported in people previously infected with *Salmonella* (Gradel *et al.*, 2009). In small intestinal epithelial cells and in the mouse intestine, an increase in WNT11 expression at both the mRNA and protein level was observed following *Salmonella* infection. Further investigations revealed that increased WNT11 reduced the number of *Salmonella* bacteria in HCT-116 cells and inhibited the inflammatory response, suggesting that WNT11 plays a role in protecting against bacterial infections and in the regulation of inflammation.

WNT11 has been implicated in colorectal carcinogenesis and has been proposed to be involved in cancer progression through the induction of proliferation, migration and invasion (Nishioka *et al.*, 2013). In addition, in mouse intestinal epithelial cells, WNT11 stimulates proliferation and migration (Ouko *et al.*, 2004). Furthermore, a reduction in cell migration has been reported in HCT-116 cells with abolished WNT11 expression (Dwyer *et al.*, 2010). Kirikoshi and colleagues have proposed that upregulation of *WNT11* in colorectal adenocarcinomas may play an important role in colorectal tumourigenesis through the stimulation of WNT signalling (Kirikoshi *et al.*, 2001).

5.4.1.3 WNT11 in the colorectum of people with ulcerative colitis

Differential expression of *WNT11* in the mucosa of people with UC has only been reported in one study. *WNT11* expression, assessed by microarray analysis, was eleven-fold higher in the uninflamed mucosa of patients with UC compared with healthy controls (You *et al.*, 2008).

An earlier study investigated expression of *WNT11*, amongst other WNT ligands and receptors, in myofibroblasts isolated from patients with UC but found that *WNT11* expression was too low to be analysed (Hughes *et al.*, 2011).

5.4.1.4 WNT11 in the colorectum of people with polyps

Similar to investigations in patients with UC, the number of studies investigating *WNT11* expression in patients with polyps are limited and *WNT11* methylation in these patients has not been analysed previously.

Lai *et al.* investigated expression of *WNT11*, amongst other genes, in a mouse model of Peutz-Jegher's syndrome, an inherited disease characterised by the development of polyps in the digestive tract (Lai *et al.*, 2011). Peutz-Jegher's patients are at increased risk of developing cancers of the GI tract, amongst others. Expression of a number of WNT pathway-related genes differed significantly in gastric polyp tissue compared with the healthy mucosa, including *WNT5A*, *WNT11* and *FZD4*. *WNT11* expression was more than two-fold greater in gastric polyps compared with the healthy mucosa.

5.4.2 Hypotheses, aims and objectives

5.4.2.1 Hypotheses

The hypothesis for this investigation was that the differential expression of *WNT11* observed in 'Polyp' participants, who are at higher risk of CRC, may be a consequence of differences in *WNT11* methylation. In particular, it was hypothesised that the increased expression of *WNT11* in these participants may have resulted from a reduction in *WNT11* methylation.

5.4.2.2 Aims

This study aimed to test this hypothesis by investigating the differences in *WNT11* methylation levels in the macroscopically-normal mucosa of people at differential CRC risk, i.e. those with quiescent UC, those with a prior history of adenomatous polyps and those at normal risk of developing CRC.

5.4.2.3 Objectives

- To obtain colorectal biopsies from the macroscopically-normal mucosa of three groups of DISC Study participants at differential risk of CRC, i.e. those with quiescent UC, those with a prior history of adenomatous polyps and "normal" participants;
- To select an adequate primer to be used to quantify *WNT11* methylation;
- To bisulphite modify DNA previously extracted by the DISC Study Team;
- To amplify the region to be analysed by PCR;
- To perform an assay validation analysis;
- To quantify *WNT11* methylation at each CpG site by pyrosequencing;
- To compare *WNT11* methylation at each CpG site and the mean across all sites in the 'Normal' participants and in the two higher-risk groups ('UC' and 'Polyp' participants);
- To investigate the effects of key epidemiological factors including age and gender on *WNT11* methylation.

5.4.3 Methods

The methods for this study are described in section 2.2.3. Amplification of BM-DNA by PCR and subsequent quantification of *WNT11* methylation by pyrosequencing was undertaken under my supervision by Idoia Ibero, an Erasmus Project student, to whom I am very grateful. A commercially-available primer assay was used for the methylation analysis of *WNT11*. The sequence analysed (please see below) comprised five CpG sites (highlighted in yellow), was within a CpG island and was located 1,436bp away from the promoter region (Figure 5.10).

GGAGA**CG**GGGTCCT**CG**GGGGA**CG**CA**CG**G**CG**A

The ANOVA GLM was used to investigate differences in *WNT11* mean methylation and methylation at each CpG site with the exception of site 4, where the data were not normally-distributed and consequently the non-parametric test Kruskal-Wallis was used. The Dunnett post-hoc test was used for multiple comparisons with the control ('Normal') group and the Bonferroni method was used for pairwise comparisons.

Figure 5.10 *WNT11* nucleotide sequence (-) illustrating promoter sequences, CpG islands and sequence analysed by pyrosequencing.

CCTGTGCGCGTGGACGCGGGGTCCCTACTCACAGCCACTTGATGCCATAGCACACGCCGGTCT
GGAGCGCCAGGGCGAAGAGCAGCGCCTCGCAGACCTGCGGCCGCGCCCTCATCGTCGCGCG
GCGGGCGCGCCCG**GGGTCACACCCAGGAGGAGCCGCGCCGAAGTCCTCCGCCTGCACG**
GCCGCCGCTGGTCCTGCACGCCGCCTGCAGCCGGGGAGAGCGGAGGCGGCGGGTTAA
GGCGGCGCGCGGGCGGGGGAGGCGTTTTATTCAAATTACAAAGGAGGGGTCGGGGCCC
GGGGAGGCCGAGCGCGGCGGGCGTCCCCTCCCGCTCCGCCCGGCCGGGGGACGCGTC
CCGCGCCTGGCTCGAATTAGGCGCGGCCGAAGGCGTGTCTCCCAGCGTGGCCCCGGGCC
CCAACGCGCCTTCGAGCCCCTCTGGCCGGGGCAGCCGGAAGGAGGGACAGGAGGCGGCAGA
ACCGGAGACCCAGGAGAGACCGGGCAGACACAGCGAGAGGGAGAAGGACGCTCAGAGGACA
AGGCTGAGAGCCACGGAGACAGAGGGGGAAACCCGGACAGACGCAACCTGGGACGGAAAGG
AGAGGAATGAGGAATCAGGAGTCCAGGCGAGGCAGCAGAGTCGAGGACCCGGAGCTGGGGC
TTATCCCTGCAATATTGAGCATTAGTTAATAGGGCCTCGGCAATTCTCCTTCCCAAACGTGTTTC
AGTTTACCTCCTCAGTAAGCTGGGGCAATTCGGGCACAATTCCGCTTTTATAAAGAACGTTCCC
TGGCCGGGCAGGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGTCGAGGCGGGCGGGT
CACCTGAGGTCAGGAGCTCGAGACCAGCCTGCCCAACATGGCAAAACCCCGTCTCTACTACGA
ATACAAAAAATTAGCCGGGCGTGGTGGCGGGCGCCTGTAATCCCAGCTACTCGGGAGGCTGA
GGCAGGAGAACCGCTTGAACCCGGGAGGCGGAGGTTGCAGCGAGCGGAGATTGCGCCACTG
CACTCTAGCCTGGGCGACAAGAGCGAAACTCCGTCTCAAACAAACAGACAAACAAAAAAAGTTC
CCTAACACGATCGCATCTGCTCCTCCCAGCACCCCGTGAGATAGGCACTATCGCTGCCTCATCT
CACAGATGAGGAAACTGAGGCACAGAGGTGGGGTTCAGCTAGGGAGCGGCAAGCCGGGTGTC
CGGCGAAATCAGGAAATGGGGCTTGCTCGGGGCCCTGCTTTCTCGGTGTGGGAAGGGGAGGA
TGGGAAACGGGATGGTAGGGCTCTTTGTACAGTGAGGATTCCGAGTCTCCAAGTCCGTCTTTA
GCCGCTCCACTTTGGAGGGTGGGAGTGGAAGCATGCGGGCTGGGACTGGGCAGCCCCGCCTT
AGCGAAGCGGGGAAGCTCGCGGGGCTGGCGCGGTGTTGCCTCTGGCCTCCAGGCGGCGCCC
TCGGGCCGCGGAGGAGAGGCTGCCCCGCCCGCCCGCAGCCCGCGGAGCAGCTATCTCCGCT
TTTTTTCTCCTTCACTTCATTCTCACCTCATTAACTCCCTTCCCAGCTTCTCCCAAATTTACCAAC
ACCTGGCGTTCGCCCGGAGTCAGACACAGCCCCAGCCCCTCTCCCCTCGGGAGGAGCAGAGA
CAAAGGGATTCCTTTCTAAGGGTTTGGGGAGTTGGAAAAGGAGGCGCCCACGGTAGTGGGGG
CGCTGGAGCAGAAAAGGGACGTTCTTATCTGGCTCTGTGAGCTTGGGCAAGCCCTCAGCCACC
CCAGCCTCGGTGTCCCCTTCCATAAGAACGAGGAATGGGGCT**TCGCCGTGCGTCCCCCGAG**
GACCCCGTCTCCCTGCAGCCTGCGGGGCTCAGCGCCCCTCTGCTATTTGCACCGCGACGCCA
CCTGCGTGGCGCGTTCCTGCGCGTGGGTAGTCAGCACGCAGAGATGGGAAGCCCCACTGTTT
TCTGGAGCCTGTCGGCAAAACCCACAGGAAGGCGAGGAGAAGCCTGGATCGTACAGTTTTTTT
CCTCCGCAGAGCCCCGCAGCTAGCGCCCAGCACAGGGGCGCCTGCGACAAACATGCCGTCAA
GGCTGGGGGTCTC

CpG islands are highlighted in yellow and the promoter sequence is in bold and underlined. The sequence analysed by the PyroMark WNT11 assay (Qiagen) is in red and underlined.

5.4.4 Results

DNA methylation was quantified at five CpG sites within a CpG island by pyrosequencing. Differences between the three participant groups were analysed at each of the five CpG sites as well as for the mean methylation across all five sites.

WNT11 methylation was quantified in a total of 58 participants, comprising 20 'Normal' participants, 12 'UC' participants and 26 'Polyp' participants. The participant characteristics are described in Table 5.9. Participants were matched for age, gender, BMI, smoking status and endoscopy procedure as best as possible and the participants were all Caucasian.

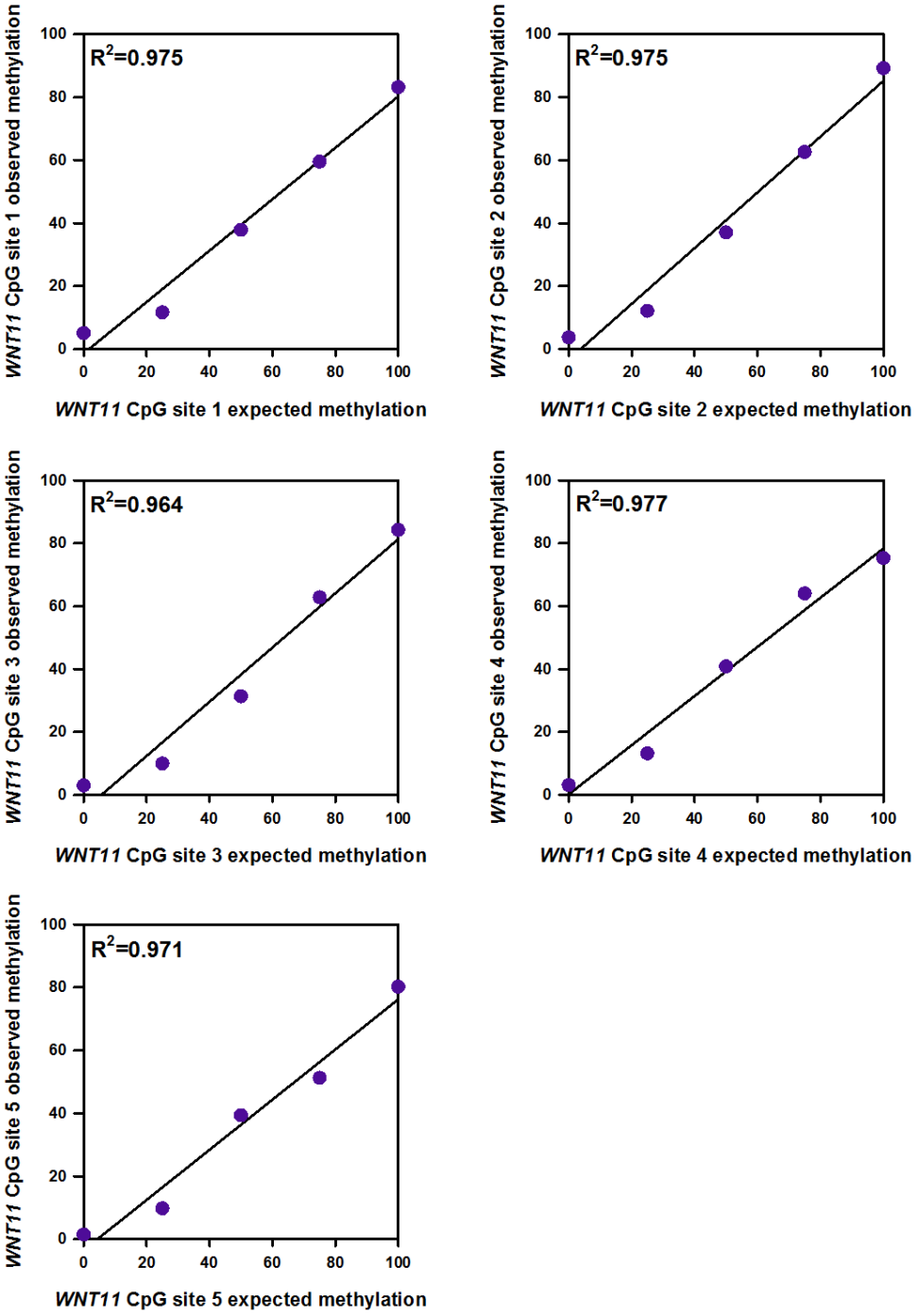
Table 5.9 Participant characteristics for *WNT11* methylation analysis.

		Higher-risk	
Risk group	**Normal**	**UC**	**Polyp**
Number of participants	20	12	26
Gender			
Female (%)	5 (25)	3 (25)	6 (23)
Male (%)	15 (75)	9 (75)	20 (77)
Age			
Mean (years)	59.6	56.50	61.88
Range	41-78	38 – 71	44 – 79
Smoking status			
Never (%)	10 (50)	3 (25)	8 (31)
Former (%)	7 (35)	8 (67)	11 (42)
Current (%)	3 (15)	1 (8)	5 (19)
BMI			
Mean (kg/m^2)	28.9	29.3	28.8
Range	23.2 – 35.7	22.2 – 37.6	19.8 – 40.3
Procedure			
Colonoscopy (%)	4 (20)	12 (100)	24 (92)
Flexi Sigmoidoscopy (%)	16 (80)	0 (0)	2 (8)

5.4.4.1 WNT11 pyrosequencing assay validation

Similarly to that performed prior to quantification of *SFRP1*, standard curves were run to validate the commercially-available *WNT11* pyrosequencing assay. The correlations between expected and observed methylation were optimal, with R^2 values greater than 0.96 achieved for all five CpG sites (Figure 5.11).

Figure 5.11 Standard curves for *WNT11* pyrosequencing assay validation at all five CpG sites.



5.4.4.2 WNT11 methylation in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants

WNT11 methylation was low across all three participant groups at all five CpG sites, ranging from 0% to 13%, ('Normal' 0% - 3.4%, 'Polyp' 0% - 13.0% and 'UC' 0.6% - 3.3%). The CpG site with the greatest methylation was CpG site 2 (mean methylation 2.1%) and the site with lowest methylation was CpG site 3 (mean methylation 1.5%).

Table 5.10 Mean *WNT11* methylation in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants at each CpG site and the mean methylation across all five sites.

CpG site	Normal	UC	Polyp	P value
1[1] N=50	1.37 (0.80 – 1.94)	1.58 (0.94 – 2.21)	2.18 (1.70 – 2.66)	0.184
2[1] N=47	1.51 (0.98 – 2.33)	1.05 (0.69 – 1.59)	2.12 (1.54 – 2.92)	**0.016**
3[1] N=47	1.18 (0.80 – 1.71)	1.36 (0.11 – 0.82)	1.53 (1.10 – 2.13)	0.660
4[2] N=53	1.88 (0.85 – 2.23)	1.97 (1.69 – 2.63)	1.98 (1.40 – 2.66)	0.325
5[1] N=47	1.77 (1.15 – 2.72)	1.86 (1.13 – 3.08)	1.49 (1.02 – 2.18)	0.694
Mean[1] N=51	1.20 (0.82 – 1.74)	1.73 (1.15 – 2.59)	2.01 (1.50 – 2.69)	0.225

Data are expressed as percentage methylation. [1]Data are presented as LSMs (95% CIs). The ANOVA GLM was used to compare methylation between the three participant groups. Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method was used for pairwise comparisons. [2]Data are presented as medians (lower and upper quartiles). The non-parametric Kruskal-Wallis test was used to compare methylation between the three participant groups.

WNT11 methylation differed significantly between the three participant groups at CpG site 2 (p=0.016). *WNT11* methylation at CpG site 2 was approximately two-fold greater in 'Polyp' participants compared with those in the 'UC' group. In addition, *WNT11* methylation at CpG site 2 also appeared to be increased in the 'Polyp' group compared with the 'Normal' group. However, when applying

Bonferroni's correction for multiple testing and adjusting the p-value threshold to 0.008, the differences in *WNT11* methylation at CpG site 2 (p=0.016) lose statistical significance.

5.4.4.3 Exploration of additional factors influencing WNT11 methylation

The effects of age, gender, endoscopy procedure, BMI and smoking status on *WNT11* methylation at each CpG site and on the mean methylation across all five sites were also explored. With the exception of endoscopy procedure, there were no effects of any these additional factors on *WNT11* methylation (Table 5.11).

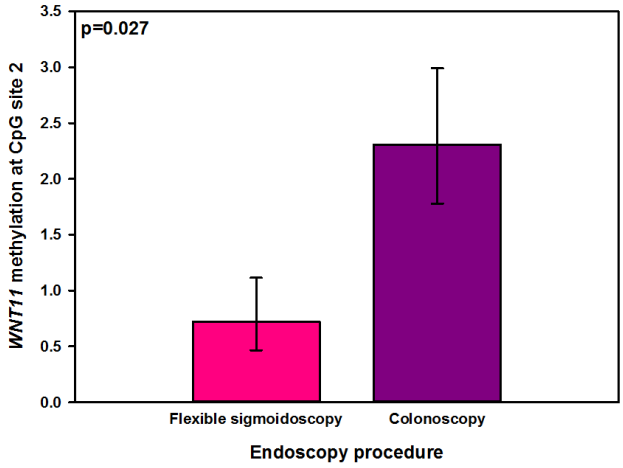
Table 5.11 P values for the effects of age, gender, endoscopy procedure, BMI and smoking status on *WNT11* methylation at each CpG site and on the mean methylation across all sites.

CpG site	Age	Gender	Endoscopy procedure	BMI	Smoking status
1[1]	0.776	0.895	0.259	0.543	0.082
2[1]	0.529	0.510	**0.027**	0.916	0.525
3[1]	0.978	0.642	0.457	0.749	0.259
4[2]	0.249	0.508	0.114	0.445	0.078
5[1]	0.616	0.660	0.367	0.718	0.886
Mean[1]	0.175	0.846	0.405	0.571	0.429

[1]Analysed as covariates in the ANOVA GLM. [2]Analysed using the non-parametric Kruskal-Wallis test.

WNT11 methylation at CpG site 2 was more than three-fold higher in participants who had a colonoscopy compared with a flexible sigmoidoscopy (p=0.027) (Figure 5.12).

Figure 5.12 Effect of endoscopy procedure on *WNT11* methylation at CpG site 2.

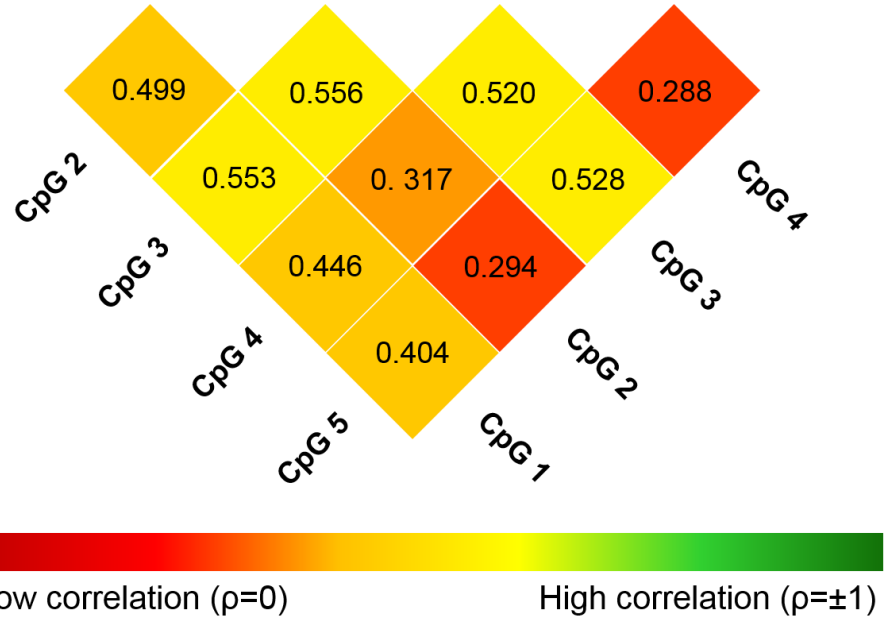


Data are expressed as LSMs and error bars represent 95% CIs (ANOVA GLM). N=47.

5.4.4.4 Correlation of WNT11 methylation between CpG sites

Spearman's correlation analysis was applied to investigate the correlation of *WNT11* methylation levels between the five CpG sites. Correlation between CpG sites ranged from moderate, for example between CpG site 3 and 1, 2, 4 and 5, to low, such as that observed between CpG site 5 and sites 2 and 4 (Figure 5.13).

Figure 5.13 Correlation of *WNT11* methylation between CpG sites.



Values represent Spearman correlation coefficients (ρ). (N=47). P<0.05.

5.4.4.5 Correlation between WNT11 methylation and expression

To investigate whether *WNT11* expression at the mRNA level was associated with *WNT11* methylation, Spearman rank order correlation analysis was run on methylation at each CpG site and the mean methylation across all five CpG sites. The results from these analyses are shown in Table 5.6.

There were no statistically significant correlations between *WNT11* methylation and expression at any of the CpG sites or the mean methylation across all five CpG sites. For two of the sites (CpG sites 1 and 5), *WNT11* methylation and expression were inversely correlated. However, for the mean methylation and the remainder of the CpG sites, a positive correlation was observed. Interestingly, at CpG site 2, where significant differences in methylation were found between the three participant groups, a close to significant positive correlation between *WNT11* methylation and expression was observed (p=0.071).

Table 5.12 Correlation between *WNT11* methylation and expression.

CpG site	Spearman Correlation Coefficient	P value
1	-0.0237	0.876
2	0.2740	0.071
3	0.0246	0.875
4	0.0208	0.891
5	-0.2050	0.186
Mean	0.0170	0.912

Spearman correlation coefficients and corresponding p values for correlation between *WNT11* methylation and expression. N=46.

5.4.5 Discussion

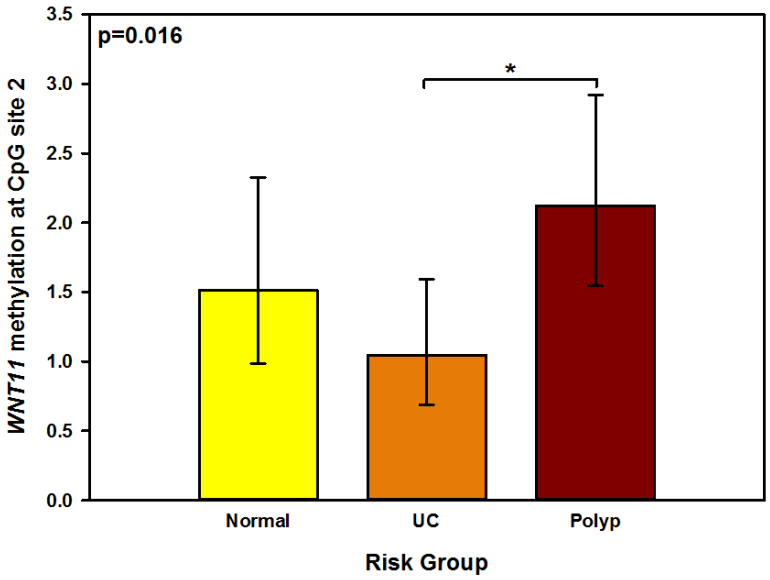
WNT11 is a WNT ligand that binds Frizzled receptors, however its effects on the WNT signalling pathway are not certain. Some studies have reported that it is only involved in activation of the non-canonical WNT pathway (Rao and Kuhl,

2010), whilst others have suggested that it is also able to activate or inhibit the canonical WNT signalling pathway (Maye *et al.*, 2004; Tao *et al.*, 2005).

Few studies have investigated the role of WNT11 in colorectal carcinogenesis and a small number of studies have reported upregulation of *WNT11* in CRC (Kirikoshi *et al.*, 2001; Nishioka *et al.*, 2013). Furthermore, *WNT11* expression is increased in the uninflamed tissue of patients with UC (You *et al.*, 2008) and in polyps from a mouse model of Peutz-Jegher's syndrome (Lai *et al.*, 2011). However, these studies have not investigated differences in *WNT11* methylation in these patients.

In the present study, *WNT11* methylation was assessed at five CpG sites in the macroscopically-normal mucosa of healthy participants, participants with quiescent UC and participants with a prior history of adenomatous polyps by pyrosequencing. A significant difference in *WNT11* methylation at CpG site 2 was observed, where *WNT11* methylation was higher in 'Polyp' participants compared with 'Normal' and 'UC' participants (p=0.016) (Figure 5.14). At the remaining four CpG sites, *WNT11* methylation was comparable between the three participant groups.

Figure 5.14 *WNT11* methylation at CpG site 2 in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.



Data are expressed as percentage methylation. Data are presented as LSMs (95% CIs). The ANOVA GLM was used to compare methylation between the three groups. Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method for pairwise comparisons.N=47.

Contrary to findings from a previous study where an increase in *WNT11* expression was reported (Lai *et al.*, 2011), an increase in methylation in 'Polyp' participants would be indicative of a reduction in *WNT11* expression. Interestingly, the results from the analysis of *WNT11* expression in the higher-risk participants (Section 5.2.4.1) showed that *WNT11* was significantly increased in 'Polyp' participants compared with the 'Normal' group. This suggests that the differences in *WNT11* expression in participants with a prior history of adenomatous polyps result from genetic or epigenetic mechanisms other than DNA methylation.

In addition, to test the hypothesis that *WNT11* expression is regulated by DNA methylation, the correlation between *WNT11* methylation and expression was investigated using Spearman's rank order correlation test. There were no significant correlations observed between methylation and expression at any of the CpG sites or the mean methylation. However, a close to significant correlation was observed for methylation at CpG site 2, where *WNT11* methylation correlated positively with expression. This is contradictory to the normal association of an increase in methylation resulting a reduction in gene expression.

5.5 *SFRP1* DNA methylation in the macroscopically-normal mucosa of people at differential risk of CRC

5.5.1 Introduction

SFRP1 encodes a member of the SFRP family that antagonises WNT signalling through competitive inhibition of Frizzled receptors (Bafico *et al.*, 1999). *SFRP1* is a tumour suppressor (Caldwell *et al.*, 2004) that is downregulated in CRCs as a result of DNA hypermethylation (Chen *et al.*, 2014b). Furthermore, an increase in *SFRP1* methylation, resulting in epigenetic silencing of *SFRP1,* has been proposed to be an early event in the adenoma-carcinoma sequence (Suzuki *et al.*, 2004).

5.5.1.1 SFRP1 methylation in the colorectum of ulcerative colitis

In inflamed rectal tissue from 36 UC patients, Kim *et al.* investigated the methylation levels of four genes, including *SFRP1*, as markers of CRC risk (Kim *et al.*, 2013). Methylation-specific PCR revealed that *SFRP1* was frequently methylated in the inflamed tissue from UC patients. Furthermore, *SFRP1* methylation was significantly higher in participants aged over 40 years old compared with those younger than 20 years old. When comparing *SFRP1* methylation in the tissue from UC and CRC patients, *SFRP1* methylation was approximately three-fold lower in UC patients compared with CRC patients. However, this study did not examine *SFRP1* methylation in healthy controls and therefore did not compare *SFRP1* methylation between UC and normal tissue.

Within UC patients, *SFRP1* methylation levels have also been reported to be increased in inflamed tissue compared with adjacent normal tissue (Wang *et al.*, 2013b). Furthermore, Wang *et al.* (2013) reported that this increase was greater than 50% in a quarter of the UC patients analysed.

An increase in *SFRP1* methylation, by over 60%, has also been found in IBD-associated neoplasia compared with tissue from UC patients, and this was increased in older compared with younger study participants (Dhir *et al.*, 2008). Similar to the findings from the study by Kim *et al.* (2013) where *SFRP1* methylation was lower in tissue from UC patients compared with that from CRC patients, *SFRP1* methylation in UC-related neoplasia has been observed to be

significantly lower than that in sporadic CRC tissue by almost two-fold (Konishi *et al.*, 2007).

5.5.1.2 SFRP1 methylation in the colorectum of people with polyps

Shao *et al.* have reported higher *SFRP1* methylation in patients with adenomatous polyps (55% methylation) compared with healthy controls, where they did not observe any methylation of *SFRP1* (Shao *et al.*, 2012). *SFRP1* methylation was significantly increased in CRC tissue (68.9% methylation) compared with that in adenomatous polyps. Belshaw *et al.* have also observed a two-fold increase in *SFRP1* methylation in tissue from patients with adenomatous polyps compared with healthy participants, however this difference was not statistically significant (Belshaw *et al.*, 2008). Similar to the findings from other studies, a positive correlation between age and *SFRP1* methylation was found.

An increase in *SFRP1* methylation has been observed in both sporadic adenomas and those occurring in patients with FAP, compared with matched healthy tissue (Caldwell *et al.*, 2010). When the authors compared sporadic adenomas with those arising in FAP patients, they observed significantly higher *SFRP1* methylation in sporadic cases. As well as quantifying *SFRP1* methylation, the study also examined *SFRP1* expression at the mRNA level to investigate any associations between the two. Indeed, *SFRP1* expression was reduced in both sporadic and FAP-related adenomas, where an increase in *SFRP1* methylation was observed, compared with healthy tissue. This was more pronounced in sporadic adenomas compared with FAP-related adenomas.

Caldwell *et al.* have also reported reduced *SFRP1* expression by approximately ten-fold in adenoma tissue compared with healthy controls (Caldwell *et al.*, 2006). Protein levels of SFRP1, assessed by immunohistochemistry, were also lower in adenomas compared with normal-adjacent tissue, and in the normal-adjacent tissue compared with healthy controls. They further investigated this finding by quantifying DNA methylation by combined bisulphite restriction analysis (COBRA) and observed significantly higher *SFRP1* methylation by approximately two-fold in adenoma tissue compared with healthy controls.

SFRP1 methylation has been observed in 88% of sporadic adenoma cases compared with 53% of healthy and normal-adjacent tissues (Qi *et al.*, 2006). Significantly increased *SFRP1* methylation was found in adenoma tissue compared with both normal-adjacent tissues and healthy controls, however there were no differences in *SFRP1* methylation observed between adenomas and carcinomas. Gene silencing or a significant reduction in *SFRP1* expression were observed in 76% of sporadic adenoma cases, and this was significantly higher when compared with that observed in healthy controls. These findings suggest that the loss of or reduction in *SFRP1* expression is observed early in the progression from normal tissue to adenoma and ultimately carcinoma and results from hypermethylation of *SFRP1*.

5.5.2 Hypotheses, aims and objectives

5.5.2.1 Hypotheses

The hypothesis for this study was that participants at higher risk of CRC, i.e. those with quiescent UC or a prior history of adenomatous polyps, would have increased *SFRP1* methylation.

5.5.2.2 Aims

This study aimed to test this hypothesis by quantifying *SFRP1* methylation levels in the macroscopically-normal mucosa of people at differential CRC risk i.e. those with quiescent UC, those with a prior history of adenomatous polyps and those at normal risk of developing CRC.

5.5.2.3 Objectives

- To obtain colorectal biopsies from the macroscopically-normal mucosa of three groups of DISC Study participants at differential risk of CRC i.e. those with quiescent UC, those with a prior history of adenomatous polyps and “normal” participants;
- To select an adequate primer within the promoter region of *SFRP1* and encompassing as many CpG sites as possible to quantify *SFRP1* methylation;

- To bisulphite modify DNA previously extracted by the DISC Study Team;
- To amplify the region to be analysed by PCR;
- To perform an assay validation analysis;
- To quantify *SFRP1* methylation at each CpG site by pyrosequencing;
- To compare *SFRP1* methylation at each CpG site and the mean across all sites in the 'Normal' participants and in the two higher-risk groups ('UC' and 'Polyp' participants);
- To investigate the effects of key epidemiological factors including age and gender on *SFRP1* methylation.

5.5.3 Methods

SFRP1 methylation was quantified at seven CpG sites by pyrosequencing using a commercially-available, optimised Qiagen assay (please see section 3.5). The methods for this study can be found in section 2.2.3.

Differences in *SFRP1* methylation between the three participant groups were analysed at each CpG site and for the mean methylation across all seven CpG sites using the ANOVA GLM with the inclusion of age, gender, endoscopy procedure, BMI and smoking status as covariates. Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method was used for pairwise comparisons.

5.5.4 Results

SFRP1 methylation was moderate at all seven CpG sites, but the inter-participant variability was large, with methylation ranging from 7.3% to 70.9% across all risk groups ('Normal' 13.6% - 45.9%, 'UC' 7.3% - 54.2% and 'Polyp' 13.6% - 70.9%). Methylation across all three participant groups was lowest at CpG site 5 (mean methylation 24.9%) and highest at CpG site 4 (mean methylation 32.4%).

5.5.4.1 SFRP1 methylation in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants

A significant difference in *SFRP1* methylation between the three participant groups was observed for the majority of the CpG sites (bar CpG site 1) and for the mean methylation across the seven sites ($p<0.05$) (Table 5.13). However, differences in *SFRP1* methylation at CpG site 1 between the three participants was close to reaching statistical significance ($p=0.055$). Dunnett's post-hoc test revealed that, in all cases, *SFRP1* methylation was significantly higher in the 'Polyp' compared with the 'Normal' group.

Mean *SFRP1* methylation across all seven CpG sites increased with increasing CRC risk, from 'Normal' participants to that observed in participants with quiescent UC and ultimately in those with a prior history of adenomatous polyps. Mean *SFRP1* methylation was 1.2-times higher in the 'Polyp' group compared with the 'Normal' group. A similar pattern was observed at each CpG site.

When applying Bonferroni's correction for multiple testing and adjusting the p-value threshold to 0.006, the differences in *SFRP1* methylation observed at CpG sites 2 to 7 and on the mean methylation across all sites is lost.

Table 5.13 Mean *SFRP1* methylation in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants at each CpG site and the mean across all sites.

CpG site	Normal	UC	Polyp	P value
1 N=103	29.8 (28.3 – 31.5)	31.4 (27.5 – 25.8)	34.8 (31.4 – 38.6)	0.055
2 N=104	23.9 (22.4 – 25.4)	27.0 (23.1 – 31.5)	30.1 (26.7 – 34.0)	**0.011**
3 N=102	22.3 (20.9 – 23.9)	25.1 (21.1 – 29.7)	28.2 (24.6 – 32.3)	**0.025**
4 N=105	30.4 (28.8 – 30.9)	34.7 (30.6 – 38.8)	36.4 (33.3 – 39.5)	**0.010**
5 N=103	22.4 (21.1 – 23.8)	25.8 (22.2 – 30.0)	27.5 (24.6 – 30.9)	**0.014**
6 N=100	28.13 (26.2 – 30.0)	32.0 (27.3 – 36.7)	34.9 (31.2 – 38.6)	**0.016**
7 N=101	29.1 (27.1 – 31.1)	32.3 (27.4 – 37.2)	36.1 (32.2 – 39.9)	**0.018**
Mean N=103	26.4 (24.9 – 27.9)	28.9 (26.1 – 32.0)	31.8 (27.6 – 36.6)	**0.027**

5.5.4.2 Exploration of additional factors influencing SFRP1 methylation

The effects of age, gender, endoscopy procedure, BMI and smoking status on *SFRP1* methylation at each CpG site and on the mean across all sites were analysed using the ANOVA GLM. There were no associations between gender, endoscopy procedure, BMI or smoking status and *SFRP1* methylation at any of the CpG sites or the mean across all sites (Table 5.14). Age was a determinant of *SFRP1* methylation at all seven CpG sites and the mean methylation across all seven CpG sites ($p<0.001$).

Table 5.14 P values for the effects of age, gender, endoscopy procedure, BMI and smoking status on *SFRP1* methylation at each CpG site and on the mean methylation across all sites.

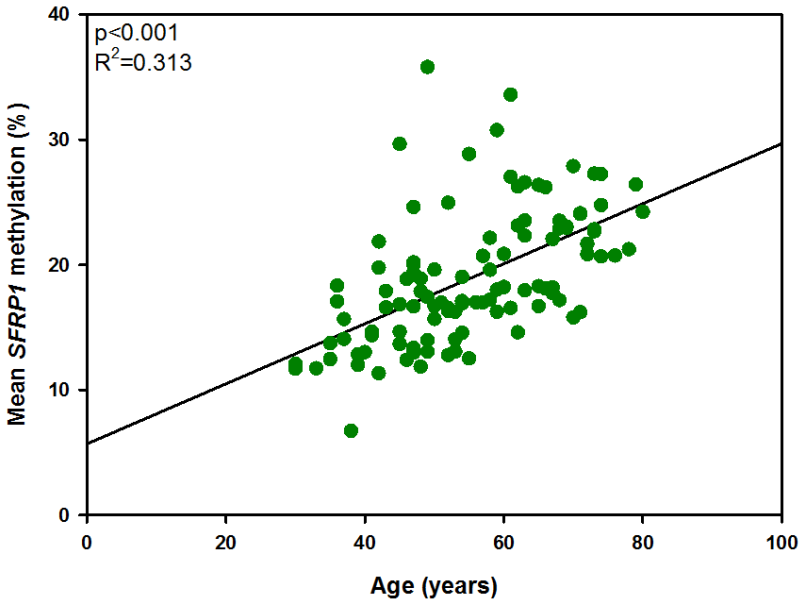
CpG site	Age	Gender	Endoscopy procedure	BMI	Smoking status
1	**<0.001**	0.578	0.752	0.404	0.666
2	**<0.001**	0.551	0.610	0.451	0.906
3	**<0.001**	0.839	0.801	0.580	0.952
4	**<0.001**	0.787	0.766	0.263	0.802
5	**<0.001**	0.826	0.661	0.646	0.674
6	**<0.001**	0.971	0.822	0.310	0.659
7	**<0.001**	0.931	0.802	0.259	0.782
Mean	**<0.001**	0.802	0.694	0.418	0.664

A positive correlation was found between age and *SFRP1* methylation at each CpG site and for the mean *SFRP1* methylation (Table 5.15). A Spearman correlation coefficient of 0.614 and a R^2 value of 0.313 were found for the correlation between age and mean *SFRP1* methylation (Figure 5.15).

