Do dietary induced pro-mutagenic DNA adducts increase risk for colorectal cancer?

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19 December 2014

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Summary

Colorectal cancer (CRC) is a major burden on public health in developed countries with high incidence and mortality rates globally. A major driving force of CRC is related to lifestyle factors, in particular dietary choices. Consumption of red meat has been identified as a risk factor for developing CRC by the World Cancer Research Fund. Increased DNA adducts in the colon via excess endogenous N-nitrosation is one mechanism thought to play a role in colorectal oncogenesis. Haem iron in red meat has also been implicated in development of CRC in humans. A dietary component that can protect against CRC, called resistant starch (RS), is the component of starch undigested in the small intestine and fermented in the colon. It is thought that preferential fermentation of carbohydrate over protein, when RS is incorporated into high protein diets, leads to a reduction in DNA changes that might initiate CRC. Green tea is a common beverage in East Asian countries and evidence from rodent and cell culture studies shows green tea as a preventative agent against CRC, although human studies are somewhat conflicting.

The global aim of this thesis is to determine whether dietary-induced DNA adducts by red meat consumption act as bio-markers for risk of CRC. The studies presented will endeavour to validate and extend previous studies demonstrating that red meat can induce pro-mutagenic adducts. Furthermore, RS and green tea will be employed in combination with red meat to ascertain any protective role they might have against pro-mutagenic formation in the colon. The risk of developing CRC with high red meat consumption will also be explored, and RS will be evaluated as a protective food against CRC formation. The hypotheses are that red meat will increase DNA adducts, but that RS and green tea consumption can reduce red meat-induced DNA adducts. Also, red meat and haem from red meat will increase risk for developing CRC, but RS will reduce the CRC risk posed by red meat. In addition to the mouse experiments, the effects of red meat

and red meat in combination with RS will be translated to the human setting, by feeding high red meat and high red meat with RS diets to healthy human volunteers. It is hypothesised that red meat will significantly increase DNA adducts in the colorectal tissue of humans consuming a high red meat diet, but that co-consumption with RS will ameliorate these adducts.

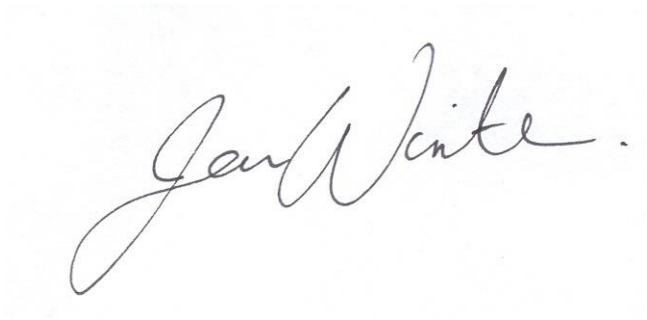
Red meat and haem increased DNA adducts of the distal colon in all mouse models and in human rectal epithelial tissue. However, there was no clear link between DNA adducts and risk for oncogenesis of the colon in the mouse models tested. RS increased fermentation of beneficial microbial metabolites, but reduced production of potentially toxic fermentation products. RS reduced proliferation rates in the distal colon of wild type and *Msh2* knockout mice, but this did not reduce pre-cancerous lesions in the colon. RS supplementation could reduce formation of pro-mutagenic adducts in wild type mice and in humans after short term consumption, but this did not translate over long term RS consumption in the Western diet mouse model. Green tea did not reduce DNA adducts either alone or in the presence of red meat, either in wild type or *MGMT* knockout mice.

In conclusion, chronic consumption of a high red meat diet can generate DNA lesions in colonic epithelial cells and RS consumption can ameliorate this affect in the short term, but this does not lead to consequent changes in risk after long term consumption in the mouse models tested. Consequently, dietary-induced DNA O^6MeG and 8-oxo adducts could perhaps be described as a marker for exposure to alkylating and oxidative agents in the diet, including red meat and its associated components such as haem, and not necessarily described as a bio-marker for CRC risk.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed:



Date: 18th December 2014

Acknowledgments

A big thank you to my supervisor and co-supervisors Richard Le Leu, Ying Hu and Graeme Young. During my PhD, and over the last 8 years, you have been extremely supportive, always encouraging and have taught me everything that I know today - without your guidance none of my successes so far would have been achievable. To my laboratory colleagues and technical staff (past and present), in particular Laura Nyskohus, Joanne Wilkins and Roshini Somashekar; together you all created a fun, exciting and team orientated environment, which made my time as a PhD student an enjoyable and memorable experience. Thanks to the clinical research nurses Jane Upton and Libby Bambacas, your help with the human intervention trial was outstanding. Appreciation to the collaborators involved in the projects, including scientists at the CSIRO in Adelaide, Silvia Gratz at University of Aberdeen, Maija Kohonen-Corish at Garvan Institute and Leona Samson at MIT in the USA. A special thanks to the School of Medicine Animal Facility Staff at Flinders University for their assistance with the animals.

To my parents Danny and Sue McShane and my sister Christine McShane for reviewing, proof reading and editing my thesis. To my entire circle of family and friends for your constant support and for always asking me "how's the PhD going?". Finally, the most important people in the world to me: Alex, Orlando and Emanuel - you are the drivers for me to achieve my potential. Without the unwavering support from my husband Alex, undertaking a PhD, writing a thesis and raising two young children would have been near impossible to do. I will forever be grateful to you, thank you.

Abbreviations

8-oxo	8-hydroxy-2'-deoxyguanosine
AAR	Acute apoptotic response
ACF	Aberrant crypt foci
AIHW	Australian Institute of Health and Welfare
AIN	American Institute of Nutrition
AOM	Azoxymethane
ATase	Alkyl-guanine-alkyl-transferase
BER	Base excision repair
BG	O^6benzylguanine
BMI	Body mass index
BCFA	Branched chain fatty acids
CD	Chrohn's disease
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CRC	Colorectal cancer
CSIRO	Commonwealth Scientific and Industrial Organisation
DAB	3'-diaminobenzamine
DMH	1,2-dimethylhydrazine
DSS	Dextran sodium sulphate
EGCG	(-)-epicatechin-3-gallate
FAP	Familial adenomatous polyposis
FCC-X	Familial colorectal cancer type X
FIT	Faecal immunochemical test
H_2O_2	Hydrogen peroxide
H&E	Haematoxylin and Eosin
HAMSB	Butyrylated high amylose maize starch
HCA	Heterocyclic amine
HNPCC	Hereditary non-polyposis colorectal cancer
HR	Homologous recombination
HRM	High red meat
HRP	Horse radish peroxidase
IACR	International Agency on Cancer Research

IBD	Inflammatory bowel disease
IHC	Immunohistochemical
IQ	2-amino-3methylimidazo [4,5-f] quinoline
IS	Internal standard
LOH	Loss of heterozygosity
MSS	Microsatellite stable
MSI	Microsatellite unstable
MIN	Microsatellite instability
MMR	Mismatch repair
MAM	Methylazoxymethanol
MeIQ	2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline
MGMT	Methyl-guanine-methyl-transferase
MNU	Methylnitrosourea
MoM	Mouse-on-mouse
mutSα	*Msh2-Msh6* MMR heterodimer complex
NHEJ	Non-homologous end joining
NOCs	N-nitroso compounds
O_2^-	Superoxide anion
O^6CMG	O^6-Carboxymethlyguanosine
O^6meG	O^6methyl-2-deoxyguanasine
PAH	Polyaromatic hydrocarbon
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
RoB	Red over blue
ROS	Reactive oxygen species
RS	Resistant starch
RT	Room temperature
SCFA	Short chain fatty acid
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labelling
UC	Ulcerative colitis
WCRF	World Cancer Research Fund

Published material and presentations arising from this thesis

Peer reviewed publications

1. *Karen J Humphreys, Michael A Conlon, Graeme P Young, David L Topping, Ying Hu, **Jean Winter**, Anthony R Bird, Lynne Cobiac, Nicholas A Kennedy, Michael Z Michael and Richard K Le Leu. (2014) Dietary manipulation of oncogenic microRNA expression in human rectal mucosa: a randomised trial. *Can. Prev. Res.* 7(8); 786–95
2. **Jean Winter**, Graeme P Young, Ying Hu, Silvia W Gratz, Michael A Conlon and Richard K Le Leu (2013) Accumulation of promutagenic DNA adducts in the mouse distal colon after consumption of heme does not induce colonic neoplasms in the western diet model of spontaneous colorectal cancer. *Mol. Nut. Food. Res.* 58(3):550-8.
3. **Jean M Winter**, Ying Hu, Graeme P Young, Maija RJ Kohonen-Corish, Richard K Le Leu, Role of red meat and resistant starch in pro-mutagenic adduct formation, thymic lymphoma and intestinal tumourigenesis in *Msh2* deficient mice. *Jrnl. Nutrigenetics and Nutrigenomics,* In press 2015

Abstract publications

1. **Jean M. Winter**, Ying Hu, Graeme P Young, Maija RJ Kohonen-Corish, Richard K Le Leu. Diverse effects of resistant starch and red meat on proliferation and O^6Methyl-2-deoxyguanasine adduct formation in the distal colon of Msh2 deficient mice: Consequences for colorectal carcinogenesis. 38th Congress of the International Society of Nutrigenetics/Nutrigenomics (ISNN). May 2-3, 2014 Gold Coast, Australia: Abstracts. J Nutrigenetics Nutrigenomics 2014;7:1-38 (DOI:10.1159/000362615) (Abstract Only)
2. Richard K Le Leu, **Jean M Winter**, Ying Hu, Laura S Nyskohus, Michael Conlon, Anthony R Bird, David L Topping, Graeme P Young. M1181 Red Meat Diets Increase the Formation of O^6Methyl2Deoxyguanosine Adducts in the Mouse Colon: Attenuation by Resistant Starch. Gastroenterology. 2010; 138(5). DOI:10.1016/S0016-5085(10)61607-1 (Abstract Only)

Publications currently under peer review

1. Richard K Le Leu, **Jean M Winter**, Karen J Humphreys, Graeme P Young, Claus T Christophersen, Ying Hu, Silvia W Gratz, Rosalind B Miller, David L Topping, Anthony R Bird, Michael A Conlon, Butyrylated starch intake can prevent red meat induced O^6-methyl-2-deoxyguanosine adducts in human rectal tissue: a randomised clinical trial. *British J. Nut.*

*J. Winter performed DNA adduct and proliferation laboratory analyses of rectal epithelial tissue samples for this project

National conference presentations

1. Poster Presentation: **Australian Health and Medical Research Congress**, Melbourne, November 2014, Methyl-guanine-methy-transferase repairs pro-mutagenic adducts and influences the acute apoptotic response to alkylating agents: Interactions of dietary red meat, green tea and gender.
2. Oral Presentation: **Australian Society for Medical Research SA Annual Scientific Meeting**, Adelaide, June 2014, Diverse effects of resistant starch and red meat on proliferation and O^6Methyl-2-deoxyguanasine adduct formation in the distal colon of Msh2 deficient mice: Consequences for colorectal carcinogenesis.
3. Oral Presentation: **Australian Society for Medical Research SA Annual Scientific Meeting**, Adelaide, June 2013, Accumulation of pro-mutagenic and oxidative DNA adducts in the distal colon after consumption of dietary haem does not increase colorectal cancer in the mouse.
4. Oral Presentation: **Australian Society for Medical Research SA Annual Scientific Meeting**, Adelaide, June 2012, Induction of Pro-mutagenic Adducts in the Colon and Risk for Colorectal Cancer: Regulation by Resistant Starch.

International conference presentations

1. Oral Presentation (Registration waived): **8th Congress of International Society of Nutrigenetics/Nutrigenomics (ISNN),** Gold Coast, QLD, Australia, May 2014 Diverse effects of resistant starch and red meat on proliferation and O^6Methyl-2-deoxyguanasine adduct formation in the distal colon of Msh2 deficient mice: Consequences for colorectal carcinogenesis.
2. Oral and Poster Presentation: **Environmental Mutagen Society Annual Meeting**, Seattle, Washington, USA, September 2012, High Dietary Protein and DNA Damage in the Mouse Colon and Human Rectal Epithelium: Regulation by Resistant Starch**.**

Chapter 1

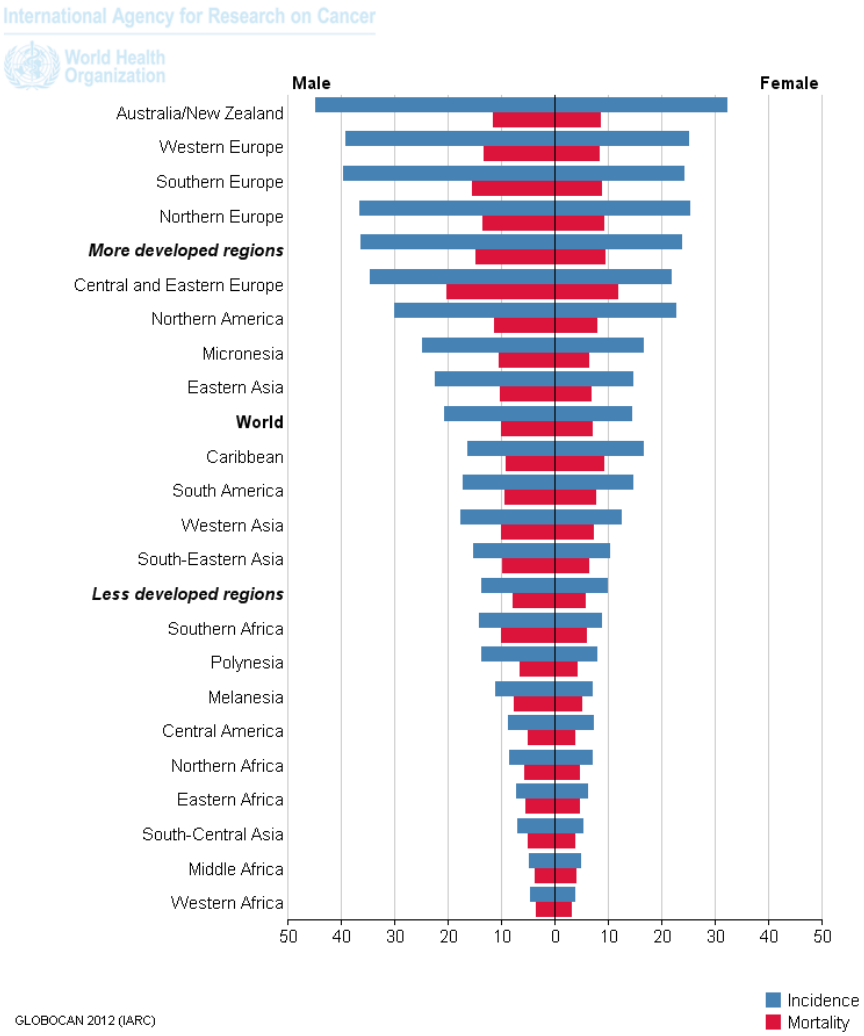
Introduction

1.1 Colorectal cancer

Colorectal cancer (CRC) is a major public health burden in many Western societies around the globe. Worldwide it ranks as the second most commonly diagnosed cancer in 2012, equal with breast and prostate cancer, and the second most common cancer causing death (Forman D et al.). In Australia, CRC is the second most commonly diagnosed cancer with over 14,000 new cases in 2007 reported by the Australian Institute of Health and Welfare (AIHW). The International Agency on Cancer Research (IACR) states that developed countries living a Western lifestyle have higher CRC incidence rates compared to developing countries (Figure 1.1). Epidemiological data summarised by the World Cancer Research Fund (WCRF) identifies the key components of a Western lifestyle as the major contributors to risk of the disease, including poor diet, alcohol consumption and lack of physical activity (WCRF/AICR 2007). Taken together, these numbers highlight the importance and need for research in CRC prevention, particularly primary prevention through modification of lifestyle choices.

Although CRC incidence rates are substantially high, the chance of survival, if lesions of the colon are identified early, is remarkably good. Primarily, the method for CRC screening is to undergo colonoscopy surveillance to identify

Figure 1.1: World Health Organisation (WHO) incidence and mortality rates of CRC according to global region[1]



Estimated age-standardised rates (World) per 100,000.
[1]This image was reproduced from the website of the International Agency for Research on Cancer, WHO, GLOBCAN 2012 estimated cancer incidence, mortality and prevalence worldwide in 2012 at http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx

polyps or cancers and surgically remove them, a highly invasive procedure that is not well accepted by the general public. Secondary prevention through screening programs such as the National Bowel Cancer Screening program in Australia, which implements the faecal immunochemical test (FIT) have been quite successful (Bobridge et al. 2013, Cole et al. 2013), however participation rates are still relatively low particularly for minority communities, the socioeconomically disadvantaged and males are less likely to screen than women (Ward et al. 2011,

Ward et al. 2014). The FIT screening can assist in diagnosing CRC at much earlier stages compared to individuals who did not participate (Cole et al. 2013), where early detection can lead to a better outcome for survival. The incidence of CRC is steadily decreasing in older populations (>50 years) but increasing in young onset CRC patients people (<50 years), a population where bowel screening programs are not targeted (Ahnen et al. 2014) and therefore addressing the risk of the younger population for better preventative strategies is essential. Due to an aging population in Australia and around the globe, the burden of CRC could soon become very costly, both economically and in terms of quality of life. This emphasises the need for research into CRC and its prevention at the earliest stages – *primary prevention* through lifestyle changes, particularly dietary choices.

To understand the influences diet has on CRC risk in humans, we first need to recognise the underlying mechanisms of cancer in the colon. The following sections will outline the carcinogenic process in the colon, give a brief overview of how normal cells transform into tumour cells, outline the risk factors associated with acquiring CRC and highlight animal models used in pre-clinical testing for studying CRC in the laboratory.

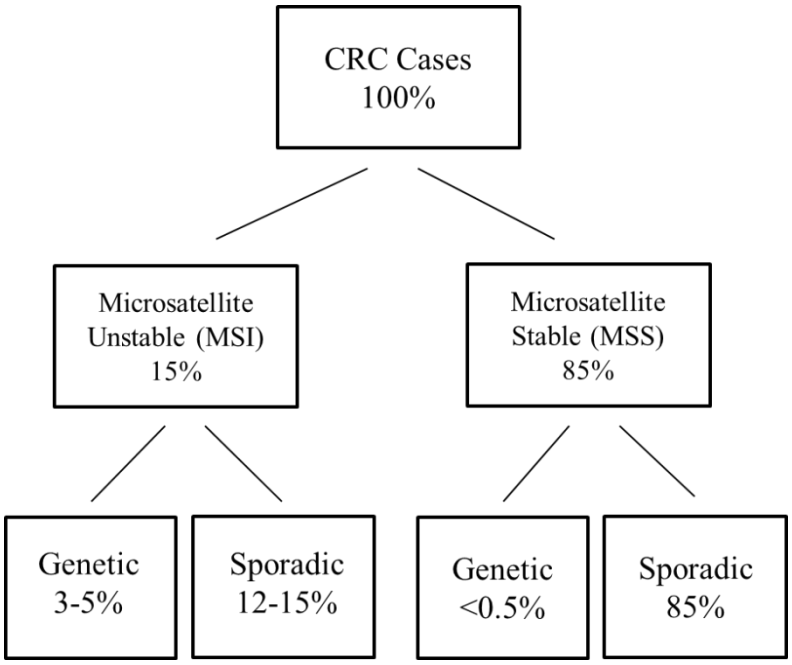
1.1.1. The process of CRC formation

The process of tumour formation has been understood for many decades to be a long and complex process (Foulds 1958). In particular, colorectal oncogenesis can take decades, beginning with some initiating event that alters the DNA, followed by a promotional stage leading to progression of metastatic cancer. Over the last 4-5 decades there has been extensive research into the

pathways associated with colon tumour formation. The concept of a multi-step pathway was generated in the early 1990s (Fearon and Vogelstein 1990). The multi-step pathway of CRC requires accumulation of changes within proto-oncogenes and tumour suppressor genes over the course of several years, as well as loss of heterozygosity (LOH). These changes create genomic instability within the epithelial cells, followed by a state of uncontrolled proliferation and replication, ultimately resulting in the generation of a mass of dysplastic cells that give rise to a tumour.

