Methylation of NOTCH genes in normal and at-risk colorectal epithelium

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This thesis represents my own work. Where the work of others has been included it has been correctly acknowledged in accordance with university and school guidance on good academic conduct. No part of the material offered has been previously submitted for a degree or other qualification in this or any other University.

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October 2012

Abstract

Introduction

Colorectal cancer (CRC) arises from genetic defects in stem cells. NOTCH signalling plays a key role in stem cell replication control. NOTCH-related genes are overexpressed in CRC. The mechanism for this is not known but could include epigenetic activation of NOTCH oncogenes via promoter hypomethylation. Methylation can be modulated by environmental stimuli including dietary factors such as butyrate, produced by bacterial fermentation of non-digestible carbohydrates in the colon. Butyrate exerts potent anti-oncogenic effects in the colorectal mucosa.

Methods

Participants were recruited at endoscopy and included those at normal risk of CRC (n=75), or higher risk of CRC because of previous adenomatous polyps (n=28) or ulcerative colitis (n=12). Participants provided 9 rectal biopsies. Normal risk participants were randomised to resistant starch (Hi-maize 260) or polydextrose supplementation in a 2x2 factorial placebo controlled trial for 50 days.

Methylation of several CpG sites in the promoters of *JAG1* (NOTCH pathway ligand) and *RBP-J* (NOTCH intracellular activator) was quantified using pyrosequencing.

Results

For *JAG1* there was trend towards lower methylation at all CpG sites in those at higher CRC risk. Methylation at *RBP-J* CpG 11 was lower in polyp patients than in controls (18.0(1.5) vs. 23.6% (0.8), p=0.011). At *JAG1* CpG 4, methylation increased following polydextrose supplementation compared to placebo (3.1(0.4) vs. 1.7%(0.4), p=0.009). A similar, but non-significant, trend was observed at other CpG sites for *JAG1*.

Conclusions

DNA methylation of NOTCH signalling genes is altered in macroscopically normal colorectal mucosa of patients at higher CRC risk. The observed changes in *JAG1*

methylation after polydextrose supplementation are consistent with a protective effect against carcinogenesis.

This thesis is dedicated to all my family who have supported and encouraged me throughout my three years. My parents have always encouraged me in any and all my endeavours and continue to do so. My wife, Johanne, has been a great support throughout and very understanding, kind and loving through all the unique challenges involved in such an undertaking. Finally I must acknowledge, my son Toby who arrived half way through this project. He may not have treated us all to a full night's sleep since he arrived, but has been a joy to watch grow and change every day!

Acknowledgements

Many people have assisted with the completion of this project. My supervisory team: John Mathers, Seamus Kelly and Mike Bradburn, have provided great support and guidance throughout.

Recruitment for the study and managing all the participants through the trial was a huge undertaking for which I am indebted to Naomi Willis and Long Xie for their hard work. Naomi Willis has project managed the DISC study with fantastic ability and organization, and she in particular needs to be acknowledged for the successful recruitment that was completed ahead of schedule.

Throughout the recruitment we were constantly surprised and encouraged by the altruism of our recruits who volunteered for all sorts of reasons to participate in a trial that required quite intensive participation on their part. During 18 months of recruitment, the endoscopy staff at both Wansbeck and North Tyneside hospitals were a great help, providing a friendly atmosphere, encouraging us, and potential recruits also. Consultants and nurses happily extended endoscopy lists to accommodate recruitment of participants.

In the laboratory Wan Adnan Omar was a fantastic teacher. Getting someone who arrived unable to use a pipette, to the stage where they are teaching others how pyrosequence is no small undertaking!

Many others have contributed ideas and guidance at various stages, Professor Sir John Burn and Professor Caroline Relton during my university progression reviews and Ian Johnstone and Nigel Belshaw of the Institute of Food Research as collaborators in the DISC study. To all those listed above I offer my sincere thanks. Throughout this project I have gained a great deal, both academically and in many other areas, without all the assistance and inspiration I received this would not have been possible.

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Abbreviations

AICR - American Institute for Cancer Research

ALDH1 - aldehyde dehydrogenase 1

ANOVA – analysis of variance

APC – adenomatous polyposis coli

ASC – adult stem cell

ATP – adenosine triphosphate

BrdUrd–bromodeoxyuridine

BMI – body mass index

CAPP- concerted action polyp prevention

CDC – cytoplasmic destruction complex

CEA - carcinoembryonic antigen

CI – confidence interval

CK - cytokeratin

COX - cytochrome c oxidase

CRC – colorectal cancer

CRP – C-reactive protein

CSC – cancer stem cell

DAB - 3,3'Diaminobenzidine

DCC – deleted in colorectal cancer

DISC – Dietary Intervention, Stem cells and Colorectal cancer

DKK - Dickkopf

DNA – deoxyribonucleic acid

DNMT - DNA methyltransferases

DLL – Delta like (protein)

DSH – Dishevelled (protein)

EDTA - ethylenediaminetetracetic acid

EpCAM – epithelial cell adhesion molecule

EPIC - European Prospective Investigation into Cancer and Nutrition

FAP – familial adenomatous polyposis

FFQ – food frequency questionnaire

GLM – General linear model

HCC – hepatocellular carcinoma

HDACi - histone deacetylase inhibitor

HES – hairy enhancer of split

^{3}HTdR - tritiated thymidine

HPLC – high performance liquid chromatography

HNPCC - hereditary non-polyposis colorectal cancer

hsCRP - high sensitivity C-reactive protein

IBD – inflammatory bowel disease

ISH – in situ hybridisation

IL – interleukin

JAG – jagged (receptor)

LINE-1 – long interspersed nucleotide element-1

LGR5 - leucine-rich-repeat containing G-protein-coupled receptor 5

LSM - least squares mean

MAP – MYH associated polyposis

MIN – multiple intestinal neoplasia

miRNA – micro riboxynucleic acid

MMR - mismatch repair

MSI – microsatellite instability

NDC – non-digestible carbohydrate

NF-κB - nuclear factor kappa B

NICD – Notch intracellular domain

NRE – Notch response element

NSAID -non-steroidal anti-inflammatory

OCT- optimal cutting temperature (compound)

OR – odds ratio

PCR – polymerase chain reaction

PD –polydextrose

PRESS – prediction error sum of squares

ROC – receiver operator characteristic

RCT – randomised controlled trial

RNA – riboxynucleic acid

RR – relative risk

RS – resistant starch

RT-PCR - real time polymerase chain reaction

SCCAI - Short clinical colitis activity index

SCID – severe combined immunodeficiency

SCFA – short chain fatty acids

SDS – sodium dodeyl sulphate

Se - Selenium

SEM – standard error of the mean

SNP – single nucleotide polymorphism

SUERC - Scottish Universities Environmental Research Centre

TNF – tumour necrosis factor

TNM – tumour, nodes, metastasis

TSG – tumour suppressor gene

UC – ulcerative colitis

WCRF - World Cancer Research Fund

WHO – World Health Organisation

CHAPTER 1. INTRODUCTION

1.1 COLORECTAL CANCER - INCIDENCE

Colorectal cancer (CRC) is common in the UK. In 2009 there were 41 142 new cases diagnosed in the UK. (CRUK 2009) It is the 2nd most common cancer in women and the 3rd in men, overall the third most common type of cancer in the UK. (Figure 1-1)(CRUK 2009) The overall lifetime risk of an individual developing CRC is estimated to be 1 in 15 for men and 1 in 19 for women. (CRUK 2009)

CRC is primarily a disease of older age with 72% of new diagnoses made in patients aged 65 or over. Incidence rates between males and females are similar until age fifty when the rate of CRC in males increases more rapidly. This trend continues until age eighty when rates equalize due to the greater life expectancy of women. (Figure 1-2) The overall male to female ratio for all age groups is 17:10. (CRUK 2009)

UK incidence has remained broadly stable since 1975. Overall incidence in Europe, particularly southern Europe, is increasing. Incidence in Asian countries such as Japan is increasing as diet becomes more 'westernised'. (CRUK 2009) Incidence in the United States rose steadily until around 1980 and has since been slowly declining. (CRUK 2009)

Worldwide there were 1.2 million new CRC diagnoses in 2010 (Jemal, Bray et al. 2011) which equates to approximately 10% of all new cancer diagnoses. (ISD 2010; Jemal, Bray et al. 2011) CRC incidence rates vary over a tenfold range from low to high incidence areas of the world. (Figure 1-3) Two-thirds of CRCs occur in developed countries. (CRUK 2009) Migrant studies have demonstrated that CRC risk increases toward that of the new country in individuals relocating from low to high incidence areas. (Stirbu, Kunst et al. 2006; Nasseri, Moulton et al. 2009) This effect is more pronounced in second generation migrants and is particularly pronounced for CRC compared with other cancers. (Flood, Weiss et al. 2000; Stirbu, Kunst et al. 2006)

The wide variation in CRC risk between countries, predominance in the most developed countries, coupled with increased individual risk when migrants move from low to high incidence countries suggest that environmental exposure plays a strong role in CRC carcinogenesis. A strong influence of environmental exposure on risk would suggest a proportion of CRCs are potentially preventable – a proportion which has been estimated to be upto 50%. (Platz, Willett et al. 2000)

FIGURE 1-1. UK INCIDENCE OF MOST COMMON TYPES OF CANCER (2009).

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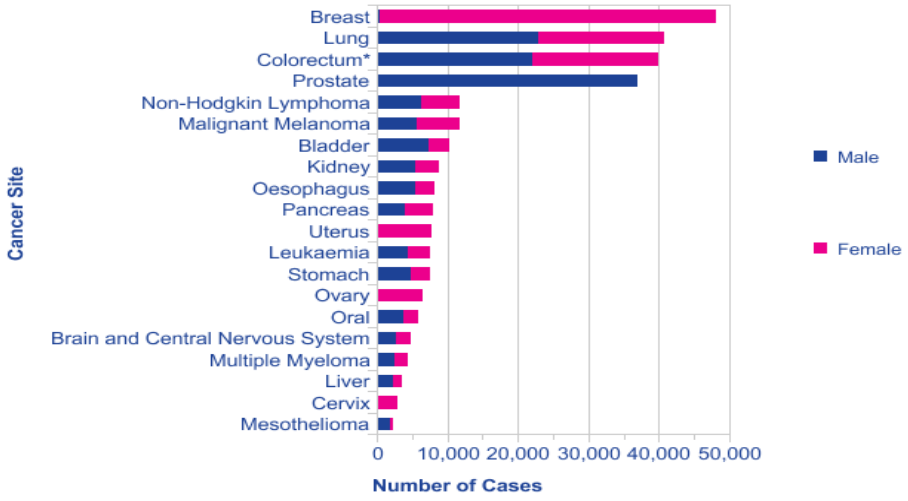

FIGURE 1-2. UK INCIDENCE OF CRC BY AGE GROUP IN MALES AND FEMALES (2009).

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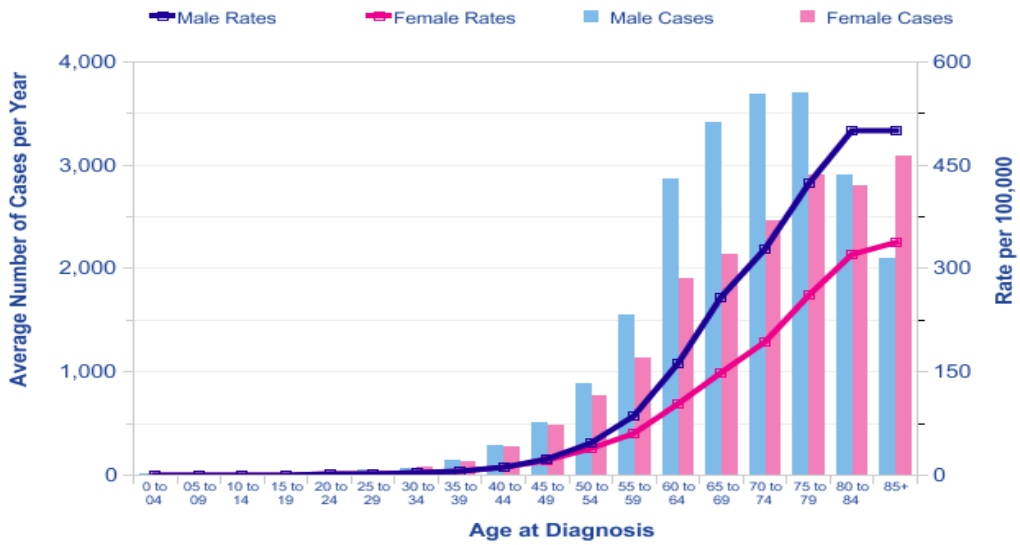

FIGURE 1-3. AGE STANDARDISED INCIDENCE AND MORTALITY OF CRC IN MEN BY WORLD REGIONS.

REPRODUCED WITH PERMISSION FROM CANCER RESEARCH UK.

HTTP://INFO.CANCERRESEARCHUK.ORG/CANCERSTATS/WORLD/COLORECTAL-CANCER-WORLD/

FIGURE 1-4. AGE STANDARDISED MORTALITY FROM CRC BY SEX, UK, 1971-2010.

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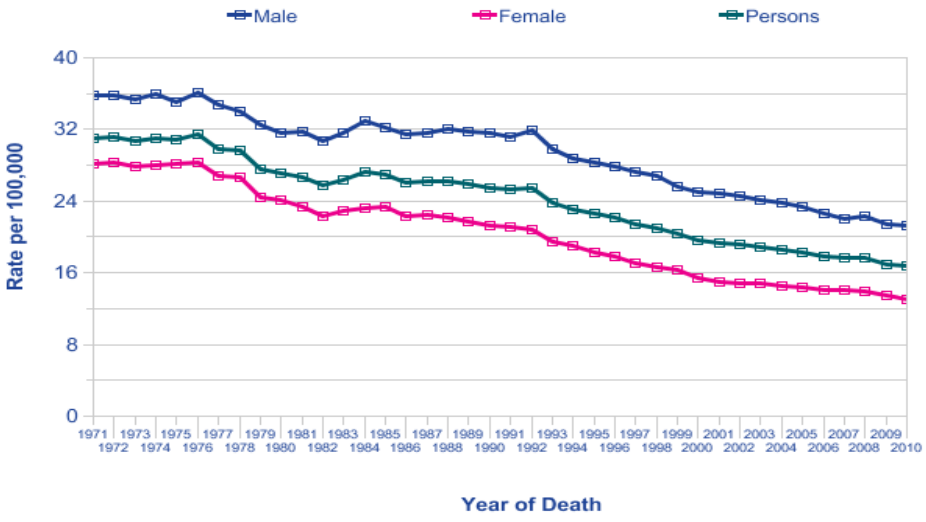


1.2 MORTALITY & SURVIVAL

As with CRC incidence rate, CRC mortality increases sharply with age. However mortality rates have decreased steadily over time with the largest decreases noted in younger patients. (Figure 1-4) Between the early 1970's and 2010 age standardised mortality from CRC has decreased by between 40 and 50% in the UK. (CRUK 2009)

Worldwide mortality from CRC is over 600,000 per year. (Jemal, Bray et al. 2011) Mortality rates are highest in high incidence areas such as southern and eastern Europe. (CRUK 2009) Mortality rates overall are decreasing in Europe. Further decreases are expected as therapy improves and bowel cancer screening programmes are introduced.

Survival is closely linked to patient age and cancer stage at diagnosis. Older patients have poorer survival. (CRUK 2009) Tumour stage can be described using the American Joint Committee on Cancer TNM (tumour, nodes & metastases) system, however the Dukes' staging originally described in 1932 (Dukes 1932) is more commonly used and well validated. Dukes' stages are described in Table 1-1. (NCIN 2009)

TABLE 1-1. DISTRIBUTION OF CRC BY DUKES' STAGE AND ASSOCIATED 5 YEAR SURVIVAL RATES

(NATIONAL CANCER INTELLIGENCE NETWORK, 2009)

Dukes' stage	Description	% of tumours	5-year survival (%)
A	Invasive carcinoma not breaching the muscularis propria	8.7	93.2
B	Invasive carcinoma breaching the muscularis propria, but not involving regional lymph nodes	24.2	77.0
C	Invasive carcinoma involving regional lymph nodes	23.6	47.7
D	Metastatic disease	9.2	6.6

1.3 NATURAL HISTORY OF COLORECTAL CANCER

1.3.1 NORMAL COLONIC MUCOSA

The normal gut mucosa consists of a specialised single layer of columnar epithelial cells which form both a barrier between the external environment of the gut lumen and the the body, and a hydrolytic and absorptive surface for digestion and absorption of nutrients and other food-derived molecules. In the proximal gut (stomach, duodenum and small bowel) food is mixed with digestive secretions so that foods can be broken down into molecular entities which are suitable for absorption. The colon receives food residues that have escaped digestion and absorption in the proximal gastrointestinal tract. The primary functions of the colon are water reabsorption and energy salvage from the end-products of colonic microbial fermentation of carbohydrates such as short-chain fatty acids (SCFA), and to a lesser extent, proteins. Colonic microbiota also synthesise amino acids, several B vitamins and vitamin K. (Manas, Martinez de Victoria et al. 2009)

As most absorption of nutrients has occurred proximally less absorptive area is required in the colon. The functional unit of the proximal gut epithelium is the crypt (invagination of the epithelium) and its associated villi (protrusions of the epithelium) which increase surface area for hydrolysis and absorption. The colon has no villi, just flat mucosa and crypts. (Figure 1-5) The crypt protrudes into the lamina propria of the colonic wall and, together with the associated flat mucosa, forms the basic functional unit of the colon epithelium.

1.3.2 NORMAL CRYPT STRUCTURE AND CELLULAR TURNOVER

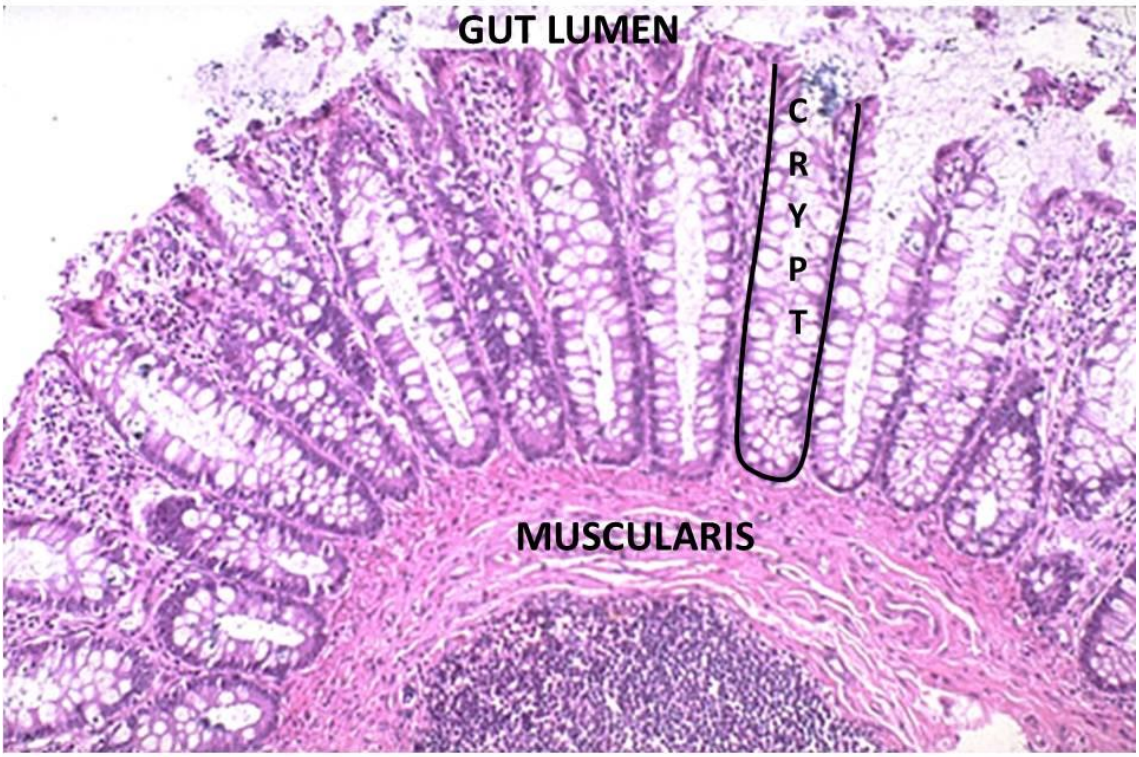
Each colonic crypt contains three terminally differentiated cell types:

- colonocytes - the absorptive cells of the colon
- goblet cells – mucous secreting cells
- entero-endocrine cells – peptide hormone secreting endocrine cells

This is in contrast to small bowel where there are four terminally differentiated cells: those mentioned above plus Paneth cells. Paneth cells contribute to host defenses by secretion of antimicrobial and other peptides. These secreted peptides moderate the intestinal bacterial flora, contributing to host immunity in the colon. Dysregulation of this system is associated with gastrointestinal diseases such as inflammatory bowel disease. (Murphy, Kwon et al. 2012)

The differentiated cells of the colonic crypt are generated at the crypt base by division of adult colonic stem cells. Immediately above the crypt base is the transit-amplifying compartment. Within the transit-amplifying compartment cells there is rapid mitotic division of partially differentiated cells which then differentiate progressively to their final phenotype as they migrate towards the gut lumen where they apoptose and are shed into gut. Colon cell turnover occurs frequently. (see section 1.7.1) In mice the time for a new cell to ascend and be shed from a crypt is only one day. (Creamer, Shorter et al. 1961) Radio-labelling of proliferative cells in normal colonic and rectal mucosa demonstrates that this process takes five days in humans. (Lipkin, Bell et al. 1963)

FIGURE 1-5. NORMAL COLON HISTOLOGY.



1.3.3 DEVELOPMENT OF COLORECTAL CANCER

Cancer is caused by alterations in gene expression which result in unregulated cellular replication. The unchecked growth of cells leads to tumour formation – neoplasia - and invasion of these transformed cells through basement membranes is the hallmark of cancer. Multiple alterations in genetic expression are usually required to give rise to a cancer. (Pecorino 2008) Pre-malignant genetic changes, occurring predictably in tandem with macroscopic neoplasia, have been well described in CRC and the macroscopic and genomic changes associated with the polyp-cancer sequence in CRC is the paradigm for carcinogenesis in other epithelial cancers.

Polyps are neoplastic lesions defined as: 'a circumscribed mass of tissue arising from the bowel wall.' (Bond 1993) Evidence that CRC arises from pre-existing polyps includes:

- The prevalence of adenomas and carcinomas are similar, with peak prevalence of adenomas being 5-10 years younger than that of carcinomas. (Muto, Bussey et al. 1975; Winawer, Zauber et al. 1987)
- Adenomatous tissue often accompanies carcinomatous tissue and small cancers are rarely found without surrounding adenomatous tissue. (Morson 1966; Green 1983)
- Sporadic adenomas are histologically identical to adenomas in familial adenomatous polyposis (FAP), which have unequivocal malignant potential. (Bussey 1975)
- The larger an adenoma becomes the more likely it is to show evidence of cellular atypia and genetic abnormalities (Muto, Bussey et al. 1975; Vogelstein, Fearon et al. 1988)
- The distribution of adenomas and carcinomas is similar throughout the colon with predominance towards distal lesions. (Granqvist 1981; Green 1983)

- Adenomas are found in up to one third of specimens resected for CRC. (Chu, Giacco et al. 1986; Eide 1986)
- Bowel screening programmes reduce CRC mortality through early diagnosis at the polyp stage. (Towler, Irwig et al. 2000; Morris, Whitehouse et al. 2012)

1.4 GENETICS & INHERITED RISK

1.4.1 INHERITED AND FAMILIAL CRC

Inherited factors play a strong role in the aetiology of CRC. In the general population lifetime risk is approximately 5-6% (CRUK 2009), but lifetime risk increases to 20% in an individual with an affected first or second degree relative and may reach 80-100% in inherited CRC syndromes. (Rustigi 2007; Kastrinos and Syngal 2011)

Familial CRC refers to a familial predisposition where no genetic abnormality has been found (and the possibility of stochastic cause remains). Hereditary CRC refers to a syndrome where an inherited mutation has been identified. Of all CRCs, 15-20% are familial and 6-7% are inherited. Approximately 5% of CRCs are due to hereditary non-polyposis colorectal cancer (HNPCC) also known as Lynch syndrome, 1% is due to familial adenomatous polyposis (FAP) and 1% due to other inherited syndromes such as MYH-associated polyposis, the hamartomatous polyposis syndromes and hyperplastic polyposis. (Rustigi 2007; Kastrinos and Syngal 2011)

1.4.2 FAMILIAL ADENOMATOUS POLYPOSIS

FAP is an autosomal dominant condition with near 100% penetrance. Sufferers develop multiple polyps throughout the gastrointestinal tract, especially the colon, in adolescence or early adult life. These individuals are at increased risk of several cancers including thyroid, stomach and duodenal but have nearly 100% risk of developing a CRC if left untreated. (Rustigi 2007; Kastrinos and Syngal 2011) FAP is caused by an inherited mutation of the *Adenomatous Polyposis Coli (APC)* gene which was defined in 1991, facilitating many developments in the understanding of the

genetic basis of CRC. (Groden, Thiveris et al. 1991) The 15 exon *APC* gene codes for the 2843 amino acid cytoplasmic APC protein which is central to the WNT signalling pathway which is in turn, intrinsic to the development of FAP and sporadic CRC.

1.4.3 WNT SIGNALLING, B-CATENIN & APC

APC is a classical tumour suppressor gene (TSG) which indirectly controls transcription of genes regulating cellular proliferation via its interaction with β-catenin. APC binding to β-catenin, together with the other components (GSK-3, CKI and Axin) of the cytoplasmic destruction complex (CDC), results in ubiquitination of β-catenin and its destruction, decreasing expression of β-catenin's target genes. (Tirnauer 2005)

Whilst *APC* function is key to the negative regulation of β-catenin activation, WNT signalling is the key positive regulator. (Clevers 2006) In the 'on state', secreted WNT proteins bind to Frizzled transmembrane receptors. This results in intracellular expression of LRP5/6 and Frizzled. Frizzled and LRP5/6 activate Dishevelled (DSH) (a cytoplasmic protein). Released DSH binds to CK1, GSK-3 and axin to the WNT/LRP5/6 complex and prevents formation of the CDC. B-catenin accumulates in the cytoplasm driving transcription of its target genes. (Clevers 2006) (Figure 1-6)

If WNT is not activated, the 'off state', then DSH, CK1, GSK-3 and axin are no longer bound to the WNT/LRP5/6 complex and the CDC can be formed. The CDC binds, ubiquitinates and destroys cytoplasmic β-catenin. (Clevers 2006) WNT is inhibited by the Dickkopf (DKK) protein which cross-links LRP6 to another transmembrane protein, Kremen, preventing its intracellular expression.(Mao, Wu et al. 2002)

Within the nucleus, transcription of WNT target genes is repressed by the complex that is formed by TCF and Groucho. If cytoplasmic β-catenin accumulates and migrates into the nucleus it displaces Groucho from TCF and the resulting β-catenin/Groucho and β-catenin/TCF complexes, especially β-catenin/TCF-4, activate target gene transcription. (Clevers 2006) The absence of APC results in constitutive activation of β-catenin/TCF-4 complex which is restored to wild type function on restoration of *APC.* (Korinek, Barker et al. 1997) (Figure 1-6)

1.4.4 WNT TARGET GENES

In health, WNT target genes have a role in stem cell maintenance and differentiation of stem cell progeny (see section 1.8.8) (Nusse 2008). In tumourigenesis, intranuclear β-catenin signalling causes increased expression of c-MYC and cyclin D1 which are key regulators of cellular growth, regulating the progress of the cell cycle at the G1/S checkpoint. Both proteins are overexpressed in CRC. (Smith, Myint et al. 1993; Arber, Hibshoosh et al. 1996) Heinen et al. showed that transfection with wild type *Apc* gene in *Apc* mutated rats restored the normal cell cycle regulation phenotype. However this effect was nullified if rats were co-transfected with β-catenin or c-myc and cyclin D1 demonstrating that the loss of G1/S phase checkpoint is mediated finally by these proteins. (Heinen, Goss et al. 2002)

FIGURE 1-6. DIAGRAMATIC REPRESENTATION OF WNT SIGNALLING IN THE ON AND OFF STATES.

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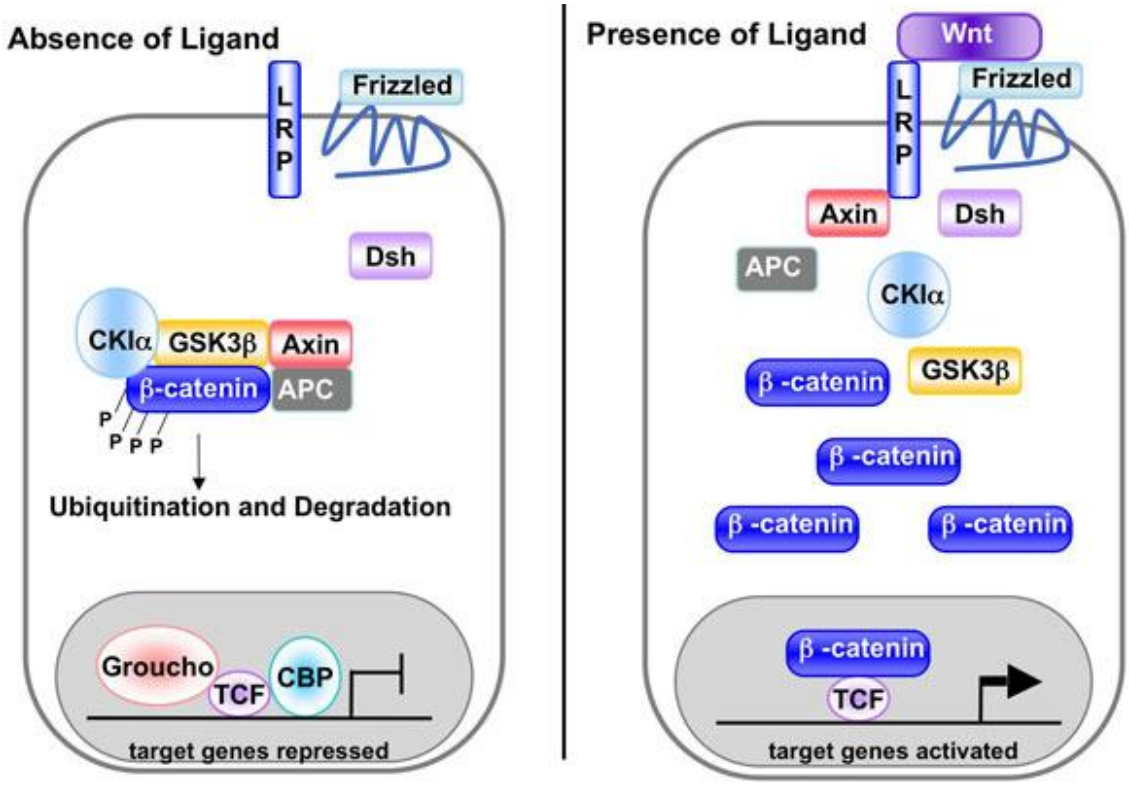


1.4.5 HEREDITARY NON-POLYPOSIS COLORECTAL CANCER (HNPCC)

Also known as Lynch syndrome, HNPCC is the most common cause of inherited CRC accounting for an estimated 1-6% of all CRC. HNPCC is an autosomal dominant trait and is associated with other epithelial tumours including ovarian, gastric and endometrial cancer. (Beggs and Hodgson 2008) These cancers are characterised by a germline defect in one of the DNA mismatch repair (MMR) genes which detect and repair DNA replication errors during mitosis. Mutations in *hMLH1*, *hMLH2* and *hMLH6* are causal for HNPCC with the former two genes accounting for the majority of tumours. (Beggs and Hodgson 2008)

Microsatellite instability (MSI) is the characteristic biomarker of HNPCC. Microsatellites are small repeating units of two to five base pairs. (Leslie, Carey et al. 2002) These repeat regions are highly vulnerable to replication errors which are usually repaired by DNA repair mechanisms within the cell. Defects in one of the seven MMR proteins encoded by the mismatch repair genes, usually MSH-1, MSH-2 and MSH-6, results in a defunct mismatch repair system allowing base mismatches and strand misalignments which occur during replication to be retained in daughter cells. (Medina-Arana, Delgado et al. 2012) When a cell is MMR deficient, microsatellites become mutated with time and the degree of mutation or MSI can be characterised as being high or low; MSI-H or MSI-L respectively. Whilst the changes occurring at microsatellites have little consequence for the genome or for the changes in gene expression which are causal for tumourigenesis, MSI acts as a surrogate marker for other genetic damage that is going unrepaired by the defunct MMR system. (Beggs and Hodgson 2008) Deficient MMR leads to rapid acquisition of other genetic mutations and accelerated cancer development with a median age of CRC diagnosis of 45 years. (Kastrinos and Syngal 2011; Medina-Arana, Delgado et al. 2012)

1.4.6 THE SPORADIC COLORECTAL CANCER SEQUENCE

Understanding of the molecular and genetic basis of sporadic CRC has been facilitated by knowledge of the abnormalities responsible for inherited CRC syndromes. The initial

description of the sequence of genetic mutations in sporadic CRC was provided by Vogelstein & Fearon who demonstrated progressive acquisition of four genetic defects as tissue progressed macroscopically from normal colorectal epithelium to small adenoma, to large adenoma and to a carcinoma. (Vogelstein, Fearon et al. 1988)

Haploinsufficiency of *APC* is thought to occur early in the sporadic cancer sequence as *APC* mutations are present in approximately the same percentage of polyps and cancers. (Beggs and Hodgson 2008) Allelic loss has been shown to increase as phenotype progresses from adenoma to carcinoma with *APC* mutation detectable in around 80% of all CRCs. (Powell, Zilz et al. 1992; CGAN 2012)(Figure 1-7)

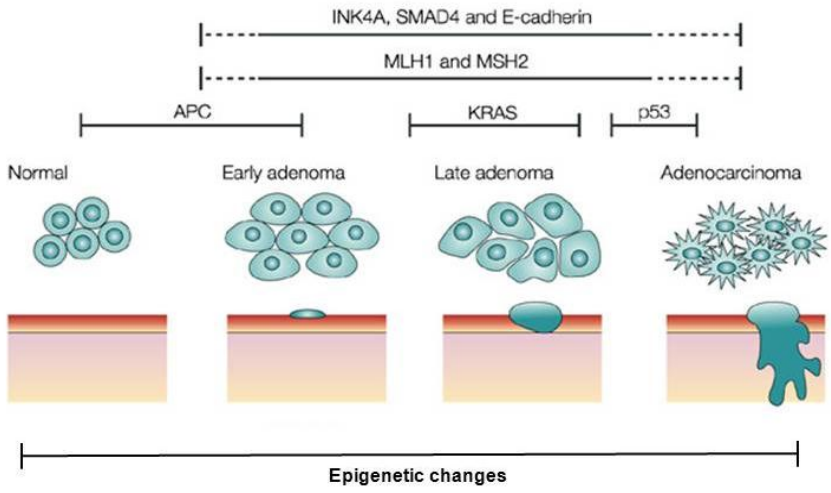
K-RAS is an oncogenic regulator of cell differentiation and mutations in this gene act later than those in *APC* in CRC carcinogenesis. (Figure 1-7) Mutation of *K-RAS* results in permanent activation of the RAS protein and uncontrolled cellular proliferation. (Scott, Bell et al. 1993) *K-RAS* mutations are found in 35-45% of colorectal adenomas and carcinomas (Beggs and Hodgson 2008; CGAN 2012) Mutations in *K-RAS* are found less frequently in small adenomas suggesting that oncogenic activation of *K-RAS* is not an initiating event in CRC development. (Leslie, Carey et al. 2002) In a study of small polyps, *K-RAS* mutation was frequent in both benign and dysplastic adenomatous polyps but was always associated with *APC* mutation when dysplasia was observed. (Jen, Powell et al. 1994)

p53 has multiple functions including DNA repair, cell cycle arrest in the presence of DNA damage and induction of apoptosis. (Pecorino 2008) It is the gene most frequently altered in any human cancer. (Caron de Fromentel and Soussi 1992) *p53* mutations lead to accumulation of mutated p53 proteins in the cell and functional inactivation of its tumour suppressor role. (Beggs and Hodgson 2008) The probability of alteration of *p53* seems to be a cumulative process and genetic mutations have been detected in 4-26% of colorectal adenomas, 50% of invasive foci in adenomas and 75% of carcinomas. (Beggs and Hodgson 2008) It has been postulated that *p53* mutation is a critical process in the phenotypic change from adenoma to carcinoma. (Leslie, Carey et al. 2002)

18q loss has long been recognised in CRC. It is present in 70% of cancers, 10-30% of early adenomas and around 60% of late adenomas. (Vogelstein, Fearon et al. 1988; Boland, Sato et al. 1995) Initially this was thought to act via the *DCC* (deleted in colorectal cancer) gene but further characterisation of the genes in this locus revealed two further putative tumour suppressor genes: *SMAD2* and *SMAD4.* Their protein products are parts of the inhibitory pathway TGF-β (Leslie, Carey et al. 2002) which acts to control cell growth, differentiation and apoptosis. (Leslie, Carey et al. 2002) Mutations in *SMAD4* have been observed in approximately 15% of CRC cell lines and 10% of CRCs (Thiagalingam, Lengauer et al. 1996; CGAN 2012). Serrated polyps – polyps containing foci of both hyperplasia and adenoma - have been created in a murine model with an induced *SMAD4* mutation, implying that this gene may have an early role in the adenoma-carcinoma sequence. (Hohenstein, Molenaar et al. 2003)

FIGURE 1-7. SUMMARY OF SELECTED GENETIC AND EPIGENTIC CHANGES IN CRC AND THEIR RELATIONSHIP TO MACROSCOPIC STAGE.

REPRODUCED WITH PERMISSION. (KERR 2003)



1.4.7 EPIGENETIC ALTERATIONS IN CRC CARCINOGENESIS

Mutations are not the only events causing alteration of gene expression in CRC development. Epigenetic events also play an important role in alterations of gene expression required for a cancer phenotype.