Table 5.15 Correlation between age and *SFRP1* methylation.

CpG site	Spearman Correlation Coefficient	P value
1	0.590	**<0.001**
2	0.596	**<0.001**
3	0.584	**<0.001**
4	0.608	**<0.001**
5	0.588	**<0.001**
6	0.605	**<0.001**
7	0.553	**<0.001**
Mean	0.614	**<0.001**

Figure 5.15 Positive correlation between age and mean *SFRP1* methylation across all seven CpG sites. N=101.

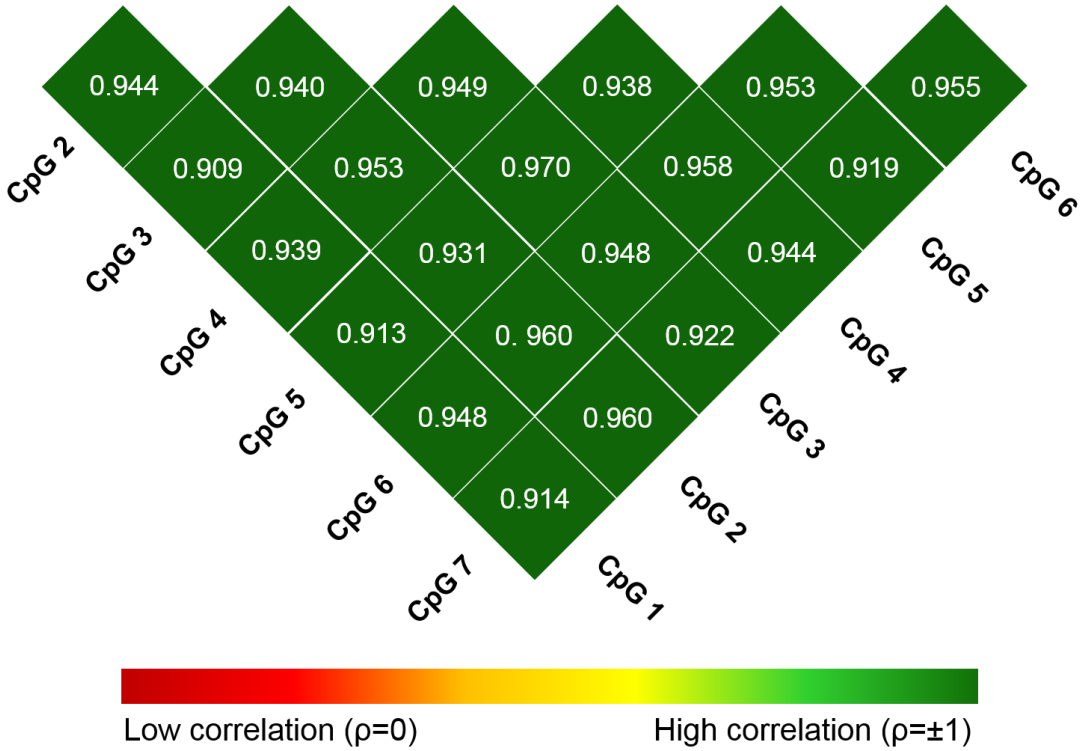


5.5.4.3 Correlation of SFRP1 methylation between CpG sites

Spearman correlation analyses were used to investigate the correlation between levels of *SFRP1* methylation between the seven CpG sites in all three groups. A strong correlation in *SFRP1* methylation levels was found between all

seven CpG sites, as indicated by Spearman correlation coefficients >0.900 (Figure 5.16).

Figure 5.16 Correlation of *SFRP1* methylation between CpG sites.



Values represent Spearman correlation coefficients (ρ). (N=100). P<0.001.

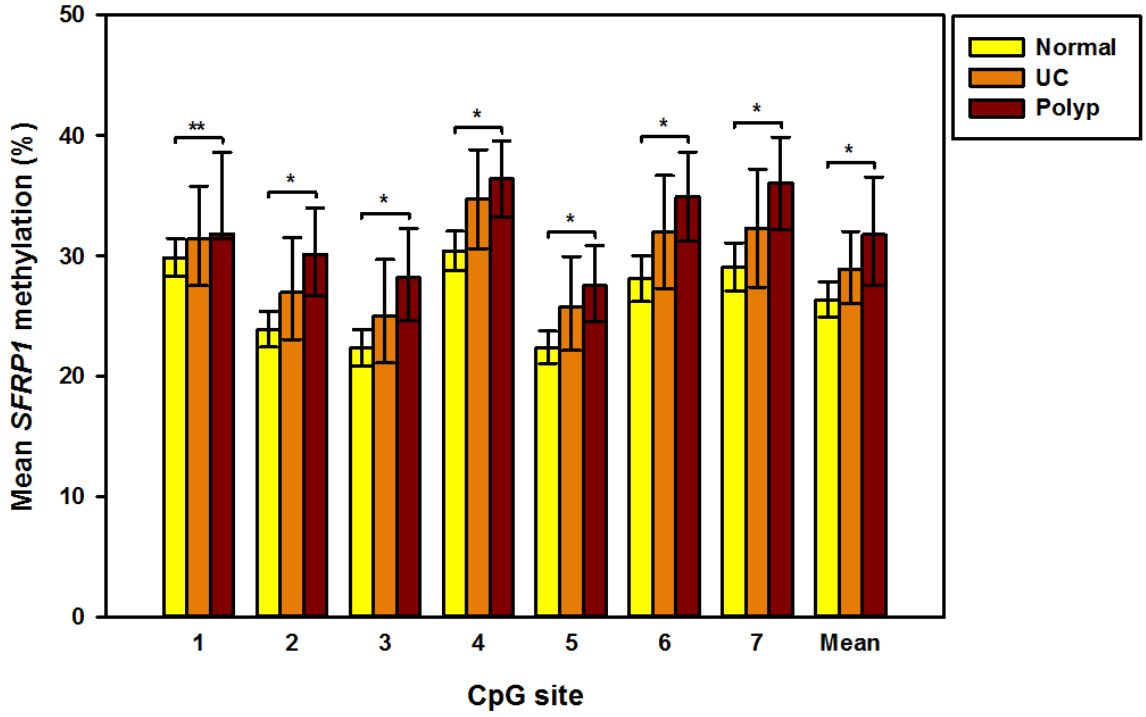
5.5.5 Discussion

SFRP1, as a consequence of DNA hypermethylation, is frequently downregulated in CRCs (Chen *et al.*, 2014b). Increased *SFRP1* methylation has also been reported in the inflamed colorectal tissue of patients with UC (Kim *et al.*, 2013) and in UC-associated cancers (Konishi *et al.*, 2007). Several studies have also observed hypermethylation of *SFRP1* in adenomas (Caldwell *et al.*, 2006; Qi *et al.*, 2006; Belshaw *et al.*, 2008; Shao *et al.*, 2012), including those in patients with FAP (Caldwell *et al.*, 2010), suggesting that this is an early event in the adenoma-carcinoma sequence.

In the present study, *SFRP1* methylation was quantified in the macroscopically-normal mucosa of healthy participants, participants with quiescent UC and participants with a prior history of adenomatous polyps at seven CpG sites within the *SFRP1* promoter region by pyrosequencing. At all seven CpG sites,

an increase in *SFRP1* methylation was observed with increasing CRC risk, from 'Normal' to 'UC' and, ultimately, to 'Polyp' groups (Figure 5.17). These differences were statistically significant at all CpG sites with the exception of CpG site 1, where a close to significant difference was observed (p=0.055), and for the mean methylation across all seven CpG sites.

Figure 5.17 *SFRP1* methylation in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.



Data are expressed as percentage methylation. Data are presented as LSMs (95% CIs). The ANOVA GLM was used to compare methylation between the three groups and Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method was used for pairwise comparisons. * p<0.05, ** p<0.10.

The effects of age, gender, endoscopy procedure, BMI and smoking status on *SFRP1* methylation were assessed as covariates using the ANOVA GLM. An association between age and *SFRP1* methylation was observed at all seven CpG sites and on the mean methylation across all sites. In all cases, age and *SFRP1* methylation correlated positively. This is consistent with findings from previous studies where a significant increase in *SFRP1* methylation has been reported with increasing age in healthy, UC and adenoma tissues (Belshaw *et al.*, 2008; An *et al.*, 2010; Rawson *et al.*, 2011; Kim *et al.*, 2013). Furthermore, this finding is consistent with that observed in the results from similar analyses in the intervention arm of the DISC Study (section 4.4.4.3). An increase in

SFRP1 methylation with age suggests a reduction in *SFRP1* expression, resulting in reduced inhibition and therefore an increase in WNT signalling activity. This could be one of the mechanisms contributing to an increase in CRC incidence with age.

5.6 Colonic crypt cell proliferative state in the macroscopically-normal mucosa of people at differential risk of CRC

5.6.1 Introduction

In this study, differences in cell proliferation, a functional outcome of the WNT pathway that is dysregulated in CRC, in the macroscopically-normal mucosa of people at differential risk of CRC were assessed following whole crypt microdissection of Schiff reagent-stained biopsies.

5.6.1.1 Cell proliferation in the colorectum of people with ulcerative colitis

Studies investigating cell proliferation in patients with UC, predominantly human studies, have been consistent in finding an increase in colonic crypt cell proliferation with UC and have also observed an altered distribution of proliferating cells.

In a rat model of induced colitis, which structurally resembles UC in humans, the cell proliferation index, assessed by Ki-67 immunoreactivity, was 40 to 60-fold higher in the colonic mucosa of colitis rats compared with controls (Vetuschi *et al.*, 2002). The authors concluded that the hyperproliferative state observed with UC may contribute to the frequent development of cancers in these patients.

One of the earliest studies investigating cell kinetics in patients with UC reported an increase in the proportion of mitotic cells in the upper compartments of the crypt in rectal biopsies from patients with active UC compared with healthy controls (Serafini *et al.*, 1981). The proliferating index in patients in remission was also significantly higher than that in healthy controls but comparable to that observed in patients with active UC.

A later study utilised autoradiography to compare proliferation between healthy controls, patients with UC, patients with adenomas and CRC patients (Kanemitsu *et al.*, 1985). The number of proliferating cells was approximately double in patients with UC, who also had a larger proliferative compartment, which is normally found in the basal half of the colonic crypt, compared with healthy controls. That same year, Franklin *et al.* also investigated differences in colonic crypt kinetics in patients with quiescent and active UC using

immunohistochemical techniques (Franklin *et al.*, 1985). A significantly higher proportion of proliferating cells was observed in the proliferative compartment (the lower two-thirds of the crypt) and the upper quarter of the crypt in patients with active UC compared with those with quiescent UC and healthy controls. In addition, a greater proportion of proliferating cells in the proliferative compartment were found in patients with quiescent UC compared with healthy controls.

In the rectum of patients with UC, although Biasco *et al.* did not observe a significant difference in the total number of proliferating cells, a four-fold increase in proliferation was observed in the upper 40% of the crypt compared with healthy controls (Biasco *et al.*, 1990). A similar later study also reported a shift in the proliferative compartment towards the mouth of the crypt in patients with UC compared with healthy controls (Paganelli *et al.*, 1993).

Sipos *et al.* have shown increasing cell proliferation with increasing inflammation in IBD, including patients with UC, compared with healthy controls, suggesting that stimulation of proliferation is a consequence of the inflammation associated with this disease (Sipos *et al.*, 2005). The group also reported differences in cell proliferation in patients with varying degrees of UC (Sipos *et al.*, 2002). Proliferation, assessed by PCNA staining, was significantly higher in all degrees of UC compared with healthy controls. Furthermore, proliferation was greater in moderate compared with mild UC, and higher in severe UC compared with both moderate and mild UC. In UC patients in remission, cell proliferation has also been found to be increased. The proliferation index of 23 patients with UC (mean 42%) exceeded that observed in the healthy epithelium (Buczynski *et al.*, 2012).

5.6.1.2 Cell proliferation in the colorectum of people with polyps

A very early study by Kanemitsu *et al.*, whose study was described previously, found that, similar to findings observed in UC patients, cell proliferation was approximately two-fold higher in patients with adenomas compared with healthy controls (Kanemitsu *et al.*, 1985). Furthermore, proliferating cells were counted in all parts of the crypt and there was no longer a defined proliferative

compartment. The investigators also observed a positive correlation between tumour grade and cell proliferation.

A similar finding in the alteration of the distribution of mitotic cells within the crypts of patients with adenomas has been shown by Biasco and colleagues (Biasco *et al.*, 1990). Although a significant difference was not observed in the total number of proliferating cells, cell proliferation in the upper 40% of the crypt was more than two-fold greater in the rectum of patients with adenomas compared with healthy controls.

Microradiography has also been used to investigate changes in proliferative activity in patients with adenomatous polyps and carcinomas in the colon (Ponz de Leon *et al.*, 1988). The number of mitotic cells within the crypts was greater in patients with polyps and carcinomas compared with healthy controls. Polyp and carcinoma patients also had a significantly greater number of proliferating cells in the upper sections of the crypt (top three fifths) compared with healthy controls. These findings demonstrated that differences in proliferation in the progression of normal colonic tissue to carcinoma may be detected in adenomatous polyps. Furthermore, it was not only the levels of proliferation that differed but also the distribution of mitotic cells, with the proliferative compartment enlarging towards the mouth of the crypt in both adenomatous polyps and carcinomas.

A later study also investigated levels of proliferation in patients with colonic polyps using immunohistochemical assessment of bromodeoxyuridine incorporation (Wilson *et al.*, 1990). However, in this study the analysed tissue was removed from a minimum of five centimetres away from the adenoma(s). It was observed that proliferation in these tissues was significantly higher than that observed in healthy controls and, consistent with findings from the study by Ponz de Leon *et al.* (1988), the proliferative compartment extended towards the mouth of the crypt.

Roncucci and colleagues evaluated cell proliferation in the normal mucosa of patients with different types of colonic polyps, including tubular and tubulovillous polyps, as well as evaluating whether differences could be observed between different clinical characteristics or outcomes (Roncucci *et al.*, 1991). Proliferation was significantly higher in the normal tissue from all of the polyp

participants compared with healthy controls, but the authors did not observe differences between clinical characteristics or outcomes, such as adenoma size or number. These findings demonstrated that the increase in cell proliferation and enlargement of the proliferative compartment towards the mouth of the crypts in adenoma patients may be observed in normal-appearing tissue and is detectable regardless of adenoma type or size.

Proliferation in lesions of the colorectum, including hyperplastic polyps, adenomas and carcinomas, has been assessed by Ki-67 labelling (Kikuchi *et al.*, 1997). In hyperplastic polyps, proliferation levels and distribution were comparable to that in the crypt base of the healthy colon but was increased in adenomas. A similar study compared cell proliferation between three different polyp types: serrated, hyperplastic and tubular adenomas, in an attempt to distinguish between characteristics of the three (Kang *et al.*, 1997). Proliferation in the middle and upper sections of the crypt increased from hyperplastic polyps to serrated adenomas and was ultimately greatest in tubular adenomas. A more recent study also assessed proliferation in different polyp types by measuring phospho-histone H3, which specifically detects mitosis (Endo *et al.*, 2013). An altered distribution of mitotic cells was observed in all three diseased tissue types when compared with the normal crypt, where proliferating cells were predominantly found in the lower third of the crypt. Proliferating cells in sessile serrated and in microvesicular hyperplastic polyps were also found in the middle and upper half of the crypt, and those in tubular adenomas were recorded in all areas of the crypt. Mitotic indices in sessile serrated polyps and tubular adenomas were significantly higher than those in both normal tissue and in microvesicular hyperplastic polyps.

Shpitz and colleagues have assessed cell proliferation at each sequential stage of colorectal carcinogenesis: ACF, hyperplastic polyps, dysplastic polyps and carcinomas (Shpitz *et al.*, 1997). When the crypts were divided into thirds, an increase in cell proliferation at each compartment was observed with increasing stage of carcinogenesis (i.e. from ACF to carcinomas), with the exception of hyperplastic polyps.

5.6.2 Hypotheses, aims and objectives

5.6.2.1 Hypotheses

The hypothesis for this investigation was that participants at higher risk of CRC, i.e. those with quiescent UC or a prior history of adenomatous polyps, would have increased colonic crypt cell proliferation and that a greater proportion of mitotic cells would be observed in the upper half of the crypts.

5.6.2.2 Aims

This study aimed to test this hypothesis by quantifying colonic crypt cell proliferation in the macroscopically-normal mucosa of people at differential CRC risk i.e. those with quiescent UC, those with a prior history of adenomatous polyps and those at normal risk of developing CRC.

5.6.2.3 Objectives

- To obtain colorectal biopsies from the macroscopically-normal mucosa of three groups of DISC Study participants at differential risk of CRC i.e. those with quiescent UC, those with a prior history of adenomatous polyps and “normal” participants;
- To compare colonic crypt cell proliferation in the ‘Normal’ participants and in the two higher-risk groups (‘UC’ and ‘Polyp’ participants);
- To assess differences in crypt measurements (length, width and volume) in ‘Normal’ participants and in the two higher-risk groups (‘UC’ and ‘Polyp’ participants);
- To investigate the effects of key epidemiological factors including age and gender on colonic crypt cell proliferation.

5.6.3 Methods

Assessment of colonic crypt cell kinetics was performed as described in section 2.2.4. Briefly, rectal biopsies were hydrated, hydrolysed and stained in Schiff reagent prior to crypt microdissection. Crypts were divided into ten equal-sized

compartments and the number of mitotic cells were counted in each compartment in addition to the recording of crypt measurements. Ten intact crypts were analysed per participant.

The ANOVA GLM was used to investigate differences in the measured outcomes between the three groups, with the inclusion of age, gender, endoscopy procedure, BMI and smoking status as covariates. The Dunnett post-hoc test was used for multiple comparisons with the control group and the Bonferroni method was used for pairwise comparisons.

5.6.4 Results

Using rectal mucosal biopsies from the macroscopically-normal mucosa of participants from the 'Normal', 'UC' and 'Polyp' groups, colonic crypt cell proliferation was assessed following whole crypt microdissection of Schiff reagent-stained biopsies. The mean total number of mitotic cells per crypt and the mean percentage of mitotic cells in the top half of the crypt were calculated by calculating the average across the 10 crypts for each participant. In addition, the crypt volumes were calculated by assuming that crypts resembled the shape of a cylinder.

5.6.4.1 Colonic crypt cell proliferation in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants

Significant differences between the three participant groups were observed for the mean total number of mitotic cells ($p=0.009$) and all three crypt measurements (length, width and volume) (Table 5.16). The total number of mitotic cells was approximately two-fold lower in 'Polyp' participants compared with 'Normal' participants. Participants with quiescent UC also appeared to have a reduced total number of mitotic cells compared with the 'Normal' group.

Although 'Normal' participants had a higher total number of mitotic cells per crypt compared with 'Polyp' participants, the opposite was observed for the percentage of mitotic cells in the top half of the crypt. The latter appeared to be highest in the two higher-risk groups, however this was not statistically significant.

Significant differences in all three crypt measurements (length, width and volume) were observed between the three participant groups. The crypts were significantly longer in ‘UC’ participants, who had a mean crypt length of 630µm compared with 557µm and 545µm in the ‘Polyp’ and ‘Normal’ groups respectively. ‘UC’ participants also had significantly wider crypts than the other two groups, with a mean crypt width of 139µm compared with 121µm and 113µm in the ‘Normal’ and ‘Polyp’ groups respectively. Furthermore, the crypt volume, calculated by assuming the shape of a cylinder, was also significantly greater in the ‘UC’ group compared with participants in the ‘Normal’ and ‘Polyp’ groups.

When applying Bonferroni’s correction for multiple testing and adjusting the p-value threshold to 0.01, the differences in total mitoses, crypt width and crypt volume between the three participant groups remain statistically significant. However, this is lost for the differences in crypt length between groups, but remains close to reaching statistical significance (p=0.014).

Table 5.16 Colonic crypt cell proliferation and crypt measurements in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.

	Normal	UC	Polyp	P value
Total mitoses	7.69 (6.22 – 9.51)	5.09 (3.50 – 7.42)	4.20 (3.27 – 5.41)	**0.009**
Mitoses in top half of crypt	4.85 (3.27 – 7.91)	9.21 (4.58 – 18.52)	5.71 (3.57 – 9.13)	0.338
Crypt length (µm)	545 (518 – 572)	630 (598 – 663)	557 (509 – 605)	**0.014**
Crypt width (µm)	121 (115 – 127)	139 (128 – 152)	113 (107 – 120)	**<0.001**
Crypt volume (μm^3)	6.04×10^6 ($6.80 – 5.40 \times 10^6$)	8.81×10^6 ($7.53 \times 10^6 – 1.04 \times 10^7$)	5.48×10^6 ($4.60 – 6.65 \times 10^6$)	**0.001**

Data are presented as LSMs (95% CIs). The ANOVA GLM was used to compare methylation between the three groups. Dunnett's and Bonferroni post-hoc tests were used for multiple comparisons. N=71.

5.6.4.2 Exploration of additional factors influencing colonic crypt cell proliferation

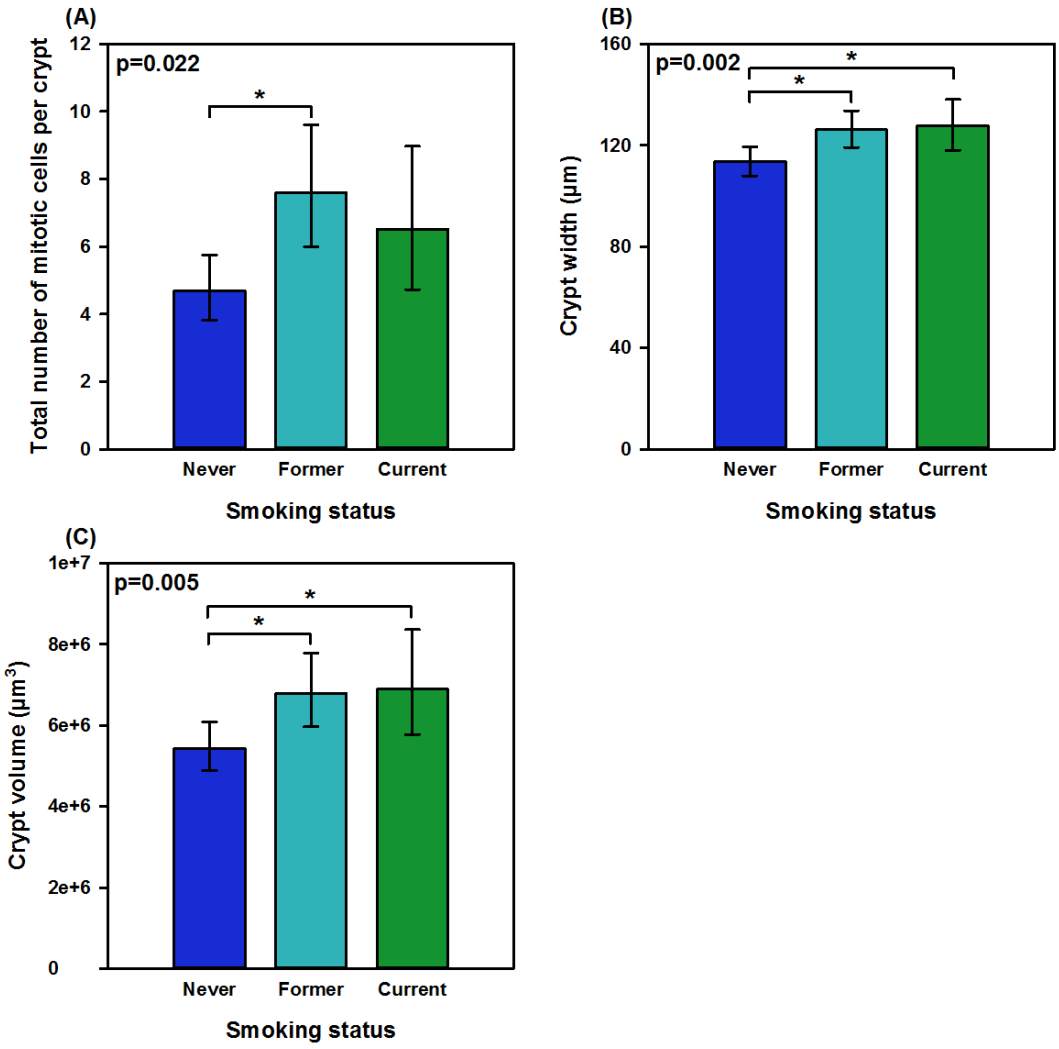
The effects of age, gender, endoscopy procedure, BMI and smoking status on the measured outcomes were also explored. Age, gender, endoscopy procedure and BMI did not have an effect on colonic crypt cell proliferation or crypt measurements (Table 5.17). However, a significant effect of smoking status was observed for the total number of mitotic cells per crypt, crypt width and crypt volume.

Table 5.17 P values for the effects of age, gender, endoscopy procedure, BMI and smoking status on colonic crypt cell proliferation and crypt measurements.

	Age	Gender	Endoscopy procedure	BMI	Smoking status
Total mitoses	0.934	0.748	0.363	0.964	**0.022**
Mitoses in top half of crypt	0.990	0.244	0.249	0.192	0.408
Crypt length	0.212	0.111	0.328	0.729	0.534
Crypt width	0.352	0.637	0.324	0.685	**0.002**
Crypt volume	0.384	0.931	0.263	0.923	**0.005**

The mean number of mitotic cells was almost two-fold higher in former smokers compared with participants who never smoked (Figure 5.18 (A)). Both former and current smokers had significantly wider and larger crypts compared with participants who had never smoked (Figure 5.18 (B) and (C)).

Figure 5.18 Effect of smoking on the total number of mitotic cells per crypt (A), crypt width (B) and crypt volume (C).



Data are presented as LSMs and error bars represent 95% CIs (ANOVA GLM). N=71.

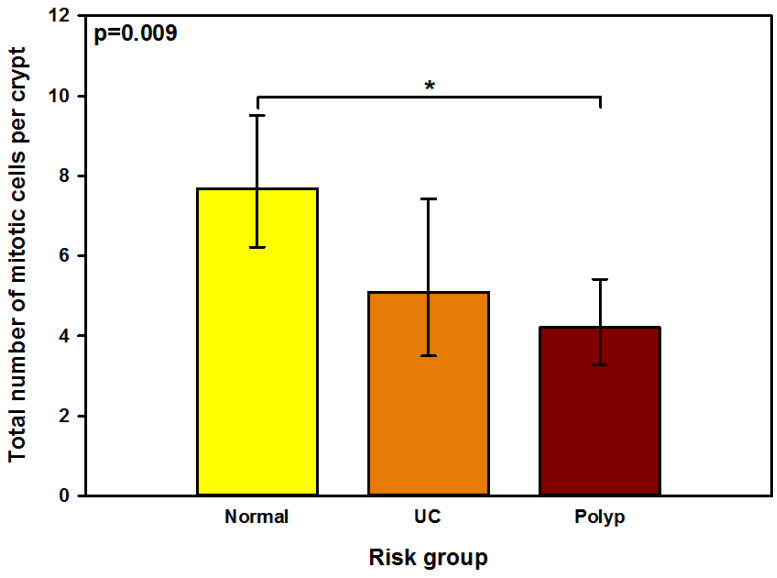
5.6.5 Discussion

Differences in the CCPS in the macroscopically-normal mucosa of people at differential risk of CRC were assessed following whole crypt microdissection of Schiff reagent-stained crypts. In ten crypts per participant, the total number of mitotic cells per crypt were counted and the proportion of mitotic cells in the upper half of the crypt were calculated as an indicator of crypt health. Furthermore, crypt measurements in the form of length and width were recorded and the crypt volume was calculated.

Unexpectedly, a reduction in the total number of mitotic cells per crypt was observed in higher-risk participants, and this was statistically significant for the

comparison between the ‘Polyp’ and ‘Normal’ groups (Figure 5.19). This finding is contrary to previous reports in the literature, where an increase in cell proliferation has been observed in both UC (Sipos *et al.*, 2005; Buczynski *et al.*, 2012) and polyp patients (Ponz de Leon *et al.*, 1988; Wilson *et al.*, 1990). However, in the present study, the proportion of mitotic cells in the top half of the crypt appeared to be greater in higher-risk participants, particularly those with quiescent UC. The latter is a better indicator of crypt health as proliferation is normally observed in the proliferative compartment in the lower half of the crypt in the healthy colon. This observation is consistent with previous findings that have reported an altered distribution of mitotic cells within the crypt, with an enlargement of the proliferative compartment and an increase in the number of mitotic cells found towards the mouth of the crypt, in both UC (Serafini *et al.*, 1981; Biasco *et al.*, 1990; Paganelli *et al.*, 1993) and polyp patients (Kanemitsu *et al.*, 1985; Roncucci *et al.*, 1991). Furthermore, an altered distribution of mitotic cells is one of the earliest detectable alterations in the apparently-normal mucosa prior to the development of malignancies (Roncucci *et al.*, 1991).

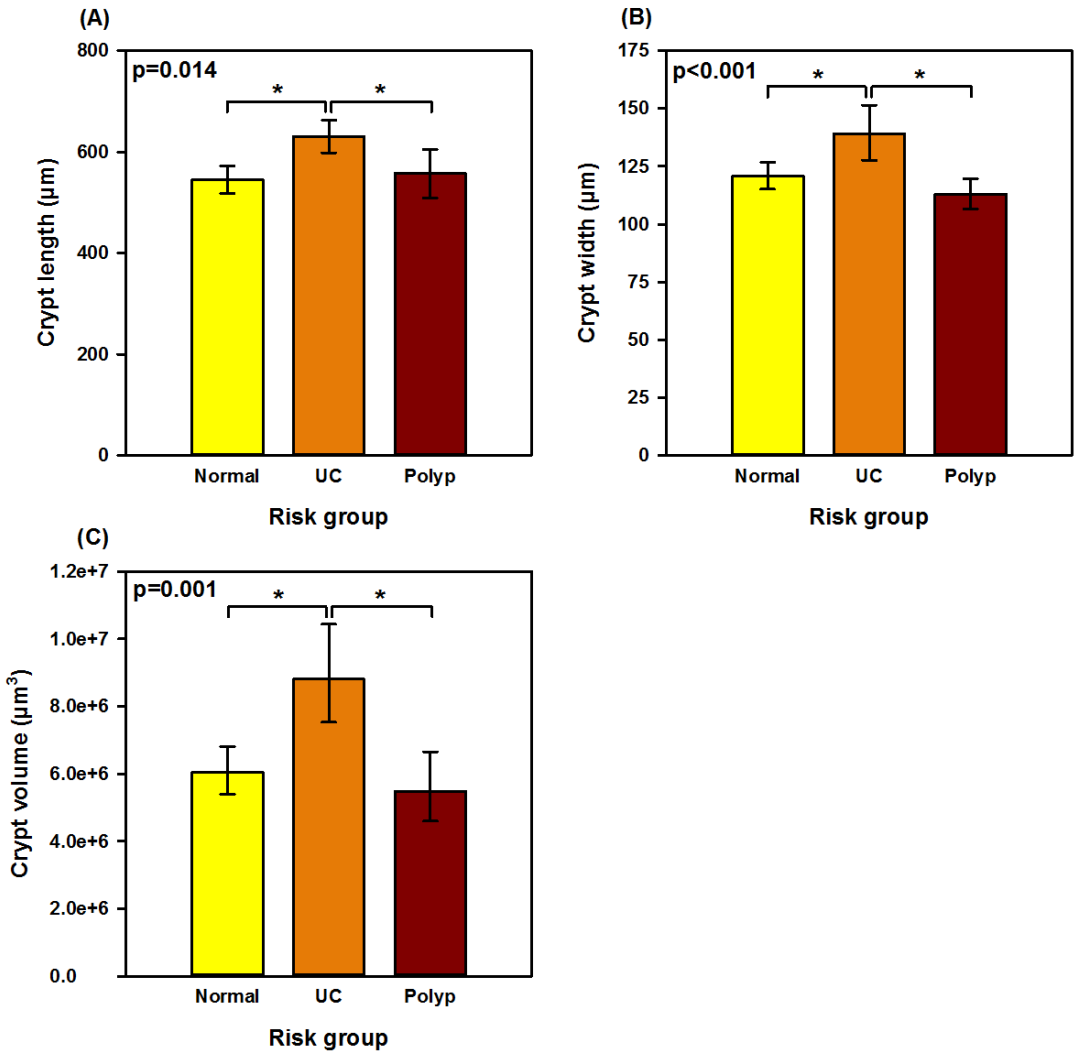
Figure 5.19 Total number of mitotic cells per crypt in the macroscopically-normal mucosa of ‘Normal, ‘UC’ and ‘Polyp’ participants.



Data are presented as LSMs (95% CIs). The ANOVA GLM was used to compare the number of mitotic cells per crypt between the three groups and Dunnett’s post-hoc test was used for multiple comparisons with the control group and the Bonferroni method was used for pairwise comparisons. N=71.

To my knowledge, this is the first study to report that crypts in people with quiescent UC are longer, wider and larger in volume compared with 'Normal' and 'Polyp' participants (Figure 5.20).

Figure 5.20 Crypt measurements and volumes in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.



Data are expressed as µm for crypt length and width and as μm^3 for crypt volume. Data are presented as LSMs (95% CIs). The ANOVA GLM was used to compare methylation between the three groups and Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method for pairwise comparisons. N=71.

Exploration of additional factors influencing colonic crypt cell proliferation and crypt dimensions revealed a significant effect of smoking status on the total number of mitotic cells and crypt width and volume. Smokers, particularly former smokers, had a greater number of proliferating cells. They also had wider crypts that were greater in volume which, although this was not paralleled

by an increase in the total number of mitotic cells, could be a consequence of the increased proportion of mitotic cells in the top half of the crypt. This would be consistent with findings from a previous study that reported stimulation of proliferation by nicotine in Caco-2 and HCT-8 CRC cell lines (Cucina *et al.*, 2012).

5.7 Expression of *BAX* and *BCL-2* apoptotic genes in the macroscopically-normal mucosa of people at differential risk of CRC

5.7.1 Introduction

As the technique utilised to assess the colonic CCPS was not adequate for the assessment of apoptosis, the expression of *BAX*, a pro-apoptotic gene, and *BCL-2*, an anti-apoptotic gene, were quantified as markers of apoptosis. These genes are described in more detail in section 4.6.1.

5.7.1.1 Apoptosis in the colorectum of people with ulcerative colitis

Epithelial dysfunction in UC is thought to result mainly from aberrant levels of apoptosis (Seidelin and Nielsen, 2006). Studies investigating the rates of apoptosis in the colonic epithelium of patients with active UC have observed significantly increased levels of apoptosis in the inflamed tissue. Enhanced apoptosis has also been reported in the uninflamed tissue or in patients with quiescent UC. Furthermore, UC patients also have an altered distribution of apoptotic cells, with cells undergoing apoptosis being detected in all parts of the crypt in contrast to the healthy colon where apoptosis occurs mainly in the upper sections of the crypt. It has been suggested that the induction of apoptosis in UC results from the levels of inflammation in these patients (Seidelin and Nielsen, 2009) and that it jeopardises the function of the epithelium as a barrier and increases the possibility of infiltration of molecules such as enterotoxins and antigens (Vetuschi *et al.*, 2002; Seidelin and Nielsen, 2009).

An increase in apoptosis has been observed in a DSS-induced rat model of colitis (Vetuschi *et al.*, 2002). The apoptotic index, determined using the TUNEL assay, was up to 120-fold higher in colitis rats compared with controls. Furthermore, expression of pro-apoptotic BAX, assessed by immunohistochemical analysis, was also significantly increased. The authors concluded that the enhanced levels of apoptosis observed in UC could lead to a breakdown of the epithelial barrier function and consequently initiate mucosal invasion (Vetuschi *et al.*, 2002).

Iwamoto and colleagues have investigated levels of apoptosis in both the inflamed and uninflamed tissue from patients with UC using various measurements such as the TUNEL assay to detect DNA breaks and IHC to quantify Fas and Fas-ligands (Iwamoto *et al.*, 1996). A significantly greater number of TUNEL-positive cells were observed in the inflamed compared with uninflamed tissue of UC patients, and significantly more TUNEL-positive cells in UC patients compared with controls. Both uninflamed and inflamed tissues from UC patients were positive for markers indicative of apoptosis, such as Fas and DNA breaks, in the lower half of the crypts compared with normal controls, where apoptosis was mainly observed towards the mouth of the crypts.

A correlation between apoptosis and increasing inflammation in patients with IBD, including UC patients, compared with healthy controls has been reported (Sipos *et al.*, 2005). This group have also reported a correlation between levels of apoptosis and the severity of UC, where patients with severe UC had significantly higher levels of apoptosis compared with those with moderate UC and patients with moderate UC had greater levels of apoptosis compared with healthy controls (Sipos *et al.*, 2002).

A study by Seidelin and Nielsen was the first to investigate rates of apoptosis in UC patients with different disease characteristics (Seidelin and Nielsen, 2009). Flow cytometry was used to quantify apoptosis in 20 patients with UC, of whom 12 had the active form of the disease, and 20 controls. Within the UC patients, the investigators observed significantly increased rates of apoptosis in the inflamed compared with uninflamed tissue, suggesting that the enhancement of apoptosis is driven by local inflammation. Consistent with the findings from the study by Sipos *et al.* (2005), a positive correlation between microscopic inflammation grade and apoptosis rates was found. However, contrary to previous findings, although rates of apoptosis were significantly higher in the inflamed tissue of UC patients, there were no differences observed in the uninflamed tissue compared with healthy controls. Overall, the authors did not observe relationships between any of the markers of disease characteristics or clinical outcomes and epithelial rates of apoptosis.

5.7.1.2 BAX and BCL-2 expression in the colorectum of people with ulcerative colitis

Although numerous studies have reported an increase in apoptosis in patients with UC, studies suggest that this is mediated through alternative apoptotic pathways to the BAX/BCL-2 pathway.

A study by Iimura *et al.* was the first to show that, unexpectedly, both *BAX* mRNA expression and protein levels were reduced in the inflamed colonic epithelium of UC patients, which would be indicative of a reduction in apoptosis (Iimura *et al.*, 2000). BAX expression in the uninflamed tissue of UC patients was comparable to that measured in healthy controls. BCL-2 expression did not differ between healthy controls and UC patients. The authors concluded that the BAX/BCL-2 pathway was not involved in the regulation of the altered levels of apoptosis observed in UC. However, they suggested that, although altered expression of BAX did not contribute to the induction of apoptosis in UC, this could be one of the factors that results in dysregulated apoptosis in the colonic epithelium.

A similar conclusion was drawn in a prospective study by Karamanolis and colleagues, who examined expression of BAX and BCL-2 by IHC and assessed apoptosis using the TUNEL method in 23 UC patients and 11 healthy controls (Karamanolis *et al.*, 2007). The investigators concluded that apoptosis was not mediated through the BAX/BCL-2 pathway as they did not find a correlation between levels of apoptosis and the expression of either protein. However, an overall increase in expression of both BAX and BCL-2 proteins was observed.

In a study described earlier, Buczynski *et al.* also assessed levels of apoptosis semi-quantitatively in UC patients in remission through immunohistochemical staining of BAX (Buczynski *et al.*, 2012). The authors established that apoptosis in UC patients was between 2% - 75%, which was significantly higher than the 0.5% - 11% normally observed in the healthy colon. In addition, staining of pro-apoptotic BAX, which would normally be observed towards the mouth of the crypt, was found at the base of the crypt.