Colorectal tumours can be classified as microsatellite stable (MSS) or having microsatellite unstable (MSI), where some clones of cells contain different numbers (more or less) of microsatellite repeats (Figure 1.2). Mostly, mutations in the DNA arise spontaneously after exposure to some external insult and these are termed sporadic or spontaneous CRC. Inheritance of genetic defects can also lead to generation of CRC, but these are not as common as individuals acquiring mutations in colon cells sporadically. There are 2 major pathways within the multi-step process of colorectal carcinogenesis: 1) Chromosomal instability (CIN) pathway, and 2) Microsatellite instability (MIN) pathway. Other pathways have also been described including CpG island methylator phenotype (CIMP), inflammation and other epigenetic changes (DNA methylation and microRNAs), all of which may be representative of sporadic or inherited CRC cases.

Figure 1.2: Overview of CRC cases by subtype

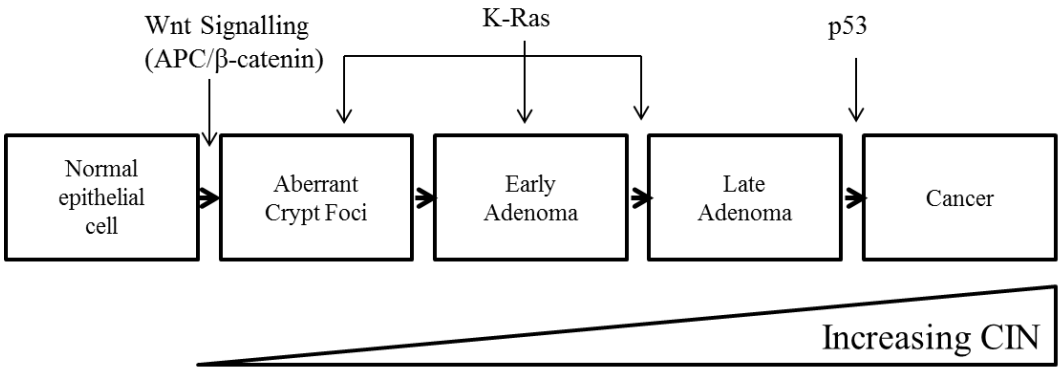


A majority of CRC cases are defined as having microsatellite stability (MSS) and are the result of mutations arising in genes sporadically from some external insult or due to inherited genetic mutation. Tumours with MSI are less common but can also be from sporadic or inherited genetic mutations. Image modified from (de la Chapelle and Hampel 2010).

By far the most common pathway for tumours to develop is through the CIN pathway (Figure 1.3). Tumours form as a result of normal epithelial cells gaining mutations that confer a hyper-proliferative advantage, leading to generation of aberrant crypt foci (ACF), adenomas (or polyps) and ultimately cancer. Early steps of the CIN pathway are characterised by genetic mutations in the *APC* gene (Powell et al. 1992), a major component of the Wnt signalling complex required for suppression of tumour growth. Another commonly mutated proto-oncogene is *K-ras*, with around 60% of CRC primary and metastatic tumours harbouring the mutation (Miglio et al. 2013). The tumour suppressor protein p53 is important for the cellular responses to DNA damage and controlling cell growth, DNA replication and cell division, and is also commonly mutated in CRC (Vogelstein et al. 2000). Over time, accumulation of genetic

defects in these genes (and several others) leads to an increasing level of chromosomal instability within the cell, which increases the likelihood that the dysplastic cells will progress to cancer.

Figure 1.3: Synopsis of the colorectal oncogenesis pathway



A brief overview of the multi-step process of CRC, highlighting the most common genes that are mutated at varying stages of the oncogenesis pathway. Modified from (Pino and Chung 2010).

1.1.2. Risk factors for CRC

A number of risk factors for developing CRC are identified as both environmental/lifestyle and genetic causes. The overwhelming majority of CRC can be attributed to environmental/lifestyle manifestation, with dietary choices having the greatest impact (dietary impacts will be explained in more detail later in section 1.4) with exercise and smoking also being influential. Alcohol consumption is regarded as one of the leading causes of overall cancer, including CRC, by the International Agency on Cancer Research (IACR), with risk being dose-dependent (Fedirko et al. 2011). Physical inactivity and obesity is common in developed countries and also in populations from developing nations who adopt a Western lifestyle. A higher BMI has been associated with increased risk of developing CRC (Laiyemo 2014) and increases in blood insulin and glucose levels, as a direct consequence of increased adipose tissue in obese individuals,

can increase risk of CRC by 2-fold (Schoen et al. 1999). Smoking is well known to cause lung cancer and heart disease, but has also been associated with CRC risk (Chan and Giovannucci 2010). All these risk factors are elements of human behaviour which are avoidable. Therefore, the individual can essentially have some element of control over their own CRC risk by making better dietary choices, cessation of smoking, reducing or avoiding alcohol and maintaining a healthy weight through regular physical activity. On the other hand, there are several risk factors which are, by their very nature, unavoidable. Due to the fact that CRC initiation and progression can take decades to occur (as previously described), as a person ages their risk for developing CRC increases dramatically, with that risk rising rapidly after the age of 50 (Day and Velayos 2014). Males typically have a higher incidence of CRC compared to females, with evidence to suggest the female sex hormone estrogen and hormone replacement therapy in women undergoing menopause can regulate transcription factors via genetic and epigenetic interactions, preventing progression of the oncogenic pathway (Barzi et al. 2013).

There are a few well characterised inherited syndromes and gastrointestinal diseases associated with having an increased lifetime risk of developing CRC. However, these are not nearly as influential as the fore mentioned environmental exposures, accounting for approximately 5% of all CRC cases. Familial adenomatous polyposis (FAP) is a genetically inherited form of CRC, resulting in the loss of one *APC* allele via germ-line transmission. FAP patients develop several hundred polyps in the gastrointestinal tract at a much earlier age of onset than sporadic CRC cases, with a 100% lifetime risk of acquiring the disease without treatment (Laurent et al. 2011). Hereditary non-

polyposis CRC (HNPCC or Lynch Syndrome) is another familial inherited form of CRC and is characterised by the loss of a single allele of one of the mismatch repair (MMR) genes, comprising approximately 50% of HNPCC cases. MMR proteins are involved in repair of single nucleotide mutations or loops that form, mostly in microsatellite repeats, thus most HNPCC patients have tumours displaying MSI and MMR loss. However, the other 50% of HNPCC cases, although showing typical inheritance traits of HNPCC, polyps are generally MSS and display MMR capability, which have recently been termed ‘‘Familial colorectal cancer type X’’ (FCC-X) (Garre et al. 2014). Genetic polymorphisms within the general population (non-familial cases) have also been associated with increased risk to CRC, including TP53 (Naccarati et al. 2012) and *MGMT* (Loh et al. 2010) and polymorphisms for some genes encoding microRNAs (Schmit et al. 2014), small non-coding segments of DNA that can regulate transcription of genes. Inflammation is another risk factor implicated in promoting the colorectal oncogenesis pathway. In particular, inflammation associated with gastrointestinal inflammatory diseases can modify risk of CRC, particularly with long term disease. Ulcerative colitis (UC) and Chrohn’s disease (CD) are both inflammatory bowel diseases (IBD) where patients suffer from chronic inflammation of the intestinal lining usually resulting in painful ulcers, bleeding and diarrhoea. Reports of an increased risk of CRC are common in IBD patients and subsequently this population are urged to undergo surveillance more frequently than the general population (Rubin et al. 2013).

Overall, risk factors for CRC can, for the most part, be modified through changes in lifestyle and habits. However, considering the ease of access to unhealthy food, cigarettes and alcohol in Western demographics, changing the

ingrained lifestyle patterns of millions of people living in developing countries is emerging as a great challenge. This highlights the need for quality, peer-reviewed and evidenced based research to guide education and health policies in cancer prevention, particularly CRC prevention. Understanding the basic mechanisms of genetic, inflammatory and environmental interactions within the colon and its influence on CRC will allow for all individuals at risk of CRC, particularly those in high risk populations, to understand how to reduce their lifetime risk of developing CRC.

1.2 Animal models of CRC

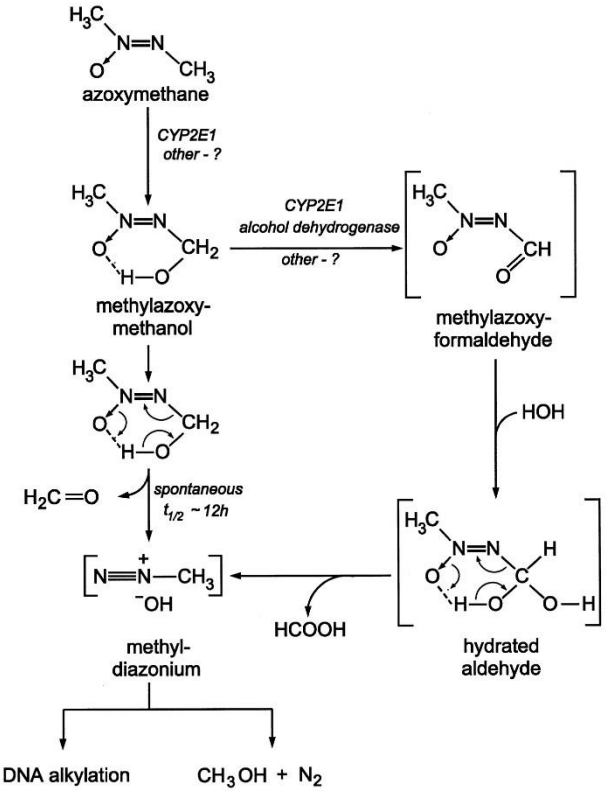
A useful method for identifying risk factors and the associated mechanisms of disease progression in CRC is by implementing pre-clinical trials in rodent models of CRC. The following section of this chapter will outline common models of CRC used for intervention studies and their relevance for translation in the human, as well as detailing three rodent models that will be implemented for this thesis.

1.2.1 The chemical carcinogenic model of CRC

There are several different chemical carcinogens that have been used over the years for inducing pre-neoplastic lesions, adenomas and adenocarcinomas of the colon in rodents. These include 1,2-dimethylhydrazine (DMH), azoxymethane (AOM), methylazoxymethanol (MAM) acetate, methylnitrosourea (MNU) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine(PhIP),2-amino-3methylimidazo [4,5-f]quinoline(IQ),2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline (MeIQ)(Suzui et al. 2013). By far the most widely employed is the chemical pre-carcinogen AOM. It has been known for over 4 decades that repeated exposure to the

chemically derived compound AOM is carcinogenic, particularly to the large intestine. AOM is an intermediate metabolite of DMH, which can be found naturally in the Cycad plant originating from tropical and sub-tropical regions (Hirono 1981). AOM is a pre-carcinogen, whereby it requires activation by the host before it is considered carcinogenic. The metabolic pathway of AOM (Figure 1.4) has been described by several and is summarised by Sohn et al (2001). The CYP2E1 liver enzyme converts AOM to the intermediate MAM, whereby MAM can either spontaneously or enzymatically convert to the methyldiazonium ion: the primary molecule that alkylates the DNA base (Sohn et al. 2001). DNA alkylation can act to initiate carcinogenesis that closely mimics the molecular and pathological pathway seen in humans. This includes inactivation of the Wnt signalling pathway (Zhang et al. 2013), generation of the pre-neoplastic lesions aberrant crypt foci (ACF) (Ochiai et al. 2014), induction of mutations in oncogene *K-ras* (Erdman et al. 1997, McKinzie and Parsons 2011), and development of colon tumours predominately within the distal colorectal epithelia, where most sporadic human CRC are located. More recently, the AOM model has been modified to incorporate treatment with an inflammatory chemical agent dextran sodium sulphate (DSS) – the AOM-DSS model. The AOM-DSS model still presents with characteristics typical of the AOM model but can generate tumours in a much shorter time frame, closely resembling the human form of chronic colitis-associated CRC (Clapper et al. 2007).

Figure 1.4: Summary of the metabolic pathways for AOM activation in the liver



Metabolic activation of AOM is achieved through enzymatic reactions with CYP2E1 in the liver, where it is converted to MAM. CYPE21 can further convert MAM into methylazoxyformaldehyde, which in converted to the ultimate alkylating agent the methyldiazonium ion. MAM can also spontaneous convert to the methyldiazonium ion. Image reproduced from Sohn et al (2001) page 8436

The AOM and AOM-DSS models of CRC present an ideal situation for generating significant tumours in the colon in a relatively short time frame with relatively low sample numbers. Mutations commonly found in human colon tumours, including the *k-ras* gene, are known to be the result of DNA methylation, as a consequence of inadequate alkyl repair (Mokarram et al. 2013). However, debate remains as to the relevance of these models to sporadic tumours observed in humans. Certainly, humans are not normally exposed to AOM (if at all) in the typical environment, as the Cycad plant where AOM is derived is not commonly found in many parts of the world. Furthermore, although patients with

IBD have an enhanced risk of CRC compared to individuals without IBD, only a very small subset of CRC cases can be attributed to chronic colitis (Rogler 2014). Thus, although the AOM and AOM-DSS model of CRC is very useful, implementing this model for pre-clinical chemoprevention trials examining spontaneous CRC (the most common form of CRC) is somewhat debatable.

1.2.2. The Western diet model of spontaneous CRC

The Western-style diet serves as a model for spontaneous CRC. It encompasses a combination of Western dietary risk factors, shown to be implicated in CRC initiation and promotion.

It has been known for several decades that key vitamins and minerals are protective against CRC formation. Particularly for CRC; calcium, vitamin D, vitamin B12 and folate have all been shown to be protective against CRC (Yang and Newmark 1987). A diet high in fat is associated with an increased risk of CRC, likely from increased lipid peroxidation generating oxidative stress and DNA damage within the colon (Bartsch and Nair 2002, Marnett 2002). High fat diets in combination with low minerals and vitamins can cause dysregulation of colonic lipid metabolism, oxidative stress, and the immune response, and is thought to be one part of the mechanism of Western diet induced CRC (Erdelyi et al. 2009). Dietary fibre has a significant and convincing protective effect on CRC and will be explained in extensive detail in section 1.4. Briefly, the physical and physiological interactions within the colonic lumen by dietary fibre can reduce carcinogen availability and thereby reduce risk of CRC (McIntyre et al. 1993, Young et al. 2005), and low levels of fibre is one component of the Western diet. High sugar diets in big blue rats can significantly increase mutation frequency

rates in the colon but not in the liver, independent of oxidative and DNA repair pathways, indicating that sugar is directly or indirectly geno-toxic to the colon (Dragsted et al. 2002) and therefore is also incorporated into the Western diet model of CRC.

The Western diet model can enhance tumour formation in wild type mice at 18 and 24 months compared to mice that consume a standard diet, without any form of chemical induction/promotion or underlying knockout of genes associated with CRC risk (Newmark et al. 1990, Newmark et al. 1991, Newmark et al. 2001, Newmark et al. 2009). Although the time to generate tumours is relatively long compared to the carcinogen models already mentioned, the tumours which form are spontaneous and, therefore, ideally represent the *majority* of human CRC cases. Therefore, this model could be very useful in identifying other dietary risk factors, and to allow characterisation of the mechanism of action of those risk factors in the colonic epithelia.

1.2.3. Genetically modified mice for modelling CRC

As discussed previously, a small proportion of all diagnosed CRC cases are due to inheriting defective genes which are associated with CRC progression in sporadic cases as well, including the *APC* gene and genes involved in MMR pathways. Several genetically modified mouse models have been created to mimic these conditions to identify risk factors, treatments and preventative dietary compounds for high risk populations.

The Apc min mouse has a single *APC* allele knocked out and as a consequence several intestinal polyps form, particularly after exposure to AOM (Orner et al. 2002, Greenspan et al. 2010). This model mimics the familial

inherited syndrome FAP, which is characterised by a young onset of disease and up to a 100% lifetime risk. However, most polyps in Apc min mice develop in the small intestine, therefore relevance to the human CRC setting is speculative because small intestinal cancer is relatively rare in humans (Neugut et al. 1997). More recently, targeted *APC* knockout mice (*CPC;Apc* mice) have been developed, whereby a single *APC* allele is knocked out in cells along from the distal small intestine to the rectum, where tumours mostly develop in the distal colon (Hinoi et al. 2007). The *CPC;Apc* mouse shows promise as a pre-clinical model for FAP, as well as for translation into sporadic CRC mechanisms as sporadic tumours in the distal colon commonly harbour *APC* mutations.

The *Msh2* knockout mouse model has been used in CRC intervention studies that mimic a familial form of CRC called HNPCC (or Lynch syndrome). HNPCC is a genetically inherited syndrome resulting from the germ-line transmission of one or more of mutated MMR genes, including *Msh2*. The *Msh2* knockout mouse is deficient in MMR capabilities and compared to wild type mice they develop significantly more spontaneous deletion/insertion and transition/transversion mutations throughout the length of the large intestine (Hegan et al. 2006), generate more colorectal tumours when exposed to AOM (Mladenova et al. 2011) and increase the risk of inflammatory associated colorectal tumours forming after DSS exposure (Kohonen-Corish et al. 2002).

The *MGMT* knockout mouse is deficient in methyl-guanine-methyl-transferase (MGMT) activity. Briefly, the MGMT enzyme is responsible for repairing alkylated DNA (Margison et al. 2003) as a consequence of exposure to alkylating agents (such as AOM), but will be explained in more detail in section 1.3. Mice without functioning MGMT as a result of genetic inactivation display

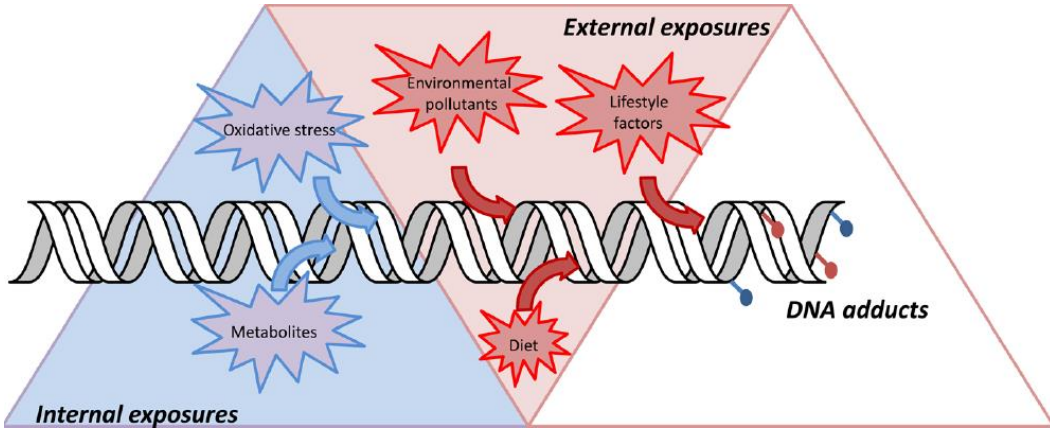
higher rates of CRC in the AOM-DSS model compared to normal mice (Kawate et al. 1998, Bugni et al. 2009) and are sensitive to repeated exposure to high doses of AOM (Glassner et al. 1999).

Overall, using genetically modified mice which have lost important genetic material that is required for adequate protection against CRC progression in rodent models of CRC is promising. These mouse models will allow investigators to determine the roles these genes play in colon carcinogenesis and how dietary interventions might alter the genetic environment within the colon. However, the use of knockout mice for dietary interventions has not been widely implemented (Tammariello and Milner 2010), and not at all in the *MGMT* knockout mouse to date. Considering the relevance to human cancer processes in the colon that the *Msh2* and *MGMT* genes have, these mice represent likely models for investigating mechanisms of risk for dietary interventions. More specifically, they depict a likely model for identifying risk of CRC after exposures to alkylating agents (either chemically or dietary derived) and their mechanism of repair. This is because *MGMT* and *Msh2* are key components involved in the protection against alkylating DNA adducts (or lesions) (explained in section 1.3): alkyl DNA adducts are implicated as a major trigger of CRC.