The term epigenetics was first defined in 1939 by C.H. Waddington to describe, 'the causal interactions between genes and their products, which bring the phenotype into being.' (Waddington 1939) The term has subsequently been refined, and redefined as: 'as heritable changes in gene expression that are not due to any alteration in the DNA sequence.' (Holliday 1987)

Epigenetic modifications of gene expression arise by three main mechanisms: histone modification, gene methylation and micro riboxynucleic acid (RNA) (miRNA) inhibition. (Esteller 2008) Histone molecules are five small proteins exhibiting a highly positive charge which tightly bind DNA (strongly negatively charged) and form the core of all DNA packaging. DNA bound to a histone is essentially inaccessible for transcription and expression but alterations to histone proteins such as methylation or acetylation may alter conformation and binding of DNA, changing the expression of the relevant gene. (Alberts, Bray et al. 1994; Pecorino 2008) Micro RNAs are 20-30 base RNAs which are encoded in the genome and transcribed into RNA but not translated into protein products. Instead these microRNAs act to inhibit transcriptional and translational processes of other genes to regulate their expression. (Bartel 2004) Methylation of genes is also associated with transcriptional activation and silencing and is discussed in detail below (see Section 1.4.8). In a fashion similar to genomic events, epigenetic events may be cumulative, and different types of epigenetic modification such histone modification and promoter methylation may act together to alter gene expression. (Mossman and Scott 2011)

1.4.8 GENE METHYLATION

In mammals, methylation of the genome refers to the presence of a methyl group at the 5' position in cytosine residues within CpG dinucleotides. The addition of methyl groups is mediated by DNA methyltransferases (DNMT) 1, 3a and 3b. (Zaidi, Young et al. 2011) Methylation may alter transcription through a number of mechanisms including: alteration of promoter binding sites for transcription factors and other protein components of the transcriptional machinery, by nucleosomal occupancy alteration or through recruitment of histone deacetylases to remodel the chromatin structure to an inactive state. (Lim, Neilsen et al. 2011; Zaidi, Young et al. 2011; Wang, Maurano et al. 2012)

CpGs are not evenly distributed within the genome. Around one half of genes contain a short segment which is CpG dense – a CpG island - whilst the remainder of the genome is relatively CpG deficient. (Jones 2012) Functional control of expression by methylation is largely thought to occur when CpG islands are present within gene promoters although new roles are being observed for telomeric, exonic and inter-genic areas of the genome. (ENCODE 2012; Jones 2012) Promoter methylation is generally highly correlated with gene expression although there are recent data suggesting that exonic methylation status is more highly correlated, although mechanistic relationships underpinning this finding are not understood. (Bell, Pai et al. 2011)

The recent publication of data from the ENCODE project which profiled CpG methylation for an average number of 1.2 million CpGs in 82 cell lines found that 96% of CpGs were differentially methylated in at least one cell type. (Meissner, Mikkelsen et al. 2008) Similar to Bell et al. (above) ENCODE suggested that the most variability in methylation occurred out with gene promoters at intergenic and coding segments of the genome. (ENCODE 2012) Unmethylated coding regions were found to bind the enhancer protein P300 which has suggested new mechanisms for the methylation dependent control of transcription. (Ogryzko, Schiltz et al. 1996; ENCODE 2012) In normal tissues methylation of promoters was suggested to occur as a passive

phenomenon in response to lack of transcription factor binding to promoter regions. The association that suggested this passive role for methylation was reversed in cancer tissues (although CRC cell lines were not tested) suggesting that gene specific methylation may take on a more active role in determination of transcription during carcinogenesis. (Thurman, Rynes et al. 2012)

Whilst the relationship of CpG methylation and transcription is well established mechanisms regulating gene methylation, particularly those causing demethylation of CpGs are less well characterised. (Branco, Ficz et al. 2012; Jones 2012) Potential pathways have been proposed including hyper hydroxylation of methyl cytosines by TET enzyme systems and subsequent replacement with unmethylated cytosines by base excision repair mechanisms, although this has only been demonstrated in non-CpG rich areas of the genome. (He, Li et al. 2011; Ito, Shen et al. 2011; Maiti and Drohat 2011; Nabel and Kohli 2011)

TSG hypermethylation silencing and hypomethylation based activation of oncogenes are now recognised as hallmark epigenetic defects in CRC and ageing. (Jones and Baylin 2007; Belshaw, Pal et al. 2010; Berdasco and Esteller 2010; Sunami, de Maat et al. 2011) Promoter hypermethylation is such a prevalent defect in CRC carcinogenesis that many consider it to be the third hit in Knudson's model for inactivation of TSGs. (Herman and Baylin 2003) A selection of genes known to exhibit promoter hypermethylation in CRC is listed in Table 1-2. (van Engeland, Derks et al. 2011) Several TSGs are also known to be silenced in adenomatous polyps via promoter hypermethyaltion. (Jones and Baylin 2002; Chan and Rashid 2006) Importantly, subtle changes in methylation of TSG promoters have been detected in macroscopically normal mucosa in individuals with CRC and also in the normal mucosa of individuals at higher risk of CRC. (Al-Ghnaniem, Peters et al. 2007; Belshaw, Elliott et al. 2008) These findings suggest that methylation changes may be precursors of tumourigenesis and may contribute to the field defects which are present in the vulnerable, or higher risk, mucosa. (Belshaw, Elliott et al. 2008) Progressive promoter hypermethylation is also associated with normal ageing, independent of other risk factors, which may give rise

to some of the increased CRC risk with older age. (Shen, Kondo et al. 2005; Belshaw, Elliott et al. 2008)

Aberrant methylation is present in the majority of CRCs (van Engeland, Derks et al. 2011) and as methylation is modifiable (section 1.4.10) restoration of normal tissue methylation represents an exciting therapeutic target. Agents which alter methylation are currently used routinely for haematological malignancies and myelodysplastic syndromes. However, until recently, applications in solid organ tumours have been limited by effects in non-cancerous tissues. The development of targeted agents and the discovery of the de-methylating properties of current anti-arrhythmic drugs such as procainamide and hydralazine have revived research into solid organ tumour therapies. (Ren, Singh et al. 2011) In pre-clinical CRC models de-methylating agents including boswellic acid can decrease hypermethylation of TSGs such as *SAMD14* and *SMPD3* and restore normal expression. (Fini, Selgrad et al. 2007; Shen, Takahashi et al. 2012) A phase 1 clinical trial has demonstrated proof of concept in humans showing that global methylation can be altered in metastatic cancer patients (several of whom had CRC) using 5-Azacytidine (de-methylating agent) and valproic acid (histone deacetylase inhibitor). (Braiteh, Soriano et al. 2008)

Whilst drug discovery for agents that modulate methylation holds future prospect, methylation can currently predict response to existing therapies. *DEXI* hypermethylation predicts poor tumour response to irinotecan chemotherapy (Miyaki, Suzuki et al. 2012) and methylation of *TFAP2E–DKK4* predicts response to fluorouracil and other chemotherapeutic strategies with response of 15% in hypomethylated tumours and 80% in hypermethylated tumours in prospective human studies. (Ebert, Tänzer et al. 2012)

TABLE 1-2. GENE PATHWAYS AND GENES KNOWN TO BE AFFECTED BY PROMOTER HYPERMETHYLATION IN CRC. ADAPTED FROM VAN ENGELAND ET AL. 2011 (VAN ENGELAND, DERKS ET AL. 2011)

Pathway	Genes with known hypermethylation
WNT	*APC, SFRP1, SFRP2, SFRP4, SFRP5*, *SOX17, WNT5a*, *DKK1*, *DKK3*, *WIF1*, *AXIN2*
NOTCH	*NEURL*
Cell cycle regulation	*P16^{INK4A}*, *KLF4*
Transcription regulation	*GATA4, GATA5*, *RUNX3*, *CDX1*, *HLTF*, *FOXL2, ALX4*
DNA repair/stability	*MLH1*, *MGMT, WRN, CHFR*
Invasion and metastasis	*TIMP3*, *RECK*, *CXCL12, TFPI2*

Whilst hypermethylation of TSGs is the more frequently reported pathway for epigenetic mediated carcinogenesis, hypomethylation of oncogene promoters also acts as a carcinogenic pathway, although hypomethylation is more established in non-CRC epithelial cancers. For example, hypomethylation of miRNA-191 is responsible for its over-expression in hepatocellular carcinoma (HCC) which contributes to epithelial to mesenchymal transformation. (He, Cui et al. 2011) Promoter hypomethylation leading to oncogene upregulation in HCC has also been observed for *CD147*. (Kong, Liao et al. 2011) Whole epigenome array data in HCC has found that an equal number of promoters are affected by hypomethylation (3,689 promoters corresponding to 1,974 genes) and hypermethylation (3,517 promoters, 1,894 genes) in cancer tissues compared to controls. Functional clustering of pathways of the over-expressed genes showed that they controlled: cell growth, cell adhesion and communication, signal

transduction, mobility, and invasion. Genes which were overexpressed in HCC were compared to published data in other epithelial cancers (breast and ovarian) and 42 genes were found to be commonly overexpressed with associated hypomethylation. Attempts were made to correlate overexpressed genes due to hypomethylation in HCC to those overexpressed in CRC by hypomethylation but none were found due to the paucity of such data in CRC. (Stefanska, Huang et al. 2011)

In breast cancer upregulation of the NOTCH1 protein (section 1.9, Figure 1-8) has been observed as cancers progress in stage and is associated with poor tumour differentiation. This increased expression has been correlated with progressive hypomethylation of the *NOTCH1* promoter. (Zhang, Sun et al. 2011) Similar associations of expression, tumour stage and hypomethylation have been reported for the circadian gene *TIMELESS* in breast cancer. (Fu, Leaderer et al. 2011)

Hypomethylation of oncogene promoters has additionally been reported in salivary gland adenoid cystic carcinoma, oesophageal carcinoma and glioma. (Alvarez, Opalinska et al. 2011; Liu, Tang et al. 2011; Shao, Sun et al. 2011)

In CRC, hypomethylation of parts of the Sonic hedgehog pathway are the mechanism by which folate depletion can enhance the invasiveness of cultured CRC cells. (Wang, Hsu et al. 2012) *RAPGEF1,* which has a role in regulation of proliferation, differentiation and apoptosis, has been found to be hypomethylated in 40% of CRCs compared to normal controls. (Samuelsson, Alonso et al. 2011) In CpG methylator phenotype tumours, a type of CRC characterised by a high degree of methylation dependent gene silencing, (Issa 2004) DNMT3a is frequently hypomethylated, potentially leading to its overexpression and increased methylation at multiple loci observed in this tumour subtype. (Drini, Wong et al. 2011)

1.4.9 GLOBAL DNA METHYLATION

In addition to specific gene promoter methylation changes, there is overall global DNA hypomethylation associated with CRC. (Hiraoka, Kato et al. 2010; Khare and Verma 2012) Global hypomethylation occurs with age and promotes genomic instability.

(Belshaw, Pal et al. 2010; Beck, Garcia-Perez et al. 2011) Whilst global methylation is measured on a genome wide basis, most of the genome is CpG deplete (section 1.4.8) and reported global methylation actually occurs predominantly in repeat elements rather than evenly throughout the genome. (Jones 2012) Whilst global hypomethylation was originally thought to represent a bystander effect on non-coding DNA, genomic instability is actually mediated by the demethylation and transcriptional activation of previously silenced transposable elements which make up 45% of the human genome. Activation of transposable elements results in insertions of mutated DNA and is implicated in the initiation of several human diseases including various cancers. (Beck, Garcia-Perez et al. 2011) Transposable elements in the human genome are primarily long interspersed element 1 (LINE-1) (18% of the human genome) and short interspersed elements (27% of the human genome). (Beck, Garcia-Perez et al. 2011)

1.4.10 METHYLATION AND THE MICROENVIRONMENT

Methylation status can be affected by the tissue microenvironment. (Arasaradnam, Commane et al. 2008) Many environmental exposures and lifestyle factors have been shown to affect the epigenome including: folate consumption, tobacco smoke exposure, alcohol exposure, ageing, hormonal exposures, air pollution and heavy metal exposure. (Christensen, Marsit et al. 2012) Environmental exposures affect different tissues in a context specific pattern and the interaction of environmental factors and the epigenome may depend on factors such as transcription factor availability or pre-existing methylation status of a tissue. (Rakyan, Down et al. 2010; Alegría-Torres, Baccarelli et al. 2011; Christensen, Marsit et al. 2012) In colorectal cells there is evidence that methylation status and genetic expression can be modulated by dietary supplementation with resistant starch (section 1.12) and its colonic fermentation product, butyrate. (Dronamraju, Coxhead et al. 2008; Dronamraju, Coxhead et al. 2009) Further details of promoter methylation modulation by environmental factors are detailed in section 1.12.7.3.

1.5 ULCERATIVE COLITIS

Ulcerative colitis (UC) is an inflammatory bowel disease characterised by diffuse superficial mucosal inflammation that is essentially confined to the colon. The incidence of UC is 10-120 per 100 000 per year and prevalence is 100-200 per 100 000 with approximately 100 000 people thought to be affected in the UK. (Carter, Lobo et al. 2004) Ethnic groups have varying prevalences with Ashkenazi Jews and Indian immigrants having a particularly high rate of UC. (Carter, Lobo et al. 2004; Nicholls and Tekkis 2005) The disease is predominantly incident in younger people with an equal sex distribution. (Nicholls and Tekkis 2005)

1.5.1 ULCERATIVE COLITIS AND COLORECTAL CANCER

The aetiology and pathogenesis of UC have not been fully defined. Best evidence suggests that it is a combination of a response to an environmental factor in a genetically susceptible individual. (Nanau and Neuman 2012; Neuman and Nanau 2012) UC confers an increased risk of CRC which increases with time from diagnosis. Individual reports of the magnitude of CRC risk vary between 60% risk at 40 years and a risk of CRC similar to the normal population. (Zisman and Rubin 2008) The best current estimate is based on a meta-analysis of published evidence performed in 2001 which showed the incidence of CRC was 2% after 10 years, 8% after 20 years, and 18% after 30 years following diagnosis of UC with lifetime risk 2.4 times that of the general population. (Eaden, Abrams et al. 2001; Jess, Rungoe et al. 2012) More recent, smaller, reports have suggested lower risks of CRC leading to the suggestion that UC associated CRC may be decreasing possibly due to therapeutic improvements in UC management. (Bernstein, Blanchard et al. 2001; Winther, Jess et al. 2004; Rutter, Saunders et al. 2006)

Disease specific risk factors modify an individual's risk of CRC. Incidence of UC associated CRC increases with time since diagnosis (Gong, Lv et al. 2011; Higashi, Futami et al. 2011) and with longer segments of colonic involvement (relative risk 1.7 for proctitis, 2.8 for left-sided disease and 14.8 for pancolitis) (Ekbom, Helmick et al.

1990) Several studies have found the depth of mucosal inflammation to be independently predictive of CRC development. (Rutter, Saunders et al. 2004; Rubin 2006; Gupta, Harpaz et al. 2007) Other risk factors include a family history of CRC independent of a family history of colitis (Askling, Dickman et al. 2001; Velayos, Loftus et al. 2006) and a young age at disease onset (Ekbom, Helmick et al. 1990). Co-existing primary sclerosing cholangitis is strongly associated with CRC development and increased surveillance is recommended in such patients. (Mowat, Cole et al. 2011)

1.5.2 PATHOLOGY OF CRC IN UC

The pathology of cancer in UC differs from the pathology of sporadic CRC both at a macroscopic level and also at a molecular level. The microscopic phenotype of pre-malignant UC lesions follows a similar progression to the sporadic polyp-cancer sequence progressing from, indeterminate to low grade to high grade dysplasia and, with basement membrane invasion, to carcinoma. However dysplastic lesions associated with UC are more commonly flat rather than raised polyps therefore detection and removal are more complex (Neumann, Vieth et al. 2011). Technologies have developed to improve diagnostic accuracy of pre-malignant lesions in UC such as narrow-band imaging or chromoendoscopy as adjuncts to standard colonoscopic surveillance. (Zisman and Rubin 2008; Neumann, Vieth et al. 2011) Finding areas of dysplasia usually mandates colectomy as 19% of patients with a low grade dysplasia will have synchronous high grade dysplasia and approximately fifty percent will develop CRC within five years. Of those with high grade dysplasia 43% will have a synchronous CRC. (Bernstein, Shanahan et al. 1994; Ullman, Croog et al. 2003)

Most cancers related to UC are adenocarcinomas, however, there is an increased frequency of cancer types less frequently seen as sporadic CRCs such as mucinous carcinomas or signet cell adenocarcinomas. (Harpaz and Polydorides 2010) Synchronous CRCs also occur more frequently in the context of UC. Sporadic CRC has a frequency of two synchronous tumours in less than five percent of all cases. Three or more synchronous tumours occur so infrequently that data are not available on their prevalence. In contrast the incidence of two synchronous tumours in UC is between

ten and thirty percent and three or more synchronous tumours are more common. (Harpaz and Polydorides 2010)

1.5.3 GENETIC CHANGES IN UC ASSOCIATED CANCER

At a molecular level the genetic changes in UC associated CRC are similar but the sequence in which they are acquired in premalignant lesions is often different. (Itzkowitz and Harpaz 2004) *APC* mutation is generally considered to be an initiating event in sporadic CRC however in UC associated CRC it is a late and infrequent event only occurring in high grade dysplasia or cancer. (Kern, Redston et al. 1994; Aust, Terdiman et al. 2002) *K-RAS* mutation is relatively infrequent in UC and, as it is thought to confer a polypoid phenotype, may account for the increased incidence of flat dysplasia observed in UC. (Burmer, Levine et al. 1990; Yashiro, Carethers et al. 2001) *p53* is a late event in sporadic CRC but is thought to be an initiating lesion in UC associated CRC as it is often present in macroscopically normal mucosa before dysplasia can be detected. (Burmer, Levine et al. 1990; Burmer, Rabinovitch et al. 1992)

1.5.4 EPIGENETIC CHANGES IN UC AND UC ASSOCIATED CANCER

Epigenetic factors influence the UC disease process independently of carcinogenic potential. DNA methylation changes have been shown at multiple loci when patients with inflammatory bowel disease are matched to normal controls. A sub-set of genes displaying aberrant methylation further differentiates Crohn's colitis from UC. (Lin, Hegarty et al. 2011) When patients with UC for more than seven years are compared to normal controls global DNA hypomethylation and increased proliferation index within crypts – a phenotype associated with higher cancer risk – are observed. (Glória, Cravo et al. 1996; Konishi, Shen et al. 2007) Maternal supplementation with a methyl donor in mice, increases methylation in 59 gene promoters and decreases methylation in 96 gene promoters in the offspring mice. These mice are more susceptible to chemical induction of colitis, a situation not found in the offspring of mice on a

maternal control diet subsequently fed a methyl donor in the neonatal period. (Schaible, Harris et al. 2011)

DNA methylation changes are also described in the pathogenesis of UC associated CRC. DNA methylation changes are likely to be founder mutations in UC associated CRC and epigenetic field change (see section 1.8) may explain the more rapid acquisition of further somatic mutations and also the preponderance for synchronous lesions. In murine models promoter methylation changes in many genes have been noted as early as eight weeks after colitis induction and these changes are independent of inflammatory cell presence or absence. (Katsurano, Niwa et al. 2012) Promoter hypermethylation of two TSGs; *ESR-1* and *N-33* have been shown to be higher in patients with UC compared to matched controls. (Arasaradnam, Khoo et al. 2010) When *APC* mutations are examined in IBD related cancers, somatic mutations, common in sporadic CRC (present in 74% of tumours), are rare in IBD associated CRC (6% of all tumours). (Tarmin, Yin et al. 1995) Promoter methylation of ten key WNT pathway genes has been examined in non-UC controls, long-standing UC patients and patients with UC associated cancer. *APC2, SFRP1, SFRP4, SFRP5, DKK1, WIF1* all showed significantly increased promoter methylation in long-standing UC compared to normal controls. *APC2, SFRP1* and *SFRP2* also showed increased promoter methylation in UC associated CRC compared to longstanding UC controls suggesting that this maybe the mechanism controlling altered WNT pathway expression where genetic mutation of *APC* is not present. (Dhir, Montgomery et al. 2008) Hypermethylation of *ARF* has also been shown to be associated with duration of UC and predicts of risk of developing UC associated CRC. (Moriyama, Matsumoto et al. 2007) The promoter site of the E-cadherin gene (*CDH1*), related to cell adhesion properties, has also been shown to be hypermethylated and transcriptionally silenced in dysplastic versus non-dysplastic UC tissue. (Azarschab, Porschen et al. 2002) Wider whole genome methylation arrays have identified multiple aberrantly methylated loci in UC sufferers which correlated highly with methylation abnormalities also detected in a sporadic CRC cell line. (Koizumi, Alonso et al. 2012)

1.6 SUMMARY – CLINICAL CHARACTERISTICS OF CRC AND AETIOLOGY

CRC represents a major disease burden both in the UK and worldwide. Survival has improved with improved therapeutics however incidence is largely unchanged. A minority of CRC (6-7%) has a defined heritable genetic cause (hereditary CRC) whilst another minority (15-20%) has a genetically undefined heritable increased risk (familial CRC). The majority of CRC is sporadic and data showing variation between populations depending on environmental and particularly dietary exposures, with rapid conversion to a host nations risk in migrant individuals, suggest that environmental exposures are critical in CRC aetiology. This strong environmental influence suggests that a proportion of sporadic CRC may be preventable - estimated to be up to 50%. (Platz, Willett et al. 2000)

The genetic 'hits' required for tumourigenesis are relatively well understood, largely due to the study of hereditary syndromes, particularly FAP. In sporadic CRC carcinogenesis, genetic mutations occur predictably in tandem with macroscopic development of neoplasia and invasion – the polyp-cancer sequence. (Vogelstein, Fearon et al. 1988)

Also contributing to the CRC phenotype are epigenetically driven changes in gene expression. Methylation is a key pathway altering gene expression by hypomethylation based activation of oncogenes, hypermethylation based silencing of TSGs or global hypomethylation activating previously silenced transposable elements resulting in genomic instability. Multiple pathways are subject to methylation changes in CRC. Whilst DNA methylation signatures are heritable they are also plastic to environmental factors.

UC associated CRC represents a distinct CRC subtype. Epigenetic alterations play a key role in the field change which predisposes to rapid acquisition of mutations and the increased incidence of synchronous tumours. Methylation changes potentially account for a degree of the predisposition to developing UC and, once the disease is

established, provide the founder mutation for those individuals who go on to develop UC associated CRC.

1.7 COLONIC CRYPT BIOLOGY AND STEM CELLS

1.7.1 THE NORMAL COLONIC CRYPT

CRC is a disease of genetic expression at the molecular level which translates to a disease of cellular proliferation. (Pecorino 2008) Knowledge of cellular proliferation and the resulting dynamics of the functional unit of the colon – the crypt - are therefore required to understand CRC pathogenesis.

Colonic crypts are invaginations of the colorectal mucosa, opening into the lumen of the gut. New crypt cells originate at the crypt base from the division of stem cells and migrate lumenwards. As cells progress lumen-wards they differentiate progressively and lose their proliferative capacity through the transit amplifying region to become terminally differentiated cells (either secretory goblet cells or absorptive colonocytes) in the upper third of the crypt. (Potten, Kellett et al. 1992) The average crypt is approximately 80 cells high and 40-45 cells in circumference with between 2000 and 2200 cells per crypt. (Potten, Kellett et al. 1992)

Bromodeoxyuridine (BrdUrd) labelling of proliferating cells (S-phase) shows 10% of crypt cells to be in S phase at any time in normal mucosa. Ninety percent of BrdUrd labelled cells are found between position 4 and 43 (from the crypt base) and maximal labelling occurs at position 15. (Potten, Kellett et al. 1992) Thus the zone of proliferation is located in the lower half of the crypt with the greatest proliferation occurring in the lower third - the crypt base and transit amplifying compartment.

1.7.2 THE DISEASED COLONIC CRYPT

Altered colorectal cellular proliferation related to tumour development was first reported in carcinogen treated mice. (Thurnherr, Deschner et al. 1973) Initially there was a shift in proliferation to include the entire crypt length although the majority of proliferation was retained in the lower half. With further genotoxic insults the main area of proliferation shifted to the upper half of the crypt. The shift of the proliferative compartment occurs in tandem with an overall increase in the proportion of S-phase

cells. (Roncucci, Pedroni et al. 2000) Similar shifts in the zone of maximal proliferation and increase of the proportion of S-phase cells are seen in macroscopically normal tissue adjacent to tumours and in patients with FAP and HNPCC. (Roncucci, Pedroni et al. 2000)

1.7.3 THE STEM CELL

Cells in colorectal crypts, as with all cells in the body, arise from stem cells. Adult stem cells (ASC) are cells which satisfy two criteria:

Longevity – long lived cells able to undergo multiple divisions

Multipotency – the ability of progeny to differentiate into all the cell types of that organ (*cf*. pluripotency which is the ability of an embryonic stem cell to differentiate into any cell type of the organism). (Barker, van de Wetering et al. 2008)

Barker et al. have provided a gold standard method for identification of colorectal ASCs. ASCs in the intestinal mucosa stain positively for Leucine-rich-repeat containing G-protein-coupled receptor 5 (Lgr5) in mouse colon and small intestine. (Barker, van Es et al. 2007) Lgr5 is a protein whose transcription is increased in response to an activated Wnt pathway. WNT is a key pathway in the control of the stem cell differentiation and activity (see section 1.8.8). (Barker and Clevers 2006; Van der Flier, Sabates-Bellver et al. 2007) Lgr5 expression is limited to the base of the intestinal crypt with differential expression from Paneth cell and transit-amplifying cell specific markers which suggested Lgr5 as a putative ASC marker in the colon. (Van der Flier, Sabates-Bellver et al. 2007) In the small bowel Lgr5 positive cells reside between the Paneth cells in the crypt base. (Barker, van Es et al. 2007) An average of 3.5 Lgr5 expressing cells are present per crypt base in the mouse. These cells are actively cycling as they stain positively for Ki67 (a proliferation marker). (Barker, van Es et al. 2007)

Labelling of the putative stem cells and their progeny using a tamoxifen activated LacZ reporter demonstrated multipotency and longevity. (Barker, van de Wetering et al.

2008) Mice with the inducible LacZ reporter were given a tamoxifen pulse and killed at 1, 5, 12, 35 and 60 days. Labelled cells were seen to be emanating lumen-wards from the Lgr5 positive cells. The proportion of labelled cells within each crypt base remained stable indicating that the Lgr5 positive cells continued to divide over a 60 day period and did not ascend the crypt as would be expected for non-stem epithelial cells. (Barker, van Es et al. 2007) These experiments have subsequently been replicated and extended over 14 months, confirming greater longevity of Lgr5 positive cells. (Barker, van Es et al. 2008) Within the progeny of Lgr5 positive cells in small bowel and colon all cell types in the gastrointestinal mucosa were identified confirming multipotency. (Barker, van Es et al. 2007) Additionally, single cell transplantation of an Lgr5 positive cell was able to grow complete crypt structures in culture containing all differentiated cell types of the intestine in the proportions expected in normal epithelium. Whilst this is not a defining feature of an ASC (see beginning of section) it confirms the progenitor capacity of the Lgr5 positive cell population. (Sato, Vries et al. 2009)

1.8 CRYPT DYNAMICS, SUCCESSION AND FIELD CHANGE

1.8.1 CLONALITY OF THE CRYPT

Experiments where stem cell progeny have been labelled demonstrate the clonal nature of differentiated crypt cell populations depending on their originator ASC within the crypt base. (Barker, van Es et al. 2007) Methylation signatures within crypts can also be used to demonstrate distinct clonal populations within a crypt. (Graham, Humphries et al. 2011) CRC arises from the acquisition of mutations within a single ASC (see section 1.8.7). The process for the development of a single mutated ASC into a CRC requires an acceleration of normal processes that occur within the crypts and epithelium as a whole. These processes and how they become dysregulated in carcinogenesis are detailed below.

1.8.2 MONOCLONAL CONVERSION AND NICHE SUCCESSION

The vast majority of ASC divisions are likely to be asymmetrical producing one ASC and one partially differentiated cell, with occasional symmetrical divisions producing either two daughter differentiated cells or two daughter ASCs. Over time random lineage extinction and matched lineage expansion will occur by the process of symmetrical divisions and several cell lineages within a crypt will eventually be succeeded by a single clonal population. (Boman, Wicha et al. 2007; Graham, Humphries et al. 2011) This clonality has been shown in XY/XO mosaic normal mucosa where each individual crypt was monoclonal with either exclusively XY or XO karyotype. (Novelli, Williamson et al. 1996)

The patterns of succession can be observed using age related methylation signatures within the crypt. The theory underpinning these observations is that whilst overall methylation of a CpG with age related change in a tissue will gradually change in an individual over time, at a cellular level the methylation at the same CpG locus is an all or nothing phenomenon. This means that methylation at a cellular level is a binary phenomenon whilst measurement of methylation in a tissue is measured on a

continuous scale, usually described in terms of percentage of cells methylated within a tissue or simply 'percentage methylation'. Methylation is heritable, so the progeny of a stem cell will display the same methylation signature as its parent stem cell with only very slight drift as differentiated cells may pick up additional methylation marks in response to environmental stimuli. This drift from daughter cells and parent stem cells is minimal as methylation signatures at the bottom and top of crypts are the same, thus methylation observed in whole crypt samples reflects methylation status of the parent stem cell. (Yatabe, Tavaré et al. 2001)

Clonal succession has been confirmed by measuring the number of populations with distinct methylation signatures on genes known to display age related change in colonic epithelium. (Yatabe, Tavaré et al. 2001) Yatabe et al. found that each succession cycle (one clonal population replaced entirely by another) would be approximately 8.2 years (95% confidence interval (CI) 2.7-19 years). (Yatabe, Tavaré et al. 2001; Kim and Shibata 2002) Crypt succession has also been shown by measurement of epigenetic changes and associated COX deficiency as a result of mitochondrial DNA mutation (see section 1.8.3), again suggesting that succession is a slow process (taking up to 10 years) in normal tissues. (Graham, Humphries et al. 2011)

In a normal colonic crypt, succession would be random, but this would not remain the case if an ASC were to acquire a genetic mutation conferring a survival or growth advantage. In such a situation the probability would be for the mutated stem cell to succeed the crypt resulting in monoclonal conversion with a pro-carcinogenic genetic mutation. (Kim and Shibata 2002) Although succession and monoclonal conversion remains macroscopically invisible if there is no phenotypic difference in populations, molecular changes do occur. Several studies have demonstrated pro-carcinogenic genetic mutations in phenotypically normal crypts of patients at higher risk of CRC. (Hanahan and Weinberg 2000; Belshaw, Elliott et al. 2008; Belshaw, Pal et al. 2010; Fujii, Katake et al. 2010)

The number of stem cells per crypt giving rise to clonal populations can also be inferred by counting the number of distinct age-related epigenetic signatures per

crypt. As these epigenetic marks are involved in ageing but not carcinogenesis, and therefore do not confer a survival advantage in themselves, measurement of these signatures remains valid in epithelium under pro-carcinogenic influence. Morphologically normal colon crypts from resected FAP and non-FAP colon controls have been analysed to assess the number of distinct methylation lineages per crypt. Fewer distinct methylation signatures were observed in non-FAP controls whilst a greater number of distinct signatures were present per crypt in macroscopically normal FAP epithelium. (Kim and Shibata 2004) Mathematical modeling suggested that an increase in the number of stem cell reproductive cycles in FAP crypts did not explain the increased number of distinct signatures observed, however an increase in the number of ASCs per crypt did explain the observed data. (Kim, Calabrese et al. 2004) These data confirm subtle mucosal kinetic changes preceding morphological change in at risk epithelium and suggest that a founder change is likely to be a change in ASC number as demonstrated by an increase in the number of distinct clonal populations.

1.8.3 CRYPT FISSION AND FIELD CHANGE

Epithelium at risk of CRC, such as in longstanding UC, have large areas of tissue containing thousands of crypts with single clonal populations implying that ASCs with an acquired survival advantage can expand to populate more than a single crypt. (Humphries and Wright 2008) Branched crypts are observed occasionally (<1%) in normal human colon leading to the suggestion that intestinal crypts divide by a process called fission, similar to somatic cells dividing by mitosis.

Lineage tracing using cytochrome c oxidase (COX) status (either present or absent for any one cell) has been used to demonstrate the process of crypt fission. The COX status of a cell is encoded through mitochondrial DNA. Multiple different mitochondrial mutations may result in COX deficiency as COX status is encoded by multiple repeated sequences which are subject to relatively frequent errors in replication. (Tariq, Stuart et al. 2009) Specific mutations causing COX deficiency can be used to trace ancestry in a similar fashion to age-related methylation marks. Double immuno-histochemical staining can track both COX status and its specific causal

mitochondrial mutation in gastrointestinal tissue. (McDonald, Preston et al. 2006) This technique had been utilised to confirm monoclonal conversion of crypts in both colon (Taylor, Barron et al. 2003) and stomach crypts. (McDonald, Greaves et al. 2008)

Both Greaves et al. and Graham et al. have shown that in fissioning crypts, single cells from either branching arm shared the same specific mitochondrial mutation that resulted in COX deficiency (the odds of this occurring randomly are less than one in 2.48×10^9). (Kim and Shibata 2004; Greaves, Preston et al. 2006) They also showed that in adjacent non-fissioning COX deficient crypts, single cells shared the same mitochondrial mutation and that COX negative crypts were clustered in the colon. This field effect was associated particularly with increasing age. These data suggest that crypt fission is indeed the method by which field changes occur in the colon. (Greaves, Preston et al. 2006; Graham, Humphries et al. 2011) Similar patches of identically mutated mitochondrial DNA have also been identified in the small intestine. (Lydia, Maesha et al. 2009)

Whilst Graham et al. confirmed crypt 'relatedness' using COX mutation they could not confirm 'relatedness' of crypts sharing a COX mutation using methylation signatures of non-coding areas of the genome. (Graham, Humphries et al. 2011) This lack of methylation 'relatedness' within age related gene promoters has also been observed in adjacent crypts presumed to share common ancestry by proximity, but not shown by other means to do so. (Kim and Shibata 2004) As these data are derived from macroscopically normal appearing mucosa this can be accounted for by the long time periods over which crypt fission is thought to occur. As fission is such an infrequent process in normal tissue, sampling of adjacent crypts reflects a temporally distant fission where two daughter clonal crypts have subsequently developed entirely different methylation signatures. (Graham, Humphries et al. 2011) As the epigenetic defects studied did not confer a survival advantage to the cell (non-coding DNA or ageing related gene promoters) this would explain such data as the lineage expansion and extinction associated with such defects would be balanced and random.

In higher risk epithelium pro-carcinogenic field change has been confirmed by various techniques. Proteomic data has demonstrated differential protein expression in normal mucosa from patients with no colonic abnormality and normal appearing flat mucosa from those with either adenomatous polyps (206 proteins) or cancer (61 proteins). (Polley, Mulholland et al. 2006) Multiple other reports have found epigenetic alterations associated with carcinogenesis in macroscopically normal mucosa adjacent to tumours. (Suzuki, Gabrielson et al. 2002; Shen, Kondo et al. 2005; Milicic, Harrison et al. 2008)

Crypt fission may account for colonisation of large fields of epithelium arising, originally, from a single ASC with a mutation conferring a survival advantage. However, the time that is proposed for monoclonal conversion within a crypt (8.2 years) (Yatabe, Tavaré et al. 2001) and crypt fission (estimated to occur every 17 years and taking 27 months) (Totafurno, Bjerknes et al. 1987) in normal tissue makes field change of large areas of epithelium and subsequent carcinogenesis over a human lifetime impossible. However, there are data suggesting that at risk epithelium may have an increased rate of crypt fission.

In at risk epithelium there is evidence of increased ASCs per crypt as higher numbers of distinct age-related methylation signatures are present in higher risk epithelium indicating a greater number of clonal populations. (Kim, Calabrese et al. 2004) Using a non-specific marker of all crypt basal cells Boman et al. have shown an expansion in the proliferative component lumenwards in human FAP colons. The noted changes were progressive as the pathology progressed from normal to aberrant crypt foci to adenomatous polyp. (Boman, Walters et al. 2004) Mathematical modelling of the observed data suggested that as part of a general increase in the proliferative compartment there would be an increase in the ASC number per crypt. An increase in size, and shift lumenwards, of the proliferative compartment has also been identified in sporadic colorectal adenomas. (Wong, Mandir et al. 2002; Dronamraju, Coxhead et al. 2009)

This overpopulation of the ASCs appears to specifically drive an increased rate of crypt fission. (Brittan and Wright 2002; He, Zhang et al. 2004) The initial genetic change in sporadic cancer is the *APC* mutation. (Beggs and Hodgson 2008) Haploinsufficiency for the *APC* allele in isolation results in no macroscopic epithelial change. (Hanahan and Weinberg 2000) Microscopically, increased rates of crypt fission are observed in FAP patients with haploinsufficiency, with a nineteen fold increase in the number of crypts in fission compared to non-FAP controls. (Wasan, Park et al. 1998) Thus a rapidly dividing ASC which has lost growth checkpoint characteristics would clonally expand by increased symmetrical division. This would increase the number of ASCs in the crypt base and shift the zone of proliferation towards the lumen. An overpopulation of ASCs would stimulate rapid fission, propagating its clonal daughter population exponentially across large areas of epithelium.

1.8.4 STEM CELLS AND CARCINOGENESIS

Several observations in CRC suggest the presence of a distinct subpopulation of cancer stem cells (CSC). (Boman and Wicha 2008) Firstly, therapies (chemotherapy and radiotherapy) targeted at rapidly reproducing cells may decrease tumour bulk but often do not lead to complete cure with high incidence of local or metastatic recurrence. This occurs after apparently curative therapy suggesting that a sub-population can 'escape' such treatments and repopulate a tumour in the absence of clinically detectable disease at the completion of therapy. Secondly the vast majority of cells (e.g. differentiated cells in the gut, reproductive tract and breast) are rapidly turning over. These cells are present for days or weeks but not the years required to accrue all the genetic changes required to induce cancer phenotype. (Boman and Wicha 2008) Data such as this suggests that the cellular population of cancer is heterogeneous, with a distinct subpopulation of cells which is able to initiate carcinogenesis and often evade current therapeutic strategies - the cancer stem cell.

The concept of a founder cell population is not new (Virchow 1855) however the term 'cancer stem cell' is more modern. (Carney, Gazdar et al. 1982) Since the initial discovery of a CSC in lung cancer (Carney, Gazdar et al. 1982), confirmation of CSCs in

other tissues has followed rapidly. CSCs have been identified in brain (Singh, Hawkins et al. 2004), breast (Al-Hajj, Wicha et al. 2003), haematological malignancies (Bonnet and Dick 1997), head and neck cancers (Zhang, Filho et al. 2012), pancreatic cancer (Li, Heidt et al. 2007), melanoma (Monzani, Facchetti et al. 2007) and colon cancer (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007).

Much of what is known about CSC biology has been derived from study of haematological malignancies as CSCs have been characterised definitively in this form of cancer for the longest time. (Bonnet and Dick 1997; Wang and Dick 2005) It has been shown that most circulating cells in acute myeloid leukaemia do not have a proliferative potential. There is only a small subset of cells – the CSCs - which are able to replicate *in vitro* after extraction from a patient. (Wang and Dick 2005) This system is analogous to normal hematopoiesis and also analogous to current understanding of crypt biology. (Wang and Dick 2005) These CSCs are thought to be relatively slow-cycling which allows them to evade cancer therapies which primarily target rapidly replicating cells. (Boman and Wicha 2008) CSCs produce rapidly cycling, partially differentiated cells (analogous to transit amplifying cells) which produce terminally differentiated cells to form the tumour bulk. (Wang and Dick 2005)

1.8.5 EVIDENCE FOR THE COLORECTAL CSC

Two groups published data at approximately the same time which confirmed the existence of CRC-CSCs. Both used CD133 as the marker to identify potential CSCs. CD133 is an antigen expressed by normal primitive endothelial, haematopoietic and epithelial cells (Yin, Miraglia et al. 1997; Uchida, Buck et al. 2000; Salven, Mustjoki et al. 2003) and has been shown to enrich for CSCs in multiple other tumours. (Ferrandina, Petrillo et al. 2009) High expression of CD133 is associated with poor prognosis in CRC. (Horst, Kriegl et al. 2008; Maeda, Shinchi et al. 2008; Li, Li et al. 2009; Artells, Moreno et al. 2010)

The initial publication demonstrating colon CSCs was by Ricci-Vitiani et al. in 2007. Flow cytometry was used to separate $CD133^+$ cells in 19 human CRC specimens. The

$CD133^+$ population made up around 2.5% (±1.4%) of the total tissue volume and did not express cytokeratin 20 (CK20) which is expressed in differentiated cells. (Ricci-Vitiani, Lombardi et al. 2007) CD133 expression was barely detectable in normal adjacent colonic epithelium.

To test the potential of CD133 as a colon CSC marker the tumourigenic potential was compared of $CD133^+$ and $CD133^-$ cells in severe combined immunodeficient (SCID) mice. After separation, both $CD133^+$ and $CD133^-$ expressed similar phenotypes to the parent tumour in terms of *APC*, carcinoembryonic antigen (CEA) and *p53*. Transplantation of any number of $CD133^-$ cells did not recreate tumour in the recipient mice however transplantation of 3,000 $CD133^+$ cells was able to generate new tumour in recipient mice as was transplantation of 10^6 unseparated cells. (Ricci-Vitiani, Lombardi et al. 2007)

$CD133^+$ cells were also transferred to serum free culture media (used to grow undifferentiated cells) and serum containing culture medium (used to grow differentiated cells). In serum containing media $CD133^-$ cells were able to survive for around 14 days whilst viable $CD133^+$ cell numbers declined rapidly. In serum free conditions there was rapid growth and replication to form colonospheres of $CD133^+$ cells from five of the 15 tumours tested. $CD133^-$ cells rapidly died in such conditions and progressive selection of $CD133^+$ cells from the colonospheres seemed to lead to an increase in replication with a decrease in doubling time observed with progressive passages. The proportion of $CD133^+$ cells within the colonospheres remained high and CK20 expression remained low indicating an undifferentiated or 'primitive' phenotype. (Ricci-Vitiani, Lombardi et al. 2007)

Implantation of these colonospheres into SCID mice led to rapid tumour formation. Serial transplantation of $CD133^+$ cells from the induced tumour induced quaternary tumours. When colonospheres were placed in culture media containing 5% serum differentiation was observed with differentiated cells acquiring similar morphology and immunological phenotype to the original tumour. The colonospheres began to widely

express CK20 and other markers of differentiation. After this, colonospheres lost their ability to initiate tumour growth in SCID mice. (Ricci-Vitiani, Lombardi et al. 2007)

In this series of experiments it was shown that all $CD133^+$ cells from a tumour were undifferentiated and that this population had the ability to initiate tumour growth. Whilst CD133 appears to be a sensitive marker it may not be specific as not all positive populations were able to generate tumour. Interestingly the determination of CD133 status appeared to be plastic, with a change in phenotype of $CD133^+$ colonospheres when exposed to different environmental conditions. (Ricci-Vitiani, Lombardi et al. 2007; Yang, Wang et al. 2012)

Further evaluation of CD133 as a marker for CSCs was performed by O'Brien et al. who also transplanted CRC tumour samples enriched for $CD133^+$ cells into SCID mice. They observed new tumour growth with similar molecular profiles to parent tumours in all 17 mice. (O'Brien, Pollett et al. 2007)

Using serial dilution techniques before transfer to SCID mice of unfractionated cells they estimated one CSC per 5.7×10^4 unselected tumour cells (95% confidence interval (CI) $3.4 \times 10^4 – 9.3 \times 10^4$ cells). When they repeated this after enrichment based on CD133 status, $CD133^-$ cells again were unable to generate tumour in SCID mice whilst $CD133^+$ cells did initiate tumours with much lower numbers transplanted than that required to initiate tumour using unfractionated cells. Tumours were consistently generated with transplantation of $1x\ 10^3$ $CD133^+$ cells and generated after injection of only 100 cells in one in four mice. Serial dilution of the $CD133^+$ fraction suggested that there were one CSC per 262 $CD133^+$ cells (95% CI 129-534) suggesting that $CD133^+$ status enriches 216 fold for CSCs. (O'Brien, Pollett et al. 2007)

In addition to CD133 there are other antigenic combinations which have been suggested to mark CRC-CSCs more specifically. Dalerba et al. found epithelial cell adhesion molecule $(EpCAM)^{high}/CD44^+$ epithelial cells were able to initiate new tumours in SCID mice consistently while $EpCAM^{low}/CD44^-$ cells could not. (Dalerba, Dylla et al. 2007) High expression of CD133 and CD44 in combination is able to predict

poor prognosis in CRC possibly suggesting a higher ratio of stem cells to differentiated cells in such tumours. (Galizia, Gemei et al. 2012)

1.8.6 CHARACTERISTICS OF COLON CANCER STEM CELLS

Tumours are characterised by increased growth and replication compared with normal surrounding tissue. Boman et al. have sought to define the characteristics of the CSC compartment which give rise to this increased growth and replication. They mathematically modeled tumour replication assuming CSCs represent a constant 0.25 - 2.5% (O'Brien, Pollett et al. 2007; Ricci-Vitiani, Lombardi et al. 2007) of the total cellular population (10^8-10^{13} cells). (Boman, Wicha et al. 2007) The model demonstrated a significant expansion in ASC number from healthy epithelium was required, taking into account even the largest estimations of ASC numbers per crypt previously suggested (largest estimates are 40-60 ASCs per crypt based on indirect evidence compared to the more widely accepted estimate of 4-6 ASCs per crypt based on direct observations). (Issa 2000; Barker, van Es et al. 2007) Assuming that CSCs arise from ASCs, tumour development required a twenty fold increase in symmetrical division of ASCs each producing two daughter ASCs. Symmetrical division of transit amplifying cells, increased symmetrical division of ASCs to produce two daughter transit amplifying cells and other permutations of division and replication did not fit observed tumour characteristics. (Boman, Wicha et al. 2007)

1.8.7 ORIGINS OF CANCER STEM CELLS

CSCs have the ability to initiate and drive tumour growth and have accrued several genetic and epigenetic mutations to achieve this phenotype. ASCs are the only cell with the longevity to accrue these mutations so it is likely that ASCs are the origins of CSCs – known as the ‘bottom-up’ hypothesis. However the alternative hypothesis states that CSCs originate out with the stem cell compartment from a more differentiated cell type, potentially within the transit amplifying compartment – the top-down hypothesis (Wright and Poulsom 2002)

Most biological data logically supports the bottom-up hypothesis such as: stem cell longevity, crypt monoclonal conversion and propagation of mutation by crypt fission. Barker et al. sought to definitively prove ASCs as the originators of CSCs. Mice with an inducible floxed *Apc* allele linked to *Lgr5* allowed the *Apc* gene to be knocked down after administration of β-naphthoflavone specifically in the colonic $Lgr5^{+}$ ASC population. On induction with β-naphthoflavone there was rapid proliferation of ASCs with increased cell numbers ascending the crypts compared to cell counts prior to *Apc* knockdown. Although the $Lgr5^{+}$ stem cells initially remained in the crypt base microadenomas soon formed. Large multi-villus adenomas developed at 14 days. Within the adenomas fluorescence-activated cell sorting demonstrated that at $Lgr5^{+}$ cells made up approximately 6% of the total cells (an increased proportion compared to that observed prior to *Apc* knockdown). Similar patterns were observed in both the small bowel and the colon. (Barker, Ridgway et al. 2009) This accelerated model of CRC carcinogenesis shows that an initiating mutation specifically applied to ASCs generates a neoplastic phenotype as observed in sporadic CRC carcinogenesis, strongly suggesting that CSCs originate from ASCs.