5.7.1.3 Apoptosis in people with polyps

Polyps were previously thought to result primarily from a hyperproliferative state until it was suggested that dysregulation in apoptosis may also be implicated and that a balance between the two processes was important in determining the overall proliferative state of the crypt (Bedi *et al.*, 1995).

In the healthy crypt, the majority of the apoptotic cells are found in the mouth of the crypt (Hall *et al.*, 1994), however, in adenomatous polyps the inverse has been observed, with apoptosis being reported at the base of the crypt (West *et al.*, 2009). Controversially, the majority of studies have reported an increase in the levels of apoptosis in the progression of the normal mucosa to adenomas and ultimately carcinomas (Koornstra *et al.*, 2003), with only a limited number of studies reporting a reduction in apoptosis in adenomas (Bedi *et al.*, 1995; Moss *et al.*, 1996a; Moss *et al.*, 1996b). More recently, studies have focussed on comparing levels of apoptosis in different polyp types such as serrated or hyperplastic polyps.

A reduction in apoptosis in adenomatous polyps was observed in a study by Moss *et al.*, which assessed levels of apoptosis in surgically-resected tissues taken from 10 healthy participants and 22 patients with adenomatous polyps using the TUNEL method (Moss *et al.*, 1996b). In the healthy mucosa, the majority of cells undergoing apoptosis were located in the base of the crypt but in tissue from adenomatous polyps a gradient with increasing apoptosis from the luminal surface towards the base of the crypt was observed. Overall, apoptosis levels in adenomatous polyps were approximately half that observed in healthy controls. A more recent, large prospective study observed similar findings in 226 adenoma patients and 493 healthy controls from the Diet and Health Study III (Martin *et al.*, 2002). Patients with adenomas had lower apoptotic scores (mean 2.46 apoptotic cells per crypt) compared with healthy controls (mean 2.94 apoptotic cells per crypt).

As concluded by Koornstra *et al.* (2003) in their review, the majority of studies have consistently reported an increase in apoptosis in patients with polyps. Sinicrop *et al.* examined apoptotic rates in tissue taken from normal participants, those with polyps (hyperplastic and adenomatous) and carcinomas using the TUNEL method and observed significantly greater levels of apoptosis

in the mucosa of patients with polyps (Sinicrope *et al.*, 1996). Furthermore, the number of apoptotic cells in the base of the crypt was also significantly increased. The authors concluded that a progressive increase in apoptotic rates was found during the progression from the normal mucosa to adenomas and ultimately adenocarcinomas. An increase in apoptosis with colorectal tumour progression has also been reported by Hawkins *et al.*, who used an *in situ* end-labelling assay to detect apoptosis in samples from 26 adenomas and 83 carcinomas (Hawkins *et al.*, 1997).

Several studies have also investigated apoptosis in the crypts of varying polyp types and the majority have reported an increase in apoptosis with increasing severity or grades of dysplasia of the polyps. The assessment of apoptosis in different types of polyps, including sessile serrated, microvesicular hyperplastic polyps and tubular adenomas, has been assessed through the detection of cleaved caspase 3, an effector caspase of apoptosis (Endo *et al.*, 2013). Similar to that observed in healthy tissue, cells undergoing apoptosis in sessile serrated polyp and microvesicular hyperplastic polyp tissues were located at the mouth of the crypts. However, in tissue from tubular adenomas, apoptotic cells were observed in all parts of the crypts. Apoptotic indices were comparable and lowest in normal and microvesicular hyperplastic polyps tissue but were increased in sessile serrated polyps and in tubular adenomas.

An increase in rates of apoptosis with progressing severity of polyp types has also been reported by Ladas *et al.*, who detected apoptosis using the TUNEL method in 75 polyp patients, comprising patients with serrated polyps, hyperplastic polyps and tubulovillous adenomas (Ladas *et al.*, 2005). Apoptosis was lowest in hyperplastic polyps and highest in tubulovillous adenomas.

5.7.1.4 BAX and BCL-2 expression in people with polyps

Studies investigating expression of BAX and BCL-2 proteins in polyps have predominantly used IHC. The results from these studies have yielded conflicting findings, with some reporting an increase in BCL-2 expression in adenomas compared with healthy tissue (Bosari *et al.*, 1995; Nakamura *et al.*, 1995; Flohil *et al.*, 1996) and others have observed that expression of BAX and BCL-2 is dependent on polyp type (Ladas *et al.*, 2005; Endo *et al.*, 2013).

An early study by Nakamura *et al.* reported significantly higher expression of anti-apoptotic BCL-2 in colorectal polyps (adenomas) compared with adjacent normal tissue and healthy controls, suggesting that, in adenoma tissue, BCL-2 is involved in the regulation of apoptosis (Nakamura *et al.*, 1995). That year, another study also quantified BCL-2 expression in the healthy mucosa, hyperplastic polyps, adenomas and carcinomas (Bosari *et al.*, 1995). The authors observed comparable levels of BCL-2 expression in the healthy mucosa and in hyperplastic polyps, but increased BCL-2 expression in adenomas compared with the latter two. The findings from a later study were consistent with that observed by Nakamura *et al.* (1995) and Bosari *et al.* (1995) where BCL-2 expression was greatest in carcinoma tissue and was also higher in adenoma tissue compared with hyperplastic polyps (Flohil *et al.*, 1996).

In addition to investigating apoptosis using the TUNEL method, Ladas *et al.* also quantified BAX and BCL-2 by IHC in patients with serrated polyps, hyperplastic polyps and adenomas (Ladas *et al.*, 2005). Expression of the anti-apoptotic BCL-2 protein was comparable in serrated and hyperplastic polyps, but significantly higher in tubulovillous adenomas. Expression of the pro-apoptotic protein, BAX, was lowest in hyperplastic polyps and highest in tubulovillous adenomas. Endo *et al.* also examined expression of BAX and BCL-2 in sessile serrated polyps, hyperplastic polyps and tubular adenomas and, contrary to the previous literature, they observed significantly reduced expression of both BCL-2 and BAX in serrated and hyperplastic polyps (Endo *et al.*, 2013). However, the expression of both proteins was significantly increased in tubular adenomas compared with healthy controls. A reduction in BAX expression in hyperplastic polyps has also been reported by Tateyama *et al.*, who also observed an increase in BAX protein levels in serrated and tubular adenomas (Tateyama *et al.*, 2002).

Giarnieri *et al.* have investigated differences in BAX and BCL-2 expression in varying degrees of dysplasia in rectal adenomatous polyps (Giarnieri *et al.*, 2000). Reduced expression of anti-apoptotic BCL-2 was observed in dysplastic polyps of moderate and severe degree, compared with those of low degree and hyperplastic polyps. However, there were no differences in the expression of pro-apoptotic BAX observed. Similar findings were reported by Hawkins *et al.*, who reported an inverse correlation between BCL-2 expression and the

increase in levels of apoptosis they observed in the progression of colorectal neoplasia (Hawkins *et al.*, 1997).

5.7.2 Hypotheses, aims and objectives

5.7.2.1 Hypotheses

The hypothesis for this study was that participants at higher risk of CRC, i.e. those with quiescent UC or a prior history of adenomatous polyps, would have greater levels of apoptosis, indicated by an increase in expression of *BAX* (pro-apoptotic) and a decrease in expression of *BCL-2* (anti-apoptotic) compared with normal participants.

5.7.2.2 Aims

This study aimed to test this hypothesis by quantifying expression of *BAX* and *BCL-2* apoptotic genes in the macroscopically-normal mucosa of people at differential risk of CRC i.e. those with quiescent UC, those with a prior history of adenomatous polyps and "normal" participants.

5.7.2.3 Objectives

- To obtain colorectal biopsies from the macroscopically-normal mucosa of DISC Study participants at differential risk of CRC i.e. those with quiescent UC, those with a prior history of adenomatous polyps and "normal" participants;
- To utilise previously extracted RNA to synthesise cDNA;
- To quantify the expression of *BAX* and *BCL-2* by qPCR;
- To compare *BAX* and *BCL-2* expression in the 'Normal' participants and in the two higher-risk groups ('UC' and 'Polyp' participants);
- To investigate the effects of key epidemiological factors including age and gender on expression of *BAX* and *BCL-2*.

5.7.3 Methods

RNA previously extracted for the quantification of WNT pathway-related gene expression was used to synthesise cDNA for the quantification of *BAX* and *BCL-2* expression as described in section 2.2.5. Reverse transcription and quantification of *BAX* and *BCL-2* expression by qPCR were undertaken under my supervision by Idoia Ibero, an Erasmus Project student, to whom I am very grateful.

Differences in *BAX* and *BCL-2* expression and in the ratio of *BAX* to *BCL-2* between the three participant groups were investigated using the ANOVA GLM, with the inclusion of age, gender, endoscopy procedure, BMI and smoking status as covariates. Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method was used for pairwise comparisons. Data for *BAX* expression was not normally-distributed despite transformation and consequently the non-parametric Kruskal-Wallis test was used to investigate differences between the three groups.

5.7.4 Results

5.7.4.1 BAX and BCL-2 expression in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants

Expression of *BAX* and *BCL-2* was low in the colorectal mucosa of all three participants groups. The results from this study showed that expression of both *BAX* and *BCL-2* ($p<0.001$) and the ratio of *BAX*/*BCL-2* ($p=0.027$) differed significantly between the three participant groups (Table 5.18). Expression of both *BAX* and *BCL-2* was significantly higher in 'Polyp' participants compared with 'Normal' and 'UC' participants. 'Normal' participants had a significantly higher ratio of *BAX* to *BCL-2* by approximately 10% compared with the two higher-risk groups.

When applying Bonferroni's correction for multiple testing and adjusting the p-value threshold to 0.017, the differences in *BAX* and *BCL-2* expression between the three participant groups remain statistically significant ($p<0.001$). However, statistical significance for differences in the ratio of *BAX/BCL-2* is lost ($p=0.027$).

Table 5.18 *BAX* and *BCL-2* expression in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.

	Normal	UC	Polyp	P value
***BAX*[1]** N=81	1.37 (0.74 – 2.86)	2.25 (1.34 – 3.09)	11.12 (3.89 – 21.14)	**<0.001**
***BCL-2*[2]** N=70	0.420 (0.306 – 0.576)	0.238 (0.119 – 0.475)	1.301 (0.736 – 2.300)	**<0.001**
***BAX/BCL-2*[2]** N=68	0.878 (0.856 – 0.900)	0.800 (0.750 – 0.850)	0.815 (0.772 – 0.858)	**0.027**

BAX and *BCL-2* expression data are expressed as adjusted copies. *BAX/BCL-2* is expression as the ratio of *BAX* ΔCt to *BCL-2* ΔCt. [1]Data are presented as median (lower and upper quartiles). The non-parametric Kruskal-Wallis test was used to compare methylation between the three groups. [2]Data are presented as LSMs (95% CIs). The ANOVA GLM was used to compare methylation between the three groups and Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method was used for pairwise comparisons.

5.7.4.2 Exploration of additional factors influencing BAX and BCL-2 expression

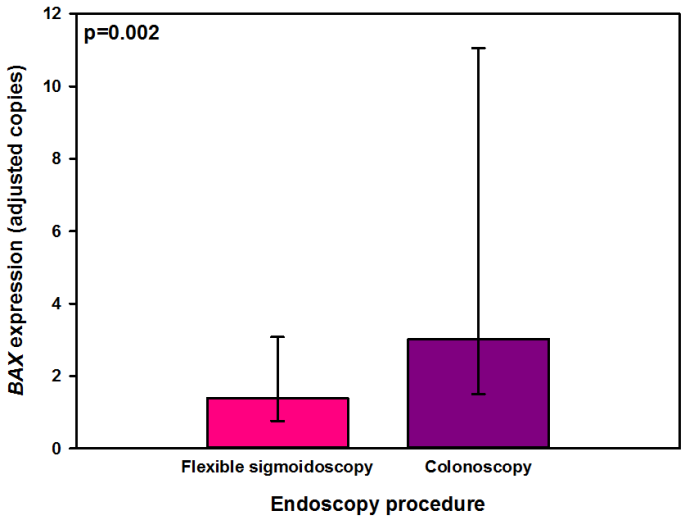
The effects of age, gender, endoscopy procedure, BMI and smoking status on *BCL-2* expression and on the ratio of *BAX* to *BCL-2* were assessed as covariates using the ANOVA GLM. As *BAX* expression was not normally-distributed, the non-parametric Kruskal-Wallis test was used. There were no effects of age, gender, BMI or smoking status on expression of *BAX* or *BCL-2.* Endoscopy procedure was found to have a significant effect on *BAX* expression (Table 5.19).

Table 5.19 P values for the effects of age, gender, endoscopy procedure, BMI and smoking status on *BAX* and *BCL-2* expression.

	Age	Gender	Endoscopy procedure	BMI	Smoking status
BAX	0.812	0.891	**0.002**	0.437	0.494
BCL-2	0.991	0.496	0.996	0.502	0.744
BAX/BCL-2	0.864	0.191	0.795	0.217	0.924

Participants who underwent endoscopic examination by colonoscopy had significantly higher expression of pro-apoptotic *BAX*, by approximately two-fold, compared with those who had the flexible sigmoidoscopy procedure (Figure 5.21).

Figure 5.21 Effect of endoscopy procedure on *BAX* expression.



Data are expressed as adjusted copies. Data are presented as medians and error bars represent lower and upper quartiles (Kruskal-Wallis). N=81.

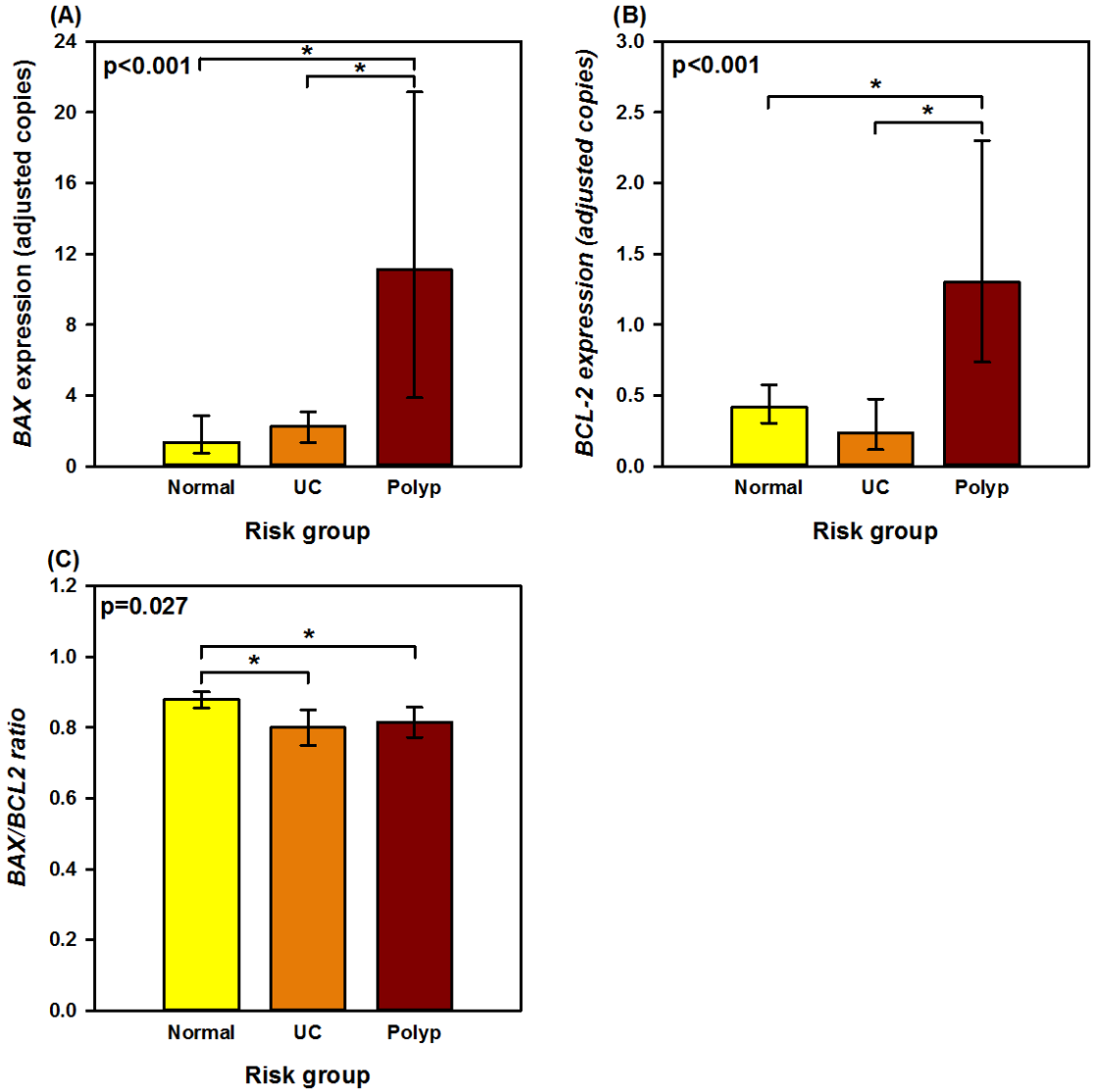
5.7.5 Discussion

Induced apoptosis has been observed in the colorectum of patients with UC, however, studies suggest that this is not mediated by the BCL-2 family. Whilst one study observed a reduction in *BAX* expression in the inflamed tissue of UC patients (Iimura *et al.*, 2000), other studies have found an increase in both *BAX* and *BCL-2* expression with UC (Karamanolis *et al.*, 2007). *BAX* expression was approximately eight-fold greater in participants with a prior history of polyps compared with 'Normal' participants, and five times greater than that quantified in participants with quiescent UC (Figure 5.22 (A)). Expression of BAX was also approximately two-fold greater in 'UC' participants compared with 'Normal' participants, but this was not statistically significant. An increase in expression of pro-apoptotic *BAX* in these higher-risk participants would suggest an increase in apoptosis, which is consistent with findings from the literature.

Expression of anti-apoptotic *BCL-2* was also highest in 'Polyp' participants compared with the other two groups (Figure 5.22(B)), which, contrary to that

insinuated by an increase in pro-apoptotic *BAX*, suggests a reduction in apoptosis in participants with a prior history of adenomatous polyps.

Figure 5.22 Expression of *BAX* and *BCL-2* in the macroscopically-normal mucosa of 'Normal', 'UC' and 'Polyp' participants.



Expression of *BAX* (A) (N=81) and *BLC-2* (B) (N=70) are expressed as adjusted copies. The *BAX/BCL-2* ratios (C) (N=68))were calculated using the ΔCt values for each gene. Data for *BAX* (A) are presented as medians and error bars represent lower and upper quartiles (Kruskal-Wallis). Data for *BCL-2* (B) and *BAX/BCL-2* ratio (C) are presented as LSMs and error bars represent 95% CIs (ANOVA GLM). Dunnett's post-hoc test was used for multiple comparisons with the control group and the Bonferroni method for pairwise comparisons.

Controversially, as reviewed by Koornstra *et al.* (2003), the majority of studies investigating apoptosis in polyps have observed an increase in apoptosis compared with the healthy mucosa (Koornstra *et al.*, 2003), with only a limited number of studies reporting a reduction (Bedi *et al.*, 1995; Moss *et al.*, 1996a;

Moss *et al.*, 1996b). However, several studies have reported an alteration in the distribution of apoptotic cells with an increase in the number of cells undergoing apoptosis at the base of the crypt (Hall *et al.*, 1994).

Previous studies investigating *BAX* and *BCL-2* expression have primarily quantified protein levels by immunohistochemical analyses. *BAX* expression has been reported to be increased in adenomas, however differences between colorectal lesions have been observed with increasing expression associated with polyps of greater severity such as tubular adenomas (Ladas *et al.*, 2005; Endo *et al.*, 2013). Consistent with findings from the present study, several studies have suggested that BCL-2 expression is increased in adenomas (Nakamura *et al.*, 1995), however a study that quantified BCL-2 in the rectum reported reduced expression in lesions of moderate and severe degrees (Giarnieri *et al.*, 2000).

The findings from the present study where expression of both pro-apoptotic *BAX* and *BCL-2* was increased in the macroscopically-normal mucosa of participants with prior history of polyps is consistent with findings from previous studies (Giarnieri *et al.*, 2000; Ladas *et al.*, 2005; Endo *et al.*, 2013). Although an increase in *BAX* suggests an increase in apoptosis, an increase in *BCL-2* expression is representative of the contrary. The finding that the *BAX/BCL-2* ratio was significantly lower in the ‘Polyp’ group compared with the ‘Normal’ group suggests that apoptosis is reduced in these participants (Figure 5.22 (C)).

Altogether, the higher expression of pro-apoptotic *BAX* and reduced inhibition associated with lower expression of *BCL-2* in participants with quiescent UC compared with ‘Normal’ participants is consistent with findings from previous studies that have reported increased apoptosis in these patients. However, the observation that the *BAX/BCL-2* ratio was significantly lower in participants with quiescent UC compared with ‘Normal’ participants contradicts this finding (Figure 5.22 (C)).

Chapter 6 General Discussion

6.1 Main findings

This Ph.D. project was embedded within the DISC Study, which is the largest randomised, placebo-controlled dietary intervention investigating the effects of supplementing healthy participants with RS and/or PD on colorectal function. This Ph.D. project focused on testing the hypothesis that these NDCs modulate the WNT signalling pathway, primarily the expression of WNT pathway components, and two of its functional outcomes viz. cell proliferation and apoptosis. In addition, I tested the hypothesis that effects on WNT signalling resulted from altered epigenetic mechanisms specifically DNA methylation and miRNA expression. To determine the potential implications of these WNT signalling changes, I also investigated similar outcomes in the macroscopically-normal mucosa of people at higher risk of CRC i.e. those with quiescent UC or with a prior history of adenomatous polyps. The main findings from both studies (the dietary intervention and investigations in people at differential risk of CRC) are summarised in Table 6.1.

For these purposes, I quantified the expression of twelve WNT pathway-related genes which were selected because of their involvement in the WNT signalling pathway, evidence that they were dysregulated in CRC and that their expression is modulated by butyrate (see section 3.3). Furthermore, a pilot study was conducted to compare the expression of a larger panel of WNT pathway-related genes in people at normal CRC risk and in the macroscopically-normal mucosa of those with a prior history of adenomatous polyps, at higher risk of CRC. A subset of these genes showing the largest fold-difference and/or statistically significant differences between the groups was chosen (see section 3.2).

I observed that both RS and PD affected expression of WNT pathway components and/or target genes at the mRNA level and the main findings were the following:

1. RS supplementation downregulated expression of β-catenin, encoded by *CTNNB1*, and the target gene *c-MYC*, suggesting that it reduced WNT

pathway activity. This is consistent with previous findings that treatment of SW837 and HCT-116 CRC cells with butyrate, a product of RS and NDC fermentation, decreased c-MYC expression at both the mRNA (Wilson *et al.*, 2002; Lazarova *et al.*, 2014b) and protein levels (Taylor *et al.*, 1992). In contrast, other studies have observed no effect of RS on *CTNNB1* expression in a rat model of CRC (AOM-treated rats) (Cray *et al.*, 2013) or of butyrate treatment on *CTNNB1* expression in SW620 and HCT-116 CRC cells (Bordonaro *et al.*, 2002) and one study reported increased levels of β-catenin protein in eight CRC cell lines (SW48, SW620, LS174T, HCT-116, DLD-1, COLO201, HT29 and LoVo) after 5mM butyrate treatment (Bordonaro *et al.*, 2007).

2. I observed that expression of *SFRP1,* an antagonist of WNT signalling, was lower in participants who consumed RS compared with placebo. Less SFRP1 would suggest less repression of WNT signalling and, consequently, enhanced pathway activity. However, *SFRP1* antagonises WNT signalling competitively either by binding to WNT ligands or to Frizzled receptors and so an increase in WNT ligands may overcome these effects of repressed activity. Interestingly, PD also reduced the expression of both *SFRP1* (p=0.053) and *SFRP2* (p=0.010). To my knowledge, the effects of RS or PD (or of butyrate) on *SFRP1* and *SFRP2* expression within the colorectal mucosa have not been investigated previously. However, a study using gastric cancer cell lines reported that butyrate increases the expression of SFRP1 and SFRP2 proteins (Shin *et al.*, 2012).

3. At the mRNA level, I observed that expression of *c-JUN* (p=0.046), a target of the WNT pathway, and of *WNT11* (p=0.040), a WNT ligand, was increased in those at higher CRC risk with the expression of both genes being greatest in ‘Polyp’ participants. These findings suggest that those at greater CRC risk experienced enhanced WNT pathway activity as *WNT11* is an activator of WNT signalling and *c-JUN* is a target gene. Whilst it has been suggested that *WNT11* can activate (Tao *et al.*, 2005) or repress the WNT pathway (Maye *et al.*, 2004), my observation of greater *c-JUN* expression in ‘Polyp’ participants suggests higher WNT pathway activity in higher-risk individuals. Both *c-JUN* and *WNT11* are

upregulated in colorectal adenocarcinomas (Magrisso *et al.*, 1993; Kirikoshi *et al.*, 2001). Interestingly, although not statistically significant, RS supplementation lowered *WNT11* expression, suggesting that RS may have the potential to reduce the aberrant expression of this gene in people at higher risk of CRC.

4. To test the hypothesis that altered WNT gene expression may result from epigenetic mechanisms, I explored the epigenetic regulation of *SFRP1*, which was modified by both RS and PD, by quantifying by qPCR the expression of a panel of eight miRNAs implicated in CRC, modulated by butyrate and predicted to target *SFRP1*. Neither RS nor RS had significant effects on expression of *miR-17, miR-19a, miR-19b, miR-20a, miR-25, miR-93, miR-106b* or *miR-424.* However, there was a trend for lower expression of three miRNAs from the miR-17-92 oncogenic cluster, *miR-17, miR-19a* and *miR-19b*, with RS supplementation. This observation is consistent with the reports of down-regulated expression of these three miRNAs by butyrylated RS in humans (Humphreys *et al.*, 2014) and by butyrate *in vitro* in HT-29 and HCT-116 CRC cells (Humphreys *et al.*, 2013). Additional miRNAs may be involved in the modulation of *SFRP1* expression by RS or PD. The deciphering of miRNAs that target specific genes is complex because a single gene may be regulated by a large number of miRNAs and, likewise, a single miRNA may target numerous genes (Lewis *et al.*, 2005). The panel of miRNAs quantified in this study were selected by reviewing findings from studies that used expression techniques and bioinformatic databases which, based on sequence homology, predicted those miRNAs which would target *SFRP1.* However, the fact that, to my knowledge, there are no studies that have investigated the regulation of *SFRP1* expression by miRNAs directly is a limitation of the evidence base.

5. In the analyses of the participants at differential risk of CRC, a panel of five miRNAs was selected based on evidence that the expression of each miRNA has been reported to be dysregulated in CRC and that the miRNA is involved in regulation of the WNT signalling pathway. I observed no significant differences in the expression of these miRNAs between ‘Normal’, ‘UC’ and ‘Polyp’ participants. In addition, in these

participants at differential CRC risk, I quantified the expression of the same panel of eight miRNAs selected for the RS and PD intervention study analyses. This showed that *miR-424* expression was increased in participants with quiescent UC compared with both 'Normal' and 'Polyp' participants. This is consistent with findings from a study by Lin *et al.*, who reported dysregulated expression of *miR-424* in the colonic mucosa of both UC and Crohn's disease patients (Lin *et al.*, 2014).

6. To investigate the potential regulation of *SFRP1* by promoter methylation, I quantified DNA methylation at seven CpG sites within the promoter region of *SFRP1* by pyrosequencing in both intervention participants and in the three groups of participants at differential risk of CRC. This investigation was built on evidence of lower *SFRP1* expression by promoter hypermethylation in CRCs (Chen *et al.*, 2014b). I observed no significant effects of neither RS nor PD on methylation of *SFRP1* at any of the seven CpG sites quantified or on the mean methylation across all 7 sites. In both the intervention and higher-risk participants, there was a strong correlation of *SFRP1* methylation between the seven CpG sites. However, as effects may only be observed on methylation at a single CpG site (Weaver *et al.*, 2004), effects of the intervention at each of the seven CpG sites as well as on the mean methylation across all sites were investigated. Moreover, in cross-section analysis, there was no evidence of a correlation between *SFRP1* methylation and expression (see section 4.4.4.4). Because of the critical role played by promoters in regulation of gene expression, *SFRP1* methylation was examined at CpG site-rich regions known as CpG islands within the promoter region. Recent evidence suggests that methylation at additional genomic domains known as CpG shores, which are found up to 2kb upstream of the promoter region, may also be important in the regulation of gene expression and altered methylation of CpG island shores has been reported in colon cancer tissue (Irizarry *et al.*, 2009). In addition, RS and PD may regulate *SFRP1* by other epigenetic mechanisms with or without the involvement of DNA methylation. Butyrate is a well-established HDACi (Berni Canani *et al.*, 2012) and so altered histone acetylation may be an alternate mechanism through which RS and PD could have modulated *SFRP1* expression.

7. Greater methylation of *SFRP1* at all seven CpG sites investigated was observed in 'UC' and, even higher methylation, in 'Polyp' participants compared with normal controls. This is consistent with reports in the literature that *SFRP1* methylation is increased in both UC and adenoma patients (Caldwell *et al.*, 2006; Qi *et al.*, 2006; Belshaw *et al.*, 2008; Shao *et al.*, 2012; Kim *et al.*, 2013). Higher methylation implies lower *SFRP1* expression and, consequently, an increase in WNT pathway activity would be anticipated. However, a limitation of this study is that, due to unreliable data for expression of *SFRP1* at the mRNA level, it was not possible to investigate the relationship between *SFRP1* methylation and expression directly.

8. Based on my observation of significant differences in expression of *WNT11* in people at differential risk of CRC (see section 5.2.4.1), I undertook the first known study of the methylation state of *WNT11* in the colorectum. Spearman correlation analyses revealed only a weak to moderate correlation between methylation levels at the different CpG sites, strengthening the rationale to analyse differences between risk groups both at each of the five CpG sites and on the methylation across all sites. This showed that methylation at CpG site 2 was significantly lower in participants with quiescent UC compared with 'Normal' and 'Polyp' participants. However, mean methylation across all five CpG sites did not differ between participant groups.

9. Dysregulation of the WNT pathway during colorectal carcinogenesis affects cell proliferation (which is usually increased (Risio *et al.*, 1991; Bostick *et al.*, 1997)). In the present study, I quantified colorectal crypt cell proliferation by whole crypt microdissection of Schiff reagent-stained biopsies as described and validated previously by Mills *et al.* (Mills *et al.*, 2001). This revealed that RS supplementation increased total crypt cell proliferation by approximately 33%. This agrees with the literature where a number of studies in healthy participants have shown an increase in cell proliferation in healthy tissue with RS supplementation (Mentschel and Claus, 2003), although others have not observed an effect of RS (Wacker *et al.*, 2002; Worthley *et al.*, 2009). However, there were no effects of either RS or PD on the proportion of mitotic cells observed in

the upper half of the crypt, suggesting that neither NDC had positive, or adverse, effects on a functional marker of CRC risk in these healthy participants. In contrast, Dronamraju *et al.* reported that RS supplementation for up to four weeks in patients prior to surgery for CRC removal reduced cell proliferation in the upper half of the crypt (p=0.028 for Study 1) (Dronamraju *et al.*, 2009). Taken together, these findings suggest that any “protective” effects of RS on crypt cell proliferation may be evident only in those with CRC.

10. In the present study, cell proliferation was lower in ‘UC’ and ‘Polyp’ participants compared with ‘Normal’ participants. This is contrary to findings from previous studies which have reported consistently increased levels of cell proliferation in both UC and polyp patients (Serafini *et al.*, 1981; Kanemitsu *et al.*, 1985; Biasco *et al.*, 1990; Wilson *et al.*, 1990). However, although not statistically significant, the proportion of mitotic cells in the top half of the crypt appeared to be increased in higher-risk participants, particularly in those with quiescent UC (see section 5.6.4.1). This finding is consistent with those from the literature which show dysregulation of the distribution of mitotic cells in such patients (Franklin *et al.*, 1985; Ponz de Leon *et al.*, 1988; Biasco *et al.*, 1990). The latter is a superior indicator of bowel health as altered distribution of mitotic cells within the crypt is one of the earliest detectable alterations in the mucosa prior to the development of malignancies (Roncucci *et al.*, 1991).

11. Attempts were made to use microdissected crypts for the assessment of apoptosis but very few apoptotic cells were detected so that this technique was insufficiently sensitive for investigating potential treatment differences in apoptosis. As an alternative approach, expression of *BAX* and *BCL-2*, (positive and negative regulators of the apoptotic response, respectively), was quantified by qPCR. In the intervention participants, there was no evidence that either RS or PD modulated expression of *BAX* or *BCL-2* at the mRNA level. Since the protein products of these two genes have opposing effects on regulation of apoptosis, the ratio of pro-apoptotic *BAX* to anti-apoptotic *BCL-2* was calculated as this may be more meaningful in the regulation of apoptosis than the expression of

either gene individually. Again, this showed no significant effects of RS nor PD and these findings suggest that neither RS nor PD affects the mitochondrial, or intrinsic, pathway of apoptosis which is mediated by the BCL-2 family of apoptotic genes. However, whether RS and/or PD affected levels of apoptosis overall remains to be discovered since there are additional apoptotic pathways such as the extrinsic pathway (Watson, 2004).

12. Expression of both BAX and BCL-2 was greater in 'Polyp' participants compared with 'Normal' participants and with quiescent UC patients. Furthermore, both 'Polyp' and 'UC' participants had lower *BAX/BCL-2* ratios than that observed in healthy controls. A lower ratio of *BAX/BCL-2* suggests a reduction in apoptosis which is contrary to findings from the majority of previous studies showing increased levels of apoptosis in patients with polyps (Koornstra *et al.*, 2003). Whilst there is evidence that apoptosis is increased in the tissue of UC patients (Iwamoto *et al.*, 1996; Sipos *et al.*, 2005), previous investigations of the expression of *BAX* and *BCL-2* in UC patients have yielded conflicting results. Whilst some studies have reported downregulation of *BAX* in tissue from UC patients, others have observed upregulation of *BAX* and *BCL-2* (Iimura *et al.*, 2000; Karamanolis *et al.*, 2007).

Table 6.1 Summary of main findings from dietary intervention study and CRC differential risk study.

	Change in CRC	Findings from dietary intervention study	Findings from CRC differential risk study
WNT pathway-related gene expression			
c-JUN	Increased	No effect	Increased in 'Polyp' group
c-MYC	Increased	Decreased with RS	No differences
CTNNB1	Increased	Decreased with RS	No differences
SFRP1	Decreased	Decreased with RS and trend for decrease with PD	No differences
SFRP2	Decreased	Decreased with PD	No differences
WNT11	Increased	No effect	Increased in 'Polyp' group
DNA methylation			
SFRP1	Increased	No effect	Increased in 'Polyp' group
WNT11	Not reported previously	-	Increased at CpG site 2 in 'Polyp' group
miRNA expression			
miR-424	Increased	No effect	Increased in 'UC' group

	Change in CRC	Findings from dietary intervention study	Findings from CRC differential risk study
Colonic crypt cell proliferative state			
Total proliferation	Increased	Increased with RS	Decreased in 'Polyp' group
Crypt dimensions	Increased	No effect	Increased in 'UC' group
Expression of regulators of apoptosis			
BAX	Decreased	No effect	Increased in 'Polyp' group
BCL-2	Increased	No effect	Increased in 'Polyp' group
BAX/BCL-2 ratio	Decreased	No effect	Decreased in 'UC' and 'Polyp' groups

6.2 Conclusions

This Ph.D. study aimed to test the hypothesis that the NDCs RS and PD are protective against CRC through the genetic and epigenetic modulation of the WNT signalling pathway, which is frequently aberrantly expressed in CRC. I also hypothesised that two functional outcomes of this pathway, cell proliferation and apoptosis, would be modulated as a consequence of altered WNT signalling. In addition, I hypothesised that participants at higher risk of CRC, i.e. those with quiescent UC or a prior history of adenomatous polyps, would have aberrantly expressed WNT signalling and dysregulated proliferation and apoptosis.

At first sight, my major findings on the effects of NDC supplementation on WNT pathway gene expression appear to be in conflict. The reduced expression of *CTNNB1* and *c-MYC* following RS supplementation suggests a reduction in WNT pathway activity. However, the downregulation of two WNT antagonists, *SFRP1* and *SFRP2*, by RS and/or PD suggests that NDC supplementation may have reduced a brake on WNT signalling activity. Given that RS induced cell proliferation but had no detectable effect on BCL-2-mediated apoptosis, it seems probable that the net effect of RS supplementation was increased WNT pathway activity. Bordonaro *et al.* have proposed that butyrate, a fermentation product of RS and other NDCs, stimulates the WNT signalling pathway and that moderate levels of WNT signalling pathway stimulation are associated with increased cell proliferation (Bordonaro *et al.*, 2008a). We may therefore conclude that the observed increase in cell proliferation following RS supplementation may have resulted from stimulation of the WNT pathway, as indicated by a decrease in *SFRP1* expression.

Further exploration of these findings is warranted. For example, there is a need to confirm that the modulation in expression of WNT pathway-related genes at the mRNA level, reported here, is also evident at the levels of both protein concentration and activity. It would be useful to investigate other doses of these agents to determine the dose which maximised effects on WNT signalling and its associated functional outcomes. In addition, extending the duration of intervention with RS and/or PD would help to reveal whether the observed effects are i) increased with exposure time or ii) temporary and diminish with

time. The latter would be evidence that, in healthy participants, changes in WNT signalling in the colorectal mucosa are part of a homeostatic response to increased intake of NDCs and, therefore, of limited relevance to longer-term CRC risk. If the effects increased with time of exposure, this would suggest that RCTs with interventions lasting 50 days (as in the present study) may be inadequate to reveal the full effects of NDC supplementation. Longer-term interventions are more challenging in terms of logistical issues and in maintaining participant compliance.

Furthermore, the relatively large inter-participant variability, particularly when examining gene and miRNA data, made it difficult to detect statistically significant effects of the interventions or of differences between CRC risk groups. Some of this inter-individual variation may be inevitable (a consequence of individual genotype or previous environmental exposures) but some variance may be within the experimenter's control. For example, the DISC Study recruited both men and women over a wide range of ages and with considerable variation in adiposity, smoking behaviour and habitual diet. I attempted to control for the most obvious potential confounders e.g. age, sex, BMI and smoking behaviour by including these factors as covariates in my analyses but it is probable that other unmeasured, or unknown, factors contributed to inter-individual variation in WNT pathway gene expression. A better understanding of these factors would allow the design of future intervention studies which were more sensitive i.e. capable of detecting smaller treatment effects.

In addition, some of the observed inter-individual variation may have been due to differences in the cellular composition of the colorectal mucosal biopsies collected in the DISC Study. We attempted to minimise inter-individual differences by ensuring that all biopsies were collected from the same anatomical site i.e. 10 centimetres from the ano-rectal margin. This is important because my earlier Masters' project had shown differences in WNT gene expression when biopsies were collected from different, but defined, sites in the large bowel (Malcomson, 2011). In addition, previous work from the Mathers' laboratory has shown that there are systematic gradients in crypt cell proliferation along the human large bowel (Mills *et al.*, 2001). Visual inspection of the biopsies used in this study showed that they differed in size and this may

reflect differences in the depth of the “bite” taken by the biopsy forceps. Different depths of biopsy “bite” may result in differences in the proportions of columnar epithelial cells and of the underlying stroma in different biopsies. Only a small number of studies have reported expression of WNT pathway components, predominantly WNT ligands such as *WNT2* and *WNT5A* (Smith *et al.*, 1999; Fodde and Brabletz, 2007; Najdi *et al.*, 2011), in stromal compartments, suggesting that potential differences in the proportion of stromal tissue between samples may have implications on the estimates of expression of these genes.