1.3 DNA adducts

A DNA adduct is the covalent interaction of a molecule with the DNA base resulting in a structural change of the DNA (Jeffrey 1985). DNA adducts can arise by an external insult (e.g. diet or environmental exposure, such as cigarette smoke) or an internal insult (e.g. oxidative stress) (Figure 1.5). Exploration of DNA adducts and their consequence for health is growing, leading

Figure 1.5: Sources of DNA adducts



Schematic diagram representing the external and internal routes of exposure to agents that can lead to DNA adduct formation. Image reproduced from Balbo et al (2014) page 356.

to the investigative approach of "DNA adductomics" recently emerging (Balbo et al. 2014). There are many classifications of DNA adducts, which can be pro-mutagenic if they are left in the cell unrepaired; mis-incorporation of DNA bases by the polymerase complex generates DNA mutations that can initiate oncogenesis (Swenberg et al. 2011). Therefore, the DNA adduct plays an important role in carcinogenesis. This is particularly important in CRC: the colorectal epithelium is exposed to exogenous agents generating DNA adducts from the diet on a daily basis, throughout one's lifetime. The following sections of this chapter will detail two classes of adducts found in colon cells: 8-hydroxy-2'-deoxyguanosine and O^6methyl-2-deoxyguanasine. Detail will be presented on what they are, how they are repaired and consequences of their persistence within the cell, highlighting their potential use as a bio-marker of risk for CRC.

1.3.1. 8-hydroxy-2'-deoxyguanosine

The 8-hydroxy-2'-deoxyguanosine (8-oxo) adduct is an oxidative adduct. There are many agents that can generate 8-oxo adducts in the DNA. Most 8-oxo adducts are a result of inflammatory agent exposures that generate reactive

oxygen species (ROS) such as superoxide anion (O_2^-) and the non-radical oxidant hydrogen peroxide (H_2O_2) (Ba et al. 2014). Radiation sources (e.g. UV) can also generate the DNA 8-oxo adduct at levels that can cause significant DNA damage (Kozmin et al. 2005).

1.3.1.1.Repair and mutagenesis of 8-oxo adduct

The major repair pathway for the 8-oxo DNA adduct is via base excision repair (BER), with the DNA glycosylase enzyme OGG1 having an affinity for excising the 8-oxo adduct from the DNA (Maynard et al. 2009). The OGG1 is a bi-functional DNA glycosylases: it has both glycosylase activity and an intrinsic 3' AP lyase activity. This allows for excision of the damaged base and incision of the DNA backbone creating a single strand break, which is then ligated together to generate a newly synthesised and repaired DNA strand (Iyama and Wilson 2013). A common polymorphism within the human population (Cys326-hOGG1) has shown to significantly inhibit OGG1 repair activity of cells under oxidative stress (Kershaw and Hodges 2012). Thus, activity of OGG1 plays an important role for clearance of oxidative DNA damage in the cell.

In the absence of BER, or due to failed BER, the 8-oxo adduct can be mutagenic. The 8-oxo adduct allows the DNA polymerase complexes to insert the incorrect base during replication, leading to generation of mutations (Shibutani et al. 1991). The most abundant mutation incorporated as a result of 8-oxo adducts are G:C → T:A transversion mutations (Le Page et al. 1995). Mice that are mutant for the OGG1 gene show a high rate of spontaneous mutations, inflammation and increased adenoma and adenocarcinoma formation in the lung, liver stomach and colorectum (Sampath 2014). In humans, large chromosomal deletions within the

OGG1 gene have been linked to a variety of different cancers, including CRC (Shinmura and Yokota 2001). The 8-oxo adduct presents as a good marker for oxidative stress, which have the capacity to initiate oncogenesis in cells exposed to exogenous and endogenous inflammatory agents.

1.3.1.2.The 8-oxo adduct and CRC

The 8-oxo adduct has been linked to increased risk for CRC. In CRC patients, quantity of DNA 8-oxo adducts in adenocarcinoma samples was found to be higher than in corresponding normal tissue (Plachetka et al. 2013). Patients with CRC and inflammatory bowel disease present with higher levels of 8-oxo measured in their plasma (Gackowski et al. 2002, Dincer et al. 2007) and CRC patients show higher levels of 8-oxo DNA adducts measured in colon tissue compared to patients without CRC (Obtulowicz et al. 2010, Psofaki et al. 2010). Also, an increase in mitochondrial mutations was associated with increased levels of 8-oxo adducts in patients with ulcerative colitis (Nishikawa et al. 2005), an inflammatory bowel disease that is a known risk factor for development of CRC.

In rodent models of CRC there is a strong association with changes in 8-oxo adducts in fluids and target tissues and correlations with risk for tumour development. Decreases in tumour burden and ACF formation of F344 rats exposed to AOM corresponded to marked reduction in urinary output of 8-oxo adducts (Harris et al. 2001). In F344 rats exposed to DMH (20mg/kg), 8-oxo levels in the colonic mucosa 24 hours later were significantly higher compared to rats who received vehicle treatment only (Lodovici et al. 2000).

There is strong evidence to suggest generation of 8-oxo adducts, or an inherent lack of repair capacity for 8-oxo adducts, is implicated in cancer, in

particular inflammatory associated CRC. Therefore, 8-oxo DNA adducts might be useful as a potential bio-marker for CRC as a consequence of exposure to inflammatory agents from dietary exposures that are known risk factors for CRC.

1.3.2. O^6methyl-2-deoxyguanosine

The O^6methyl-2-deoxyguanosine (O^6MeG) adduct is due to an alkyl group being incorporated into the DNA by alkylating agents (Saffhill et al. 1985). More specifically, it is when a methyl group attaches to the 6th oxygen on the guanine base, where the phosphate backbone in the DNA helix subsequently changes structure, ultimately changing the capacity for the polymerase complex to accurately incorporate the correct DNA base during replication.

1.3.2.1.Repair of O^6MeG by methyl-guanine-methyl-transferase

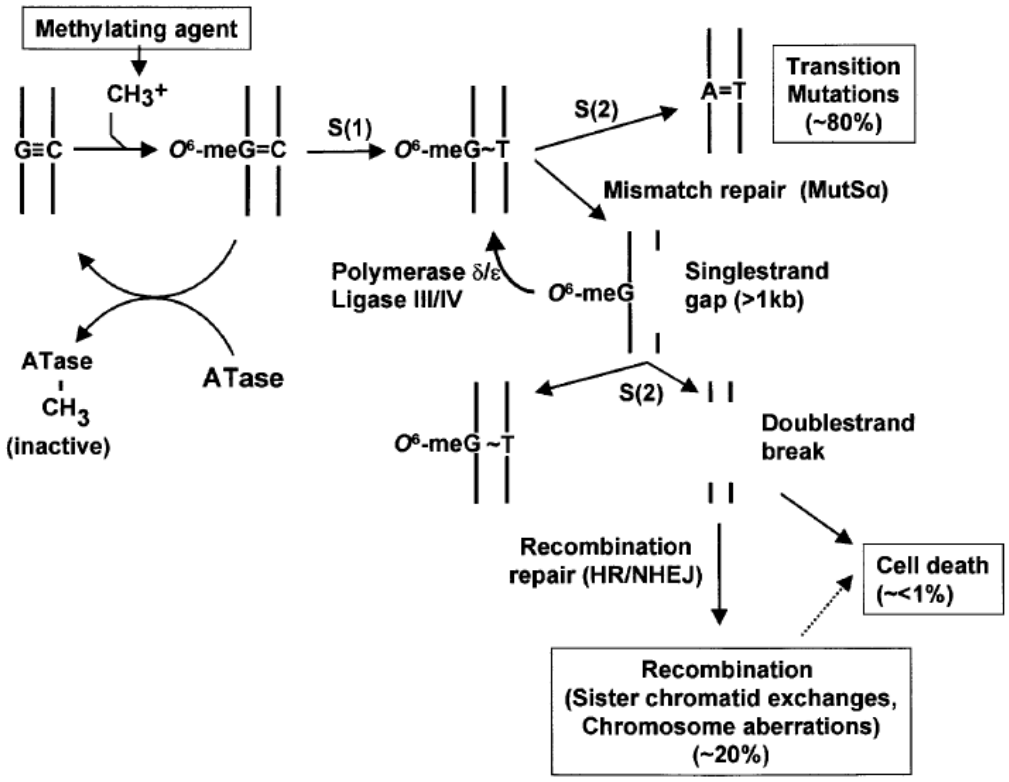
The first line of defence against the O^6MeG adduct is activity of methyl-guanine-methyl-transferase (MGMT) enzyme, also known as alkyl-guanine-alkyl-transferase (ATase). The MGMT enzyme removes the methyl group from the G base to a cysteine residue in the active region of the protein, without any interruption of the DNA strand or the replication process (Povey et al. 2002). Once the MGMT enzyme cysteine residue is methylated, the enzyme is targeted proteolysis by ubiquitous degradation, as the *S*-alkyl cysteine formed cannot be regenerated (Fang et al. 2005). Persistence of the O^6MeG base as a consequence of inactivation of the MGMT enzyme can enhance tumours in the distal colon of mice after exposure to the alkylating agent DMH (Jackson et al. 2000). Mice deficient in MGMT activity can increase tumour numbers in the colon of the AOM-DSS model (Bugni et al. 2009). In humans, genetic polymorphisms found in the *MGMT* gene can also affect the activity of MGMT and has been associated

with increasing risk of several cancers, including CRC (Liu et al. 2013). Epigenetic methylation of the *MGMT* promoter region (which affects its activity) can significantly increase mutations in the *K-ras* gene of CRC patients (de Vogel et al. 2009). Rat studies have shown *K-ras* is targeted by the alkylating agent AOM (McKinzie and Parsons 2011), where AOM induces a potent dose of O^6MeG adducts in the distal colon, inactivating MGMT activity within as little as 2 hours after exposure (Nyskohus et al. 2013). Therefore, repair of the O^6MeG adduct by MGMT, and the prospect of downstream mutagenesis and risk for CRC, is somewhat dependent on the activity of MGMT within the target tissue.

1.3.2.2.Consequences of O^6MeG persistence within the cell after failed MGMT repair

The mutagenic capacity of the O^6MeG adduct (Figure 1.6) has been widely reported and is summarised comprehensively in Margison et al (2002). The mutagenesis process of the O^6MeG adduct begins in the first round of replication after exposure to alkylating agents, in the absence of MGMT repair. When the polymerase complex arrives at the adducted G base, a T is incorporated instead of the usual C, due to the changed structure in the DNA phosphate backbone (Loechler et al. 1990). Without recognition of this mis-incorporated base, a second round of replication causes the polymerase to insert an A base opposite the T: the result is a GC→AT transition mutation. However, the *Msh2-Msh6* MMR heterodimer complex (mutSα) has the ability to recognise the O^6MeG-T mis-pair. MMR excises the single point mutation on one strand from the DNA, generating a single strand gap in the process (Margison et al. 2002).

Figure 1.6: Summary of the mutagenic process of O^6MeG adduct after exposure to alkylating agents



Failed repair of O^6MeG adducts by MGMT can lead to GC→AT transition mutations, cell death (apoptosis) or chromosomal aberrations/exchanges leading to persistent mutations and chromosomal instability within the cell. ATase: alkyl-transferase, S (1): 1st S phase in replication, S (2): 2nd S phase in replication, HR: Homologous recombination, NHEJ: Non-homologous end joining. Image reproduced from Margison et al (2002) page 484.

The single strand gap in the DNA can be repaired by DNA ligases, returning the DNA to a normal sequence. However, because the G base still has the O^6MeG adduct, the cell will essentially undergo a constant repetition of MMR recognition and repair, termed the "futile" repair process (Branch et al. 1993). The other option is an opposing O^6MeG base is also excised from the DNA, thereby generating a double strand DNA break after 2 rounds of replication (Figure 1.6). This ultimately leads to cell death or double strand break repair via homologous recombination (HR) or non-homologous end joining (NHEJ). Such processes can

lead to genomic instability via the production of chromatid exchanges and chromosomal rearrangements.

1.3.2.3.The O^6MeG adduct and CRC

Since unrepaired O^6MeG adducts can initiate mutagenic pathways in the cell after exposure to potent alkylating agents, it seems reasonable to advocate the O^6MeG adduct as a bio-marker for CRC risk in humans, for low and high risk populations.

Animal models have certainly shown that an increase in O^6MeG adducts *generated by an alkylating carcinogen* is associated with increased risk of colon tumours. Repeated exposure of the alkylating carcinogen DMH in mice by i.p. injection can significantly enhance O^6MeG adducts, more so in the distal colon, with the cumulative sum of O^6MeG correlating with overall tumour yield (Jackson et al. 2003). Rats treated with AOM in combination with the MGMT inhibitor O^6benzylguanine (BG) show an increase in colon tumour incidence by over 30% compared to AOM only treated rats and over 51 tumours in the AOM with BG treated group harbouring *K-ras* mutations compared to zero *K-ras* mutations in the AOM only group (Wali et al. 1999). Rats treated with mesenchymal stem cells show lowered colonic O^6MeG adducts at 8 hours after AOM exposure, which translated to a lowered acute apoptotic response (AAR) after AOM, and subsequently less colon tumours compared to rats receiving AOM treatment only (Nasuno et al. 2014). The link between increasing colonic O^6MeG adducts and risk for tumours in chemical rodent carcinogen studies is clearly defined, with an increase in O^6MeG adducts equating to an increase in risk for CRC.

In humans, the association of colonic DNA adducts in general and risk for CRC has been identified (Pfohl-Leszkowicz et al. 1995, Jonsson et al. 2010). However, the link of O^6MeG adducts more specifically and their influence on CRC risk within the human population is not clear and the data is limited. DNA O^6MeG adducts can be detected in several sites of the body including the colorectal tissue, with a substantial variation between tissue sites and between individuals (Hall et al. 1991). In the colon, the amount of DNA O^6MeG adducts has also been identified as highly variable throughout the length of the colon (of CRC patients), with the highest levels seen in the distal colon and rectum – the location of highest risk for CRC to occur (Povey et al. 2000). However, in a previous study no link was shown between colonic O^6MeG levels in normal and non-normal tumour tissue and CRC, nor any link between increasing O^6MeG adducts and increased risk for mutations in the k-ras gene (Jackson et al. 1996). The evidence that O^6MeG adduct accumulation in humans is a risk factor for CRC is conflicting. In light of the pre-clinical data, which presents a clearer picture of increased CRC risk as a consequence of O^6MeG adduct accumulation, further analysis of this adduct in human colorectal tissue is warranted. Certainly, O^6MeG adducts are generated to detectable levels in the colon and therefore still present as a possible marker to risk of CRC as a consequence of alkylating exposure in humans.

Using O^6MeG as a bio-marker for CRC risk, as opposed to simply a bio-marker of exposure to alkylating agents, is still somewhat debatable and not entirely convincing. More investigation is needed in humans and in rodent models that give a more relevant and appropriate route exposure to alkylating carcinogens from the environment, as opposed to artificial chemical elements. This will allow

for better determination of risk of oncogenesis, as a direct consequence of increasing O^6MeG DNA adducts in the colon from dietary agents and to translate these findings into the human setting.

1.4 Diet and colorectal cancer

Consumption of foods that harbour the potential to initiate or promote oncogenesis poses as the most substantial risk factor for developing CRC. Indeed, there is a plethora of literature on pre-clinical animal models and human intervention trials assessing dietary risk factors, and protective factors, of colorectal oncogenesis. The following section will discuss, in particular, three food components in more detail: Red meat, resistant starch (RS) and green tea. These three foods will be the major dietary focus of this thesis and form a substantial part of the overall aims and hypotheses. Here an overview of the evidence will be given in rodent and human trials identifying effects of red meat, RS and green tea on colonic oncogenesis. There will also be details on the potential mechanisms of action that have been proposed for these nutritional compounds, and how they link with risk for developing CRC.

1.4.1. Red meat

It is well accepted that red meat (and processed red meat) are risk factors for developing CRC and the WCRF reported this in a review of the evidence in 2007. In 2011 the WCRF updated this report, and their conclusion still supports red meat as a convincing risk factor for CRC. A search of the literature published in only the last 2 years (from beginning 2013 and all of 2014) demonstrate at least 12 human studies and 4 detailed reviews or meta-analyses (Table 1.1). All but 2 of the mentioned studies support an increased risk of CRC with increased red

meat consumption. It is important to note that due to the complexity of studying dietary interactions in the gut on CRC risk, randomised controlled trials are not common. In addition to this, there are several confounding factors that will have significant influence on associations of diet and risk for CRC (including smoking, alcohol consumption and physical activity) that can impact the outcome. Nonetheless, the evidence in humans advocating that red meat is associated with increased CRC is persuasive.

Although it can be said with a good degree of certainty that red meat increases risk of CRC in humans, the mechanism(s) by which this happens is unclear. Furthermore, some rodent studies contradict epidemiological data that red meat can increase CRC, where rats fed high red meat diets as beef have significantly less DMH-induced colorectal adenocarcinomas compared to rats fed casein milk protein (Nutter et al. 1983, Pence et al. 1995). There have been plausible theories put forward, with no one mechanism really portraying a decisive role in oncogenesis of the colon. There are four major areas of research into mechanisms of risk of CRC: 1) Carcinogen formation by cooking red meat at high temperatures; 2) High protein diets delivering substrates to the colon for fermentation by bacteria into toxic by-products; 3) Oxidative stress caused by haem iron from the haemoglobin in red meat; and 4) N-nitroso compounds (NOCs) generating pro-mutagenic alkylating adducts. The following paragraphs will explain how of each of these components contribute to CRC risk.