In addition to mouse evidence there are indirect data in human tissue linking the origins of the CSC to ASCs. Boman et al. performed immuno-histochemical staining of adenomatous polyps from patients undergoing prophylactic colectomy for FAP. Crypts were stained with a variety of markers known to stain crypt basal cells (MSH2, Bcl-2 and survivin), proliferating cells (Ki-67 and topoisomerase II) and differentiated cells ($p21^{WAF1/C1P1}$ and $p27^{kip1}$). These were compared with two types of normal control: macroscopically normal FAP crypts and crypts from non-FAP patients. (Boman, Walters et al. 2004) In general they found that the proportion of all stained cell types (basal and proliferative) to total cell number increased from normal mucosa to macroscopically normal FAP crypts to FAP adenomatous crypts. The most marked difference occurred in the proportions of cells positive for crypt basal markers. In FAP adenoma tissue these cells made up the majority of all cells however in normal crypts these cells comprised the smallest proportion. Crypt basal marker staining cells were

confined to the lowest crypt positions in the normal mucosa whilst they occupied the entire length of the crypt in FAP adenomas. (Boman, Walters et al. 2004)

The findings of this study are consistent with modeling work suggesting that stem cell over-population is the initiating step in tumour development. (Boman, Fields et al. 2008) However the work discussed above uses a stain for crypt basal cells, i.e not a specific ASC stain, so the results shown may represent an over-population of another basal cell population such as transit amplifying cells. Barker et al.'s work does show specific expansion of $Lgr5^{+}$ ASCs though this has not been replicated in a human tissue. (Barker, Ridgway et al. 2009)

Several studies have now documented increased expression of the stem cell marker Lgr5 in CRC cell lines (Uchida, Yamazaki et al. 2010), tumours and metastasis (Kleist, Xu et al. 2011) and in peripheral blood of CRC patients (Valladares-Iyerbes, Blanco-Calvo et al. 2012) with increased expression linked to poor prognosis. (Kleist, Xu et al. 2011; Valladares-Iyerbes, Blanco-Calvo et al. 2012)

1.8.8 SIGNALLING PATHWAYS IN CANCER STEM CELLS

WNT signalling is implicitly involved in CRC carcinogenesis as discussed in section 1.4. As CRC is a disease of the stem cells it is likely that the transition of ASC to CSC is mediated in part at least by WNT's role in stem cell maintenance, where it prevents differentiation in the crypt base and promotes differentiation towards secretory (goblet cell) lineages luminally. (Scoville, Sato et al. 2008). Blocking Tcf-4, a downstream transcriptional effector of Wnt, in mouse embryo results in direct transformation of endoderm into differentiated gut epithelium composed almost entirely of enterocytes, with complete absence of the proliferative compartment and death. (Korinek, Barker et al. 1998) This effect is replicated in upstream silencing of Wnt by Dkk (a soluble Wnt inhibitor) which results in loss of crypts and decreased villus size in mice. (Pinto, Gregorieff et al. 2003; Kuhnert, Davis et al. 2004). Growth patterns can be returned to normal by removing Wnt inhibition. (Kuhnert, Davis et al. 2004)

Other signalling pathways such as NOTCH, BMP, P10 and PI3K have been shown to have both independent and inter-related roles in the maintenance and activity of the intestinal stem cell population. (Scoville, Sato et al. 2008).

1.9 NOTCH SIGNALLING

1.9.1 NOTCH SIGNALLING IN NORMAL MUCOSA

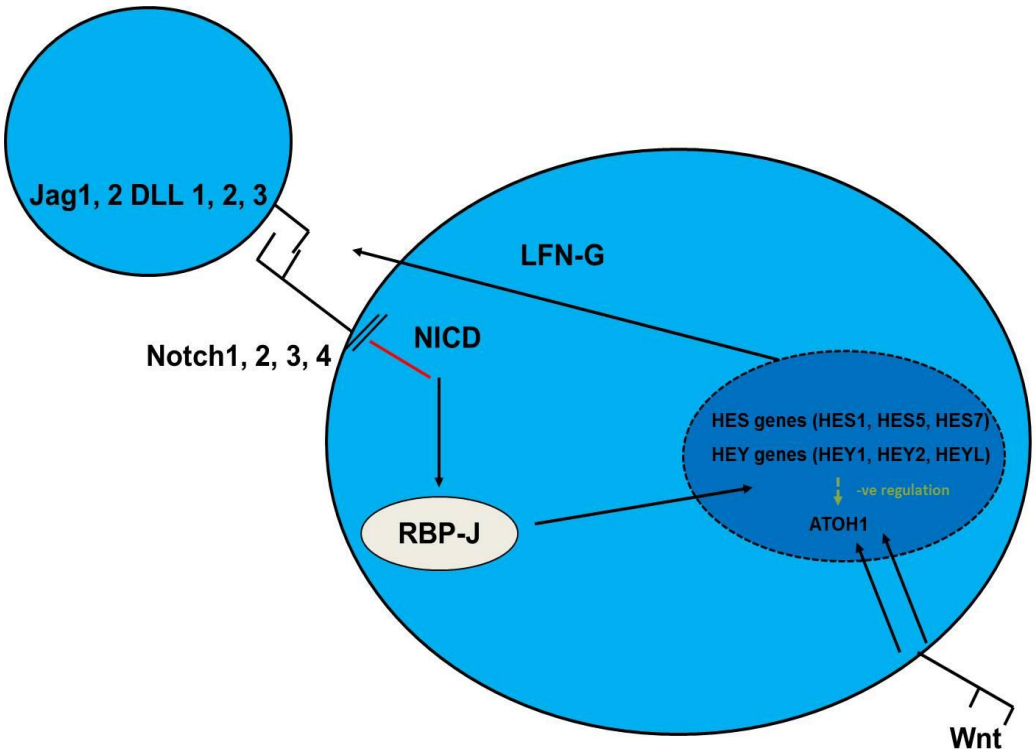
The NOTCH receptors are a family of transmembrane cell surface proteins which regulate the NOTCH pathway. The *Notch* gene was initially identified in 1917 and was so named because mutation in *Drosophila* resulted in a notched wing phenotype. (Morgan 1917) Core components of the NOTCH pathway in humans are: the ligands on the sending cell, Delta-like (DLL) 1, 2 and 3 and Jagged (JAG) 1 and 2; receptors on the receiving cell, NOTCH 1, 2, 3 and 4; and the transcription factor RBP-J, a sequence specific DNA binding protein. (Lai 2004)

NOTCH is a relatively simple cascade activated by direct cell to cell contact of NOTCH ligands and NOTCH receptors. (Borggrefe and Oswald 2009) When JAG or DLL interact with NOTCH receptors a two stage, γ-secretase dependent, proteolysis is instituted resulting in cleavage of the Notch intracellular domain (NICD) which binds (heterodimerises) the nuclear transcription factor RBP-J. RBP-J activates transcription of target genes with an RBP-J binding site. In the absence heterodimerisation of the RBP-J protein by NICD, RBP-J acts as a transcriptional silencer of NOTCH target genes which led to it being considered as a transcriptional silencing molecule initially. (Borggrefe and Oswald 2009) Downstream target genes regulated by NOTCH include: hairy enhancer of split (HES) family, *HES1, HES5, HES7, HEY1, HEY2* and *HEYL* (Borggrefe and Oswald 2009); *NRARP* and *Deltex-1* (negative regulators); *C-MYC*; *CYCLIN D-1* and *P21-WAF1* which are also implicated in human carcinogenesis. (Borggrefe and Oswald 2009) (Figure 1-8)

NOTCH signalling was first identified as a key pathway for carcinogenesis in human T-cell acute lymphoblastic leukaemia where a chromosomal translocation results in constitutive NOTCH activation (Ellisen, Bird et al. 1991). Aberrant NOTCH signalling has been identified in several other human cancers including breast, prostate, skin and cervical. (Lai 2004) In several cases the mechanism of aberrant NOTCH signalling is interaction of the RBP-J complex with viruses such as Epstein-Barr resulting in

activation of the NOTCH pathway by constitutive binding of RBP-J to transcriptional co-factors. (Allenspach, Maillard et al. 2002) NOTCH signalling has been more extensively investigated in breast cancer where there is promise as a therapeutic target, both to promote differentiation of stem cells, enhancing radio and chemo sensitivity, and as a key pathway in carcinogenesis. (Harrison, Farnie et al. 2010)

FIGURE 1-8. DIAGRAMMATIC REPRESENTATION OF THE NOTCH SIGNALLING PATHWAY.



NICD – NOTCH INTRACELLULAR DOMAIN

1.9.2 NOTCH SIGNALLING AND STEM CELLS

NOTCH has been shown to be an implicit signalling pathway in determining cell fate and differentiation of ASCs in the colon. (Katoh 2011) Whilst WNT drives differentiation to secretory cell lineages, NOTCH maintains ASCs in an undifferentiated state in the crypt base (Kazanjian and Shroyer 2011) and counterbalances WNT's action to promote cells to a secretory fate towards the lumen. (van Es, Jay et al. 2005; Andreu, Peignon et al. 2008; Katoh 2011) Luminally NOTCH has dual functions promoting differentiation to absorptive lineages whilst actively decreasing differentiation to secretory lineages. In mice Notch promotes differentiation to absorptive cell lineages via the effectors *Hes1* and *Ascl1*. (van Es, van Gijn et al. 2005) *Hes1* negatively regulates *Atoh1* transcription. Increased *Atoh1* promotes differentiation to secretory lineages via *Math1* (human ortholog *HATH1*) and other proteins. (Jensen, Pedersen et al. 2000; Yang, Bermingham et al. 2001). Gain of function studies demonstrate that increased Notch increases the number of undifferentiated crypt basal cells together with an increased number of differentiated absorptive cells towards the lumen. (Fre, Huyghe et al. 2005; van Es, van Gijn et al. 2005)

Several other strategies have been used to block or enhance Notch signalling further defining the pathway. Mice born with constitutively active *Notch1* live to three days. These mice have an absence of goblet cells throughout the intestine and a relative lack of Paneth cells. In addition the proliferative compartment expands to encompass the whole crypt and villus in the small bowel. (Fre, Huyghe et al. 2005)

Cleavage of the NICD, and activation of RBP-J as a pro-transcription factor via co-factor recruitment in response to the NICD, is a γ-secretase dependent process. Γ-secretase inhibitors have been used in the treatment of Alzheimer's disease and toxicological studies of its effects in rats have shown increased numbers of goblet (secretory) cells as would be expected with Notch inhibition. (Milano, McKay et al. 2004; Wong, Manfra et al. 2004) Subsequent work has aimed to elucidate the mechanisms of Notch mediated effects that have been observed. Van Es et al. used mice with an inducible

Rbp-j knockout and found bowel histology was not noticeably different at 2 days whilst levels of *Hes1* were decreased and levels of *Atoh1* were increased. Math1 protein was also found to be expressed throughout the crypt when it is usually only expressed in differentiated secretory cells. (van Es, van Gijn et al. 2005) After five days the transit-amplifying compartment had been entirely replaced by goblet cells expressing Math1 whilst Ki67 and BrdU inclusion (markers of ongoing proliferation) had become undetectable. (van Es, van Gijn et al. 2005)

1.9.3 NOTCH SIGNALLING AND COLORECTAL CANCER

NOTCH is a critical pathway in CRC carcinogenesis. Increased *NOTCH1* expression and decreased *NOTCH2* expression are both associated with features of an advanced CRC phenotype such as advanced stage, poor differentiation and vascular invasion. Multivariate analysis showed both were independent predictors of decreased survival with co-expression predicting a sub group with very short survival. (Chu, Zhang et al. 2011) Decreased NOTCH2 expression in association with poor differentiation has been replicated in an independent dataset. (Jin, Zhang et al. 2012) NOTCH is activated in the majority of primary CRCs together with WNT although the precise mechanism of NOTCH activation remains unclear. (section 1.9.5) (Veenendaal, Kranenburg et al. 2008; Kazanjian and Shroyer 2011)

Notch signalling activation has been investigated in murine adenomas. Heterozygous $Apc^{Multiple\ intestinal\ neoplasia\ (Min)}$ mice showed increased expression of Notch receptors and ligands throughout polyp tissue and in crypt bases of macroscopically normal tissue. Increased *Hes1* expression was also noted throughout the adenomatous tissue. (van Es, van Gijn et al. 2005; Guilmeau, Flandez et al. 2009) Inhibition of Notch in these Apc^{Min} mice by γ-secretase induced differentiation of cells in variable proportions with over 50% of adenomas responding and generating $Math1^{+}/Ki67^{-}$ cells (differentiated, non-proliferative cells), a situation never observed in untreated mice. (van Es, van Gijn et al. 2005) These findings suggest that active Wnt and Notch are both required to maintain the proliferative phenotype of neoplastic lesions.

Downstream NOTCH targets (*HES1*) have been found to be considerably upregulated in CRC tissue when compared with patient matched normal tissue. (Peignon, Durand et al. 2011) Ligand and receptor expression studies showed several that were unchanged between cancer and non-cancer controls (DLL-1, DLL-3 and NOTCH 3) however DLL-4, JAG-1, JAG-2, NOTCH 1 and NOTCH 2 were all upregulated. (Peignon, Durand et al. 2011) *JAG1* overexpression in human CRC has been confirmed in several other studies and has been correlated with adverse features such as poor differentiation. (Guilmeau, Flandez et al. 2009; Jin, Zhang et al. 2012)

Similar results in a larger panel of human CRC have been reported by Reedijk et al. (Reedijk, Odorcic et al. 2008) They found *HES1* and NOTCH 1 were consistently upregulated in all of the tumours (n=20) whilst JAG1 and JAG2 were upregulated inconsistently and DLL1, DLL3, DLL4 and NOTCH 2 were expressed at levels found in normal crypt bases. *LFNG*, a member of the Frizzled family of genes known to affect NOTCH through alteration of the specificity of the ligand/receptor interaction, was found to be consistently upregulated in human CRCs whilst other members of the Frizzled family retained normal expression. (Reedijk, Odorcic et al. 2008)

Based on the observation that CRC is goblet cell deficient (Ho, Niehans et al. 1993), *HATH1* (as the final mediator of secretory differentiation in response to increased *ATOH1* and decreased NOTCH) expression was quantified by Leow et al. (Leow, Romero et al. 2004) Using the gene logic database they determined that *HATH1* expression was high in normal colon compared to other tissues and downregulated in CRC, a phenomenon not observed in other cancer tissues. This was subsequently confirmed in prospectively collected human CRC tumours and CRC cell lines. *HATH1* induction in CRC cell lines was able to decrease replication with a tenfold reduction in number of colonies formed on culture in soft agar. On xenograft into SCID mice there was absent tumour growth of *HATH1* induced cells, compared with expected growth in non-*HATH1* expressing controls. (Leow, Romero et al. 2004) The ability of NOTCH knock-down to promote differentiation and decrease proliferation has been replicated in other cell line based studies. (Sikandar, Pate et al. 2010; Yeung, Gandhi et al. 2011)

ATOH1 (which commits cells to a secretory lineage via HATH1) can also be considered as an independent TSG. (Leow, Romero et al. 2004; Bossuyt, Kazanjian et al. 2009) *ATOH1* is silenced in approximately 70% of CRCs although point deletions accounting for this are only detectable in half of silenced cases. (Leow, Romero et al. 2004; Bossuyt, Kazanjian et al. 2009) Re-induction of silenced *ATOH1* in CRC cell cultures greatly reduces proliferation. (Leow, Romero et al. 2004)

In CRC-CSCs NOTCH signalling has been found to be increased 10-30 fold. This overexpression was found to prevent apoptosis via *ATOH1*, an effect that could be reversed on *ATOH1* knock-down. (Sikandar, Pate et al. 2010)

The therapeutic implications of these data have been tested in three studies. Human CRC explants have been transplanted into SCID mice and the effects of irinotecan and γ-secretase inhibitors tested. Sensitivity to chemotherapy was enhanced when both agents were provided with the greatest effect observed in tumours over-expressing *NOTCH1*. (Arcaroli, Powell et al. 2012) In CRC cell lines oxaliplatin, 5-fluoruracil and irinotecan have been shown to upregulate NOTCH1 and activate pro-survival cell pathways suggesting a potential mechanism by which NOTCH may mediate resistance to chemotherapy. This effect was abrogated by γ-secretase inhibitors in addition to chemotherapy. (Meng, Shelton et al. 2009) In NOTCH over-expressing CRC cell lines blocking of DLL4 using a monoclonal antibody greatly reduces tumour growth and enhances the radio-sensitivity of tumours in mouse explants.(Liu, Bham et al. 2011)

1.9.4 EPIGENETIC REGULATION OF NOTCH

There are few data on epigenetic regulation of NOTCH in CRC. In gastric cancer NOTCH pathway activation has been shown to be under the influence of *DLL1* methylation in cell lines and *DLL1* methylation is specifically associated with certain tumour subtypes. (Piazzi, Fini et al. 2011) *NOTCH1* and *NOTCH3* have been shown to be under methylation dependent control of expression in hepatic ASCs. (Reister, Kordes et al. 2011) In breast cancer, hypomethyaltion of *NOTCH1* has been correlated with NOTCH1 overexpression and advanced cancer stage. (Zhang, Sun et al. 2011) Although there are

no data to date demonstrating methylation as the mechanism of altered NOTCH signalling in CRC, analysis of *NOTCH 1,2,3,4* has only identified genetic mutation in 2% of all CRCs. (Lee, Jeong et al. 2007)

1.9.5 NOTCH AND ITS RELATIONSHIP WITH WNT

WNT is classically regarded as the key pathway in CRC carcinogenesis (section 1.4.3) Both WNT and NOTCH are crucial for maintenance of undifferentiated cells in the stem cell compartment with inactivation of either resulting differentiation and loss of proliferation. (Korinek, Barker et al. 1998; van Es, van Gijn et al. 2005) Stem cell overpopulation (section 1.8.3) is an initiating event in CRC carcinogenesis so signalling pathways controlling stem cell proliferation (WNT and NOTCH), which are known to be overexpressed in CRC, are likely to be implicitly involved in CRC carcinogenesis. Recent work suggests that there is a degree of cross-talk between the NOTCH and WNT pathways in CRC.

Peignon et al. assessed whether upregulation of Notch targets such as *Hes1* was due to increased β-catenin related transcription. Induced loss of *Apc* in a murine model resulted in immediate upregulation of *Hes1* expression together with the expected acquisition of a pre-malignant phenotype (increased proliferation and expansion of the proliferative zone lumenwards). (Peignon, Durand et al. 2011) Mechanisms of the relationship of Wnt/β-catenin and the Notch pathway were then explored. After induced loss of *Apc*, expression of *Dll4*, *Jag1*, *Jag2*, *Notch1*, *Notch2* and *Notch4* were all increased at five days. However, on restoration of wild type only *Jag1* returned immediately to normal, suggesting that the interaction of β-catenin and the Notch pathway maybe mediated by *Jag1.* (Peignon, Durand et al. 2011)

Similar data has been found using *APC* deficient human CRC cells lines when both WNT and NOTCH were activated, and then, with inactivation of either pathway or both. (Rodilla, Villanueva et al. 2009). Both pathways had several downstream transcriptional pathways in common. Additionally increased WNT signalling increased *JAG1* transcription and inhibition of WNT returned *JAG1* levels to normal. *Jag1* deletion

in *Apc* ^{Min} mice was sufficient to reduce the size of adenomas in mice at four months. (Rodilla, Villanueva et al. 2009)

Intermediate steps in this pathway have been further defined with the observation that progastrin inhibition produces a phenotype similar to that of Notch inhibition (differentiation towards secretory phenotype) but not reminiscent of Wnt silencing (absorptive cell differentiation). (Pannequin, Delaunay et al. 2007; Pannequin, Bonnans et al. 2009) Progastrin is over expressed in human CRC (Siddheshwar, Gray et al. 2001) and is a downstream target of the WNT pathway. (Koh, Bulitta et al. 2000) This suggests that progastrin is the downstream target of Wnt which acts to promote *Jag1*, activating canonical Notch signalling. (Pannequin, Bonnans et al. 2009)

To define whether *Apc* loss could be rescued by normalisation of Notch expression, *Rbp-j* was also knocked down to silence Notch. In this case mice with both *Apc* and *Rbp-j* loss had no difference in numbers, size or morphology of polyps or protein expression analysis compared to those with induced *Apc* loss only. The ability of downregulated Notch to rescue *Apc*$^{-}$ phenotype was not observed. However the phenotype was normalised when expression of Math1 was specifically induced, suggesting that this downstream Notch target gene may also be regulated via Wnt. Specific inhibition of *Math1* increased tumourigenesis in mice. (Peignon, Durand et al. 2011) Similar results have been observed in human cells with the additional re-expression of *HATH1* after reactivation of *APC* in an *APC* deficient human CRC cell line. (Leow, Romero et al. 2004)

Loss of Math1 has been shown to increase tumourigenesis in murine models and loss of *ATOH1* function (Math1 and HATH1's upstream regulator) is present in around 70% of human tumours and potentially under epigenetic control. (Bossuyt, Kazanjian et al. 2009) High levels of *ATOH1* methylation were detected in both the promoter region and coding genome CpG islands in CRC samples compared to controls. *ATOH1* expression could be reactivated with the addition of DNA methyltransferase to CRC cell lines with epigenetic silencing of *ATOH1.* (Bossuyt, Kazanjian et al. 2009). Downstream targets of WNT signalling, which contribute to cell cycle control, cyclin D-1 and p27

can be up-regulated by induction of HATH1 without change to WNT signalling. (Leow, Romero et al. 2004)

Another mechanism of NOTCH/WNT linkage has been proposed by Tsuchiya et al. (Tsuchiya, Nakamura et al. 2007) to account for tumours where *ATOH1* downregulation is not observed despite a goblet cell deficient phenotype. (Aragaki, Tsuchiya et al. 2008) In these cases, cell line work showed that HATH1 suppression was not mediated by transcriptional silencing of *ATOH1* and that normal *HATH1* mRNA levels were observed with decreased HATH1 protein levels. This suggests that a post-transcriptional mechanism may account for the goblet cell deficient phenotype. Exploration of the mechanism showed that HATH1 was degraded by GSK3β (a dephosphorylating enzyme that acts on β-catenin as part of the CDC). The degradation of HATH1 by GSK3β and evidence that this effect was reversed on silencing of the WNT pathway suggests that GSK3β directly modifies HATH1 levels post-transcriptionally. (Tsuchiya, Nakamura et al. 2007)

The actions of WNT on NOTCH have been extensively investigated whilst the reverse causal pathway, the action of NOTCH on WNT has been less well documented. The balance of data in the literature available therefore seems to suggest a one way relationship where WNT signalling controls NOTCH. Some data does support this as when Notch is constitutively activated in transgenic mice the intestinal phenotype is grossly altered and death occurred within three days, however Wnt signalling, defined by nuclear β-catenin, TCF-4 and LEF1, was unchanged. (Fre, Huyghe et al. 2005) Notch inhibition therefore seems to have an independent phenotype which is not due to any influence on Wnt signalling.

However there are suggestions of feedback of NOTCH on WNT. Katoh et al. screened WNT inhibitor promoter regions for the Notch responsive element (NRE). NREs were found on *DKK2* (a secreted WNT inhibitor). Promoter and expression analysis suggested *DKK2* as a NOTCH target gene. (Katoh 2007) This negative feedback on WNT signalling may be lost in CRC as DKKs are frequently found to be silenced by promoter hypermethylation. (Sato, Suzuki et al. 2007) More direct feedback on the WNT has

been demonstrated by Kwon et al. who have shown that NOTCH1 can directly bind Ser37 phosphorylated β-catenin (the small proportion of total cellular β-catenin that is biologically active). This occurs independently of all downstream NOTCH factors and is an effect limited to NOTCH1 rather than other NOTCH receptors. (Kwon, Cheng et al. 2011)

1.9.6 NOTCH SIGNALLING AND INFLAMMATORY BOWEL DISEASE

Studies examining the role of NOTCH signalling in IBD are limited. In mouse models, differences between IBD and controls and between Crohn's disease and UC have been investigated. With comparable levels of inflammation, Math1 was increased in Crohn's disease but not in UC mice. Although the proportion of goblet cells was reduced in both IBDs, goblet cell depletion was more marked in UC mice (in the upper third of the crypt). (Gersemann, Becker et al. 2009) Both goblet cell depletion in UC, and decreased expression of HATH1, have been replicated in human UC sufferers. (Zheng, Tsuchiya et al. 2011) Mice with induced colitis show overexpression of *Jag1*, *Dll1* and *Dll4* compared to normal controls. (Imaeda, Andoh et al. 2011) In an induced colitis mouse model, blocking of Notch signalling with dibenzazepine is able to prevent goblet cell and Math1 depletion compared to controls and increases the rates of mucosal healing. (Shinoda, Shin-Ya et al. 2010)

1.10 SUMMARY – MICROSCOPIC AND MOLECULAR BIOLOGY OF CRC

NOTCH signalling is a highly conserved pathway directing differentiation to absorptive differentiated cells and maintaining basal stem cells in an undifferentiated proliferative state. There is a high degree of interaction with the WNT signalling system which has yet to be fully defined. Both pathways are implicit in embryological development of the gut and subsequently in regulating cellular homeostasis.

CRC is a disease of stem cells. CRC displays significant aberrations in both WNT and NOTCH signalling. It is likely that altered NOTCH signalling drives carcinogenesis by conferring an embryological phenotype of maintained de-differentiation and

proliferation on CRC-CSCs. Aberrant NOTCH signalling is required to initiate, promote and maintain a neoplastic phenotype.

Whilst WNT abnormalities have been relatively well described in CRC the causal lesions for abnormalities in expression of NOTCH signalling components in CRC have not been well characterised. The limited evidence to date suggests that there may be genetic mutations, epigenetic control or interactions between WNT and NOTCH driving changes in NOTCH expression. This project aims to explore potential epigenetic regulation of NOTCH.

1.11 COLORECTAL CANCER AND THE ENVIRONMENT

Less than a quarter of CRCs can be accounted for by direct inheritance of genetic mutation. (Rustigi 2007) Migration studies show that migrant individuals rapidly take on the host countries risk of CRC. This 'migration effect' is stronger for CRC than for many other cancer types indicating the strong influence of environmental exposure. (Flood, Weiss et al. 2000; Stirbu, Kunst et al. 2006; Nasseri, Moulton et al. 2009) Large prospective studies evaluating general good health measures have demonstrated an incremental risk in CRC associated with poorer lifestyle factors. (Macfarlane, Macfarlane et al. 2006)

Several environmental and personal factors affect the risk of developing CRC. Smoking has been shown to have a marked effect, increasing CRC risk by around 20% (Relative risk (RR) 1.19; 95% CI 1.09-1.24, current smokers vs. non-smokers) in a meta-analysis of 22 prospective cohort studies including 23 500 participants. The pro-carcinogenic effect of smoking was more pronounced for rectal cancer compared to colon cancer. (Huxley, Ansary-Moghaddam et al. 2009) Additionally mortality after CRC diagnosis is increased in smokers and there is a dose-response relationship for both incidence and mortality with increased overall cigarette exposure. (Liang, Chen et al. 2009) The biology of CRCs between smokers and non-smokers is different with smokers more likely to have *K-RAS* mutation negative tumours. (Samadder, Vierkant et al. 2012)

In a meta-analysis of physical activity and CRC risk, increased physical activity is protective against CRC. Data were available from 27 studies including approximately 27 500 participants. Physical activity reduced CRC risk by around by 20% (RR 0.81; 95% CI 0.77-0.86). The observed effect was stronger for colon cancer compared to rectal cancer and for men. (Huxley, Ansary-Moghaddam et al. 2009)

Meta-analysis of obesity as a risk factor included 58 000 individuals and concluded that individuals with a body mass index (BMI) of greater than 30 kg/m^2 had a 20% increase in CRC risk (RR 1.19; 95% CI 1.11 – 1.29). Obesity may be considered as a combined

measure of both poor dietary choice and lower physical activity, but obesity also increases cancer risk by independent means.

Adipose tissue is recognised as an endocrine organ as well as a fat storage tissue. Adipose tissue releases pro-inflammatory cytokines and increased adiposity is associated with higher circulating levels of interleukin 6 (IL6), tumour necrosis factor alpha (TNF-alpha) and C reactive protein (CRP). (Das 2001) Concentrations of these cytokines decrease with weight loss. (Esposito, Pontillo et al. 2003; Dietrich and Jialal 2005; Imayama, Ulrich et al. 2012) Proposed mechanisms by which inflammatory cytokines promote cancer include: paracrine regulation of tumour cells, inflammation creating reactive oxygen species, apoptotic suppression and pro-angiogenic response. (Le Blay, Michel et al. 2003)

Additionally, with increased adiposity and energy intake, metabolic adaptions occur in response to high circulating levels of free fatty acids resulting in insulin resistance and hyperinsulinaemia. (Calle and Kaaks 2004) Hyperinsulinaemia and increased adipokine levels have been shown to correlate with increased CRC risk. (Ho, Wang et al. 2012) Hyperinsulinaemia inhibits apoptosis and stimulates proliferation in CRC cell lines and murine studies have shown promising effects of metformin (a biguanide hypoglycaemic agent) as a chemopreventative agent for CRC. (Heijnen, van Amelsvoort et al. 1996; Algire, Amrein et al. 2010)

Whilst there appears to be a correlation between CRP levels (and other inflammatory cytokines) and several epithelial cancers, including CRC, it has been difficult to determine if elevated CRP is causal, a response to early carcinogenesis or represents a confounder in response to other CRC risk factors known to increase systemic inflammation such as obesity or smoking. (Allin and Nordestgaard 2011) Difficulty in establishing this relationship has been due to long time course and large number of participants required to demonstrate a temporal relationship prospectively. Recent data has supported a causal role, as the temporal relationship between increased systemic inflammatory parameters followed by increased rate of CRC carcinogenesis has been demonstrated in a large cohort study originally recruited to study

cardiovascular risk factors. (Prizment, Anderson et al. 2011) Whatever the precise relationship CRP can be used to augment the accuracy of CRC screening (Tao, Haug et al. 2012) and predict metastatic disease in locally advanced CRC. (Ishizuka, Nagata et al. 2012)

1.12 DIET AND COLORECTAL CANCER

The impact of diet on colonic health is most aptly demonstrated by migrant studies when populations relocate from low to high risk areas.(Stirbu, Kunst et al. 2006; Nasseri, Moulton et al. 2009) This effect is more apparent for CRC than other epithelial tumours. (Flood, Weiss et al. 2000; Stirbu, Kunst et al. 2006)

The World Cancer Research Fund (WCRF)/American Institute for Cancer Research (AICR) report on 'Food, Nutrition and the Prevention of Cancer' has judged that 'food and nutrition has a highly important role in the prevention and causation of colorectal cancer.' (WCRF/AICR. 2007) The report combined results for 752 publications concerning the role of diet and CRC. It found strong evidence that increased red meat, processed meat and alcohol consumption, body fatness and abdominal fatness all increased the risk of CRC. The evidence was graded as probable that dietary fibre, garlic, milk and calcium were protective against the development of CRC. (WCRF/AICR. 2007) In 2010 WCRF/AICR released their CRC update report which added 263 papers to the meta-analysis and the evidence of a protective effect of dietary fibre was upgraded to convincing. (Dagfinn, Doris et al. 2011; Perera, Thompson et al. 2012; WCRF/AICR. 2012) (Table 1-3)

TABLE 1-3. DIETARY AND LIFESTYLE RISK AND PROTECTIVE FACTORS FOR CRC.

(REDRAWN FROM WCRF/AICR CRC UPDATE REPORT 2010)

	Decreases risk	**Increases risk**
Convincing	Physical activity Foods containing dietary fibre	Red meat Processed meat Alcoholic drinks (men) Body fatness Abdominal fatness Adult attained height
Probable	Garlic Milk Calcium	Alcoholic drinks (women)

1.12.1 DIETARY FIBRE AND COLORECTAL CANCER

There is considerable debate on the actual definition of dietary fibre. The World Health Organisation (WHO) Codex Alimentarius Committee on Nutrition and Foods for Special Dietary Uses has been debating a universally acceptable definition for 20 years and separate definitions currently exist for the UK government, the EU, and WHO. Most debate revolves around the relative importance of chemical structure vs. physiological effect and/or health benefit. A current pragmatic definition would be that dietary fibre is a heterogeneous group of dietary components composed principally of non-digestible carbohydrates (NDC) usually derived naturally from plant cell walls. NDCs are primarily non-starch polysaccharides such as cellulose and pectin. (Heijnen, van Amelsvoort et al. 1996; Saemann, Bohmig et al. 2000; Asp and Bender 2006) Whilst the

consensus view on dietary fibre and CRC risk is now clear there are variations in effect found across different studies which may be accounted for by difficulties studying dietary effects.

The WCRF/AICR report concluded that there was a ten percent decrease in risk of cancer for every increase in fibre intake of 10g/day. (Dagfinn, Doris et al. 2011) The European Prospective Investigation into Cancer and Nutrition (EPIC), a large prospective study which included over 1700 cases of CRC, also found a protective effect for CRC with increasing dietary fibre intake (RR 0.79; 95% CI 0.63-0.99). (Bingham, Day et al. 2003)

There are several proposed mechanisms by which dietary fibre could exert its protective effect. Firstly increased dietary fibre may dilute carcinogen exposure. Those with higher levels of dietary fibre have increased colonic transit times and greater stool bulk which may limit the time and concentration of toxins the colonic epithelium is exposed to. (Cummings 1981) It has also been proposed that fibre molecules are able to bind potentially carcinogenic compounds preventing exposure to the colonic mucosa. (Bingham, Day et al. 2003)

Whilst these mechanisms may have a contributory effect there is strong evidence to suggest that benefit is derived from a sub-group of dietary fibre – resistant starch (RS). RS is defined as any starch or products of starch digestion that are not absorbed in the small bowel. (Nilsson, Ostman et al. 2008) It is subdivided into four main types:

RS1 - physically inaccessible starch due to an intact cell wall, mostly present in whole grains

RS2 – resistant starch granules (ungelatinised), found in green bananas and high amylose corn starch

RS3 – retrograded starch (after cooking and cooling a proportion of starch crystallises rendering it resistant to amylases), found in cooked cooled potatoes

RS4 – chemically modified starches often used as food additives (Asp and Bender 2006)

1.12.2 RESISTANT STARCH AS A DIETARY COMPONENT

RS is present in the everyday diet in foods such as wheat bran, rye bread, kidney beans, potatoes and green bananas. (Table 1-4) Average daily RS consumption is estimated to be around 2.8g/day in the UK (Wacker, Wanek et al. 2002) compared to 20g/day, the amount proposed to produce health benefits. (Englyst, Kingman et al. 1992) It is feasible to supplement a range of carbohydrate containing foods with manufactured RS without detectable change to taste or consistency of food stuffs. (Cook and Sellin 1998)

TABLE 1-4. RS CONTENT OF VARIOUS FOODS.

FROM THE NATIONAL STARCH FOOD INNOVATION DATABASE OF RESISTANT STARCH.

Food	**Serving size**	**Resistant starch content (g)**
Navy beans (baked beans)	½ cup cooked	9.8
Banana, raw	1 medium, peeled	4.7
Cold potato	1 small	3.2
Lentils	½ cup cooked	2.5
Cold pasta	1 cup	1.9
Pearl barley	½ cup, cooked	1.6
Oatmeal	1 cup, cooked	0.7
Wholegrain bread	2 slices	0.5

1.12.3 SAFETY AND STATUTORY STATUS OF RS

RS is found naturally in many foods as discussed above. No allergic reactions to RS have ever been reported and there are no safety issues. (Goldring 2004; Nugent 2005) Side effects such as flatulence, bloating, abdominal pain and laxative effects have been reported with higher doses (>30g/day) however these usually settle with time.

(Heijnen, van Amelsvoort et al. 1996; Grabitske and Slavin 2009) One small study of eight participants has reported an increase of DNA adducts in individuals receiving very high doses of RS, however these results have not been replicated subsequently. (Wacker, Wanek et al. 2002)

Currently RS has no specific legal status and naturally occurring RSs would be defined as dietary fibres for food labelling purposes in the UK. (Nugent 2005) The status of synthetically produced RSs is more complex as they must satisfy the accepted criteria for the structure of a fibre (polysaccharide with polymerisation degree >3) and have a proven health benefit to be classed as dietary fibre. (Stowell 2009) Health benefit claims are accepted nationally leading to variation in the classification of type 4 RSs on a country by country basis. Additionally, standard analytical methods to determine fibre content of whole foods are not often able to distinguish synthetic or naturally occurring fibres once they are mixed in a final product. In the UK, labelled dietary fibre content is determined by the AOAC (997.08) method which includes RSs and fructans. Different measurement methodologies and definitions of a products status make comparisons of RS intake in different populations problematic.

1.12.4 HEALTH BENEFITS OF RESISTANT STARCH

Several trials have examined the effect of RS on satiety. In a randomised double-blind crossover trial, Nilsson et al. found that consumption of RS at breakfast increased subjective satiety compared with a low fibre meal. (Nilsson, Ostman et al. 2008) RS supplementation was also shown to decrease food intake for subsequent meals. (Nilsson, Ostman et al. 2008) This effect has not been consistently demonstrated in other studies of RS supplementation and there may be variation depending on the format in which the RS supplement is taken and dose of supplement. (Willis, Eldridge et al. 2009; Monsivais, Carter et al. 2011; Karalus, Clark et al. 2012)

Several trials have demonstrated that ingestion of RS has a beneficial effect on glycaemic control. (Robertson 2012) Al-Tamimi et al. showed lower levels of insulin release and decreased peak blood glucose concentrations after consumption of a

standard glucose drink with RS versus glucose and a control calorie matched fibre supplement. (Rycroft, Jones et al. 2001) A cross-over study of healthy young adults has shown similar results when a standard glucose load was given with either RS supplemented rice versus normal rice. (Le Blay, Michel et al. 2003) In patients with metabolic syndrome and type 2 diabetes, RS supplementation has been shown to improve insulin sensitivity when compared to placebo in a randomised controlled trial. (Aoyama, Kotani et al. 2010)

Local colonic effects include lowering the pH of stool preventing the formation of potentially carcinogenic bile salt metabolites and promoting absorption of calcium. (Asp and Bender 2006) RSs most potent colonic effect is likely through its action as a prebiotic and the products of its bacterial fermentation. The definition of a prebiotic is: 'a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health.'(Gibson, Probert et al. 2004) Almost any carbohydrate that reaches the colon will act as a substrate for bacterial fermentation and may alter the composition of the commensal microbiota. Specific beneficial change is thought to result from increase in *bifidobacteria* and *lactobacilli* rather than less beneficial species such as *Bacteroides* and *clostridia*. (Macfarlane, Macfarlane et al. 2006; Bird, Conlon et al. 2010) It is also recognised that an important characteristic of a prebiotic is to provide a substrate for bacterial fermentation which generates short chain fatty acids (SCFAs) which improve colonic health. (Macfarlane, Macfarlane et al. 2006; Bird, Conlon et al. 2010)

Although specific effects of different carbohydrates are difficult to characterise due to dietary heterogeneity there is evidence that specific molecules have differential effects on bacterial populations and fermentation. (Rycroft, Jones et al. 2001) RS is one of the more complex prebiotics in the colon compared with shorter chain carbohydrates. RS has been shown to increase products of fermentation to a greater extent in the distal colon compared to the proximal colon of rats. Less complex carbohydrates had the opposite effect with the increase in fermentation products primarily in the proximal

colon. (Le Blay, Michel et al. 2003) Products of RS fermentation include carbon dioxide, methane and SCFAs. SCFAs (acetate, proprionate and butyrate), particularly butyrate, have a strongly beneficial effect on the colonic mucosa. Overall propionate and acetate are produced in greater quantity, but butyrate is the preferred fuel of colonocytes, contributing to 60% of their energy requirements, and is absorbed preferentially to acetate and proprionate. (Cook and Sellin 1998; D'Argenio and Mazzacca 1999) RS has been noted to have the property of making greater quantities of SCFAs, particularly butyrate, in contrast to other colonic bacterial substrates. (Bird, Conlon et al. 2010)

1.12.5 POLYDEXTROSE

Polydextrose (PD) is a complex synthetically manufactured polysaccharide generated by bulk melt polycondensation of glucose and sorbitol. (Stowell 2009) PD is 96% undigested within the small bowel and is also fermented by the colonic microbiota to SCFAs. (Stowell 2009) It has appetite suppressing effects similar to RS (Hull, Re et al. 2012; Ranawana, Muller et al. 2012). Consumption of PD in humans at a dose of 21g/day has a prebiotic effect. (Hooda, Boler et al. 2010) In healthy human subjects consumption of 8g/day has demonstrated that PD is a prebiotic which is slowly fermented over the length of the colon and fermentation of PD reduces the genotoxicity of faecal water. (Costabile, Fava et al. 2012) When PD is fermented in an *in vitro* colonic model and the products applied to colon cancer cells in culture, up regulation of protective pathways regulating apoptosis and down regulation of pro-carcinogenic cell cycle pathways are observed. (Putaala, Mäkivuokko et al. 2011)

1.12.6 BUTYRATE EFFECTS ON COLORECTAL EPITHELIUM

Butyrate has been shown to have several different anti-inflammatory properties in the gut. (Vinolo, Rodrigues et al. 2011) *In vitro* it has been shown to increase rates of neutrophil apoptosis; decreased neutrophil apoptosis is usually associated with chronic inflammation. (Aoyama, Kotani et al. 2010) Modulation of the inflammatory mechanisms of the epithelium has been shown by butyrate's ability to restore normal expression of immune modulators in inflamed tissue such as: IL-10, IL-12 (Saemann,

Bohmig et al. 2000), IL-8 (Kwon, Kim et al. 2010), Fas (Zimmerman, Singh et al. 2012) and nuclear factor kappa B (NF-κB) (Andersen, Christensen et al. 2010; Russo, Luciani et al. 2012) NF-κB is one of the principal transcription factors which regulates the inflammatory response (Bonizzi and Karin 2004) and is constitutively activated in 40% of CRC. (Sakamoto, Maeda et al. 2009; Machado, Constantino et al. 2012) Array data after induced blocking of NF-κB has demonstrated that many of the genes down-regulated upon inhibition of NF-κB are known to control tumour progression and metastasis. (Agarwal, Das et al. 2005) High NF-κB expression in CRC has been linked with poor prognosis. (Kwon, Kim et al. 2010) Certain polymorphisms of NF-κB may modulate an individual's response to dietary factors. In a large, prospective Danish population there was a 3% increase risk of CRC per 25g red or processed meat consumed per day in individuals with an NF-κB 94 deletion and no effect of increased meat consumption in individuals without this mutation. (Andersen, Christensen et al. 2010)

Butyrate has actions on the colorectal epithelium additional to its anti-inflammatory properties. Cancer cells display foreign antigenic signatures and so induce an immune response. Evasion of this immune response is required for a cancer to progress. The degree of tumour infiltration by immune cells is a prognostic marker in a variety of cancers. (Prestwich, Errington et al. 2008) Immuno-editing is the process of natural selection of tumour cells that progress vs. those that do not by their ability or otherwise to evade the host immune response. (Dunn, Bruce et al. 2002; Prestwich, Errington et al. 2008) Dietary components that enhance the immune system's ability to destroy tumour cells may have a chemoprotective role. (Armstrong and Mathers 2000) Butyrate was initially reported by Perrin et al. to increase the susceptibility of cancer cells to IL-2 activated natural killer cells in rats. (Perrin, Cassagnau et al. 1994) Enhanced natural killer cell lysis of tumour cells has also been shown in human CRC cell lines after supplementation with butyrate. (Zhang, Wang et al. 2009) Other mechanisms by which butyrate may enhance the immune response have been demonstrated, such as enhancement of human leucocyte antigens (Kvale and

Brandtzaeg 1995; Siavoshian, Blottiere et al. 1996) or inhibition of decay accelerating factor. (Andoh, Shimada et al. 2002)

Additionally butyrate promotes apoptosis in cancer cells. Butyrate is able to induce caspase cascade mediated apoptosis in CRC cell lines. (Medina, Edmonds et al. 1997; Pajak, Gajkowska et al. 2009) Butyrate has also been shown to enhance the cellular kill in CRC cancer cell lines treated with the cytotoxic cisplatin. (Koprinarova, Markovska et al. 2010) Caco-2 cell line studies have demonstrated that this effect is greater with butyrate than other SCFAs and that these changes are mediated via redox status and D-glucose metabolism alterations. (Matthews, Howarth et al. 2012) Butyrate's ability to induce apoptosis in *in vitro* cancer models has been replicated *in vivo*, where a dose-dependent increase in apoptosis with butyrate was shown in rats with induced CRC. (Clarke, Young et al. 2012) Whilst enhanced apoptosis has been demonstrated in CRC models this effect is not present in normal mucosa. (Hass, Busche et al. 1997) Mentschel et al. found that apoptosis was reduced by one third in RS supplemented pigs with normal mucosa vs. controls whilst mitotic rates remained the same. Changes in the apoptotic rate were not consistent throughout the crypt. Apoptosis decreased in the upper two thirds of the crypt and increased in the lower third. (Mentschel and Claus 2003) The differential effects of butyrate appear to be mediated by the differentiation status of cells with apoptosis occurring primarily in undifferentiated cells at the crypt base. Comalada et al. found no effect in apoptosis in normal differentiated colonocytes or well differentiated CRC cells whilst there was a large increase in apoptosis in poorly differentiated CRC cells supplemented with butyrate. (Comalada, Bailon et al. 2006) Similar results have been shown in an *in vivo* model of rat CRC carcinogenesis. (Clarke, Young et al. 2012) Cumulatively, these data suggest a maximal effect of butyrate in undifferentiated crypt basal cells; the stem cell compartment.