The results from this study suggest that WNT signalling is aberrantly expressed in participants at higher risk of CRC and that this is detectable even in the macroscopically-normal mucosa of these patients, suggesting a field effect within the colorectum. This is demonstrated by the upregulation of *WNT11*, a WNT ligand that can activate the WNT pathway, and *c-JUN*, a target of WNT signalling. However, these findings at the mRNA level require confirmation at the protein level. Unexpectedly, whilst both ‘UC’ and ‘Polyp’ participants had reduced total numbers of mitotic cells per crypt compared with ‘Normal’ participants, the proportion of mitotic cells in the upper (luminal) half of the crypt appeared to be increased, which suggests dysregulation in this process. The lower *BAX/BCL-2* expression ratio which I observed is evidence that apoptosis mediated by the BCL-2 family was reduced in higher-risk participants. Lazarova *et al.* have previously shown a correlation between WNT pathway activity and levels of apoptosis (Lazarova *et al.*, 2004), although others have reported the opposite (Groden *et al.*, 1995; Morin *et al.*, 1996; Zhang *et al.*, 2001), underlining the importance of undertaking further studies of the WNT signalling pathway, and related functional outcomes, in the macroscopically-normal human colorectal mucosa.

6.3 Strengths and limitations of the study

To date, the DISC Study is the largest double-blind, randomised-controlled dietary intervention investigating the effects of NDCs in healthy subjects. Previous studies such as the CAPP1 and CAPP2 studies have investigated the effects of RS in patients with FAP and HNPCC who are at much higher-risk of CRC because of inherited defects in the *APC* gene and in those encoding MMR genes respectively (Burn *et al.*, 2011a; Mathers *et al.*, 2012). Both CAPP1 (113 participants) and CAPP2 (463 participants), were large, long-term studies that supplemented participants with 30g of RS daily for up to 12 and four years respectively. Another study supplemented 65 CRC patients with RS or ordinary starch for up to four weeks and investigated the effects of RS on colonic crypt cell kinetics and expression of cell cycle regulatory genes in both tumour and adjacent normal tissues from these participants (Dronamraju *et al.*, 2009). Other trials in healthy subjects include a randomised, placebo-controlled, crossover study in which 20 participants were given 12.5g of RS and/or *Bifidobacterium lactis* for four weeks (Worthley *et al.*, 2009) and a randomised, crossover trial supplementing 18 participants with 28g of RS for seven days (van Munster *et al.*, 1994).

I observed a significant effect of RS on some of the measured outcomes, with a 25% difference being the smallest effect size for *CTNNB1* expression and total mitoses for the comparison of RS and placebo. Consequently, I calculated the sample size which would be required to detect a similar-sized effect on other outcomes measured. For example, in my study there were no significant effects of RS or PD on miRNA expression. A sample size calculation (α 0.05 and power of the test 0.80) revealed that I would have needed at least 91 participants to detect a 25% difference in expression of *miR-17*, a miRNA with intermediate levels of variation between participants. However, for *miR-19b*, which showed the largest inter-participant variation in expression, a sample size of 2067 would have been required to detect a 25% difference in expression due to RS or PD. Finally, for the analysis of *SFRP1* methylation, a *post hoc* power analysis revealed that the probability of correctly rejecting a false null hypothesis was only 0.20 and was consequently underpowered.

A strength of this project is that all of the participants in the DISC Study, including both those in the intervention and the higher-risk groups, were recruited from two hospitals in the North East of England and samples were collected, processed and stored using identical methods which minimises potential confounders. Stringent inclusion and exclusion criteria, such as the exclusion of people taking anti-inflammatories, minimised the effects of possible confounders on the analysed outcomes. Furthermore, where possible, participant characteristics, such as age, gender and BMI, were included as covariates during statistical analyses to prevent any effects on the measured outcomes. Randomisation of participants across the study groups aims to remove the effect of such confounders, however this did not apply for the investigations in the 'UC' and 'Polyp' participants. The double-blind nature of the DISC Study was maintained until the completion of all analyses (both laboratory and statistical), reported in this Ph.D., consequently preventing any bias.

This Ph.D. utilised mucosal biopsies collected from the mid-rectum. The fact that changes were observed within the rectum, which is not as active in respect of bacterial fermentation of NDCs as more proximal areas in the large bowel, may suggest that the effects of treatments (and differences between CRC risk groups) may have been more pronounced in other sites, such as the caecum and transverse colon. On the other hand, because the majority of CRCs develop in the rectum, [between 2007 and 2009, 27% of CRC cases in the UK were rectal (CRUK, 2014a)], analyses performed at this site may be more relevant from a disease prevention perspective. With the notable exceptions of *AES, CTNNB1* and *FBXW4*, previous work demonstrated that overall there were no differences in the expression of WNT pathway components in the caecum, transverse colon, sigmoid colon and rectum in a panel of 35 WNT pathway-related genes (Malcomson, 2011). In that study, I found that the expression of *AES* and *CTNNB1* was significantly greater in the rectum and that *FBXW4* expression was reduced in the rectum compared with the sigmoid colon, suggesting that WNT activity is enhanced in the rectum. It is important to note that *CTNNB1* expression was altered by RS supplementation in the present study. These findings suggest that the effects on WNT signalling observed in this study may be representative of the whole colorectum but this remains to be demonstrated experimentally.

The fact that all the biopsies used in this study were collected from the same anatomical site is a strength since it implies that the data are likely to be directly comparable and not subject to confounding by (unknown) differences due to sample site. In addition, for the analyses in the higher-risk participants who had quiescent UC or a prior history of adenomatous polyps, all biopsies were of macroscopically-normal mucosa i.e. not tissue with active inflammation and not from the polyp tissues themselves. The majority of previous studies utilising patients with UC or polyps have analysed the diseased tissue i.e. the inflamed or polyp tissue itself. The present study facilitated detection of any field effects that may be present in the normal-appearing tissue in the mucosa of people at higher risk of CRC. It must be noted, however, that not all analyses were undertaken in the same biopsy sample from the individual. For example, gene expression and methylation analyses were performed in OCT-embedded biopsies whereas CCPS analyses were in Carnoy's-fixed biopsies.

The collection of rectal biopsies post-intervention by rigid sigmoidoscopy is a less invasive procedure than flexible sigmoidoscopy and colonoscopy and does not require bowel preparation. Logistically, it would not have been ideal to ask the participants to undergo another flexible sigmoidoscopy or colonoscopy procedure to retrieve biopsies from other sites within the colon post-intervention and, in any case, it would not have been possible to recruit the number of volunteers in the specified timeframe. However, the fact that bowel preparation was required prior to the collection of pre-intervention samples, or baseline samples for the higher-risk participants, but not post-intervention could have had an effect on some of the results. To minimise this possibility and to avoid any confounding effects of bowel preparation, participants were randomised to NDC intervention according to the endoscopy procedure used at baseline and the latter was included as a covariate during statistical analyses.

With regard to analytical techniques, robust methodology was utilised in each aspect of the laboratory work undertaken in this study. Where applicable, technique optimisation and validation assays were run prior to the main analyses to test the accuracy, specificity and/or reproducibility of the procedures. For example, thorough optimisation of the method utilised to isolate RNA from rectal mucosal biopsies was performed which aimed to achieve the greatest yield and quality possible from these small samples. Other examples

showing the robustness of the methodological techniques used include the analysis of melt (dissociation) curves for each qPCR experiment to test the specificity of the experiment and the running of standard curves for each assay analysed by pyrosequencing prior to quantification.

The quantification of gene and miRNA expression could have been improved by the use of TaqMan® assays, which provide greater detection specificity than is possible with DNA-binding dye-based methods such as SYBR® Green which was used in the present study. However, as noted above, the melt curves for each experiment were checked, and if they were found to be inadequate, the experiment was repeated and/or data were excluded from subsequent analyses. A limitation to the *SFRP1* and *WNT11* methylation analyses by pyrosequencing is that this is limited to a specific site, for example within the promoter region, and does not exclude effects or differences at additional sites within the gene.

Furthermore, suitable methods were selected for statistical analyses following examination of the distribution of each dataset. When data were normally-distributed, the parametric ANOVA GLM test was used and included age, gender, endoscopy procedure, BMI and smoking habit as covariates. Not only did this adjust for these factors as possible confounders, but it also allowed for the exploration of the effects of these factors on the measured outcomes. For the analyses of the intervention data, the pre-intervention measurement was also included as a covariate, which adjusted for any differences between the groups at baseline.

6.4 Future research

Statistically significant differences in the expression of certain WNT pathway genes were observed following RS and PD supplementation in the intervention study and in the comparisons between 'Normal', 'UC' and 'Polyp' participants. Although there are correlations between expression at the mRNA level and the abundances of the corresponding proteins, this correlation can be as little as 40%, suggesting that the remaining 60% may result from post-transcriptional regulation (Vogel and Marcotte, 2012). To confirm that the effects that I have observed at the mRNA level are mirrored phenotypically, protein levels should be quantified for example by immunohistochemical analyses or semi-quantitatively using Western Blotting. This should include quantification of SFRP1 protein expression for which I observed significantly greater promoter methylation in higher-risk participants compared with normal-risk participants.

A panel of 12 WNT pathway-related genes was selected carefully to be quantified in the intervention study and higher-risk participants. It would be very interesting to explore differences in a larger number of genes using microarray technology, in at least a smaller sample of the participants. WNT signalling pathway PCR arrays are available and allow for the quantification of 84 WNT pathway-related genes. Similarly, additional arrays could be used to investigate differences in other non-WNT pathway-related genes such as the Human Cancer Pathway Finder PCR array (Qiagen) that quantifies expression of genes encompassing nine pathways implicated in transformation and tumourigenesis. This approach could also be applied to quantify the expression of additional miRNAs implicated in carcinogenesis.

To confirm the effects of the observed differences in the expression of WNT pathway-related genes on WNT signalling, measurements of active, dephosphorylated β-catenin by IHC could be used as an indicator of WNT activity. For example, I speculated that lower WNT pathway activity would be responsible for the observed reduction in *c-MYC* found in participants who were supplemented with RS, and I hypothesise that these participants would also show a reduction in β-catenin, particularly the dephosphorylated active form, at the protein level. In addition, it would be possible to detect the sub-cellular

location of β-catenin through IHC staining. More nuclear β-catenin staining would be indicative of greater WNT pathway activity.

Levels of apoptosis were assessed by quantification of the expression of *BAX* and *BCL-2,* two members of the BCL-2 family of apoptosis-regulators. However, differences in the expression of *BAX* and *BCL-2* only reflect effects on apoptosis mediated by the BCL-2 family of proteins and may not be a true indicator of apoptosis levels within the crypt. Furthermore, it is the BAX and BCL-2 proteins which are regulators of apoptosis and, therefore, it would be important to quantify expression of these at the protein level. In addition, apoptosis within the colon may be mediated via other pathways such as the extrinsic pathway (Watson, 2004). Consequently, further investigations into levels of apoptosis in the crypts of participants at higher-risk and those who underwent the intervention are merited. For example, the TUNEL assay could be used to detect DNA fragmentation, a consequence of apoptosis, as an indicator of apoptosis.

Appendices

Appendix A. Confirmation of ethical opinion

Newcastle & North Tyneside 2 Research Ethics Committee
Room 002
TEDCO Business Centre
Rolling Mill Road
Jarrow
NE32 4BW

Telephone: 0191 428 3565
Facsimile: 0191 428 3432
Email: gillian.mayer@sotw.nhs.uk

10 December 2009

Professor John Mathers
Professor of Human Nutrition
Institute of Ageing and Health
William Leech Building
Newcastle University
Newcastle upon Tyne
NE2 4HH

Dear Professor Mathers

Study Title:	**Diet related biomarker of colorectal cancer risk - the impact of non-digestible carbohydrates**
REC reference number:	**09/H0907/77**
Protocol number:	**v 1**

Thank you for your letter of 4 December 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair who noted that this is a good, thorough response.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a **Favourable** ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. I will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.
It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*	
Covering Letter	Iain McCallum	23 October 2009	
REC application	28026/70673/1/201	20 October 2009	
Protocol	v 1	21 October 2009	
Investigator CV	John Mathers	20 October 2009	
Referees or other scientific critique report	Ref 49395, 49396, 49425	12 December 2008	
Summary/Synopsis	v 1	16 October 2009	
CV for student/key investigator	Iain McCallum	20 October 2009	
CV for key investigator	Seamus Kelly	20 October 2009	
CV for key investigator	David Bradburn	20 October 2009	
Letter of support from Danisco	Julian Stowell	30 March 2009	
Letter of support from SUERC	Tom Preston	19 March 2009	
Details of subcontractors	Danisco and SUERC		
Letter from Newcastle University re peer reviews	Helen Rodgers	12 December 2008	
Response to reviewers' comments	Seamus Kelly		
Response to Request for Further Information	John Mathers	04 December 2009	
Participant Information Sheet: Normals	v 2	21 November 2009	
Participant Information Sheet: Polyps	v 2	21 November 2009	
Participant Information Sheet: UC	v 2	21 November 2009	
Participant Consent Form	v 2	21 November 2009	
Letter of invitation to participant	(Normals) v 2	21 November 2009	

Letter of invitation to participant	(Polyps) v 2	21 November 2009	
Letter of invitation to participant	(UC) v 2	21 November 2009	
GP/Consultant Information Sheets	(Normals) v 2	21 November 2009	
GP/Consultant Information Sheets	(UC) v 2	21 November 2009	
GP/Consultant Information Sheets	(Polyps) v 2	21 November 2009	

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.npsa.nhs.uk.

09/H0907/77	**Please quote this number on all correspondence**

Yours sincerely

Professor Philip M Preshaw
Chair

Appendix B. Letter sent to potential DISC Study participants

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

Dear Mr./Mrs. X,

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

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 C. Patient screening

Patient screening

Age <16	○	Therapy with aspirin/other NSAID	○
Age >85	○	Familial polyposis syndrome	○
Lynch syndrome	○	Known colorectal tumour	○
Previous colorectal resection	○	Pregnancy	○
Chemotherapy in last 6 months	○	Active colonic inflammation	○
Other immunosuppressive medication/steroid	○	Colorectal carcinoma found	○
Incomplete left sided examination	○	Iatrogenic perforation	○
Colorectal cancer on histology	○	Crohn's disease	○

UC group ONLY (score should be less than 5)

Bowel frequency (day)

1-3	0
4-6	1
7-9	2
>9	3

Bowel frequency (night)

1-3	1
4-6	2

Blood in Stool

Trace	1
Occasionally frank	2
Usually frank	3

Urgency of defecation

Hurry	1
Immediately	2
Incontinence	3

General Well Being

Very well	0
Slightly below par	1
Poor	2
Very poor	3
Terrible	4

Extracolonic manifestation, 1 per manifestation

Patient name ……..... Patient date of birth ……..

Assessed by …….. Date of assessment ……..

Suitable Yes/No **Informed consent Yes/No** **Study ID DISC ……**

Appendix D. DISC Study consent form

I freely consent to participate in this project	Yes	No
I have had the opportunity to ask questions	Yes	No
I understand that my care will not be affected if I choose not to participate	Yes	No
I understand I can withdraw at any time	Yes	No
I give permission for my GP to be informed	Yes	No
I am happy for the samples to be stored after the study has finished in case further testing can be carried out later	Yes	No
I understand that relevant sections of my medical notes And data collected during the study, may be looked at by Individuals from regulatory authorities or from the NHS trust, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my records.	Yes	No

Participant signature …….. Date ……

Participant name (print) …….

Researcher signature …….. Date ……

Researcher name (print) …….

1 copy to patient; 1 to research file; 1 to notes

Appendix E. DISC Study food frequency questionnaire

Food Frequency Questionnaire

Name:_____

Date:_____

Thank you for agreeing to take part in the DISC study. This is a simple questionnaire designed to help us understand what kinds of food you normally eat. It is not a test, so there are no right or wrong answers. It is your usual diet we are interested in.

All information will be treated in the strictest confidence.

Thank you for your time

Please turn over and read the instructions for answering questions before completing the questionnaire.

FFQ Questionnaire

About the food you eat

The following questions are about the food you usually eat and how often you eat certain foods. Please read the following instructions before answering the questions.

For each food there is an amount shown, either a "medium serving" or a common household unit such as a slice or teaspoon. Please put a tick in the box to indicate how often, **on average**, you have eaten the specified amount of each food **during the past year.**

EXAMPLE:

For white bread the amount is one slice, so if you ate 4 or 5 slices a day, you should put a tick in the column headed "4-5 per day".

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
BREAD & SAVOURY BISCUITS (one slice or biscuit)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
White bread and rolls								✓	

EXAMPLE:

For chips, the amount is a "medium serving", so if you had a helping of chips twice a week you should put a tick in the column headed "2-4 per week".

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
POTATOES, RICE & PASTA (medium serving)	Never or less than once/month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Chips				✓					

It is your turn to answer now!

Please put a tick in each box to indicate how often, **on average**, you have eaten each food **during the past year.**

Please estimate your average food use as best you can, and please answer every question - do not leave **ANY** lines blank. **Please put a tick on every line.**

FOODS & AMOUNTS	**AVERAGE USE LAST YEAR**								
19. MEAT & FISH ***(medium serving)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Beef: roast, steak, mince, stew casserole, curry or bolognese	1	2	3	4	5	6	7	8	9
Beefburgers	1	2	3	4	5	6	7	8	9
Pork: roast, chops, stew, slice or curry	1	2	3	4	5	6	7	8	9
Lamb: roast, chops, stew or curry	1	2	3	4	5	6	7	8	9
Chicken, turkey or other poultry: including fried, casseroles or curry	1	2	3	4	5	6	7	8	9
Bacon	1	2	3	4	5	6	7	8	9
Ham	1	2	3	4	5	6	7	8	9
Corned beef, Spam, luncheon meats	1	2	3	4	5	6	7	8	9
Sausages	1	2	3	4	5	6	7	8	9
Savoury pies, e.g. meat pie, pork pie, pasties, steak & kidney pie, sausage rolls, scotch egg	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Please check that you have a tick on EVERY line									

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
19. MEAT & FISH, (continued) ***(medium serving)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Liver, liver pate, liver sausage	1	2	3	4	5	6	7	8	9
Fried fish in batter, as in fish and chips	1	2	3	4	5	6	7	8	9
Fish fingers, fish cakes	1	2	3	4	5	6	7	8	9
Other white fish, fresh or frozen, e.g. cod, haddock, plaice, sole, halibut	1	2	3	4	5	6	7	8	9
Oily fish, fresh or canned, e.g. mackerel, kippers, tuna, salmon, sardines, herring	1	2	3	4	5	6	7	8	9
Shellfish, e.g. crab, prawns, mussels	1	2	3	4	5	6	7	8	9
20. BREAD & SAVOURY BISCUITS ***(one slice or biscuit)***									
White bread and rolls	1	2	3	4	5	6	7	8	9
Scones, teacakes, crumpets, muffins or croissants	1	2	3	4	5	6	7	8	9
Brown bread and rolls	1	2	3	4	5	6	7	8	9
Wholemeal bread and rolls	1	2	3	4	5	6	7	8	9
Cream crackers, cheese biscuits	1	2	3	4	5	6	7	8	9
Pitta bread, naan bread, chapati	1	2	3	4	5	6	7	8	9
Garlic bread	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
21. CEREALS ***(one bowl)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Porridge, Readybrek	1	2	3	4	5	6	7	8	9
Sugar coated cereals e.g. Sugar Puffs, Cocoa Pops, Frosties	1	2	3	4	5	6	7	8	9
Non-sugar coated cereals e.g. Cornflakes, Rice Crispies	1	2	3	4	5	6	7	8	9
All Bran, Bran Flakes, Muesli	1	2	3	4	5	6	7	8	9
Wholegrain cereals *e.g.* Cheerios, Weetabix, Shredded Wheat	1	2	3	4	5	6	7	8	9
22. POTATOES, RICE & PASTA ***(medium serving)***									
Boiled, mashed, instant or jacket potatoes	1	2	3	4	5	6	7	8	9
Chips, potato waffles	1	2	3	4	5	6	7	8	9
Roast potatoes	1	2	3	4	5	6	7	8	9
Yorkshire pudding, pancakes, dumpling	1	2	3	4	5	6	7	8	9
Potato salad	1	2	3	4	5	6	7	8	9
White rice	1	2	3	4	5	6	7	8	9
Brown rice	1	2	3	4	5	6	7	8	9
White or green pasta, e.g. spaghetti, macaroni, noodles	1	2	3	4	5	6	7	8	9
Tinned pasta, e.g. spaghetti, ravioli, macaroni	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
22. POTATOES, RICE & PASTA (continued) ***(medium serving)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Super noodles, pot noodles, pot savouries	1	2	3	4	5	6	7	8	9
Wholemeal pasta	1	2	3	4	5	6	7	8	9
Lasagne, moussaka, cannelloni	1	2	3	4	5	6	7	8	9
Pizza	1	2	3	4	5	6	7	8	9
23. DAIRY PRODUCTS & FATS									
Single or sour cream *(tablespoon)*	1	2	3	4	5	6	7	8	9
Double or clotted cream *(tablespoon)*	1	2	3	4	5	6	7	8	9
Low fat yoghurt, fromage frais *(125g carton)*	1	2	3	4	5	6	7	8	9
Full fat or Greek yoghurt *(125g carton)*	1	2	3	4	5	6	7	8	9
Dairy desserts *(125g carton),* e.g. mousse	1	2	3	4	5	6	7	8	9
Cheese, e.g. Cheddar, Brie, Edam *(medium serving)*	1	2	3	4	5	6	7	8	9
Cottage cheese, low fat soft cheese *(medium serving)*	1	2	3	4	5	6	7	8	9
Eggs as boiled, fried, scrambled, omelette etc. *(one)*	1	2	3	4	5	6	7	8	9
Quiche *(medium serving)*	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
23.(b) The following on bread or vegetables *(teaspoon)*	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Butter	1	2	3	4	5	6	7	8	9
Block margarine, e.g. Stork, Krona	1	2	3	4	5	6	7	8	9
Polyunsaturated margarine, e.g. Flora sunflower	1	2	3	4	5	6	7	8	9
Other soft margarine, dairy spreads, e.g. Blue Band, Clover	1	2	3	4	5	6	7	8	9
Low fat spread, e.g. Gold	1	2	3	4	5	6	7	8	9
24. SWEETS & SNACKS									
Sweet biscuits, chocolate, e.g. digestive *(one)*	1	2	3	4	5	6	7	8	9
Sweet biscuits, plain, e.g. Nice, ginger *(one)*	1	2	3	4	5	6	7	8	9
Cakes e.g. fruit, sponge, sponge pudding *(medium serving)*	1	2	3	4	5	6	7	8	9
Sweet buns & pastries e.g. flapjacks, doughnuts, Danish pastries, cream cakes *(medium serving)*	1	2	3	4	5	6	7	8	9
Fruit pies, tarts, crumbles *(medium serving)*	1	2	3	4	5	6	7	8	9
Milk puddings, e.g. rice, custard, trifle *(medium serving)*	1	2	3	4	5	6	7	8	9
Chocolates *(small bar or ¼ pound of chocolates)*	1	2	3	4	5	6	7	8	9
Ice cream, choc ices *(one)*	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
24. SWEETS & SNACKS (continued)	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Sweets, toffees, mints *(one packet)*	1	2	3	4	5	6	7	8	9
Sugar added to tea, coffee, cereal *(teaspoon)*	1	2	3	4	5	6	7	8	9
Crisps or other packet snacks e.g. Wotsits *(one packet)*	1	2	3	4	5	6	7	8	9
Peanuts or other nuts *(one packet)*	1	2	3	4	5	6	7	8	9
25. SOUPS, SAUCES AND SPREADS									
Vegetable soups *(bowl)*	1	2	3	4	5	6	7	8	9
Meat soups *(bowl)*	1	2	3	4	5	6	7	8	9
Sauces, e.g. white sauce, cheese sauce, gravy *(medium serving)*	1	2	3	4	5	6	7	8	9
Tomato based sauces, e.g. pasta sauces *(medium serving)*	1	2	3	4	5	6	7	8	9
Tomato ketchup, brown sauce *(tablespoon)*	1	2	3	4	5	6	7	8	9
Relishes, e. g. pickles, chutney, mustard *(tablespoon)*	1	2	3	4	5	6	7	8	9
Low calorie, low fat salad cream or mayonnaise *(tablespoon)*	1	2	3	4	5	6	7	8	9
Salad cream, mayonnaise *(tablespoon)*	1	2	3	4	5	6	7	8	9
French dressing *(tablespoon)*	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
25. SOUPS, SAUCES AND SPREADS (continued)	Never or less than once/ month	1-3 per month	Once A Week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Other salad dressing *(tablespoon)*	1	2	3	4	5	6	7	8	9
Marmite, Bovril *(teaspoon)*	1	2	3	4	5	6	7	8	9
Jam, marmalade, honey, syrup *(teaspoon)*	1	2	3	4	5	6	7	8	9
Peanut butter *(teaspoon)*	1	2	3	4	5	6	7	8	9
Chocolate spread, chocolate nut spread *(teaspoon)*	1	2	3	4	5	6	7	8	9
Dips, e.g. houmous, cheese and chive *(tablespoon)*	1	2	3	4	5	6	7	8	9
26. DRINKS									
Tea *(cup)*	1	2	3	4	5	6	7	8	9
Coffee, instant or ground *(cup)*	1	2	3	4	5	6	7	8	9
Coffee whitener, e.g. Coffee-mate *(teaspoon)*	1	2	3	4	5	6	7	8	9
Cocoa, hot chocolate *(cup)*	1	2	3	4	5	6	7	8	9
Horlicks, Ovaltine *(cup)*	1	2	3	4	5	6	7	8	9
Wine *(glass)*	1	2	3	4	5	6	7	8	9
Beer, lager or cider *(half pint)*	1	2	3	4	5	6	7	8	9
Port, sherry, vermouth, liqueurs *(glass)*	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

Please check that you have a tick on EVERY line

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
26. DRINKS (continued)	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Spirits, e.g. gin, brandy, whisky, vodka *(single)*	1	2	3	4	5	6	7	8	9
Low calorie or diet fizzy soft drinks *(glass)*	1	2	3	4	5	6	7	8	9
Fizzy soft drinks, e.g. Coca cola, lemonade *(glass)*	1	2	3	4	5	6	7	8	9
Pure fruit juice (100%), e.g. orange, apple juice *(glass)*	1	2	3	4	5	6	7	8	9
Fruit squash or cordial *(glass)*	1	2	3	4	5	6	7	8	9
27. FRUIT *(1 fruit or medium serving)* *For very seasonal fruits such as strawberries, please estimate your average use when the fruit is in season									
Apples	1	2	3	4	5	6	7	8	9
Pears	1	2	3	4	5	6	7	8	9
Oranges, satsumas, mandarins, tangerines, clementines	1	2	3	4	5	6	7	8	9
Grapefruit	1	2	3	4	5	6	7	8	9
Bananas	1	2	3	4	5	6	7	8	9
Grapes	1	2	3	4	5	6	7	8	9
Melon	1	2	3	4	5	6	7	8	9
*Peaches, plums, apricots, nectarines	1	2	3	4	5	6	7	8	9
*Strawberries, raspberries, kiwi fruit	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
27. FRUIT (continued) ***(1 fruit or medium serving)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Tinned fruit	1	2	3	4	5	6	7	8	9
Dried fruit, e.g. raisins, prunes, figs	1	2	3	4	5	6	7	8	9
28. VEGETABLES Fresh, frozen or tinned ***(medium serving)***									
Carrots	1	2	3	4	5	6	7	8	9
Spinach	1	2	3	4	5	6	7	8	9
Broccoli	1	2	3	4	5	6	7	8	9
Brussels sprouts	1	2	3	4	5	6	7	8	9
Cabbage	1	2	3	4	5	6	7	8	9
Peas	1	2	3	4	5	6	7	8	9
Green beans, broad beans, runner beans	1	2	3	4	5	6	7	8	9
Marrow, courgettes	1	2	3	4	5	6	7	8	9
Cauliflower	1	2	3	4	5	6	7	8	9
Parsnips, turnips, swedes	1	2	3	4	5	6	7	8	9
Leeks	1	2	3	4	5	6	7	8	9
Onions	1	2	3	4	5	6	7	8	9
Garlic	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day

PLEASE PUT A TICK ON EVERY LINE.

FOODS & AMOUNTS	AVERAGE USE LAST YEAR								
28. VEGETABLES Fresh, frozen or tinned (continued) ***(medium serving)***	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Mushrooms	1	2	3	4	5	6	7	8	9
Sweet peppers	1	2	3	4	5	6	7	8	9
Beansprouts	1	2	3	4	5	6	7	8	9
Green salad, lettuce, cucumber, celery	1	2	3	4	5	6	7	8	9
Mixed vegetables (frozen or tinned)	1	2	3	4	5	6	7	8	9
Watercress	1	2	3	4	5	6	7	8	9
Tomatoes	1	2	3	4	5	6	7	8	9
Sweetcorn	1	2	3	4	5	6	7	8	9
Beetroot, radishes	1	2	3	4	5	6	7	8	9
Coleslaw	1	2	3	4	5	6	7	8	9
Avocado	1	2	3	4	5	6	7	8	9
Baked Beans	1	2	3	4	5	6	7	8	9
Dried lentils, beans, peas	1	2	3	4	5	6	7	8	9
Tofu, soya meat, TVP, Vegeburger	1	2	3	4	5	6	7	8	9
	Never or less than once/ month	1-3 per month	Once a week	2-4 per week	5-6 per week	Once a day	2-3 per day	4-5 per day	6+ per day
Please check that you have a tick on EVERY line									

29. (a) What type of milk did you most often use?

Select one only

Full cream……❒1
Channel Islands……❒2
Dried milk……❒3
Semi-skimmed……❒4
Skimmed……❒5
Soya……❒6
Other……❒7
None……❒8

4/52

29. (b) Approximately, how much milk did you drink each day, including milk with tea, coffee, cereals etc?

None……❒1
Quarter of a pint (roughly 125mls)……❒2
Half a pint (roughly 250mls) ……❒3
Three quarters of a pint (roughly 375mls) ……❒4
One pint (roughly 500mls) ……❒5
More than one pint (more than 500mls) ……❒6

4/53

30. What kind of fat did you most often use for frying, roasting, grilling etc?

Select one only

Butter……❒1
Lard/dripping……❒2
Solid vegetable fat……❒3
Margarine……❒4
Vegetable oil……❒5
Olive oil……❒6
None……❒7

4/54

31. How often did you eat food that was fried at home?

Select one only

Daily……❒1
1-3 times a week……❒2
4-6 times a week……❒3
Less than once a week……❒4
Never……❒5

4/55

32. How often did you eat fried food *away* from home?

Select one only

Daily……❒1
1-3 times a week……❒2
4-6 times a week……❒3
Less than once a week……❒4
Never……❒5

4/56

33. (a) How often did you add salt to food while cooking?
Select one only

Always……❒1
Usually……❒2
Sometimes……❒3
Rarely……❒4
Never……❒5

4/57

33. (b) How often did you add salt to any food at the table?

Select one only

Always……❒1
Usually……❒2
Sometimes……❒3
Rarely……❒4
Never……❒5

4/58

34. Do you follow a special diet?
Please tick all that apply.

No……❒1
Yes, due to a medical condition/allergy……❒2
Yes, to lose weight……❒3
Yes, because of personal beliefs ……❒4
Yes other……❒5

4/63

35. Over the last year, how often have you eaten organic foods?
Select one only.

Most days……❒1
Once or twice a week……❒2
Once a month……❒3
Never/hardly ever……❒4

4/64

36. Have you taken any of the following during the past year?

a) Vitamins (e.g. multivitamins, vitamin B, vitamin C, folic acid)

Yes……❒1
No……❒2

If YES, what type and when _____

4/65

b) Minerals (e.g. iron, calcium, zinc, magnesium)

Yes……❒1
No……❒2

If YES, what type and when _____

4/66

c) Fish oils (e.g. cod liver oil, omega-3)

Yes……❒1
No……❒2

If YES, what type and when _____

4/67

d) Other food supplements (e.g. oil of evening primose, starflower oil, royal jelly, ginseng)

Yes……❒1
No……❒2

If so, what type and when _____

4/68

37. During the course of last year, on average, how many times did you eat the following foods?

Food Type	**Times/week**	**Portion size**	
Vegetables (not including potatoes)		medium serving	5/41
Salads		medium serving	5/43
Fruit and fruit products (not including fruit juice)		medium serving or 1 fruit	5/45
Fish and fish products		medium serving	5/47
Meat, meat products and meat dishes (including bacon, ham and chicken)		medium serving	5/49

38. How often do you eat fruit or vegetables from a garden or allotment?

Select one only.

Most days……❒1
Once or twice a week……❒2
Once a month……❒3
Never/hardly ever……❒4

5/50

39. How would you most commonly eat the following vegetables? If you have not eaten the vegetable listed within the last year, please tick the 'Not eaten ' box.

Select one box only for each vegetable;

	Raw	Boiled < 10mins	Boiled >10mins	Steamed	Fried	Not eaten
Asparagus	❒	❒	❒	❒	❒	❒
Artichoke	❒	❒	❒	❒	❒	❒
Beansprouts	❒	❒	❒	❒	❒	❒
Beetroot	❒	❒	❒	❒	❒	❒
Broad beans	❒	❒	❒	❒	❒	❒
Broccoli	❒	❒	❒	❒	❒	❒
Brussel Sprouts	❒	❒	❒	❒	❒	❒
Cauliflower	❒	❒	❒	❒	❒	❒

	Raw	Boiled < 10mins	Boiled >10mins	Steamed	Fried	Not eaten
Cabbage	❒	❒	❒	❒	❒	❒
Chick Peas	❒	❒	❒	❒	❒	❒
Courgette	❒	❒	❒	❒	❒	❒
Curly Kale	❒	❒	❒	❒	❒	❒
Green Beans	❒	❒	❒	❒	❒	❒
Leeks	❒	❒	❒	❒	❒	❒
Lentils	❒	❒	❒	❒	❒	❒
Lettuce	❒	❒	❒	❒	❒	❒
Mixed veg frozen	❒	❒	❒	❒	❒	❒
Mixed veg canned	❒	❒	❒	❒	❒	❒
Parsnip	❒	❒	❒	❒	❒	❒
Peas	❒	❒	❒	❒	❒	❒
Red Kidney Beans	❒	❒	❒	❒	❒	❒
Runner Beans	❒	❒	❒	❒	❒	❒
Spinach fresh	❒	❒	❒	❒	❒	❒
Spinach frozen	❒	❒	❒	❒	❒	❒
Sweetcorn fresh	❒	❒	❒	❒	❒	❒
Sweetcorn canned	❒	❒	❒	❒	❒	❒

40. For the following foods, please list the top three makes and/or brands you most commonly consume.
If you do not eat this type of food please tick the 'not eaten' box.

Bread: 1:_____

2:_____

3:_____ Not eaten ❒

Breakfast Cereal

1:_____

2:_____

3:_____ Not eaten ❒

About you and your health

41. For your age, would you say that your health was:
Please tick one box on the scale of 1 to 5:

	1	2	3	4	5	
very good	❒	❒	❒	❒	❒	very poor

5/51

42. Which of the following best describes your daily work or other daytime activity that you usually do?
Please tick one box only.

I am usually sitting and do not walk about much……❒1

I stand or walk about quite a lot, but do not have to carry or lift things very often ……❒2

I usually lift or carry light loads or have to climb stairs or hills often ……❒3

I do heavy work or carry heavy loads often ……❒4

5/52

43. Please give the average number of hours per week you spend doing sports and other activities.

Please write in the amount for each; if none write "0"

a) Mildly energetic

(e.g. walking, gardening, playing darts, general housework) _____ hour/s

b) Moderately energetic

(e.g. heavy housework or gardening, dancing, golf, cycling, leisurely swimming) _____ hour/s

c) Vigorous

(e.g. running, competitive swimming or cycling, tennis, football, squash, aerobics) _____ hour/s

5/64

44. Do you smoke?

Yes, I smoke daily .....❒1
Yes, I smoke occasionally .....❒2
No, I used to smoke .....❒3
No, I have never smoked .....❒4

5/65

44. If yes or you used to smoke, how much, on average, do you (or did you) smoke a day?
Please write in the amount for each; if none write "0"

Cigarettes _____
Cigars _____
Ounces tobacco _____

5/74

45. In the past 12 months have you taken an alcoholic drink:
Please tick one box.

Twice a day or more .....❒1
Almost daily .....❒2
Once or twice a week .....❒3
Once or twice a month .....❒4
Special occasions only .....❒5
Not at all .....❒6

5/75

46. In a typical 7-day week, including the weekend, how many standard drinks of alcohol do you drink? *(see the table below)*
Please write the number in the box below.

I usually drink ____ standard drinks of alcohol per week

5/76

ONE STANDARD DRINK = ½ pint of beer or ½ pint cider or ½ pint lager or 1 glass of wine, martini, or cinzano or 1 small glass of Sherry or Port or 1 measure of Spirits (gin, whiskey, vodka etc.) or 1 measure liquor **A PINT OF BEER, CIDER, OR LAGER COUNTS AS TWO STANDARD DRINKS** 1 A DOUBLE MEASURE OF SPIRITS COUNTS AS TWO STANDARD DRINKS

47. (a) Do you have any long-term illness, physical or mental health problem or handicap?

Yes……❒1
No……❒2

5/91

48. (b) If yes, does this limit your daily activity in any way?

Yes……❒1
No……❒2

5/92

THANK YOU FOR TAKING TIME TO COMPLETE THIS QUESTIONNAIRE

Appendix F. DISC Study lifestyle questionnaire

Life style Questionnaire

Name:_____

Date:_____

Thank you for agreeing to take part in this study. This is a simple life style questionnaire to give us an idea of your physical activity. It is not a test, so there are no right or wrong answers.

All information will be treated in the strictest confidence.

Thank you for your time

Please turn over to complete the questionnaire.

1. We would like to know the type and amount of physical activity involved in your work. Please tick what best corresponds to your present activities from the following four possibilities:

- Sedentary occupation _____

You spend most of your time sitting (such as an office)

- Or Standing occupation _____

You spend most of your time standing or walking. However, your work does not require intense physical effort (e.g. shop assistant, hairdresser, guard etc.)

- Or Physical work _____

The work involves some physical effort including handling of heavy objects and use of tools (e.g. plumber, cleaner, nurse, instructor, electrician, carpenter etc.)

- Or Heavy Manual work _____

This involves very vigorous physical activity including handling of very heavy objects (e.g. docker, miner, bricklayer, construction worker, etc.)

2. In a typical week, during the past 12 months, how many hours did you spend on each of the following activities? (Put 0 if none)

- Walking, including walking to work, shopping and leisure

In summer _____ hours per week

In winter _____ hours per week

- Cycling, including cycling to work and during leisure time

In summer _____ hours per week

In winter _____ hours per week

- Gardening

In summer _____ hours per week

In winter _____ hours per week

- Housework such as cleaning, washing, cooking, childcare

_____ hours per week

- Do-it-yourself

  _____ hours per week

- Other physical exercise such as keep fit, aerobics, swimming, jogging

In summer _____ hours per week

In winter _____ hours per week

3. In a typical week during the past year did you practise any of the above activities vigorously enough to cause sweating or a faster heartbeat?