Table 1.1: Human studies identifying CRC risk associated with red meat consumption

Change in risk of CRC in human studies of increasing red meat and processed red meat consumption		
Increase	**No Change**	**Decrease**
(Ramzi et al. 2014) (Figueiredo et al. 2014) (Pham et al. 2014)* (Nimptsch et al. 2014) (Pericleous et al. 2013)* (Helmus et al. 2013) (Rohani-Rasaf et al. 2013) (Miller et al. 2013) (Johnson et al. 2013)* (Parr et al. 2013)	(Key et al. 2014) (Egeberg et al. 2013)	*No published studies*

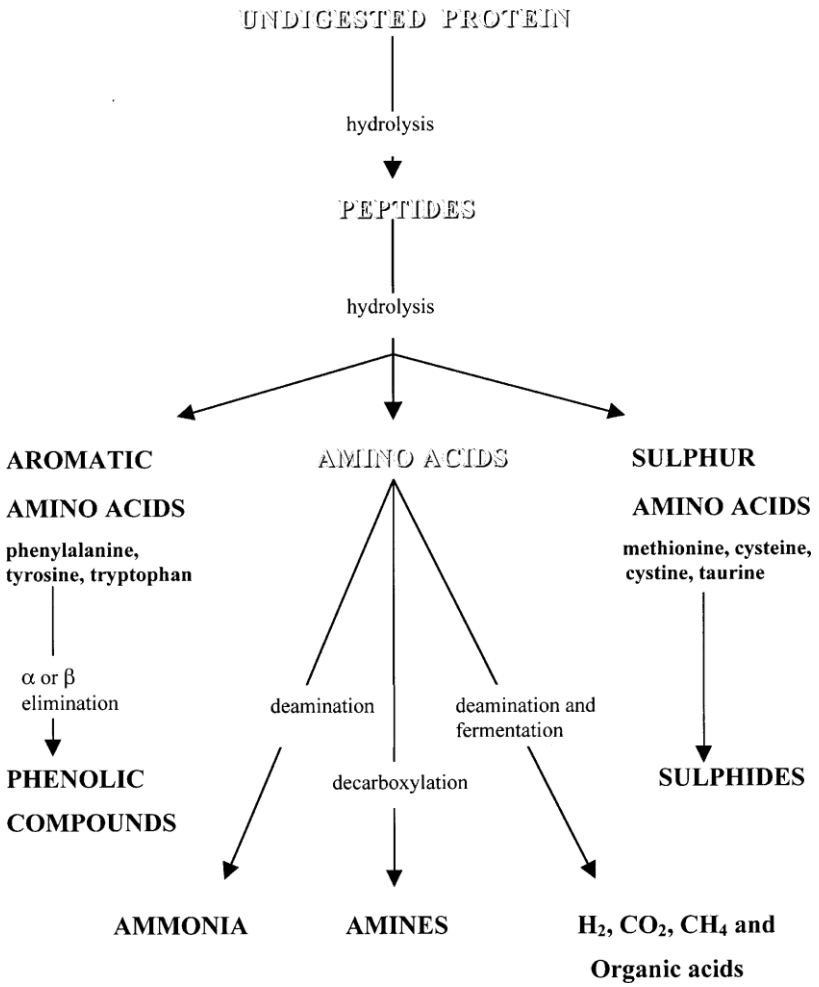
* Indicates review or meta-analysis

When cooked at high temperatures red meat can form carcinogens and mutagens called heterocyclic amines (HCAs) and polyaromatic hydrocarbons (PAHs). The heat changes the chemical bonds of the amino acids within the meat such that they form highly carcinogenic structures. PAHs and HCAs can induce large, bulky DNA adducts known to induce mutations in the DNA (Nakagama et al. 2005), leading to cancer formation in several target organs (Shirai et al. 2002). Rodent models demonstrate how exposure to chemically derived carcinogens found in cooked meat can substantially increase pre-cancerous lesions, adenomas and adenocarcinomas, particularly in the distal colon (Xu and Dashwood 1999). In humans, epidemiological data identifies increased consumption of "well-done" meat and thus an increased intake of meat carcinogens as a risk factor for CRC (Rohrmann et al. 2009, Fu et al. 2012, {Rohrmann, 2009 #116). However, these carcinogens are also formed at high temperatures when cooking white meat such as chicken and fish at levels equal to that seen in red meat such as beef (Puangsombat et al. 2012). White meat does not have the same associations of

CRC as does red meat (Norat et al. 2005), with fish actually having a protective effect against CRC (Gonzalez 2006). It seems counterintuitive that those carcinogens which induce cancer would also be formed in meat products that offer protection against cancer. Thus, other mechanisms besides HCA and PAH induced oncogenesis may contribute further to the process of CRC formation after red meat consumption.

Protein is metabolised in the gut by the microbiota generating toxic metabolites (Figure 1.7). Depending on the diversity of the microbiota in the colonic lumen, the transit time of the digestive content and the availability of amino acids, different fermentation paths will be taken producing a variety of metabolites such as amines, ammonia, sulphides or phenols (Hughes et al. 2000). Increasing dietary protein intake can increase the amount of protein reaching the colon and therefore increase the level of protein metabolites excreted in the faeces. There has been suggestion that such geno-toxic protein fermentation products listed in Figure 1.7 could cause damage to colon cells and subsequently induce or promote CRC. In rats, diffusing ammonia into the colon at physiological concentrations can increase epithelial cell proliferation of the distal colon (Ichikawa and Sakata 1998). Feeding high protein diets to rats as casein (a purified dairy protein source) increased cell proliferation in the colon and was associated with higher faecal ammonia concentrations (Lin and Visek 1991). In contrast, chemically induced tumours in rats fed a high casein diet showed no link between ammonia and phenol levels in the faeces and ACF formation of the colon (Corpet et al. 1995)

Figure 1.7: Possible pathways of protein fermentation in the colonic lumen



Microbial metabolic pathways of undigested protein fermentation in the gut and the final toxic metabolites produced. Adapted from Hughes et al 2000.

High potato protein diets can increase urinary cresol concentration and increase small intestinal tumours, suggesting a promoting effect of dietary protein on tumourigenesis (Le Leu et al. 2007), however, these changes in protein fermentation did not result in increased risk of colon tumours in those rats. Although dietary protein can increase protein fermentation and generate toxic metabolites, there is still uncertainty as to whether these products are one of the causative factors of CRC as a consequence of high protein diets.

Haem from haemoglobin in red meat has been connected to development of CRC in humans (Tappel 2007, Cross et al. 2010). Haem iron is readily bio-available for absorption in the small intestine, but there is a limitation to its absorption and consequently a large proportion still reaches the colon (Roberts et al. 1993). Several animal studies have revealed that diets with relatively high haem concentrations (above average human consumption levels (Lombardi-Boccia et al. 2002) at 0.25>1.5 µmol/g) contribute to cell toxicity, hyper-proliferation, reduced apoptosis, and increased rates of CRC (de Vogel et al. 2008, Ishikawa et al. 2010, Santarelli et al. 2010, Ijssennagger et al. 2012, Ijssennagger et al. 2013). A diet incorporating low levels of haem more typical of a high red meat diet (at 0.25 µmol/g) actually showed a reduction the total number of ACF per colon, although the ACF size was larger with haem diets compared to a control diet (Pierre et al. 2003). The intake of haem from a typically high red meat diet as beef would equate to an average concentration of 0.14µmol/g (Lombardi-Boccia et al. 2002). This indicates that 0.25 µmol/g of haem used in the latter study more closely resembles that of a high red meat intake in humans (although still much higher) and therefore may not actually be a risk factor for tumour development in the colon. Consequently, it still remains speculative as to whether dietary haem acts to induce or promote CRC, with many rodent trials to date using haem as the "carcinogen" employing unrealistically high doses and for very short periods of feeding (e.g. 14 days).

Generation of DNA alkyl adducts that can generate mutations in proto-oncogenes have been suggested as a significant contributor to the aetiology of CRC. NOCs are agents that can alkylate the DNA. They are found at high levels in processed red meat and are also endogenously generated from bacterial

fermentation of amino acids in the lumen (Hughes et al. 2001). Furthermore, haem itself can be nitrosylated and acts as a catalyst of NOC formation, which is thought to be why red meat can generate significantly more NOCs compared to white meat (Bingham et al. 2002). Bingham and colleagues (Bingham et al. 1996) first suggested that DNA alkylation by NOCs from red meat could be responsible for initiation of mutations. Since then there have been a few publications in humans and rodents indicating that NOC exposure in the colon as a consequence of dietary red meat can cause significant damage to the colon, and therefore, likely increasing risk for CRC (Hughes et al. 2001, Bingham et al. 2002, Hughes et al. 2002, Lewin et al. 2006, Winter et al. 2011). Significant associations have been found between high red meat consumption and an increase in *K-ras* mutations of patients with CRC (Wark et al. 2006), although another study showed no such associations (O'Brien et al. 2000). In a human randomised control trial it was shown for the first time that high red meat diets could significantly increase the alkyl adduct O^6-Carboxymethlyguanosine (O^6CMG) in exfoliated colon cells found in the faecal matter, and this was associated with faecal NOC output (Lewin et al. 2006). However, in contrast to the O^6MeG adduct, which has been well studied and well established as a pro-mutagenic adduct, the mutagenic potential of the O^6CMG adduct is less well defined (Zhang et al. 2013). Furthermore, using exfoliated colon cells that are already dead and not attached to the colon in anyway do not ideally represent the physiology of epithelial cells *in situ*. In a mouse study feeding red meat for only 4 weeks, significant increases of the pro-mutagenic O^6MeG adduct was identified in colorectal cells *in vivo* (Winter et al. 2011), although there is no data available in humans identifying if red meat can induce O^6MeG DNA adducts. There is

substantial evidence indicating red meat can generate significant levels of DNA alkylation in the colon, likely as a consequence of N-nitrosation in the lumen acting as the source of alkylating agent, which might lead to increased mutation rates and thus cancer progression. However, there is still no direct evidence linking NOC exposure to CRC.

1.4.2. Resistant starch

Burkitt originally speculated that dietary fibre could decrease CRC risk (Burkitt 1971), although the concept of resistant starch was not known at the time. Resistant starch (RS) is a type of fibre that escapes digestion in the small intestine where it is fermented in the colon, and classified according to their physiochemical properties (Topping and Clifton, 2001). RS has several properties both physical and physiological that have been linked with protection of CRC in humans. These include increasing faecal bulk to dilute likely carcinogens and lowering faecal pH (Young and Le Leu 2004); increasing production of potentially beneficial molecules called short chain fatty acids (SCFAs) (Cummings et al. 1996); and reducing toxic products of protein fermentation in the colon (Birkett et al. 1996). In comparison to many sources of dietary fibre that reach the colon, RS generates substantially higher ratios of the SCFA butyrate in the lumen, where it is rapidly fermented by the microbiota (Noakes et al. 1996).

Although RS is linked to beneficial effects in the colon, some human intervention studies show no correlation between cell proliferation and levels of RS intake (Grubben et al. 2001, Wacker et al. 2002, Dronamraju et al. 2009). In others, cancer incidence in high risk populations of CRC (Lynch syndrome patients) was not affected (Burn et al. 2008), and one particular study actually

showed an increase in lipid peroxidation-induced DNA adducts after RS consumption (Wacker et al. 2002). The inconsistent results from these studies might be attributed to dosing complexities of RS. Intake of overall fibre in the trials was mostly recorded at over 30g per day. However, actual RS intake would differ as various sources of RS can have different amounts of total RS and fibre content. For example, Hylon VII contains 18% dietary fibre but 48% RS, whereas Hi-Maize 240 contains 40% dietary fibre but as much as 53% RS (Le Leu et al. 2009). This implies dosing of RS was insufficient in almost all of the fore mentioned studies, and subsequently the effect this might have on the colon could be insufficient. As such, higher doses of RS in human intervention trials are needed.

In the United States, where CRC risk is high, the average daily intake of RS is only 5g, varying between 3-8g daily (Murphy et al. 2008) and RS intake is comparable in Australia at 3-9 g of RS per adult per day (Roberts 2004). Compared to African Americans, native Africans have a substantially higher intake of maize (corn) that is rich in RS, and as a consequence generate significantly more SCFAs in their colons (O'Keefe et al. 2009). Consumption of starchy foods with high levels of RS, as seen in native Africans, is likely to be a contributing factor for their low CRC incidence (Ahmed et al. 2000, O'Keefe et al. 2007). However, studying the effects of RS in the colonic environment in human subjects is complex, as there are a variety of RS sources that can be used, and maintaining an adequate dose in the individual is difficult. Thus, interpreting the protective effect RS in CRC in humans is challenging and studies may need to be focussed in pre-clinical models to ascertain any protective effects on CRC.

Rodent studies utilising RS fermentation to change epithelial cell kinetics and risk of CRC are promising. Chemical carcinogen models of CRC have shown reductions in cell proliferation and ACF, lower tumour incidence rates, increases in apoptotic removal of damaged cells, and which all correlate with abundant increases in SCFA production within the colon of rats (Le Leu et al. 2007, Clarke et al. 2008, Liu and Xu 2008, Clarke et al. 2012). Furthermore, in short term feeding trials in rats, RS can ameliorate DNA single strand breaks generated by consumption of high protein diets (including red meat) as measured by the Comet Assay (Toden et al. 2006, Toden et al. 2007, Toden et al. 2007, Bajka et al. 2008). A dose dependent decrease of DNA single strand breaks was identified in rats consuming increasing amounts of RS, although as little as 10% of a RS source was enough to exert a protective effect (Toden et al. 2007). Furthermore, RS could protect against red meat-induced O^6MeG DNA adducts in the colon of mice after only 4 weeks of feeding (Winter et al. 2011). These data illustrate the potential role of RS fermentation of the colon and its ability to protect against DNA damage that could initiate oncogenesis in the colon. Considering RS can reach the colon undigested and carbohydrates are preferentially fermented by bacteria over protein sources, the potentially damaging effects of protein intake, particularly red meat intake, might be minimised by the addition of RS to the diet. Overwhelmingly, RS is seen as a protective food source for the colon. However, more *in vivo* evidence determining the effect of high dietary protein, in particular high red meat diets, in combination with RS are needed to identify potential mechanisms of associated risks of CRC.

1.4.3. Green tea

Green tea has been implicated as a chemo-preventative dietary agent against development of CRC. Evidence from *in vivo* rodent and *in vitro* cell culture studies shows the capability of green tea to act as a preventative agent against CRC, although human intervention trials are somewhat conflicting (Fujiki et al. 2012, Butt et al. 2013). Several mechanisms of action of green tea in the colon have been presented, both *in vivo* and *in vitro*, including anti-inflammatory properties (Barnett et al. 2013, Sadik 2013), protection against oxidative stressors (Dashwood et al. 1999), anti-proliferative effects (Du et al. 2012) and it has been suggested to induce apoptosis in response to high levels of DNA damage (Xiao et al. 2008, Du et al. 2012). Green tea consumption *in vivo* has been shown to reduce ACF and tumour burden in rodents after exposure to chemically derived alkylating carcinogens (Ju et al. 2003, Hu et al. 2013) and HCA carcinogens (Dashwood et al. 1999, Xu and Dashwood 1999), with the active constituent being polyphenol compounds, mainly (-)-epicatechin-3-gallate (EGCG). Certainly, protection against HCA induced CRC is linked to a reduction of bulky DNA adducts, which are thought to be "quenched" *in vitro* by the phenolic compounds that are found in green tea (Xu and Dashwood 1999, Schut and Yao 2000).

There is evidence proposing that green tea could protect against the alkylating abilities of red meat, namely NOCs. Phenolic compounds in green tea can act to inhibit NOC formation both *in vitro* and as measured in urine excreted from humans consuming green tea (Wu et al. 1993). Excretion of NOCs in the urine of human volunteers consuming nitrate in the drinking water was significantly decreased after consumption green tea (Stich 1992, Vermeer et al. 1999). However, when higher intakes of green tea (up to 8 cups or 4g equivalent)

actually increased NOC formation, suggesting that high doses of phenolic compounds might catalyse nitrosation in the gut, thereby increasing NOC output (Vermeer et al. 1999). To date, one study has examined red meat in combination with tea consumption (but not green tea), measuring NOCs excretion in the faeces from human volunteers. Hughes et al (2002) found that supplementing a 420g/day red meat diet with 3g/day of freeze dried tea extract could reduce excretion of nitrite in the faeces compared to a red meat only or red meat with vegetable diet, but did not reduce total faecal NOC excretion (Hughes et al. 2002). There have been no studies in rodents identifying the effects of red meat in combination with green tea. Preliminary data shown in rats with 0.5% green tea in a standard diet up-regulates MGMT activity in the colon (Hu et al. unpublished). It might be plausible that green tea could protect against alkylating effects of red meat not only by sequestering NOCs but also by suppressing DNA alkylating adducts via enhancing MGMT activity.

1.5 General aims and hypotheses

Induction of pro-mutagenic adducts from exogenous agents in the diet present as a likely candidate for generating genomic instability and thus initiating the cancer process. Here, the global aim is to determine whether dietary-induced DNA adducts by red meat consumption act as bio-markers for risk of CRC. The studies presented in this proposal will endeavour to validate and extend previous studies demonstrating that red meat can induce pro-mutagenic adducts. Furthermore, RS and green tea will be employed in combination with red meat to ascertain any protective role they might have against pro-mutagenic formation in the colon. The risk of developing CRC with high red meat consumption will also be explored, and RS will be evaluated as a protective food against CRC

formation. The hypotheses are that red meat will increase DNA adducts, but that RS and green tea consumption can reduce red meat-induced DNA adducts. Also, red meat and haem from red meat will increase risk for developing CRC, but RS will reduce the CRC risk posed by red meat. To test these aims and hypotheses, three mouse models that are susceptible to CRC development will be used: 1) The Western diet model of spontaneous CRC; 2) The *Msh2* knockout mouse model; and 3) The *MGMT* mouse model.

In addition to the mouse experiments, the effects of red meat and red meat in combination with RS will be translated to the human setting, by feeding high red meat and high red meat with RS diets to healthy human volunteers. This trial will aim to determine if red-meat induced DNA adducts generated in mice are also generated in the colonic epithelial cells of humans, and whether RS offers any protection against these adducts. It is hypothesised that red meat will significantly increase DNA adducts in the colorectal tissue of humans consuming a high red meat diet, but that co-consumption with RS will ameliorate these adducts.

Overall, this thesis intends to offer insight into the effects of different diets on the risk of CRC under different genetic backgrounds, and how modulating the diet can influence an individual's risk of developing CRC over their lifetime.

Chapter 2

Materials and methods

2.1. Animal ethics

All mouse procedures were approved by the Flinders University Animal Welfare committee (approval numbers 809/12, 824/12 and 860/13). Research with animals was conducted according to the Australian code for the care and use of animals for scientific purposes. Mice were weighed regularly to monitor for any significant body weight loss that might be attributed to ill health or development of tumours. Clinical monitoring sheets (see example in Appendix A) were recorded daily to monitor the overall health of the animals including weight loss, scratching, diarrhoea, blood in stool, coat condition, posture, activity and any other relevant comments on the welfare of the animals.

2.2. Animal models of CRC

2.2.1 Western diet model of spontaneous CRC

The Western-style diet mouse model was introduced in the early 90's (Risio et al. 1996) and has since been used by others to study the effects of a typical western diet on spontaneous CRC (Newmark et al. 2001, Yang et al. 2008, Erdelyi et al. 2009, Newmark et al. 2009). Unique components of this diet include low levels of key minerals and vitamins, particularly vitamin D and calcium, which are demonstrated to have a protective effect on CRC (Newmark et al. 2009). The diet is also high in fat and sugar and contains low dietary fibre, all which have been

implicated in CRC development. This model more closely mimics the human path of CRC, where dietary exposure is an important risk factor for a majority of sporadic colon cancers. There is also no application of chemical carcinogens or inflammatory molecules to initiate or promote CRC, which do not ideally represent carcinogenic exposure from the environment.

The components of vitamins and minerals used in this thesis were adapted from Newmark et al (2009) with some slight modifications. For a detailed list of the Western diet refer to Appendix B. All mice were male c57Bl/J strain, purchased from Animal Resource Centre, Perth, Western Australia. They were acclimatised for at least 1-2 weeks before being placed on experimental diets.

2.2.2. Msh2 knockout mouse model

The M*sh2* mouse model was chosen for a few reasons. Firstly, loss of MMR function by deletion of one or more of the MMR gene complex is a trait seen in the familial form of CRC, called hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome), and therefore the *Msh2* model has some similarities to the human disease. Secondly, the O^6MeG adduct (which forms a large part of the hypotheses of this thesis) can generate single nucleotide mutations, and these mutations can be recognised by the MMR complex (including the *Msh2* protein). Thirdly, *Msh2* knockout mice develop significantly more colon tumours in response to chemical carcinogens compared to wild type mice (Mladenova et al. 2011). Taken together, it seemed reasonable that if a diet high in red meat increased O^6MeG adducts in *Msh2* knockout mice could have downstream consequences on carcinogenesis, and any protective effects of RS could also be ascertained.

All *Msh2* knockout mice were ordered from Australian Bio Resources (ABR), Sydney, Australia, with the help from A/Prof. Maija Koonen-Corish of the Garvan Institute, Sydney, Australia. The mice were initially bred on a 129/OLA background (de Wind et al. 1998) but had been backcrossed with C57Bl6J strain mice for multiple generations. Breeding at ABR was carried out with $Msh2^{+/-}$ breeding pairs to produce wild type, heterozygote and homozygous knockout litters. $Msh2^{-/-}$ and $Msh2^{+/+}$ mice at approximately 4 weeks of age, both males and females, were used in the experiments in equal numbers.