Butyrate has consistently been shown to have an anti-neoplastic effect on CRC cell lines. (Rycroft, Jones et al. 2001; Le Blay, Michel et al. 2003) Animal studies have also shown a protective effect of butyrate against cancer formation. (Medina, Afonso et al.

1998; Le Leu, Brown et al. 2007; Aoyama, Kotani et al. 2010) In rats fed a diet high in red meat, high numbers of DNA mutations were found in a dose dependent fashion. However supplementation of the rats' diet with RS caused increases in colonic butyrate concentrations and ameliorated DNA mutations caused by the red meat diet. (Toden, Bird et al. 2007) High fibre diets not rich in RS, did not have the protective effect of RS supplemented diets. (Toden, Bird et al. 2007)

1.12.6.1 Butyrate as a modulator of response in colonic injury

Butyrate delivered directly to the human colon has been shown to increase cellular replication rates in injured epithelium in UC and produce healing. (Freeman 1986) Protein synthesis in cells from patients with UC is increased significantly with butyrate supplementation compared with cells from normal controls or cancer patients. (Agarwal, Das et al. 2005) Several rat studies have shown that anastomotic strength is increased after colorectal resection when the animals are given butyrate enemas rather than placebo. (Dunn, Bruce et al. 2002; Prestwich, Errington et al. 2008; Bloemen, Schreinemacher et al. 2010; Mathew, Wann et al. 2010) Similar improvements in strength of a colonic anastomosis have been reported when butyrate is supplemented intravenously to rats. (Rolandelli, Buckmire et al. 1997)

When butyrate has been administered to animals with induced CRC a protective effect has not been noted either in terms of fewer or less aggressive CRCs. (Freeman 1986; Deschner, Ruperto et al. 1990; Caderni, Luceri et al. 2001) Several possibilities exist to explain these data such as timing of butyrate administration with respect to tumour development stage, dose of butyrate and other independent effects of dietary fibre. (Lupton 2004) However the simplest explanation is that the individual effects of butyrate act at a much more subtle level than methods used for induction of CRCs and that the effects of butyrate are not appreciable in the face of such severe genotoxic insults. In humans diagnosed with CRC and administered RS supplementation as part of a randomised controlled trial prior to surgery, RS patients had decreased crypt proliferation and beneficial changes in methylation of several TSGs compared to

controls indicating that there are butyrate effects in established CRC although not at a macroscopic level. (Dronamraju, Coxhead et al. 2009)

1.12.6.2 BUTYRATE METABOLISM IN ULCERATIVE COLITIS

Whilst the exact aetiology of UC remains to be elucidated it is clear that butyrate metabolism has a role in healing in UC and potentially in the aetiology of the condition.

Butyrate metabolism is impaired in UC mucosa with deficient mitochondrial β-oxidation of butyrate resulting in an energy deficiency for colonocytes. (Roediger 1980; Chapman and Grahn 1994) It has been suggested that this could represent the primary lesion in UC development as induced blocking of mitochondrial β-oxidation in rats results in a similar clinical and pathological disease to human UC. (Roediger and Nance 1986) However when *in vivo* butyrate metabolism was measured in human patients with UC, butyrate metabolism was found to be deficient only in actively inflamed mucosa and returned to normal with disease remission suggesting altered metabolism was a consequence rather than an initiating factor. (Den Hond, Hiele et al. 1998)

Several studies have addressed the role of butyrate in treating active UC or maintaining UC remission. Dietary supplementation with *Plantagoovata* – a rapidly fermentable RS - has been shown in a controlled clinical trial to maintain UC remission with similar efficacy to mesalazine. (Fernandez-Banares, Hinojosa et al. 1999) Another RS – germinated barley fraction – has been shown to assist mucosal healing in active flares of UC and maintain remission. (Kanauchi, Suga et al. 2002; Kanauchi, Mitsuyama et al. 2003; Hanai, Kanauchi et al. 2004) To achieve greatest effect in UC it seems that SCFA generation should occur throughout the colon; that is gradual fermentation is preferential to rapid caecal fermentation as provision of less complex, rapidly fermentable RSs do not show the magnitude of beneficial effects of slowly fermented RSs. (Galvez, Rodriguez-Cabezas et al. 2005)

1.12.7 POTENTIAL MECHANISMS OF BUTYRATE ACTION

1.12.7.1 BUTYRATE AND WNT SIGNALLING

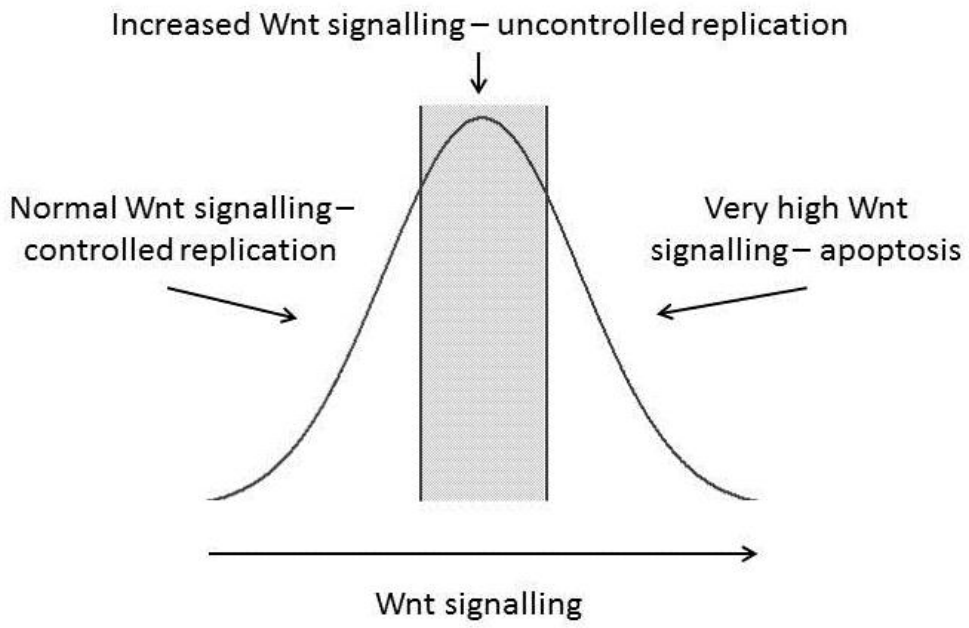
Butyrate has been shown to affect WNT signalling which may contribute to its anti-tumourigenic properties. In studies of CRC cell lines, the addition of butyrate has been shown to increase WNT signalling – a finding that would be expected to be pro-carcinogenic. (Bordonaro, Lazarova et al. 2002; Lazarova, Bordonaro et al. 2004) However this was associated with increased apoptosis and decreased clonal growth proportional to the increase in WNT signalling with no effect noted on cellular differentiation. (Lazarova, Bordonaro et al. 2004) Within each cell line, there was intra-cellular heterogeneity of response with apoptotic cells showing higher levels of WNT activation than live cells. (Lazarova, Bordonaro et al. 2004) When upregulation of WNT signalling was repressed so was the apoptotic effect of butyrate. They concluded that the response of a cell to butyrate was mediated by the level of WNT expressed by that cell. (Lazarova, Bordonaro et al. 2004)

These data appear contradictory as whilst WNT was increased by butyrate administration, an unexpected finding for a protective agent, apoptosis was also enhanced especially in the most highly expressing WNT cells. (Barker and Clevers 2006; Clevers 2006; Katoh 2011; White, Chien et al. 2012)

Bordonaro et al. propose this may be due to a relationship whereby WNT has an optimum level of activation for carcinogenesis, before and after which, WNT results in either controlled replication (normal tissue homeostasis) or apoptosis respectively. (Figure 1-9) (Bordonaro, Lazarova et al. 2008) 'Optimal' WNT levels for cancer result in uncontrolled proliferation and tumourigenesis. This theory is supported by an observation in FAP where the second allelic hit to *APC* which allows progression of a polyp to cancer has to be 'correct' depending on the specific germline *APC* mutation. Mutations or 'hits' are complimentary as many second hits do not actually result in progression of neoplasia. (Medina, Edmonds et al. 1997) For progression to cancer the 'correct' mutation maintains low levels of *APC* expression so that downstream WNT

expression is partially regulated but increased - the 'just right' hypothesis. (Albuquerque, Breukel et al. 2002)

FIGURE 1-9. CELLULAR REPLICATION IN RESPONSE TO WNT SIGNALLING ACTIVITY AS PROPOSED BY BORDONARO ET AL.



1.12.7.2 BUTYRATE AS A HISTONE DEACETYLASE INHIBITOR

Butyrate also acts as a histone deacetylase inhibitor (HDACi). This action is likely to both contribute to alterations in WNT and via other mechanisms. HDACis are a potential therapy in CRC and are already in clinical use for haematological cancers as vorinostat (Zolinza™, Merck, NJ, USA) which has been licenced in the U.S. as third line therapy for cutaneous T-cell lymphoma since 2006. (Tan, Cang et al. 2010) Pre-clinical data has shown that HDACis are active against CRC and act synergistically with established therapies such as 5-FU and irinotecan to enhance chemosensitivity of tumours. (Kim, Kim et al. 2009) The metastatic potential of CRC cell lines can be

abolished using butyrate to restore histone acetylation towards normal level. (Li and Chen 2012)

HDACis act epigenetically by modifying histone structure thus altering gene expression (section 1.4.7). (Pajak, Gajkowska et al. 2009) HDACis promote hyperacetylation of histones and increase transcription of CRC TSGs and reduce transcription of CRC oncogenes both *in vitro* and *in vivo*. (Dronamraju, Coxhead et al. 2009) One mechanism by which butyrate suppresses NF-κB activation in the colon is via its action as an HDACi. (Bonizzi and Karin 2004)

Butyrate's action as an HDACi has also been shown to influence the alterations in WNT signalling discussed previously. Butyrate and other HDACis have been shown to directly increase the levels of active β-catenin in the cell leading to apoptosis. Where β-catenin formation is blocked, or in cells resistant to production of β-catenin, the apoptotic effects of butyrate and other HDACis is abolished. (Pajak, Gajkowska et al. 2009)

1.12.7.3 Butyrate and methylation

Butyrate, via its action as an HDACi, is also able to reverse hypermethylation of silenced TSGs and induce differentiation of colorectal cancer cells *in vivo*. (Sgambato, Puglisi et al. 2010; Sarkar, Abujamra et al. 2011) In gastric cancer butyrate is able to reverse hypermethylation of silenced WNT inhibitors and restore their expression. (Shin, Kim et al. 2012)

Conversely in normal human fibroblasts (both adult and foetal) butyrate is able induce de-differentiation, increasing the harvest of stem cells from culture. Genome wide microarray shows that the mechanism for this is demethylation of gene promoter regions. (Mali, Chou et al. 2010) Although this action to de-differentiate cells may seem contradictory these differential effects could be accounted for by the different biological systems (normal and cancer tissue) and the fact that the initial methylation pattern of these tissues would be different as methylation is tissue specific. (Thompson, Atzmon et al. 2010; Liang, Song et al. 2011)

1.13 HUMAN CLINICAL TRIALS INVOLVING RESISTANT STARCH

Despite strong evidence *in vitro* and epidemiologically for the benefits of dietary fibre and specifically RS in CRC chemoprevention, clinical trials have not consistently demonstrated similar effects. Several large trials have enrolled human volunteers after diagnosis of a colorectal adenomatous polyp and randomised them to either high or low RS diets to examine the effect on polyp recurrence. The Toronto Polyp prevention trial was small (n=201) and used dietary advice rather than a supplement to change diet for two years. They reported no significant difference in polyp recurrence between the groups although with dietary advice only, changes in terms of fat and fibre consumed by participants fell short of those that had been defined *a priori*. (McKeown-Eyssen, Bright-See et al. 1994) Similar results were reported from the Australian polyp prevention trial. In this trial of 411 patients, participants were assigned to one of eight groups. High fibre, low fat and high beta-carotene were tested in all combinations against placebo. No overall effect was noted for any of the interventions tested. Only one group taking a combination of low fat/high fibre had a significant decrease in large adenomas at both 24 and 48 months. The reliability of these data are questionable given very small sample size within a single group. (MacLennan, Macrae et al. 1995)

Larger trials have similarly failed to show benefits of dietary fibre on polyp prevention. Alberts et al. found no difference in polyp recurrence in adults taking high wheat-bran fibre vs. a low fibre supplement, although baseline fibre consumption was high in both groups, compliance in the high fibre arm was poor and follow-up was only 3 years. (Alberts, Martinez et al. 2000)

The polyp prevention trial randomised patients to counseling to achieve a low fat, high fibre, high fruit and vegetable intake diet vs. standard healthy eating advice. This trial also failed to show benefit of such a diet compared with controls at both four (Schatzkin, Lanza et al. 2000) and eight years (Lanza, Yu et al. 2007) after adenomatous polyp resection. Sustained dietary changes did occur but these drifted with longer follow-up. (Lanza, Schatzkin et al. 2001) Sub analysis of the polyp prevention trial has

shown that those who were deemed 'super-compliers' with advice to maintain a low fat, high fruit and vegetable, high fibre diet over four years had a 35% lower risk of polyp recurrence than controls. (Sansbury, Wanke et al. 2009)

The Concerted Action Polyp Prevention (CAPP) studies were large randomised controlled trials (RCT) to test the chemoprotective effects of RS and aspirin (www.ncl.ac.uk/capp). The trials investigated two populations at high risk of CRC; CAPP1 examined the effects of RS and aspirin in patients with FAP whilst CAPP2 examined the effects in patients with HNPCC. (Burn, Chapman et al. 1998) Participants were randomised to take either: aspirin (600mg/day), RS (15g/day raw potato starch, 15g/day Hylon VII (CAPP1), 15g/day Novelose 240, 15g/day Novelose 330 (CAPP2)), aspirin and RS or double placebo. (Mathers, Mickleburgh et al. 2003) CAPP1 recruited 133 participants and followed them up for 1 year. Although there was a trend towards smaller polyps in both the aspirin and RS treated group there was no effect on polyp recurrence. (Burn, Bishop et al. 2011) CAPP2 has reported results of 703 HNPCC who were followed up for a mean of 29 months. There was no difference in either the rate of polyp formation or the rate of cancer diagnosis between any of the groups. (Burn, Bishop et al. 2008)

1.14 SUMMARY – THE ROLE OF RS IN MODULATING CRC RISK

The human trial data seems to contradict the basic science, animal and epidemiological evidence showing protective effects for fibre, RS and butyrate discussed earlier.

It is clear that, via butyrate, RS has potent effects on both normal colonic mucosa and CRC cells in addition to other general health benefits. Butyrate directly affects WNT signalling, one of the core functional pathways in CRC pathogenesis. The effects that butyrate has on colorectal epithelium suggest strongly that it should act as a chemoprotective agent in humans, however this has not been bourne out in human trials. There are several potential reasons for this. Firstly the effects generically attributed to fibre (used in several of the human trials) are likely derived from a

specific subset of fibre such as RS. Therefore trials which generically increase dietary fibre may not increase the 'correct' fibre resulting in no overall effect. Secondly it is possible that patients who are recruited to trials on the basis of previous adenomatous polyps or high risk conditions such as FAP and HNPCC have too great a mutation load for butyrate's action to be detectable using polyp recurrence as the outcome measure. Where more subtle mucosal kinetic changes and methylation marks have been measured in CRC patients supplemented with RS whilst awaiting surgery, beneficial effects are noted. (Dronamraju, Coxhead et al. 2009) It has also been shown that the effects of butyrate alter depending on the phenotypic status of the cell (Comalada, Bailon et al. 2006). The polyp forming stage of CRC carcinogenesis may not be sensitive to the chemopreventative effect of butyrate, although this seems less likely as adenomatous change would be associated with an increase in butyrate sensitive, undifferentiated cells (section 1.8.3). It is also likely that in patients who are at high risk of forming further polyps, either due to genetic mutations or because they have had a prior adenomatous polyp, may require longer periods of dietary change than those used in some of the human trials. Indeed when a subset of good compliers in one trial was examined at longer follow-up there does appear to be a reduction in polyp formation. (Sansbury, Wanke et al. 2009)

For these reasons it is likely that meaningful data on the chemoprotective effects of RS is likely to be obtained from those at normal risk of CRC. The use of cancer or polyp development as end points is impractical as these occur infrequently in a normal population and time periods for development are long. Therefore surrogate endpoints of cancer risk (biomarkers) are required so valid new knowledge can be discovered contemporaneously and practically.

1.15 HYPOTHESES, AIMS AND OBJECTIVES

1.15.1 HYPOTHESES

1. Selected NOTCH genes (*ATOH1*, *NOTCH1*, *NOTCH3*, *JAG1*, *LFNG*, *RBP-J*) display hypomethylation of promoter regions in those at higher risk of CRC compared to normal controls.

2. Global genome methylation, as measured by LINE-1, will be decreased in those at higher risk of CRC compared to normal controls.

3. Systemic inflammation, as measured by hsCRP, will be elevated in those at higher risk of CRC compared to normal controls.

4. Dietary intervention in individuals at normal risk of CRC with either polydextrose or Hi-maize 260® will increase methylation of NOTCH gene promoters.

5. Dietary intervention in individuals at normal risk of CRC with either polydextrose or Hi-maize 260® will increase global DNA methylation.

6. Dietary intervention in individuals at normal risk of CRC with either polydextrose or Hi-maize 260® will decrease systemic inflammation.

7. Pro-carcinogenic and protective anthropometric and environmental factors will influence methylation of NOTCH genes in normal risk individuals with increased methylation associated with protective exposures and decreased methylation associated with harmful exposures.

1.15.2 AIMS

Broadly the aims of the project are to test the hypotheses detailed above by collecting biological samples (including rectal biopsies), anthropometric, demographic and dietary data from individuals at higher risk of CRC due to a previous diagnosis of adenomatous polyps within the colon or a diagnosis of UC for longer than 7 years and

comparing these to a control population at normal risk of CRC. Methylation assays for NOTCH genes and for global methylation will be developed and used to estimate methylation in DNA extracted from rectal mucosal biopsies of recruited participants. Individuals at normal risk will additionally be recruited and allocated to 50 day dietary intervention with RS or polydextrose using a 2x2 factorial allocation. Before and after analysis of rectal biopsies using the methylation assays developed will test the plasticity of methylation of NOTCH genes.

1.15.3 OBJECTIVES

The objectives of this study are:

1. To screen several candidate NOTCH genes for variation in promoter methylation in DNA from colorectal mucosal biopsies from a subset of trial participants at differential CRC risk i.e. normal controls, polyp patients and UC patients. Screening will identify the most variably methylated genes between risk groups and inform selection of genes to be analysed in the whole study sample.

2. To describe variation in the selected NOTCH gene methylation in rectal biopsies between individuals at normal lifetime risk of CRC and individuals at higher lifetime risk of CRC (previous adenomatous polyp or UC >7years).

3. To describe associations between lifestyle, dietary and anthropometric factors known to increase risk of CRC and methylation of NOTCH genes.

4. To investigate differences in global DNA methylation (LINE-1 assay) in DNA from rectal biopsies between individuals at normal lifetime risk of CRC and individuals at higher lifetime risk of CRC (previous adenomatous polyp or UC >7years).

5. To describe associations between lifestyle, dietary and anthropometric exposures known to increase risk of CRC and global gene methylation.

6. To undertake a randomised, double-blind, placebo-controlled human dietary intervention study with resistant starch (Hi-maize 260) and polydextrose given in a 2x2 factorial design in those at normal lifetime risk of CRC.

7. To investigate the effect of dietary supplementation with resistant starch and with polydextrose on methylation of selected NOTCH genes in the colorectal mucosa of humans at normal risk of CRC.

8. To investigate the effect of dietary supplementation with resistant starch and with polydextrose on global DNA methylation in humans at normal risk of CRC.

CHAPTER 2. METHODS

2.1 CLINICAL TRIAL PROTOCOLS

2.1.1 STATUTORY APPROVALS

Prior to recruiting any participants ethical approval was sought and gained from Newcastle and North Tyneside Research Ethics Committee 2 (09/H0907/77). (Appendix 1) Caldicott approval for storage of data was sought and gained from Northumbria NHS Foundation trust (C1792). The trial was registered with clinicaltrials.gov (NCT01075893)

2.1.2 FUNDING AND TASK ALLOCATION

The DISC study was funded by the Biotechnology and Biological Science Research Council. This funding included the appointment of Dr Naomi Willis as a research associate and Dr Long Xie as a laboratory technician to the team. All statutory approvals, trial protocols and standard operating procedures were undertaken by myself prior to their joining the study. Participant recruitment and post procedure processing was undertaken by all team members although I was responsible for all invitations to potential participants. Dr Willis undertook all home visits to volunteers and repeat biopsies were undertaken by all team members. All laboratory work; DNA extraction, bisulfite modification, assay development, sample processing and analysis was undertaken by myself with the exception of the LINE-1 assay development which was undertaken by Dr Wan Adnan Omar who also provided my laboratory training.

2.1.3 PARTICIPANT GROUPS

Allied to the research questions there were two patient groups:

1. Those at higher risk of CRC – this group comprised participants with either ulcerative colitis or a previous history of adenomatous polyp.

2. Those at 'normal' risk of CRC - this group comprised participants with no macroscopic or microscopic abnormality in their colon or other factors likely to increase their risk of CRC. These participants were invited to participate in a dietary intervention study (the DISC Study) in which they took a food supplement for 50 days. Their baseline samples were used as comparators for the higher risk group (described above).

2.1.4 SAMPLE SIZE CALCULATION

The DISC study aimed to recruit 75 patients. No formal power calculation was undertaken due the exploratory or pilot nature of the analyses being undertaken and hence the paucity of relevant data to undertake such a calculation. The sample size was based on a previous RCT undertaken within the group which demonstrated significant changes in cell proliferation and expression of cell cycle regulatory genes with RS supplementation at a similar dose over a shorter time period. (Dronamraju, Coxhead et al. 2009)

2.2 RECRUITMENT

2.2.1 APPROACH TO PATIENTS

All patients were recruited via endoscopy lists at Wansbeck General Hospital, Ashington, UK or North Tyneside General Hospital, North Shields, UK. Review of hospital databases and prior endoscopy reports allowed most patients with a history of UC or prior adenomatous polyps to be identified prior to their appointment. Potential participants were sent a detailed description of the study at least five days prior to their appointment. (Appendix 2)

Potential recruits were identified at their endoscopy visit. Suitable participants were invited to participate and screened for potential exclusion criteria (Table 2-1) (Appendix 3) before full informed consent was obtained. (Appendix 4) Sample acquisition and data collection depended on the group (higher risk of CRC or intervention study) and so these are described separately henceforth.

TABLE 2-1. PARTICIPANT EXCLUSION CRITERIA.

Exclusion criteria - general	Age <16 or >85 years Prisoner at the time of initial endoscopy Familial polyposis syndrome Hereditary non-polyposis colorectal cancer (HNPCC) Known colorectal tumour Previous colorectal resection Pregnancy Chemotherapy in last 6 months Therapy with aspirin/other non-steroidal anti-inflammatory drug (NSAID) Other immunosuppressive medication Active colonic inflammation at endoscopy Incomplete left sided colonic examination Colorectal carcinoma found at endoscopy Iatrogenic perforation at endoscopy Colorectal cancer on histology
Additional exclusion criteria for intervention group	Warfarin or other anticoagulant therapy Diabetes mellitus
Additional exclusion criteria for UC group	Short clinical colitis activity index (SCCAI) score greater than 5

2.2.2 JUSTIFICATION OF EXCLUSION CRITERIA

Many exclusion criteria are self-explanatory and were chosen for pragmatic reasons e.g. age and pregnancy. Other criteria were selected to ensure that intervention participants had no underlying cause to be at higher risk of CRC. High risk inherited conditions such as FAP and HNPCC resulted in exclusion. To screen for possible HNPCC the Amsterdam II criteria 3-2-1 rule was used: at least **3** relatives with an HNPCC associated cancer, **2** generations affected and **1** cancer diagnosed before age 50. (Vasen, Mecklin et al. 1991)

Similarly, therapy with aspirin or other NSAID resulted in exclusion as even small daily doses (75mg) of aspirin have been shown to have a protective effect against CRC development. (Fowkes, Price et al. 2010; Rothwell, Fowkes et al. 2011)

To be deemed at normal risk of CRC, subjects had to have a normal left-sided (to the splenic flexure) examination of the colon rather than a complete colonoscopy. Sigmoidoscopy reliably excludes cancer and other relevant colonic pathology depending on presenting symptoms and other risk markers for an individual. Where sigmoidoscopy is indicated the chance of missing a lesion is approximately one percent, less than the additional risk of whole colonic imaging. (Thompson, Flashman et al. 2008)

Those with active colonic inflammation, recent chemotherapy or immunosuppressive medication were excluded due to the influence of these factors on immune function and systemic immune status, as change in inflammatory markers was a study end point.

Potential participants for the dietary intervention study who were taking warfarin or other therapeutic anti-coagulant agents were excluded due to increased risk of bleeding at their repeat biopsies. Potential participants with diabetes mellitus were excluded due to potential adverse effects on glycaemic control if they were randomised to take one of the placebo carbohydrates (maltodextrin or amioca starch).

The Short Clinical Colitis Activity Index (SCCAI) is a simple validated tool to assess clinically whether potential participants have quiescent colitis at the time of endoscopy. (Walmsley, Ayres et al. 1998) Active colitis was excluded pragmatically as histological analysis would have been complicated and to ensure sample homogeneity. An SCCAI score greater than five predicts active colitis with 92% sensitivity and 91% specificity. (Jowett, Seal et al. 2001)

2.2.3 TRIAL PARTICIPANT JOURNEY - HIGHER RISK OF COLORECTAL CANCER PARTICIPANTS

2.2.3.1 COLLECTION OF DEMOGRAPHIC, ANTHROPOMETRIC, LIFESTYLE AND DIETARY DATA

Participants in the higher risk of CRC group had all measurements and samples taken at the initial (and only) visit. Demographic data were collected including: age, sex, medical history and medications. Dietary choices and habitual physical activity were assessed using questionnaires. The food frequency questionnaire (FFQ) was a locally adapted version of the validated questionnaire used in the EPIC study. (Bingham, Gill et al. 1997; Kroke, Klipstein-Grobusch et al. 1999) The physical activity questionnaire was identical to that used in the EPIC study which has been validated against objective accelerometer data. (Cust, Smith et al. 2008) It is considered suitably sensitive to detect epidemiological associations between physical activity and health outcomes when both recent and long-term activity data are required. (Cust, Smith et al. 2008) Participants also had height, weight, waist, hip and thigh circumferences measured. (see section 2.2.4.4)

2.2.3.2 BLOOD SAMPLE COLLECTION

Venepunture was performed at the time of recruitment to the study. 4 x 7.2mg EDTA (K3E) tubes (Becton Dickinson, Oxford, UK) and 1 x gel-coated (SST II) advance (Becton Dickinson, Oxford, UK) were collected. Vitamin B_{12}, plasma folate and high sensitivity C-reactive protein (hsCRP) estimation were performed at the Freeman Hospital laboratories (Newcastle-upon-Tyne, UK). Plasma selenium was measured at Liverpool

University (Liverpool, UK) after plasma was separated by centrifugation (5 minutes, 4°C, 3100g)

2.2.3.3 Biopsy collection and processing

After the endoscopy had been completed to its proximal extent, and no exclusion criteria had been found, 9 mucosal biopsies were taken from the rectum, 10cm from the anal verge, with 2.3mm spiked flexible endoscopy forceps (Biobite forceps, Medical Innovations, Essex, UK). The first biopsy was placed in 1ml RNA*later*® (Ambion, USA), two biopsies were immediately flattened and fixed in 10% formalin for later paraffin embedding and sectioning, 1 biopsy was placed in 1ml of Carnoy's solution (30% acetic acid, 70% ethanol) and 5 were snap frozen in liquid nitrogen cooled isopentane after being suspended in Tissue-tek® optimal cutting temperature (OCT) compound (Sakura, Netherlands).

Biopsies for paraffin embedding were transferred to the pathology laboratories at Northumbria Healthcare NHS Foundation trust for paraffin embedding and blocking. OCT frozen biopsies and RNA*later*® biopsies were stored at -80°C. Carnoy's samples were stored at 4°C overnight then transferred to 70% ethanol for long-term storage.

2.2.4 TRIAL PARTICIPANT JOURNEY - INTERVENTION PARTICIPANTS

Those participants who consented to take part in the intervention trial had a more complex journey through the study. (Figure 2-2)

2.2.4.1 ENDOSCOPY

At the initial endoscopy visit demographic data, blood samples and mucosal biopsies were taken, identical to those taken for participants at higher risk of CRC. Participants were given equipment for collection of urine and stool samples at home. A 'washout' period of at least seven days before urine and stool sample collection was used to mitigate against possible effects of bowel preparation on these samples.

2.2.4.2 BIOPSY PROCESSING

Biopsy collection and processing were as described previously for participants in the higher risk group. (see section 2.2.3.1)

2.2.4.3 BLOOD SAMPLE COLLECTION

Venepuncture was performed at the time of recruitment to the study. 8 x 7.2mg EDTA tubes and 1 x gel-coated were collected. Post venepuncture processing was as for higher risk of CRC participants (see section 2.2.3.2). The remaining 4 EDTA tubes were centrifuged (5 minutes, 4°C, 3100g) and plasma extracted and stored at -80°C. These samples were used to estimate plasma SCFA concentrations (Scottish Universities Environmental Research Centre (SUERC), UK).

2.2.4.4 HOME VISITS

The first visit after endoscopy was arranged in the patient's home after the seven day washout period. Participants were asked to collect urine and stool samples immediately prior to the visit. Height was measured with an ultrasound stadiometer to the nearest 0.1cm (Soehnle, Germany) and weight to the nearest 0.1kg with Tanita digital scales (TanitaEurope B.V., Amsterdam, The Netherlands). Waist, hip and thigh circumference measurements were recorded to the nearest 0.1cm. All measurements

were performed as per methods previously described. (Marfell-Jones, Olds et al. 2006) All measurements were performed twice or repeated until agreement tolerances were achieved (0.1kg weight, 0.1cm height and 1cm for waist, hip and thigh circumference).

During the course of the 50 day intervention participants were visited on two further occasions to supply additional intervention sachets, check compliance and provide support. Prior to the end of the intervention period participants were provided with apparatus to collect further urine and stool samples. Some were also supplied with an accelerometer to validate physical activity data reported in the lifestyle questionnaire.

2.2.4.5 RANDOMISATION & BLINDING

Randomisation of participants was stratified for the initial endoscopic procedure (colonoscopy or flexible sigmoidoscopy) to mitigate against the effect of different bowel preparation regimes. Participants selected an opaque sealed envelope from a box for the relevant procedure. This was coded A, B, C or D. Blinding was maintained until data collection and analysis was complete.

2.3 INTERVENTIONS

Two active ingredients and two placebos were tested in a 2x2 factorial design. The active ingredients were Hi-maize 260 (National Starch, USA) and polydextrose (Danisco, Finland). Maltodextrin and Amioca starch acted as placebos.

2.3.1 HI-MAIZE 260 (RS)

Hi-maize 260 is a granular RS with an energy content of 5.7kJ/g (*cf*. 16.4 kJ/g white flour). Approximately 40% of Hi-maize 260 is digestible in the small bowel so 60% is RS available for fermentation in the colon.
(http://www.foodinnovation.com/Downloads/Applications/HimaizeBrochure.pdf)

2.3.2 POLYDEXTROSE

PD was supplied by Danicso (Finland) as Litesse® ultra (http://www.danisco.com/wps/wcm/connect/www/corporate/products/product_range/sweeteners/litesse). It is a complex synthetically manufactured polysaccharide generated by bulk melt polycondensation of glucose and sorbitol which is essentially tasteless and can be incorporated into foods to enhance texture. (Stowell 2009) It has an energy content of 4.1 kJ/g and is less than 5% digested in the small bowel (>95% NDC available for fermentation in the colon).

2.3.3 DIGESTIBLE CARBOHYDRATES- MALTODEXTRIN AND AMIOCA STARCH

Two carbohydrates which are completely digested and absorbed within the small bowel were used within the DISC study. They were provided together to create a placebo arm within the trial and were combined with active agents in the single agent arms so that blinding could be maintained. These agents were:

- maltodextrin which is produced by partial hydrolysis of starch molecules. It is rapidly digested and absorbed in the small bowel as glucose.
- amioca starch is derived from maize corn and consists mainly of amylopectin (low amylose content), a highly branched glucose polymer. It is completely digested and absorbed as glucose in the small bowel. (http://eu.foodinnovation.com/docs/AMIOCA.pdf)

2.3.4 INTERVENTION COMBINATION

Interventions were combined so that each participant was taking either (Figure 2-1):

- Double intervention agent (Hi-maize 260 & PD)
- Hi-maize 260 (combined with amioca starch)
- PD (combined with matched maltodextrin)
- Placebo (maltodextrin and amioca starch)

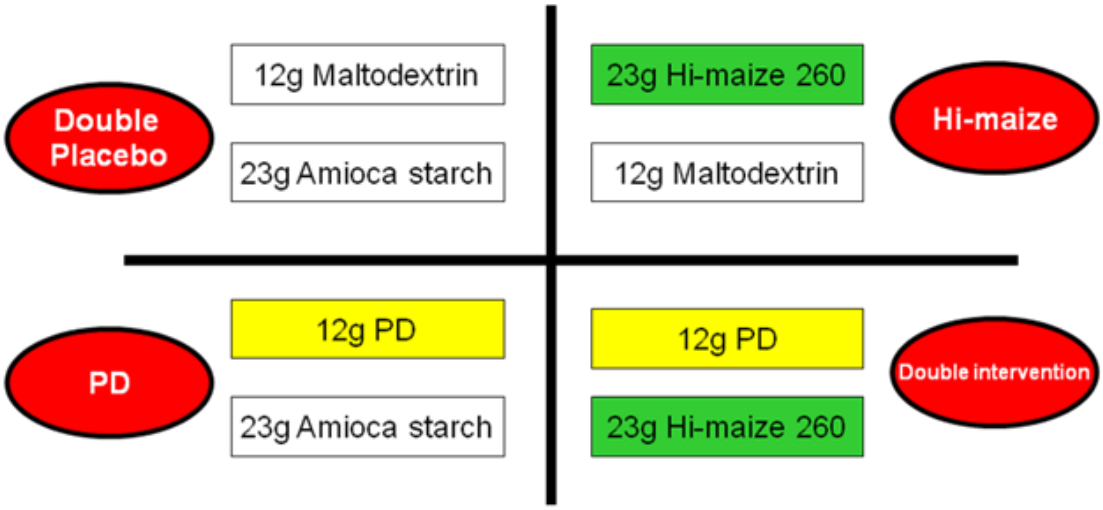
The amount of intervention agent supplied per day was calculated to deliver the same dose of non-digestible carbohydrate to the colon for PD and Hi-maize 260 based on known proportion digested in the small bowel i.e. 12 and 23g/d respectively. The dose of 23g/day RS has been previously shown to modulate cell proliferation and gene expression as part of an RCT and 12g/day PD was greater than the dose (8g/day) which has been shown to reduce the genotoxity of faecal water in humans. (Dronamraju, Coxhead et al. 2009; Putaala, Mäkivuokko et al. 2011) The quantity of maltodextrin or amioca starch supplied was matched to the quantity of the corresponding intervention agent so that the total weight of supplement consumed each day was 35g/day for each of the four treatment limbs. (Figure 2-1)

All interventions were supplied in 8x8cm opaque silver sachets coded depending upon contents. Sachets were combined to produce each intervention and packed in boxes to contain one week's supply of a supplement. The daily dose of each agent was divided between two sachets so each day each participant was asked to consume four sachets daily.

Participants were asked to consume the supplement on cold food or mixed with cold water or juice. Participants were free to divide the doses as they saw fit throughout the day. Participants were asked to retain both eaten and uneaten sachets for collection and counting to give an estimate of compliance.

FIGURE 2-1. COMBINATIONS OF INTERVENTIONS AND PLACEBOS

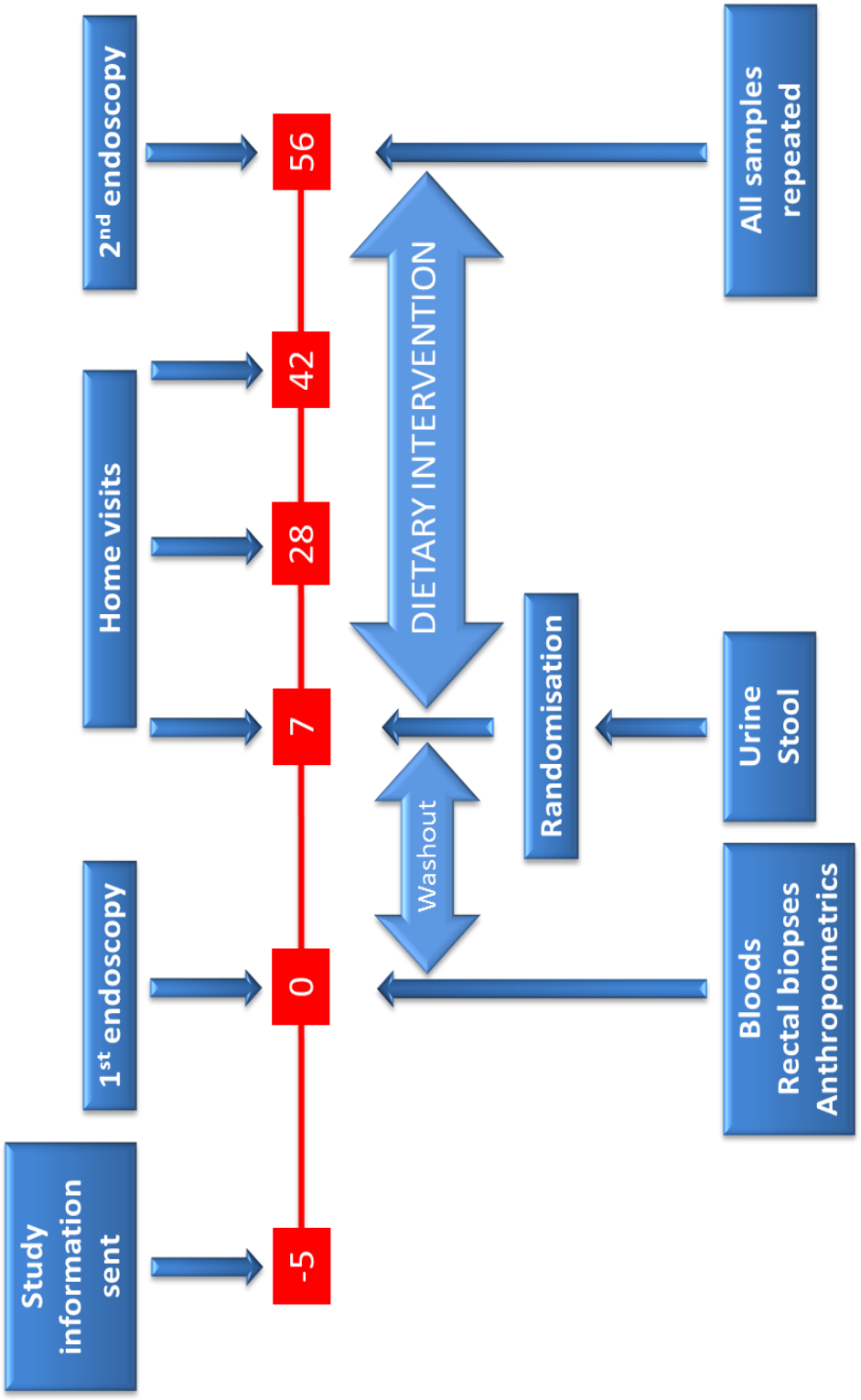
(PD – POLYDEXTROSE)



2.3.4.1 REPEAT BIOPSIES

At the end of the 50 day period the participants re-attended the hospital for their end of intervention repeat biopsies and measurements. All initial measurements were repeated (blood samples, anthropometry, biopsies, stool and urine). Mucosal biopsies were taken using a rigid sigmoidoscope from the mid rectum using rigid 4mm Sarratt biopsy forceps (Stericom, UK) (10cm from the anal verge). Rigid sigmoidoscopy avoided the need for bowel preparation prior to the repeat biopsies. All repeat samples were processed identically to the samples obtained at the start of the study (see section 2.2.3.2, 2.2.3.3).

FIGURE 2-2. SCHEMATIC REPRESENTATION OF PARTICIPANTS PROGRESS THROUGH THE DISC STUDY



2.4 LABORATORY METHODS

2.4.1 MEASURING METHYLATION

Several methods exist to assess the degree of methylation of promoter sequences of the genome. Pyrosequencing is a 'sequencing by synthesis' technique for quantifying the methylation status of individual CpG sites. (Tost and Gut 2007)

2.4.2 BISULFITE MODIFICATION

Sequencing to measure methylation is achieved by generating a methylation specific sequence alteration. This utilises the desulphonation reaction between pyrimidines and sodium bisulfite initially described in the 1970s. (Hayatsu, Wataya et al. 1970; Hayatsu, Wataya et al. 1970) Whilst the desulphonation reaction with unmethylated cytosine is rapid, the desulphonation of methylated cytosine happens at a much slower rate, such that the deamination of unmethylated cytosine will be will be complete before the deamination of methylated cytosine has begun. (Frommer, McDonald et al. 1992)

Bisulfite modification utilises this reaction to convert unmethylated cytosine residues to uracil, whilst methylated cytosines remain as cytosine. The region of interest within a genome can then be amplified using polymerase chain reaction (PCR) and the pattern of original methylation can be measured by sequencing the complimentary product and determining the ratio of cytosines to thymines at each CpG site; hence determining the ratio of methylated to unmethylated cytosines in the original sample.