Yes ___ No ___ Don't know ____

- If yes, for how many hours per week in total did you practise such vigorous physical activity? (Put 0 if none)

  _____hours per week

4. In a typical day during the past 12 months, how many floors of stairs did you climb? (Put '0' if none)

_____ floors per day

Appendix G. Example of results from ANOVA GLM analysis

General Linear Model: AXIN2 POST versus National Starch, Danisco

```
Factor           Type   Levels  Values
National Starch  fixed       2  0, 1
Danisco          fixed       2  0, 1

Analysis of Variance for AXIN2 POST, using Adjusted SS for Tests

Source                   DF   Seq SS   Adj SS   Adj MS      F      P
AXIN2 PRE                 1  12.6233  11.1931  11.1931  11.71  0.001
Age                       1   0.1863   0.0001   0.0001   0.00  0.990
Procedure code            1   0.2551   0.1275   0.1275   0.13  0.716
Gender code               1   0.1923   0.0166   0.0166   0.02  0.896
Smoking code              1   0.8134   0.4350   0.4350   0.46  0.503
Pre BMI                   1   0.7768   1.0708   1.0708   1.12  0.294
National Starch           1   1.3843   1.4046   1.4046   1.47  0.230
Danisco                   1   0.1274   0.1622   0.1622   0.17  0.682
National Starch*Danisco   1   1.6936   1.6936   1.6936   1.77  0.188
Error                    56  53.5112  53.5112   0.9556
Total                    65  71.5638
S = 0.977526   R-Sq = 25.23%   R-Sq(adj) = 13.21%

Term             Coef  SE Coef      T      P
Constant        0.858    1.127   0.76  0.450
AXIN2 PRE     0.24165  0.07061   3.42  0.001
Age           0.00015  0.01192   0.01  0.990
Procedure co   0.1000   0.2739   0.37  0.716
Gender code    0.0343   0.2605   0.13  0.896
Smoking code  -0.1068   0.1583  -0.67  0.503
Pre BMI       0.02644  0.02498   1.06  0.294

Unusual Observations for AXIN2 POST
Obs  AXIN2 POST      Fit   SE Fit  Residual  St Resid
 36     5.38027  3.70281  0.51278   1.67746      2.02 R
 38     4.77401  2.22279  0.39065   2.55123      2.85 R
 42     0.79307  2.71488  0.47059  -1.92182     -2.24 R
R denotes an observation with a large standardized residual.

Means for Covariates
Covariate          Mean    StDev  Covariate        Mean   StDev
AXIN2 PRE        2.9273   1.8312  Gender code    0.5000  0.5038
Age             51.3333  11.4789  Smoking code   0.7727  0.8189
Procedure code   0.7121   0.4562  Pre BMI       29.7700  5.1639

Least Squares Means for AXIN2 POST
National Sta           Mean  SE Mean
0                     2.514   0.1747
1                     2.217   0.1691
Danisco
0                     2.313   0.1719
1                     2.418   0.1775
National Sta*Danisco
0            0        2.635   0.2428
0            1        2.394   0.2560
1            0        1.992   0.2525
1            1        2.442   0.2480

Grouping Information Using Bonferroni Method and 95.0% Confidence
National
Starch     N  Mean  Grouping
0         32   2.5  A
1         34   2.2  A
Means that do not share a letter are significantly different.
```

Grouping Information Using Bonferroni Method and 95.0% Confidence

Danisco	N	Mean	Grouping
1	32	2.4	A
0	34	2.3	A

Means that do not share a letter are significantly different.

Grouping Information Using Bonferroni Method and 95.0% Confidence

National Starch	Danisco	N	Mean	Grouping
0	0	17	2.6	A
1	1	17	2.4	A
0	1	15	2.4	A
1	0	17	2.0	A

Means that do not share a letter are significantly different.

Appendix H. Evidence from the literature identifying miRNAs modified by butyrate treatment and whose expression is altered in CRC and that may target *SFRP1*

miRNA	Effect of butyrate on expression	References	Expression change in CRC	References	Evidence that miRNA targets *SFRP1*	References
miR-7	↓	*(Hu et al., 2011)*	↑ in stool of CRC patients	(Ahmed *et al.*, 2013)	Significant inverse correlation	(Fu *et al.*, 2012)
			↓ in CRC tumours	(Zhang *et al.*, 2013)		
			↓ in plasma of CRC patients	(Wang *et al.*, 2013d)		
			↑ in adenocarcinoma cases	(Hamfjord *et al.*, 2012)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		

miR-17	↓	(Hu *et al.*, 2011) (Humphreys *et al.*, 2013)	↑ in CRC tissue/cells	(Ma *et al.*, 2012), (Earle *et al.*, 2010), (Diosdado *et al.*, 2009)	Significant inverse correlation	(Fu *et al.*, 2012)
			↑ in colon cancer	(Tsuchida *et al.*, 2011)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
			↑ in rectal cancer	(Gaedcke *et al.*, 2012)		
miR-18a	↓	(Hu *et al.*, 2011) (Humphreys *et al.*, 2013)	↑ in plasma of CRC patients	(Luo *et al.*, 2013)		
			↑ in CRC tissue/cells	(Arndt *et al.*, 2009), (Diosdado *et al.*, 2009), (Motoyama *et al.*, 2009)		
			↑ in colon cancer	(Tsuchida *et al.*, 2011), (Wang *et al.*, 2010)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
			↑ in rectal cancer	(Gaedcke *et al.*, 2012)		

miR-18b	↓	(Hu *et al.*, 2011) (Humphreys *et al.*, 2013)	↑ in colon cancer	(Wang *et al.*, 2010)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
miR-19a	↓	(Hu *et al.*, 2011) (Humphreys *et al.*, 2013)	↑ in colon cancer	(Wang *et al.*, 2010), (Zhang *et al.*, 2012), (Tsuchida *et al.*, 2011)	Significant inverse correlation	(Fu *et al.*, 2012)
			↑ in CRC tissue/cells	(Diosdado *et al.*, 2009), (Monzo *et al.*, 2008), (Bandres *et al.*, 2006)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
miR-19b	↓	(Hu *et al.*, 2011) (Humphreys *et al.*, 2013)	↑ in colon cancer	(Tsuchida *et al.*, 2011)	Significant inverse correlation	(Fu *et al.*, 2012)

miR-20a	↓	(Hu *et al.*, 2011) (Humphreys *et al.*, 2013)	↑ in plasma of CRC patients	(Luo *et al.*, 2011), (Luo *et al.*, 2013)	Significant inverse correlation	(Fu *et al.*, 2012)
			↑ in CRC tissue	(Ma *et al.*, 2011), (Motoyama *et al.*, 2009), (Earle *et al.*, 2010), (Arndt *et al.*, 2009), (Diosdado *et al.*, 2009)		
			↑ in colon cancer	(Schetter *et al.*, 2008), (Volinia *et al.*, 2006), (Schepeler *et al.*, 2008), (Tsuchida *et al.*, 2011)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
miR-20a*	↓	(Hu *et al.*, 2011)	↑ in colon cancer	(Wang *et al.*, 2010)		

miR-20b	↓	(Hu *et al.*, 2011) (Humphreys *et al.*, 2013)	↓ in colon cancer	(Sarver *et al.*, 2009)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012),		
miR-25	↓	(Hu *et al.*, 2011)	↑ in CRC tissue/cells	(Earle *et al.*, 2010), (Arndt *et al.*, 2009), (Monzo *et al.*, 2008)	Significant inverse correlation	(Fu *et al.*, 2012)
			↓ in colon cancer	(Li *et al.*, 2013)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
miR-29a	↓	(Hu *et al.*, 2011)	↑ in plasma of CRC patients	(Luo *et al.*, 2013), (Huang *et al.*, 2010)	Targets *SFRP1* (validated)	(Fu *et al.*, 2012)
			↑ in CRC tissue/cells	(Arndt *et al.*, 2009), (Monzo *et al.*, 2008)		

miR-34a	↓	(Hu *et al.*, 2011)	↑ in CRC tissue/cells	(Arndt *et al.*, 2009), (Monzo *et al.*, 2008), (Nugent *et al.*, 2012), (Wang *et al.*, 2012)		
			↓ in colon cancer tissue/cells	(Roy *et al.*, 2012)		
miR-92a	↓	(Hu *et al.*, 2011) (Humphreys *et al.*, 2013)	↑ in plasma of CRC patients	(Luo *et al.*, 2013) ,(Huang *et al.*, 2010)		
			↑ CRC tissue/cells	*(Yamada et al., 2013a)*		
			↑ in colon cancer	(Tsuchida *et al.*, 2011), (Ma *et al.*, 2011)		
			↑ in plasma of CRC patients	(Huang *et al.*, 2010)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		

miR-93	↓	(Hu *et al.*, 2011)	↑ in CRC tissue	(Ma *et al.*, 2011), (Earle *et al.*, 2010) ,(Arndt *et al.*, 2009)	Significant inverse correlation	(Fu *et al.*, 2012)
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
			↓ in colon cancer	(Xiao *et al.*, 2013)		
miR-95	↑	(Hu *et al.*, 2011)	↑ in CRC tissue/cells	(Arndt *et al.*, 2009), (Monzo *et al.*, 2008), (Huang *et al.*, 2011)	Significant inverse correlation	(Fu *et al.*, 2012)
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
miR-96	↑	(Hu *et al.*, 2011)	↑ in colon cancer	(Sarver *et al.*, 2009),		
			↑ in CRC tissue/cells	(Arndt *et al.*, 2009), (Monzo *et al.*, 2008), (Xu *et al.*, 2012), (Bandres *et al.*, 2006), (Hamfjord *et al.*, 2012)		

miR-106a	↓	(Hu *et al.*, 2011) (Humphreys *et al.*, 2013)	↑ in CRC tissue/cells	(Ma *et al.*, 2011), (Monzo *et al.*, 2008)		
			↑ in colonic tumours	(Schetter *et al.*, 2008), (Volinia *et al.*, 2006)		
			↑ in stool of CRC patients	(Link *et al.*, 2010)		
miR-106b	↓	(Hu *et al.*, 2011)	↑ in plasma of CRC patients	(Luo *et al.*, 2013)		
			↑ in colon cancer	(Wang *et al.*, 2010)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		

miR-145	↓	(La Rocca *et al.*, 2011)	↑ in plasma of CRC patients	(Luo *et al.*, 2013),	Significant inverse correlation	(Fu *et al.*, 2012)
			↓ in CRC tissue/cells	(Wang *et al.*, 2009a), (Motoyama *et al.*, 2009), (Earle *et al.*, 2010), (Arndt *et al.*, 2009), (Slaby *et al.*, 2007), (Bandres *et al.*, 2006), (Hamfjord *et al.*, 2012), (Luo *et al.*, 2011), (Ma *et al.*, 2011) (Chen *et al.*, 2009),	
			↓ in colon cancer	(Schepeler *et al.*, 2008)		
			↓ in rectal cancer	(Gaedcke *et al.*, 2012)		

miR-183	↑	(Hu *et al.*, 2011)	↑ in CRC tissue/cells	(Motoyama *et al.*, 2009), (Earle *et al.*, 2010), (Arndt *et al.*, 2009), (Bandres *et al.*, 2006),		
			↑ in colon cancer	(Sarver *et al.*, 2009)		
			↑ in rectal cancer	(Li *et al.*, 2012)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
miR-194	↑	(Hu *et al.*, 2011)	↑ in CRC tissue/cells	(Monzo *et al.*, 2008)		
			↓ in CRC tissue/cells	(Chiang *et al.*, 2012), (Gaedcke *et al.*, 2012)		
miR-196b	↓	(Hu *et al.*, 2011)	↑ in colon cancer	(Wang *et al.*, 2010)		

miR-215	↑	(Hu *et al.*, 2011)	↓ in CRC tissue/cells	(Earle *et al.*, 2010), (Faltejskova *et al.*, 2012)		
			↑ in CRC tissue/cells	(Monzo *et al.*, 2008)		
			↓ in rectal cancer	(Gaedcke *et al.*, 2012), (Chiang *et al.*, 2012)		
miR-221	↓	(Hu *et al.*, 2011)	↑ in CRC tissue/cells	(Pu *et al.*, 2010), (Wang *et al.*, 2011b)		
			↑ in colonic tumours	(Volinia *et al.*, 2006)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
miR-222	↓	(Hu *et al.*, 2011)	↑ in CRC tissue/cells	(Monzo *et al.*, 2008)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
miR-300	↑	(Hu *et al.*, 2011)	↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
miR-381	↑	*(Hu et al., 2011)*	↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		

miR-424	↑	(Hu *et al.*, 2011)	↑ in colon cancer	(Wang *et al.*, 2010)		
			↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		
			↑ in CRC tissue/cells	(Wang *et al.*, 2013c)		
miR-487b	↑	(Hu *et al.*, 2011)	↑ in CRC stromal tissue	(Nishida *et al.*, 2012)	Significant inverse correlation	(Fu *et al.*, 2012)
miR-492	↑	(Hu *et al.*, 2011)	↑ in rectal cancer	(Gaedcke *et al.*, 2012)		
miR-874	↑	(Hu *et al.*, 2011)	↑ in CRC stromal tissue	(Nishida *et al.*, 2012)		

Bibliography

Abbasi, A.M., Chester, K.A., Talbot, I.C., Macpherson, A.S., Boxer, G., Forbes, A., Malcolm, A.D. and Begent, R.H. (1993) 'CD44 is associated with proliferation in normal and neoplastic human colorectal epithelial cells', *Eur J Cancer*, 29A(14), pp. 1995-2002.

Aguilera, O., Fraga, M.F., Ballestar, E., Paz, M.F., Herranz, M., Espada, J., Garcia, J.M., Munoz, A., Esteller, M. and Gonzalez-Sancho, J.M. (2006) 'Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer', *Oncogene*, 25(29), pp. 4116-21.

Ahmed, F.E., Ahmed, N.C., Vos, P.W., Bonnerup, C., Atkins, J.N., Casey, M., Nuovo, G.J., Naziri, W., Wiley, J.E., Mota, H. and Allison, R.R. (2013) 'Diagnostic microRNA markers to screen for sporadic human colon cancer in stool: I. Proof of principle', *Cancer Genomics Proteomics*, 10(3), pp. 93-113.

Ahuja, N., Li, Q., Mohan, A.L., Baylin, S.B. and Issa, J.P. (1998) 'Aging and DNA methylation in colorectal mucosa and cancer', *Cancer Res*, 58(23), pp. 5489-94.

Akiyama, Y., Watkins, N., Suzuki, H., Jair, K.W., van Engeland, M., Esteller, M., Sakai, H., Ren, C.Y., Yuasa, Y., Herman, J.G. and Baylin, S.B. (2003) 'GATA-4 and GATA-5 transcription factor genes and potential downstream antitumor target genes are epigenetically silenced in colorectal and gastric cancer', *Mol Cell Biol*, 23(23), pp. 8429-39.

An, B., Kondo, Y., Okamoto, Y., Shinjo, K., Kanemitsu, Y., Komori, K., Hirai, T., Sawaki, A., Tajika, M., Nakamura, T., Yamao, K., Yatabe, Y., Fujii, M., Murakami, H., Osada, H., Tani, T., Matsuo, K., Shen, L., Issa, J.P. and Sekido, Y. (2010) 'Characteristic methylation profile in CpG island methylator phenotype-negative distal colorectal cancers', *Int J Cancer*, 127(9), pp. 2095-105.

Andersen, N.N. and Jess, T. (2013) 'Has the risk of colorectal cancer in inflammatory bowel disease decreased?', *World J Gastroenterol*, 19(43), pp. 7561-8.

Anderson, C.B., Neufeld, K.L. and White, R.L. (2002) 'Subcellular distribution of Wnt pathway proteins in normal and neoplastic colon', *Proc Natl Acad Sci U S A*, 99(13), pp. 8683-8.

Arndt, G.M., Dossey, L., Cullen, L.M., Lai, A., Druker, R., Eisbacher, M., Zhang, C., Tran, N., Fan, H., Retzlaff, K., Bittner, A. and Raponi, M. (2009) 'Characterization of global microRNA expression reveals oncogenic potential of miR-145 in metastatic colorectal cancer', *BMC Cancer*, 9, p. 374.

Astbury, N.M., Taylor, M.A. and Macdonald, I.A. (2013) 'Polydextrose results in a dose-dependent reduction in ad libitum energy intake at a subsequent test meal', *Br J Nutr*, 110(5), pp. 934-42.

Aune, D., Chan, D.S., Lau, R., Vieira, R., Greenwood, D.C., Kampman, E. and Norat, T. (2011) 'Dietary fibre, whole grains, and risk of colorectal cancer: systematic review and dose-response meta-analysis of prospective studies', *BMJ*, 343, p. d6617.

Aust, D.E., Terdiman, J.P., Willenbucher, R.F., Chew, K., Ferrell, L., Florendo, C., Molinaro-Clark, A., Baretton, G.B., Lohrs, U. and Waldman, F.M. (2001) 'Altered distribution of beta-catenin, and its binding proteins E-cadherin and APC, in ulcerative colitis-related colorectal cancers', *Mod Pathol*, 14(1), pp. 29-39.

Bafico, A., Gazit, A., Pramila, T., Finch, P.W., Yaniv, A. and Aaronson, S.A. (1999) 'Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling', *J Biol Chem*, 274(23), pp. 16180-7.

Baghurst, P.A., Baghurst, K.I. and Record, S.J. (1996) 'Dietary fibre, non-starch polysaccharides and resistant starch- a review.', *Food Aust*, 48((Suppl)), pp. S3-S35.

Bandres, E., Cubedo, E., Agirre, X., Malumbres, R., Zarate, R., Ramirez, N., Abajo, A., Navarro, A., Moreno, I., Monzo, M. and Garcia-Foncillas, J. (2006) 'Identification by Real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues', *Mol Cancer*, 5, p. 29.

Bandzar, S., Gupta, S. and Platt, M.O. (2013) 'Crohn's disease: a review of treatment options and current research', *Cell Immunol*, 286(1-2), pp. 45-52.

Bannister, A.J. and Kouzarides, T. (2011) 'Regulation of chromatin by histone modifications', *Cell Res*, 21(3), pp. 381-95.

Bariol, C., Suter, C., Cheong, K., Ku, S.L., Meagher, A., Hawkins, N. and Ward, R. (2003) 'The relationship between hypomethylation and CpG island methylation in colorectal neoplasia', *Am J Pathol*, 162(4), pp. 1361-71.

Bassaganya-Riera, J., DiGuardo, M., Viladomiu, M., de Horna, A., Sanchez, S., Einerhand, A.W., Sanders, L. and Hontecillas, R. (2011) 'Soluble fibers and resistant starch ameliorate disease activity in interleukin-10-deficient mice with inflammatory bowel disease', *J Nutr*, 141(7), pp. 1318-25.

Bauer-Marinovic, M., Florian, S., Muller-Schmehl, K., Glatt, H. and Jacobasch, G. (2006) 'Dietary resistant starch type 3 prevents tumor induction by 1,2-dimethylhydrazine and alters proliferation, apoptosis and dedifferentiation in rat colon', *Carcinogenesis*, 27(9), pp. 1849-59.

Baylin, S.B. and Herman, J.G. (2000) 'DNA hypermethylation in tumorigenesis: epigenetics joins genetics', *Trends Genet*, 16(4), pp. 168-74.

Beards, E., Tuohy, K. and Gibson, G. (2010) 'A human volunteer study to assess the impact of confectionery sweeteners on the gut microbiota composition', *Br J Nutr*, 104(5), pp. 701-8.

Bedi, A., Pasricha, P.J., Akhtar, A.J., Barber, J.P., Bedi, G.C., Giardiello, F.M., Zehnbauer, B.A., Hamilton, S.R. and Jones, R.J. (1995) 'Inhibition of apoptosis during development of colorectal cancer', *Cancer Res*, 55(9), pp. 1811-6.

Belshaw, N.J., Elliott, G.O., Foxall, R.J., Dainty, J.R., Pal, N., Coupe, A., Garg, D., Bradburn, D.M., Mathers, J.C. and Johnson, I.T. (2008) 'Profiling CpG island field methylation in both morphologically normal and neoplastic human colonic mucosa', *Br J Cancer*, 99(1), pp. 136-42.

Ben, Q., Sun, Y., Chai, R., Qian, A., Xu, B. and Yuan, Y. (2014) 'Dietary fiber intake reduces risk for colorectal adenoma: a meta-analysis', *Gastroenterology*, 146(3), pp. 689-699 e6.

Bentwich, I., Avniel, A., Karov, Y., Aharonov, R., Gilad, S., Barad, O., Barzilai, A., Einat, P., Einav, U., Meiri, E., Sharon, E., Spector, Y. and Bentwich, Z. (2005) 'Identification of hundreds of conserved and nonconserved human microRNAs', *Nat Genet*, 37(7), pp. 766-70.

Berni Canani, R., Di Costanzo, M. and Leone, L. (2012) 'The epigenetic effects of butyrate: potential therapeutic implications for clinical practice', *Clin Epigenetics*, 4(1), p. 4.

Bian, Z., Li, L., Cui, J., Zhang, H., Liu, Y., Zhang, C.Y. and Zen, K. (2011) 'Role of miR-150-targeting c-Myb in colonic epithelial disruption during dextran sulphate sodium-induced murine experimental colitis and human ulcerative colitis', *J Pathol*, 225(4), pp. 544-53.

Biasco, G., Paganelli, G.M., Miglioli, M., Brillanti, S., Di Febo, G., Gizzi, G., Ponz de Leon, M., Campieri, M. and Barbara, L. (1990) 'Rectal cell proliferation and colon cancer risk in ulcerative colitis', *Cancer Res*, 50(4), pp. 1156-9.

Biden, K.G., Simms, L.A., Cummings, M., Buttenshaw, R., Schoch, E., Searle, J., Gobe, G., Jass, J.R., Meltzer, S.J., Leggett, B.A. and Young, J. (1999) 'Expression of Bcl-2 protein is decreased in colorectal adenocarcinomas with microsatellite instability', *Oncogene*, 18(5), pp. 1245-9.

Bienz, M. and Clevers, H. (2000) 'Linking colorectal cancer to Wnt signaling', *Cell*, 103(2), pp. 311-20.

Bingham, S.A., Day, N.E., Luben, R., Ferrari, P., Slimani, N., Norat, T., Clavel-Chapelon, F., Kesse, E., Nieters, A., Boeing, H., Tjonneland, A., Overvad, K., Martinez, C., Dorronsoro, M., Gonzalez, C.A., Key, T.J., Trichopoulou, A., Naska, A., Vineis, P., Tumino, R., Krogh, V., Bueno-de-Mesquita, H.B., Peeters, P.H., Berglund, G., Hallmans, G., Lund, E., Skeie, G., Kaaks, R. and Riboli, E. (2003) 'Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study', *Lancet*, 361(9368), pp. 1496-501.

Bird, A. (2002) 'DNA methylation patterns and epigenetic memory', *Genes Dev*, 16(1), pp. 6-21.

Birkett, A., Muir, J., Phillips, J., Jones, G. and O'Dea, K. (1996) 'Resistant starch lowers fecal concentrations of ammonia and phenols in humans', *Am J Clin Nutr*, 63(5), pp. 766-72.

Bisgaard, M.L., Fenger, K., Bulow, S., Niebuhr, E. and Mohr, J. (1994) 'Familial adenomatous polyposis (FAP): frequency, penetrance, and mutation rate', *Hum Mutat*, 3(2), pp. 121-5.

Bodinham, C.L., Frost, G.S. and Robertson, M.D. (2010) 'Acute ingestion of resistant starch reduces food intake in healthy adults', *Br J Nutr*, 103(6), pp. 917-22.

Bogaert, J. and Prenen, H. (2014) 'Molecular genetics of colorectal cancer', *Ann Gastroenterol*, 27(1), pp. 9-14.

Boler, B.M., Serao, M.C., Bauer, L.L., Staeger, M.A., Boileau, T.W., Swanson, K.S. and Fahey, G.C., Jr. (2011) 'Digestive physiological outcomes related to polydextrose and soluble maize fibre consumption by healthy adult men', *Br J Nutr*, 106(12), pp. 1864-71.

Bordonaro, M., Lazarova, D.L., Augenlicht, L.H. and Sartorelli, A.C. (2002) 'Cell type- and promoter-dependent modulation of the Wnt signaling pathway by sodium butyrate', *Int J Cancer*, 97(1), pp. 42-51.

Bordonaro, M., Lazarova, D.L. and Sartorelli, A.C. (2007) 'The activation of beta-catenin by Wnt signaling mediates the effects of histone deacetylase inhibitors', *Exp Cell Res*, 313(8), pp. 1652-66.

Bordonaro, M., Lazarova, D.L. and Sartorelli, A.C. (2008a) 'Butyrate and Wnt signaling: a possible solution to the puzzle of dietary fiber and colon cancer risk?', *Cell Cycle*, 7(9), pp. 1178-83.

Bordonaro, M., Lazarova, D.L. and Sartorelli, A.C. (2008b) 'Hyperinduction of Wnt activity: a new paradigm for the treatment of colorectal cancer?', *Oncol Res*, 17(1), pp. 1-9.

Bordonaro, M., Mariadason, J.M., Aslam, F., Heerdt, B.G. and Augenlicht, L.H. (1999) 'Butyrate-induced apoptotic cascade in colonic carcinoma cells: modulation of the beta-catenin-Tcf pathway and concordance with effects of sulindac and trichostatin A but not curcumin', *Cell Growth Differ*, 10(10), pp. 713-20.

Bordonaro, M., Tewari, S., Cicco, C.E., Atamna, W. and Lazarova, D.L. (2011) 'A switch from canonical to noncanonical Wnt signaling mediates drug resistance in colon cancer cells', *PLoS One*, 6(11), p. e27308.

Bosari, S., Moneghini, L., Graziani, D., Lee, A.K., Murray, J.J., Coggi, G. and Viale, G. (1995) 'bcl-2 oncoprotein in colorectal hyperplastic polyps, adenomas, and adenocarcinomas', *Hum Pathol*, 26(5), pp. 534-40.

Bostick, R.M., Fosdick, L., Grandits, G.A., Lillemoe, T.J., Wood, J.R., Grambsch, P., Louis, T.A. and Potter, J.D. (1997) 'Colorectal epithelial cell proliferative kinetics and risk factors for colon cancer in sporadic adenoma patients', *Cancer Epidemiol Biomarkers Prev*, 6(12), pp. 1011-9.

Bouhnik, Y., Raskine, L., Simoneau, G., Vicaut, E., Neut, C., Flourie, B., Brouns, F. and Bornet, F.R. (2004) 'The capacity of nondigestible carbohydrates to stimulate fecal bifidobacteria in healthy humans: a double-blind, randomized, placebo-controlled, parallel-group, dose-response relation study', *Am J Clin Nutr*, 80(6), pp. 1658-64.

Bradburn, D.M., Mathers, J.C., Gunn, A., Burn, J., Chapman, P.D. and Johnston, I.D. (1993) 'Colonic fermentation of complex carbohydrates in patients with familial adenomatous polyposis', *Gut*, 34(5), pp. 630-6.

Brittan, M. and Wright, N.A. (2004) 'Stem cell in gastrointestinal structure and neoplastic development', *Gut*, 53(6), pp. 899-910.

Bronner, M.P., Culin, C., Reed, J.C. and Furth, E.E. (1995) 'The bcl-2 proto-oncogene and the gastrointestinal epithelial tumor progression model', *Am J Pathol*, 146(1), pp. 20-6.

Brown, I., Warhurst, M., Arcot, J., Playne, M., Illman, R.J. and Topping, D.L. (1997) 'Fecal numbers of bifidobacteria are higher in pigs fed Bifidobacterium longum with a high amylose cornstarch than with a low amylose cornstarch', *J Nutr*, 127(9), pp. 1822-7.

Brunt, K.R., Zhang, Y., Mihic, A., Li, M., Li, S.H., Xue, P., Zhang, W., Basmaji, S., Tsang, K., Weisel, R.D., Yau, T.M. and Li, R.K. (2012) 'Role of WNT/beta-catenin signaling in rejuvenating myogenic differentiation of aged mesenchymal stem cells from cardiac patients', *Am J Pathol*, 181(6), pp. 2067-78.

Buczynski, J., Spychalski, M., Lawska-Wierzchniewska, A. and Dziki, A. (2012) 'Higher apoptosis index and proliferation index in colonocytes of patients with ulcerative colitis in remission', *Pol Przegl Chir*, 84(6), pp. 271-5.

Burkitt, D.P. (1971) 'Possible relationships between bowel cancer and dietary habits', *Proc R Soc Med*, 64(9), pp. 964-5.

Burmer, G.C., Rabinovitch, P.S., Haggitt, R.C., Crispin, D.A., Brentnall, T.A., Kolli, V.R., Stevens, A.C. and Rubin, C.E. (1992) 'Neoplastic progression in ulcerative colitis: histology, DNA content, and loss of a p53 allele', *Gastroenterology*, 103(5), pp. 1602-10.

Burn, J., Bishop, D.T., Chapman, P.D., Elliott, F., Bertario, L., Dunlop, M.G., Eccles, D., Ellis, A., Evans, D.G., Fodde, R., Maher, E.R., Moslein, G., Vasen, H.F., Coaker, J., Phillips, R.K., Bulow, S. and Mathers, J.C. (2011a) 'A randomized placebo-controlled prevention trial of aspirin and/or resistant starch in young people with familial adenomatous polyposis', *Cancer Prev Res (Phila)*, 4(5), pp. 655-65.

Burn, J., Bishop, D.T., Mecklin, J.P., Macrae, F., Moslein, G., Olschwang, S., Bisgaard, M.L., Ramesar, R., Eccles, D., Maher, E.R., Bertario, L., Jarvinen, H.J., Lindblom, A., Evans, D.G., Lubinski, J., Morrison, P.J., Ho, J.W., Vasen, H.F., Side, L., Thomas, H.J., Scott, R.J., Dunlop, M., Barker, G., Elliott, F., Jass, J.R., Fodde, R., Lynch, H.T. and Mathers, J.C. (2008) 'Effect of aspirin or resistant starch on colorectal neoplasia in the Lynch syndrome', *N Engl J Med*, 359(24), pp. 2567-78.

Burn, J., Gerdes, A.M., Macrae, F., Mecklin, J.P., Moeslein, G., Olschwang, S., Eccles, D., Evans, D.G., Maher, E.R., Bertario, L., Bisgaard, M.L., Dunlop, M.G., Ho, J.W., Hodgson, S.V., Lindblom, A., Lubinski, J., Morrison, P.J., Murday, V., Ramesar, R., Side, L., Scott, R.J., Thomas, H.J., Vasen, H.F., Barker, G., Crawford, G., Elliott, F., Movahedi, M., Pylvanainen, K., Wijnen, J.T., Fodde, R., Lynch, H.T., Mathers, J.C., Bishop, D.T. and Investigators, C. (2011b) 'Long-term effect of aspirin on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial', *Lancet*, 378(9809), pp. 2081-7.

Bushati, N. and Cohen, S.M. (2007) 'microRNA functions', *Annu Rev Cell Dev Biol*, 23, pp. 175-205.

Bustin, S.A. (2000) 'Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays', *J Mol Endocrinol*, 25(2), pp. 169-93.

CAC (2009) 'Codex Alimentarius Commission; Food and Agriculture Organization; World Health Organization. Report of the 30th session of the Codex Commitee on nutrition and foods for special dietary uses. http://www.codexalimentarius.net/download/report/710/al32_26e.pdf (accessed August 2014)'.

Caldwell, G.M., Jones, C., Gensberg, K., Jan, S., Hardy, R.G., Byrd, P., Chughtai, S., Wallis, Y., Matthews, G.M. and Morton, D.G. (2004) 'The Wnt antagonist sFRP1 in colorectal tumorigenesis', *Cancer Res*, 64(3), pp. 883-8.

Caldwell, G.M., Jones, C.E., Ashley, A.M., Wei, W., Hejmadi, R.K., Morton, D.G. and Matthews, G.M. (2010) 'Wnt signalling in adenomas of familial adenomatous polyposis patients', *Br J Cancer*, 103(6), pp. 910-7.

Caldwell, G.M., Jones, C.E., Taniere, P., Warrack, R., Soon, Y., Matthews, G.M. and Morton, D.G. (2006) 'The Wnt antagonist sFRP1 is downregulated in premalignant large bowel adenomas', *Br J Cancer*, 94(6), pp. 922-7.

Cao, Y.C., Yang, F., Liu, X.H., Xin, X., Wang, C.C. and Geng, M. (2012) '[Expression of Wnt5a, APC, beta-catenin and their clinical significance in human colorectal adenocarcinoma]', *Zhonghua Zhong Liu Za Zhi*, 34(9), pp. 674-8.

Cassand, P., Maziere, S., Champ, M., Meflah, K., Bornet, F. and Narbonne, J.F. (1997) 'Effects of resistant starch- and vitamin A-supplemented diets on the promotion of precursor lesions of colon cancer in rats', *Nutr Cancer*, 27(1), pp. 53-9.

Chalitchagorn, K., Shuangshoti, S., Hourpai, N., Kongruttanachok, N., Tangkijvanich, P., Thong-ngam, D., Voravud, N., Sriuranpong, V. and Mutirangura, A. (2004) 'Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis', *Oncogene*, 23(54), pp. 8841-6.

Chen, B., She, S., Li, D., Liu, Z., Yang, X., Zeng, Z. and Liu, F. (2013) 'Role of miR-19a targeting TNF-alpha in mediating ulcerative colitis', *Scand J Gastroenterol*, 48(7), pp. 815-24.

Chen, K. and Rajewsky, N. (2007) 'The evolution of gene regulation by transcription factors and microRNAs', *Nat Rev Genet*, 8(2), pp. 93-103.

Chen, L.H., Song, J.L., Qian, Y., Zhao, X., Suo, H.Y. and Li, J. (2014a) 'Increased preventive effect on colon carcinogenesis by use of resistant starch (RS3) as the carrier for polysaccharide of Larimichthys crocea swimming bladder', *Int J Mol Sci*, 15(1), pp. 817-29.

Chen, X., Guo, X., Zhang, H., Xiang, Y., Chen, J., Yin, Y., Cai, X., Wang, K., Wang, G., Ba, Y., Zhu, L., Wang, J., Yang, R., Zhang, Y., Ren, Z., Zen, K., Zhang, J. and Zhang, C.Y. (2009) 'Role of miR-143 targeting KRAS in colorectal tumorigenesis', *Oncogene*, 28(10), pp. 1385-92.

Chen, Y.Z., Liu, D., Zhao, Y.X., Wang, H.T., Gao, Y. and Chen, Y. (2014b) 'Aberrant promoter methylation of the SFRP1 gene may contribute to colorectal carcinogenesis: a meta-analysis', *Tumour Biol*.

Chiang, Y., Song, Y., Wang, Z., Liu, Z., Gao, P., Liang, J., Zhu, J., Xing, C. and Xu, H. (2012) 'microRNA-192, -194 and -215 are frequently downregulated in colorectal cancer', *Exp Ther Med*, 3(3), pp. 560-566.

Chung, D.C. and Rustgi, A.K. (2003) 'The hereditary nonpolyposis colorectal cancer syndrome: genetics and clinical implications', *Ann Intern Med*, 138(7), pp. 560-70.

Cicek, B., Arslan, P., Kelestimur, F. (2009) 'The effects of oligofructose and polydextrose on metabolic control parameters in Type-2 Diabetes', *Pakistan Journal of Medical Sciences*, 25, pp. 573-578.

Clarke, J.M., Young, G.P., Topping, D.L., Bird, A.R., Cobiac, L., Scherer, B.L., Winkler, J.G. and Lockett, T.J. (2012) 'Butyrate delivered by butyrylated starch increases distal colonic epithelial apoptosis in carcinogen-treated rats', *Carcinogenesis*, 33(1), pp. 197-202.

Clevers, H. (2006) 'Wnt/beta-catenin signaling in development and disease', *Cell*, 127(3), pp. 469-80.

Clevers, H. (2013) 'The intestinal crypt, a prototype stem cell compartment', *Cell*, 154(2), pp. 274-84.

Clevers, H. and Batlle, E. (2006) 'EphB/EphrinB receptors and Wnt signaling in colorectal cancer', *Cancer Res*, 66(1), pp. 2-5.

Coleman, L.J., Landstrom, E.K., Royle, P.J., Bird, A.R. and McIntosh, G.H. (2002) 'A diet containing alpha-cellulose and fish oil reduces aberrant crypt foci formation and modulates other possible markers for colon cancer risk in azoxymethane-treated rats', *J Nutr*, 132(8), pp. 2312-8.

Comalada, M., Bailon, E., de Haro, O., Lara-Villoslada, F., Xaus, J., Zarzuelo, A. and Galvez, J. (2006) 'The effects of short-chain fatty acids on colon epithelial proliferation and survival depend on the cellular phenotype', *J Cancer Res Clin Oncol*, 132(8), pp. 487-97.

Coskun, M., Bjerrum, J.T., Seidelin, J.B., Troelsen, J.T., Olsen, J. and Nielsen, O.H. (2013) 'miR-20b, miR-98, miR-125b-1*, and let-7e* as new potential diagnostic biomarkers in ulcerative colitis', *World J Gastroenterol*, 19(27), pp. 4289-99.

Costabile, A., Fava, F., Roytio, H., Forssten, S.D., Olli, K., Klievink, J., Rowland, I.R., Ouwehand, A.C., Rastall, R.A., Gibson, G.R. and Walton, G.E. (2011) 'Impact of polydextrose on the faecal microbiota: a double-blind, crossover, placebo-controlled feeding study in healthy human subjects', *Br J Nutr*, pp. 1-11.

Cray, N., Pillatzki, A., Zhao, Y., Birt, D.F. and Whitley, E.M. (2013) 'Effects of resistant starch on beta catenin in azoxymethane-treated colonic epithelium', *The FASEB Journal*, 27, p. 874.9.

Cray, N.L. (2013) *Effects of diets containing digestion-resistant starch on Wnt pathway control of proliferation and differentiation of the colorectal mucosa*. Iowa State University.

CRUK (2012) 'Cancer Research UK bowel cancer survival statistics'. 2nd September 2014. Available at: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/bowel/survival/.

CRUK (2013) *Cancer Research UK. The bowel.* Available at: http://www.cancerresearchuk.org/about-cancer/type/bowel-cancer/about/the-bowel#large (Accessed: 14th September 2014).

CRUK (2014a) *Cancer Research UK bowel cancer incidence statistics*. Available at: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/bowel/incidence/ (Accessed: 2nd September 2014).

CRUK (2014b) *Cancer Research UK bowel cancer key facts*. Available at: http://www.cancerresearchuk.org/cancer-info/cancerstats/keyfacts/bowel-cancer/ (Accessed: 2nd September 2014).

CRUK (2014c) *Cancer Research UK bowel cancer mortality statistics*. Available at: http://www.cancerresearchuk.org/cancer-info/cancerstats/types/bowel/mortality/ (Accessed: 2nd September 2014).

CRUK (2014d) *Cancer Research UK worldwide cancer incidence statistics*. Available at: http://www.cancerresearchuk.org/cancer-info/cancerstats/world/incidence/ (Accessed: 2nd September 2014).

Cucina, A., Dinicola, S., Coluccia, P., Proietti, S., D'Anselmi, F., Pasqualato, A. and Bizzarri, M. (2012) 'Nicotine stimulates proliferation and inhibits apoptosis in colon cancer cell lines through activation of survival pathways', *J Surg Res*, 178(1), pp. 233-41.

Cui, H., Onyango, P., Brandenburg, S., Wu, Y., Hsieh, C.L. and Feinberg, A.P. (2002) 'Loss of imprinting in colorectal cancer linked to hypomethylation of H19 and IGF2', *Cancer Res*, 62(22), pp. 6442-6.