2.2.3. MGMT knockout mouse model

The *MGMT* knockout mouse was selected due to its lack of DNA O^6MeG repair. A major focus of this thesis is red meat induced DNA O^6MeG adducts and their association with CRC risk. Therefore, a mouse deficient in O^6MeG repair seemed potentially useful to study the effects of dietary red meat on colorectal oncogenesis. Initially, research plans included a long term tumour study, feeding red meat and/or resistant starch to *MGMT* knockout mice over the course of 12 months. It was hypothesised that if red meat-induced DNA adducts were not efficiently repaired (and these adducts are linked to cancer formation), an increase in colon tumours would be observed in *MGMT* deficient mice. However, due to circumstances (within the animal facility) that were out of my control, the time taken for the *MGMT* mice to be imported from the USA was much longer than anticipated. These mice were not available anywhere else in Australia. Consequently, only a short term feeding study could be carried out in the time constraints of the candidature.

The particular *MGMT* mouse strain used in this thesis was originally generated by Glassner et al (1999) with a targeted construct inserted within the *MGMT* gene, deleting the sequence coding for the active alkylating repair site (Glassner et al. 1999). The MGMT activity of progeny mice is at extremely low levels or not detectable. For this thesis, breeding pairs (2 male and 3 female) were imported on a material transfer agreement between Dr Richard Le Leu and Dr Leona Samson of the Massacheuttus Institute of Technology (MIT) in the USA. All imported male and female mice were *MGMT*$^{-/-}$ and were confirmed void of any detectable MGMT activity by scientists at MIT. The School of Medicine Animal Facility (SOMAF) carried out breeding within SOMAF at Flinders Medical Centre, Bedford Park, South Australia. Mice were weaned at 21 days and acclimatised for 2 weeks before beginning any experimental procedures.

2.3. Animal experimental dietary compositions

2.3.1. Red meat

All preparation methods of red meat used for animal experiments in Chapters 4, 6 and 7 are detailed in this section. Red meat was sourced from a local butcher in Adelaide. It was supplied as top-side beef with excess fat removed, before being ground into mince-meat and sealed in plastic bags for storage at 4°C. The following day, meat was cooked on a flat gas BBQ plate at the lowest temperature setting (approximately 150°C), while being continuously turned to avoid any prolonged frying or burning of the meat. This method of cooking avoids formation of heterocyclic amines and poly aromatic hydrocarbons, which have also been associated with CRC risk (Rohrmann et al. 2009). After cooking, the meat was dried in an oven overnight at approximately

35°C and ground to a fine powder before mixing in with the dry ingredients for preparation of experimental diets. Saturated fat content of the red meat powder was 10% and analysed by standard lipid extraction gravimetrically with a mixture of chloroform: methanol (1:1, v/v) according to the method of Daugherty and Lento (Daugherty and Lento 1983)(see Appendix C for detailed methods). Allowrie lard was used as the saturated fat source in the non-red meat groups to control for saturated fat content of the red meat diet. The lard was heated in a standard microwave on low-medium heat until it was liquefied, and mixed into the diet with other ingredients. To calculate moisture content of the final meat preparation, representative powdered samples were pre-weighed in Petri dishes and then heated in a dry oven at 110°C for 3 hrs. Samples were then cooled to room temperature in an evacuated desiccator prior to weighing, with the total loss in moisture calculated at 5%. Casein (Inpak Foods) was used as a non-meat protein source to control for the red meat protein.

2.3.2. Haem

Haem content of the final red meat preparation was analysed using a method modified from Hornsey (Hornsey 1956). Weighed 5g portions of powdered meat were measured in triplicate and placed into 50ml tubes. A total of 12.5ml of acetone solution (Appendix D) was added to each triplicate and mixed with a vortex for 15 seconds. A further 12.5ml of acetone solution was added to each tube and mixed again thoroughly. Tubes were capped tightly and let stand for 1 hour in the dark at room temperature before centrifugation for 10 minutes at 2200g. The supernatant was used for reading absorbance at 640nm, with an incubation temperature of 24.6°C, on a Benchmark Plus reader against a reagent blank. Calculation of the final haemin content of red meat and final concentration

of haemin required to add to the diets is detailed in Appendix E. The source of haemin was haemin chloride, purchased from Sigma Aldrich (catalogue # 51280), which was chosen because it only contains the haem molecule of interest and not the entire haemoglobin protein. A total of 0.01034g was added per 100g to get a final concentration of 0.2µmol/g of diet preparation.

2.3.3. Resistant starch

This section details the RS used for all animal studies. High amylose maize starch (HAMS; Hi-maize 260), type 2 RS, was used as the source of RS and was supplied by the National Starch and Chemical Company (now known as Ingerdion™). Hi-maize 260 has been shown to contain approximately 50% RS (Le Leu et al. 2009) and was added at a level of 10 g/100 g diet; therefore, a total of 5% in each diet preparation. This level of RS was chosen because it has been shown previously to induce significant amounts of SCFA and reduce adenomas in the colon of rodents (Le Leu et al. 2007), it is at a level more suitable for use in human studies and it has been shown to generate SCFA in the presence of red meat previously (Winter et al. 2011). Rodent studies have typically used 20% or more of RS sources (Le Leu et al. 2009), which is not an ideal level to compare with human intake. A 20% RS diet in humans would be near impossible to consume on a daily basis by any one individual, particularly if it is not part of their regular diet. Excessive flatulence, abdominal cramping and bloating after consumption of a high non-digestible carbohydrate diet is not well tolerated (Grabitske and Slavin 2009).

2.3.4. Green tea

The source of green tea used was polyphenon 60 (Sigma catalogue # P1204). This product contains ≥60% total catechins from green tea and was added at 0.5% of the total diet preparation. The green tea was weighed and mixed in with dry ingredients before preparing final diet preparations. The level of green tea used (0.5% of the diet) was selected as it has been shown previously to reduce large ACF formation in the colon of rats after exposure to alkylating carcinogens (Hu et al. 2013). A preliminary trial in rats (performed in our laboratory) showed that green tea mixed with the diet generated a higher MGMT activity level in the colon compared to green tea in the drinking water (Hu et al unpublished data). In addition, for humans it would be challenging to consume 0.5% of green tea in beverage form (requiring around 4-5 cups per day), compared to one pill containing the green tea extract per day. Therefore, it was decided to add the green tea in powdered form to the diet so that results might be translated to the human setting.

2.4. Faecal collections and biological assays

2.4.1. Faecal collections

Each group of mice were moved from their housing cage into a clean cage to collect fresh faecal samples. Over 5 days mice were handled gently to collect and weigh faecal matter and immediately collected into 5ml tubes. At least 0.3g of faecal matter was pooled from mice in each cage before the mice were placed back into their housing cages. A total of 2 separate faecal samples (≥ 0.3g) were collected; one for measurement of pH and ammonia (Chapter 2.7.2), the other for a combined measurement of SCFA and phenols (Chapter 2.7.3). For measurement of pH and ammonia, 2 days of pooled faecal samples were placed into 3 times the

weight to volume of cold saline solution (0.9%) for immediate measure of faecal pH, and stored at -20°C for later analysis of ammonia. For measurement of SCFA faeces were stored in 2ml Eppendorf tubes at -20ºC ready for preparation of distillation.

2.4.2. Faecal pH and ammonia assay

A two point calibration was performed on a pH meter and glass embodied electrode (Eutech Instruments). The faecal sample in 3 × weight to volume of saline was measured for pH and recorded. Faecal samples for ammonia assay were defrosted on ice and vortexed before centrifuging for 10 minutes at 3000 rpm. A 100µl supernatant aliquot was diluted to make a 1 in 3 (×4) dilution for Ammonia measurement using Janus Robotic Liquid handling System (Bajka et al. 2008). Results were calculated as µmol/ml in the original diluted faecal sample in saline. All ammonia assay measurements and calculations were carried out by the CSIRO, Food and Nutritional Sciences, Adelaide.

2.4.3. Faecal SCFA, phenols and p-cresol assay

Collected faecal matter was weighed into 5ml tubes then 2 ml of internal standard (IS) (see Appendix D) and left on ice for 30 minutes to soften then mixed thoroughly with a vortex. Mixed faecal samples were then centrifuged at 3000rpm for 15 minutes at 5-10°C and the supernatant transferred to a clean 5ml tube. A vacuum distillation manifold (Labglass) was lubricated with petroleum jelly on the Y pieces and 2 quick fast flasks were attached to the rear side (receiving flasks). In two duplicate quick fast flasks, 200µl of faecal supernatant was added with 40µl of 1M phosphoric acid (sample flasks). The sample flasks were dipped and swirled into ice cold ethanol (at -80°C) for about 10 seconds,

creating an even layer of sample around the base of the flask. The sample flasks were attached to the front of the Y piece, leaving the base of the flask in a cup with ice cold ethanol. A Wombat high vacuum pump (Javac) was attached and turned on to create a vacuum in the manifold, then the Y taps were turned off to isolate each flask. Sample flasks were transferred out of the ice cold ethanol and replaced with the receiving flask to start the distillation process. To speed the reaction, the sample flasks were placed in warm water at approximately 60°C until the distillate had formed in the receiving flasks. Once the distillate was thawed, 100µl from each duplicate was transferred to a Gas Chromatography vial for SCFA analysis or a HPLC vial for phenols and cresols analysis.

Both SCFA and phenol analysis of prepared samples were carried out by technicians at CSIRO, Food and Nutritional Sciences, Adelaide, following protocol as described previously (Murray and Adams 1988, Bajka et al. 2006). Once each faecal sample had been distilled, 100µl was injected into an Agilent Technologies 6890N Network Gas Chromatograph System fitted with a Zebron ZB-FFAP column (0.53 mm × 30 mm). Another 100µl of distillate was injected into a Shimadzu LC-10AD HPLC machine with RF-10AXL fluorescence detector set at excitation 284nm, emission 310nm. With 2ml of IS added, there was 100µg of o-cresol and 1.68 µmol of heptanoic acid to each sample. The calibration tables for both HPLC and GC were set to give a relationship of areas of the peaks of known amounts of test substance and also of the IS. HPLC gave final results as µg of phenol or *p*-cresol per ml of faecal sample. The GC gave results as µmol of SCFA in total weight of the sample compared to the 1.68 µmol of heptanoic acid added to the sample. SCFA results were divided by the total weight of faeces added to the sample to get values as per g of faeces.

2.5. Animal tissue analyses

Mice were euthanized with carbon dioxide asphyxiation followed by cervical dislocation. Colon tissue and extra-colonic tissue were collected for fixation prior to analysis. For ACF and tumour quantification, the entire length of the colon was cut open longitudinally, stretched out and affixed to Hi-Bond™ membrane (GE Healthcare) prior to fixation. For distal and proximal colon analysis, 10mm sections from each end of the colon were cut in a cross section before processing and embedding.

2.5.1. Tissue fixation, processing and embedding

Tissue was placed into a solution of 10% buffered formalin (Orion Laboratories catalogue # C924) containing 3.6% w/v of formaldehyde, phosphate buffered to pH 7. After 24 hours, the formalin solution was removed and replaced with a 70% solution of ethanol for storage. Before processing, colon and tumour tissue samples were cut into cross section lengths of approximately 5mm and placed into an embedding cassette (Tehnoplas catalogue # CAS LID-02) with biopsy pad to secure in place. Approximately 30 cassettes were processed at any one time through gradient ethanol solutions, xylene and paraffin wax using a Microm STP120 tissue processor (Microm, Germany) (details of protocol in Appendix F). Cassettes were then transferred to a Thermo Fisher (Shandon Histostar) embedding centre paraffin wax holding tank kept at 60°C. A stainless steel mould was filled with fresh melted wax, and the tissue was orientated to create a transverse cross section in the final tissue mould. The tissue and wax mould were cooled on the cold plate of the embedding centre for approximately

10-15 minutes. All paraffin embedded moulds were stored in a sealed container at room temperature until required.

2.5.2. Apoptosis evaluation in Western diet models (Chapter 3 and 4)

Paraffin embedded distal colons were cut on a Leica RM2235 Microtome (Leica Microsystems, Germany) at 5μm and stained on Microscope slides (HD Scientific Supplies). Sections were independently coded, rehydrated through gradient alcohols and stained for DNA double strand breaks using the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay, following the manufacturer's instructions (Appendix G). Slides were independently and randomly coded and evaluated under light microscopy with an Olympus BX41 microscope. Positive apoptotic cells were assessed as dark brown nuclear staining and were calculated as the number of positive cells per crypt.

2.5.3. Apoptosis evaluation in response to AOM (Chapter 7 and 8)

Paraffin embedded distal colons were cut on a Leica RM2235 Microtome (Leica Microsystems, Germany) at 5μm and stained on Microscope slides (HD Scientific Supplies). Sections were rehydrated in gradient ethanol, stained with JJ Harris Haematoxylin (see Appendix G) and evaluated under light microscopy with an Olympus BX41 microscope. Slides were independently coded and apoptosis was identified in 20 randomly chosen crypts by morphological changes such as cell shrinkage, condensed chromatin and sharply defined borders with a clear rounded halo (Le Leu et al. 2003). Positive apoptotic nuclei were calculated as the mean number of apoptotic cells per colonic crypt.

2.5.4. Determination of cell proliferation

Proliferative cells in the distal colonic epithelium were measured using an antibody specific for the nuclear proliferating antigen Ki-67 in combination with an IHC detection method. 5µm cut sections on pre-coated SuperFrost® Plus slides (Menzel-GAser, Germany) were independently coded and rehydrated in gradient ethanol (Appendix G). Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in 50% ethanol (Appendix D) for 15 minutes at RT. Antigen retrieval was performed using citrate buffer pH 6.5 (Appendix D) for 60 minutes in a 2100 Antigen Retriever (Pick Cell Laboratories). Slides were cooled at RT in the citrate buffer. To block non-specific binding, 30 minute incubation with a protein blocking solution (catalogue #SIG-3226, Signet Laboratories) was carried out. A monoclonal rat-anti-mouse Ki-67 antigen (SP6 catalogue# 120-16667, Sapphire Bioscience) was diluted 1:500 in phosphate buffered saline (PBS, pH 7.4) (Sigma-Aldrich). Incubation of the primary antibody was overnight at 4°C in a humidified chamber.

The next day slides were washed 3 times for 2 min each in PBS, then a horse radish peroxidase (HRP) linking reagent (catalogue# SIG-3226, Signet Laboratories) was applied to each slide for 30 minutes at RT and rinsed again with PBS 3 times for 2 min each. The sections were labelled with an avidin/biotinylated peroxidase complex (catalogue# SIG-3226, Signet Laboratories) for 20 minutes at RT and rinsed 3 times with PBS for 2 mins each. Slides were visualised by incubating with 40µl of 3'-diaminobenzamine (DAB) chromagen in 1ml of DAB substrate buffer (Signet Laboratories) for 5 minutes at RT, counterstained with JJ Harris Haematoxylin for 1 minute before dehydration through gradient alcohols and cover slipped (Appendix G). Slides were visualised

under light microscopy with an Olympus BX41 microscope by brown nuclear staining and assessed as Ki-67 positive cells per crypt.

2.5.5. DNA adduct quantification

The level of DNA alkylation and oxidation was quantified using an immunohistological antibody for the DNA adducts O^6-Methyl-2-deoxyguanosine (O^6MeG) and 8-hydroxy-2-deoxyguanosine (8-oxo) on paraffin embedded distal colons. On pre-coated SuperFrost® Plus slides (Menzel-GAser, Germany), 4µm cut sections were rehydrated (Appendix G), incubated in 3% H_2O_2 in 50% ethanol (Appendix G) for 15 minutes at RT. Antigen retrieval was performed using citrate buffer pH 6.5 (Appendix D) for 60 minutes in 2100 Antigen Retriever (Pick Cell Laboratories). RNase (Appendix D) treatment of sections was carried out for 1 hour in a 37°C oven followed with a 5 minute treatment of 140mm NaCl solution at 4°C to stop the RNase reaction. Alkali treatment (Appendix D) was performed for a total of 5 minutes at 4°C to unwind the DNA and expose the lesions of interest. Sides were washed 3 times with PBS for 2 min each before applying Special Block A (Covance Laboratories) for 30 min at RT. The O^6MeG primary monoclonal antibody (Clone EM 2-3, Squarix Biotechnology, Germany) was diluted to 1:1000 in PBS and applied to the slides overnight at RT (or overnight at 4°C for proximal colon sections in Chapter 6). The mouse monoclonal 8-oxo adduct antibody (Isotope IgG2b, catalogue# 4354-MC-050, Trevigen) was diluted to 1:2000 in PBS and applied to slides overnight at 4°C. The following day, sections were washed 3 times with PBS for 2 mins and incubated with Special Block B (Covance Laboratories) for 30 minutes at RT. A Poly-HRP anti-mouse IgG (Covance Laboratories) was incubated on the sections for 30 minutes before washing in PBS 3 times for 2 minutes each. Positive cells were visualised using

40µl of DAB chromagen in 1ml of DAB substrate buffer (Signet Laboratories) for 1 minute, before counterstaining with JJ Harris haematoxylin, dehydrated in gradient ethanol and cover slipped (Appendix G) for observation under light microscopy.

Computer image analysis of the adduct staining intensity was measured using pictures taken with the Q Imaging Micropublisher 3.3 RTV digital camera attached to an Olympus BX41 Microscope at 20 × optical zoom. The software was provided by Paul Jackway from the department of Mathematical and Information Sciences, CSIRO. The program Olysia Bioreport Imaging System 5.0 was used for image capture of an intact crypt column. Image brightness, exposure and white balance all remained the same for each image that was captured. The programs R for windows 2.1.0 and Q capture suite 2.68.6.0 was used for quantifying the captured images. These programs convert the level of positive staining to negative staining for the O^6MeG adduct into a quantifiable number by using the intensity of "red" colour divided by the intensity of "blue" colour of each individual cell's nucleus. This gives a ratio of positive staining intensity to negative staining intensity called the red over blue (RoB) ratio. A higher ratio indicates a more intense positive stain, which correlates to a higher DNA adduct load. Each cell along the crypt was measured for RoB ratio, and 20 crypts per mouse were counted. Total sum of adduct formation was calculated by summing each RoB ratio for the average crypt height. This was performed for each mouse and then averaged for each treatment group to give a final measure of "Sum of staining intensity". For Chapters 6, 7 and 8, total SUM of DNA adduct values were normalised by subtracting from the mean of control group then divided by the standard deviation of the control group.

2.5.6. Aberrant crypt foci and tumour analysis

The entire colon length was analysed for tumours and ACF. Excess mucus and debris were washed from the colon with 0.9% solution of saline and gently wiped clean with a cotton tip. A 0.4% solution of methylene blue diluted with a 0.9% saline solution was made up. The entire colon was submerged in the 0.4% methylene blue solution for approximately 1-2 minutes. Colons were immediately visualised for tumours and ACF under a dissecting microscope at 10× optical zoom. ACF are identified as having crypts with large openings, unusual shape and stained darker than the normal surrounding crypts. ACF were classified as small (≤3 ACs), large (≥4 ACs) and total ACF. Any suspected colonic or extra colonic tumours were taken for fixation and tissue processing (Appendix F) before staining with Haematoxylin and Eosin (H&E) stain for histological confirmation (Appendix G). Slides were visualised under light microscopy. Colorectal and small intestinal tumours were classified as adenoma or adenocarcinoma and classification was confirmed by Dr Ying Hu from Flinders University. Adenomas characterised by expansion of the mucosal layer, reduction in goblet cell number, moderate loss of mucosal architecture by glandular growth and dilated cysts. Adenomas were further classified into 2 grades of dysplasia (low and high). Adenocarcinoma was identified when there was severe distortion of cytological and glandular architecture, loss of cell polarity, prominent cellular atypia and invasion through the muscularis mucosa.