2.4.3 PYROSEQUENCING

Primer sets are designed to amplify a region of interest in the gene which is known to contain several CpG sites. This region is then amplified by PCR and the product is denatured to single DNA strands. The pyrosequencer then sequences the complementary strand to the PCR product which is incubated with DNA polymerase, ATP (adenosine triphosphate), sulfurylase, luciferase, and apyrase as well as the

substrates, adenosine 5' phosphosulfate, and luciferin. Bases are sequentially added to the reaction and if a base is incorporated it is accompanied by release of pyrophosphate. (Figure 2-3) Pyrophosphate is converted to ATP by ATP sulfurylase which drives conversion of luciferin to oxyluciferin generating visible light in proportion to the amount of ATP produced. The light produced is therefore also proportional to the number of nucleotides incorporated into the sequence and is recorded by the pyrosequencer. (Figure 2-4) At the completion of this reaction unincorporated bases are cleared by apyrase before further bases are added. The incorporation ratio of cytosine to thymine at the completion of sequencing is directly related to the cytosine/thymine ratio in the PCR product and hence the methylated to unmethylated CpG ratio in the original sample.

FIGURE 2-3. SEQUENCING OF COMPLIMENTARY STRAND TO SINGLE-STRANDED PCR PRODUCT IN THE PYROSEQUENCER.

(REPRODUCED WITH PERMISSION FROM WWW.PYROSEQUENCING.COM)

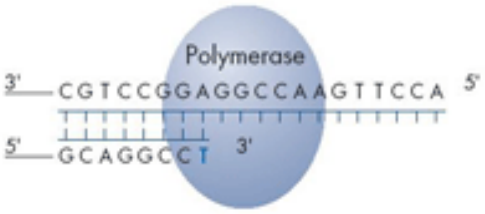
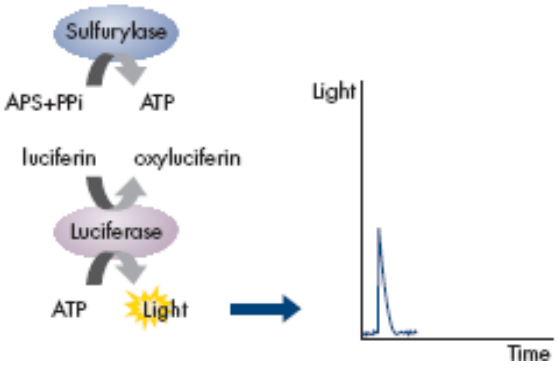


FIGURE 2-4. INCORPORATION OF A NUCLEOTIDE IN THE COMPLIMENTARY SEQUENCE RESULTS IN LUCIFERASE MEDIATED RELEASE OF LIGHT MEASURED BY THE PYROSEQUENCER.

(REPRODUCED WITH PERMISSION FROM WWW.PYROSEQUENCING.COM)



2.5 BIOINFORMATIC ANALYSIS & PRIMER DESIGN

Genes were selected initially based on their proposed biological relevance. (section 1.9.3). The list selected was: *ATOH1, JAG1, LFNG, NOTCH1, NOTCH2, NOTCH3* and *RBP-J*.

The gene2promoter function on the genomatix website (www.genomatix.de) was used to identify likely promoter sequences for relevant transcripts. Promoter sequences were copied into methprimer (http://www.urogene.org/methprimer). A 200 base pair window including, CG ratio >0.5 and CpG observed to expected ratio >0.6, was used to identify promoter zones rich in CpGs. (Gardiner-Garden and Frommer 1987; Feinberg and Tycko 2004) CpG rich promoter regions were chosen as regions of interest for assay of methylation status and bisulfite modified to be suitable for analysis in PSQ assay design (Biotage AB, Uppsala, Sweden). In PSQ assay design, multiple CpG rich target zones were tested to generate the best primer sets based on the suitability score generated by PSQ and verified on direct examination of the sequence. Eighteen potential primer sets were identified and tested. (Table 2-2) The final assays that were used to screen the test sample population are detailed in Table 2-3.

TABLE 2-2. SUMMARY OF PRIMER SEQUENCES TESTED

(PCR- POLYMERASE CHAIN REACTION)

Assay	PCR	Validated	Reproducible	Screened	Excluded due to
NOTCH1(1)	No	No	No	No	No PCR product generated
NOTCH 1(2wob)	Yes	No	No	No	Unable to establish consistent optimal annealing temperature
NOTCH1(3)	Yes	No	No	No	Inconsistent validation
NOTCH1(4)	Yes	No	No	No	Inconsistent validation
NOTCH1(5)	Yes	Yes	Yes	Yes	
NOTCH2(1)	No	No	No	No	No PCR product generated
NOTCH2(2)	Yes	No	No	No	Would not sequence past 1st CpG site on pyrosequencing
NOTCH2(3)	Yes	No	No	No	Inconsistent validation
NOTCH3(1)	Yes	No	No	No	Few CpGs included, good result with Notch3 (3)
NOTCH3(2)	Yes	No	No	No	Few CpGs included, good result with Notch3 (3)
NOTCH3(3)	Yes	Yes	Yes	Yes	
NOTCH3(4)	Yes	Yes	Yes	No	Same CpGs included as Notch 3(3)
LFNG (1)	Yes	No	No	No	Inconsistent validation
LFNG (2)	No	No	No	No	No PCR product generated
LFNG (3)	Yes	Yes	Yes	Yes	
RBP-J	Yes	Yes	Yes	Yes	
ATOH1	Yes	Yes	Yes	Yes	
JAG1	Yes	Yes	Yes	Yes	

2.6 TECHNIQUES

2.6.1 DNA EXTRACTION

DNA was extracted from half a biopsy using a standard phenol-chloroform methodology. Samples were homogenized in a solution of 500µl SET-SDS (25ml 50mM Tris, 12.5 mM ethylenediamine tetracetic acid (EDTA), 0.5% sodium dodeyl sulphate (SDS)) and 16µl proteinase K (Fermentas) overnight at 55°C. DNA was precipitated by the addition of 600µl 24:1 chloroform:isoamyl alcohol (Fermentas) and the supernatant separated using phaselock gel tubes (5-prime, Hamburg, Germany). The supernatant was incubated with 16µl RNAase/T1 (Fermentas) at 37°C for 30 minutes. 42µl 3M sodium acetate was added, followed by 400µl isopropanolol and 2.5µl (20mg/ml solution) glycogen (Fermentas). The DNA precipitated was separated by centrifugation at 13 000 rpm for 5 minutes. The DNA pellet was washed with 70% ethanol twice before air drying and re-suspending in 50µl of 2mM Tris.

2.6.2 BISULFITE MODIFICATION

DNA was bisulfite modified using EZ DNA methylation Gold™ kit (Zymo, CA, USA) according to the manufacturer's protocol.

2.6.3 PCR

All PCRs were performed on a Sensoquest Thermocycler (Göttingen, Germany). Standard PCR contents were: 12.5µl Hotstar Taq (Qiagen), 9.5µl nuclease free water, 1µl forward primer, 1µl reverse primer and 1µl template DNA. Where required Q-solution (Qiagen) was added, 2µl was substituted for 2µl nuclease free water. Standard cycling conditions were: enzyme activation (95°C for 5 minutes), 50 cycles of denaturation (15s 95°C), annealing (30s optimal annealing temperature, Table 2-3) and extension (15s 72°C) followed by a final extension phase (5 minutes 72°C) and termination of the reaction by cooling to 4°C indefinitely.

2.6.4 PYROSEQUENCING

Pyrosequencing was performed using a Pyromark Q96 ID (Qiagen) using Pyromark Gold Q96 reagents (Qiagen). 10µl of PCR product was placed in each well of a standard 96 well plate together with 38µl binding buffer (Qiagen), 30µl nuclease free water and 2µl Streptavidin Sepharose High Performance beads (GE Healthcare, Uppsala, Sweden). Plates were covered and shaken (15rpm) to prevent bead sedimentation until ready for analysis. In a corresponding pyrosequencing plate (Qiagen), 11.5µl of annealing buffer and 0.5µl of sequencing primer were aliquoted for each sample. Samples were drawn up by vacuum probes on the Q96 vacuum work station (Qiagen), washed in 70% ethanol (5s), denatured in 0.8% NaOH solution (10s) and washed in Pyromark wash buffer (Qiagen) (5s) before being transferred to the pyrosequencing plate. The pyrosequencing plate was heated to bind the sequencing primer (80°C for 2 minutes) prior to analysis on the pyrosequencer.

2.7 ASSAY DEVELOPMENT

2.7.1 PCR OPTIMISATION

The designed primer sets were synthesised by Eurofins (Ebersberg, Germany). Lyophilised primers were reconstituted using nuclease-free water to a concentration of 10pmol/µl. Gradient PCR was performed around the estimated optimal annealing temperature (T_m-2°C) to identify the actual optimal PCR conditions. PCR products were compared against the anticipated product size using gel electrophoresis for correct product size and product purity. Where required, further optimisation was conducted using additional Q-solution (Qiagen, Crawley, UK) or by varying cycle number and timings. Final assay conditions are summarised in Table 2-3.

2.7.2 ASSAY VALIDATION AND REPRODUCIBILITY

After PCR optimisation, biotin-labelled (5') reverse primers were used for pyrosequencing. PCR was performed using control DNA of predetermined degrees of methylation (Epitect Control DNA, Qiagen). 100%, 75%, 50%, 25% and 0% methylated control DNA was amplified and pyrosequenced to determine the relationship between observed methylation levels and known methylation levels. Both pre- and post-PCR dilutions were performed and the results plotted using Microsoft Excel 2010. (White, Durston et al. 2006) A best fit line was applied and the coefficient of determination (R^2) calculated. R^2>0.95 was deemed to validate the assay at each individual CpG site.

The reproducibility of assays was tested using the assays for three genes (*ATOH1, LFNG,* and *RBP-J*). The proportion of samples in which variability was greater than 5% in duplicate samples was 6.3% for duplicate bisulfite modification procedures (13 samples, 142 CpG sites), 4.3% for duplicate PCR reactions (12 samples, 139 CpG sites) and 2.2% for duplicate pyrosequencer runs (9 samples, 90 CpG sites).

2.7.3 ASSAY SCREENING

To identify gene assays which were likely to yield biologically interesting results, 6 assays for 6 genes (Table 2-3) were applied to DNA from a sub-group of study participants. This screening sub-group comprised eight polyp participants, eight normal participants (age, sex and smoking status matched to the polyp sub-group), and eight UC participants. Due to the small size of the UC group matching was not possible. Data from the screening phase were used to determine those genes displaying differential methylation signatures in those at higher risk of cancer and those at normal risk. These data were used in conjunction with other factors to determine which genes would be analysed for the whole study population. (section 3.2.8)

2.7.4 GLOBAL DNA METHYLATION

Global DNA methylation was assessed using an assay which quantified CpG methylation in the long interspersed nucleotide element-1 (LINE 1). Around 18% of the human genome consists of LINE-1 repeats with 500 000 truncated and 5 000 full length sequences (Kazazian 2000) LINE-1 methylation status is thought to represent the genome-wide DNA methylation status and has been shown to be the best surrogate for the current gold standard assay: high performance liquid chromatography (HPLC). (Lisanti, Omar et al. 2012)

2.8 SAMPLE PROCESSING

RBP-J and *JAG1* methylation and LINE-1 (global DNA methylation) were quantified on the entire study population. All samples were processed in duplicate using independent PCR reactions. Each participant's duplicate PCR samples were analysed in the same pyrosequencing run to minimise batch effect. For intervention participants, before and after intervention samples were analysed in the same run. Duplicate data were deemed acceptable if there was agreement within 5% (absolute values). If there was not agreement, a third sample was run. The mean value of these data was used for analysis. If after 3 values there was a significant outlying value (>15% difference

from others values within 5%) this value was discarded from the analysis. Mean values were used for statistical analysis.

TABLE 2-3. SEQUENCES AND PCR CONDITIONS FOR VALIDATED PRIMERS.

Assay	Forward primer	Reverse primer	Sequencing primer	Annealing temperature (°C)	Reaction additives	Cycle adjustments
ATOH1	GTTTGGGAGTTTTTTGTATA	GTTTGGGAGTTTTTTGTATA	GTTTGGGAGTTTTTTGTATA	49	Q- sol.	None
JAG1	TTGGGTAGAGGTGGTTAG	CCCTTTTAAATCAACTACA	TTGGGTAGAGGTGGTTA	49	Q- sol.	None
LFNG	GGTTAAGTTTTTGTTTTGTATAAATAA	AAAAAAACCCAACCTAACCAAC	GAGGAAGTAGGATGTTTT	53	Nil	None
NOTCH1	ATGGGGGTTGTTTTGGAGATG	ATGGGGGTTGTTTTGGAGATG	GGGTTGTTTTGGAGAT	57	Nil	None
NOTCH3	TGTATTGGGGGATGGGGATG	CCTCCTCCCTCCTTCCCTAAACT	GGGTGGGTAGGGATT	56	Nil	None
RBP-J	GGTTTTTAGGGAAGGTAG	CCTTTTTCCTCACTCCTC	GAGTAGGATTTTTTATTTT	49	Q-sol	None

2.9 NON-LABORATORY DATA

2.9.1 FFQ AND LIFESTYLE QUESTIONNAIRE DATA

All questionnaire data were checked for completion before the end of the study. Missing data were checked with study participants. Data were entered into customised databases in Microsoft Access 2010. Thirteen percent of all data entries were checked for accuracy which was found to be >98%. Data were extracted from the FFQ database to provide estimates of consumption for dietary fibre (Englyst, Kingman et al. 1992), red meat, processed meat, alcohol, garlic and calcium. Calcium was used to estimate dairy consumption as technical issues did not allow extraction of dairy intakes from the database and calcium is a reasonable surrogate as cow's milk is the basis for most dairy consumed in the UK. (DEFRA 2010). Estimates of physical activity were extracted from the lifestyle questionnaire. Physical activity was coded depending on recreational and occupational activity levels. (Figure 2-5) (Wareham, Jakes et al. 2003)

2.9.2 BLOOD AND ANTHROPOMETRIC DATA

Blood results were received in Microsoft Excel spreadsheet format from the analyzing institution and were transferred directly into the custom built DISC study database (Xentec Ltd., Wallsend, UK). Paper based records of volunteer anthropometrics were entered directly into the DISC database. Techniques used by collaborators to obtain additional data used in the present analysis will not be described here.

FIGURE 2-5. CALCULATION OF PHYSICAL ACTIVITY CATEGORY.

Recreational activity	Occupational activity: Sedentary	Standing	Physical	Heavy manual
0 h/day	1	2	3	4
< 0.5 h/day	2	3	4	4
0.5-1 h/day	3	4	4	4
>1 h/day	4	4	4	4

FROM (WAREHAM, JAKES ET AL. 2003). 1- INACTIVE, 2 - MODERATELY INACTIVE, 3 – MODERATELY ACTIVE, 4 - ACTIVE

2.10 STATISTICAL ANALYSIS

Statistical analysis was performed in Minitab 16.0. $p<0.05$ was considered significant throughout unless otherwise stated.

2.10.1 NORMALITY OF DATA AND TRANSFORMATION

Several datasets within the study had significant positive skew. In these cases transformations were attempted. Natural log transformation[1] was found to be the best transformation though frequently did not normalise datasets to a degree to pass tests of normality. Reciprocal, arcsin, log10 and square root transformations were also tested. The General Linear Model (GLM) was used for subsequent analysis. GLM incorporates several statistical tests: primarily analysis of variance (ANOVA) and multiple linear regression. Normality of the data set is not an assumption for multiple linear regression (Miles and Shevlin 2001) and ANOVA is reasonably robust even in the presence of non-normally distributed data. (Box and Anderson 1955; Lindman 1974). Therefore data were analysed without transformation and significant results were

[1] Where all original values were > 1.0; $X_T = \log_e (X_R)$ where X_T = transformed value and X_R = original value. Where there were values <1.0; $X_T = \log_e(X_R+C)$ where C is the lowest value to ensure that $X_R+C=1$

confirmed with non-parametric equivalent tests when significant. Non-parametric testing could not be used throughout as GLM was required to obtain measures of treatment effect from the 2x2 factorial design.

2.10.2 ANALYSIS OF BASELINE VARIABLES

The GLM was used to investigate possible differences between the different risk of CRC groups (normal, polyps and UCs). Dunnett's test was used to identify the origin of significant variance comparing healthy controls with polyp and UC groups. Where categorical data (smoking status, sex etc.) were required within the analysis these were binary coded. Standard covariates used within all analyses were age, sex and smoking status. Chi-square tests were used to compare groups at baseline where these data were of a proportional nature.

2.10.3 IDENTIFICATION OF FACTORS PREDICTING METHYLATION OF THE SELECTED GENES

Hypothesis based stepwise linear regression with forward selection was used to model factors predicting methylation of the examined genes. Required factors in all models were age, sex, smoking status and waist circumference. Free predictors were hsCRP, plasma vitamin B_{12}, plasma folate, fibre consumption, red meat consumption, processed meat consumption, alcohol intake, calcium intake and garlic intake. α to enter was set at 0.25.

2.10.4 IDENTIFICATION OF FACTORS PREDICTING POLYP SEVERITY

Nominal logistic regression was used to identify factors able to predict severity of disease in the polyp group. Factors tested were anthropometric variables, systemic inflammation, dietary exposure data and methylation of the analysed genes. Modelling was attempted for each individual group with an α of 0.05 for the test for all slopes demonstrating a significantly predictive factor within the group.

2.10.5 IDENTIFICATION OF FACTORS FOR POTENTIAL DIAGNOSTIC TESTING

To identify whether measured factors within the study had the potential for use as a diagnostic test, factors were entered into binary logistic regression to define variation between polyps and normals. Those factors with the greatest variation were analysed using Receiver Operator Characteristic (ROC) curves – a method of varying cut-off for factors in a diagnostic test to optimise sensitivity and specificity. A contingency table of true positives, false positives, true negatives and false negatives was created allowing sensitivity and specificity for the selected factors to be calculated.

2.10.6 EFFECTS OF DIETARY INTERVENTIONS

The effect of each of the interventions (PD and Hi-maize 260) was tested by analysing outcome data (after intervention) using the GLM with the baseline values as covariates. This analysis examined both the main effects of each intervention agent and also potential interactions between the intervention agents. Least squares mean (LSM) for each intervention are presented.

Although there were some factors that differed between groups at baseline (age, plasma selenium, processed meat consumption (section 4.1.2)) these were not included as covariates during analysis. Dietary, demographic and anthropometric factors are only known to effect baseline values of methylation. Methylation at the end of the study was analysed for intervention effect with baseline methylation as a covariate. This accounts for variation in baseline values and analyses change (β value) from baseline. As there are no data to suggest that any baseline values measured moderate methylation *change* in response to dietary intervention, no other factors have been included as covariates for the analysis of effects of intervention.

CHAPTER 3. RESULTS - BASELINE

3.1 RECRUITMENT

In total, 92 participants (healthy controls) were recruited to the dietary intervention study. Of these, 75 participants completed the dietary intervention and provided samples at the end of the study. For all participants, >95% of biological samples, anthropometric data and dietary data were collected.

In addition, 26 participants with previous adenomatous polyps and 12 participants with UC were recruited.

3.1.1 CHARACTERISTICS OF THE PARTICIPANT GROUPS

There was a significant difference in the sex distribution between groups with a greater proportion of males in the polyp and UC groups (χ^2=9.825, p=0.007). There was a significant difference in the mean age of the groups (p=0.003) and Dunnett's test showed that the participants in the polyp group were, on average, older than the normal controls by approximately 9 years. No differences were detected in the anthropometric data between the normal, polyp and UC groups in terms of weight, BMI, hip circumference and waist circumference. (Table 3-1) There was a difference in thigh circumference (p=0.041) with both the UC and polyp groups having smaller thigh circumferences than the controls. (Table 3-1)

Baseline hsCRP differed significantly between groups (p=0.014). Post-hoc Dunnett's test showed that the UC group differed from the control group (p<0.05). This result was largely influenced by a single UC outlier with a CRP of 141 (presumably representing an intercurrent illness). After removing this outlier a significant difference remained; these data are presented in Table 3-2. Post-hoc Dunnett's test showed higher hs-CRP in the polyp group compared to normal (8.05 vs. 3.28mg/l). There was no difference in nutritional indices sampled in blood (vitamin B_{12}, folate and selenium). (Table 3-2)

No significant differences were noted in the consumption between groups of any of the dietary factors to be tested for effect on methylation of NOTCH pathway genes. (Table 3-3)

TABLE 3-1. DEMOGRAPHIC AND ANTHROPOMETRIC DATA AT BASELINE FOR INTERVENTION, UC AND POLYP GROUPS.

		Controls (n=91)	**Polyp (n=26)**	**UC (n=12)**	p value
Sex (M:F)	(M:F)	35:40	20:6	9:3	0.007
Age (years)	LSM (SEM)	52.6 (1.3)	61.8 (2.3)	56.4 (3.5)	0.003
Smoking status	(Current/ Ex/Never)	37:20:16	8:10:5	3:10:1	0.091
Weight (kg)	LSM (SEM)	83.0 (1.8)	81.6 (3.0)	80.9 (4.6)	0.862
Body mass index (kg/m^2)	LSM (SEM)	30.1 (0.6)	28.9 (1.0)	28.4 (1.6)	0.401
Hip circumference (cm)	LSM (SEM)	107.1 (1.3)	104.0 (2.2)	102.2 (3.3)	0.246
Waist circumference (cm)	LSM (SEM)	99.6 (1.5)	99.7 (2.5)	98.5 (3.8)	0.964
Thigh circumference (cm)	LSM (SEM)	60.0 (0.8)	56.6 (1.3)	56.4 (2.0)	0.041

TABLE 3-2. BLOOD MEASUREMENTS FOR INTERVENTION, POLYP AND UC GROUPS AT BASELINE.

	Controls (n=90)	**Polyp (n=26)**	**UC (n=12)**	
Assay	LSM (SEM)			p value
C-reactive protein (mg/l)	3.3 (0.8)	8.0 (1.4)	3.4 (2.2)	0.014
Serum B_{12} (ng/l)	425.6 (21.5)	439.0 (39.3)	477.9 (60.4)	0.702
Red cell folate (µg/l)	269.5 (13.8)	255.8 (25.3)	285.5 (38.9)	0.792
Plasma selenium (µmol/l)	1.20 (0.22)	1.30 (0.08)	1.27 (0.09)	0.264

TABLE 3-3. DIETARY INTAKES OF GROUPS ESTIMATED BY FOOD FREQUENCY QUESTIONNAIRE.

PORTIONS ARE SELF-REPORTED 'MEDIUM SIZED SERVINGS'

	Normal (n=84)	**Polyp (n=26)**	**UC (n=11)**	
Assay	LSM (SEM)			p value
Energy intake (kJ/day)	11408 (584)	12692 (1086)	10455 (1536)	0.290
Total fat intake (g/day)	100.5 (6.6)	111.1 (12.4)	92.3 (17.4)	0.636
Fibre intake (g/day) (Englyst, Kingman et al. 1992)	22.5 (10.3)	24.4 (10.4)	20.0 (8.3)	0.524
Red meat (portions/day)	0.48 (0.04)	0.59 (0.07)	0.36 (0.11)	0.290
Processed meat (portions/day)	0.60 (0.06)	0.74 (0.11)	0.62 (0.15)	0.567
Alcohol intake (g/day)	22.3 (3.6)	33.2 (6.4)	22.0 (9.1)	0.314
Calcium intake (mg/day)	1193 (51)	1260 (96)	1177 (136)	0.813
Garlic intake (portions/day)	0.27 (0.03)	0.15 (0.05)	0.22 (0.08)	0.221

3.2 RESULTS OF SCREENING OF CANDIDATE NOTCH GENES

3.2.1 CHARACTERISTICS OF STUDY PARTICIPANTS USED FOR SCREENING NOTCH GENES

Eight participants from each patient group were selected to test the panel of NOTCH gene promoter methylation assays (section 2.7.3). These data were used to inform selection of genes for evaluation in the whole study population based on variability of gene methylation between higher and normal risk of CRC groups.

The sample was age, sex and smoking status matched as far as possible between the polyp and normal groups. Best-matched UC participants were selected on this basis although the group size limited accurate matching. Characteristics of matched variables are reported in Table 3-4. The genes screened were as follows:

ATOH1, *JAG1*, *LFNG*, *NOTCH1*, *NOTCH3* and *RBP-J*.

TABLE 3-4. CHARACTERISTICS OF THE PARTICIPANTS FROM EACH PATIENT GROUP USED FOR ASSAY SCREENING.

		Normal (n=8)	**Polyp (n=8)**	**UC (n=8)**	p value
Sex (M:F)	(M:F)	3:5	3:5	6:2	0.829
Age (years)	Mean (SD)	65.4 (8.1)	65.1 (8.4)	56.5 (12.4)	0.879
Smoking status	(Current/ Ex/Never)	4:4:0	3:1:2 (2 missing)	3:4:1	Not calculated

3.2.2 *ATOH1* METHYLATION RESULTS

ATOH1 is negatively regulated by NOTCH signalling via *HES* and *HEY*. Upregulation of *HES* and *HEY* downregulates *ATOH1* and promotes differentiation of epithelial cells to absorptive lineages. The *ATOH1* assay examined 6 CpG sites in the promoter region. Data for one UC participant were not available due to wide variation in methylation recorded between four repeat pyrosequencer measurements. Low level methylation (range 2.3 – 7.1%) was observed at all 6 CpG sites and in all 3 patient groups with no significant differences between patient groups observed. (Table 3-5)

TABLE 3-5.SUMMARY OF *ATOH1* METHYLATION BY PARTICIPANT GROUP FOR THE SCREENING SAMPLE.

	Normal (n=8)	**Polyp (n=8)**	**UC (n=7)**	
CpG site	LSM (SEM)			p value
1	3.2 (0.6)	2.5 (0.5)	2.6 (0.6)	0.536
2	6.9 (0.9)	7.1 (0.7)	6.6 (0.9)	0.918
3	3.5 (0.7)	3.0 (0.6)	4.0 (0.7)	0.573
4	3.7 (0.7)	4.3 (0.6)	4.5 (0.7)	0.735
5	5.7 (0.9)	4.9 (0.7)	4.6 (0.9)	0.611
6	3.0 (0.6)	2.3 (0.5)	2.8 (0.6)	0.576

3.2.3 *JAG1* METHYLATION RESULTS

JAG1 expression is upregulated in human CRC (Reedijk, Odorcic et al. 2008; Peignon, Durand et al. 2011) and higher expression is linked to poor tumour differentiation and prognosis. (Jin, Zhang et al. 2012) The assay developed for *JAG1* examined 5 CpG sites. Most CpG sites showed methylation levels between 2 and 10%. Methylation of all CpGs tended to be lower in the polyp group and a significant difference was observed in methylation at site 4 with mean values of 4.4, 2.8 and 3.6% for normals, polyps and UC groups respectively. However, Dunnett's test did not show a difference between UC or polyp groups and controls (normals) ($p>0.05$). (Table 3-6)

TABLE 3-6. SUMMARY OF *JAG1* METHYLATION BY PARTICIPANT GROUP FOR THE SCREENING SAMPLE.

	Normal (n=8)	**Polyp (n=8)**	**UC (n=8)**	
CpG site	LSM (SEM)			p value
1	4.9 (1.0)	3.9 (1.0)	4.6 (1.0)	0.561
2	4.3 (1.1)	2.8 (1.1)	4.0 (1.1)	0.440
3	4.2 (0.7)	3.6 (0.7)	4.4 (0.7)	0.322
4	4.4 (0.6)	2.8 (0.6)	3.6 (0.6)	0.027
5	3.9 (0.8)	2.9 (0.8)	3.0 (0.8)	0.399

3.2.4 *LFNG* METHYLATION RESULTS

LFNG affects NOTCH signalling by altering the specificity of NOTCH receptor and sending cell ligand interaction. *LFNG* expression is upregulated in most human CRCs. (Reedijk, Odorcic et al. 2008). Levels of methylation of this gene were low at all 10 CpG sites measured (all values <5.4%). Where statistical significance was achieved or approached by parametric testing (sites 4, 6 and 7) the distributions were not normal. These data were unsuitable for transformation and Kruskal-Wallis testing showed differences between patient groups which approached significance at site 4 only. (site 4 p=0.061, site 6 p=0.172, site 7 p=0.101) (Table 3-7)

TABLE 3-7.SUMMARY OF *LFNG* METHYLATION BY PARTICIPANT GROUP FOR THE SCREENING SAMPLE.

	Normal (n=8)	**Polyp (n=8)**	**UC (n=8)**	P value
CpG site	LSM (SEM)			
1	1.0 (0.4)	1.0 (0.4)	1.4 (0.4)	0.772
2	2.6 (0.6)	2.8 (0.6)	2.2 (0.6)	0.748
3	2.0 (0.5)	1.2 (0.5)	0.9 (0.5)	0.347
4	0.0 (0.5)	0.8 (0.5)	1.6 (0.5)	0.085
5	5.2 (0.6)	5.3 (0.6)	5.4 (0.6)	0.971
6	4.3 (0.8)	5.7 (0.8)	6.9 (0.8)	0.082
7	0.7 (0.5)	0.6 (0.5)	2.4 (0.5)	0.034
8	2.0 (1.2)	2.0 (1.2)	4.7 (1.2)	0.222
9	1.8 (0.7)	1.6 (0.7)	3.2 (0.7)	0.269
10	0.4 (0.5)	1.0 (0.5)	0.3 (0.5)	0.571

3.2.5 *NOTCH1* METHYLATION RESULTS

NOTCH1 is over expressed in CRC and high expression predicts poor prognosis (Chu, Zhang et al. 2011; Jin, Zhang et al. 2012). *NOTCH1* mutation has not been detected in human CRC. (Lee, Jeong et al. 2007) Data from one UC patient was not available due to wide variation between four repeat pyrosequencer assays resulting in this sample being rejected from the analysis. Methylation values were less than 5.5% at all sites with no differences detected between patient groups. (Table 3-8)

TABLE 3-8. SUMMARY OF *NOTCH1* METHYLATION BY PARTICIPANT GROUP IN THE SCREENING SAMPLE.

	Normal (n=8)	**Polyp (n=8)**	**UC (n=7)**	P value
CpG site	LSM (SEM)			
1	1.3 (0.8)	1.2 (0.9)	2.7 (0.9)	0.321
2	1.1 (0.6)	1.0 (0.7)	1.9 (0.6)	0.549
3	2.7 (0.5)	2.6 (0.5)	3.3 (0.5)	0.453
4	0.7 (0.4)	0.4 (0.4)	1.2 (0.4)	0.339
5	4.4 (0.5)	3.1 (0.6)	4.3 (0.5)	0.613
6	1.3 (0.3)	0.8 (0.3)	1.1 (0.3)	0.648
7	1.9 (0.5)	0.8 (0.5)	1.0 (0.5)	0.215
8	1.9 (0.3)	1.8 (0.3)	2.0 (0.3)	0.667
9	1.5 (0.4)	0.7 (0.4)	0.8 (0.4)	0.459
10	5.5 (1.0)	4.1 (1.0)	4.5 (1.0)	0.592

3.2.6 *NOTCH3* METHYLATION RESULTS

NOTCH3 is significantly upregulated in the majority of human CRCs. Downregulation of NOTCH3 in tumour xenografts decreases tumour growth. (Serafin, Persano et al. 2011) Mutational analysis has only identified a *NOTCH3* mutation in 2% of CRCs. (Lee, Jeong et al. 2007). The *NOTCH3* assay examines 9 CpG sites. Data for one UC participant were not included due to wide variation in assay values after four repeat measurements.

There was significant variation in this dataset at CpG site 9 and variation approaching statistical significance at site 5. (Table 3-9) Data were not normal and not suitable for transformation. Similar levels of significance were found using Kruskal-Wallis (site 5 – p=0.083, site 9 – p=0.008). Post-hoc Dunnett's test showed significant differences between the UC and normal group at site 5 and between the polyp and normal group at site 9.

TABLE 3-9. SUMMARY OF *NOTCH3* METHYLATION BY PARTICIPANT GROUP IN SCREENING SAMPLE.

	Normal (n=8)	**Polyp (n=8)**	**UC (n=7)**	P value
CpG site	LSM (SEM)			
1	3.7 (0.8)	3.1 (0.8)	4.9 (0.8)	0.323
2	11.5 (1.2)	9.4 (1.3)	11.9 (1.3)	0.359
3	4.8 (0.8)	3.2 (0.9)	3.6 (0.9)	0.442
4	5.3 (1.3)	5.7 (1.3)	7.1 (1.3)	0.644
5	2.0 (0.7)	3.4 (0.8)	4.7 (0.8)	0.063
6	5.1 (1.0)	5.6 (1.0)	5.6 (1.0)	0.888
7	7.9 (1.3)	5.5 (1.4)	7.4 (1.4)	0.444
8	7.8 (1.4)	7.4 (1.5)	9.2 (1.5)	0.690
9	2.5 (0.6)	5.0 (0.6)	4.2 (0.6)	0.013

3.2.7 *RBP-J* METHYLATION RESULTS

The assay developed for *RBP-J* examined 12 CpG sites. RBP-J is the intranuclear transcription factor for the NOTCH pathway and may either facilitate or repress NOTCH gene transcription depending upon the co-transcription factor recruited. Co-transcription factor recruitment is defined by the presence or otherwise of NICD within the nucleus. Knock-down of *RBP-J* induces near complete conversion of undifferentiated crypt cells to goblet phenotype both in normal epithelium and adenomas. (van Es, van Gijn et al. 2005)

Most sites, except site 11, were minimally methylated (values <6.9%). However significant differences between patient groups are observed for CpGs 1, 4 and 9. These data were not normally distributed and significant differences using parametric testing were replicated with Kruskal-Wallis testing ($p<0.05$). Post-hoc Dunnett's test did not demonstrate significant differences of the polyp or UC group from the normal group ($p>0.05$). There was an observed trend towards higher methylation in the UC group which was not statistically significant in the screening sample. (Table 3-10)

TABLE 3-10. SUMMARY OF *RBP-J* METHYLATION BY PARTICIPANT GROUP IN SCREENING SAMPLE.

	Normal (n=8)	**Polyp (n=8)**	**UC (n=8)**	
CpG site	LSM (SEM)			p value
1	1.0 (0.5)	0.5 (0.5)	1.6 (0.5)	0.049
2	0.4 (0.9)	0.7 (0.9)	2.2 (0.9)	0.309
3	1.0 (1.2)	0.9 (1.2)	3.0 (1.2)	0.718
4	0.8 (1.4)	0.4 (1.4)	3.3 (1.4)	0.047
5	1.1 (1.5)	1.4 (1.5)	3.5 (1.5)	0.656
6	2.2 (2.3)	2.4 (2.3)	6.9 (2.3)	0.143
7	0.9 (1.5)	0.8 (1.5)	3.2 (1.5)	0.310
8	0.7 (1.2)	0.7 (1.2)	2.7 (1.2)	0.554
9	1.4 (1.7)	0.5 (1.7)	2.7 (1.7)	0.021
10	0.7 (1.6)	0.7 (1.6)	3.7 (1.6)	0.127
11	17.5 (2.3)	20.3 (2.3)	24.1 (2.3)	0.112
12	2.3 (1.6)	2.9 (1.6)	4.9 (1.6)	0.501

3.2.8 GENE SELECTION FOR MAIN STUDY

The screening results suggested that there are unlikely to be significant between patient group differences for the assays developed for *NOTCH1, LFNG* and *ATOH1*. The final gene selection to be run on all study samples was:

1. *RBP-J*
2. *JAG1*
3. *NOTCH3*

In addition to these genes a LINE-1 assay was run on all samples to estimate global gene methylation between groups and in response to the dietary intervention.

Complete data on *NOTCH3* was not able to be collected within the time period of the study. Initial runs on the complete study set revealed high variation in methylation values for duplicate samples. This variability meant that high numbers of repeat measurements were required to define final methylation values for statistical analysis for each sample (section 2.8). High variability within duplicate samples for the *NOTCH3* assay also raises doubts as to the test, re-test validity of this assay.

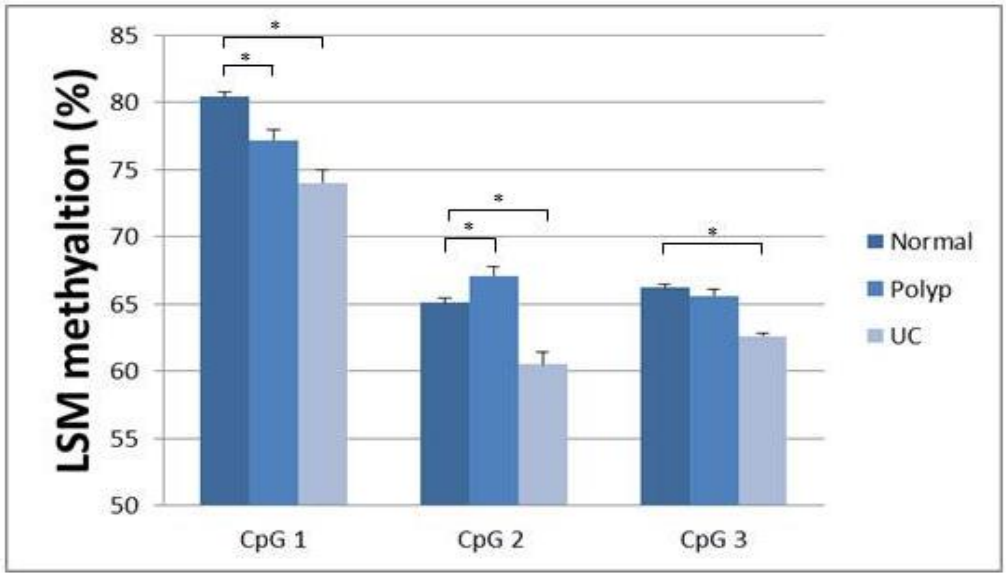
3.3 METHYLATION OF NOTCH GENES AND GLOBAL METHYLATION IN POLYP, UC AND NORMAL PARTICIPANTS

3.3.1 LINE-1 (GLOBAL) METHYLATION

Data for LINE-1 were normally distributed. Results are presented as least squares mean (LSM) with standard error of the mean (SEM). Smoking status, sex, age and adiposity (waist circumference) were included as covariates for all analyses.

Global methylation as measured by LINE-1 is shown in Figure 3-1 (and appendix 5). Methylation values at all three CpG sites within the assay were lower for UC participants than polyp and normal controls ($p<0.05$). Methylation of polyp patients was lower at CpG site 1 compared to normal controls (77.2 vs. 80.4%, $p<0.05$), higher at CpG site 2 (67.1 vs. 65.1%, $p<0.05$) and no significant difference was detected at site 3 (65.6 vs. 66.2%, $p>0.05$)

FIGURE 3-1. LINE-1 METHYLATION VALUES BY GROUP (NORMALS, POLYPS AND UC) FOR THE WHOLE STUDY SAMPLE.



PERCENTAGE METHYLATION LEVELS AT CPGS 1,2 &3 OF THE LINE-1 PYROSEQUENCING ASSAY BETWEEN THOSE AT NORMAL RISK (N=91) OF CRC AND THOSE AT HIGHER RISK (POLYP (N=26) AND UC (N=12) GROUPS). *=P<0.05 USING GLM AND DUNNETT'S POST-HOC TEST FOR BETWEEN GROUP DIFFERENCES.

3.3.2 PREDICTION OF LINE-1 METHYLATION

Forward stepwise regression was undertaken using all normal risk participant samples with LINE-1 data (n=72). Required predictors were age, sex, waist circumference and smoking status. Free predictors were dietary factors (section 2.10.3) hsCRP, plasma B_{12} and plasma folate. The final model selected included seven factors:

Mean LINE-1 methylation = 70.5 + 1.11 smoking code + 0.07 age (years) - 0.52 sex code - 0.02 waist circumference (cm) - 0.05 fibre intake (g/day) - 0.002 serum B_{12} (ng/l) - 0.08 pre CRP (mg/l) (R^2=30.2%, R^2(adj)=22.6, PRESS=323.4)

Where smoking code value was 0 for never smokers, 1 for ex-smokers, 2 for current smokers and sex code value was 0 for female and 1 for male.

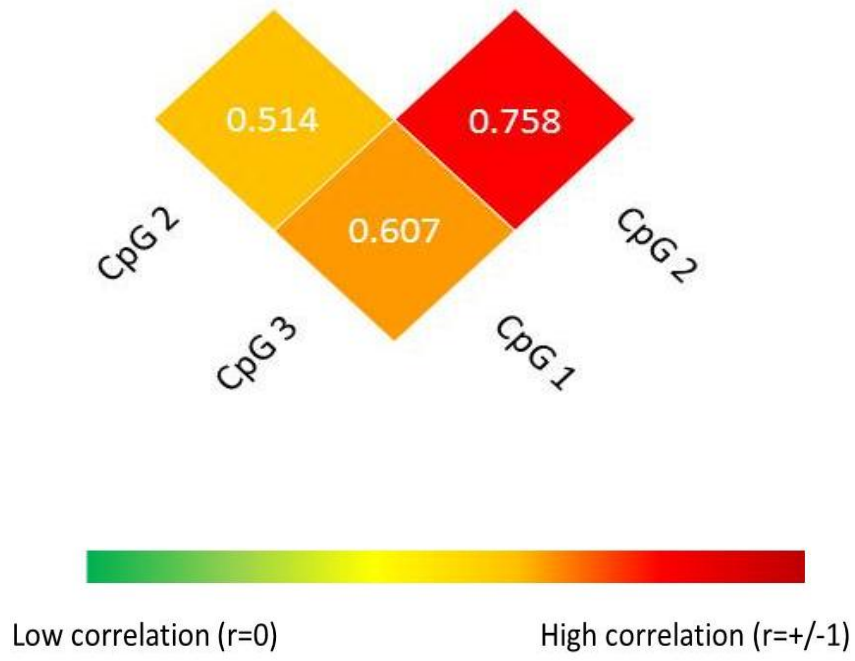
R^2 (coefficient of multiple determination) indicates the proportion of variation that is explained by the factors within the model. Increasing the number of factors within the multiple linear regression model necessarily increases the fit and hence the R^2 value increases. Adjusted R^2 values adjust for the number of variables and will decrease with the addition factors which have a poor fit for the data set. PRediction Error Sum of Squares (PRESS) estimates the ability of the model to predict future results. PRESS residuals are calculated for each data point by removing that data point, recalculating the regression equation and determining the new equation residual for that data point. The PRESS statistic compares all the residuals for the regression equation to the PRESS residuals. Higher PRESS values indicate better predictive power. (Miles and Shevlin 2001)

3.3.2.1 CORRELATION OF LINE-1 METHYLATION BETWEEN CPGS

Good correlations were observed between all three CpG sites on the LINE-1 assay using Pearson's product moment correlation coefficient. High correlation (r>0.6) was observed between sites 1 and 3 and sites 2 and 3. (Figure 3-2)

FIGURE 3-2. PEARSON'S CORRELATION (R) OF VALUES AT CPG SITES WITHIN THE LINE-1 ASSAY.