Cummings, J.H., Beatty, E.R., Kingman, S.M., Bingham, S.A. and Englyst, H.N. (1996) 'Digestion and physiological properties of resistant starch in the human large bowel', *Br J Nutr*, 75(5), pp. 733-47.

Cummings, J.H. and Stephen, A.M. (2007) 'Carbohydrate terminology and classification', *Eur J Clin Nutr*, 61 Suppl 1, pp. S5-18.

Dai, W.B., Ren, Z.P., Chen, W.L., Du, J., Shi, Z. and Tang, D.Y. (2007) '[Expression and significance of APC, beta-catenin, C-myc, and Cyclin D1 proteins in colorectal carcinoma]', *Ai Zheng*, 26(9), pp. 963-6.

Daly, K., Cuff, M.A., Fung, F. and Shirazi-Beechey, S.P. (2005) 'The importance of colonic butyrate transport to the regulation of genes associated with colonic tissue homoeostasis', *Biochem Soc Trans*, 33(Pt 4), pp. 733-5.

Danisco (2011) *An introduction to Litesse® polydextrose, the better fibre*. Available at: http://www.danisco.com/fileadmin/user_upload/danisco/documents/Danisco_Litesse_Qleaflet.pdf (Accessed: 9th September 2014).

Daroqui, M.C. and Augenlicht, L.H. (2010) 'Transcriptional attenuation in colon carcinoma cells in response to butyrate', *Cancer Prev Res (Phila)*, 3(10), pp. 1292-302.

Davies, R.J., Miller, R. and Coleman, N. (2005) 'Colorectal cancer screening: prospects for molecular stool analysis', *Nat Rev Cancer*, 5(3), pp. 199-209.

De Queiroz Rossanese, L.B., De Lima Marson, F.A., Ribeiro, J.D., Coy, C.S. and Bertuzzo, C.S. (2013) 'APC germline mutations in families with familial adenomatous polyposis', *Oncol Rep*, 30(5), pp. 2081-8.

DeVries, J.W. (2003) 'On defining dietary fibre', *Proc Nutr Soc*, 62(1), pp. 37-43.

Dhir, M., Montgomery, E.A., Glockner, S.C., Schuebel, K.E., Hooker, C.M., Herman, J.G., Baylin, S.B., Gearhart, S.L. and Ahuja, N. (2008) 'Epigenetic regulation of WNT signaling pathway genes in inflammatory bowel disease (IBD) associated neoplasia', *J Gastrointest Surg*, 12(10), pp. 1745-53.

Diosdado, B., van de Wiel, M.A., Terhaar Sive Droste, J.S., Mongera, S., Postma, C., Meijerink, W.J., Carvalho, B. and Meijer, G.A. (2009) 'MiR-17-92 cluster is associated with 13q gain and c-myc expression during colorectal adenoma to adenocarcinoma progression', *Br J Cancer*, 101(4), pp. 707-14.

Djuranovic, S., Nahvi, A. and Green, R. (2012) 'miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay', *Science*, 336(6078), pp. 237-40.

Dolmans, G.H., Werker, P.M., Hennies, H.C., Furniss, D., Festen, E.A., Franke, L., Becker, K., van der Vlies, P., Wolffenbuttel, B.H., Tinschert, S., Toliat, M.R., Nothnagel, M., Franke, A., Klopp, N., Wichmann, H.E., Nurnberg, P., Giele, H., Ophoff, R.A. and Wijmenga, C. (2011) 'Wnt signaling and Dupuytren's disease', *N Engl J Med*, 365(4), pp. 307-17.

Dorak, M.T. (2006) *Real-time PCR*. 1 edn. Taylor and Francis.

Dronamraju, S.S., Coxhead, J.M., Kelly, S.B., Burn, J. and Mathers, J.C. (2009) 'Cell kinetics and gene expression changes in colorectal cancer patients given resistant starch: a randomised controlled trial', *Gut*, 58(3), pp. 413-20.

Du, S.J., Purcell, S.M., Christian, J.L., McGrew, L.L. and Moon, R.T. (1995) 'Identification of distinct classes and functional domains of Wnts through expression of wild-type and chimeric proteins in Xenopus embryos', *Mol Cell Biol*, 15(5), pp. 2625-34.

Dupont, C., Armant, D.R. and Brenner, C.A. (2009) 'Epigenetics: definition, mechanisms and clinical perspective', *Semin Reprod Med*, 27(5), pp. 351-7.

Dwyer, M.A., Joseph, J.D., Wade, H.E., Eaton, M.L., Kunder, R.S., Kazmin, D., Chang, C.Y. and McDonnell, D.P. (2010) 'WNT11 expression is induced by estrogen-related receptor alpha and beta-catenin and acts in an autocrine manner to increase cancer cell migration', *Cancer Res*, 70(22), pp. 9298-308.

Eaden, J. (2003) 'Review article: the data supporting a role for aminosalicylates in the chemoprevention of colorectal cancer in patients with inflammatory bowel disease', *Aliment Pharmacol Ther*, 18 Suppl 2, pp. 15-21.

Eaden, J.A., Abrams, K.R. and Mayberry, J.F. (2001) 'The risk of colorectal cancer in ulcerative colitis: a meta-analysis', *Gut*, 48(4), pp. 526-35.

Earle, J.S., Luthra, R., Romans, A., Abraham, R., Ensor, J., Yao, H. and Hamilton, S.R. (2010) 'Association of microRNA expression with microsatellite instability status in colorectal adenocarcinoma', *J Mol Diagn*, 12(4), pp. 433-40.

Eberhart, C.E., Coffey, R.J., Radhika, A., Giardiello, F.M., Ferrenbach, S. and DuBois, R.N. (1994) 'Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas', *Gastroenterology*, 107(4), pp. 1183-8.

Eden, A., Gaudet, F., Waghmare, A. and Jaenisch, R. (2003) 'Chromosomal instability and tumors promoted by DNA hypomethylation', *Science*, 300(5618), p. 455.

Edwards, K., Logan, J. and Saunders, N. (2004) *Real-time PCR: an essential guide*. U.K.: Horizon Bioscience.

Elinav, E., Nowarski, R., Thaiss, C.A., Hu, B., Jin, C. and Flavell, R.A. (2013) 'Inflammation-induced cancer: crosstalk between tumours, immune cells and microorganisms', *Nat Rev Cancer*, 13(11), pp. 759-71.

Emenaker, N.J., Calaf, G.M., Cox, D., Basson, M.D. and Qureshi, N. (2001) 'Short-chain fatty acids inhibit invasive human colon cancer by modulating uPA, TIMP-1, TIMP-2, mutant p53, Bcl-2, Bax, p21 and PCNA protein expression in an in vitro cell culture model', *J Nutr*, 131(11 Suppl), pp. 3041S-6S.

Endo, A., Koizumi, H., Takahashi, M., Tamura, T., Tatsunami, S., Watanabe, Y. and Takagi, M. (2013) 'A significant imbalance in mitosis versus apoptosis accelerates the growth rate of sessile serrated adenoma/polyps', *Virchows Arch*, 462(2), pp. 131-9.

Englyst, H.N., Kingman, S.M. and Cummings, J.H. (1992) 'Classification and measurement of nutritionally important starch fractions', *Eur J Clin Nutr*, 46 Suppl 2, pp. S33-50.

Esteller, M. (2007) 'Cancer epigenomics: DNA methylomes and histone-modification maps', *Nat Rev Genet*, 8(4), pp. 286-98.

Esteller, M., Sparks, A., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M.A., Gonzalez, S., Tarafa, G., Sidransky, D., Meltzer, S.J., Baylin, S.B. and Herman, J.G. (2000) 'Analysis of adenomatous polyposis coli promoter hypermethylation in human cancer', *Cancer Res*, 60(16), pp. 4366-71.

Evertsson, S., Bartik, Z., Zhang, H., Jansson, A. and Sun, X.F. (1999) 'Apoptosis in relation to proliferating cell nuclear antigen and Dukes' stage in colorectal adenocarcinoma', *Int J Oncol*, 15(1), pp. 53-8.

Faltejskova, P., Svoboda, M., Srutova, K., Mlcochova, J., Besse, A., Nekvindova, J., Radova, L., Fabian, P., Slaba, K., Kiss, I., Vyzula, R. and Slaby, O. (2012) 'Identification and functional screening of microRNAs highly deregulated in colorectal cancer', *J Cell Mol Med*, 16(11), pp. 2655-66.

Fang, Y., Wang, L., Zhang, Y., Ge, C. and Xu, C. (2014) '[Wif-1 methylation and beta-catenin expression in colorectal serrated lesions]', *Zhonghua Bing Li Xue Za Zhi*, 43(1), pp. 15-9.

Farkas, S.A., Vymetalkova, V., Vodickova, L., Vodicka, P. and Nilsson, T.K. (2014) 'DNA methylation changes in genes frequently mutated in sporadic colorectal cancer and in the DNA repair and Wnt/beta-catenin signaling pathway genes', *Epigenomics*, 6(2), pp. 179-91.

Fava, F., Makivuokko, H., Siljander-Rasi, H., Putaala, H., Tiihonen, K., Stowell, J., Tuohy, K., Gibson, G. and Rautonen, N. (2007) 'Effect of polydextrose on intestinal microbes and immune functions in pigs', *Br J Nutr*, 98(1), pp. 123-33.

Feinberg, A.P., Gehrke, C.W., Kuo, K.C. and Ehrlich, M. (1988) 'Reduced genomic 5-methylcytosine content in human colonic neoplasia', *Cancer Res*, 48(5), pp. 1159-61.

Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D., Forman, D. and Bray, F. (2013) *Globocan 2012 v1.0 cancer incidence and mortality worldwide: IARC CancerBase No. 11*. Available at: http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx (Accessed: 2nd September 2014).

Feulgen, R. (1914) 'Über die Kohlenhydratgruppe in der echten Nucleinsäure. Vorläufige Mitteilung.', *Z Physiol Chem*, 92, pp. 154-158.

Fevr, T., Robine, S., Louvard, D. and Huelsken, J. (2007) 'Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells', *Mol Cell Biol*, 27(21), pp. 7551-9.

Figdor, S.K. and Bianchine, J.R. (1983) 'Caloric utilization and disposition of [14C]polydextrose in man', *J Agric Food Chem*, 31(2), pp. 389-93.

Figdor, S.K. and Rennhard, H.H. (1981) 'Caloric utilization and disposition of [14C]polydextrose in the rat', *J Agric Food Chem*, 29(6), pp. 1181-9.

Fink, L., Seeger, W., Ermert, L., Hanze, J., Stahl, U., Grimminger, F., Kummer, W. and Bohle, R.M. (1998) 'Real-time quantitative RT-PCR after laser-assisted cell picking', *Nat Med*, 4(11), pp. 1329-33.

Fleisher, A.S., Esteller, M., Harpaz, N., Leytin, A., Rashid, A., Xu, Y., Liang, J., Stine, O.C., Yin, J., Zou, T.T., Abraham, J.M., Kong, D., Wilson, K.T., James, S.P., Herman, J.G. and Meltzer, S.J. (2000) 'Microsatellite instability in inflammatory bowel disease-associated neoplastic lesions is associated with hypermethylation and diminished expression of the DNA mismatch repair gene, hMLH1', *Cancer Res*, 60(17), pp. 4864-8.

Flohil, C.C., Janssen, P.A. and Bosman, F.T. (1996) 'Expression of Bcl-2 protein in hyperplastic polyps, adenomas, and carcinomas of the colon', *J Pathol*, 178(4), pp. 393-7.

Flood, D.M., Weiss, N.S., Cook, L.S., Emerson, J.C., Schwartz, S.M. and Potter, J.D. (2000) 'Colorectal cancer incidence in Asian migrants to the United States and their descendants', *Cancer Causes Control*, 11(5), pp. 403-11.

Fodde, R. and Brabletz, T. (2007) 'Wnt/beta-catenin signaling in cancer stemness and malignant behavior', *Curr Opin Cell Biol*, 19(2), pp. 150-8.

Ford, A.C., Moayyedi, P. and Hanauer, S.B. (2013) 'Ulcerative colitis', *BMJ*, 346, p. f432.

Franklin, W.A., McDonald, G.B., Stein, H.O., Gatter, K.C., Jewell, D.P., Clarke, L.C. and Mason, D.Y. (1985) 'Immunohistologic demonstration of abnormal colonic crypt cell kinetics in ulcerative colitis', *Hum Pathol*, 16(11), pp. 1129-32.

Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L. and Paul, C.L. (1992) 'A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands', *Proc Natl Acad Sci U S A*, 89(5), pp. 1827-31.

Fu, J., Tang, W., Du, P., Wang, G., Chen, W., Li, J., Zhu, Y., Gao, J. and Cui, L. (2012) 'Identifying microRNA-mRNA regulatory network in colorectal cancer by a combination of expression profile and bioinformatics analysis', *BMC Syst Biol*, 6, p. 68.

Fu, X., Li, J., Li, K., Tian, X. and Zhang, Y. (2009) 'Hypermethylation of APC promoter 1A is associated with moderate activation of Wnt signalling pathway in a subset of colorectal serrated adenomas', *Histopathology*, 55(5), pp. 554-63.

Fung, K.Y., Cosgrove, L., Lockett, T., Head, R. and Topping, D.L. (2012) 'A review of the potential mechanisms for the lowering of colorectal oncogenesis by butyrate', *Br J Nutr*, 108(5), pp. 820-31.

Gaedcke, J., Grade, M., Camps, J., Sokilde, R., Kaczkowski, B., Schetter, A.J., Difilippantonio, M.J., Harris, C.C., Ghadimi, B.M., Moller, S., Beissbarth, T., Ried, T. and Litman, T. (2012) 'The rectal cancer microRNAome--microRNA expression in rectal cancer and matched normal mucosa', *Clin Cancer Res*, 18(18), pp. 4919-30.

Galiatsatos, P. and Foulkes, W.D. (2006) 'Familial adenomatous polyposis', *Am J Gastroenterol*, 101(2), pp. 385-98.

Gaspar, C., Cardoso, J., Franken, P., Molenaar, L., Morreau, H., Moslein, G., Sampson, J., Boer, J.M., de Menezes, R.X. and Fodde, R. (2008) 'Cross-species comparison of human and mouse intestinal polyps reveals conserved mechanisms in adenomatous polyposis coli (APC)-driven tumorigenesis', *Am J Pathol*, 172(5), pp. 1363-80.

Gaudier, E., Jarry, A., Blottiere, H.M., de Coppet, P., Buisine, M.P., Aubert, J.P., Laboisse, C., Cherbut, C. and Hoebler, C. (2004) 'Butyrate specifically modulates MUC gene expression in intestinal epithelial goblet cells deprived of glucose', *Am J Physiol Gastrointest Liver Physiol*, 287(6), pp. G1168-74.

Genomatix (2014) *Genomatix software suite v3.2*.

Germann, A., Dihlmann, S., Hergenhahn, M., Doeberitz, M.K. and Koesters, R. (2003) 'Expression profiling of CC531 colon carcinoma cells reveals similar regulation of beta-catenin target genes by both butyrate and aspirin', *Int J Cancer*, 106(2), pp. 187-97.

Giarnieri, E., Nagar, C., Valli, C., Brunetti, E., Palmieri, M.B. and Vecchione, A. (2000) 'BCL2 and BAX expression in hyperplastic and dysplastic rectal polyps', *Hepatogastroenterology*, 47(31), pp. 159-62.

Gibson, G.R., Scott K.P., Rastall R.A., Tuohy K.M., Hotchkiss A., Dubert-Ferrandon A., Gareau M., Murphy E.F., Saulnier D., Loh G., Macfarlane S., Delzenne N., Ringel Y., Kozianowski G., Dickman R., Lenoir-Wijnkoop I., Walker C., Buddington R. (2010) 'Dietary prebiotics: current status and new definition', *Food Sci Technol Bull*, 7(1), pp. 1-19.

Goelz, S.E., Vogelstein, B., Hamilton, S.R. and Feinberg, A.P. (1985) 'Hypomethylation of DNA from benign and malignant human colon neoplasms', *Science*, 228(4696), pp. 187-90.

Goodlad, R.A., Levi, S., Lee, C.Y., Mandir, N., Hodgson, H. and Wright, N.A. (1991) 'Morphometry and cell proliferation in endoscopic biopsies: evaluation of a technique', *Gastroenterology*, 101(5), pp. 1235-41.

Grabitske, H.A. and Slavin, J.L. (2009) 'Gastrointestinal effects of low-digestible carbohydrates', *Crit Rev Food Sci Nutr*, 49(4), pp. 327-60.

Gradel, K.O., Nielsen, H.L., Schonheyder, H.C., Ejlertsen, T., Kristensen, B. and Nielsen, H. (2009) 'Increased short- and long-term risk of inflammatory bowel disease after salmonella or campylobacter gastroenteritis', *Gastroenterology*, 137(2), pp. 495-501.

Gregorieff, A., Pinto, D., Begthel, H., Destree, O., Kielman, M. and Clevers, H. (2005) 'Expression pattern of Wnt signaling components in the adult intestine', *Gastroenterology*, 129(2), pp. 626-38.

Groden, J., Joslyn, G., Samowitz, W., Jones, D., Bhattacharyya, N., Spirio, L., Thliveris, A., Robertson, M., Egan, S., Meuth, M. and et al. (1995) 'Response of colon cancer cell lines to the introduction of APC, a colon-specific tumor suppressor gene', *Cancer Res*, 55(7), pp. 1531-9.

Groden, J., Thliveris, A., Samowitz, W., Carlson, M., Gelbert, L., Albertsen, H., Joslyn, G., Stevens, J., Spirio, L., Robertson, M. and et al. (1991) 'Identification and characterization of the familial adenomatous polyposis coli gene', *Cell*, 66(3), pp. 589-600.

Gross, A., McDonnell, J.M. and Korsmeyer, S.J. (1999) 'BCL-2 family members and the mitochondria in apoptosis', *Genes Dev*, 13(15), pp. 1899-911.

Grubben, M.J., van den Braak, C.C., Essenberg, M., Olthof, M., Tangerman, A., Katan, M.B. and Nagengast, F.M. (2001) 'Effect of resistant starch on potential biomarkers for colonic cancer risk in patients with colonic adenomas: a controlled trial', *Dig Dis Sci*, 46(4), pp. 750-6.

Gujral, T.S. and MacBeath, G. (2010) 'A system-wide investigation of the dynamics of Wnt signaling reveals novel phases of transcriptional regulation', *PLoS One*, 5(4), p. e10024.

Ha, M.A., Jarvis, M.C. and Mann, J.I. (2000) 'A definition for dietary fibre', *Eur J Clin Nutr*, 54(12), pp. 861-4.

Hackl, M., Brunner, S., Fortschegger, K., Schreiner, C., Micutkova, L., Muck, C., Laschober, G.T., Lepperdinger, G., Sampson, N., Berger, P., Herndler-Brandstetter, D., Wieser, M., Kuhnel, H., Strasser, A., Rinnerthaler, M., Breitenbach, M., Mildner, M., Eckhart, L., Tschachler, E., Trost, A., Bauer, J.W., Papak, C., Trajanoski, Z., Scheideler, M., Grillari-Voglauer, R., Grubeck-Loebenstein, B., Jansen-Durr, P. and Grillari, J. (2010) 'miR-17, miR-19b, miR-20a, and miR-106a are down-regulated in human aging', *Aging Cell*, 9(2), pp. 291-6.

Hall, P.A., Coates, P.J., Ansari, B. and Hopwood, D. (1994) 'Regulation of cell number in the mammalian gastrointestinal tract: the importance of apoptosis', *J Cell Sci*, 107 (Pt 12), pp. 3569-77.

Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F.J. and Brummer, R.J. (2008) 'Review article: the role of butyrate on colonic function', *Aliment Pharmacol Ther*, 27(2), pp. 104-19.

Hamfjord, J., Stangeland, A.M., Hughes, T., Skrede, M.L., Tveit, K.M., Ikdahl, T. and Kure, E.H. (2012) 'Differential expression of miRNAs in colorectal cancer: comparison of paired tumor tissue and adjacent normal mucosa using high-throughput sequencing', *PLoS One*, 7(4), p. e34150.

Hanahan, D. and Weinberg, R.A. (2000) 'The hallmarks of cancer', *Cell*, 100(1), pp. 57-70.

Hanahan, D. and Weinberg, R.A. (2011) 'Hallmarks of cancer: the next generation', *Cell*, 144(5), pp. 646-74.

Hass, R., Busche, R., Luciano, L., Reale, E. and Engelhardt, W.V. (1997) 'Lack of butyrate is associated with induction of Bax and subsequent apoptosis in the proximal colon of guinea pig', *Gastroenterology*, 112(3), pp. 875-81.

Hawkins, N., Lees, J., Hargrave, R., O'Connor, T., Meagher, A. and Ward, R. (1997) 'Pathological and genetic correlates of apoptosis in the progression of colorectal neoplasia', *Tumour Biol*, 18(3), pp. 146-56.

He, L. and Hannon, G.J. (2004) 'MicroRNAs: small RNAs with a big role in gene regulation', *Nat Rev Genet*, 5(7), pp. 522-31.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B. and Kinzler, K.W. (1998) 'Identification of c-MYC as a target of the APC pathway', *Science*, 281(5382), pp. 1509-12.

Hengst, C., Ptok, S., Roessler, A., Fechner, A. and Jahreis, G. (2009) 'Effects of polydextrose supplementation on different faecal parameters in healthy volunteers', *Int J Food Sci Nutr*, 60 Suppl 5, pp. 96-105.

Herbst, A., Wallner, M., Rahmig, K., Stieber, P., Crispin, A., Lamerz, R. and Kolligs, F.T. (2009) 'Methylation of helicase-like transcription factor in serum of patients with colorectal cancer is an independent predictor of disease recurrence', *Eur J Gastroenterol Hepatol*, 21(5), pp. 565-9.

Hernandez-Blazquez, F.J., Habib, M., Dumollard, J.M., Barthelemy, C., Benchaib, M., de Capoa, A. and Niveleau, A. (2000) 'Evaluation of global DNA hypomethylation in human colon cancer tissues by immunohistochemistry and image analysis', *Gut*, 47(5), pp. 689-93.

Hernot, D.C., Boileau, T.W., Bauer, L.L., Middelbos, I.S., Murphy, M.R., Swanson, K.S. and Fahey, G.C., Jr. (2009) 'In vitro fermentation profiles, gas production rates, and microbiota modulation as affected by certain fructans, galactooligosaccharides, and polydextrose', *J Agric Food Chem*, 57(4), pp. 1354-61.

Herold, K.M. and Rothberg, P.G. (1988) 'Evidence for a labile intermediate in the butyrate induced reduction of the level of c-myc RNA in SW837 rectal carcinoma cells', *Oncogene*, 3(4), pp. 423-8.

Heruth, D.P., Zirnstein, G.W., Bradley, J.F. and Rothberg, P.G. (1993) 'Sodium butyrate causes an increase in the block to transcriptional elongation in the c-myc gene in SW837 rectal carcinoma cells', *J Biol Chem*, 268(27), pp. 20466-72.

Higgins, J.A., Brand Miller, J.C. and Denyer, G.S. (1996) 'Development of insulin resistance in the rat is dependent on the rate of glucose absorption from the diet', *J Nutr*, 126(3), pp. 596-602.

Higgins, J.A., Higbee, D.R., Donahoo, W.T., Brown, I.L., Bell, M.L. and Bessesen, D.H. (2004) 'Resistant starch consumption promotes lipid oxidation', *Nutr Metab (Lond)*, 1(1), p. 8.

Hlubek, F., Brabletz, T., Budczies, J., Pfeiffer, S., Jung, A. and Kirchner, T. (2007) 'Heterogeneous expression of Wnt/beta-catenin target genes within colorectal cancer', *Int J Cancer*, 121(9), pp. 1941-8.

Hockenbery, D.M. (1992) 'The bcl-2 oncogene and apoptosis', *Semin Immunol*, 4(6), pp. 413-20.

Hodin, R.A., Meng, S., Archer, S. and Tang, R. (1996) 'Cellular growth state differentially regulates enterocyte gene expression in butyrate-treated HT-29 cells', *Cell Growth Differ*, 7(5), pp. 647-53.

Hogan, N.M., Joyce, M.R. and Kerin, M.J. (2012) 'MicroRNA expression in colorectal cancer', *Cancer Biomark*, 11(6), pp. 239-43.

Hooda, S., Boler, B.M., Serao, M.C., Brulc, J.M., Staeger, M.A., Boileau, T.W., Dowd, S.E., Fahey, G.C., Jr. and Swanson, K.S. (2012) '454 pyrosequencing reveals a shift in fecal microbiota of healthy adult men consuming polydextrose or soluble corn fiber', *J Nutr*, 142(7), pp. 1259-65.

Hu, S., Dong, T.S., Dalal, S.R., Wu, F., Bissonnette, M., Kwon, J.H. and Chang, E.B. (2011) 'The microbe-derived short chain fatty acid butyrate targets miRNA-dependent p21 gene expression in human colon cancer', *PLoS One*, 6(1), p. e16221.

Huang, N., Katz, J.P., Martin, D.R. and Wu, G.D. (1997) 'Inhibition of IL-8 gene expression in Caco-2 cells by compounds which induce histone hyperacetylation', *Cytokine*, 9(1), pp. 27-36.

Huang, Z., Huang, D., Ni, S., Peng, Z., Sheng, W. and Du, X. (2010) 'Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer', *Int J Cancer*, 127(1), pp. 118-26.

Huang, Z., Huang, S., Wang, Q., Liang, L., Ni, S., Wang, L., Sheng, W., He, X. and Du, X. (2011) 'MicroRNA-95 promotes cell proliferation and targets sorting Nexin 1 in human colorectal carcinoma', *Cancer Res*, 71(7), pp. 2582-9.

Hughes, K.R., Sablitzky, F. and Mahida, Y.R. (2011) 'Expression profiling of Wnt family of genes in normal and inflammatory bowel disease primary human intestinal myofibroblasts and normal human colonic crypt epithelial cells', *Inflamm Bowel Dis*, 17(1), pp. 213-20.

Humphreys, K.J., Cobiac, L., Le Leu, R.K., Van der Hoek, M.B. and Michael, M.Z. (2013) 'Histone deacetylase inhibition in colorectal cancer cells reveals competing roles for members of the oncogenic miR-17-92 cluster', *Mol Carcinog*, 52(6), pp. 459-74.

Humphreys, K.J., Conlon, M.A., Young, G.P., Topping, D.L., Hu, Y., Winter, J.M., Bird, A.R., Cobiac, L., Kennedy, N.A., Michael, M.Z. and Le Leu, R.K. (2014) 'Dietary Manipulation of Oncogenic MicroRNA Expression in Human Rectal Mucosa: A Randomized Trial', *Cancer Prev Res (Phila)*, 7(8), pp. 786-95.

Humphries, A. and Wright, N.A. (2008) 'Colonic crypt organization and tumorigenesis', *Nat Rev Cancer*, 8(6), pp. 415-24.

Hussain, S.P., Amstad, P., Raja, K., Ambs, S., Nagashima, M., Bennett, W.P., Shields, P.G., Ham, A.J., Swenberg, J.A., Marrogi, A.J. and Harris, C.C. (2000) 'Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease', *Cancer Res*, 60(13), pp. 3333-7.

Hylla, S., Gostner, A., Dusel, G., Anger, H., Bartram, H.P., Christl, S.U., Kasper, H. and Scheppach, W. (1998) 'Effects of resistant starch on the colon in healthy volunteers: possible implications for cancer prevention', *Am J Clin Nutr*, 67(1), pp. 136-42.

Iacopetta, B., Grieu, F., Phillips, M., Ruszkiewicz, A., Moore, J., Minamoto, T. and Kawakami, K. (2007) 'Methylation levels of LINE-1 repeats and CpG island loci are inversely related in normal colonic mucosa', *Cancer Sci*, 98(9), pp. 1454-60.

Iimura, M., Nakamura, T., Shinozaki, S., Iizuka, B., Inoue, Y., Suzuki, S. and Hayashi, N. (2000) 'Bax is downregulated in inflamed colonic mucosa of ulcerative colitis', *Gut*, 47(2), pp. 228-35.

Ilyas, M., Tomlinson, I.P., Rowan, A., Pignatelli, M. and Bodmer, W.F. (1997) 'Beta-catenin mutations in cell lines established from human colorectal cancers', *Proc Natl Acad Sci U S A*, 94(19), pp. 10330-4.

Inestrosa, N.C. and Toledo, E.M. (2008) 'The role of Wnt signaling in neuronal dysfunction in Alzheimer's Disease', *Mol Neurodegener*, 3, p. 9.

Ingredion (2014) *Why Hi-maize® resistant starch.* Available at: http://www.foodinnovation.com/foodinnovation/en-us/Hi-maize/Pages/Why-Hi-maize.aspx (Accessed: 9th September 2014).

Ireland, H., Kemp, R., Houghton, C., Howard, L., Clarke, A.R., Sansom, O.J. and Winton, D.J. (2004) 'Inducible Cre-mediated control of gene expression in the murine gastrointestinal tract: effect of loss of beta-catenin', *Gastroenterology*, 126(5), pp. 1236-46.

Irizarry, R.A., Ladd-Acosta, C., Wen, B., Wu, Z., Montano, C., Onyango, P., Cui, H., Gabo, K., Rongione, M., Webster, M., Ji, H., Potash, J.B., Sabunciyan, S. and Feinberg, A.P. (2009) 'The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores', *Nat Genet*, 41(2), pp. 178-86.

Ishizuka, S., Nagai, T. and Hara, H. (2003) 'Reduction of aberrant crypt foci by ingestion of polydextrose in the rat colorectum', *Nutrition Research*, 23(1), pp. 117-22.

Itzkowitz, S.H. and Yio, X. (2004) 'Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation', *Am J Physiol Gastrointest Liver Physiol*, 287(1), pp. G7-17.

Iwamoto, M., Ahnen, D.J., Franklin, W.A. and Maltzman, T.H. (2000) 'Expression of beta-catenin and full-length APC protein in normal and neoplastic colonic tissues', *Carcinogenesis*, 21(11), pp. 1935-40.

Iwamoto, M., Koji, T., Makiyama, K., Kobayashi, N. and Nakane, P.K. (1996) 'Apoptosis of crypt epithelial cells in ulcerative colitis', *J Pathol*, 180(2), pp. 152-9.

Jacobasch, G., Schmiedl, D., Kruschewski, M. and Schmehl, K. (1999) 'Dietary resistant starch and chronic inflammatory bowel diseases', *Int J Colorectal Dis*, 14(4-5), pp. 201-11.

Jacobs, D.R., Jr., Marquart, L., Slavin, J. and Kushi, L.H. (1998) 'Whole-grain intake and cancer: an expanded review and meta-analysis', *Nutr Cancer*, 30(2), pp. 85-96.

Jess, T., Gamborg, M., Matzen, P., Munkholm, P. and Sorensen, T.I. (2005) 'Increased risk of intestinal cancer in Crohn's disease: a meta-analysis of population-based cohort studies', *Am J Gastroenterol*, 100(12), pp. 2724-9.

Jie, Z., Bang-Yao, L., Ming-Jie, X., Hai-Wei, L., Zu-Kang, Z., Ting-Song, W. and Craig, S.A. (2000) 'Studies on the effects of polydextrose intake on physiologic functions in Chinese people', *Am J Clin Nutr*, 72(6), pp. 1503-9.

Johns, L.E. and Houlston, R.S. (2001) 'A systematic review and meta-analysis of familial colorectal cancer risk', *Am J Gastroenterol*, 96(10), pp. 2992-3003.

Kalimutho, M., Del Vecchio Blanco, G., Di Cecilia, S., Sileri, P., Cretella, M., Pallone, F., Federici, G. and Bernardini, S. (2011) 'Differential expression of miR-144* as a novel fecal-based diagnostic marker for colorectal cancer', *J Gastroenterol*, 46(12), pp. 1391-402.

Kameue, C., Tsukahara, T., Yamada, K., Koyama, H., Iwasaki, Y., Nakayama, K. and Ushida, K. (2004) 'Dietary sodium gluconate protects rats from large bowel cancer by stimulating butyrate production', *J Nutr*, 134(4), pp. 940-4.

Kanaan, Z., Roberts, H., Eichenberger, M.R., Billeter, A., Ocheretner, G., Pan, J., Rai, S.N., Jorden, J., Williford, A. and Galandiuk, S. (2013) 'A plasma microRNA panel for detection of colorectal adenomas: a step toward more precise screening for colorectal cancer', *Ann Surg*, 258(3), pp. 400-8.

Kane, M.F., Loda, M., Gaida, G.M., Lipman, J., Mishra, R., Goldman, H., Jessup, J.M. and Kolodner, R. (1997) 'Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines', *Cancer Res*, 57(5), pp. 808-11.

Kanemitsu, T., Koike, A. and Yamamoto, S. (1985) 'Study of the cell proliferation kinetics in ulcerative colitis, adenomatous polyps, and cancer', *Cancer*, 56(5), pp. 1094-8.

Kang, M., Mitomi, H., Sada, M., Tokumitsu, Y., Takahashi, Y., Igarashi, M., Katsumata, T. and Okayasu, I. (1997) 'Ki-67, p53, and Bcl-2 expression of serrated adenomas of the colon', *Am J Surg Pathol*, 21(4), pp. 417-23.

Karamanolis, D.G., Kyrlagkitsis, I., Konstantinou, K., Papatheodoridis, G.V., Karameris, A., Mallas, E., Ladas, S.D. and Raptis, S. (2007) 'The Bcl-2/Bax system and apoptosis in ulcerative colitis', *Hepatogastroenterology*, 54(76), pp. 1085-8.

Karczmarski, J., Rubel, T., Paziewska, A., Mikula, M., Bujko, M., Kober, P., Dadlez, M. and Ostrowski, J. (2014) 'Histone H3 lysine 27 acetylation is altered in colon cancer', *Clin Proteomics*, 11(1), p. 24.

Kennedy, J.F., Knill, C.J. and Taylor, D.W. (1995) 'Maltodextrins', in Dziedzic, S.Z. and Kearsley, M.W. (eds.) *Handbook of starch hydrolysis products and their derivatives*.

Kerr, J.F., Wyllie, A.H. and Currie, A.R. (1972) 'Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics', *Br J Cancer*, 26(4), pp. 239-57.

Kien, C.L., Blauwiekel, R., Bunn, J.Y., Jetton, T.L., Frankel, W.L. and Holst, J.J. (2007) 'Cecal infusion of butyrate increases intestinal cell proliferation in piglets', *J Nutr*, 137(4), pp. 916-22.

Kikuchi, Y., Dinjens, W.N. and Bosman, F.T. (1997) 'Proliferation and apoptosis in proliferative lesions of the colon and rectum', *Virchows Arch*, 431(2), pp. 111-7.

Kim, T.O., Park, J., Kang, M.J., Lee, S.H., Jee, S.R., Ryu, D.Y., Yang, K. and Yi, J.M. (2013) 'DNA hypermethylation of a selective gene panel as a risk marker for colon cancer in patients with ulcerative colitis', *Int J Mol Med*, 31(5), pp. 1255-61.

King, N.A., Craig, S.A., Pepper, T. and Blundell, J.E. (2005) 'Evaluation of the independent and combined effects of xylitol and polydextrose consumed as a snack on hunger and energy intake over 10 d', *Br J Nutr*, 93(6), pp. 911-5.

Kinzler, K.W., Nilbert, M.C., Su, L.K., Vogelstein, B., Bryan, T.M., Levy, D.B., Smith, K.J., Preisinger, A.C., Hedge, P., McKechnie, D. and et al. (1991) 'Identification of FAP locus genes from chromosome 5q21', *Science*, 253(5020), pp. 661-5.

Kinzler, K.W. and Vogelstein, B. (1996) 'Lessons from hereditary colorectal cancer', *Cell*, 87(2), pp. 159-70.

Kinzler, K.W. and Vogelstein, B. (1997) 'Cancer-susceptibility genes. Gatekeepers and caretakers', *Nature*, 386(6627), pp. 761, 763.

Kirikoshi, H., Sekihara, H. and Katoh, M. (2001) 'Molecular cloning and characterization of human WNT11', *Int J Mol Med*, 8(6), pp. 651-6.

Klaus, A. and Birchmeier, W. (2008) 'Wnt signalling and its impact on development and cancer', *Nat Rev Cancer*, 8(5), pp. 387-98.

Kleessen, B., Stoof, G., Proll, J., Schmiedl, D., Noack, J. and Blaut, M. (1997) 'Feeding resistant starch affects fecal and cecal microflora and short-chain fatty acids in rats', *J Anim Sci*, 75(9), pp. 2453-62.

Kloosterman, W.P. and Plasterk, R.H. (2006) 'The diverse functions of microRNAs in animal development and disease', *Dev Cell*, 11(4), pp. 441-50.

Kobori, A., Bamba, S., Imaeda, H., Ban, H., Tsujikawa, T., Saito, Y., Fujiyama, Y. and Andoh, A. (2010) 'Butyrate stimulates IL-32alpha expression in human intestinal epithelial cell lines', *World J Gastroenterol*, 16(19), pp. 2355-61.

Kolonel, L.N. (1980) 'Cancer patterns of four ethnic groups in Hawaii', *J Natl Cancer Inst*, 65(5), pp. 1127-39.

Konishi, K., Shen, L., Wang, S., Meltzer, S.J., Harpaz, N. and Issa, J.P. (2007) 'Rare CpG island methylator phenotype in ulcerative colitis-associated neoplasias', *Gastroenterology*, 132(4), pp. 1254-60.

Kono, S. (2004) 'Secular trend of colon cancer incidence and mortality in relation to fat and meat intake in Japan', *Eur J Cancer Prev*, 13(2), pp. 127-32.

Koornstra, J.J., de Jong, S., Hollema, H., de Vries, E.G. and Kleibeuker, J.H. (2003) 'Changes in apoptosis during the development of colorectal cancer: a systematic review of the literature', *Crit Rev Oncol Hematol*, 45(1), pp. 37-53.

Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P.J. and Clevers, H. (1998) 'Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4', *Nat Genet*, 19(4), pp. 379-83.

Koukos, G., Polytarchou, C., Kaplan, J.L., Morley-Fletcher, A., Gras-Miralles, B., Kokkotou, E., Baril-Dore, M., Pothoulakis, C., Winter, H.S. and Iliopoulos, D. (2013) 'MicroRNA-124 regulates STAT3 expression and is down-regulated in colon tissues of pediatric patients with ulcerative colitis', *Gastroenterology*, 145(4), pp. 842-52 e2.

Krause, W.F. and DuBois, R.N. (2001) 'The molecular basis for prevention of colorectal cancer', *Clin Colorectal Cancer*, 1(1), pp. 47-54.

Kripke, S.A., Fox, A.D., Berman, J.M., Settle, R.G. and Rombeau, J.L. (1989) 'Stimulation of intestinal mucosal growth with intracolonic infusion of short-chain fatty acids', *JPEN J Parenter Enteral Nutr*, 13(2), pp. 109-16.

Kucerova, D., Sloncova, E., Tuhackova, Z., Uhlirova, M., Kos, M. and Sovova, V. (1999) 'Changes of E-cadherin and beta-catenin levels during induced differentiation of colorectal carcinoma cells', *Int J Mol Med*, 4(5), pp. 541-4.

Kuhnert, F., Davis, C.R., Wang, H.T., Chu, P., Lee, M., Yuan, J., Nusse, R. and Kuo, C.J. (2004) 'Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1', *Proc Natl Acad Sci U S A*, 101(1), pp. 266-71.