2.6. Human randomised cross over trial

2.6.1. Study design and participants

The human study was a randomised crossover trial consisting of two intervention periods of four weeks, preceded by a 4-week run-in (baseline) and separated by a 4-week (washout) period (for study design refer to Appendix H). Twenty-three healthy volunteers participated in the trial. Ten volunteers completed the high red meat (HRM) intervention as first diet period; while 13 volunteers completed the high red meat with butyrylated high amylose maize starch (HRM+HAMSB) intervention first. Exclusion criteria included evidence of active mucosal bowel disease, intolerance to high-fibre foods or any perceived contraindication to consumption of the test products. During the entry (baseline) and washout periods, participants consumed their habitual diets. For the interventions, they were allocated randomly to a HRM diet or to a HRM diet supplemented with 40 g of butyrylated high amylose maize starch (HAMSB) per day (HRM+HAMSB diet). During the HRM intervention, the participants consumed 300 g (raw weight) of cooked lean red meat per day which was supplied frozen in 100 g packs of lean mince, beef strips, or lamb strips with three packs to be consumed each day. During HRM+HAMSB intervention participants were also required to consume a total of two pre- packed 20 g sachets of HAMSB daily, one in the morning and one in the evening by mixing the powder into 250 mL reduced fat milk or orange juice. The HAMSB was manufactured by Ingredion (Bridgewater, New Jersey). Participants on the HRM arm of the study were asked to consume 250 mL of reduced fat milk or orange juice per day to match the HRM+HAMSB intervention. Participants were instructed to maintain their usual diet during the study but to avoid consuming high levels of protein or fibre, or probiotic supplements, except those prescribed for the study.

Participants were asked to avoid consuming, or record the use of, any medication that could interfere with bowel function (including antibiotics). Participants were monitored by a trial nurse (weekly) and dietician (at end of each 4-week period) to ensure that diet and intervention guidelines were followed, and weight was kept stable. Details of medical history and medications, weight, bowel health and symptoms and adverse events were collected by the trial nurse throughout the study. Composition of the participants' diets and compliance with the interventions was assessed using weighed food diaries which were completed by participants at the end of each 4-week dietary period, 3 days prior to each clinic visit. Food diaries were entered into Foodworks Professional 7 nutritional calculation software (Xyris Software, Kenmore Hills, Queensland, Australia) by a dietician, to calculate energy and macronutrient intake based on Australian food composition tables and food manufacturers' data. The study was approved by Flinders Clinical Research Ethics Committee (reference no. 155/09; Flinders Medical Centre, Bedford Park, SA, Australia) and all volunteers gave written informed consent.

2.6.2. Sample collection

Stool and rectal pinch biopsy specimens were obtained at the completion of the 4-week entry period (baseline) and at the end of each intervention and the washout period. A complete faecal collection was conducted by all the participants for the last 48 h of each dietary period, and samples were stored in portable freezers (-20˚C). At each visit to the Flinders Medical Centre clinic an experienced gastroenterologist collected 4 rectal mucosal biopsies at each visit with rigid forceps through sigmoidoscopic examination performed without bowel preparation or prior dietary restriction. Biopsies were formalin-fixed and

dehydrated through gradient alcohols and xylene before being embedded in paraffin wax (Appendix F).

2.6.3. Stool analyses

All SCFA, ammonia and phenols analyses were carried out by technicians at the Food and Nutrition Flagship laboratories, CSIRO, Adelaide. Faecal samples were thawed at 4°C, pooled, homogenized, and then subsampled for analysis. Weighed portions for the determination of SCFA and phenols were analysed using gas chromatography (Chapter 2.4.3). Ammonia concentration in faeces was measured using the indophenol blue method (Chapter 2.4.2). All NOCs were analysed by scientists at the Rowett Institute of Nutrition and Health, University of Aberdeen, United Kingdom. Faecal samples were diluted 5-fold with distilled water, and the content of NOCs analysed by measuring the chemical release of nitric oxide detected on a thermal energy analyser (Holtrop et al. 2012) and values were expressed as apparent total NOCs (ng/mL faecal water sample).

2.6.4. Rectal biopsies

Rectal biopsies were embedded in paraffin, sectioned at 4 μm and their O^6MeG adduct load was quantified using an anti O^6MeG antibody (Squarix Biotechnology Marl, Germany) as described in Chapter 2.5.5. All slides were independently and randomly coded before quantitation of nuclear staining for O^6MeG with a computer image analysis protocol (Chapter 2.5.5).

Proliferation status of cells in the rectal crypts was determined by standard immunohistochemical techniques using the proliferating cell nuclear antigen (PCNA) antibody (PC10, Santa Cruz, CA, USA) and mouse-on-mouse (MoM) polymer linking kit (Covance Laboratories). First, 5μm cut sections on pre-coated

SuperFrost® Plus slides (Menzel-GAser, Germany) were independently coded and rehydrated in gradient ethanol (Appendix G). Endogenous peroxidase activity was quenched by incubation in 3% H_2O_2 in 50% ethanol (Appendix G) for 15 minutes at RT. Antigen retrieval was performed using citrate buffer pH 6.5 (Appendix D) for 60 minutes in a 2100 Antigen Retriever (Pick Cell Laboratories). Slides were cooled at RT in the citrate buffer. To block non-specific binding, 30 minute incubation with a protein blocking solution (Signet Laboratories) was carried out. A 1 in 50 dilution of the PCNA antibody (PC10, Santa Cruz, CA, USA) in PBS was used to incubate the sections at 4°C overnight before rinsing off in PBS the next day. Slides were incubated with boost reagent (Covance) for 30 minutes, rinsed 3 x in PBS before a 30 minute incubation with polymer-HRP (Covance) at room temperature and rinsed again 3 x in PBS. Positive cells were visualised using 40µl of DAB chromagen in 1ml of DAB substrate buffer (Signet Laboratories) for 3 minutes, before counterstaining with JJ Harris haematoxylin, dehydrated in gradient ethanol and cover slipped (Appendix G) for observation under light microscopy. Positive cells were assessed as the number of PCNA positive cells per crypt. Serial sections 50µm apart were carried out where necessary, in order to get an adequate number of whole crypts to count for each protocol.

2.7. Statistical analysis

All statistical analyses were carried out using IBM SPSS statistics software, version 20, unless otherwise stated. Statistical significance was accepted at $p<0.05$ level. Data are represented as the mean ± standard error (SE).

2.7.1. Chapter 3

Tumour outcomes are expressed as incidence. General linear model univariate analysis was used to determine the main effect of RS addition and haem on all outcomes measured. Independent sample t-tests were performed on each variable to determine the difference between short term and long term feeding of each diet on DNA markers and cell kinetics. Colon tumour incidence was analysed by Crosstabs Fishers Exact test.

2.7.2. Chapter 4

All bodyweight, faecal analyses and IHC measurements were analysed first by One-way ANOVA to determine any differences between dietary groups. All IHC analyses were also tested for the main effects of protein level (15% vs 30%), red meat and RS intake by full factorial analysis. Interaction of red meat and RS on IHC analyses were tested using general linear model univariate analysis. Colon tumour incidence was analysed using crosstabs with Fischer's exact test.

2.7.3. Chapter 5

Dr Rosalind B. Miller at CSIRO Mathematics, Informatics and Statistics, performed all statistical tests carried out. The statistical analyses were performed with scripts in R, version 3.0.1, using the R statistical package. All statistical analyses were carried out with the non-linear mixed effect (nlme) package in R using a linear mixed effects model with Subjects as the random effect on either base-10 logarithm-transformed data, where necessary, or on untransformed data for each of the variables. Initial analyses were undertaken using both periods of the trial, including baseline and washout periods. However, the data analyses of the initial study showed that some of the response variables had carry-over

effects, including the primary end-point O^6MeG but not SCFA (Appendix I). This was indicated by a significant difference between the baseline level and the washout level of the response variable or a significant interaction between the week of diet consumption and the response variable. Therefore the data analyses used for this chapter were only those of the first period of the study (i.e. only measurement weeks 0 and 4). As a result, the analysis performed and reported here was undertaken using only the baseline and the first period data. A comparison between the groups in the first period of the trial was using a linear mixed effects model, testing for changes from the baseline and a difference between treatments. The final column of each table gives the p-value for the difference between the two treatments at week 4, and these were tested using either the original data or the log-10 transformed data as appropriate.

2.7.4. Chapter 6

Cumulative survival of *Msh2*$^{-/-}$ mice are presented in Kaplan-Meier curves and generated over the experiment form 100 *Msh2*$^{-/-}$ mice. Overall survival between all groups and control vs. red meat were analysed with log-rank (Mantel-Cox) test. To avoid variation of adduct staining attributed to aging (Winter et al. 2014), IHC measurements of the colonic epithelia and final mouse bodyweights were analysed in 12 mice from each dietary group (both *Msh2*$^{-/-}$ and wild type) that survived beyond 5 months. Data for O^6MeG and MGMT were standardised to control diet for each mouse within their genotype before comparison tests were carried out. One-way ANOVA was used as an initial comparison between all dietary groups for bodyweight and IHC analyses. Subsequent univariate analysis was used to identify effects of RS, red meat or genotype on final bodyweight. Differences in proliferation, O^6MeG and MGMT, depending on genotype or diet

treatment, were analysed using independent sample t-test. An effect of RS supplementation on proliferation in the colon was determined using univariate analysis of variance. Correlations of normalised O^6MeG and MGMT data for red meat group were determined using bivariate Pearson correlation (1-tailed). Due to the very small number of mice that had ACF, an effect of RS or red meat was analysed using generalised linear model (Wald chi-squared test). A test of diet effects on small intestinal tumour incidence were analysed using Generalized Linear Model: Ordinal Logistic (chi squared test) and for lymphoma incidence by Crosstabs (Pearson chi squared test). Overall tumour burden identified as the mean number of tumours per mouse was compared using independent sample t-test between the red meat group and control, RS and red meat + RS groups.

2.7.5. Chapter 7

Mouse final bodyweights, apoptosis, DNA O^6MeG adducts and Ki-67 were first analysed by General Linear Model Univariate Analysis of Variance to determine the main effects of treatment type (vehicle vs AOM), Genotype (wild type vs *MGMT*$^{-/-}$), Gender (male vs female), red meat consumption (no red meat vs red meat diets) and green tea consumption (no green tea vs green tea). Significant parameters were used in 2-way interaction analysis by Univariate Analysis and any significant interactions were analysed for 3 way interactions.

2.7.6. Chapter 8

One way ANOVA was performed for each parameter measured in the colon to identify any changes over time. Subsequently, independent sample t-tests were performed at each time point between wild type and *MGMT*$^{-/-}$ mice, for each parameter measured in the colon.

Chapter 3

Accumulation of pro-mutagenic DNA adducts in the mouse distal colon after consumption of haem does not induce colonic neoplasms in the Western diet model of spontaneous colorectal cancer.

3.1. Introduction

Increased DNA alkyl adducts in the colon via excess endogenous N-nitrosation is one mechanism that is thought to play a role in colorectal oncogenesis, although little is known about DNA adduct formation in the colon in response to red meat consumption (Kuhnle and Bingham 2007, Winter et al. 2011). Recent studies have shown that red meat can enhance the production of the pro-mutagenic adduct O^6-Methyl-2-deoxyguanosine (O^6MeG) after feeding of high red meat diets to mice, without chemical carcinogenic intervention (Winter et al. 2011) The O^6MeG adduct is a known mutagenic lesion in both animals and humans resulting from exposure to alkylating agents and is repaired by the enzyme O^6-methylguanine-DNA methyltransferase (MGMT). Persistence of unrepaired O^6MeG adduct changes the chemical bonds in the DNA backbone and thus the DNA polymerase reads the G as an A after one round of replication. If this mismatch is not repaired, further changes in the DNA backbone lead to the polymerase incorporating a T instead of a C, therefore a GC:AT transition mutation arises following a second round of replication (Margison et al. 2002).

This transition mutation has been identified in several genes including the *K-ras* gene, where mutation of this gene is a known mechanism of human oncogene activation in CRC (Margison et al. 2002).

The DNA oxidative adduct 8-hydroxy-2 deoxyguanosine (8-oxo) has been implicated in development of cancer for several years (Floyd 1990). 8-oxo is a pro-mutagenic adduct that if not repaired or removed from the cell can incorporate base insertions into the DNA, with G → T tranversions being the most abundant (Shibutani et al. 1991). The major repair pathway for this DNA adduct is via base excision repair, with the DNA glycosylase enzyme OGG1 having an affinity for excising the 8-oxo adduct from the DNA (Maynard et al. 2009). Patients with CRC and inflammatory bowel disease present with higher levels of 8-oxo measured in plasma (Gackowski et al. 2002, Dincer et al. 2007) and CRC patients show higher levels of 8-oxo DNA adducts measured in colon tissue compared to patients without CRC (Obtulowicz et al. 2010, Psofaki et al. 2010). 8-oxo adducts are linked with carcinogenesis although the significance of the 8-oxo adduct and consumption of red meat or haem in CRC has not been studied extensively.

Haem iron in red meat is potentially damaging to the colonic epithelium and has been implicated in development of CRC in humans (Cross et al. 2010) There are many different proteins regulating haem iron absorption in the small intestine and although it is readily bio-available there is a limitation to its absorption, therefore some will reach the colon (Roberts et al. 1993). Various animal studies have shown that diets with haem concentrations above average human consumption levels (0.25-1.5µmol/g) (Lombardi-Boccia et al. 2002) contribute to cell toxicity, hyper-proliferation, reduced apoptosis and increased

rates of CRC (Sesink et al. 1999, Pierre et al. 2003, Tappel 2007, de Vogel et al. 2008, Santarelli et al. 2010, Ijssennagger et al. 2012, Ijssennagger et al. 2013). A diet incorporating low levels of haem (at 0.25µmol/g) has shown a reduction in total number of aberrant crypt foci (ACF) per colon, although the ACF size was larger with haem diets compared to a control diet (Pierre et al. 2003). The intake of haem from a 30% red meat diet as beef would equate to an average concentration of 0.14µmol/g (Lombardi-Boccia et al. 2002), indicating that 0.25µmol/g of haem more closely resembles that of a high red meat intake in humans. Besides causing damage to the colon cells directly, haem-iron from red meat also triggers the formation of potentially damaging DNA alkylating NOCs either via increasing bacterial nitrate reductase activity or via reacting with nitrite or nitric oxide in the lumen (Cross et al. 2003). In humans, consumption of high levels of red meat has been directly related to increased alkyl adducts in the colon, and this was correlated to increased faecal NOC output (Lewin et al. 2006). There are strong indications that haem contributes to development of CRC. However, there have been no rodent models to date utilising a haem-containing diet at levels relevant to human consumption to identify its long term effects on the colon, without utilising other chemical carcinogens to induce cancer.

On the opposing side, a dietary component that has been implicated in protection against CRC is resistant starch (RS), the component of starch that is undigested in the small intestine and fermented rapidly in the colon. It is thought that the preferential fermentation of carbohydrate over protein by luminal microflora, when RS is incorporated into high protein diets, leads to a reduction in DNA changes that might initiate CRC (Le Leu et al. 2007, Le Leu and Young 2007, Winter et al. 2011). This fermentation increases the production of short

chain fatty acids (SCFA) (Cummings et al. 1996), in particular butyrate, which is important for large bowel function and is the major energy source for colon epithelial cells. RS has also been shown to reduce production of toxic protein fermentation products (Birkett et al. 1996, Winter et al. 2011). *In vivo* animal models demonstrate the ability of butyrate to increase the acute apoptotic response to a chemical carcinogen and reduce cellular proliferation to allow for repair or removal of highly damaged cells (Le Leu et al. 2007).

3.2. Aims and hypotheses

As haem is considered to be oncogenic, either directly or indirectly, and RS has the capacity to reduce oncogenic DNA lesions in the colon, this chapter investigated whether feeding haem (at human-relevant amounts) to mice for short or long periods of time could induce pro-oncogenic adducts or colon tumours, and if epithelial events relevant to formation of DNA lesions were affected. It also aimed to determine whether adding RS at levels appropriate for human dietary intake could manipulate these parameters. The hypotheses are that consumption of dietary haem will increase DNA adduct accumulation in the distal colon, but that RS consumption with or without haem will reduce DNA adducts. Also, that dietary haem will increase the incidence of colonic tumours in the mouse but RS intake will reduce colon tumours both with and without haem consumption. The specific aims are as follows:

1. To ascertain if consumption of haem, as hemin chloride, will enhance accumulation of DNA O^6MeG and DNA 8-oxo adducts in the colon of mice over time, and if co-consumption with RS will reduce adduct formation in the colon.

2. Identify if dietary haem, as hemin chloride, will increase spontaneous tumour formation in the colon of mice after 18 months consuming a Western diet, and if co-consumption of haem with RS will protect against tumour formation.
3. To determine if accumulation of colonic DNA adducts are associated with spontaneous tumour formation in the colon of mice.

3.3. Materials and methods

3.3.1. Animals and diets

A total of 208 eight week old wild type c57BL/J male mice were obtained from the Animal Resource Centre, Perth, Western Australia. The Flinders University of South Australia Animal Welfare Committee approved all experimental procedures (ethics approval number 809/12). Animals were randomly placed four mice per cage and divided into dietary groups (long term study n=40, short term study n=12) under controlled conditions of 22 ± 2ºC (SD), 80 ± 10% humidity and 12h light/dark cycle. Animals had free access to food and water and were weighed once weekly throughout the study period. 60 mice consumed diets for four weeks (short term) and 160 mice were on diets for 18 months (long term).

The experimental diets were based on the American Institute of Nutrition (AIN) diet AIN-76 with modified amounts of vitamins and minerals for rats and mice adapted to resemble a typical Western diet and known to lead to spontaneous tumours in rats (Newmark et al. 2009) (Table 3.1). Experimental diets were a modification of the AIN-76 diet and are presented in more detail in

Chapter 2.3. Final diet preparations were placed into air sealed containers, stored at 4°C in the dark, with fresh food in the mouse cages replaced daily.

Table 3.1: Composition of Experimental Diets in g/100g

Ingredient	Control	Control + RS	Haem	Haem + RS
Casein	17.6	17.6	17.6	17.6
Corn starch	10	-	10	-
Hi-Maize™	-	10	-	10
Sucrose	45.5	45.5	45.5	45.5
Sunflower Seed oil	16.8	16.8	16.8	16.8
Lard	3.2	3.2	3.2	3.2
α-cellulose	2	2	2	2
L-cysteine	0.3	0.3	0.3	0.3
Choline	0.12	0.12	0.12	0.12
Minerals*	3.5	3.5	3.5	3.5
Vitamins*	1	1	1	1
DL-Methionine	0.15	0.15	0.15	0.15
Hemin#	-	-	0.01304	0.01304

* Western diet vitamin and mineral mixtures containing modified calcium at 0.5 mg/g, phosphorus at 3.6 mg/g, folic acid at 0.23 mg/g, and vitamin D3 at 0.11 IU/g.(see appendix B)
# Hemin is at 0.2µmol/g of total diet (see Appendix E).

3.3.2. Faecal analysis

In the fourth week (short term) or sixth month (long term) of experimental diets two separate fresh faecal samples were collected from each mouse for measurement of faecal pH (Chapter 2.4.2) and SCFA, phenols and *p*-cresols (Chapter 2.4.3).

3.3.3. Tissue sample collection

All colon samples were fixed with 10% buffered formalin solution containing 3.6% formaldehyde for 24 hours then transferred to 70% ethanol for processing. On completion of short term experimental diets 1cm of distal colon was used for analysis. After the long term feeding experiment the entire colon

was cut longitudinally for analysis of tumours under a dissecting microscope before a 1cm distal colon segment was taken for analysis and all tumours were collected and processed for H&E staining for histological confirmation. Distal colonic tissue and tumours were processed through gradient alcohols and xylene, embedded in paraffin wax (Appendix G) before sectioning on a microtome at 4µm for IHC analysis.

3.3.4. Cell proliferation and apoptosis measurement

Proliferative activity of distal colonic epithelial cells was measured using an antibody specific for the nuclear proliferating antigen Ki-67 (rat-anti-mouse clone TEC-3, Dako, USA) (Chapter 2.5.4). Slides were visualised under light microscopy by brown nuclear staining and proliferation was assessed as the ki-67 positive cells per crypt column length. Apoptosis was measured using a terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay kit (Chapter 2.5.1). Slides were visualized under light microscopy by brown nuclear staining and apoptosis in the distal colon was assessed as the number of positive apoptotic nuclei per crypt column length. All slides were independently and randomly coded.