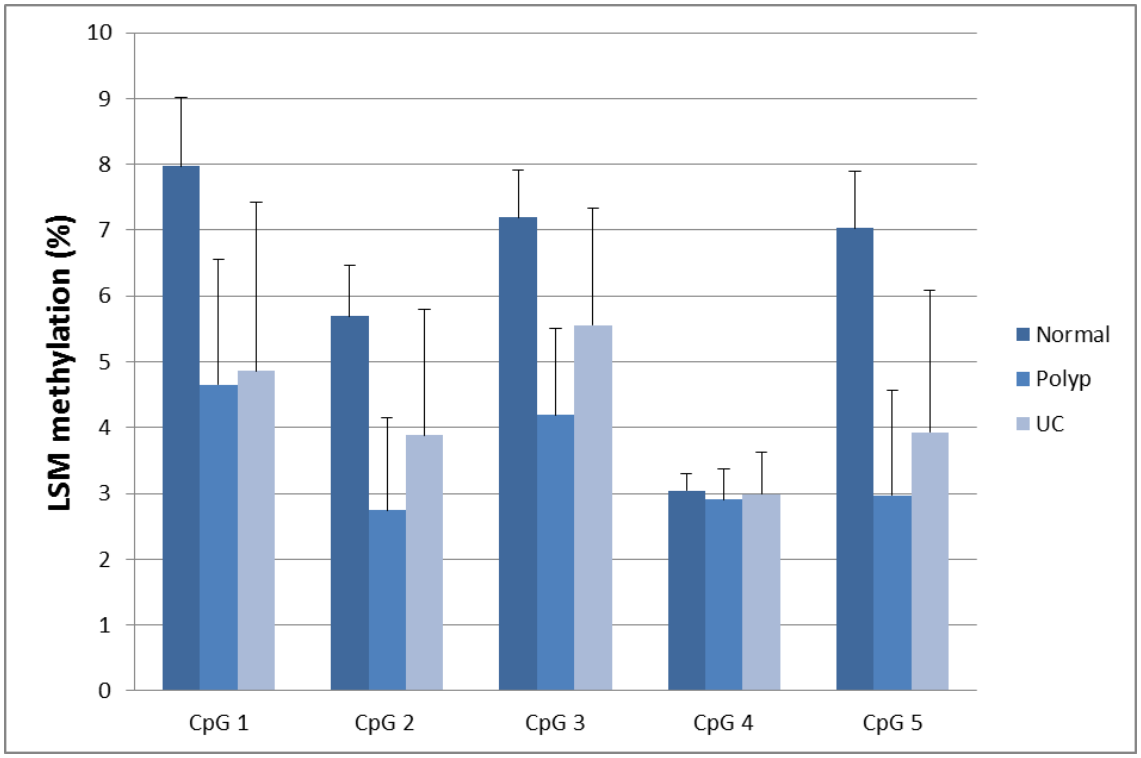
(ALL P VALUES<0.001)



3.3.3 *JAG1* METHYLATION

Data for *JAG1* are presented in Appendix 6 and Figure 3-3. Smoking status, sex, age and adiposity (waist circumference) were included as covariates for all analyses. A wider range of methylation values was observed compared to the screening values obtained earlier (whole population range 2.9-8.0%, screening sample range 2.8-4.9%). These higher values occurred in the normal group. Whilst no significant difference was demonstrated between the three groups there was a trend towards lower methylation in the higher risk of CRC groups (polyp and UC) with the lowest methylation at all 5 CpG sites observed in the polyp group.

FIGURE 3-3. *JAG1* METHYLATION VALUES BY GROUP (NORMALS, POLYPS AND UC) FOR WHOLE STUDY SAMPLE.



PERCENTAGE METHYLATION LEVELS AT CPGS 1-5 OF THE *JAG1* PYROSEQUENCING ASSAY BETWEEN THOSE AT NORMAL RISK (N=75) OF CRC AND THOSE AT HIGHER RISK (POLYP (N=26) AND UC (N=12) GROUPS). *=P<0.05 USING GLM AND DUNNETT'S POST-HOC TEST FOR BETWEEN GROUP DIFFERENCES.

3.3.4 PREDICTION OF *JAG1* METHYLATION

Forward stepwise regression was undertaken using all normal risk participant samples with mean *JAG1* methylation (n=72). Required predictors were age, sex, waist circumference and smoking status. Free predictors were dietary factors (section 2.10.3), hsCRP, plasma B_{12} and plasma folate. The final model included six factors:

Mean *JAG1* methylation (%) = 7.3+ 1.7 smoking code + 0.007 age (years) + 2.1 sex code - 0.01 waist circumference (cm) + 4.4 garlic consumption (portions/day) - 0.003 calcium intake (mg/day)

(R^2=12.84%, R^2(adj)=5.61, PRESS=3489.7)

Where smoking code value was 0 for never smokers, 1 for ex-smokers and 2 for current smokers and sex code value was 0 for female and 1 for male.

Although age, waist circumference and sex were variables included in the model to account for possible confounding effects their predictive value within the model was poor with p values of 0.913, 0.849 and 0.179 respectively. Overall the model explained a relatively small proportion of the mean methylation status of *JAG1* with low R^2 value. Removing age, waist circumference and sex did not significantly improve the predictive value of the model (R^2=12.95%, R^2(adj)=8.12, PRESS=3298.1)

As this model did not explain much of the variation within mean *JAG1* methylation, modelling was attempted using *JAG1* CpG 5 as it had shown the greatest degree of variation in those at normal and higher risk of CRC. Forward stepwise regression selected the same predictors to include within the model:

JAG1 CpG 5 methylation (%) = 7.9 + 2.6 smoking code + 3.0 sex code + 0.001 age (years) - 0.009 waist circumference (cm) + 5.9 garlic consumption (portions/day) - 0.004 calcium intake (mg/day)

(R^2=15.6%, R^2(adj)=7.62, PRESS=5244.9)

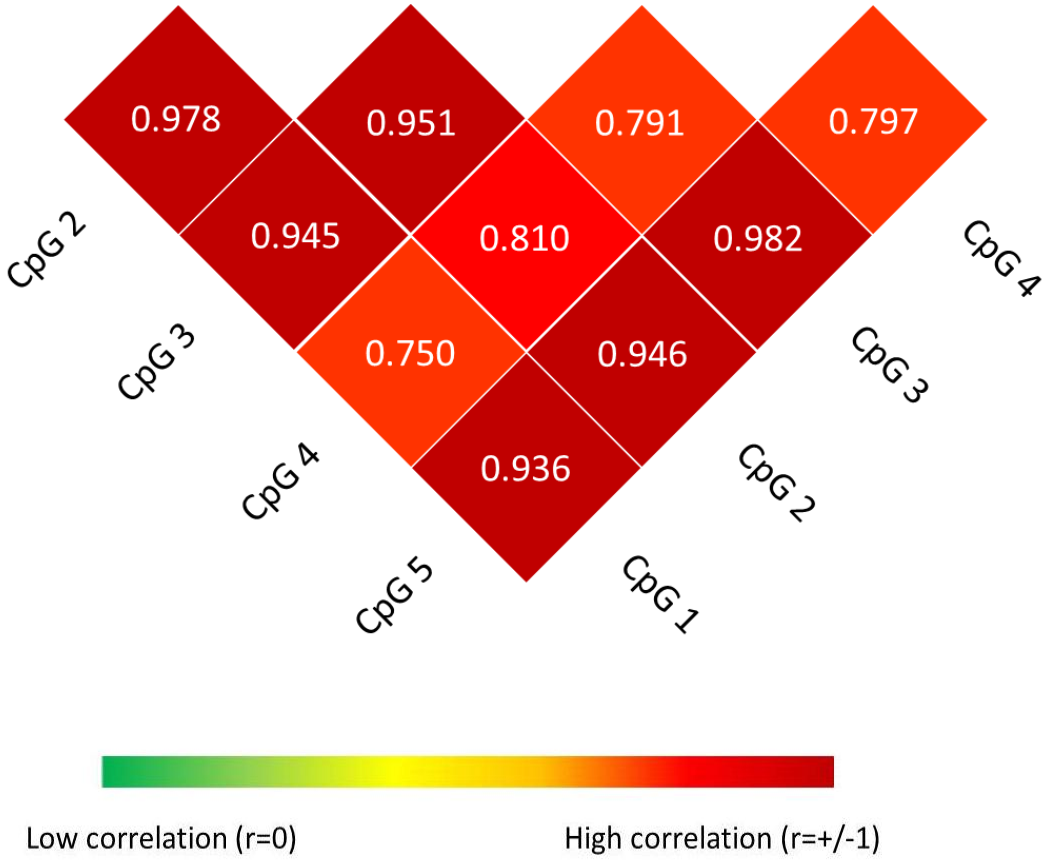
Within this second model the effect sizes and directions of effects were similar. Also there was a low R^2 value with a very high PRESS, suggesting an equation that could not explain much of the variance of a value but could predict methylation extremely accurately. This suggested a model demonstrating statistical instability and review of the residual distribution of both equations revealed non-normal distributions which could not be normalised with removal of outliers. Therefore the results of these *JAG1* analyses must be treated with caution as they may be subject to statistical error.

3.3.4.1 Correlation of JAG1 methylation between CpGs

The heterogeneity between models was likely to be due to very high levels of correlation between the CpG sites of the *JAG1* as all values for Pearson's correlation were >0.75 (Figure 3-4).

FIGURE 3-4. PEARSON'S CORRELATION (R) OF VALUES AT CPG SITES WITHIN THE *JAG1* ASSAY.

(ALL P VALUES<0.001)



3.3.5 *RBP-J* METHYLATION

There was minimal methylation (<6.3%) at all CpG sites in all groups except at CpG site 11. CpG site 11 showed higher methylation with individual participant's values ranging from 6.8-49.0%. The data for *RBP-J* promoter methylation at CpG site 11 were normally distributed.

GLM testing showed a significant difference between the three groups at this site ($p=0.006$). Post-hoc Dunnett's test showed lower methylation in the polyp group compared to control (normal) (18.0 vs. 23.6%, $p<0.05$). Mean methylation in the UC group was also higher than the normal group (22.7%) but this value did not differ significantly from the control group. (Table 3-11, Appendix 7)

TABLE 3-11. *RBP-J* METHYLATION VALUES BY GROUP (NORMALS, POLYPS AND UC) FOR WHOLE STUDY SAMPLE.

CpG site	Normal (n=86)	Polyp (n=25)	UC (n=12)	P-value
	LSM (SEM)			
1	1.2 (0.2)	0.9 (0.3)	1.4 (0.4)	0.525
2	1.9 (0.3)	1.4 (0.5)	2.4 (0.7)	0.629
3	2.2 (0.4)	2.0 (0.7)	3.0 (0.9)	0.684
4	2.1 (0.4)	1.8 (0.8)	3.2 (1.1)	0.565
5	2.2 (0.5)	1.8 (0.8)	3.1 (1.2)	0.647
6	5.2 (0.7)	5.0 (1.3)	6.3 (1.7)	0.811
7	2.6 (0.5)	1.8 (0.8)	3.1 (1.2)	0.652
8	2.3 (0.4)	1.7 (0.7)	2.8 (1.0)	0.630
9	2.9 (0.5)	1.9 (1.0)	3.8 (1.3)	0.461
10	2.5 (0.5)	1.8 (0.9)	3.5 (1.3)	0.505
11	23.6 (0.8)	18.0 (1.5)	22.7 (2.0)	0.006
12	4.0 (0.5)	4.8 (1.2)	4.8 (1.2)	0.841

3.3.6 PREDICTION OF *RBP-J* METHYLATION

Forward stepwise regression was undertaken using all baseline samples with mean *RBP-J* methylation (n=71). Required predictors were age, sex, waist circumference and smoking status. Free predictors were dietary factors (section 2.10.3), hsCRP, plasma B_{12} and plasma folate. The final model included six free factors:

RBP-J mean methylation (%) = 8.3 - 0.3 smoking code + 3.0 sex code - 0.04 age (years) - 0.04 waist circumference (cm) + 3.5 garlic consumption (portions/day) - 0.001 calcium intake (mg/day) - 0.6 processed meat intake (portions/day) + 0.2 pre CRP (mg/l) + 0.003 serum B_{12} (ng/l) - 0.01 alcohol intake (units/day)

(R^2=25.5%, R^2(adj)=13.1, PRESS=632.2)

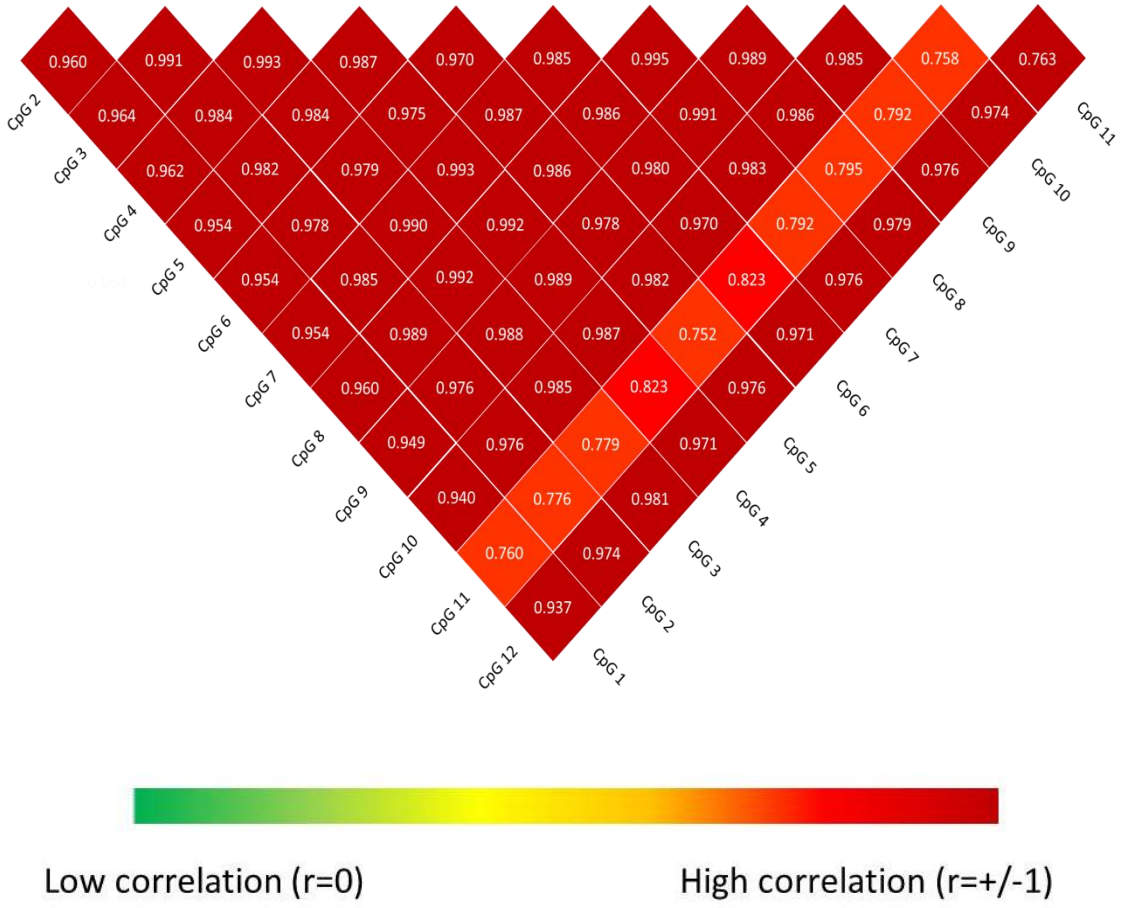
Where smoking code value was 0 for never smokers, 1 for ex-smokers, 2 for current smokers and sex code value was 0 for female and 1 for male.

3.3.6.1 CORRELATION OF *RBP-J* METHYLATION BETWEEN CPGS

High correlation (r>0.6) of methylation between was observed for all combinations of CpGs on the *RBP-J* assay. Almost all showed very high correlation, r>0.9. The exception to this was correlations with CpG site 11. At CpG 11 correlations with other sites were still high (all r>0.75) but were noticeably lower than for other CpGs in the correlation matrix. (Figure 3-5)

FIGURE 3-5. PEARSON'S CORRELATION (R) VALUES AT CPG SITES WITHIN THE *RBP-J* ASSAY.

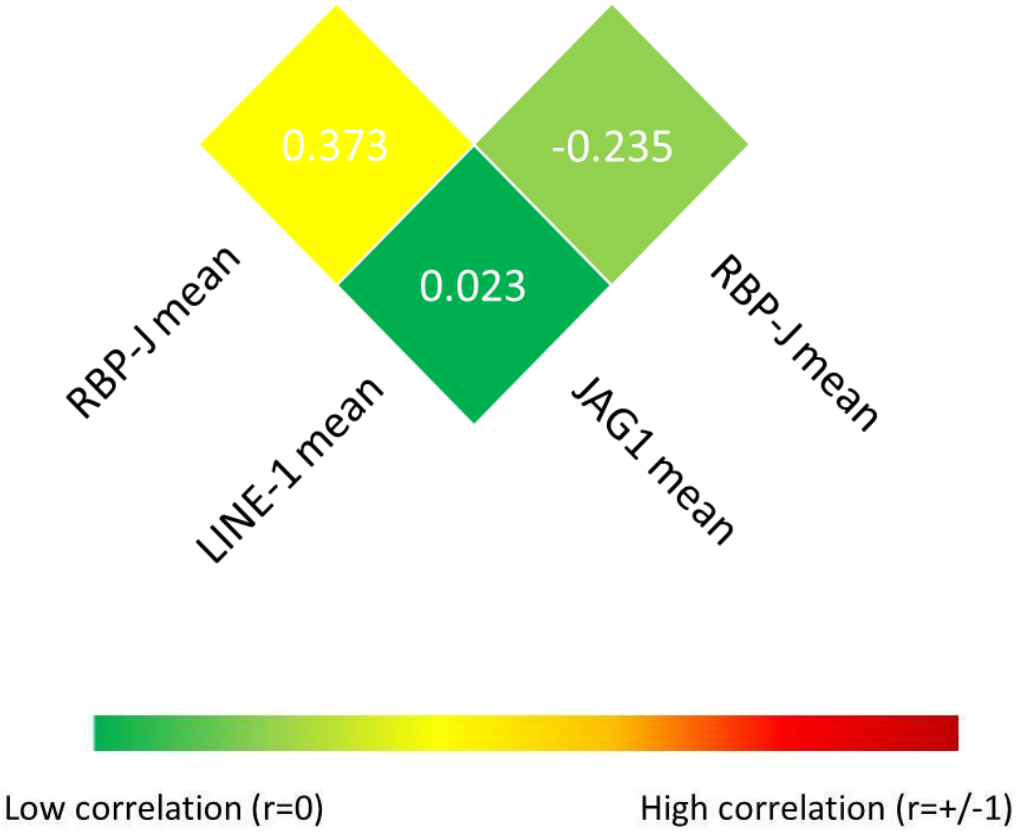
(ALL P VALUES <0.001)



3.3.7 CORRELATION BETWEEN LINE-1, *RBP-J* AND *JAG1* METHYLATION

To investigate possible relationships between the gene specific and global methylation, mean global methylation (LINE-1) was correlated with mean *RBP-J* and mean *JAG1* methylation. The three mean methylation values were not significantly correlated with one another (r<0.4). (Figure 3-6)

FIGURE 3-6. CORRELATIONS BETWEEN MEAN METHYLATION OF MEASURED GENES (LINE-1, *RBP-J* & *JAG1*)

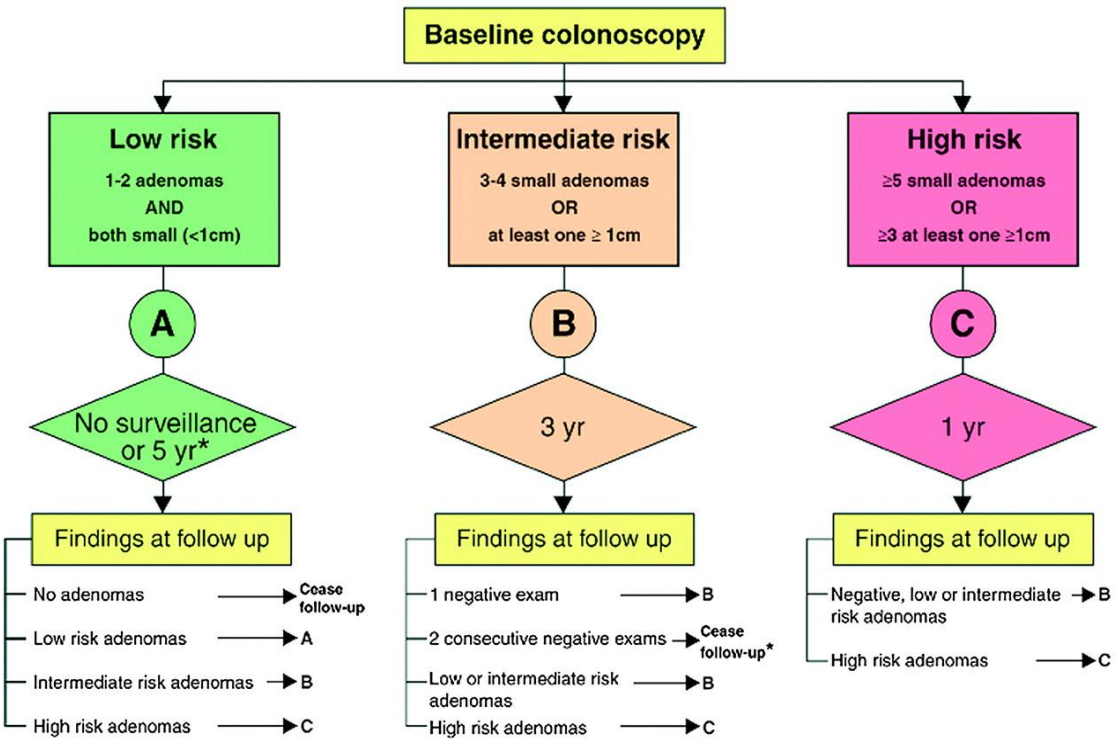


3.4 ANALYSIS OF THE POLYP GROUP

Within the polyp group only, ordinal logistical regression was undertaken to investigate predictors of future risk of CRC. (section 2.10.4) Future risk was defined by previous polyp characteristics from histopathology as per the British Society of Gastroenterology guidelines for colonoscopic surveillance after polypectomy. (Atkin and Saunders 2002) Entry into each risk category was defined by a combination of the size and polyp number (Figure 3-7). There were 11 patients deemed at low risk of CRC (Group A in Figure 3-7 below), 11 at moderate risk (Group B in Figure 3-7 below) and 4 at high risk (Group C in Figure 3-7 below).

FIGURE 3-7. DETERMINATION OF POLYP RISK STRATIFICATION.

(REPRODUCED WITH PERMISSION, ATKIN & SAUNDERS 2002)



3.4.1 INFLUENCE OF ENVIRONMENTAL EXPOSURES

Potential factors that might be associated with polyp severity were analysed in linked groups due to the small number of cases (n=26) limiting the capability of detecting significant factors when all factors were tested at once. The 'test for all slopes equal zero' defines whether there is one or more factors tested within a group of factors with a significant predictive value for the methylation outcome of interest at $p<0.05$.

Within this dataset, age, sex, smoking status, height and waist circumference were non-significant predictors of CRC risk in polyp patients (test for all slopes, p=0.156) Similarly, no association was found between hsCRP as an estimate of systemic inflammation and risk of CRC (test for all slopes, p=0.27) When red meat, processed meat, garlic, calcium, total fibre, alcohol, total energy and fat consumption were tested against CRC risk no significant associations were found (test for all slopes, p=0.068).

3.4.2 INFLUENCE OF GLOBAL AND GENE SPECIFIC METHYLATION

Neither LINE-1 methylation for any of the three CpG sites measured separately (test for all slopes, p=0.104) nor mean LINE-1 methylation (test for all slopes, p=0.197) predicted entry into the CRC risk groups. Individual *JAG1* CpG methylation values (test for all slopes, p=0.817) and mean *JAG1* methylation (test for all slopes, p=0.427) also did not predict entry into CRC risk groups.

Ordinal logistic regression showed that *RBP-J* methylation could predict entry into the risk groups for CRC development (test for all slopes, p=0.04). Within this model, the CpGs with low level methylation (CpGs 1-5, 7-10 and 12) generated instability with extremely wide 95% CIs for odds ratios (OR). The most extreme example of this being CpG 2; OR 5250 95% CI 0.43-64 624 869. The only CpG site within the model with an individual significant p value for prediction of severity was site 11 (OR 0.56, 95%CI-0.34-0.95, p=0.03). The Pearson test for goodness-of-fit test for the whole model (including unstable factors) was highly significant ($p<0.001$) indicating the whole model did not accurately predict entry into groups.

Removing the statistically unstable values left a model which included CpG sites 6 and 11 only. This model predicted entry into the CRC risk categories (test for all slopes, p=0.05, CpG site 6 OR 1.23 (95% CI 1.01-1.51) p=0.044, CpG site 11 OR 0.85 (95% CI 0.73-1.00) p=0.045)). The coefficients of effect were 0.21 for CpG site 6 and -0.15 for CpG site 11 suggesting that higher methylation of site 6 was associated with higher risk category for CRC in polyp patients with the opposite effect for site 11. Mean *RBP-J* methylation did not predict entry into CRC risk categories (test for all slopes, p=0.454).

3.5 PREDICTION OF DIAGNOSIS OF ADENOMATOUS POLYPS

To investigate whether the methylation values obtained in rectal mucosal samples could accurately predict the presence or otherwise of adenomatous polyps elsewhere in the colon binary logistic regression was undertaken using the polyp and normal populations. Potential predictive methylation factors were selected based on the variation detected between the polyp and normal groups; large variations in methylation between groups at a CpG site meant that site was more likely to be able to distinguish between groups. Therefore the parameters selected were: age, *JAG1* CpG 5 methylation, LINE-1 CpG 1 methylation and *RBP-J* CpG 11 methylation.

Analysis of all parameters revealed a significant relationship (test for all slopes, $p<0.001$. Age ($p=0.003$), LINE-1 ($p=0.016$) and *RBP-J* ($p=0.009$) showed strong predictive relationships whilst *JAG1* did not ($p=0.655$). *JAG1* was dropped from the final model.

The final model therefore included:

Age -	OR 1.08 95% CI 1.03-1.13, coefficient 0.08, p=0.002
LINE-1 CpG 1 methylation -	OR 0.80 95% CI 0.68-0.94, coefficient -0.21, p=0.007
RBP-J CpG 11 methylation -	OR 0.88 95% CI 0.80-0.97, coefficient -0.12, p=0.002

This suggests that older age, lower LINE-1 CpG 1 methylation and lower *RBP-J* CpG 11 methylation would predict a diagnosis of an adenomatous polyp. LINE-1 showed the strongest effect size (coefficient) followed by *RBP-J* CpG 11 methylation and age.

ROC curve analysis revealed an area under the curve of 0.82 suggesting a good clinical test however the poor sensitivity (38.5%) would mean this was not suitable for clinical practice. (Table 3-12)

TABLE 3-12. SUMMARY OF PROSPECTIVE 'DIAGNOSTIC ABILITY' OF THE LOGISTIC REGRESSION MODEL BASED ON ROC ANALYSIS.

	Test positive	Test negative	Total
Polyp	10	16	26
Normal	6	85	91
Total	16	101	117

Sensitivity: true positive/ (true positive + false negative) x100

10/26 x100 = 38.5%

Specificity: true negative/ (true negative + false positive)

85/91 x100 = 93.4%

3.6 DISCUSSION OF BASELINE RESULTS

3.6.1 LIMITATIONS, BIAS AND ROLE OF CHANCE

3.6.1.1 SELECTION OF GENES TO BE INVESTIGATED

Throughout the project there has been a significant attrition of assays. This attrition was initially due genetic sequences that were incompatible with primer design for pyrosequencing usually due to very high density of CpG sites and inability to validate assays for reasons such as primer dimer generation on testing of the assay. (Table 2-2) Subsequently validated assays were selected based on pragmatic criteria to identify genes likely to be differentially methylated in those at normal and higher risk of CRC. Criteria applied at this stage were:

- Biological – literature suggesting a role for the gene in carcinogenesis
- Assay based – assay reliability, i.e. need for minimal repetition of samples to achieve acceptable tolerances
- Likely to display variable methylation between groups at different risk of CRC – based on the gene screening process

These criteria have limitations. Biological data available is incomplete, especially in a pathway such as NOTCH which is less well characterised than others such as WNT in CRC carcinogenesis. Data are more widely available on the role of NOTCH signalling in breast cancer (Harrison, Farnie et al. 2010; Reedijk 2012). Although there are many similarities between these two epithelial cancers, applicability of data from one cancer type to the other is not guaranteed. In this study *JAG1* was the only gene selected for analysis in the whole study population known to have altered expression on CRC. (Jin, Zhang et al. 2012)

Assay based criteria were purely pragmatic. Assay validity was demonstrated individually by comparison of pyrosequencer results with control DNA of known methylation and production of standard curves. Test-retest validity was demonstrated between different bisulfite modifications, different PCR reactions and different

pyrosequencer runs prior to commencing actual study measurements. (section 2.7.2) Despite acceptable validation parameters some assays, most notably *NOTCH3,* were found to be unstable at the screening stage meaning that a high proportion of the wells in a single 96-well plate on each pyrosequencer run failed and variation between repeat samples was higher than acceptable tolerances between matched samples. *NOTCH3* analysis was attempted on the full study population but initial pyrosequencer runs showed very few samples pairs with agreement within 5%, so *NOTCH3* was given lower priority for complete evaluation.

Finally the likelihood of variable methylation between groups was considered based on results from a small 'screening' sample from each group. To avoid confounding effects, these samples of participants were matched as far as possible for age, sex and smoking status. However, the small sample size increased the likelihood of type II errors. In this case a type II error would result rejection of a gene from full evaluation due to a non-significant value, when evaluation in the full study population would have demonstrated a significant difference. Therefore for the purposes of deciding genes to be included the level of significance was raised to $p<0.1$ and selection of genes was undertaken based on statistical differences in conjunction with biological and assay based criteria discussed above.

3.6.1.2 Measurement and analysis limitations

Many of the CpGs within the current study had mean methylation values <5%. Due to the hard lower end point of these datasets (0%) positive skew was common, increasing the chance of spurious significant statistical tests. The use of non-parametric tests throughout was considered and rejected as parametric tests were required to take proper account of covariates as baseline comparability of the groups was not perfect (Table 3-1).

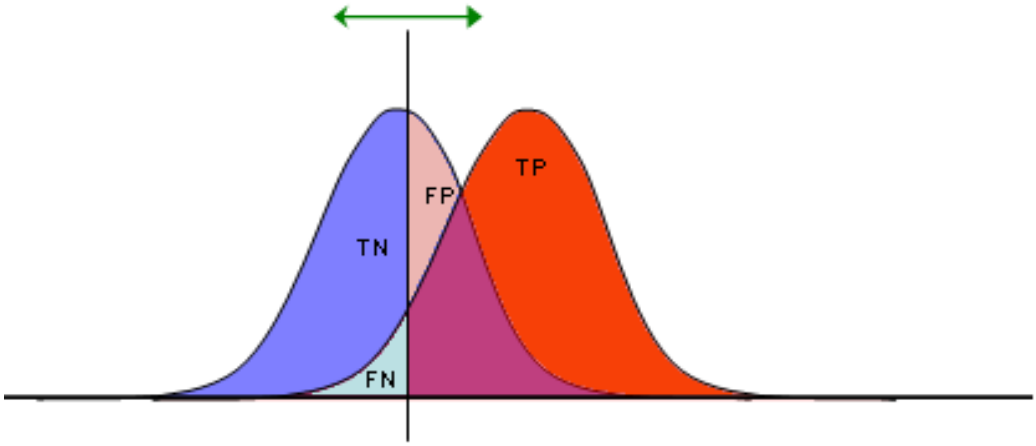
The biological action of the NOTCH pathway is procarcinogenic i.e. increased expression would be noted in carcinogenesis which, if related to gene methylation, would be shown as hypomethylation. Therefore when initial levels of methylation are low it becomes difficult to detect differences between groups without increasing the power of the statistical test and therefore also the sample size.

However small changes may be relevant. Estimation of methylation reflects the global status of a tissue whilst within each cell methylation at an individual CpG site is a binary phenomenon. Thus methylation effects at a cellular level are not continuous but binary and so small changes measured in tissue may have a profound impact at a cellular level. This is potentially very relevant as NOTCH appears to define the number of undifferentiated stem cells within a crypt base (in conjunction with other signalling pathways) and overpopulation of this compartment is the initial microscopic lesion in carcinogenesis. However stem cell specific methylation analysis was not possible within this project and would not be justified without appropriate preliminary data.

A parametric approach was selected to give a consistent statistical approach as this would be required for subsequent analyses of treatment effects in the factorially designed dietary intervention study. Parametric tests were used in isolation where data were normally distributed but where data were not normally distributed and parametric tests had shown a significant result this was confirmed or refuted using non-parametric equivalents. (see 3.2.4 for example)

Data on gene specific and global methylation and age were used to create a predictive model where these factors could be used to predict an adenomatous polyp diagnosis. The predictive factors tested within the model were those that had shown the greatest variation between groups. Using factors showing greatest variation may imply that the final model would inevitably predict group entry as a 'self-fulfilling prophecy'. Whilst this may be true, it is pragmatic to pick factors as predictors which are different between groups as these are more likely to discriminate groups, although this is not always the case. Comparative analysis between groups determines differences in terms of location and spread of the data. Predictive analyses (such as ROC) also compare these attributes of the group datasets but rely more heavily on overlap to define factors which discriminate consistently between groups. (Figure 3-8) Thus the location of data may be significantly different but a pattern of overlap may exist that means a factor does not discriminate reliably between groups. The data presented within this project must also be interpreted cautiously as all predictive models must be replicated in at least one independent population prospectively before they can be considered validated.

FIGURE 3-8. THE CHARACTERISTICS OF MODELLING A PREDICTIVE STATISTICAL TOOL.



THE HORIZONTAL LINE REPRESENTS THE SCALE OF MEASUREMENT FOR EACH PREDICTIVE FACTOR (EG. AGE, GLOBAL OR GENE SPECIFIC METHYLATION). THE VERTICAL BAR REPRESENTS THE 'CUT-OFF' ABOVE WHICH A TEST IS DETERMINED POSITIVE AND BELOW WHICH A TEST IS DETERMINED NEGATIVE. THE TWO CURVES REPRESENT THE DISTRIBUTION OF TEST VALUES FOR EACH DIAGNOSIS, IN THIS CASE ADENOMATOUS POLYPS AND NORMAL. IF THE CURVES OVERLAP THERE WILL BE A PROPORTION OF INCORRECT TEST RESULTS, FALSE POSITIVES (FP) AND FALSE NEGATIVES (FN). MOVING THE CUT-OFF VALUE (GREEN ARROW) WILL ALTER THE NUMBER OF FPS AND FNS AND THERFORE ALTER THE SENSITIVITY AND SPECIFICITY OF THE TEST. (TP – TRUE POSITIVE, TN - TRUE NEGATIVE)

3.6.2 CHARACTERISTICS OF PATIENT GROUPS

3.6.2.1 ANTHROPOMETRIC, LIFESTYLE, DIETARY AND BLOOD FACTORS

Most anthropometric factors within the three patient groups were well-matched except waist measurement. The difference noted in thigh measurements (lower circumference in UC and polyp patients) in the context of similar BMIs may reflect a relative tendency towards central or abdominal obesity which is recognised as a risk factor for sporadic and UC associated CRC. (Larsson and Wolk 2007; Park, Mitrou et al. 2012). Most participants within the study were overweight or obese (17.3% healthy (BMI 20-25), 36% overweight (BMI 25-30), 40% obese (BMI 30-40) and 6.7% morbidly obese (BMI>40)). Mean BMI for all groups was greater than the healthy range (normal – 30.1, polyp – 28.9, UC – 28.4kg/m^2).

There were also significant age and sex mismatches between the groups. Polyp patients were more frequently male. Polyps and CRC are recognised to occur more

frequently in males (CRUK 2009; Leffler, Kheraj et al. 2012) In this study the degree of male preponderance in the polyp group was greater than that which would be expected. This may represent sampling error or chance. To minimise confounding, sex was included as a covariate in all comparative analyses and as a required factor in all regression equations. As expected polyp patients were older. Age is the strongest independent risk factor for adenomatous polyp diagnosis. (Leffler, Kheraj et al. 2012)

No between group differences were noted in terms of dietary intake measured by FFQ. Mean energy intake was high in the polyp (12692kJ/day) and normal groups (11408kJ/day) and potentially within recommended limits in the UC group (10455 kJ/day). The most recent report on recommended energy intake in the UK (SACN 2011) has increased previous values for average daily recommended intake. Intake recommendations are based on age, sex and physical activity and are calculated for BMI 22.5 kg/m^2. Physical activity estimates are not directly comparable between this study and the SACN report due to different measurement tools. Using the mean ages of the study participants and assuming 'average' physical activity by the SACN definition recommended daily intake of our study participants would be 10 900 kJ/day for the 'average' male within the study population and 8 600 kJ/day for the 'average' female. (SACN 2011)

These recommended values would be increased in the higher BMI participants (specific values not available) within our study groups and would vary depending upon physical activity and age of each participant. The lack of comparability of physical activity estimates and inability to adjust recommended intake values depending on BMI of participants means more detailed individualised comparisons i.e. how many participants within in each group over-consume, would be neither informative nor accurate. In addition the FFQ used in this study has been shown to underreport total energy intake suggesting that actual energy intake of participants may be even higher than calculated values. (Kroke, Klipstein-Grobusch et al. 1999) Objectively measured indices of micronutrient status: serum B_{12}, red cell folate and plasma selenium showed no significant differences between groups.

After exclusion of the significant outlier in the UC group (very high CRP likely due to an intercurrent illness) there was a significantly higher hsCRP concentration in the polyp group compared normal and UC (8.05 vs. 3.28 vs. 3.43 mg/l respectively, p=0.014) The relationship of CRP elevation and established CRC is established (Allin and Nordestgaard 2011) and recent data suggests that increased systemic inflammation is causative rather than a bystander or confounding effect. (Prizment, Anderson et al. 2011) Higher hsCRP levels within the polyp group reflect increased systemic inflammation in this group. Prizment et al.'s data suggests that this increased level of systemic inflammation would contribute to causation of CRC. No difference was detected between the UC group and normal controls (3.4 vs. 3.3 mg/l respectively). This may reflect the sampling strategy (excluding those with active disease), or that the dysplasia-cancer sequence in UC is dependent on other factors such as local inflammation rather than systemic inflammation.

3.6.2.2 GLOBAL METHYLATION BETWEEN GROUPS

Global hypomethylation is a well-documented phenomenon in cancer in general and in CRC specifically. (Hiraoka, Kato et al. 2010; Khare and Verma 2012) Progressive hypomethylation in neoplastic tissue from normal to polyp to CRC has been shown using the LINE-1 assay (Sunami, de Maat et al. 2011) and greater degrees of tumour hypomethylation are associated with poor prognosis. (Ogino, Nosho et al. 2008; Ahn, Chung et al. 2011) Global hypomethylation is consistently found in tumours in sporadic and hereditary CRC and is also often found in adjacent macroscopically normal colonic mucosa in many CRC patients. This normal tissue hypomethylation occurs more frequently in hereditary CRC than sporadic CRC and is particularly prevalent with MSI-H tumours suggesting that, especially in hereditary and MSI-H CRC, global hypomethylation is part of the pro-carcinogenic field change. (Pavicic, Joensuu et al. 2012)

In this study, significantly decreased methylation in the polyp group was found at one CpG (site 1) in the LINE-1 assay. At other sites the methylation was either greater or equivalent comparing the polyp group to normal participants. (Figure 3.1) Previous studies have usually reported mean methylation of all CpGs within the LINE-1 assay.

These studies have found that global hypomethylation in normal tissue predicts specific tumour sub-types (Kamiyama, Suzuki et al. 2012; Pavicic, Joensuu et al. 2012) and is not a consistent finding in all CRC. (Estécio, Gharibyan et al. 2007; Ogino, Kawasaki et al. 2008; Figueiredo, Grau et al. 2009) The data obtained in this study suggests that mean LINE-1 methylation may not be the most informative method of analysing global DNA methylation data and that analysis of individual CpGs within LINE-1 may detect more subtle procarcinogenic changes.

In contrast to this, there was significant global hypomethylation in macroscopically normal tissue from patients in the UC group at all CpGs compared to normal controls. (Figure 3-1) These results are similar to those observed by Glória et al. (Glória, Cravo et al. 1996) who found hypomethylation of DNA in rectal biopsies from UC patients compared with controls using a semi-quantitative methyl group incorporation assay. Their study included those with active disease and they showed a dose-response association of hypomethylation with progressive disease activity. Our findings confirm a marked decrease in global methylation independent of disease activity in a UC group with disease for greater than seven years. Group size (n=12) and inclusion criteria led to a relatively homogeneous group that did not allow further analysis of disease or patient factors predicting methylation.

3.6.2.3 METHYLATION OF NOTCH GENES IN GROUPS AT DIFFERENTIAL CRC RISK

This project is the first report of data on the methylation of NOTCH genes in the normal colorectal epithelium of patients with no colorectal pathology or in those at higher CRC risk. NOTCH is critical in colorectal ASC maintenance and differentiation (Katoh 2011; Peignon, Durand et al. 2011) and in CRC development (Veenendaal, Kranenburg et al. 2008; Chu, Zhang et al. 2011). *JAG1* (sending cell ligand) expression is upregulated in human CRC (Reedijk, Odorcic et al. 2008; Peignon, Durand et al. 2011) but there are no data on *RBP-J* expression in CRC. Depending on the presence or absence of NICD within the nucleus, RBP-J recruits either repressive co-transcription factors or activation co-transcription factors. Despite its potential dual role, data suggests it acts as an oncogene as silencing of *RBP-J* in breast cancer reduces cellular proliferation (Yong, Sun et al. 2011) and silencing in the colorectal epithelium results in terminal differentiation of all cells ablating the stem cell compartment. (van Es, van Gijn et al. 2005)

Methylation of *JAG1* was not found to be different using ANOVA analysis. (Figure 3-3) There was a trend towards lower methylation in those at higher risk of cancer, especially the polyp group, at all CpG sites except site 4. This is the direction of effect that is anticipated for oncogenic activation via methylation change. Although statistical significance was not achieved the trend observed may have transcriptional significance. However assumption of a change in transcription cannot be assumed, even though it is likely. (Bell, Pai et al. 2011) Correlation with expression data would be required to link methylation change to a functional effect.