Kumemura, M., Shimizu, S., Tanizaki, M., Kurosumi, M., Masaoka, Y., Shoji, S., Katoh, O., Fujimoto, N. and Watanabe, H. (1998) 'The early phase of colon tumorigenesis induced by dimethylhydrazine in ICR mice', *Oncol Rep*, 5(3), pp. 621-4.

La Rocca, G., Shi, B., Audia, A., Ferrari-Amorotti, G., Mellert, H.S., Calabretta, B., McMahon, S.B., Sepp-Lorenzino, L. and Baserga, R. (2011) 'Regulation of microRNA-145 by growth arrest and differentiation', *Exp Cell Res*, 317(4), pp. 488-95.

Ladas, S.D., Kitsanta, P., Triantafyllou, K., Tzathas, C., Spiliadi, C. and Raptis, S.A. (2005) 'Cell turnover of serrated adenomas', *J Pathol*, 206(1), pp. 62-7.

Lahtz, C. and Pfeifer, G.P. (2011) 'Epigenetic changes of DNA repair genes in cancer', *J Mol Cell Biol*, 3(1), pp. 51-8.

Lai, C., Robinson, J., Clark, S., Stamp, G., Poulsom, R. and Silver, A. (2011) 'Elevation of WNT5A expression in polyp formation in Lkb1+/- mice and Peutz-Jeghers syndrome', *J Pathol*, 223(5), pp. 584-92.

Lallemand, F., Courilleau, D., Sabbah, M., Redeuilh, G. and Mester, J. (1996) 'Direct inhibition of the expression of cyclin D1 gene by sodium butyrate', *Biochem Biophys Res Commun*, 229(1), pp. 163-9.

Lanham-New, S.A., Macdonald, I.A. and Roche, H.M. (2011) *Nutrition and Metabolism*. 2 edn. Wiley-Blackwell.

Laukoetter, M.G., Mennigen, R., Hannig, C.M., Osada, N., Rijcken, E., Vowinkel, T., Krieglstein, C.F., Senninger, N., Anthoni, C. and Bruewer, M. (2011) 'Intestinal cancer risk in Crohn's disease: a meta-analysis', *J Gastrointest Surg*, 15(4), pp. 576-83.

Lazarova, D., Lee, A., Wong, T., Marian, B., Chiaro, C., Rainey, C. and Bordonaro, M. (2014a) 'Modulation of Wnt Activity and Cell Physiology by Butyrate in LT97 Microadenoma Cells', *J Cancer*, 5(3), pp. 203-13.

Lazarova, D.L., Bordonaro, M., Carbone, R. and Sartorelli, A.C. (2004) 'Linear relationship between Wnt activity levels and apoptosis in colorectal carcinoma cells exposed to butyrate', *Int J Cancer*, 110(4), pp. 523-31.

Lazarova, D.L., Chiaro, C. and Bordonaro, M. (2014b) 'Butyrate induced changes in Wnt-signaling specific gene expression in colorectal cancer cells', *BMC Res Notes*, 7, p. 226.

Le Leu, R.K., Brown, I.L., Hu, Y., Esterman, A. and Young, G.P. (2007) 'Suppression of azoxymethane-induced colon cancer development in rats by dietary resistant starch', *Cancer Biol Ther*, 6(10), pp. 1621-6.

Le Leu, R.K., Hu, Y., Brown, I.L., Woodman, R.J. and Young, G.P. (2010) 'Synbiotic intervention of Bifidobacterium lactis and resistant starch protects against colorectal cancer development in rats', *Carcinogenesis*, 31(2), pp. 246-51.

Lee, B.B., Lee, E.J., Jung, E.H., Chun, H.K., Chang, D.K., Song, S.Y., Park, J. and Kim, D.H. (2009) 'Aberrant methylation of APC, MGMT, RASSF2A, and Wif-1 genes in plasma as a biomarker for early detection of colorectal cancer', *Clin Cancer Res*, 15(19), pp. 6185-91.

Lee, E., Salic, A., Kruger, R., Heinrich, R. and Kirschner, M.W. (2003) 'The roles of APC and Axin derived from experimental and theoretical analysis of the Wnt pathway', *PLoS Biol*, 1(1), p. E10.

Lee, W.J. and Hase, K. (2014) 'Gut microbiota-generated metabolites in animal health and disease', *Nat Chem Biol*, 10(6), pp. 416-24.

Leslie, A., Carey, F.A., Pratt, N.R. and Steele, R.J. (2002) 'The colorectal adenoma-carcinoma sequence', *Br J Surg*, 89(7), pp. 845-60.

Lewis, B.P., Burge, C.B. and Bartel, D.P. (2005) 'Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets', *Cell*, 120(1), pp. 15-20.

Li, L.C. and Dahiya, R. (2002) 'MethPrimer: designing primers for methylation PCRs', *Bioinformatics*, 18(11), pp. 1427-31.

Li, Q. and Chen, H. (2012) 'Silencing of Wnt5a during colon cancer metastasis involves histone modifications', *Epigenetics*, 7(6), pp. 551-8.

Li, Q., Zou, C., Han, Z., Xiao, H., Wei, H., Wang, W., Zhang, L., Zhang, X., Tang, Q., Zhang, C., Tao, J., Wang, X. and Gao, X. (2013) 'MicroRNA-25 functions as a potential tumor suppressor in colon cancer by targeting Smad7', *Cancer Lett*, 335(1), pp. 168-74.

Li, X., Zhang, G., Li, Q., Xu, M., Feng, D. and Wu, W. (2012) '[Identification of aberrantly expressed miRNAs in rectal cancer]', *Zhong Nan Da Xue Xue Bao Yi Xue Ban*, 37(7), pp. 662-8.

Liang, P.S., Chen, T.Y. and Giovannucci, E. (2009) 'Cigarette smoking and colorectal cancer incidence and mortality: systematic review and meta-analysis', *Int J Cancer*, 124(10), pp. 2406-15.

Lin, J., Welker, N.C., Zhao, Z., Li, Y., Zhang, J., Reuss, S.A., Zhang, X., Lee, H., Liu, Y. and Bronner, M.P. (2014) 'Novel specific microRNA biomarkers in idiopathic inflammatory bowel disease unrelated to disease activity', *Mod Pathol*, 27(4), pp. 602-8.

Lin, S.Y., Yeh, K.T., Chen, W.T., Chen, H.C., Chen, S.T., Chiou, H.Y. and Chang, J.G. (2004) 'Promoter CpG methylation of tumor suppressor genes in colorectal cancer and its relationship to clinical features', *Oncol Rep*, 11(2), pp. 341-8.

Link, A., Balaguer, F., Shen, Y., Nagasaka, T., Lozano, J.J., Boland, C.R. and Goel, A. (2010) 'Fecal MicroRNAs as novel biomarkers for colon cancer screening', *Cancer Epidemiol Biomarkers Prev*, 19(7), pp. 1766-74.

Lipkin, M., Reddy, B., Newmark, H. and Lamprecht, S.A. (1999) 'Dietary factors in human colorectal cancer', *Annu Rev Nutr*, 19, pp. 545-86.

Lisanti, S., Omar, W.A., Tomaszewski, B., De Prins, S., Jacobs, G., Koppen, G., Mathers, J.C. and Langie, S.A. (2013) 'Comparison of methods for quantification of global DNA methylation in human cells and tissues', *PLoS One*, 8(11), p. e79044.

Liu, R. and Xu, G. (2008) 'Effects of resistant starch on colonic preneoplastic aberrant crypt foci in rats', *Food Chem Toxicol*, 46(8), pp. 2672-9.

Liu, W., Dong, X., Mai, M., Seelan, R.S., Taniguchi, K., Krishnadath, K.K., Halling, K.C., Cunningham, J.M., Boardman, L.A., Qian, C., Christensen, E., Schmidt, S.S., Roche, P.C., Smith, D.I. and Thibodeau, S.N. (2000) 'Mutations in AXIN2 cause colorectal cancer with defective mismatch repair by activating beta-catenin/TCF signalling', *Nat Genet*, 26(2), pp. 146-7.

Liu, X., Wu, S., Xia, Y., Li, X.E., Xia, Y., Zhou, Z.D. and Sun, J. (2011) 'Wingless homolog Wnt11 suppresses bacterial invasion and inflammation in intestinal epithelial cells', *Am J Physiol Gastrointest Liver Physiol*, 301(6), pp. G992-G1003.

Louis, P., Scott, K.P., Duncan, S.H. and Flint, H.J. (2007) 'Understanding the effects of diet on bacterial metabolism in the large intestine', *J Appl Microbiol*, 102(5), pp. 1197-208.

Luo, J., Chen, J., Deng, Z.L., Luo, X., Song, W.X., Sharff, K.A., Tang, N., Haydon, R.C., Luu, H.H. and He, T.C. (2007) 'Wnt signaling and human diseases: what are the therapeutic implications?', *Lab Invest*, 87(2), pp. 97-103.

Luo, X., Burwinkel, B., Tao, S. and Brenner, H. (2011) 'MicroRNA signatures: novel biomarker for colorectal cancer?', *Cancer Epidemiol Biomarkers Prev*, 20(7), pp. 1272-86.

Luo, X., Stock, C., Burwinkel, B. and Brenner, H. (2013) 'Identification and evaluation of plasma microRNAs for early detection of colorectal cancer', *PLoS One*, 8(5), p. e62880.

Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers, H., Schlag, P.M., Birchmeier, W. and Behrens, J. (2002) 'Negative feedback loop of Wnt signaling through upregulation of conductin/axin2 in colorectal and liver tumors', *Mol Cell Biol*, 22(4), pp. 1184-93.

Lutgens, M.W., van Oijen, M.G., van der Heijden, G.J., Vleggaar, F.P., Siersema, P.D. and Oldenburg, B. (2013) 'Declining risk of colorectal cancer in inflammatory bowel disease: an updated meta-analysis of population-based cohort studies', *Inflamm Bowel Dis*, 19(4), pp. 789-99.

Lynch, H.T. and de la Chapelle, A. (2003) 'Hereditary colorectal cancer', *N Engl J Med*, 348(10), pp. 919-32.

Lyu, Z., Chen, H., Jiang, L., Zheng, H. and Hu, J. (2014) '[Detection of RASSF2 and sFRP1 promoter region methylation in sporadic colorectal cancer patients]', *Zhonghua Wei Chang Wai Ke Za Zhi*, 17(1), pp. 41-4.

Ma, Q., Yang, L., Wang, C., Yu, Y.Y., Zhou, B. and Zhou, Z.G. (2011) '[Differential expression of colon cancer microRNA in microarry study]', *Sichuan Da Xue Xue Bao Yi Xue Ban*, 42(3), pp. 344-8.

Ma, Y., Yang, Y., Wang, F., Zhang, P., Shi, C., Zou, Y. and Qin, H. (2013) 'Obesity and risk of colorectal cancer: a systematic review of prospective studies', *PLoS One*, 8(1), p. e53916.

Ma, Y., Zhang, P., Yang, J., Liu, Z., Yang, Z. and Qin, H. (2012) 'Candidate microRNA biomarkers in human colorectal cancer: systematic review profiling studies and experimental validation', *Int J Cancer*, 130(9), pp. 2077-87.

Maeda, K., Chung, Y., Kang, S., Ogawa, M., Onoda, N., Nishiguchi, Y., Ikehara, T., Nakata, B., Okuno, M. and Sowa, M. (1998) 'Cyclin D1 overexpression and prognosis in colorectal adenocarcinoma', *Oncology*, 55(2), pp. 145-51.

Maglinte, D.D., Gourtsoyiannis, N., Rex, D., Howard, T.J. and Kelvin, F.M. (2003) 'Classification of small bowel Crohn's subtypes based on multimodality imaging', *Radiol Clin North Am*, 41(2), pp. 285-303.

Magrisso, I.J., Richmond, R.E., Carter, J.H., Pross, C.B., Gilfillen, R.A. and Carter, H.W. (1993) 'Immunohistochemical detection of RAS, JUN, FOS, and p53 oncoprotein expression in human colorectal adenomas and carcinomas', *Lab Invest*, 69(6), pp. 674-81.

Maier, S., Daroqui, M.C., Scherer, S., Roepcke, S., Velcich, A., Shenoy, S.M., Singer, R.H. and Augenlicht, L.H. (2009) 'Butyrate and vitamin D3 induce transcriptional attenuation at the cyclin D1 locus in colonic carcinoma cells', *J Cell Physiol*, 218(3), pp. 638-42.

Makivuokko, H., Nurmi, J., Nurminen, P., Stowell, J. and Rautonen, N. (2005) 'In vitro effects on polydextrose by colonic bacteria and caco-2 cell cyclooxygenase gene expression', *Nutr Cancer*, 52(1), pp. 94-104.

Malcomson, F. (2011) *Site-specific differences in WNT signalling within the colon*. Masters in Research (MRes) Dissertation thesis. Newcastle University.

Mann, B., Gelos, M., Siedow, A., Hanski, M.L., Gratchev, A., Ilyas, M., Bodmer, W.F., Moyer, M.P., Riecken, E.O., Buhr, H.J. and Hanski, C. (1999) 'Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas', *Proc Natl Acad Sci U S A*, 96(4), pp. 1603-8.

Mao, W., Wordinger, R.J. and Clark, A.F. (2010) 'Focus on molecules: SFRP1', *Exp Eye Res*, 91(5), pp. 552-3.

Martin, C., Connelly, A., Keku, T.O., Mountcastle, S.B., Galanko, J., Woosley, J.T., Schliebe, B., Lund, P.K. and Sandler, R.S. (2002) 'Nonsteroidal anti-inflammatory drugs, apoptosis, and colorectal adenomas', *Gastroenterology*, 123(6), pp. 1770-7.

Martinasso, G., Oraldi, M., Trombetta, A., Maggiora, M., Bertetto, O., Canuto, R.A. and Muzio, G. (2007) 'Involvement of PPARs in Cell Proliferation and Apoptosis in Human Colon Cancer Specimens and in Normal and Cancer Cell Lines', *PPAR Res*, 2007, p. 93416.

Martinez, I., Kim, J., Duffy, P.R., Schlegel, V.L. and Walter, J. (2010) 'Resistant starches types 2 and 4 have differential effects on the composition of the fecal microbiota in human subjects', *PLoS One*, 5(11), p. e15046.

Mathers, J.C., Movahedi, M., Macrae, F., Mecklin, J.P., Moeslein, G., Olschwang, S., Eccles, D., Evans, G., Maher, E.R., Bertario, L., Bisgaard, M.L., Dunlop, M., Ho, J.W., Hodgson, S., Lindblom, A., Lubinski, J., Morrison, P.J., Murday, V., Ramesar, R., Side, L., Scott, R.J., Thomas, H.J., Vasen, H., Gerdes, A.M., Barker, G., Crawford, G., Elliott, F., Pylvanainen, K., Wijnen, J., Fodde, R., Lynch, H., Bishop, D.T., Burn, J. and Investigators, C. (2012) 'Long-term effect of resistant starch on cancer risk in carriers of hereditary colorectal cancer: an analysis from the CAPP2 randomised controlled trial', *Lancet Oncol*, 13(12), pp. 1242-9.

Mathers, J.C., Smith, H. and Carter, S. (1997) 'Dose-response effects of raw potato starch on small-intestinal escape, large-bowel fermentation and gut transit time in the rat', *Br J Nutr*, 78(6), pp. 1015-29.

Maye, P., Zheng, J., Li, L. and Wu, D. (2004) 'Multiple mechanisms for Wnt11-mediated repression of the canonical Wnt signaling pathway', *J Biol Chem*, 279(23), pp. 24659-65.

Maziere, S., Meflah, K., Tavan, E., Champ, M., Narbonne, J.F. and Cassand, P. (1998) 'Effect of resistant starch and/or fat-soluble vitamins A and E on the initiation stage of aberrant crypts in rat colon', *Nutr Cancer*, 31(3), pp. 168-77.

McIntyre, A., Gibson, P.R. and Young, G.P. (1993) 'Butyrate production from dietary fibre and protection against large bowel cancer in a rat model', *Gut*, 34(3), pp. 386-91.

McKay, R.M., Peters, J.M. and Graff, J.M. (2001) 'The casein kinase I family in Wnt signaling', *Dev Biol*, 235(2), pp. 388-96.

McMahon, F.G. (1974) *Polydextrose Study Number III*. Tulane University, New Orleans, LA.

Medina, V., Afonso, J.J., Alvarez-Arguelles, H., Hernandez, C. and Gonzalez, F. (1998) 'Sodium butyrate inhibits carcinoma development in a 1,2-dimethylhydrazine-induced rat colon cancer', *JPEN J Parenter Enteral Nutr*, 22(1), pp. 14-7.

Menezes, C.C., de Deus Souza Carneiro, J., Borges, S.V., da Silva, V.S., Brigagao, M.R. and Azevedo, L. (2012) 'Development of low-calorie guava preserves with prebiotics and evaluation of their effects on carcinogenesis biomarkers in rats', *Food Chem Toxicol*, 50(10), pp. 3719-24.

Mentschel, J. and Claus, R. (2003) 'Increased butyrate formation in the pig colon by feeding raw potato starch leads to a reduction of colonocyte apoptosis and a shift to the stem cell compartment', *Metabolism*, 52(11), pp. 1400-5.

Mescher, A.L. (2010) *Junqueira's Basic Histology*. 12th Edition edn. United States of America: The McGraw-Hill Companies.

Metcalfe, C., Ibrahim, A.E., Graeb, M., de la Roche, M., Schwarz-Romond, T., Fiedler, M., Winton, D.J., Corfield, A. and Bienz, M. (2010) 'Dvl2 promotes intestinal length and neoplasia in the ApcMin mouse model for colorectal cancer', *Cancer Res*, 70(16), pp. 6629-38.

Michael, M.Z., SM, O.C., van Holst Pellekaan, N.G., Young, G.P. and James, R.J. (2003) 'Reduced accumulation of specific microRNAs in colorectal neoplasia', *Mol Cancer Res*, 1(12), pp. 882-91.

Migheli, F., Stoccoro, A., Coppede, F., Wan Omar, W.A., Failli, A., Consolini, R., Seccia, M., Spisni, R., Miccoli, P., Mathers, J.C. and Migliore, L. (2013) 'Comparison study of MS-HRM and pyrosequencing techniques for quantification of APC and CDKN2A gene methylation', *PLoS One*, 8(1), p. e52501.

Milicic, A., Harrison, L.A., Goodlad, R.A., Hardy, R.G., Nicholson, A.M., Presz, M., Sieber, O., Santander, S., Pringle, J.H., Mandir, N., East, P., Obszynska, J., Sanders, S., Piazuelo, E., Shaw, J., Harrison, R., Tomlinson, I.P., McDonald, S.A., Wright, N.A. and Jankowski, J.A. (2008) 'Ectopic expression of P-cadherin correlates with promoter hypomethylation early in colorectal carcinogenesis and enhanced intestinal crypt fission in vivo', *Cancer Res*, 68(19), pp. 7760-8.

Miller, D.M., Thomas, S.D., Islam, A., Muench, D. and Sedoris, K. (2012) 'c-Myc and cancer metabolism', *Clin Cancer Res*, 18(20), pp. 5546-53.

Mills, S.J., Mathers, J.C., Chapman, P.D., Burn, J. and Gunn, A. (2001) 'Colonic crypt cell proliferation state assessed by whole crypt microdissection in sporadic neoplasia and familial adenomatous polyposis', *Gut*, 48(1), pp. 41-6.

Monzo, M., Navarro, A., Bandres, E., Artells, R., Moreno, I., Gel, B., Ibeas, R., Moreno, J., Martinez, F., Diaz, T., Martinez, A., Balague, O. and Garcia-Foncillas, J. (2008) 'Overlapping expression of microRNAs in human embryonic colon and colorectal cancer', *Cell Res*, 18(8), pp. 823-33.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B. and Kinzler, K.W. (1997) 'Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC', *Science*, 275(5307), pp. 1787-90.

Morin, P.J., Vogelstein, B. and Kinzler, K.W. (1996) 'Apoptosis and APC in colorectal tumorigenesis', *Proc Natl Acad Sci U S A*, 93(15), pp. 7950-4.

Mortensen, F.V., Langkilde, N.C., Joergensen, J.C. and Hessov, I. (1999) 'Short-chain fatty acids stimulate mucosal cell proliferation in the closed human rectum after Hartmann's procedure', *Int J Colorectal Dis*, 14(3), pp. 150-4.

Moss, S.F., Liu, T.C., Petrotos, A., Hsu, T.M., Gold, L.I. and Holt, P.R. (1996a) 'Inward growth of colonic adenomatous polyps', *Gastroenterology*, 111(6), pp. 1425-32.

Moss, S.F., Scholes, J.V. and Holt, P.R. (1996b) 'Abnormalities of epithelial apoptosis in multistep colorectal neoplasia demonstrated by terminal deoxyuridine nick end labeling', *Dig Dis Sci*, 41(11), pp. 2238-47.

Motoyama, K., Inoue, H., Takatsuno, Y., Tanaka, F., Mimori, K., Uetake, H., Sugihara, K. and Mori, M. (2009) 'Over- and under-expressed microRNAs in human colorectal cancer', *Int J Oncol*, 34(4), pp. 1069-75.

Murphy, N., Norat, T., Ferrari, P., Jenab, M., Bueno-de-Mesquita, B., Skeie, G., Dahm, C.C., Overvad, K., Olsen, A., Tjonneland, A., Clavel-Chapelon, F., Boutron-Ruault, M.C., Racine, A., Kaaks, R., Teucher, B., Boeing, H., Bergmann, M.M., Trichopoulou, A., Trichopoulos, D., Lagiou, P., Palli, D., Pala, V., Panico, S., Tumino, R., Vineis, P., Siersema, P., van Duijnhoven, F., Peeters, P.H., Hjartaker, A., Engeset, D., Gonzalez, C.A., Sanchez, M.J., Dorronsoro, M., Navarro, C., Ardanaz, E., Quiros, J.R., Sonestedt, E., Ericson, U., Nilsson, L., Palmqvist, R., Khaw, K.T., Wareham, N., Key, T.J., Crowe, F.L., Fedirko, V., Wark, P.A., Chuang, S.C. and Riboli, E. (2012) 'Dietary fibre intake and risks of cancers of the colon and rectum in the European prospective investigation into cancer and nutrition (EPIC)', *PLoS One*, 7(6), p. e39361.

Nagel, R., le Sage, C., Diosdado, B., van der Waal, M., Oude Vrielink, J.A., Bolijn, A., Meijer, G.A. and Agami, R. (2008) 'Regulation of the adenomatous polyposis coli gene by the miR-135 family in colorectal cancer', *Cancer Res*, 68(14), pp. 5795-802.

Naghibalhossaini, F., Zamani, M., Mokarram, P., Khalili, I., Rasti, M. and Mostafavi-Pour, Z. (2012) 'Epigenetic and genetic analysis of WNT signaling pathway in sporadic colorectal cancer patients from Iran', *Mol Biol Rep*, 39(5), pp. 6171-8.

Najdi, R., Holcombe, R.F. and Waterman, M.L. (2011) 'Wnt signaling and colon carcinogenesis: beyond APC', *J Carcinog*, 10, p. 5.

Nakamura, T., Sakai, T. and Nariya, S. (1995) 'Cell death in colorectal polyps as evaluated by in situ 3'-tailing reaction and its relationship to BCL-2 expression', *Pathol Int*, 45(10), pp. 721-8.

Nakashima, M., Meirmanov, S., Matsufuji, R., Hayashida, M., Fukuda, E., Naito, S., Matsuu, M., Shichijo, K., Kondo, H., Ito, M., Yamashita, S. and Sekine, I. (2002) 'Altered expression of beta-catenin during radiation-induced colonic carcinogenesis', *Pathol Res Pract*, 198(11), pp. 717-24.

Ng, E.K., Chong, W.W., Jin, H., Lam, E.K., Shin, V.Y., Yu, J., Poon, T.C., Ng, S.S. and Sung, J.J. (2009) 'Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening', *Gut*, 58(10), pp. 1375-81.

Nguyen, A.V., Albers, C.G. and Holcombe, R.F. (2010) 'Differentiation of tubular and villous adenomas based on Wnt pathway-related gene expression profiles', *Int J Mol Med*, 26(1), pp. 121-5.

Niba, L.L. (2002) 'Resistant starch: a potential functional food ingredient', *Nutrition & Food Science*, 32(2), pp. 62-67.

Nieminen, U., Jussila, A., Nordling, S., Mustonen, H. and Farkkila, M.A. (2014) 'Inflammation and disease duration have a cumulative effect on the risk of dysplasia and carcinoma in IBD: a case-control observational study based on registry data', *Int J Cancer*, 134(1), pp. 189-96.

Nilsson, A.C., Ostman, E.M., Holst, J.J. and Bjorck, I.M. (2008) 'Including indigestible carbohydrates in the evening meal of healthy subjects improves glucose tolerance, lowers inflammatory markers, and increases satiety after a subsequent standardized breakfast', *J Nutr*, 138(4), pp. 732-9.

Nishida, N., Nagahara, M., Sato, T., Mimori, K., Sudo, T., Tanaka, F., Shibata, K., Ishii, H., Sugihara, K., Doki, Y. and Mori, M. (2012) 'Microarray analysis of colorectal cancer stromal tissue reveals upregulation of two oncogenic miRNA clusters', *Clin Cancer Res*, 18(11), pp. 3054-70.

Nishioka, M., Ueno, K., Hazama, S., Okada, T., Sakai, K., Suehiro, Y., Okayama, N., Hirata, H., Oka, M., Imai, K., Dahiya, R. and Hinoda, Y. (2013) 'Possible involvement of Wnt11 in colorectal cancer progression', *Mol Carcinog*, 52(3), pp. 207-17.

Noakes, M., Clifton, P.M., Nestel, P.J., Le Leu, R. and McIntosh, G. (1996) 'Effect of high-amylose starch and oat bran on metabolic variables and bowel function in subjects with hypertriglyceridemia', *Am J Clin Nutr*, 64(6), pp. 944-51.

Nofrarias, M., Martinez-Puig, D., Pujols, J., Majo, N. and Perez, J.F. (2007) 'Long-term intake of resistant starch improves colonic mucosal integrity and reduces gut apoptosis and blood immune cells', *Nutrition*, 23(11-12), pp. 861-70.

Nugent, M., Miller, N. and Kerin, M.J. (2012) 'Circulating miR-34a levels are reduced in colorectal cancer', *J Surg Oncol*, 106(8), pp. 947-52.

O'Keefe, S.J. (2008) 'Nutrition and colonic health: the critical role of the microbiota', *Curr Opin Gastroenterol*, 24(1), pp. 51-8.

O'Keefe, S.J., Chung, D., Mahmoud, N., Sepulveda, A.R., Manafe, M., Arch, J., Adada, H. and van der Merwe, T. (2007) 'Why do African Americans get more colon cancer than Native Africans?', *J Nutr*, 137(1 Suppl), pp. 175S-182S.

O'Keefe, S.J., Ou, J., Aufreiter, S., O'Connor, D., Sharma, S., Sepulveda, J., Fukuwatari, T., Shibata, K. and Mawhinney, T. (2009) 'Products of the colonic microbiota mediate the effects of diet on colon cancer risk', *J Nutr*, 139(11), pp. 2044-8.

Oberg, A.L., French, A.J., Sarver, A.L., Subramanian, S., Morlan, B.W., Riska, S.M., Borralho, P.M., Cunningham, J.M., Boardman, L.A., Wang, L., Smyrk, T.C., Asmann, Y., Steer, C.J. and Thibodeau, S.N. (2011) 'miRNA expression in colon polyps provides evidence for a multihit model of colon cancer', *PLoS One*, 6(6), p. e20465.

Oltvai, Z.N., Milliman, C.L. and Korsmeyer, S.J. (1993) 'Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death', *Cell*, 74(4), pp. 609-19.

Ouko, L., Ziegler, T.R., Gu, L.H., Eisenberg, L.M. and Yang, V.W. (2004) 'Wnt11 signaling promotes proliferation, transformation, and migration of IEC6 intestinal epithelial cells', *J Biol Chem*, 279(25), pp. 26707-15.

Ouyang, H., Furukawa, T., Abe, T., Kato, Y. and Horii, A. (1998) 'The BAX gene, the promoter of apoptosis, is mutated in genetically unstable cancers of the colorectum, stomach, and endometrium', *Clin Cancer Res*, 4(4), pp. 1071-4.

Paganelli, G.M., Higgins, P.J., Biasco, G., Lipkin, M., Brandi, G., Santucci, R., Miglioli, M. and Barbara, L. (1993) 'Abnormal rectal cell proliferation and p52/p35 protein expression in patients with ulcerative colitis', *Cancer Lett*, 73(1), pp. 23-8.

Pandey, S., Gordon, P.H. and Wang, E. (1995) 'Expression of proliferation-specific genes in the mucosa adjacent to colon carcinoma', *Dis Colon Rectum*, 38(5), pp. 462-7.

Parkin, D.M. and Boyd, L. (2011) '8. Cancers attributable to overweight and obesity in the UK in 2010', *Br J Cancer*, 105 Suppl 2, pp. S34-7.

Parkin, D.M., Boyd, L. and Walker, L.C. (2011) '16. The fraction of cancer attributable to lifestyle and environmental factors in the UK in 2010', *Br J Cancer*, 105 Suppl 2, pp. S77-81.

Pekow, J.R., Dougherty, U., Mustafi, R., Zhu, H., Kocherginsky, M., Rubin, D.T., Hanauer, S.B., Hart, J., Chang, E.B., Fichera, A., Joseph, L.J. and Bissonnette, M. (2012) 'miR-143 and miR-145 are downregulated in ulcerative colitis: putative regulators of inflammation and protooncogenes', *Inflamm Bowel Dis*, 18(1), pp. 94-100.

Perrin, P., Pierre, F., Patry, Y., Champ, M., Berreur, M., Pradal, G., Bornet, F., Meflah, K. and Menanteau, J. (2001) 'Only fibres promoting a stable butyrate producing colonic ecosystem decrease the rate of aberrant crypt foci in rats', *Gut*, 48(1), pp. 53-61.

Peters, J.M., McKay, R.M., McKay, J.P. and Graff, J.M. (1999) 'Casein kinase I transduces Wnt signals', *Nature*, 401(6751), pp. 345-50.

Phelps, R.A., Chidester, S., Dehghanizadeh, S., Phelps, J., Sandoval, I.T., Rai, K., Broadbent, T., Sarkar, S., Burt, R.W. and Jones, D.A. (2009) 'A two-step model for colon adenoma initiation and progression caused by APC loss', *Cell*, 137(4), pp. 623-34.

Pinto, D., Gregorieff, A., Begthel, H. and Clevers, H. (2003) 'Canonical Wnt signals are essential for homeostasis of the intestinal epithelium', *Genes Dev*, 17(14), pp. 1709-13.

Ponz de Leon, M., Roncucci, L., Di Donato, P., Tassi, L., Smerieri, O., Amorico, M.G., Malagoli, G., De Maria, D., Antonioli, A., Chahin, N.J. and et al. (1988) 'Pattern of epithelial cell proliferation in colorectal mucosa of normal subjects and of patients with adenomatous polyps or cancer of the large bowel', *Cancer Res*, 48(14), pp. 4121-6.

Potten, C.S., Kellett, M., Rew, D.A. and Roberts, S.A. (1992a) 'Proliferation in human gastrointestinal epithelium using bromodeoxyuridine in vivo: data for different sites, proximity to a tumour, and polyposis coli', *Gut*, 33(4), pp. 524-9.

Potten, C.S., Kellett, M., Roberts, S.A., Rew, D.A. and Wilson, G.D. (1992b) 'Measurement of in vivo proliferation in human colorectal mucosa using bromodeoxyuridine', *Gut*, 33(1), pp. 71-8.

Powell, S.M., Zilz, N., Beazer-Barclay, Y., Bryan, T.M., Hamilton, S.R., Thibodeau, S.N., Vogelstein, B. and Kinzler, K.W. (1992) 'APC mutations occur early during colorectal tumorigenesis', *Nature*, 359(6392), pp. 235-7.

Pronczuk, A. and Hayes, K.C. (2006) 'Hypocholesterolemic effect of dietary polydextrose in gerbils and humans', *Nutrition Research*, 26(1), pp. 27-31.

Pryde, S.E., Duncan, S.H., Hold, G.L., Stewart, C.S. and Flint, H.J. (2002) 'The microbiology of butyrate formation in the human colon', *FEMS Microbiol Lett*, 217(2), pp. 133-9.

Pu, X.X., Huang, G.L., Guo, H.Q., Guo, C.C., Li, H., Ye, S., Ling, S., Jiang, L., Tian, Y. and Lin, T.Y. (2010) 'Circulating miR-221 directly amplified from plasma is a potential diagnostic and prognostic marker of colorectal cancer and is correlated with p53 expression', *J Gastroenterol Hepatol*, 25(10), pp. 1674-80.

Pulkkinen, K., Murugan, S. and Vainio, S. (2008) 'Wnt signaling in kidney development and disease', *Organogenesis*, 4(2), pp. 55-9.

Purwani, E., Iskandriati, D. and Suhartono, M. (2012) 'Fermentation product of RS3 inhibited proliferation and induced apoptosis in colon cancer cell HCT-116', *Advances in Bioscience and Biotechnology*, 3, pp. 1189-1198.

Putaala, H., Makivuokko, H., Tiihonen, K. and Rautonen, N. (2011) 'Simulated colon fiber metabolome regulates genes involved in cell cycle, apoptosis, and energy metabolism in human colon cancer cells', *Mol Cell Biochem*, 357(1-2), pp. 235-45.

Qi, J., Zhu, Y.Q., Luo, J. and Tao, W.H. (2006) 'Hypermethylation and expression regulation of secreted frizzled-related protein genes in colorectal tumor', *World J Gastroenterol*, 12(44), pp. 7113-7.

Qi, J., Zhu, Y.Q., Luo, J. and Tao, W.H. (2007) '[The role of secreted Wnt-antagonist genes hypermethylation in early detection of colorectal tumor]', *Zhonghua Yi Xue Za Zhi*, 87(28), pp. 1954-7.

Qiagen (2014) *Pyrosequencing technology and platform overview.* Available at: http://www.qiagen.com/gb/resources/technologies/pyrosequencing-resource-center/technology-overview/ (Accessed: 15th September 2014).

Ramzan, N.N., Leighton, J.A., Heigh, R.I. and Shapiro, M.S. (2002) 'Clinical significance of granuloma in Crohn's disease', *Inflamm Bowel Dis*, 8(3), pp. 168-73.

Rao, T.P. and Kuhl, M. (2010) 'An updated overview on Wnt signaling pathways: a prelude for more', *Circ Res*, 106(12), pp. 1798-806.

Rawson, J.B., Manno, M., Mrkonjic, M., Daftary, D., Dicks, E., Buchanan, D.D., Younghusband, H.B., Parfrey, P.S., Young, J.P., Pollett, A., Green, R.C., Gallinger, S., McLaughlin, J.R., Knight, J.A. and Bapat, B. (2011) 'Promoter methylation of Wnt antagonists DKK1 and SFRP1 is associated with opposing tumor subtypes in two large populations of colorectal cancer patients', *Carcinogenesis*, 32(5), pp. 741-7.

Risio, M., Lipkin, M., Candelaresi, G., Bertone, A., Coverlizza, S. and Rossini, F.P. (1991) 'Correlations between rectal mucosa cell proliferation and the clinical and pathological features of nonfamilial neoplasia of the large intestine', *Cancer Res*, 51(7), pp. 1917-21.

Roberts, J., Jones, G.P., Rutishauser, I.H.E., Birkett, A. and Gibbons, C. (2004) 'Resistant starch in the Australian diet', *Nutrition and Dietetics*, 61(2), pp. 98-104.

Robertson, K.D. (2001) 'DNA methylation, methyltransferases, and cancer', *Oncogene*, 20(24), pp. 3139-55.

Robertson, K.D. (2005) 'DNA methylation and human disease', *Nat Rev Genet*, 6(8), pp. 597-610.

Robertson, M.D., Currie, J.M., Morgan, L.M., Jewell, D.P. and Frayn, K.N. (2003) 'Prior short-term consumption of resistant starch enhances postprandial insulin sensitivity in healthy subjects', *Diabetologia*, 46(5), pp. 659-65.

Roediger, W.E. (1980) 'Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man', *Gut*, 21(9), pp. 793-8.

Rogler, G. (2014) 'Chronic ulcerative colitis and colorectal cancer', *Cancer Lett*, 345(2), pp. 235-41.

Roncucci, L., Scalmati, A. and Ponz de Leon, M. (1991) 'Pattern of cell kinetics in colorectal mucosa of patients with different types of adenomatous polyps of the large bowel', *Cancer*, 68(4), pp. 873-8.

Rosman-Urbach, M., Niv, Y., Birk, Y., Smirnoff, P., Zusman, I., Morgenstern, S. and Schwartz, B. (2004) 'A high degree of aneuploidy, loss of p53 gene, and low soluble p53 protein serum levels are detected in ulcerative colitis patients', *Dis Colon Rectum*, 47(3), pp. 304-13.

Ross, M.H. and Pawlina, W. (2011) *Histology; a text and atlas*. 6th Edition edn.

Roy, S., Levi, E., Majumdar, A.P. and Sarkar, F.H. (2012) 'Expression of miR-34 is lost in colon cancer which can be re-expressed by a novel agent CDF', *J Hematol Oncol*, 5, p. 58.

Ruby, J.G., Jan, C., Player, C., Axtell, M.J., Lee, W., Nusbaum, C., Ge, H. and Bartel, D.P. (2006) 'Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans', *Cell*, 127(6), pp. 1193-207.

Ruemmele, F.M., Dionne, S., Qureshi, I., Sarma, D.S., Levy, E. and Seidman, E.G. (1999) 'Butyrate mediates Caco-2 cell apoptosis via up-regulation of pro-apoptotic BAK and inducing caspase-3 mediated cleavage of poly-(ADP-ribose) polymerase (PARP)', *Cell Death Differ*, 6(8), pp. 729-35.

Ruemmele, F.M., Schwartz, S., Seidman, E.G., Dionne, S., Levy, E. and Lentze, M.J. (2003) 'Butyrate induced Caco-2 cell apoptosis is mediated via the mitochondrial pathway', *Gut*, 52(1), pp. 94-100.

Russo, I., Luciani, A., De Cicco, P., Troncone, E. and Ciacci, C. (2012) 'Butyrate attenuates lipopolysaccharide-induced inflammation in intestinal cells and Crohn's mucosa through modulation of antioxidant defense machinery', *PLoS One*, 7(3), p. e32841.

Rustgi, A.K. (2007) 'The genetics of hereditary colon cancer', *Genes Dev*, 21(20), pp. 2525-38.

Rutter, M.D., Saunders, B.P., Wilkinson, K.H., Rumbles, S., Schofield, G., Kamm, M.A., Williams, C.B., Price, A.B., Talbot, I.C. and Forbes, A. (2006) 'Thirty-year analysis of a colonoscopic surveillance program for neoplasia in ulcerative colitis', *Gastroenterology*, 130(4), pp. 1030-8.

Sakamoto, J., Nakaji, S., Sugawara, K., Iwane, S. and Munakata, A. (1996) 'Comparison of resistant starch with cellulose diet on 1,2-dimethylhydrazine-induced colonic carcinogenesis in rats', *Gastroenterology*, 110(1), pp. 116-20.

Sakamoto, T., Matsuda, T., Nakajima, T. and Saito, Y. (2013) 'Clinicopathological features of colorectal polyps: evaluation of the 'predict, resect and discard' strategies', *Colorectal Dis*, 15(6), pp. e295-300.