3.3.5. DNA adduct quantification

DNA alkylation was quantified using an antibody specific for the DNA adduct O^6-Methyl-2-deoxyguanosine (O^6MeG, Clone EM 2-3,Squarix Biotechnology, Germany) and the level of oxidative adducts was measured using an antibody specific for the DNA adduct 8-hydroxy-2 deoxyguanosine (8-oxo, Trevigen, USA) combined with a mouse-on-mouse polymer horse radish peroxidase (Poly-HRP) detection system (Covance Laboratories) on paraffin-

embedded distal colon sections. The primary antibody concentration for O^6MeG was 1:1000 (in PBS) and for 8-oxo the primary antibody was applied at 1:2000 (in PBS). Immunohistochemical (IHC) staining procedures and computer image analysis of stained slides were carried out as described in Chapter 2.5.5. Total sum of adduct formation was calculated along the crypt length for each mouse and averaged for each dietary treatment. All slides were independently and randomly coded so that dietary groups were not known to the counter.

3.4. Results

3.4.1. Body weight and faecal analysis

In the short term, no significant differences in body weight (g) were observed after consuming the experimental diets for four weeks, however after long term feeding mice consuming dietary haem had significantly reduced body weights compared to mice on control diet ($p<0.01$) (Table 3.2). After both short and long term feeding, addition of RS to the diet significantly lowered faecal pH compared to those diets without RS ($p<0.0001$) and markedly increased faecal total SCFA concentration ($p<0.0001$) including acetate ($p<0.006$), propionate ($p<0.0001$) and butyrate ($p<0.0001$) (Table 3.2). RS was also able to reduce concentration of faecal *p*-cresol ($p<0.0001$) and phenol ($p<0.0001$) in the short term (Table 3.2). Haem addition had no significant effect on faecal fermentation of SCFAs, phenol or p-cresols compared to the control after long term experiment there were significantly reduced concentrations of acetate ($p<0.001$), propionate ($p< 0.05$), butyrate ($p<0.05$) and total SCFAs ($p<0.01$) (Table 3.3) compared to the Control + RS diet. After long term feeding diets containing RS showed

significantly reduced fermentation of phenols but not p-cresols compared to diets without RS ($p<0.05$).

Table 3.2: Short term study measures of body weight and fermentation[1]

Variable	Unit	Experimental Diet Groups			
		Control (n=12)	**Control + RS (n=12)**	**Haem (n=12)**	**Haem + RS (n=12)**
Body Weight	g	28.5 ± 0.4	28.4 ± 0.5	29.3 ± 0.9	28.6 ± 0.5
Faecal pH	-	7.8 ± 0.2	7.1 ± 0.2[a]	7.6 ± 0.1	7.0 ± 0.1[a]
Total SCFAs	µmol/g	45.6 ±11.3	109.9 ± 10.4[a]	55.6 ± 12.2	117.3 ± 16.9[a]
Butyrate	µmol/g	1.8 ± 0.8	19.2 ± 3.2[a]	4.9 ± 0.9	20.4 ± 3.1[a]
Acetate	µmol/g	36.1 ± 8.7	61.2 ± 6.6[a]	42.0 ± 9.8	68.1 ± 10.3[a]
Propionate	µmol/g	7.6 ± 1.9	29. 6 ± 2.5[a]	8.7 ± 1.8	28.8 ± 4.1[a]
Phenol	µg/g	7.2 ± 1.6	0.8 ± 0.6[a]	7.3 ± 1.7	0.2 ± 0.1[a]
***p*-cresol**	µg/g	21.7 ± 2.5	11.8 ± 1.6[a]	21.5 ± 3.3	9.3 ± 1.1[a]

[1] Data represented as Mean ± SE
[a] Control + RS and Haem + RS is significantly different to Control and Haem

Table 3.3: Long term study measures of body weight and fermentation[1]

Variable	Unit	Experimental Diet Groups			
		Control (n=40)	**Control + RS (n=40)**	**Haem (n=40)**	**Haem + RS (n=40)**
Body Weight	g	58.3 ± 2.1	52.6 ± 1.8	48.0 ± 2.1[a]	51.7 ± 1.4
Faecal pH	-	7.7 ± 0.2	7.1 ± 0.2[a]	7.5 ± 0.1[d]	6.9 ± 0.1[a,c]
Total SCFAs	µmol/g	25.3 ± 3.8[b]	52.4 ± 5.1[a]	22.3 ± 3.1[b]	19.0 ± 2.6[b]
Butyrate	µmol/g	4.9 ±1.5[b]	18.8 ± 1.9[a]	6.8 ± 1.3[b]	6.2 ± 0.7[b]
Acetate	µmol/g	17.7 ± 2.8	15.1 ± 1.6	8.4 ± 1.0[a]	7.1 ± 1.3[a,b]
Propionate	µmol/g	2.8 ± 0.3[b]	14.8 ± 1.6[a]	4.5 ± 0.7[b]	3.5 ± 0.5[b]
Phenol	µg/g	2.0 ± 0.7	0.9 ± 0.1[a]	1.8 ± 0.2	1.2 ± 0.4
***p*-cresol**	µg/g	7.5 ± 2.1	11.1 ± 1.7	7.8 ± 0.7	6.7 ± 1.1

[1] Data represented as Mean ± SE
[a] significantly different to Control
[b] significantly different to Control + RS
[c] significantly different to Haem
[d] significantly different to Haem + RS

3.4.2. Short-term effects of haem and RS

In the short term, haem at 0.2µmol/g in the diet increased cell proliferation (Figure 3.1B) as measured by positive Ki-67 cells in the colon compared to diets without haem ($p<0.05$). RS significantly increased crypt height in the colon compared to diets without RS ($p<0.05$) but it did not increase the proportion of Ki-67-positive cells (Figure 3.2B and 3.2C). There were no significant effects of haem or RS on apoptotic cell counts in colonic epithelial cells (Figure 3.1A and 3.2A). There were no significant differences after four weeks of feeding in either the O^6MeG adduct (Figure 3.1D and 3.2D) or the 8-oxo adduct (Figure 1E and 2E) between haem and control diets or after the addition of RS to the diet.

3.4.3. Long-term effects of haem and RS

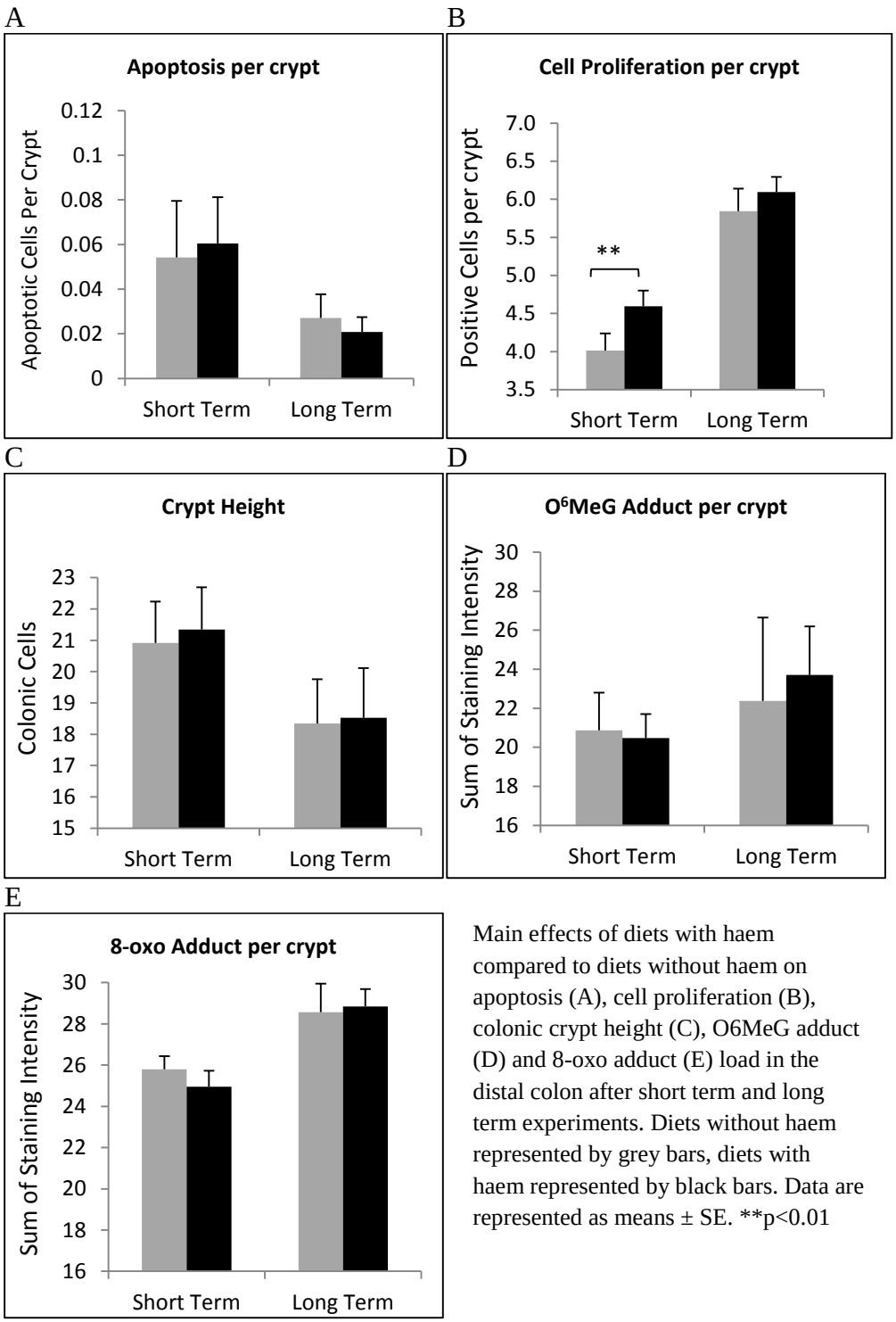
After 18 months on experimental diets, mice fed a diet containing haem showed no changes in proliferation, crypt height, apoptosis or DNA adduct levels compared to mice fed a haem-free diet (Figure 3.1A-3.1E). Diets containing RS increased colonic crypt height ($p<0.01$) as well as increasing cell proliferation ($p<0.01$) compared to diets without RS (Figure 3.2B and 3.2C). However, RS did not affect apoptosis or DNA adducts (Figure 3.2C, 3.2D and 3.2E). There were no significant differences in colon tumour incidence, which were very low, for any of the dietary interventions (Table 3.4).

Table 3.4: Effects of dietary interventions on colon tumor incidence

	Haem Effect			RS Effect		
	No Haem	Haem	p value*	No RS	RS	p value*
Total colon tumors	4	0	-	3	1	-
Colon tumor incidence (%)	4.45	0	0.134	3.35	1.1	0.523

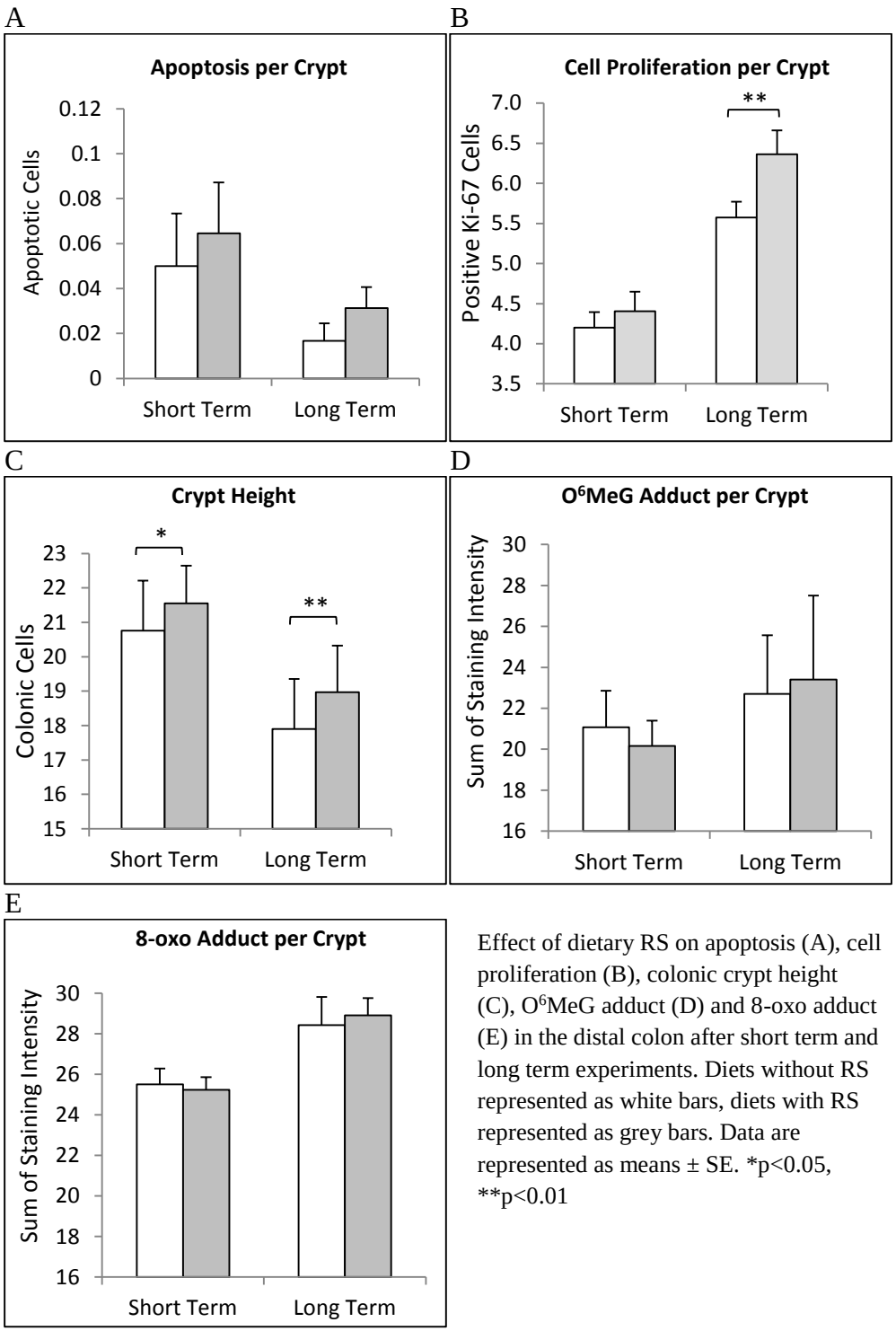
* Cross tabulation performed for colon cancer using Fishers exact test.

Figure 3.1: Epithelial changes for short term and long term haem consumption



Main effects of diets with haem compared to diets without haem on apoptosis (A), cell proliferation (B), colonic crypt height (C), O6MeG adduct (D) and 8-oxo adduct (E) load in the distal colon after short term and long term experiments. Diets without haem represented by grey bars, diets with haem represented by black bars. Data are represented as means ± SE. **$p<0.01$

Figure 3.2: Epithelial changes for short term and long term RS consumption

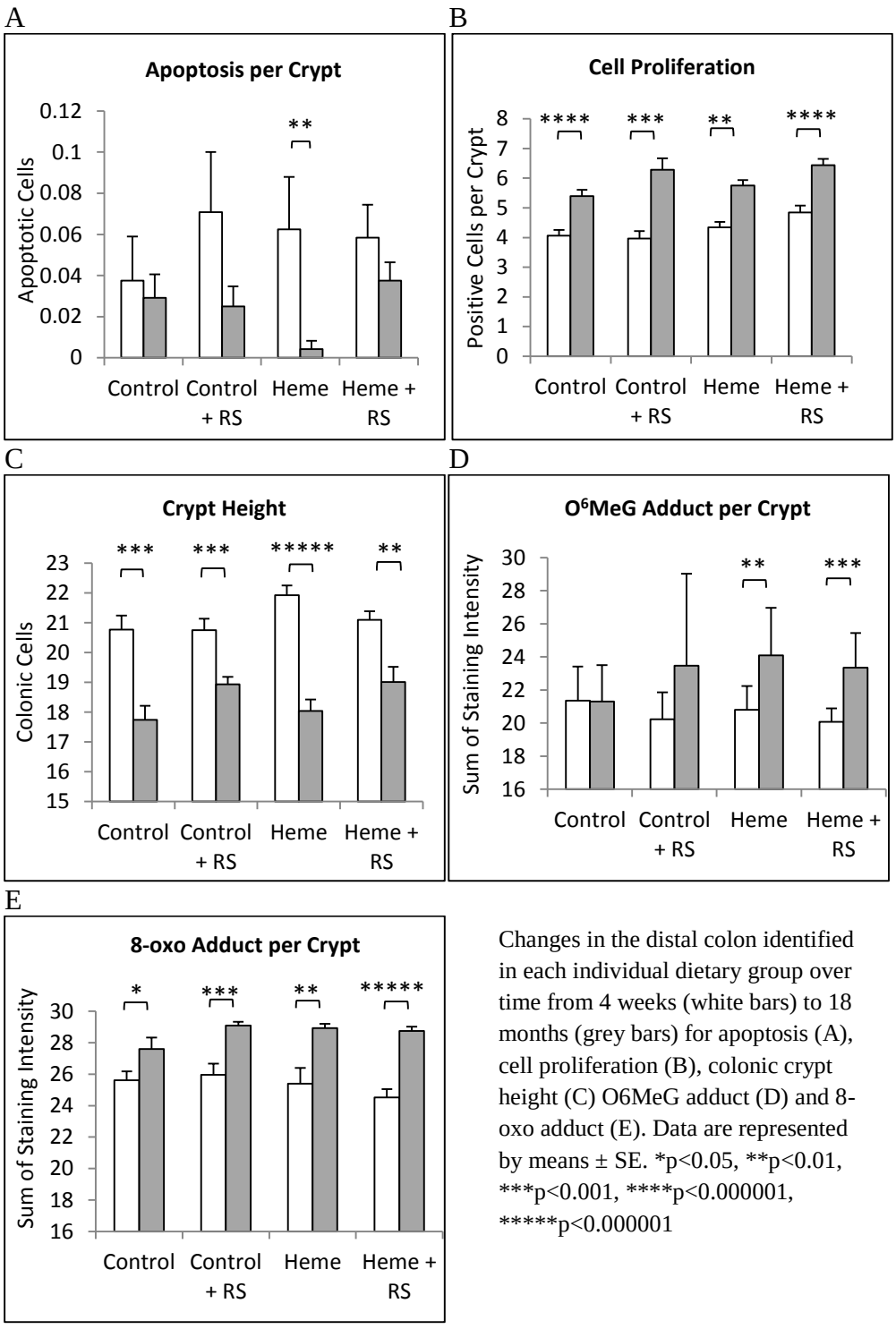


Effect of dietary RS on apoptosis (A), cell proliferation (B), colonic crypt height (C), O^6MeG adduct (D) and 8-oxo adduct (E) in the distal colon after short term and long term experiments. Diets without RS represented as white bars, diets with RS represented as grey bars. Data are represented as means ± SE. *$p<0.05$, **$p<0.01$

3.4.4. Diet-related changes over time

To ascertain if diets led to changes over time we compared short and long term feeding of haem and RS (Figure 3.3). Dietary haem significantly lowered rates of apoptosis in older mice compared to their younger counterparts (Figure 3.3A, $p<0.01$). Cell proliferation increased and crypt height decreased with age (Figure 3.3B and 3.3C) for all dietary treatments ($p<0.01$). Diets that contained haem ($p<0.01$) or haem with RS ($p<0.001$) showed a significant increase in O^6MeG DNA adduct accumulation over time; whereas diets without haem (Control and Control + RS) showed no significant difference between the two time points (Figure 3.3D). 8-oxo adducts in the colon accumulated over time (Figure 3.3E) with all 4 dietary interventions (Control: $p<0.05$, Control + RS: $p<0.001$, Haem: $p<0.01$ and Haem + RS: $P<0.000001$).