Most sites on the *RBP-J* assay were minimally methylated (<3%) in the normal population and so in terms of oncogenic activation by hypomethylation are less likely to be transcriptionally significant. In contrast, CpG site 11 was relatively highly methylated in normals and significant hypomethylation at this site was noted in the polyp group (23.6 vs. 18.0%, p=0.006). There was no statistically significant difference observed in the UC group. In normal participants, methylation at all CpG sites within the assay was highly correlated with each other (Pearson's r >0.9) except for CpG 11,

potentially suggesting different causal factors for methylation at this site. Within the polyp group, methylation of CpG site 11 and CpG site 6 predicted the disease risk category. A continuous effect within the polyp group with progressively lower *RBP-J* site 11 methylation associated with more severe disease strengthens confidence in the association observed in the between group analysis.

3.6.2.4 Prediction of global DNA methylation

Forward stepwise regression analysis was used to identify potential predictors of baseline DNA methylation levels in the normal participants using anthropometric, dietary and lifestyle factors. Forward stepwise regression has been subject to criticism when used indiscriminately, in a non-hypothesis based fashion. (Miles and Shevlin 2001) This form of data-mining may generate spurious or biologically implausible relationships. In this study only pre-specified variables based on *a priori* hypotheses from best available evidence were interrogated. (WCRF/AICR. 2012)

Since global DNA hypomethylation is linked with increasing risk of CRC, the direction of effect within the LINE-1 regression equation is expected to be negative for exposures which increase CRC risk and positive for protective exposures. The equation suggested that male sex would decrease global methylation by approximately 0.5% and an increase in waist circumference by 1 cm would decrease methylation by 0.02%. These were expected effects of known CRC risk factors. (section 1.1 and 1.11)

Age and smoking status had opposite effects to those expected. Although these were forced factors within the model, their individual p values (smoking status p=0.002 and age p=0.001) indicate strong relationships. The other forced variables in the equation (sex and waist circumference) had p values >0.05 indicating the strength of their association with global methylation was less strong. In another analysis of predictive factors of global methylation (also assessed by measurement of LINE-1 methylation in normal mucosa) of 400 polyp patients, no associations were found for age, sex, smoking status or BMI. (Figueiredo, Grau et al. 2009)

Figueiredo et al. did not find any association between circulating vitamin B_{12} concentrations and global DNA methylation whereas in the present study vitamin B_{12}

concentrations were inversely related to global DNA methylation (p=0.069). Vitamin B_{12} derivatives are used in diverse biological processes involving methyl group transfer (Kung, Ando et al. 2012) and maternal vitamin B_{12} status has been shown to correlate inversely with infant global methylation. (McKay, Groom et al. 2012) In the current study, total dietary fibre intake predicted global methylation but the direction of effect was opposite to the CRC protective effect that would be anticipated (increased fibre – decreased global methylation). (WCRF/AICR. 2012)

Inverse correlations of CRP concentration and global DNA methylation as shown in this study, have been shown in several small studies of cancer patients (Kwon, Kim et al. 2010; Allin and Nordestgaard 2011; Ishizuka, Nagata et al. 2012) and a direction of effect for the association, CRP and systemic inflammation resulting in cancer, has been suggested by demonstration of a temporal relationship. (Prizment, Anderson et al. 2011) This may represent an isolated effect in the colon as the association between systemic inflammation and decreased global methylation has not been replicated in blood. (Baccarelli, Tarantini et al. 2010; Zhang, Santella et al. 2012) However, lack of replication between tissues is not concerning as wide variations in tissue specific epigenetic marking are known to be prevalent. (Thompson, Atzmon et al. 2010; Liang, Song et al. 2011; McKay, Xie et al. 2011).

Overall, these predictive data must be interpreted with caution as the relatively limited number of cases included in the study and the small variance within the normal population make relationships both difficult to find and of uncertain significance until they are replicated in another population.

3.6.2.5 PREDICTORS OF METHYLATION OF *JAG1* AND *RBP-J*

Since there are no data on methylation of NOTCH related genes in CRC, there are no comparable data for the environmental predictors of *JAG1* and *RBP-J* methylation identified by regression analysis in this study. If methylation supresses expression, we would anticipate that methylation of oncogenes such as *JAG1* and *RBP-J* would be decreased by factors that enhance CRC risk.

For *JAG1*, smoking was found to increase methylation (coefficient 2.55, p= 0.051) as was male sex (coefficient 2.98, p=0.157), both unexpected effects. The relatively low p values associated with these parameters means they would have entered the model on the basis of free selection (α to enter 0.25) even though they were forced parameters. Even after removal of outliers and high leverage data points, the distribution of residuals within the regression equation was not normal so the assumptions of multiple regression were not satisfied and this may therefore represent a statistical error. The low R^2 together with a very high PRESS statistic suggest this may be the case. Replication in a larger independent data set would be required to test this.

The identified predictors of *RBP-J* methylation i.e. age, smoking behaviour, waist circumference, processed meat consumption, alcohol and garlic intake and hsCRP showed the expected directions of effect for most parameters (except sex and hsCRP). Garlic was noted to have a particularly strong effect on *RBP-J* methylation raising mean values by 3.5% per portion consumed daily. The standard error of 1.3 and p=0.01 suggest a robust statistical finding. No data to date suggest a specific role of garlic or other alliums in direct modulation of DNA methylation. This may represent an interesting new association, however, if it were not to be replicated in an independent data set it may be that the instrument of measurement (asking about portions per day of garlic) is inaccurate. It could be flawed due to different individual perceptions of 'a medium portion' size and participants lack of knowledge of whether they are consuming garlic if meals are prepared for them.

3.6.3 POTENTIAL BIOLOGICAL CONSEQUENCES

Description of the potential biological consequences associated with a single epigenetic signature is complex. Despite macroscopic similarity, CRC is a molecularly heterogeneous disease. Issues of confounding effects that cannot be measured or have not been adjusted for can exist and there is great difficulty in defining cause and effect in studies of epigenetic epidemiology. These concepts are discussed in greater detail in the global discussion of the study (section 5.2) (Ogino, Chan et al. 2011; Ng, Barrett et al. 2012; Relton and Davey Smith 2012)

3.6.3.1 GLOBAL DNA METHYLATION

The biological consequences of global DNA hypomethylation have been relatively well described. Global hypomethylation (assessed by LINE-1) has been associated with a family history of CRC and young age at onset (Baba, Huttenhower et al. 2010). LINE-1 is usually heavily methylated preventing retrotransposal element activation. Demethylation activates these elements promoting genomic instability and acquisition of further genomic and epigenomic alterations. (Hoffmann and Schulz 2005)

Although this points to a key causal role in the acquisition of a cancer phenotype, the global hypomethylation epitype is not a consistent finding in all CRCs and is more associated with MSI-H and hereditary cancers. (Kamiyama, Suzuki et al. 2012; Pavicic, Joensuu et al. 2012) Data from this study indicates that it may also play a role in pathogenesis of UC associated CRC. Global demethylation is also not generally considered a founder epimutation as it has been shown to be a response to preceding genetic or epigenetic changes which result in dysfunctional chromatin remodelling. (Hoffmann and Schulz 2005). Thus global demethylation acts as a promoting epigenetic alteration in carcinogenesis as well as a biomarker of specific tumour sub-types. Further investigation may also demonstrate utility as a biomarker of cancer risk in UC.

3.6.3.2 METHYLATION OF *JAG1* AND *RBP-J*

RBP-J acts as both a transcriptional repressor and an activator depending on the recruitment of co-factors in the presence or absence of NICD. Downstream genes affected by RBP-J are summarized in section 1.9. The potential consequences of suppression of *RBP-J* expression can be predicted from the phenotype and transcriptome observed in *Rbp-j* knockout experiments in mice. *Rbp-j* knockout results in decreased *Hes1* and *Math1* expression. Phenotypically the transit-amplifying and stem cell compartments of the crypt were replaced entirely by post-mitotic goblet cells without any change in apoptosis. (van Es, van Gijn et al. 2005) These changes represent complete differentiation of the stem cell compartment in the absence of Notch signalling. As CRC carcinogenesis may be associated with an initial increase in stem cell number (Boman, Walters et al. 2004; Boman, Fields et al. 2008) the direction

of effect in carcinogenesis would be increased *RBP-J* expression via hypomethylation as was observed at CpG site 11 in *RBP-J* in the present study.

Although *JAG1* overexpression has been documented in the majority of CRC (Reedijk, Odorcic et al. 2008; Peignon, Durand et al. 2011) no mechanism for this altered transcription has been identified. One plausible mechanism is methylation dependent regulation of expression. The direction of change observed in higher risk groups compared to controls would suggest that this may be the case; however biological effect cannot be defined without measurement of functional outcomes (mRNA or protein).

3.7 SUMMARY OF BASELINE FINDINGS

These data suggest that global DNA hypomethylation occurs in UC patients with long-standing disease independent of disease activity. Global hypomethylation is associated with genomic instability and increased cancer risk. Previous studies have found global hypomethylation in normal mucosa of patients with sporadic and hereditary CRC, though this is more common in hereditary CRC. In this study significant hypomethylation in the polyp group was found at CpG site 1 compared to normals, but methylation at CpG site 2 was increased and at CpG site 3 no difference was detected. This suggests that the practice of reporting mean LINE-1 methylation in the literature is not the most informative and that global demethylation may play a lesser role in sporadic CRC carcinogenesis.

JAG1 methylation was reduced at most CpG sites, except site 4, in the expected direction for oncogenic activation although significant differences were not detected statistically. Most sites on the *RBP-J* assay were minimally methylated and therefore small changes were difficult to detect in this study and such changes are less likely to be biologically significant. Where there was significant methylation (CpG site 11) this was decreased, as expected, in patients with adenomatous polyps. Additionally there was progressive decrease in methylation at this site with increasing risk of CRC defined by polyp characteristics.

Exposures that may influence methylation have been suggested using linear regression. These data are exploratory and require to be validated in a larger dataset prospectively.

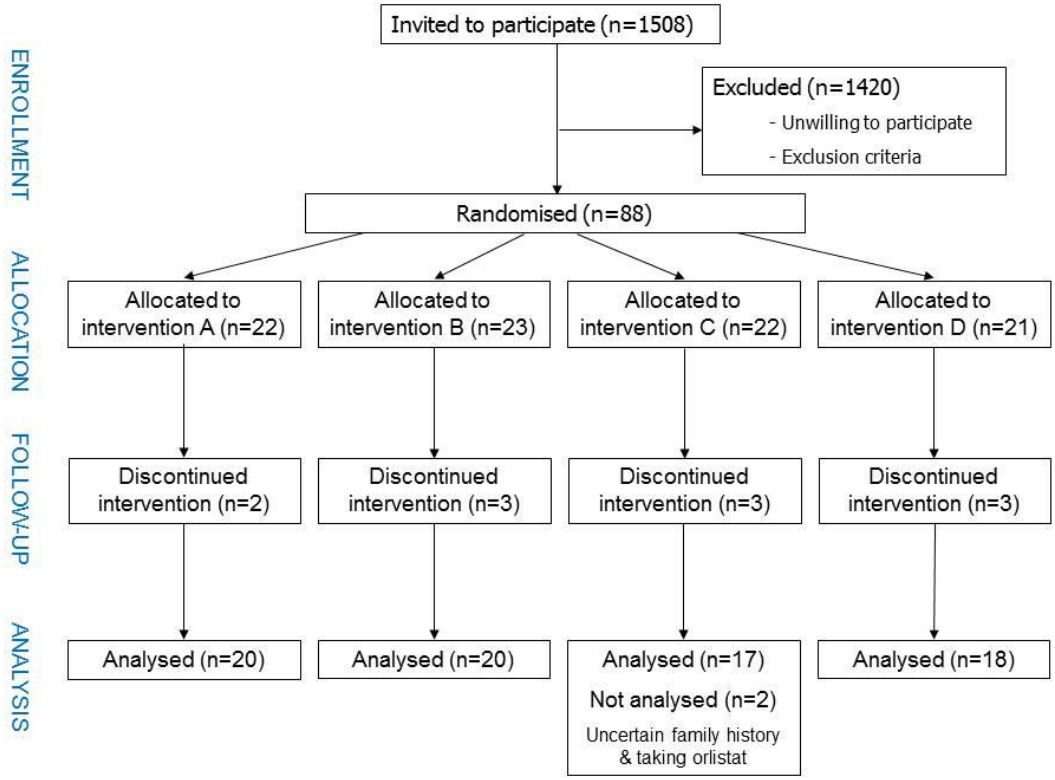
CHAPTER 4. RESULTS - INTERVENTION GROUP (THE DISC STUDY)

4.1 RECRUITMENT TO INTERVENTION

1508 potential participants attending endoscopy were invited to attend the DISC study. 1420 were excluded on the basis of non-attendance, being unwilling to participate or fulfilling exclusion criteria, most commonly aspirin therapy. 88 consented and were randomised. 11 participants dropped out post-randomisation. Reasons for drop-outs were: not liking intervention (n=2), changed mind (n=2), unable to fulfil time commitments (n=3), privacy issues with family (n=1), bloating whilst taking the intervention (n=1), becoming unwell during the intervention phase (not related to intervention) (n=1) and incorrect classification of endoscopy as normal by the study team when a polyp had been found (n=1). Drop-outs were balanced between groups.

Two participants were excluded at the analysis stage. An audit of participants revealed that one potentially fitted the Amsterdam criteria for possible HNPCC diagnosis. (Vasen, Mecklin et al. 1991) This participant was not able to be contacted to clarify family history and so was excluded. The second excluded participant was taking Orlistat. Orlistat inhibits fat digestion causing steatorrhoea. High fat content in the colon may alter microbiota populations confounding several outcome measures in the study. (Jiang, Lupton et al. 1996; Murphy, Cotter et al. 2010) A summary of recruitment is presented as a CONSORT diagram. (Schulz, Altman et al. 2010) (Figure 4-1)

FIGURE 4-1. CONSORT DIAGRAM SHOWING FLOW OF PARTICIPANTS THROUGH ENROLLMENT, ALLOCATION, FOLLOW-UP AND ANALYSIS PHASES OF THE DISC STUDY.



4.1.1 CHARACTERISTICS OF DISC PARTICIPANTS

Final diagnoses at endoscopy were classified as:

- Normal microscopically – where, in addition to a normal endoscopy, mucosal biopsies had been sent for histology and no abnormalities were reported
- Normal macroscopically – where final endoscopic diagnosis was recorded as 'normal' and no other clinical diagnosis was made or investigation planned
- Haemorrhoids – where this was the final endoscopic diagnosis and the presentation was per rectal bleeding
- Diverticular disease – visualised at endoscopy with no other abnormality
- Other perianal – which consisted of perianal skin tag, rectocoele and anal fissure and the remaining endoscopy was normal

The final diagnoses of the DISC participants are listed in Table 4-1. Twenty participants (27%) had normal histology in addition to normal endoscopy. No participants had histology samples sent and were subsequently excluded due to abnormalities detected. Only 5 participants (7%) had any detectable colonic abnormality which was diverticular disease. Some studies have reported an association between diverticular disease and CRC but these have been subject to major issues of confounding and bias and more modern and reliable evidence does not support such a link. (Ekbom 2012)

TABLE 4-1. FINAL CLINICAL DIAGNOSES IN THE DISC STUDY PARTICIPANTS.

Diagnosis	**Number of participants**
Normal microscopically	20 (27%)
Normal macroscopically	17 (23%)
Haemorrhoids	29 (39%)
Diverticular disease	5 (7%)
Other perianal	5 (7%)

4.1.2 RANDOMISATION AND BASELINE COMPARABILITY OF GROUPS

There was no significant difference in the sex distribution between groups (p=0.074) or smoking status (p=0.604). There was a significant difference in age between the groups with mean age being older for participants in Group B (p=0.043). No differences were detected in the anthropometric data between groups in terms of weight (p=0.775), BMI (p=0.879), hip circumference (p=0.262), waist circumference (p=0.781) or thigh circumference (p=0.702). (Table 4-2)

No difference was detected in hsCRP (p=0.600), serum B_{12} (p=0.528) and red cell folate (p=0.708). There was a significant difference detected in Selenium levels between groups (p=0.005) although all values lay well within the normal range of 0.8-2.0µmol/l. (Table 4-3)

Intake of energy (p=0.618), fat (p=0.737), fibre (p=0.359), red meat (p=0.840), alcohol (p=0.315), calcium (p=0.521) and garlic (p=0.177) did not vary significantly between intervention groups at baseline. In contrast, processed meat consumption was different between groups (p=0.023) at baseline. Post-hoc Tukey's test showed that group A had higher consumption (0.88 portions/day), and group C a lower consumption, (0.38 portions/day) than groups B & D (0.49 and 0.62 portions/day respectively) ($p<0.05$) (Table 4-4).

TABLE 4-2. ANTHROPOMETRIC DATA BY INTERVENTION GROUP AT BASELINE FOR DISC STUDY PARTICIPANTS.

		Group A (n=20)	**Group B (n=20)**	**Group C (n= 17)**	**Group D (n=18)**	**P value**
Age (years)	LSM (SEM)	48.2 (2.6)	58.4 (2.6)	53.5 (2.9)	50.1 (2.8)	0.043
Sex (M:F)	(M:F)	10:10	11:9	13:4	6:12	0.074
Weight (kg)	LSM (SEM)	83.43 (18.89)	80.14 (16.0)	83.09 (15.93)	85.67 (13.73)	0.775
Body mass index (kg/m^2)	LSM (SEM)	29.83 (4.72)	29.67 (6.09)	31.02 (5.36)	29.96 (5.44)	0.879
Smoking status	Current/ Ex/Never	4:4:12	3:5:12	3:6:8	6:6:6	0.604
Hip circumference (cm)	LSM (SEM)	108.0 (11.0)	102.8 (10.7)	110.1 (11.0)	107.9 (13.6)	0.262
Waist circumference (cm)	LSM (SEM)	99.3 (14.5)	97.3 (14.1)	100.7 (11.3)	101.4 (12.4)	0.781
Thigh circumference (cm)	LSM (SEM)	59.9 (5.8)	58.7 (8.2)	61.1 (6.4)	60.5 (5.8)	0.702

TABLE 4-3. CRP AND MICRONUTRIENT CONCENTRATIONS IN BLOOD BY INTERVENTION GROUP AT BASELINE FOR DISC STUDY PARTICIPANTS.

	Group A (n=20)	**Group B (n=20)**	**Group C (n= 17)**	**Group D (n=18)**	
Assay	LSM (SEM)				p value
C-reactive protein (mg/l)	2.75 (0.99)	4.56 (0.99)	3.15 (1.07)	3.81 (1.07)	0.600
Serum B_{12} (ng/l)	395.1 (47.9)	485.1 (47.9)	418.2 (51.9)	469.1 (53.5)	0.528
Red cell folate (µg/l)	263.5 (31.4)	259.1 (31.4)	290.8 (34.1)	306.9 (35.1)	0.708
Plasma selenium (µmol/l)	1.05 (0.05)	1.14 (0.05)	1.27 (0.05)	1.31 (0.05)	0.005

TABLE 4-4. DIETARY INTAKE BY GROUP AT BASELINE FOR DISC STUDY PARTICIPANTS.

	Group A (n=20)	**Group B (n=20)**	**Group C (n= 17)**	**Group D (n=18)**	
Variable	LSM (SEM)				p value
Energy intake (MJ/day)	12.53 (1.34)	10.71 (1.34)	10.70 (1.45)	12.59 (1.41)	0.618
Total fat intake (g/day)	114.6 (15.6)	91.7 (15.6)	95.8 (16.9)	105.8 (16.5)	0.737
Fibre intake (g/day)	24.4 (2.4)	21.3 (2.4)	20.0 (2.6)	25.6 (2.5)	0.359
Red meat (portions/day)	0.47 (0.08)	0.40 (0.08)	0.48 (0.08)	0.50 (0.08)	0.840
Processed meat (portions/day)	0.88 (0.12)	0.49 (0.12)	0.38 (0.13)	0.62 (0.12)	0.023
Alcohol intake (g/day)	14.4 (6.2)	28.2 (6.2)	29.3 (6.7)	21.1 (6.5)	0.315
Calcium intake (mg/day)	1266 (109.0)	1115 (109.0)	1115 (118.3)	1302 (114.9)	0.521
Garlic intake (portions/day)	0.25 (0.06)	0.19 (0.06)	0.40 (0.07)	0.26 (0.07)	0.177

4.2 EFFECTS OF INTERVENTIONS

Results are presented below for effects of the RS source Hi-maize 260 (active agent) compared with the corresponding placebo - amioca starch (control group) and for effects of the active agent PD compared with its placebo - maltodextrin (control group). The only covariate included in analysis was baseline methylation level. (section 2.10.6)

4.2.1 EFFECT SUPPLEMENTATION WITH RS AND PD ON ANTHROPOMETRIC MEASUREMENTS AND BLOOD INDICES

Most anthropometric variables i.e. weight, BMI, waist and hip circumference were unchanged by either agent after the intervention. PD intervention reduced thigh circumference slightly, but significantly, compared with controls (59.7 vs. 60.4cm, p=0.039) (Table 4-5).

Neither systemic inflammation estimated by hsCRP nor blood indices of micronutrient status (vitamin B_{12}, folate, selenium) were changed with either intervention agent alone, nor was there any evidence of significant interactions between RS and PD on any of these variables after the 50 day intervention (Table 4-6).

TABLE 4-5. EFFECT OF RS AND PD SUPPLEMENTATION ON ANTHROPOMETRIC MEASUREMENTS.

	RS allocation LSM (SEM)			**PD allocation** LSM (SEM)			**Interaction**
	Hi-maize 260	Control	*p value*	PD	Control	*p value*	*p value*
Weight (kg)	83.9 (0.3)	83.4 (0.2)	*0.191*	83.5 (0.8)	83.7 (0.8)	*0.524*	*0.502*
BMI (kg/m^2)	30.2 (0.1)	30.1 (0.1)	*0.513*	30.1 (0.1)	30.2 (0.1)	*0.523*	*0.831*
Waist (cm)	99.9 (0.4)	100.3 (0.4)	*0.546*	99.6 (0.4)	100.5 (0.4)	*0.118*	*0.319*
Hip (cm)	109.0 (1.6)	108.5 (1.5)	*0.823*	109.2 (1.5)	108.3 (1.5)	*0.673*	*0.415*
Thigh (cm)	60.1 (0.3)	60.0 (0.3)	*0.945*	59.7 (0.3)	60.4 (0.3)	*0.039*	*0.092*

TABLE 4-6. EFFECT OF RS AND PD SUPPLEMENTATION ON BLOOD CRP AND MICRONUTRIENT STATUS.

	RS allocation LSM (SEM)			**PD allocation** LSM (SEM)			**Interaction**
	Hi-maize 260	Control	*p value*	PD	Control	*p value*	*p value*
hsCRP (mg/l)	2.7 (0.3)	2.2 (0.3)	*0.274*	2.3 (0.3)	2.5 (0.3)	*0.575*	*0.727*
Serum B_{12} (ng/l)	454.2 (39.5)	428.4 (35.8)	*0.631*	418.4 (38.3)	464.2 (37.5)	*0.399*	*0.151*
Red cell folate (µg/l)	276.8 (13.2)	286.9 (11.9)	*0.573*	280.3 (12.7)	283.5 (12.4)	*0.859*	*0.593*
Plasma Se (µmol/l)	1.21 (0.04)	1.22 (0.03)	*0.785*	1.21 (0.03)	1.22 (0.03)	*0.796*	*0.535*

4.2.2 EFFECT OF RS AND PD ON LINE-1 METHYLATION

LINE-1 methylation was not affected by either RS or PD at any of the 3 CpGs within the assay (or mean methylation across the 3 sites) and there was no evidence of any interaction between RS and PD. (Table 4-7)

TABLE 4-7. EFFECTS OF RS AND PD SUPPLEMENTATION ON LINE-1 METHYLATION.

CpG site	**RS allocation** LSM (SEM)			**PD allocation** LSM (SEM)			**Interaction**
	Hi-maize 260	Control	*p value*	PD	Control	*p value*	*p value*
1	76.7 (0.8)	77.2 (0.8)	*0.673*	76.9 (0.8)	76.9 (0.8)	*0.993*	*0.422*
2	63.8 (0.6)	63.9 (0.6)	*0.869*	64.1 (0.6)	63.6 (0.6)	*0.554*	*0.843*
3	65.1 (0.5)	65.6 (0.5)	*0.482*	65.7 (0.5)	65.0 (0.5)	*0.307*	*0.360*
Mean	68.5 (0.5)	68.9 (0.5)	*0.594*	68.9 (0.5)	68.5 (0.5)	*0.149*	*0.832*

4.2.3 EFFECT OF RS AND PD ON *JAG1* METHYLATION

After 50 days of supplementation, methylation of *JAG1* at CpG site 4 was increased significantly (p=0.009) in those that allocated to PD (3.1 (PD) vs. 1.7% (control)) (Table 4-8) All other CpGs on the *JAG1* assay showed higher levels of methylation in response to PD compared to controls although these changes were not statistically significant (p>0.05). For an oncogene higher levels of methylation after treatment would potentially confer a protective effect by resulting in less gene transcription. There were no consistent, or significant, changes in *JAG1* methylation in response to RS supplementation. (Table 4-8)

TABLE 4-8. EFFECTS OF RS AND PD SUPPLEMENTATION ON *JAG1* METHYLATION.

CpG site	**RS allocation** LSM (SEM)			**PD allocation** LSM (SEM)			**Interaction**
	Hi-maize 260	Control	*p value*	PD	Control	*p value*	*p value*
1	3.8 (0.8)	4.8 (0.7)	*0.386*	4.7 (0.8)	3.9 (0.8)	*0.450*	*0.750*
2	2.6 (0.6)	3.2 (0.6)	*0.482*	3.2 (0.6)	2.6 (0.6)	*0.461*	*0.836*
3	6.7 (0.7)	6.1 (0.7)	*0.459*	7.1 (0.7)	5.5 (0.7)	*0.115*	*0.834*
4	2.5 (0.4)	2.3 (0.4)	*0.703*	3.1 (0.4)	1.7 (0.4)	0.009	*0.341*
5	4.5 (0.7)	4.7 (0.6)	*0.895*	5.0 (0.7)	4.2 (0.6)	*0.368*	*0.950*
Mean	4.0 (0.5)	4.2 (0.5)	*0.742*	4.7 (0.5)	3.6 (0.5)	*0.132*	*0.924*

4.2.4 EFFECT OF RS AND PD ON *RBP-J* METHYLATION

The results by treatment group are summarized in Table 4-9. No statistically significant differences were noted for either RS or PD supplementation in isolation compared to placebo controls.

Several of the CpG sites achieved (CpG 2) or approached (CpGs 1, 3, 4, 7, 8, 10, 11) statistical significance for an interaction effect. The effect at all sites was the same with higher methylation in the double intervention and double placebo (control) groups and lower methylation for each of the single intervention groups (data for mean *RBP-J* methylation interaction effects shown in Table 4-10). The initial analysis of results for interaction effects (mean *RBP-J* methylation) did not fulfill the necessary assumptions of GLM, specifically normal distribution of residuals. Removal of three outliers with high standardised residuals (these individuals were distributed evenly between intervention groups) from the analysis of mean *RBP-J* methylation gave a significant interaction p value of 0.044 and normalised the distribution of residuals. Mean methylation was higher in the double intervention and double placebo groups (3.1 and 3.3% respectively) and lower in the single intervention groups (Hi-maize 260 – 2.4%, PD – 2.6%). (Figure 4-2)

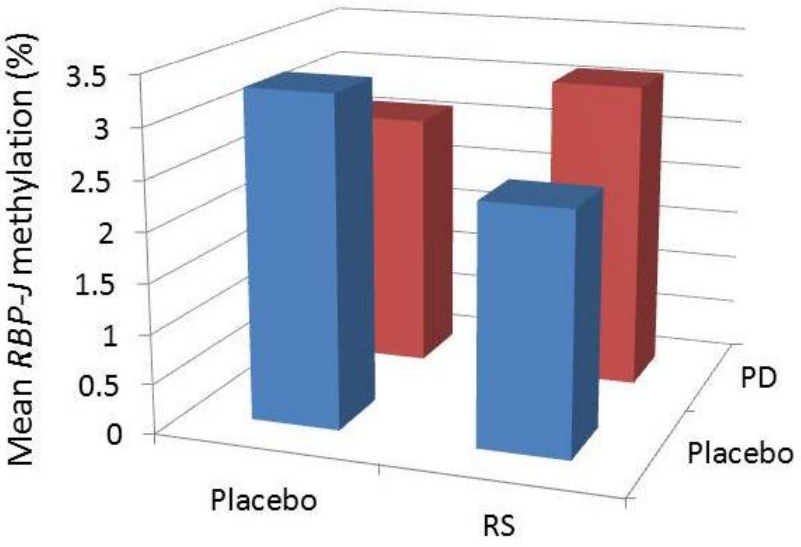
TABLE 4-9. EFFECTS OF RS AND PD SUPPLEMENTATION ON *RBP-J* METHYLATION.

CpG site	**RS allocation** LSM (SEM)			**PD allocation** LSM (SEM)			**Interaction**
	Hi-maize 260	Control	*p value*	PD	Control	*p value*	*p value*
1	0.9 (0.2)	0.9 (0.2)	*0.892*	0.7 (0.2)	1.0 (0.2)	*0.207*	*0.099*
2	1.3 (0.2)	1.3 (0.2)	*0.931*	1.3 (0.6)	1.3 (0.6)	*0.461*	*0.017*
3	1.5 (0.2)	1.4 (0.2)	*0.852*	1.4 (0.2)	1.5 (0.2)	*0.626*	*0.095*
4	1.0 (0.2)	1.3 (0.2)	*0.363*	0.9 (0.2)	1.4 (0.2)	*0.091*	*0.091*
5	1.2 (0.2)	1.3 (0.2)	*0.785*	1.0 (0.2)	1.5 (0.2)	*0.137*	*0.132*
6	4.0 (0.5)	4.2 (0.5)	*0.753*	4.1 (0.5)	4.2 (0.5)	*0.882*	*0.133*
7	1.4 (0.2)	1.4 (0.2)	*0.973*	1.2 (0.2)	1.5 (0.2)	*0.371*	*0.060*
8	1.3 (0.2)	1.3 (0.2)	*0.950*	1.2 (0.2)	1.4 (0.2)	*0.522*	*0.068*
9	1.3 (0.2)	1.5 (0.2)	*0.598*	1.1 (0.2)	1.6 (0.2)	*0.160*	*0.140*
10	1.4 (0.2)	1.5 (0.2)	*0.818*	1.2 (0.2)	1.8 (0.2)	*0.066*	*0.087*
11	17.5 (1.1)	18.3 (1.1)	*0.631*	18.1 (1.2)	17.7 (1.1)	*0.807*	*0.082*
12	2.9 (0.3)	2.8 (0.3)	*0.797*	2.7 (0.3)	2.9 (0.3)	*0.670*	*0.127*
Mean	3.0 (0.3)	3.1 (0.3)	*0.821*	2.9 (0.3)	3.1 (0.3)	*0.606*	*0.055*

TABLE 4-10. VALUES FOR INTERACTION EFFECTS AFTER RS AND PD SUPPLEMENTATION FOR MEAN *RBP-J* METHYLATION.

RS allocation	PD allocation	LSM	SEM
Hi-maize 260	PD	3.1	0.3
Hi-maize 260	Control	2.4	0.4
Control	PD	2.6	0.3
Control	Control	3.3	0.3

FIGURE 4-2. GRAPHICAL REPRESENTATION OF THE INTERACTION EFFECT NOTED IN RESPONSE TO RS AND PD SUPPLEMENTATION.



4.3 DISCUSSION OF THE DISC STUDY

4.3.1 CRITICAL APPRAISAL AS A RANDOMISED CONTROLLED TRIAL

The DISC study recruited the target number of participants. Although this was not subject to a formal power calculation it represented an achievable target likely able to detect effects based on a previous study conducted within the group which detected methylation changes in CRC patients given RS supplementation for 25 days. (Dronamraju, Coxhead et al. 2009) Groups were well matched at base-line except for age, selenium and processed meat consumption. These were not deemed to be required covariates for analysis of intervention effects as the distribution between treatment groups was unknown and there was no evidence that these would act as confounding variables for intervention effect when baseline methylation level was already being accounted for (section 2.10.6).

Most participants progressed to completion of the trial suggesting that the protocol was acceptable. 11 participants were unable to complete the study (12.5%) although only three of these were due to poor palatability of the intervention or side-effects (bloating). There was no differential loss to follow-up between groups which, if present, might suggest an unmeasured factor causing drop-out rates to increase in a particular group. Two participants were excluded after completing the trial as there were concerns that their inclusion might bias results. One participant was excluded due to potentially meeting family history criteria for HNPCC which could not be further clarified and, as such, meeting previously defined exclusion criteria. The other participant was excluded because he was taking Orlistat. This could have potential confounding effects as a large proportion of dietary fat is diverted to the colon which may affect the colonic bacteria (Thompson and Spiller 1995; Jiang, Lupton et al. 1996; Murphy, Cotter et al. 2010) although the only study specifically assessing Orlistat's effect found no effect on colorectal mucosal crypt cell proliferation. (Ahnen, Guerciolini et al. 2007) Orlistat use had not been anticipated prior to the trial

commencing and so was not included in the initial exclusion criteria. Importantly this post-hoc exclusion decision was made prior to any analyses being undertaken.

Overall participants' had final clinical diagnoses consistent with no increase in CRC risk suggesting that the predefined exclusion criteria were satisfactory as the basis for participant selection. Allocation of participants was free from investigator or participant influence and all laboratory and statistical analyses were undertaken with blinding to treatment allocation. As such the DISC study fulfils the criteria for a high quality RCT according to published guidelines. (Schulz, Altman et al. 2010)

4.3.2 EFFECTS OF DISC INTERVENTIONS ON ANTHROPOMETRIC AND BLOOD INDICES

The intervention combinations were designed based on the maximum energy intake increase being 180kJ/day in the double intervention group. This was calculated to provide only a modest increase in overall energy intake compared to recommended values in males (10.9MJ/day) and females (8.6MJ/day). There was no evidence of weight gain in any of the treatment groups so it is possible that participants (unconsciously) adjusted their energy intake or energy expenditure to maintain energy balance during the intervention study. Doses were also based on doses of RS which have previously been shown change methylation profiles in patients with CRC taking a 25 day intervention between diagnosis and surgery. (Dronamraju, Coxhead et al. 2009; Burn, Bishop et al. 2011) Supplying intervention so that similar doses of NDC reached the colon for each treatment, based on anticipated levels of small bowel digestion (section 2.3), allowed detection of differential effects of the two different NDCs, Hi-maize 260 and PD.

The only anthropometric measure which changed after fifty days supplementation was thigh circumference which was reduced in those participants taking PD (59.7 vs. 60.4cm, p=0.039). There are no data available to suggest that PD is known to cause weight change or alter body composition. There are data that show significant suppression of appetite by PD consumption at doses similar to the DISC study doses, with decreased consumption at subsequent *ad libitum* meals between 218-408kJ/day. (Hull, Re et al. 2012; Ranawana, Muller et al. 2012) RS consumed in greater quantities

and over a longer time period than in the DISC study has also been shown to confer more favourable fat distribution in conjunction with improved insulin sensitivity but without weight loss in subjects with metabolic syndrome. (Johnston, Thomas et al. 2010) Thigh circumference has been found to be a predictor of cardiovascular disease (smaller thighs associated with increased mortality) and it has been suggested that this is because it acts as a surrogate estimate of body fat distribution. (Berit and Peder 2009) Within the DISC study the FFQ was not repeated at the end of the study to attempt to measure dietary change as it has not been validated in this context. Data on subjective ratings of appetite and satiety or body composition were not collected. These may have clarified underlying reasons behind changes in thigh measurements or suggested that this was a type 1 error.

Systemic inflammation was not influenced as hypothesised by either intervention agent or combination of agents. PD has been shown to have anti-inflammatory properties in animal colitis models but data showing an anti-inflammatory effect in humans is not available. (Witaicenis, Fruet et al. 2010; Bassaganya-Riera, DiGuardo et al. 2011) RS has also been shown to decrease systemic inflammatory markers in animal models (Witaicenis, Fruet et al. 2010) but these results have not been replicated in human intervention studies. (Worthley, Le Leu et al. 2009; Johnston, Thomas et al. 2010). No change was expected in blood concentrations of the micronutrients B_{12}, selenium or folate and this is what was observed.

4.3.3 EFFECTS OF DISC INTERVENTIONS ON GLOBAL AND GENE SPECIFIC METHYLATION

Global methylation was unchanged after either intervention in the DISC study or any combination of interventions. The only other study to evaluate global methylation after dietary intervention in humans with RS used a similar dose in combination with probiotic supplementation in a placebo-controlled cross-over design. After forty days intervention they also found no change in LINE-1 methylation for active treatment compared to controls. (Worthley, Le Leu et al. 2009)

Baseline DISC study participant data suggested that LINE-1 methylation was decreased in response to higher reported dietary fibre intake, though not specifically RS. This

would be the direction of effect anticipated for a harmful environmental exposure. (section 3.3.2) Global gene methylation is known to decrease with age and is associated with increased CRC risk. (Ogino, Nosho et al. 2008; Belshaw, Pal et al. 2010; Sunami, de Maat et al. 2011) Figuerido et al. reported other dietary associations between LINE-1 methylation and dietary intake but no data regarding fibre consumption was reported. (Figueiredo, Grau et al. 2009)

There are several potential explanations for no effect of RS or PD being observed in the DISC study. Firstly, LINE-1 may respond to dietary and specifically RS or PD protective effects. However, this effect may occur over the lifespan of an individual and relatively short interventions (50 days) may not modulate global methylation sufficiently for detection. The small absolute lifetime differences noted between the higher risk and normal risk participants (largest between any high risk and normal control was 6.4% - section 3.3.1) in the current study suggest that absolute differences after a dietary intervention may be small and difficult to detect. A second explanation is that decreased global methylation, whilst associated with cancer risk and likely to be causative, may be brought about by mechanisms entirely independent of dietary factors such as stochastic genetic mutation of DNMTs, or that dietary factors may only contribute partially to the pathogenesis of global demethylation. Finally, as suggested by data within the current study, consumption of dietary fibre may actually be associated with decreased methylation although this would contradict the consensus evidence based on much larger studies. (Dagfinn, Doris et al. 2011; WCRF/AICR. 2012) It must be noted that the measurement instrument used in the exploratory analysis of LINE-1 methylation earlier was dietary fibre as defined by the Englyst method which estimates all dietary fibre and not RS specifically. (Englyst, Kingman et al. 1992) Also the previous analysis was underpowered for the number of associations examined so results must be treated with caution (see section 5.1.6). More definitive information would be gleaned from repetition in a larger independent data set.

JAG1 methylation was not affected by RS intervention in the DISC study. PD supplementation caused an increase in methylation levels at all CpG sites measured in the assay, an effect which was statistically significant at site 4 (3.1 (PD) vs. 1.7% (control), post-treatment values with pre-treatment values as control, p=0.009) (Table

4-8) An increase in methylation was hypothesised for a protective effect on an oncogenic gene. No other data are available on dietary effects on NOTCH signalling genes or methylation changes in response to PD specifically but other studies have shown that gene specific methylation can be modulated by RS intervention over a short intervention period of two weeks. (Dronamraju, Coxhead et al. 2009) It can reasonably be expected that one intervention can have an effect independent of another (or in conjunction with another) as different RSs have very different fermentation profiles, SCFA release and prebiotic effects. (Beards, Tuohy et al. 2010)

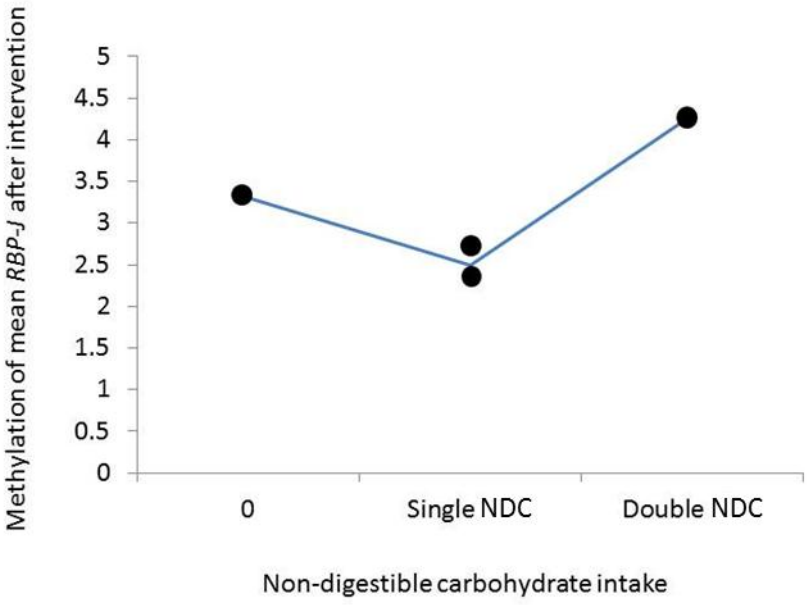
No effects of either intervention agent individually were detected on *RBP-J* methylation at any CpG site or for mean *RBP-J* methylation (Table 4-9). However there was a consistent pattern across all CpG sites for an interaction between RS and PD which was statistically significant at CpG site 2 ($p=0.017$). Mean *RBP-J* methylation interaction values are shown in Table 4-10. Higher methylation was present for double intervention (3.3%) and double placebo groups (3.1%) and lower values for Hi-maize 260 (2.4%) and PD groups (2.6%) ($p<0.05$ after removal of outliers).

Given the action of *RBP-J* as an oncogene, a similar direction of effect (increased methylation) would be expected for *JAG1* if RS or PD confers a protective effect for CRC. What has been observed is actually a curvilinear response to overall NCD dose with decreased methylation in response to a single agent and return to baseline (control group values) with double intervention. (Figure 4-3) The simplest explanation of these results is that they represent a type I error, although this seems unlikely given the reproducibility of the pattern between CpGs.

Another potential factor that could explain the results would be an unexpected placebo effect. The analysis model used does not include prospective analysis to detect a placebo effect although a post-hoc paired t-test of before and after values suggests that there was not significant change in methylation of the placebo group ($p=0.059$). If it is assumed that the placebo has no effect, then it would be concluded that individual interventions decrease methylation in an unexpected fashion and, when combined, the intervention agents cancel out each other's effect. The potential harmful effect of single agent intervention with RS or PD is unexpected and would need to be further

examined with estimation of intermediary products such as RNA and protein to see if the methylation change observed translates into a detectable change in the transcriptome or proteome. Cancellation of the effect with double intervention may be mediated by changes in the colonic microbiota in response to differing doses and types on NDC. There are previous data showing that different RSs and RS combinations have specific effects on colonic microbiotal populations (Martínez, Kim et al. 2010; Abell-J, Christophersen et al. 2011) and also that specific methods of delivery of butyrate to the colorectum have differing degrees of protective effect in terms of cancer development in carcinogen treated rats. (Clarke, Topping et al. 2008; Clarke, Young et al. 2012) Stool samples have been collected before and after intervention from all the DISC study participants and analysis of the microbiotal populations is planned as part of the DISC project. These analyses may be informative in suggesting whether changes in microbiotal colonisation of the colon mediate the cancellation effect. Confirmation of results in an independent data set would also be required validate these results.