Salehi, R., Mohammadi, M., Emami, M.H. and Salehi, A.R. (2012) 'Methylation pattern of SFRP1 promoter in stool sample is a potential marker for early detection of colorectal cancer', *Adv Biomed Res*, 1, p. 87.

Sandler, R.S., Stewart, W.F., Liberman, J.N., Ricci, J.A. and Zorich, N.L. (2000) 'Abdominal pain, bloating, and diarrhea in the United States: prevalence and impact', *Dig Dis Sci*, 45(6), pp. 1166-71.

Sarver, A.L., French, A.J., Borralho, P.M., Thayanithy, V., Oberg, A.L., Silverstein, K.A., Morlan, B.W., Riska, S.M., Boardman, L.A., Cunningham, J.M., Subramanian, S., Wang, L., Smyrk, T.C., Rodrigues, C.M., Thibodeau, S.N. and Steer, C.J. (2009) 'Human colon cancer profiles show differential microRNA expression depending on mismatch repair status and are characteristic of undifferentiated proliferative states', *BMC Cancer*, 9, p. 401.

Schee, K., Boye, K., Abrahamsen, T.W., Fodstad, O. and Flatmark, K. (2012) 'Clinical relevance of microRNA miR-21, miR-31, miR-92a, miR-101, miR-106a and miR-145 in colorectal cancer', *BMC Cancer*, 12, p. 505.

Schepeler, T., Reinert, J.T., Ostenfeld, M.S., Christensen, L.L., Silahtaroglu, A.N., Dyrskjot, L., Wiuf, C., Sorensen, F.J., Kruhoffer, M., Laurberg, S., Kauppinen, S., Orntoft, T.F. and Andersen, C.L. (2008) 'Diagnostic and prognostic microRNAs in stage II colon cancer', *Cancer Res*, 68(15), pp. 6416-24.

Scheppach, W., Bartram, P., Richter, A., Richter, F., Liepold, H., Dusel, G., Hofstetter, G., Ruthlein, J. and Kasper, H. (1992) 'Effect of short-chain fatty acids on the human colonic mucosa in vitro', *JPEN J Parenter Enteral Nutr*, 16(1), pp. 43-8.

Scheppach, W., Fabian, C., Sachs, M. and Kasper, H. (1988) 'The effect of starch malabsorption on fecal short-chain fatty acid excretion in man', *Scand J Gastroenterol*, 23(6), pp. 755-9.

Schetter, A.J., Leung, S.Y., Sohn, J.J., Zanetti, K.A., Bowman, E.D., Yanaihara, N., Yuen, S.T., Chan, T.L., Kwong, D.L., Au, G.K., Liu, C.G., Calin, G.A., Croce, C.M. and Harris, C.C. (2008) 'MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma', *JAMA*, 299(4), pp. 425-36.

Schneikert, J. and Behrens, J. (2007) 'The canonical Wnt signalling pathway and its APC partner in colon cancer development', *Gut*, 56(3), pp. 417-25.

Segditsas, S. and Tomlinson, I. (2006) 'Colorectal cancer and genetic alterations in the Wnt pathway', *Oncogene*, 25(57), pp. 7531-7.

Seidelin, J.B., Coskun, M. and Nielsen, O.H. (2013) 'Mucosal healing in ulcerative colitis: pathophysiology and pharmacology', *Adv Clin Chem*, 59, pp. 101-23.

Seidelin, J.B. and Nielsen, O.H. (2006) 'Expression profiling of apoptosis-related genes in enterocytes isolated from patients with ulcerative colitis', *APMIS*, 114(7-8), pp. 508-17.

Seidelin, J.B. and Nielsen, O.H. (2009) 'Epithelial apoptosis: cause or consequence of ulcerative colitis?', *Scand J Gastroenterol*, 44(12), pp. 1429-34.

Serafini, E.P., Kirk, A.P. and Chambers, T.J. (1981) 'Rate and pattern of epithelial cell proliferation in ulcerative colitis', *Gut*, 22(8), pp. 648-52.

Shao, S.X., Liao, X.J., Zhang, Y.X., Qiu, J.M., Zhang, X.F. and Yang, G.G. (2012) '[Multi-gene methylation detection increases positive methylation rate in colorectal cancer]', *Zhonghua Wei Chang Wai Ke Za Zhi*, 15(6), pp. 629-32.

Sharrard, R.M., Royds, J.A., Rogers, S. and Shorthouse, A.J. (1992) 'Patterns of methylation of the c-myc gene in human colorectal cancer progression', *Br J Cancer*, 65(5), pp. 667-72.

Shenoy, A.K., Fisher, R.C., Butterworth, E.A., Pi, L., Chang, L.J., Appelman, H.D., Chang, M., Scott, E.W. and Huang, E.H. (2012) 'Transition from colitis to cancer: high Wnt activity sustains the tumor-initiating potential of colon cancer stem cell precursors', *Cancer Res*, 72(19), pp. 5091-100.

Shin, H., Kim, J.H., Lee, Y.S. and Lee, Y.C. (2012) 'Change in gene expression profiles of secreted frizzled-related proteins (SFRPs) by sodium butyrate in gastric cancers: induction of promoter demethylation and histone modification causing inhibition of Wnt signaling', *Int J Oncol*, 40(5), pp. 1533-42.

Shpitz, B., Bomstein, Y., Mekori, Y., Cohen, R., Kaufman, Z., Grankin, M. and Bernheim, J. (1997) 'Proliferating cell nuclear antigen as a marker of cell kinetics in aberrant crypt foci, hyperplastic polyps, adenomas, and adenocarcinomas of the human colon', *Am J Surg*, 174(4), pp. 425-30.

Shussman, N. and Wexner, S.D. (2014) 'Colorectal polyps and polyposis syndromes', *Gastroenterol Rep (Oxf)*, 2(1), pp. 1-15.

Sierra, J., Yoshida, T., Joazeiro, C.A. and Jones, K.A. (2006) 'The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes', *Genes Dev*, 20(5), pp. 586-600.

Sikora, K., Chan, S., Evan, G., Gabra, H., Markham, N., Stewart, J. and Watson, J. (1987) 'c-myc oncogene expression in colorectal cancer', *Cancer*, 59(7), pp. 1289-95.

Singh, N., Gurav, A., Sivaprakasam, S., Brady, E., Padia, R., Shi, H., Thangaraju, M., Prasad, P.D., Manicassamy, S., Munn, D.H., Lee, J.R., Offermanns, S. and Ganapathy, V. (2014) 'Activation of Gpr109a, receptor for niacin and the commensal metabolite butyrate, suppresses colonic inflammation and carcinogenesis', *Immunity*, 40(1), pp. 128-39.

Sinicrope, F.A., Roddey, G., McDonnell, T.J., Shen, Y., Cleary, K.R. and Stephens, L.C. (1996) 'Increased apoptosis accompanies neoplastic development in the human colorectum', *Clin Cancer Res*, 2(12), pp. 1999-2006.

Sipos, F., Molnar, B., Zagoni, T., Berczi, L. and Tulassay, Z. (2005) 'Growth in epithelial cell proliferation and apoptosis correlates specifically to the inflammation activity of inflammatory bowel diseases: ulcerative colitis shows specific p53- and EGFR expression alterations', *Dis Colon Rectum*, 48(4), pp. 775-86.

Sipos, F., Zagoni, T., Molnar, B. and Tulassay, Z. (2002) '[Changes in the proliferation and apoptosis of colonic epithelial cells in correlation with histologic activity of ulcerative colitis]', *Orv Hetil*, 143(44), pp. 2485-8.

Slaby, O., Svoboda, M., Fabian, P., Smerdova, T., Knoflickova, D., Bednarikova, M., Nenutil, R. and Vyzula, R. (2007) 'Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer', *Oncology*, 72(5-6), pp. 397-402.

Slattery, M.L. (2004) 'Physical activity and colorectal cancer', *Sports Med*, 34(4), pp. 239-52.

Smith, D.R., Myint, T. and Goh, H.S. (1993) 'Over-expression of the c-myc proto-oncogene in colorectal carcinoma', *Br J Cancer*, 68(2), pp. 407-13.

Smith, E.A. and Macfarlane, G.T. (1997) 'Formation of Phenolic and Indolic Compounds by Anaerobic Bacteria in the Human Large Intestine', *Microb Ecol*, 33(3), pp. 180-8.

Smith, K., Bui, T.D., Poulsom, R., Kaklamanis, L., Williams, G. and Harris, A.L. (1999) 'Up-regulation of macrophage wnt gene expression in adenoma-carcinoma progression of human colorectal cancer', *Br J Cancer*, 81(3), pp. 496-502.

Smith, Z.D. and Meissner, A. (2013) 'DNA methylation: roles in mammalian development', *Nat Rev Genet*, 14(3), pp. 204-20.

Souleimani, A. and Asselin, C. (1993) 'Regulation of c-myc expression by sodium butyrate in the colon carcinoma cell line Caco-2', *FEBS Lett*, 326(1-3), pp. 45-50.

Sparmann, A. and van Lohuizen, M. (2006) 'Polycomb silencers control cell fate, development and cancer', *Nat Rev Cancer*, 6(11), pp. 846-56.

Stempelj, M., Kedinger, M., Augenlicht, L. and Klampfer, L. (2007) 'Essential role of the JAK/STAT1 signaling pathway in the expression of inducible nitric-oxide synthase in intestinal epithelial cells and its regulation by butyrate', *J Biol Chem*, 282(13), pp. 9797-804.

Stewart, J., Evan, G., Watson, J. and Sikora, K. (1986) 'Detection of the c-myc oncogene product in colonic polyps and carcinomas', *Br J Cancer*, 53(1), pp. 1-6.

Stowell, J.D. (2009a) 'Polydextrose', in *Fiber ingredients: food applications and health benefits*. Taylor & Francis Group, pp. 173-204.

Stowell, J.D. (2009b) 'Prebiotic Potential of Polydextrose', in Rastall, D.C.a.R.A. (ed.) *Prebiotics and Probiotics Science and Technology*. Springer Science + Business Media, LLC, p. pp. 337.

Strater, J., Koretz, K., Gunthert, A.R. and Moller, P. (1995) 'In situ detection of enterocytic apoptosis in normal colonic mucosa and in familial adenomatous polyposis', *Gut*, 37(6), pp. 819-25.

Strillacci, A., Valerii, M.C., Sansone, P., Caggiano, C., Sgromo, A., Vittori, L., Fiorentino, M., Poggioli, G., Rizzello, F., Campieri, M. and Spisni, E. (2013) 'Loss of miR-101 expression promotes Wnt/beta-catenin signalling pathway activation and malignancy in colon cancer cells', *J Pathol*, 229(3), pp. 379-89.

Struhl, K. (1998) 'Histone acetylation and transcriptional regulatory mechanisms', *Genes Dev*, 12(5), pp. 599-606.

Sun, J.Y., Huang, Y., Li, J.P., Zhang, X., Wang, L., Meng, Y.L., Yan, B., Bian, Y.Q., Zhao, J., Wang, W.Z., Yang, A.G. and Zhang, R. (2012) 'MicroRNA-320a suppresses human colon cancer cell proliferation by directly targeting beta-catenin', *Biochem Biophys Res Commun*, 420(4), pp. 787-92.

Suzuki, H., Gabrielson, E., Chen, W., Anbazhagan, R., van Engeland, M., Weijenberg, M.P., Herman, J.G. and Baylin, S.B. (2002) 'A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer', *Nat Genet*, 31(2), pp. 141-9.

Suzuki, H., Watkins, D.N., Jair, K.W., Schuebel, K.E., Markowitz, S.D., Chen, W.D., Pretlow, T.P., Yang, B., Akiyama, Y., Van Engeland, M., Toyota, M., Tokino, T., Hinoda, Y., Imai, K., Herman, J.G. and Baylin, S.B. (2004) 'Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer', *Nat Genet*, 36(4), pp. 417-22.

Suzuki, K., Suzuki, I., Leodolter, A., Alonso, S., Horiuchi, S., Yamashita, K. and Perucho, M. (2006) 'Global DNA demethylation in gastrointestinal cancer is age dependent and precedes genomic damage', *Cancer Cell*, 9(3), pp. 199-207.

Takagi, T., Naito, Y., Mizushima, K., Hirata, I., Yagi, N., Tomatsuri, N., Ando, T., Oyamada, Y., Isozaki, Y., Hongo, H., Uchiyama, K., Handa, O., Kokura, S., Ichikawa, H. and Yoshikawa, T. (2010) 'Increased expression of microRNA in the inflamed colonic mucosa of patients with active ulcerative colitis', *J Gastroenterol Hepatol*, 25 Suppl 1, pp. S129-33.

Tang, D., Liu, J., Wang, D.R., Yu, H.F., Li, Y.K. and Zhang, J.Q. (2011) 'Diagnostic and prognostic value of the methylation status of secreted frizzled-related protein 2 in colorectal cancer', *Clin Invest Med*, 34(2), pp. E88-95.

Tao, Q., Yokota, C., Puck, H., Kofron, M., Birsoy, B., Yan, D., Asashima, M., Wylie, C.C., Lin, X. and Heasman, J. (2005) 'Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos', *Cell*, 120(6), pp. 857-71.

Tarmin, L., Yin, J., Harpaz, N., Kozam, M., Noordzij, J., Antonio, L.B., Jiang, H.Y., Chan, O., Cymes, K. and Meltzer, S.J. (1995) 'Adenomatous polyposis coli gene mutations in ulcerative colitis-associated dysplasias and cancers versus sporadic colon neoplasms', *Cancer Res*, 55(10), pp. 2035-8.

Tateyama, H., Li, W., Takahashi, E., Miura, Y., Sugiura, H. and Eimoto, T. (2002) 'Apoptosis index and apoptosis-related antigen expression in serrated adenoma of the colorectum: the saw-toothed structure may be related to inhibition of apoptosis', *Am J Surg Pathol*, 26(2), pp. 249-56.

Taylor, C.W., Kim, Y.S., Childress-Fields, K.E. and Yeoman, L.C. (1992) 'Sensitivity of nuclear c-myc levels and induction to differentiation-inducing agents in human colon tumor cell lines', *Cancer Lett*, 62(2), pp. 95-105.

Thomas, M., Lieberman, J. and Lal, A. (2010) 'Desperately seeking microRNA targets', *Nat Struct Mol Biol*, 17(10), pp. 1169-74.

Thorup, I., Meyer, O. and Kristiansen, E. (1995) 'Effect of potato starch, cornstarch and sucrose on aberrant crypt foci in rats exposed to azoxymethane', *Anticancer Res*, 15(5B), pp. 2101-5.

Tice, D.A., Soloviev, I. and Polakis, P. (2002) 'Activation of the Wnt pathway interferes with serum response element-driven transcription of immediate early genes', *J Biol Chem*, 277(8), pp. 6118-23.

Toden, S., Bird, A.R., Topping, D.L. and Conlon, M.A. (2005) 'Resistant starch attenuates colonic DNA damage induced by higher dietary protein in rats', *Nutr Cancer*, 51(1), pp. 45-51.

Toden, S., Bird, A.R., Topping, D.L. and Conlon, M.A. (2006) 'Resistant starch prevents colonic DNA damage induced by high dietary cooked red meat or casein in rats', *Cancer Biol Ther*, 5(3), pp. 267-72.

Topping, D.L. and Clifton, P.M. (2001) 'Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides', *Physiol Rev*, 81(3), pp. 1031-64.

Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J.G., Baylin, S.B. and Issa, J.P. (1999) 'CpG island methylator phenotype in colorectal cancer', *Proc Natl Acad Sci U S A*, 96(15), pp. 8681-6.

Triantafillidis, J.K., Nasioulas, G. and Kosmidis, P.A. (2009) 'Colorectal cancer and inflammatory bowel disease: epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies', *Anticancer Res*, 29(7), pp. 2727-37.

Tsai, I.C., Woolf, M., Neklason, D.W., Branford, W.W., Yost, H.J., Burt, R.W. and Virshup, D.M. (2007) 'Disease-associated casein kinase I delta mutation may promote adenomatous polyps formation via a Wnt/beta-catenin independent mechanism', *Int J Cancer*, 120(5), pp. 1005-12.

Tsai, L.C., Hung, M.W., Chang, G.G. and Chang, T.C. (2000) 'Apoptosis induced by the sodium butyrate in human gastric cancer TMK-1 cells', *Anticancer Res*, 20(4), pp. 2441-8.

Tsamandas, A.C., Kardamakis, D., Petsas, T., Zolota, V., Vassiliou, V., Matatsoris, T., Kalofonos, H., Vagianos, C.E. and Scopa, C.D. (2007) 'Bcl-2, bax and p53 expression in rectal adenocarcinoma. Correlation with classic pathologic prognostic factors and patients' outcome', *In Vivo*, 21(1), pp. 113-8.

Tsikitis, V.L., White, I., Mori, M., Potter, A., Bhattcharyya, A., Hamilton, S.R., Buckmeier, J., Lance, P. and Thompson, P. (2014) 'Differential expression of microRNA-320a, -145, and -192 along the continuum of normal mucosa to high-grade dysplastic adenomas of the colorectum', *Am J Surg*, 207(5), pp. 717-22; discussion 722.

Tsuchida, A., Ohno, S., Wu, W., Borjigin, N., Fujita, K., Aoki, T., Ueda, S., Takanashi, M. and Kuroda, M. (2011) 'miR-92 is a key oncogenic component of the miR-17-92 cluster in colon cancer', *Cancer Sci*, 102(12), pp. 2264-71.

Ullman, T.A. and Itzkowitz, S.H. (2011) 'Intestinal inflammation and cancer', *Gastroenterology*, 140(6), pp. 1807-16.

Umar, A., Boland, C.R., Terdiman, J.P., Syngal, S., de la Chapelle, A., Ruschoff, J., Fishel, R., Lindor, N.M., Burgart, L.J., Hamelin, R., Hamilton, S.R., Hiatt, R.A., Jass, J., Lindblom, A., Lynch, H.T., Peltomaki, P., Ramsey, S.D., Rodriguez-Bigas, M.A., Vasen, H.F., Hawk, E.T., Barrett, J.C., Freedman, A.N. and Srivastava, S. (2004) 'Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability', *J Natl Cancer Inst*, 96(4), pp. 261-8.

Uthoff, S.M., Eichenberger, M.R., Lewis, R.K., Fox, M.P., Hamilton, C.J., McAuliffe, T.L., Grimes, H.L. and Galandiuk, S. (2001) 'Identification of candidate genes in ulcerative colitis and Crohn's disease using cDNA array technology', *Int J Oncol*, 19(4), pp. 803-10.

Uysal-Onganer, P. and Kypta, R.M. (2012) 'Wnt11 in 2011 - the regulation and function of a non-canonical Wnt', *Acta Physiol (Oxf)*, 204(1), pp. 52-64.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., Tjon-Pon-Fong, M., Moerer, P., van den Born, M., Soete, G., Pals, S., Eilers, M., Medema, R. and Clevers, H. (2002) 'The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells', *Cell*, 111(2), pp. 241-50.

van Dekken, H., Wink, J.C., Vissers, K.J., Franken, P.F., Ruud Schouten, W., WC, J.H., Kuipers, E.J., Fodde, R. and Janneke van der Woude, C. (2007) 'Wnt pathway-related gene expression during malignant progression in ulcerative colitis', *Acta Histochem*, 109(4), pp. 266-72.

van Engeland, M., Derks, S., Smits, K.M., Meijer, G.A. and Herman, J.G. (2011) 'Colorectal cancer epigenetics: complex simplicity', *J Clin Oncol*, 29(10), pp. 1382-91.

van Gorkom, B.A., Karrenbeld, A., van der Sluis, T., Zwart, N., van der Meer, R., de Vries, E.G. and Kleibeuker, J.H. (2002) 'Calcium or resistant starch does not affect colonic epithelial cell proliferation throughout the colon in adenoma patients: a randomized controlled trial', *Nutr Cancer*, 43(1), pp. 31-8.

Van Klinken, B.J., Van der Wal, J.W., Einerhand, A.W., Buller, H.A. and Dekker, J. (1999) 'Sulphation and secretion of the predominant secretory human colonic mucin MUC2 in ulcerative colitis', *Gut*, 44(3), pp. 387-93.

van Munster, I.P., Tangerman, A. and Nagengast, F.M. (1994) 'Effect of resistant starch on colonic fermentation, bile acid metabolism, and mucosal proliferation', *Dig Dis Sci*, 39(4), pp. 834-42.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A. and Speleman, F. (2002) 'Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes', *Genome Biol*, 3(7), p. RESEARCH0034.

Velazquez, O.C., Zhou, D., Seto, R.W., Jabbar, A., Choi, J., Lederer, H.M. and Rombeau, J.L. (1996) 'In vivo crypt surface hyperproliferation is decreased by butyrate and increased by deoxycholate in normal rat colon: associated in vivo effects on c-Fos and c-Jun expression', *JPEN J Parenter Enteral Nutr*, 20(4), pp. 243-50.

Vetuschi, A., Latella, G., Sferra, R., Caprilli, R. and Gaudio, E. (2002) 'Increased proliferation and apoptosis of colonic epithelial cells in dextran sulfate sodium-induced colitis in rats', *Dig Dis Sci*, 47(7), pp. 1447-57.

Vincan, E., Leet, C.S., Reyes, N.I., Dilley, R.J., Thomas, R.J. and Phillips, W.A. (2000) 'Sodium butyrate-induced differentiation of human LIM2537 colon cancer cells decreases GSK-3beta activity and increases levels of both membrane-bound and Apc/axin/GSK-3beta complex-associated pools of beta-catenin', *Oncol Res*, 12(4), pp. 193-201.

Vogel, C. and Marcotte, E.M. (2012) 'Insights into the regulation of protein abundance from proteomic and transcriptomic analyses', *Nat Rev Genet*, 13(4), pp. 227-32.

Volinia, S., Calin, G.A., Liu, C.G., Ambs, S., Cimmino, A., Petrocca, F., Visone, R., Iorio, M., Roldo, C., Ferracin, M., Prueitt, R.L., Yanaihara, N., Lanza, G., Scarpa, A., Vecchione, A., Negrini, M., Harris, C.C. and Croce, C.M. (2006) 'A microRNA expression signature of human solid tumors defines cancer gene targets', *Proc Natl Acad Sci U S A*, 103(7), pp. 2257-61.

Waby, J.S., Chirakkal, H., Yu, C., Griffiths, G.J., Benson, R.S., Bingle, C.D. and Corfe, B.M. (2010) 'Sp1 acetylation is associated with loss of DNA binding at promoters associated with cell cycle arrest and cell death in a colon cell line', *Mol Cancer*, 9, p. 275.

Wachtershauser, A. and Stein, J. (2000) 'Rationale for the luminal provision of butyrate in intestinal diseases', *Eur J Nutr*, 39(4), pp. 164-71.

Wacker, M., Wanek, P., Eder, E., Hylla, S., Gostner, A. and Scheppach, W. (2002) 'Effect of enzyme-resistant starch on formation of 1,N(2)-propanodeoxyguanosine adducts of trans-4-hydroxy-2-nonenal and cell proliferation in the colonic mucosa of healthy volunteers', *Cancer Epidemiol Biomarkers Prev*, 11(9), pp. 915-20.

Wang, C.J., Zhou, Z.G., Wang, L., Yang, L., Zhou, B., Gu, J., Chen, H.Y. and Sun, X.F. (2009a) 'Clinicopathological significance of microRNA-31, -143 and -145 expression in colorectal cancer', *Dis Markers*, 26(1), pp. 27-34.

Wang, H., Birkenbach, M. and Hart, J. (2000) 'Expression of Jun family members in human colorectal adenocarcinoma', *Carcinogenesis*, 21(7), pp. 1313-7.

Wang, H.L., Hart, J., Fan, L., Mustafi, R. and Bissonnette, M. (2011a) 'Upregulation of glycogen synthase kinase 3beta in human colorectal adenocarcinomas correlates with accumulation of CTNNB1', *Clin Colorectal Cancer*, 10(1), pp. 30-6.

Wang, J., El-Masry, N., Talbot, I., Tomlinson, I., Alison, M.R. and El-Bahrawy, M. (2013a) 'Expression Profiling of Proliferation and Apoptotic Markers along the Adenoma-Carcinoma Sequence in Familial Adenomatous Polyposis Patients', *Gastroenterol Res Pract*, 2013, p. 107534.

Wang, L.S., Kuo, C.T., Huang, T.H., Yearsley, M., Oshima, K., Stoner, G.D., Yu, J., Lechner, J.F. and Huang, Y.W. (2013b) 'Black raspberries protectively regulate methylation of Wnt pathway genes in precancerous colon tissue', *Cancer Prev Res (Phila)*, 6(12), pp. 1317-27.

Wang, M., Zhang, P., Li, Y., Liu, G., Zhou, B., Zhan, L., Zhou, Z. and Sun, X. (2012) 'The quantitative analysis by stem-loop real-time PCR revealed the microRNA-34a, microRNA-155 and microRNA-200c overexpression in human colorectal cancer', *Med Oncol*, 29(5), pp. 3113-8.

Wang, N., Chen, Y., Yang, X. and Jiang, Y. (2014) 'Selenium-binding protein 1 is associated with the degree of colorectal cancer differentiation and is regulated by histone modification', *Oncol Rep*, 31(6), pp. 2506-14.

Wang, S., Wang, L., Bayaxi, N., Li, J., Verhaegh, W., Janevski, A., Varadan, V., Ren, Y., Merkle, D., Meng, X., Gao, X., Wang, H., Ren, J., Kuo, W.P., Dimitrova, N., Wu, Y. and Zhu, H. (2013c) 'A microRNA panel to discriminate carcinomas from high-grade intraepithelial neoplasms in colonoscopy biopsy tissue', *Gut*, 62(2), pp. 280-9.

Wang, S., Xiang, J., Li, Z., Lu, S., Hu, J., Gao, X., Yu, L., Wang, L., Wang, J., Wu, Y., Chen, Z. and Zhu, H. (2013d) 'A plasma microRNA panel for early detection of colorectal cancer', *Int J Cancer*.

Wang, W., Sun, K., Wu, C.T., Lei, S.T., Zeng, J.J., Wu, Y.J. and Li, G.X. (2011b) '[Effect of miR-221-specific inhibitor on the proliferation and apoptosis of human colorectal carcinoma cells]', *Nan Fang Yi Ke Da Xue Xue Bao*, 31(4), pp. 674-7.

Wang, X., Lam, E.K., Zhang, J., Jin, H. and Sung, J.J. (2009b) 'MicroRNA-122a functions as a novel tumor suppressor downstream of adenomatous polyposis coli in gastrointestinal cancers', *Biochem Biophys Res Commun*, 387(2), pp. 376-80.

Wang, Y.X., Zhang, X.Y., Zhang, B.F., Yang, C.Q., Chen, X.M. and Gao, H.J. (2010) 'Initial study of microRNA expression profiles of colonic cancer without lymph node metastasis', *J Dig Dis*, 11(1), pp. 50-4.

Watson, A.J. (2004) 'Apoptosis and colorectal cancer', *Gut*, 53(11), pp. 1701-9.

WCRF/AICR (2007) *World Cancer Research Fund/American Institute for Cancer Research. Food, Nutrition, Physical Activity and the Prevention of Cancer: A Global Perspective*. Washington DC: AICR.

WCRF/AICR (2011) 'World Cancer Research Fund / American Institute for Cancer Research. Continuous update project report. Food, nutrition, physical activity, and the prevention of colorectal cancer'.

Weaver, G.A., Krause, J.A., Miller, T.L. and Wolin, M.J. (1988) 'Short chain fatty acid distributions of enema samples from a sigmoidoscopy population: an association of high acetate and low butyrate ratios with adenomatous polyps and colon cancer', *Gut*, 29(11), pp. 1539-43.

Weaver, I.C., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M. and Meaney, M.J. (2004) 'Epigenetic programming by maternal behavior', *Nat Neurosci*, 7(8), pp. 847-54.

Wendum, D., Masliah, J., Trugnan, G. and Flejou, J.F. (2004) 'Cyclooxygenase-2 and its role in colorectal cancer development', *Virchows Arch*, 445(4), pp. 327-33.

West, N.J., Courtney, E.D., Poullis, A.P. and Leicester, R.J. (2009) 'Apoptosis in the colonic crypt, colorectal adenomata, and manipulation by chemoprevention', *Cancer Epidemiol Biomarkers Prev*, 18(6), pp. 1680-7.

White, N.R., Mulligan, P., King, P.J. and Sanderson, I.R. (2006) 'Sodium butyrate-mediated Sp3 acetylation represses human insulin-like growth factor binding protein-3 expression in intestinal epithelial cells', *J Pediatr Gastroenterol Nutr*, 42(2), pp. 134-41.

Williams, E.A., Coxhead, J.M. and Mathers, J.C. (2003) 'Anti-cancer effects of butyrate: use of micro-array technology to investigate mechanisms', *Proc Nutr Soc*, 62(1), pp. 107-15.

Williamson, S.L., Kartheuser, A., Coaker, J., Kooshkghazi, M.D., Fodde, R., Burn, J. and Mathers, J.C. (1999) 'Intestinal tumorigenesis in the Apc1638N mouse treated with aspirin and resistant starch for up to 5 months', *Carcinogenesis*, 20(5), pp. 805-10.

Willis, H.J., Eldridge, A.L., Beiseigel, J., Thomas, W. and Slavin, J.L. (2009) 'Greater satiety response with resistant starch and corn bran in human subjects', *Nutr Res*, 29(2), pp. 100-5.

Wilson, A.J., Velcich, A., Arango, D., Kurland, A.R., Shenoy, S.M., Pezo, R.C., Levsky, J.M., Singer, R.H. and Augenlicht, L.H. (2002) 'Novel detection and differential utilization of a c-myc transcriptional block in colon cancer chemoprevention', *Cancer Res*, 62(21), pp. 6006-10.

Wilson, R.G., Smith, A.N. and Bird, C.C. (1990) 'Immunohistochemical detection of abnormal cell proliferation in colonic mucosa of subjects with polyps', *J Clin Pathol*, 43(9), pp. 744-7.

Winter, J., Nyskohus, L., Young, G.P., Hu, Y., Conlon, M.A., Bird, A.R., Topping, D.L. and Le Leu, R.K. (2011) 'Inhibition by resistant starch of red meat-induced promutagenic adducts in mouse colon', *Cancer Prev Res (Phila)*, 4(11), pp. 1920-8.

Winter, J., Young, G.P., Hu, Y., Gratz, S.W., Conlon, M.A. and Le Leu, R.K. (2014) 'Accumulation of promutagenic DNA adducts in the mouse distal colon after consumption of heme does not induce colonic neoplasms in the western diet model of spontaneous colorectal cancer', *Mol Nutr Food Res*, 58(3), pp. 550-8.

Wolter, K.G., Hsu, Y.T., Smith, C.L., Nechushtan, A., Xi, X.G. and Youle, R.J. (1997) 'Movement of Bax from the cytosol to mitochondria during apoptosis', *J Cell Biol*, 139(5), pp. 1281-92.

Wong, M.H., Huelsken, J., Birchmeier, W. and Gordon, J.I. (2002) 'Selection of multipotent stem cells during morphogenesis of small intestinal crypts of Lieberkuhn is perturbed by stimulation of Lef-1/beta-catenin signaling', *J Biol Chem*, 277(18), pp. 15843-50.

Wong, N.A., Mayer, N.J., Anderson, C.E., McKenzie, H.C., Morris, R.G., Diebold, J., Mayr, D., Brock, I.W., Royds, J.A., Gilmour, H.M. and Harrison, D.J. (2003) 'Cyclin D1 and p21 in ulcerative colitis-related inflammation and epithelial neoplasia: a study of aberrant expression and underlying mechanisms', *Hum Pathol*, 34(6), pp. 580-8.

Wong, N.A. and Pignatelli, M. (2002) 'Beta-catenin--a linchpin in colorectal carcinogenesis?', *Am J Pathol*, 160(2), pp. 389-401.

Wong, W.M. and Wright, N.A. (1999) 'Cell proliferation in gastrointestinal mucosa', *J Clin Pathol*, 52(5), pp. 321-33.

Worthley, D.L., Le Leu, R.K., Whitehall, V.L., Conlon, M., Christophersen, C., Belobrajdic, D., Mallitt, K.A., Hu, Y., Irahara, N., Ogino, S., Leggett, B.A. and Young, G.P. (2009) 'A human, double-blind, placebo-controlled, crossover trial of prebiotic, probiotic, and synbiotic supplementation: effects on luminal, inflammatory, epigenetic, and epithelial biomarkers of colorectal cancer', *Am J Clin Nutr*, 90(3), pp. 578-86.

Wu, C.W., Ng, S.C., Dong, Y., Tian, L., Ng, S.S., Leung, W.W., Law, W.T., Yau, T.O., Chan, F.K., Sung, J.J. and Yu, J. (2014) 'Identification of microRNA-135b in stool as a potential noninvasive biomarker for colorectal cancer and adenoma', *Clin Cancer Res*, 20(11), pp. 2994-3002.

Wu, C.W., Ng, S.S., Dong, Y.J., Ng, S.C., Leung, W.W., Lee, C.W., Wong, Y.N., Chan, F.K., Yu, J. and Sung, J.J. (2012a) 'Detection of miR-92a and miR-21 in stool samples as potential screening biomarkers for colorectal cancer and polyps', *Gut*, 61(5), pp. 739-45.

Wu, F., Zikusoka, M., Trindade, A., Dassopoulos, T., Harris, M.L., Bayless, T.M., Brant, S.R., Chakravarti, S. and Kwon, J.H. (2008) 'MicroRNAs are differentially expressed in ulcerative colitis and alter expression of macrophage inflammatory peptide-2 alpha', *Gastroenterology*, 135(5), pp. 1624-1635 e24.

Wu, Z.Q., Brabletz, T., Fearon, E., Willis, A.L., Hu, C.Y., Li, X.Y. and Weiss, S.J. (2012b) 'Canonical Wnt suppressor, Axin2, promotes colon carcinoma oncogenic activity', *Proc Natl Acad Sci U S A*, 109(28), pp. 11312-7.

Xiao, Z.G., Deng, Z.S., Zhang, Y.D., Zhang, Y. and Huang, Z.C. (2013) 'Clinical significance of microRNA-93 downregulation in human colon cancer', *Eur J Gastroenterol Hepatol*, 25(3), pp. 296-301.

Xie, J. and Itzkowitz, S.H. (2008) 'Cancer in inflammatory bowel disease', *World J Gastroenterol*, 14(3), pp. 378-89.

Xu, X.M., Qian, J.C., Deng, Z.L., Cai, Z., Tang, T., Wang, P., Zhang, K.H. and Cai, J.P. (2012) 'Expression of miR-21, miR-31, miR-96 and miR-135b is correlated with the clinical parameters of colorectal cancer', *Oncol Lett*, 4(2), pp. 339-345.

Yamada, N., Nakagawa, Y., Tsujimura, N., Kumazaki, M., Noguchi, S., Mori, T., Hirata, I., Maruo, K. and Akao, Y. (2013a) 'Role of Intracellular and Extracellular MicroRNA-92a in Colorectal Cancer', *Transl Oncol*, 6(4), pp. 482-92.

Yamada, N., Noguchi, S., Mori, T., Naoe, T., Maruo, K. and Akao, Y. (2013b) 'Tumor-suppressive microRNA-145 targets catenin delta-1 to regulate Wnt/beta-catenin signaling in human colon cancer cells', *Cancer Lett*, 335(2), pp. 332-42.

Yan, Z., Xiong, Y., Xu, W., Gao, J., Cheng, Y., Wang, Z., Chen, F. and Zheng, G. (2012) 'Identification of hsa-miR-335 as a prognostic signature in gastric cancer', *PLoS One*, 7(7), p. e40037.

Ying, J., Li, H., Yu, J., Ng, K.M., Poon, F.F., Wong, S.C., Chan, A.T., Sung, J.J. and Tao, Q. (2008) 'WNT5A exhibits tumor-suppressive activity through antagonizing the Wnt/beta-catenin signaling, and is frequently methylated in colorectal cancer', *Clin Cancer Res*, 14(1), pp. 55-61.

Yoshioka, M., Shimomura, Y. and Suzuki, M. (1994) 'Dietary polydextrose affects the large intestine in rats', *J Nutr*, 124(4), pp. 539-47.

You, J., Nguyen, A.V., Albers, C.G., Lin, F. and Holcombe, R.F. (2008) 'Wnt pathway-related gene expression in inflammatory bowel disease', *Dig Dis Sci*, 53(4), pp. 1013-9.

Young, G.P., McIntyre, A., Albert, V., Folino, M., Muir, J.G. and Gibson, P.R. (1996) 'Wheat bran suppresses potato starch--potentiated colorectal tumorigenesis at the aberrant crypt stage in a rat model', *Gastroenterology*, 110(2), pp. 508-14.

Zeng, H., Lazarova, D.L. and Bordonaro, M. (2014) 'Mechanisms linking dietary fiber, gut microbiota and colon cancer prevention', *World J Gastrointest Oncol*, 6(2), pp. 41-51.

Zhang, H., Zhu, Y.Q., Wu, Y.Q., Zhang, P. and Qi, J. (2014) 'Detection of promoter hypermethylation of Wnt antagonist genes in fecal samples for diagnosis of early colorectal cancer', *World J Gastroenterol*, 20(20), pp. 6329-35.

Zhang, J., Tu, Q., Bonewald, L.F., He, X., Stein, G., Lian, J. and Chen, J. (2011) 'Effects of miR-335-5p in modulating osteogenic differentiation by specifically downregulating Wnt antagonist DKK1', *J Bone Miner Res*, 26(8), pp. 1953-63.

Zhang, J., Xiao, Z., Lai, D., Sun, J., He, C., Chu, Z., Ye, H., Chen, S. and Wang, J. (2012) 'miR-21, miR-17 and miR-19a induced by phosphatase of regenerating liver-3 promote the proliferation and metastasis of colon cancer', *Br J Cancer*, 107(2), pp. 352-9.

Zhang, N., Li, X., Wu, C.W., Dong, Y., Cai, M., Mok, M.T., Wang, H., Chen, J., Ng, S.S., Chen, M., Sung, J.J. and Yu, J. (2013) 'microRNA-7 is a novel inhibitor of YY1 contributing to colorectal tumorigenesis', *Oncogene*, 32(42), pp. 5078-88.

Zhang, T., Otevrel, T., Gao, Z., Gao, Z., Ehrlich, S.M., Fields, J.Z. and Boman, B.M. (2001) 'Evidence that APC regulates survivin expression: a possible mechanism contributing to the stem cell origin of colon cancer', *Cancer Res*, 61(24), pp. 8664-7.

Zhang, W., Hart, J., McLeod, H.L. and Wang, H.L. (2005) 'Differential expression of the AP-1 transcription factor family members in human colorectal epithelial and neuroendocrine neoplasms', *Am J Clin Pathol*, 124(1), pp. 11-9.

Zhao, R. and Michor, F. (2013) 'Patterns of proliferative activity in the colonic crypt determine crypt stability and rates of somatic evolution', *PLoS Comput Biol*, 9(6), p. e1003082.

Zhu, P., Martin, E., Mengwasser, J., Schlag, P., Janssen, K.P. and Gottlicher, M. (2004) 'Induction of HDAC2 expression upon loss of APC in colorectal tumorigenesis', *Cancer Cell*, 5(5), pp. 455-63.

Zimmerman, M.A., Singh, N., Martin, P.M., Thangaraju, M., Ganapathy, V., Waller, J.L., Shi, H., Robertson, K.D., Munn, D.H. and Liu, K. (2012) 'Butyrate suppresses colonic inflammation through HDAC1-dependent Fas upregulation and Fas-mediated apoptosis of T cells', *Am J Physiol Gastrointest Liver Physiol*, 302(12), pp. G1405-15.