FIGURE 4-3. GRAPHICAL REPRESENTATION OF *RBP-J* METHYLATION IN RESPONSE TO INTERVENTION AGENTS IN THE DISC STUDY.



4.4 SUMMARY OF RESULTS FROM THE DISC STUDY

The DISC study (intervention) recruited the anticipated number of participants and inclusion and exclusion criteria selected a group who were unlikely to be at higher risk of CRC than the general population despite sampling a symptomatic population. The trial was acceptable to participants with low drop-out rates and fulfilled the criteria for a high quality RCT unlikely to be affected by bias.

PD caused a small but significant (0.7cm, p=0.039) decrease in thigh circumference compared with controls without change in other anthropometric variables. This could suggest that body fat has redistributed in response to PD supplementation but measurements to confirm this were not taken within the DISC study.

JAG1 methylation was unaffected by RS supplementation. PD supplementation caused a trend towards increased methylation at all CpG sites compared to control which reached statistical significance at CpG site 4. This is the expected direction of effect for a protective agent and demonstrates plasticity of *JAG1* methylation in response to PD dietary supplementation.

RBP-J methylation was not changed by either intervention agent. An interaction effect was noted with lower methylation in groups supplemented with a single NDC and higher in those receiving double intervention. This curvi-linear dose-response relationship appears to show a potentially harmful effect of single NDC supplementation and cancellation when double NDC dose was consumed.

CHAPTER 5. DISCUSSION

5.1 STRENGTHS AND LIMITATIONS

5.1.1 BASELINE STUDY

The baseline study describes the methylation between groups at differing risk of CRC and potential environmental factors affecting methylation. Two main analyses were undertaken; one investigating variation in NOTCH gene methylation in response to disease states that increase risk of CRC (adenomatous polyps and UC) compared to normal risk controls. The second main analysis investigated environmental exposures that may predict gene methylation in a population at normal risk of CRC. Such analyses of exposures and biomarkers are retrospective by nature meaning that associations can be defined but causality cannot be proven. However the practicalities of any prospective design, long time scale and large number of participants, made case-control the pragmatic choice for this project. In this study all groups were recruited from the same geographical and socio-economic population over the same time period and measurements were performed by the same study team minimising the risk of systematic biases being introduced.

When investigating the different methylation of NOTCH genes between higher and normal CRC risk groups a sample size calculation was not possible due to the exploratory nature of the study and the absence of data describing normal baseline methylation of NOTCH genes or anticipated difference in methylation in the higher risk CRC population. Therefore type I and type II statistical errors could have been made. The potential setting for type II errors (accepting a null hypothesis when it is false) within this study mostly concerned gene promoters with a mean methylation of less than 5% in the normal risk population. Although biologically significant demethylation may occur in response to increased risk of CRC at these CpG loci, very large samples sizes would be required to detect small absolute changes in methylation. If further data were to suggest that these CpGs are relevant in carcinogenesis then other

methods of methylation estimation would be appropriate to quantify such low level methylation accurately such as methylation specific PCR. (Belshaw, Elliott et al. 2008)

Where between-group differences have been detected in methylation of gene promoters and in whole genome methylation, these data were usually normally distributed so the use of parametric tests is valid. Where data were not normally distributed, significant differences detected using parametric tests have been confirmed using equivalent non-parametric tests (Kruskal-Wallis). In addition, within-group homogeneity of data is reassuring for generalisability of conclusions to the relevant population even when the sample size is small (as for the UC group). Confidence in the validity of results is also increased by the high correlations observed between CpGs on the same gene promoter as this is expected to be greater than 0.6 for most genes. (Bell, Pai et al. 2011) Whilst these factors are reassuring that the observed data represent real differences between those at normal and higher risk of CRC, validation can only be achieved with replication in an independent data set.

5.1.2 INTERVENTION STUDY - THE DISC STUDY

The DISC study aimed to determine the effects of the dietary intervention on putative biomarkers of CRC risk in a population of adults at normal CRC risk. Therefore, when designing this intervention study, considerable care was taken to be explicit about the inclusion and exclusion criteria to be applied before commencing recruitment to the study. Exclusion criteria were selected to minimise confounding issues which could affect study outcome measurements (eg. aspirin or other NSAID use and inflammation parameters) or result in participants at higher CRC risk, above that of the normal population, being included in the normal risk group (eg. by fulfilling the Amsterdam criteria for HNPCC). However two issues of generalisability of the DISC study outcomes to a wider normal risk population remain. The first issue is the extent that selection from a symptomatic population represents a truly ‘normal’ population. Data collected on indications for colonoscopy, final result and random histology are reassuring. A high proportion of the study sample had a final diagnosis which was not consistent with any increase in CRC risk or colorectal mucosal disease, such as haemorrhoids or anal fissures. Where biopsies were taken (27%), these were reported as normal and no

participant was excluded on the basis of these results. The final endoscopic diagnosis was listed as 'normal' with no histology for 17 participants (23%). Although no formal diagnosis has been made, it is likely that a high proportion of this group will be suffering from irritable bowel syndrome which has no association with increased CRC risk. (Norgaard, Farkas et al. 2011)

The second issue of generalisability results from the large attrition between invitation to enter the study and recruitment of participants. Overall 88 participants were randomised from 1508 invitations. Whilst a large percentage was due to pre-defined exclusion criteria, many simply refused to participate. Data are not available to suggest if, or how, this may affect the findings of the study. Studies on CRC screening have shown that patients are more likely to take up invitations using less invasive tests and a personalised invitation strategy.(Brouwers, De Vito et al. 2011; Hewitson, Ward et al. 2011) In this study rigid sigmoidoscopy represented the least invasive method of obtaining tissue for repeat samples and avoided the need for repeat bowel preparation. Potential surrogate markers such as stool and buccal cells were collected as part of the DISC study. Previous studies have shown these surrogates do not accurately replicate mucosal methylation patterns (Elliott, Johnson et al. 2012; Staley, Bradburn et al. 2012) and wide tissue specific variation in methylation is known to exist. This meant that such surrogates were not suitable to replace more invasive direct mucosal sampling. (Thompson, Atzmon et al. 2010; Liang, Song et al. 2011) Invitations were personalised to attempt to maximise participation. (Appendix 2) Recommendations about whether and how participation could be improved in future studies cannot be made as ethical approval did not allow recording reasons for refusal.

The DISC study is the largest RCT investigating the effects of RS and PD supplementation on colonic biology in people at normal risk of CRC. Previous studies using RS supplementation or placebo control in healthy humans recruited 17 and 12 participants in a cross-over design. (Wacker, Wanek et al. 2002; Worthley, Le Leu et al. 2009) The DISC study was a randomised controlled trial (RCT) using appropriate allocation concealment and double-blinding until completion of analysis, thus representing a high quality trial unlikely to be affected by bias. Well-conducted RCTs,

such as the DISC study, represent the only way to define a cause and effect relationship.

5.1.3 BIOLOGICAL SAMPLES

Biological sample collection from participants was >95% overall. Audit of samples revealed that 100% of collected samples were traceable. Samples that were not obtained were due to non-systematic factors such as loss of samples at external laboratory or inability of a participant to tolerate rigid sigmoidoscopy and were balanced equally between baseline and follow-up visits and between intervention groups. Appropriate quality and quantity of DNA was able to be extracted from all tissue samples and all blood samples received by external laboratories were suitable for analysis.

Estimations of any biomarker using pinch biopsies from the rectum are subject to the effect on an assay of non-mucosal tissue collected within the biopsy such as: stromal tissue, smooth muscle, blood vessels and blood cells which may display different biological characteristics. As epigenetic marking is tissue specific this may confound results obtained. (Thompson, Atzmon et al. 2010; Liang, Song et al. 2011) However the majority of the tissue within these biopsies is colorectal mucosa and so the results are likely to describe this tissue sufficiently.

Mucosal samples were obtained from the mid-rectum in all cases. This was a pragmatic decision based on two considerations. The first is the need to avoid potential confounding due to differences in biomarkers between different sites in the large bowel. For example, previous work from this group has shown significant, and systematic, differences in crypt cell proliferation and differential expression of WNT genes in mucosal biopsies collected from different anatomical sites in the human colo-rectum. (Mills, Mathers et al. 2001; Malcomson, Willis et al. 2012) Second was the need to use rigid sigmoidoscopy for collection of repeat biopsies due to cost implications of a second formal endoscopy, acceptability of repeat bowel preparation and endoscopy (and consequences for recruitment) and possible confounding effects that bowel preparation may have had on study outcomes.

Sampling from mid-rectum, 10cm from the anal verge ensured reproducibility of sample location between patient visits and different endoscopists as estimated location at points more proximal to this has been shown to be highly variable between endoscopists. (Vignati, Welch et al. 1994)

The rectum is the site where most CRCs and adenomatous polyps are found and so represents an informative site to sample to detect biological effect that may affect CRC risk. (Granqvist 1981; Green 1983) In UC patients the rectum provides an ideal sampling location as the rectum is involved in all cases of UC. In polyp patients the fixed sampling site may not describe the magnitude of change at the site of the disease process, which may be significantly proximal to the sampled site. Whilst additional sampling at the site of disease would add interesting data, potentially showing progressive methylation change, we hypothesised that epigenetic change would form part of a field change. The homogeneity of results in the rectal mucosa of the polyp group strongly suggests that such a field change is present. This supports the results of other studies where epigenetic field change has been confirmed in epithelium at higher risk of CRC. (Suzuki, Gabrielson et al. 2002; Shen, Kondo et al. 2005; Belshaw, Elliott et al. 2008; Milicic, Harrison et al. 2008)

5.1.4 METHYLATION ASSAY DESIGN

Within this study quantitative methylation assays were based on gene promoter regions that had been experimentally verified. Standard selection parameters for genomic areas likely to be subject to transcriptional regulation by methylation (CpG islands within experimentally verified promoter regions) were used; GC content >0.5 and observed to expected ratio of CpGs>0.6. (Gardiner-Garden and Frommer 1987; Feinberg and Tycko 2004) There are data that suggest that that regions upto 2kb upstream of promoters, known as CpG shores, may show greater variation in methylation in response to disease risk. (Irizarry, Ladd-Acosta et al. 2009) The recently published data from the ENCODE project also suggest that the greatest degree of gene methylation variation occurs in minimally methylated, intergenic (regions also known as CpG 'open sea') and exonic regions. Mechanistic processes of transcription control have been suggested, though not definitively proven, such as recruitment of P300 as a

transcription factor. The ENCODE data were not available when the current study was being undertaken and all data had been collected before the ENCODE publications on 5th September 2012. (ENCODE 2012)

Analysing these CpG shores or open sea using pyrosequencing would not be possible due to the practical limitations of pyrosequencing. This is due the disparity of to the wide regions that would need to be sequenced and the low frequency CpGs within them, and the reliable sequencing abilities of a pyrosequencer (typically <100 bases). Whilst studies have raised the possibility that transcription is more closely associated to intergenic or exonic genome methylation rather than gene promoter methylation, (Brenet, Moh et al. 2011; ENCODE 2012) these data also confirm a high correlation between promoter methylation and transcription (Brenet, Moh et al. 2011) a finding that replicates that of multiple other studies. (Hsieh 1994; Lorincz, Schübeler et al. 2002; Chevalier-Mariette, Henry et al. 2003) Thus a 'standard' assay design using experimentally verified promoters and CpG islands within these promoters was adopted in the DISC study. As such assays were practical to carry out and highly likely to be biologically relevant i.e. correlate with transcription.

5.1.5 SCREENING OF GENES FOR SELECTION TO BE ANALYSED ON THE COMPLETE DISC STUDY SAMPLE

The 'screening' of NOTCH gene promoters was designed to inform the selection of genes for the main study using a small sample of high vs. normal risk of CRC participants. Where different levels of methylation between groups were found this would suggest that a gene should be analysed in the full study population. Perhaps unsurprisingly, not all statistically significant results in the screening sample were replicated in the complete DISC study population suggesting that the small sample size increased the risk of type 1 errors at this stage. However a higher α value was applied when analysing these data and decisions made in conjunction with known biological effect and assay reliability (section 3.6.1.1). Overall, the screening study was particularly effective in identifying gene promoters which were essentially unmethylated in both those at normal and at higher CRC risk and, therefore, which are unlikely to be informative, allowing them to be rejected from the main study.

5.1.6 STATISTICAL ANALYSIS

Issues around the selection of parametric and non-parametric tests are discussed in section 2.10. Data suggesting statistically significant differences between groups were either normally distributed and thus suitable for parametric testing or not normally distributed but not suitable for transformation and non-parametric-testing was used to confirm significant results.

When considering dietary and lifestyle factors contributing to global and gene specific methylation the study is likely to be under-powered. The determination of sample sizes for multiple linear regression are defined by acceptable type I and II error rates (usually α-0.05, power-0.8) and the multiple correlation coefficient (R) – the number of variables included in the regression equation. For statistically robust results, sample sizes would have to have been much larger for *JAG1* (n≈595) and *RBP-J* (n≈252) and slightly larger for LINE-1 (n≈105). (http://www.stattools.net 2012) Although the sample sizes are small for regression analyses they are comparable to the size of other studies reporting epigenetic epidemiological associations recently reviewed by Hou et al.. (Hou, Zhang et al. 2012)

Overall these data can be viewed as exploratory only. Whilst increasing numbers in the sample would strengthen confidence in the validity of the observed associations, proof of an association would require replication in an independent data set.

5.2 ISSUES IN EPIGENETIC EPIDEMIOLOGY

5.2.1 CAUSE AND EFFECT RELATIONSHIPS

Within any epidemiological study associations between exposures and outcomes are subject to confounding and the direction of cause and effect can be difficult to ascertain. The direction of cause and effect can be rationalised logically, but not proven, in many cases. Reverse causation can be problematic in some epidemiological studies (Smith, Timpson et al. 2008) but it seems unlikely that epigenetic modifications in the genes studied here would have affected dietary and lifestyle choices. There are also data using in *in vitro* and in *in vivo* models showing the temporal relationship of

changing exposure to RS or butyrate followed by methylation change. (Dronamraju, Coxhead et al. 2008; Dronamraju, Coxhead et al. 2009; Worthley, Le Leu et al. 2009) The DISC study has also supported this temporal relationship by demonstrating a change in methylation of *JAG1* after dietary supplementation with PD as part of a high quality RCT.

Whilst the cause and effect relationship of environmental exposures and methylation can be rationalised the same is not true for differences in methylation observed between higher risk of CRC groups and normal controls. When describing these associations many other factors may moderate the different epitypes observed including: germline epigenetic variation, genetic mutation with an associated epigenetic signature, intra-uterine exposure and inheritance of the epigenome across generations reflecting distant environmental exposure. (Relton and Davey Smith 2012) Additionally the final biological effect of altered methylation in response to higher CRC risk cannot be defined without further examination or intermediary products of transcription and translation. However, the likely effect on transcription of a gene can be implied by the known relationships of gene promoter methylation and transcription in other genes. However the precise change in transcription levels for any degree of change in methylation cannot be assumed for an individual gene as methylation may be the dominant factor regulating transcription or may only play a small role in conjunction with other regulatory pathways. Thus the contribution of an epitype to the known phenotype within a group is difficult to quantify. (Relton and Davey Smith 2012)

Consideration must also be given to the potential that epigenetic traits may not be causal and may simply mark at-risk cells as a result of another biological mechanism. Recent ENCODE data has even suggested that a proportion of methylation change may actually occur passively in response to an absence of transcription factors in promoter regions of the genome. (Thurman, Rynes et al. 2012) Whilst potential plasticity of epigenetic responses makes them attractive therapeutic or chemopreventative targets, there is no therapeutic value in a non-causal epigenetic trait. However such a trait would have value as a biomarker of risk and may be useful to risk-stratify subjects using less invasive means than currently available.

5.2.2 STRATEGIES TO DEFINE CAUSALITY IN EPIGENETIC EPIDEMIOLOGY

The only experimental method that truly defines causation without confounding is a well-conducted RCT such as the DISC study as the process of adequate randomisation accounts for confounders and a temporal relationship of cause and effect can be defined. However RCTs are expensive, time consuming and may expose recruited subjects to an element of risk. Thus an RCT is the correct strategy to define a relationship where strong lower level evidence exists to support a single intervention as is the case with RS and PD. However, when analysing life-course effects of multiple exposures, potentially with interacting effects, this becomes unrealistic and other strategies must be employed.

Several other strategies exist to strengthen the likelihood of causality. These may include: demonstrating no association with implausible similar exposures, defining a biologically plausible link, measuring intermediary steps from epitype and phenotype eg. RNA or protein expression, extrapolation of animal data into humans, measurement of surrogate outcomes or linking exposures to population behaviour such as in migration studies.

Mendelian randomisation has been proposed as an additional method to link life-course exposures to outcomes whilst negating confounding effects. It relies on the random distribution of allelic variants across the population and the immunity of genetic variance from confounding influences. In this method an instrumental genetic variant highly correlated with the exposure of interest but, critically, not actually or potentially linked to the outcome of interest, is examined for an association. If the association with the 'instrumental' genetic variable with the outcome of interest is the same as the association of the exposure and outcome, then this indicates a causative link between the exposure and outcome. Genes linked with exposures are not randomly selected but often define level of exposure. An example of a useful instrumental variable gene in dietary linkages is *HFE.* This gene is involved in iron absorption and is therefore linked to serum iron concentrations implicitly (mutated *HFE* leads to low iron absorption and low serum iron concentrations). A link with *HFE* and cancer would strengthen causation in a previously defined association between

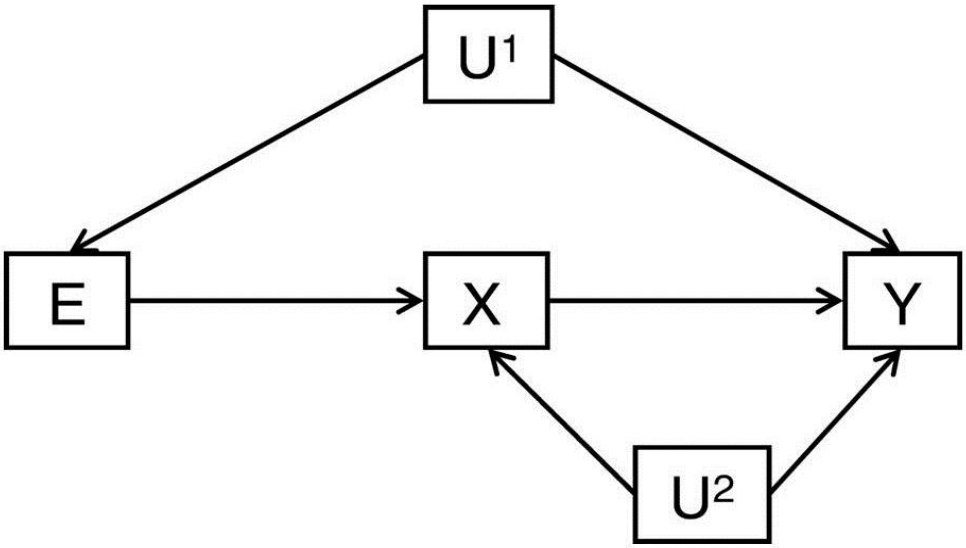
serum iron concentration and cancer. This could not be due to a confounder effect as *HFE* has no role itself in carcinogenesis. (Schatzkin, Abnet et al. 2009) Mendelian randomisation therefore represents a useful tool to define causation in epidemiology but is often limited by the lack of appropriate genetic instrumental variables. If genetic instrumental variables are available an inadequate variation either of the gene or of the outcome may make analysis complex, requiring studies with large numbers of participants. Additionally this approach maybe confounded by effects of other genetic variants associated with the outcome of interest (linkage disequilibrium) and unknown biological effect of the gene of interest on the outcome (pleiotropy). (Schatzkin, Abnet et al. 2009)

Use of the principle of Mendelian randomisation to link a single environmental exposure with an epigenetic trait and the epigenetic trait with an outcome adds an additional layer of complexity. Each link must be proven to be independent of confounders individually, so a more complex process – two-step Mendelian randomisation – has been proposed. The underlying principle is the same as Mendelian randomisation however separate instrumental genetic variables are required for both associations (Figure 5-1), increasing the complexity of experimental work. (Figure 5-2) (Relton and Davey Smith 2012) Sample sizes for such studies depend on the variation of each component in the causal relationship and the variation in instrumental genetic variables and are likely to be very large. (Schatzkin, Abnet et al. 2009)

In this study an exploratory multiple linear regression approach was used to describe potential effects of multiple dietary and lifestyle factors on methylation of selected genes. An RCT was used to define the relationship of RS and PD on methylation of these genes as this environmental exposure has very strong evidence linking it to CRC risk. (section 1.12.4)

FIGURE 5-1. DIAGRAMATIC REPRESENTATION SHOWING POSSIBLE CONFOUNDING FACTORS IN A RELATIONSHIP BETWEEN AN EXPOSURE AND OUTCOME.

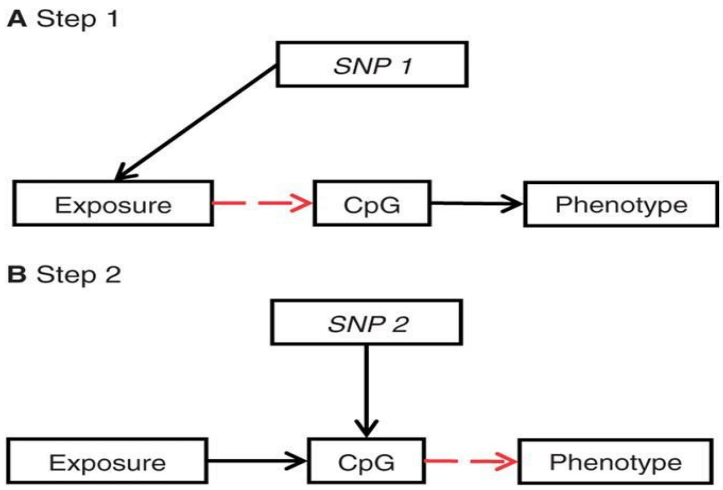
REPRODUCED WITH PERMISSION. (RELTON AND DAVEY SMITH 2012)



A MODIFIABLE CAUSAL RISK FACTOR [E] FOR DISEASE [Y] EXERTS ITS CAUSAL EFFECT (AT LEAST IN PART) VIA THE EFFECT OF E ON X (THE MEDIATOR) AND THROUGH THE CAUSAL EFFECT OF X ON Y. U1 AND U2 REPRESENT ALL CONFOUNDERS FOR THE ASSOCIATION OF E WITH Y AND X WITH Y, RESPECTIVELY. U1 AND U2 CAN INCLUDE DIFFERENT CHARACTERISTICS.

FIGURE 5-2. DIAGRAMATIC REPRESENTATION OF TWO STEP MENDELIAN RANDOMISATION TO DEFINE A CAUSAL RELATIONSHIP LINKING AN EXPOSURE, AN EPIGENETIC TRAIT AND AN OUTCOME.

REPRODUCED WITH PERMISSION. (RELTON AND DAVEY SMITH 2012)



GENETIC VARIANTS ARE USED AS INSTRUMENTAL VARIABLES IN A TWO-STEP FRAMEWORK TO ESTABLISH WHETHER DNA METHYLATION IS ON THE CAUSAL PATHWAY BETWEEN EXPOSURE AND DISEASE. (A) FIRST, A SINGLE NUCLEOTIDE POLYMORPHISM (SNP) IS USED TO PROXY FOR THE ENVIRONMENTALLY MODIFIABLE EXPOSURE OF INTEREST AND (B) SECONDLY, A DIFFERENT SNP IS USED TO PROXY FOR DNA METHYLATION LEVELS

5.3 MAIN FINDINGS

5.3.1 COMPARISON OF HIGHER VS. NORMAL RISK OF CRC PARTICIPANTS

Baseline values for anthropometric, dietary and blood values were equivalent between groups except for the polyp group being older, as expected, and a higher hsCRP in the polyp group. Higher levels of CRP have been found in both cancer and polyp patients in other studies (Allin and Nordestgaard 2011) and evidence from a large longitudinal cohort study suggests that CRP is a causative exposure. (Prizment, Anderson et al. 2011)

Global methylation was found to be decreased at all CpGs in the LINE-1 assay for UC patients compared to normal controls. This is only the second study to report such data and the only to use a fully quantitative analysis of LINE-1 methylation. (Glória, Cravo et al. 1996)

In this study differential effects were noted at the CpG sites within the LINE-1 assay between polyp and normal controls. This suggests that the usual style in the literature of reporting mean LINE-1 methylation may be inaccurate and may mask important relationships. Defining specific CpG methylation may help to promote understanding of LINE-1's direct influence on CRC carcinogenesis and on other disease processes where global hypomethylation is implicated. (Beck, Garcia-Perez et al. 2011)

Gene specific methylation was found to be altered in both of the genes examined (*JAG1* and *RBP-J*) between high risk of CRC groups and normal controls. For *RBP-J* significantly decreased methylation was observed at one CpG site (site 11) in polyp patients compared to normal controls, whilst the other CpGs were minimally methylated. Methylation of CpG 11 was able to predict entry into disease severity classifications within the polyp group. For *JAG1* all group testing did not demonstrate significant changes but a trend towards decreased methylation was seen at all CpGs (except CpG site 4) in the assay for polyp patients compared to normal controls. The direction of effect was as anticipated for oncogenes and although absolute changes were small, relative changes were large. Promoter methylation is known to correlate

with gene transcription but precise quantification of the methylation effect would require measurement of transcription or translation. (Bell, Pai et al. 2011)

In this study, the best panel of biomarkers (age, LINE-1 methylation and *JAG1* methylation) demonstrated a high specificity to define normal patients from those with adenomatous polyps but a low sensitivity making the discriminatory power of the test unsuitable for clinical practice. Other studies have reported potential blood based biomarkers for colorectal adenomas but predictive value was also unsuitable for clinical practice with low sensitivity (50%) and specificity (65%). (Cassinotti, Melson et al. 2012) Few other studies have described changes in normal epithelium in patients with colorectal adenomas. One study examined methylation of 15 genes and found that no individual gene predicted polyp patients from normal patients. Combination modelling using 10 factors only had an area under the curve (AUC) of 0.66 making it unsuitable for clinical use. (Worthley, Whitehall et al. 2010) Belshaw et al. were not able to differentiate polyp patients from normal subjects with reasonable accuracy using a methylation panel of 6 (*HPP1*, *APC*, *SFRP4*, *p16*, *ESR1* and *WIF1*) (sensitivity 38%) or 3 genes (*SFRP4*, *SFRP5* and *WIF1*) (sensitivity 61%). (Belshaw, Elliott et al. 2008) In a non-quantitative study by Ye et al. methylation of *CDKN2A/p16*, *hMLH1*, and *MGMT* were not able to predict polyps in around 200 cases and controls. However there were multiple epigenetic changes between normal tissue from polyp patients and patients at normal risk of CRC. (Ye, Shrubsole et al. 2006) More refined panels are being developed with a six gene methylation panel comprising *CNRIP1*, *FBN1*, *INA*, *MAL*, *SNCA*, and *SPG20* able to distinguish normal from polyp with 93% sensitivity and 98% specificity (AUC 0.984) and normal from cancer with 94% sensitivity and 98% sensitivity (AUC 0.986). (Lind, Danielsen et al. 2011) However this panel used tumour tissue, not normal rectal mucosa, so larger differences between actual neoplastic disease and normal methylation levels can be anticipated. If such methylation assays could be replicated in normal epithelium and detected in stool or other surrogates then accurate, non-invasive estimation of CRC risk may be possible without direct visualisation of the colon.

5.3.2 RESULTS OF THE INTERVENTION (DISC) STUDY

The DISC study results showed that LINE-1 (global) DNA methylation was not changed by a fifty day dietary intervention with either RS (Hi-maize 260) or PD. This suggests that either LINE-1 methylation is not modulated by RS intervention or that such modulation may occur over a longer time period. *JAG1* methylation did change in response to PD dietary intervention in the direction hypothesised for that of a protective effect. Whilst this occurred at all CpGs within the assay it was only statistically significant at one CpG (site 4). These data show that *JAG1* methylation is plastic to PD supplementation and that PD confers the hypothesised protective effect. Both RS and PD supplementation appeared to decrease *RBP-J* methylation (an unexpected potentially harmful effect) in isolation but this effect was cancelled with double intervention. This trend was replicated across all CpGs in the assay but was only significant at CpG site 2 and for mean *RBP-J* methylation. Such consistency suggests that this is not a spurious finding and represents a genuine effect of intervention. These results were unexpected and further analysis of stool samples collected from participants to describe bacterial population change in the colon may clarify potential mechanisms by which this effect occurred.

5.3.3 IMPLICATIONS IN COLORECTAL CANCER BIOLOGY

NOTCH signalling is known to be involved in CRC and offers a new potential therapeutic target through its actions on ASCs and CSCs. (Reedijk, Odorcic et al. 2008; Sikandar, Pate et al. 2010; Zhang, Li et al. 2010) No genetic mutation currently accounts for changes in NOTCH ligand expression observed in human CRC. (Lee, Jeong et al. 2007) If epigenetic means were found to control NOTCH signalling this could offer potential new chemopreventative and therapeutic strategies.

Studies have suggested activation of NOTCH via a direct effect of WNT pathway activation. (Rodilla, Villanueva et al. 2009) This study suggests an alternative mechanism of activation for both *RBP-J* and *JAG1* with decreased promoter methylation observed in higher risk groups of CRC. These changes occur in at-risk

epithelium, distant from the macroscopic pathology for polyps or independently of disease activity in UC, suggesting utility as biomarkers of at risk epithelium that has been subject to carcinogenic field change. If further work demonstrates that methylation levels do drive transcriptional regulation of NOTCH oncogenes, epigenetic therapeutic targets may be identified.

This study demonstrates that *JAG1* methylation is modifiable by PD intervention over a short time period at acceptable doses. *JAG1* would therefore be a potential therapeutic target as plasticity to short term intervention is proven, it is over expressed in CRC and it may moderate the link between WNT and NOTCH signalling (section 1.9.5). (Reedijk, Odorcic et al. 2008; Pannequin, Bonnans et al. 2009; Rodilla, Villanueva et al. 2009)

5.4 FUTURE RESEARCH

5.4.1 DEFINING THE ROLE OF NOTCH IN COLORECTAL CARCINOGENESIS

Whilst the role of WNT signalling is well described in CRC the specific actions and temporal relationship to phenotypic change, of the NOTCH pathway are largely unknown. NOTCH is clearly an integral pathway in CRC carcinogenesis due its role in stem cell maintenance and daughter cell differentiation in the colorectal epithelium. Whilst complete removal of NOTCH signalling has been shown to obliterate the stem cell compartment (van Es, van Gijn et al. 2005) more subtle, physiological, manipulations remain to be described. Increased expression of various NOTCH components has been described in CRC (Reedijk, Odorcic et al. 2008; Veenendaal, Kranenburg et al. 2008) but similar work in pre-malignant lesions is currently lacking.

5.4.2 DEFINING THE REGULATION OF NOTCH SIGNALLING

Control mechanisms of NOTCH pathway activation remain to be fully defined. Data suggests that genetic mutation does not account for changes in ligand expression. (Lee, Jeong et al. 2007) There does appear to be a degree of crosstalk between WNT and NOTCH. (Pannequin, Bonnans et al. 2009; Rodilla, Villanueva et al. 2009; Katoh 2011) Although the precise mechanism for activation has not been defined it seems

likely that *JAG1* may have an important role. Defining the precise role of *JAG1* in CRC carcinogenesis, in particular its role in linking NOTCH to WNT, could lead to discovery of a new therapeutic target.

This study has shown a degree of plasticity for *JAG1* to a fifty day PD intervention. Epigenetically mediated alterations in gene expression hold the potential to be manipulated by alterations in diet or by drug therapies. Dietary manipulation has greater utility in chemoprevention and further exploration of epigenetically mediated changes in carcinogenesis and their relationship to dietary exposures may improve knowledge of healthy eating. Future interventions would have to be designed so that they are acceptable and sustainable for users over long time periods perhaps by including PD into dietary products as a functional food. The difficulty of relating life course exposures to epigenetic change and outcome are discussed in section 5.2.2. Studies on the effect of dietary exposures by 2 step Mendelian randomisation or RCTs would have to be large to be adequately powered. Further work to define the role of NOTCH (section5.4.1), widen the panel of NOTCH genes examined for methylation variation (specifically *NOTCH3*) and define the relationship of methylation traits to intermediate molecular measures of expression (RNA and protein) would seem prudent initial steps before embarking on such studies.

5.4.3 MANIPULATION OF NOTCH SIGNALLING

Γ-secretase inhibitors are potent suppressors of NOTCH signalling as cleavage of the NICD is a γ-secretase dependent process. The potential utility of γ-secretase inhibitors was first realised for Alzheimer's disease where accumulation of amyloid plaques in the brain, pathognomic of this disease, is a γ-secretase dependent process. However early animal and human trials failed due to side-effects of NOTCH inhibition in the gastrointestinal tract such as bleeding and immunosuppression. (Searfoss, Jordan et al. 2003; Karran, Mercken et al. 2011) Subsequently, more specific inhibitory agents have been developed.

In breast cancer NOTCH molecular signatures predict prognosis as they do in CRC. (Reedijk, Odorcic et al. 2005) Γ-secretase inhibitors have been shown to decrease proliferation and prevent metastasis of breast cancer cells *in vitro*. (Zavadil, Cermak et

al. 2004) Pre-clinical studies suggest that NOTCH inhibition may potentiate the effect of monoclonal antibodies in the treatment of breast cancer. (Pandya, Meeke et al. 2011) Pre-clinical studies have also shown that there is potential to inhibit NOTCH signalling within other epithelial cancers specifically using monoclonal antibodies (Wu, Cain-Hom et al. 2010) and in CRC models. (Fischer, Yen et al. 2011) Currently Roche has ten agents in phase I clinical trials, Merck has nine agents and Pfizer a single agent to inhibit NOTCH signalling in a variety of cancers. (Groth and Fortini 2012)

5.4.4 DIFFERENTIATION THERAPIES FOR CRC TREATMENT

The result of NOTCH inhibition may actually result in a differentiation therapy. Differentiation therapies differentiate radio-resistant or chemo-resistant CSCs within a tumour, temporarily sensitising them to chemo or radio therapy. These approaches will become increasingly important as there is a consensus of evidence emerging that high expression of stem cells markers is related to poor prognosis, resistance to chemo and radiotherapy and recurrence in CRC. (Yasuda, Tanaka et al. 2009; Saigusa, Tanaka et al. 2011; Jao, Chen et al. 2012; Sprenger, Conradi et al. 2012)

Differentiation therapies are currently being experimented upon in breast cancer. (Harrison, Farnie et al. 2010; Pham, Phan et al. 2011) Potent differentiation effects and subsequent sensitisation to chemo and radio therapy have been observed on breast cancers when NOTCH signalling is reduced. (Harrison, Farnie et al. 2010; McGowan, Simedrea et al. 2011)

Yang et al. have shown that CD133 (a CSC marker) levels decrease in CRC cultures in response to nutrient restriction. (Yang, Wang et al. 2012) Nutrient restriction has been shown to have potent anti-cancer effects both *in vitro* and *in vivo*. (Rondini, Harvey et al. 2011) Although the authors of the paper have interpreted their data to suggest that CD133 is an unreliable stem cell marker, Yang et al. may have actually shown that the differentiation of stem cell populations in CRC cell lines is plastic in response to environmental changes. (Yang, Wang et al. 2012) To date there are no published data on manipulation of NOTCH signalling in CRC and its potential effect on stem cells. If NOTCH signalling could be suppressed and induce differentiation of CSCs this could result in improved responses to chemo and radio therapy, fewer cases of recurrent

disease and, in the case of rectal cancer, could decrease rates of surgery by increasing rates of complete pathological response after radiotherapy.

5.5 SUMMARY

NOTCH signalling plays a key role in maintenance of crypt homeostasis, particularly by maintaining ASC homeostasis. NOTCH-related genes are overexpressed in CRC but no mechanism for this has yet been described. ASC overpopulation is an early feature in CRC carcinogenesis and may be mediated by dysregulated NOTCH signalling. This project aimed to describe differences in methylation of NOTCH genes and global DNA methylation in populations at higher and normal risk of CRC, and the response of gene methylation to the provision of a potentially protective dietary supplement (RS or PD) as part of an RCT in normal risk participants.

The study recruited the anticipated number of participants and the intervention study fulfills criteria for a high quality RCT.

Between groups at higher and normal risk of CRC several differences in methylation were noted. This study is the second to report global hypomethylation in the normal mucosa of patients with longstanding UC, and is the first to do so quantitatively. Data showing differential responses of individual CpGs within the LINE-1 assay in patients with adenomatous polyps compared to controls suggest the usual practice of reporting mean LINE-1 methylation may not be informative. *JAG1* methylation was lower at 4 of the 5 CpGs tested in participants at higher risk of CRC but this was not statistically significant. *RBP-J* was minimally methylated overall at most CpGs but site 11 had higher levels of methylation and differences were observed between polyp and normal controls. Additionally methylation of CpG site 11 decreased progressively with increasing severity of polyp disease. The direction of effects noted was as anticipated for the hypothesised role of *JAG1* and *RBP-J* as oncogenes.

Dietary intervention with RS or PD did not change global methylation of DISC study participants. *JAG1* methylation did respond to PD supplementation with the anticipated protective direction of effect. *RBP-J* methylation results were unexpected with a potentially harmful effect noted with each single agent supplemented and cancellation of the effect when both were provided.

There are very few data describing the role of NOTCH signalling in CRC carcinogenesis or the factors controlling NOTCH signalling. However data to date suggest a critical role for NOTCH in ASC homeostasis and dysregulation of NOTCH leading to therapy resistant CSC phenotype. This makes NOTCH a promising future pathway for research. *JAG1* in particular holds promise a key functional regulator due to its documented overexpression in the majority of human CRCs and its potential key role linking both WNT and NOTCH.

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APPENDIX 1 – 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 2 – LETTER TO POTENTIAL STUDY PARTICIPANTS

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

Dear Mrs Thomson,

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 3 – EXCLUSION CRITERIA FOR POTENTIAL PARTICIPANTS

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 4 – 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 5 – TABULATED BASELINE LINE-1 METHYLATION DATA

LINE-1 METHYLATION VALUES BY GROUP (NORMALS, POLYPS & UC) FOR WHOLE STUDY SAMPLE.

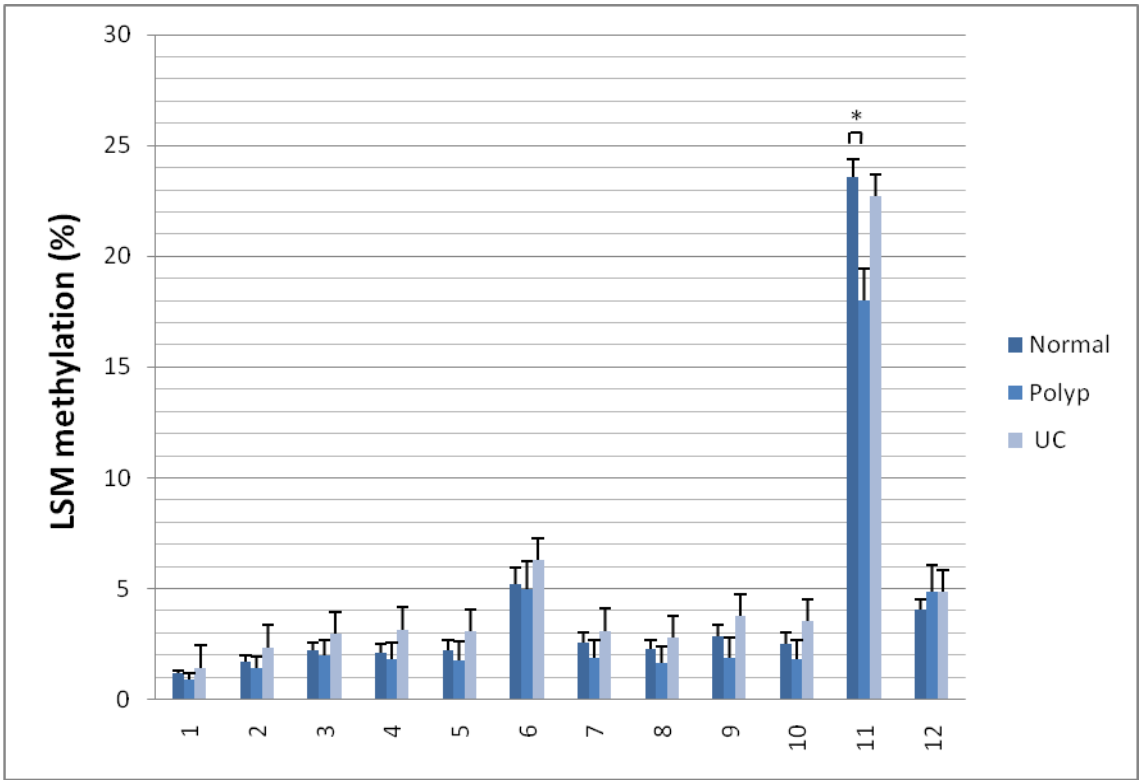
CpG site	Normal (n=91)	Polyp (n= 26)	UC (n=12)	P-value	Post-hoc test
	LSM (SEM)				
1	80.4 (0.4)	77.2 (0.7)	74.0 (1.0)	<0.001	UC < polyp < normal
2	65.1 (0.4)	67.1 (0.7)	60.5 (0.9)	<0.001	UC < normal < polyp
3	66.2 (0.3)	65.6 (0.5)	62.6 (0.3)	<0.001	UC < normal & polyp

APPENDIX 6 – TABULATED BASELINE *JAG1* METHYLATION DATA

***JAG1* METHYLATION VALUES BY GROUP (NORMALS, POLYPS AND UC) FOR WHOLE STUDY SAMPLE.**

CpG site	Normal (n=78)	Polyp (n=25)	UC (n=12)	P-value
	LSM (SEM)			
1	8.0 (1.0)	4.7 (1.9)	4.9 (2.6)	0.256
2	5.7 (0.8)	2.7 (1.4)	3.9 (1.4)	0.193
3	7.2 (0.7)	4.2 (1.3)	5.5 (1.8)	0.225
4	3.1 (0.3)	2.9 (0.5)	3.0 (0.6)	0.969
5	7.0 (0.9)	3.0 (1.6)	3.9 (2.2)	0.076

APPENDIX 7 – HISTOGRAM OF BASELINE *RBP-J* METHYLATION DATA



PERCENTAGE METHYLATION LEVELS AT CPGS 1-12 OF THE *RBP-J* PYROSEQUENCING ASSAY BETWEEN THOSE AT NORMAL RISK (N=75) OF CRC AND THOSE AT HIGHER RISK (POLYP (N=26) AND UC (N=12) GROUPS). *=P<0.05 USING GLM AND DUNNETT'S POST-HOC TEST FOR BETWEEN GROUP DIFFERENCES.