Figure 3.3: Colonic epithelial changes over time from 4 weeks to 18 months



Changes in the distal colon identified in each individual dietary group over time from 4 weeks (white bars) to 18 months (grey bars) for apoptosis (A), cell proliferation (B), colonic crypt height (C) O6MeG adduct (D) and 8-oxo adduct (E). Data are represented by means ± SE. *p<0.05, **p<0.01, ***p<0.001, ****p<0.000001, *****p<0.000001

3.5. Discussion

These experiments show that dietary haem increases proliferation in mouse colonic epithelium in the short term, inhibits colonic apoptosis over the long term and contributes to accumulation of O^6MeG pro-mutagenic adducts in the colon over time. Interestingly, these adverse changes invoked by dietary haem did not increase rates of colon cancer in this mouse model. There are a few reasons why there was no risk of haem intake on CRC: 1) Haem may need to be consumed in conjunction with other components of red meat and may not be carcinogenic on its own; 2) There were simply insufficient levels of pro-mutagenic adducts generated to initiate CRC; 3) A low level of prolonged insult gives time for the cells homeostatic mechanisms to control and/or reverse any potential adverse effects of haem consumption; and 4) Not enough time was allowed for the carcinogenic process to take place and the study would need to be extended. Each of these points will be discussed here.

There is evidence that increased haem intake from red meat will catalyse the production of more NOCs in the colonic lumen, leading to increased risk in developing colon cancer (Cross et al. 2003). Haem-induced increases in NOC can lead to increased alkylating adducts in the colon, as NOC are potent initiators of such alkylating adducts (Lewin et al. 2006). We observed an accumulation of O^6MeG adducts over time with haem consumption, however this did not accelerate colorectal oncogenesis under the conditions of the experiment. Haem alone may catalyse the production of NOC's but red meat and particularly processed meat (Santarelli et al. 2010) contain NOCs that might further contribute to alkylating DNA lesions of the colon. Due to sampling limitations, NOCs were

not measured in the current study, but this would be warranted in future studies examining dietary haem as a cancer causing agent

Clearly, the alkyl adducts generated were not sufficient to initiate CRC. Whether this was due to failure to reach a sufficient number or whether it was in the context of a low-risk setting for CRC, given that no other mutagen was given or that there was no high risk factor present, it cannot be certain. This might be further explored by feeding higher amounts of haem but such would be above what is reasonably encountered in the human diet. Whether the level of haem-induced adducts were also at a level such that the cells defence mechanism for O^6MeG repair (MGMT) and 8-oxo repair (OGG1) were adequate enough to prevent the onset of carcinogenesis in this mouse model is also undetermined. Measurements of DNA adduct repair are warranted to determine if alkyl and oxidative repair mechanisms are up-regulated in response to long term haem feeding.

It might also be that the duration of the current study may need to be extended beyond 18 months to see if there is further accumulation of O^6MeG adducts and whether this increases risk for CRC. In the current study diets were adapted from Newmark et al (2009) where at 12-18 months colon tumours formed and an end-point of 24 months saw significant increases in CRC compared to normal diets. However, due to a significant weight loss in haem-fed mice (Table 3), as well as unexpected early losses of mice from other groups (compared to loss of mice in Newmark et al (2009) study), 18 months was used as the cut-off point to avoid further loss of mice.

A high level of dietary haem has been previously shown by other researchers to increase cell proliferation and decrease apoptosis in the colon of

rats, possibly as a result of increased oxidative stress and a loss of cell feedback between damaged cells and proliferating cells (Ijssennagger et al. 2012). We also observed increased cell proliferative activity in the short term and a reduction of apoptosis over the long term in the current study (at 0.2µmol), but this did not equate to a higher CRC risk. More recently, Ijssennagger et al (Ijssennagger et al. 2013) showed 0.2µmol of haem was sufficient enough to induce acute oxidative stress and delayed cytotoxic stress in the lumen of mice after only 2-14 days of haem consumption, with a dramatic increase in proliferating colon cells. In a study where haem was used at a similar concentration of 0.25µmol/g ACF size increased, however, there was a statistically significant reduction in number of azoxymethane (AOM)-induced ACFs per colon (after 100 days) compared to the control diet (92 ± 16 compared to 125 ± 25) (Pierre et al. 2003). The study by Pierre et al (2003) utilised a different base diet to the current study, and treated mice with AOM to drive the CRC pathway, whereas the study in this chapter used only haem as the potential carcinogenic agent. This indicates that haem may be carcinogenic at higher doses but not at lower doses more relevant to human consumption over a longer period of time, and could explain why we saw no significant effect on CRC in the current study. According to two studies (Lombardi-Boccia et al. 2002, Lombardi-Boccia et al. 2002) the level of haem-iron in cooked red meat as beef is on average 2.63mg/100g of meat. This equates to an average concentration of haem in a 30% red meat diet to be 0.14µmol/g (Lombardi-Boccia et al. 2002). Most studies have used much higher doses, up to 10 times this amount of haem, ranging from 0.25µmol/g up to 1.5µmol/g of diet (Pierre et al. 2003, de Vogel et al. 2008, Ijssennagger et al. 2012), which is quite unrealistic in the context of human consumption. The concentration of haem in

our study at 0.2µmol is equivalent to that of the high red meat diet of 30% (Appendix E), representing a maximum intake of haem achieved in humans. Although haem may disrupt homeostasis of the cell by increasing proliferation and decreasing apoptosis, this had no influence in the context of our studies on initiation or promotion of CRC. The effect of haem in the form of hemin on colon carcinogenesis remains questionable, with levels of haem present in a relatively normal diet not affecting CRC risk.

These findings that dietary RS can generate a more beneficial fermentation profile in the colonic lumen support previous literature that this food source acts to reduce toxic protein fermentation and acts as a promoter of SCFA production, particularly the anti-cancer compound butyrate. There is a firm belief that the preferential fermentation of RS in the colon over protein leads to increased production of chemo-protective compounds and reduction of potentially toxic compounds in the lumen (Birkett et al. 1996). Rodent models consistently show fermentation of RS and production of SCFAs is linked to decreased rates of DNA lesions in colon cells, ACFs and tumours in chemical carcinogenic models of colorectal cancer (Le Leu et al. 2007, Le Leu et al. 2007, Liu and Xu 2008). It has been shown previously that RS is able to reduce DNA adducts induced by high red meat diets, but not high casein diets, without the use of chemical carcinogens, and this was related to increased SCFA and decreased protein fermentation (Winter et al. 2011). The levels of haem used here did not induce detectable changes in DNA adducts between individual diets (both alkylating and oxidative) in the short term to detect a protective effect of RS despite increased SCFA production. Also, over the long term, addition of RS was not able to reduce the observed increase of adducts induced by haem and one reason for this could

be the decrease in SCFA production by RS in the presence of haem after long periods. It is likely that the bacterial population in the colon has shifted with consumption of haem over longer periods, favouring a move away from beneficial bacteria fermentation provided by an RS diet. Previous studies have confirmed that consumption of dietary haem does create shifts in populations of bacteria in the colonic lumen (IJssennagger et al. 2012). Due to the small faecal sample size of the mice it was not possible to measure bacterial profile but this would be warranted in future studies to identify the interaction of haem with other dietary agents.

This study identified dietary haem as an agent that can increase DNA adducts considered to be pro-mutagenic, increase cell proliferation and reduce apoptosis rates in the colon of mice. It also confirms that RS can promote beneficial bacterial fermentation and reduce toxic protein fermentation in the colon of rodents over short periods, but SCFA production was not sustained over longer periods in combination with haem intake. All these changes observed had no influence on CRC risk. The level of haem in a typical western diet may not be sufficient to initiate CRC alone. This suggests that haem may act with other components of red and processed meat, such as NOCs, contributing to DNA damaging events in the colon that might lead to colorectal carcinogenesis.

Chapter 4

Dietary-induced colonic O^6-methyl-2-deoxyguanosine adducts do not affect risk for spontaneous colorectal cancer in the Western diet mouse model: Implications for red meat and resistant starch intake.

4.1. Introduction

High protein diets are becoming more popular in the modern age, mostly for management of body weight, but also for improving overall health including preventing against metabolic syndrome (Dong et al. 2013). However, there is also a large body of evidence that suggests high protein diets, particularly from red meat sources, affect colonic health and CRC risk (Kim et al. 2013). Consumption of high protein diets can deliver larger amounts of undigested protein to the colon, where it can be fermented into potentially geno-toxic products (Hughes et al. 2000). Animal studies have shown that increasing intake of casein, a purified dairy protein, can increase DNA stand breaks, measured by the comet assay (Toden et al. 2003, Toden et al. 2005, Toden et al. 2007). It is also emerging that red meat protein may be more harmful to the colonic environment than white meat or non-meat protein sources. Rats fed a high red meat diet compared to a white meat or casein protein diet showed higher DNA damage in colon cells, measured by DNA strand breaks, telomere shortening and DNA pro-mutagenic adducts (Toden et al. 2003, Toden et al. 2005, Toden et al. 2007, Winter et al.

2011, O'Callaghan et al. 2012). In humans, red meat has been associated with increasing CRC risk (Larsson et al. 2005, Larsson and Wolk 2006), whereas white meat such as fish and chicken show no such associations (English et al. 2004, Hu et al. 2008). There is a lot of evidence showing protein fermentation in the colon from high protein diets has the potential to be geno-toxic to the cells, but their effects on spontaneous CRC risk remains unclear.

Alkylation of the DNA from exposure to N-Nitroso compounds (NOC) from bacterial fermentation of red meat in the colon might be one mechanism by which red meat increases risk for CRC (Bingham et al. 1996). There is a dose-dependent effect of red meat intake and NOC formation in the faeces of humans (Hughes et al. 2001). The pro-mutagenic alkyl adduct O^6MeG can lead to mutations in proto-oncogenes (Jackson et al. 1997) and is formed in high concentrations within the colon after exposure to chemical alkylating carcinogens (Hong et al. 1999). Exposure to red meat can also increase O^6MeG adducts in the colon of mice fed a high red meat diet (Winter et al. 2011). Other alkylating adducts have been shown to increase in exfoliated colon cells from the faeces of human volunteers consuming a high red meat diet, and this was closely associated with faecal NOC concentrations (Lewin et al. 2006). Previously, in Chapter 4 of this thesis, it was identified that dietary haem (a component of red meat) could enhance accumulation of pro-mutagenic adducts within the colon, however this did not increase the CRC incidence. The theory that NOC production via red meat consumption might render colon cells more susceptible to developing CRC is quite plausible. To date, there are no published data on the link between high red meat induced pro-mutagenic adducts and colon cancer.

High dietary fibre intake has long been thought to be an important factor in CRC prevention. Dietary fibre is defined as the component of carbohydrate that is not digested in the small intestine (Cummings et al. 2009). Resistant starch (RS) is a type of dietary fibre, it is the component of starch in food that reaches the colon undigested where it is fermented by the resident bacteria of the colon (Topping and Clifton 2001). Fermentation of RS generates molecules called short chain fatty acids (SCFA), which can protect against DNA damage *in vitro* and *in vivo* (Hinnebusch et al. 2002, Le Leu et al. 2009). In addition to its physiochemical properties in the lumen, RS can also increase faecal bulk and decrease transit time of contents through the gut, thereby decreasing the time of contact between potential carcinogens in the lumen and the epithelial lining (Young et al. 2005). In particular, RS has proven to decrease production of protein fermentation metabolites in the colon (Birkett et al. 1996, De Peter et al. 2007, Le Leu et al. 2007, Winter et al. 2011). It is well known that RS can modify protein fermentation in the colon as a consequence of feeding a diet high in protein, but the affect this might have on risk for CRC is not clearly defined.

4.2. Aims and hypotheses

Based on the evidence of epidemiological, observational and prospective studies, we hypothesize that high protein diets, particularly a high red meat diet, will increase the risk for developing CRC by generating DNA O^6MeG adducts. Also, that a high fibre diet with a large amount of RS might be protective by compromising red meat-induced DNA adducts. The specific aims of this chapter were as follows:

1. Determine if long term feeding of high protein diets and more specifically a red meat protein source could increase O^6MeG adducts in the colonic epithelial cells of mice, and if RS consumption could lower these adducts.
2. Determine if the increased O^6MeG adducts from consuming red meat and high protein diets is associated with an increased risk of formation of colon cancer, and if resistant starch could reduce the risk of CRC associated with high protein and/or red meat diets.

4.3. Materials and methods

4.3.1. Animals and diets

A total of 270 eight week old wild type c57BL/J male mice were obtained from the Animal Resource Centre, Perth, Western Australia. The Flinders University of South Australia Animal Welfare Committee approved all experimental procedures (ethics approval number 809/12). Animals were randomly placed four mice per cage and divided into dietary groups (n=45) under controlled conditions of 22 ± 2ºC (SD), 80 ± 10% humidity and 12h light/dark cycle. Animals had free access to food and water and were weighed once weekly throughout the study period. Mice consumed diets for 18 months, or until euthanasia due to ill health.

The experimental diets were based on the American Institute of Nutrition (AIN) diet AIN-76 with modified amounts of vitamins and minerals for rats and mice adapted to resemble a typical Western diet (Newmark et al. 2009) (Table 4.1). Red meat was added at levels that resemble a typical Western diet and that has previously been shown to induce significant O^6MeG adducts in the colon of mice (Winter et al. 2011). The purified protein source casein has been shown to

dose dependently increase DNA damage in colon cells but that resistant starch could protect against such damage (Toden et al. 2005). Therefore, casein was used as a non-meat protein source in this experiment. Casein was added at levels to achieve an equal amount of protein used in the red meat diets (Table 4.1). Detail of individual dietary compositions is presented in more detail in Chapter 2.3. Final diet preparations were placed into air sealed containers, stored at 4°C in the dark, with fresh food in the mouse cages replaced daily.

Table 4.1: Composition of experimental diets (g/100g diet)

Ingredient	Low Casein (LC)	High Casein (HC)	High Casein + RS (HC+RS)	Low Red Meat (LRM)	High Red Meat (HRM)	High Red Meat +RS (HRM+ RS)
Casein	17.6	35.3	35.3	-	-	-
Red meat[1]	-	-	-	20.43	40.9	40.9
Corn starch	10	10	-	10	10	-
sucrose	45.48	27.78	27.78	46.39	29.69	29.68
Hi-Maize	-	-	10	-	-	10
Sunflower seed oil	12.5	12.5	12.5	12.5	12.5	12.5
Lard	7.5	7.5	7.5	3.76	-	-
α-cellulose	2	2	2	2	2	2
L-cysteine	0.3	0.3	0.3	0.3	0.3	0.3
Choline	0.12	0.12	0.12	0.12	0.12	0.12
Mineral mix[2]	3.5	3.5	3.5	3.5	3.5	3.5
Vitamin mix[2]	1.0	1.0	1.0	1.0	1.0	1.0

[1]Red meat dry powder added with its own moisture content
[2]Western diet vitamin and mineral mixtures; with calcium at 0.5 mg/g, phosphorus at 3.6 mg/g, Folic acid at 0.23 µg/g, vitamin D_3 at 0.11 IU/g.(see appendix B)

4.3.2. Faecal analysis

Fresh faeces from each cage of mice were collected within one month from completion of the experimental diets and measured for faecal pH and ammonia (section 2.4.2). Another fresh faecal collection was also taken from

each mouse cage for measurement faecal SCFAs including acetate, propionate and butyrate and phenols and p-cresols (section 2.4.3).

4.3.3. Immunohistochemical analysis

Distal colon segments were taken for measurement of apoptosis by the TUNEL method (section 2.5.2) and proliferation using a Ki-67 antibody (section 2.5.4). The level of DNA alkylation was measured using an antibody specific for the O^6MeG adduct and staining intensity was quantified using a computer image analysis protocol (section 2.5.5).

4.3.4. Tumours analysis

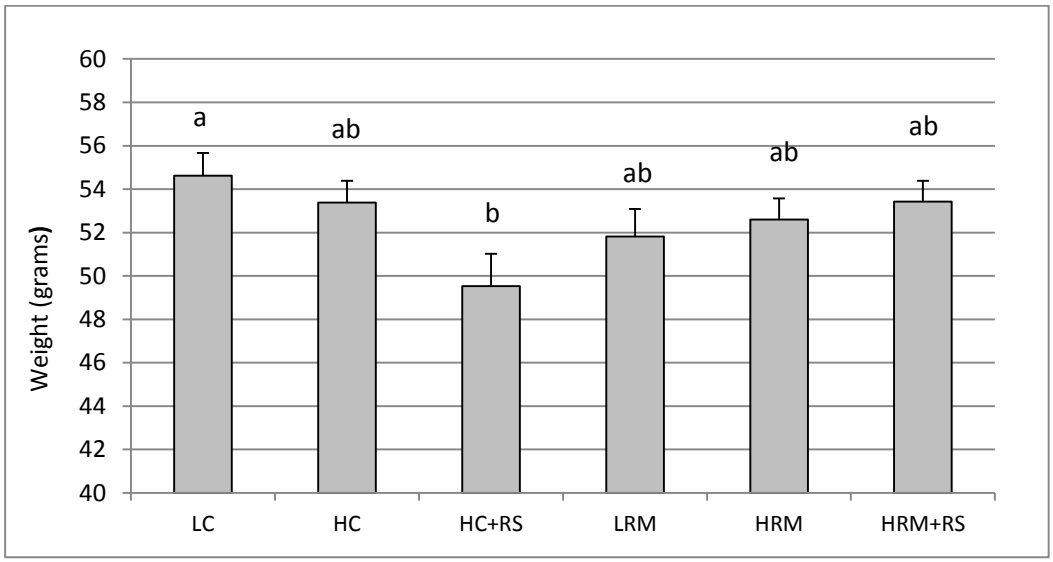
The entire colon length was analysed for tumours (adenomas and adenocarcinomas) under light microscopy, with sections taken for histological confirmation (section 2.5.6).

4.4. Results

4.4.1. Bodyweights and faecal analysis

Mice on a high casein diet with RS had lower final bodyweights compared to mice consuming a low casein diet (Figure 4.1). There were no significant differences of final bodyweights between any of the other dietary treatments. Measurements of faecal pH and concentrations of SCFA are presented in Table 4.2. Mice consuming a combination of RS with red meat resulted in a significant drop in faecal pH compared to all other non RS diets ($p<0.01$). Mice fed a high red meat diet had a significantly higher faecal pH compared to a high casein with RS ($p<0.05$). Concentrations of mouse faecal butyrate, propionate and total SCFA were significantly higher when consuming diets containing RS (both casein and

Figure 4.1: Final bodyweights of mice after 18 months



Final bodyweights of mice on the Western diet model for each dietary treatment
LC: Low Casein, HC: High Casein, HC+RS: High Casein + RS, LRM: Low Red Meat, HRM: High Red Meat, HRM+RS: High Red Meat + RS.
Groups not sharing the same subscript are significantly different at $p<0.05$.

red meat) compared to all other diets without RS ($p<0.00001$). Faecal acetate levels were significantly higher in mice consuming RS compare to high casein and high red meat diets ($p<0.0001$). Consumption of a low casein diet and low red meat diet had significantly lower levels of acetate compared to a high casein diet with RS ($p<0.05$).

Concentrations of faecal fermentation products phenol, p-cresol and ammonia are presented in Table 4.3. Mice on a diet high in red meat generated significantly higher concentrations of faecal phenols compared to all other diets, including a high red meat diet supplemented with RS ($p<0.01$). Faecal concentrations of p-cresol were also significantly higher in mice consuming a high red meat diet compared to both RS supplemented diets and low red meat and high casein diets ($p<0.05$). There were no statistically significant differences in ammonia concentrations between any of the diets.

Table 4.2: Faecal analysis of pH, total SCFAs, acetate, propionate and butyrate.

Variable	Unit	Experimental Diet Groups					
		LC	HC	HC+RS	LRM	HRM	HRM+RS
Faecal pH	-	7.7 ± 0.1	7.7 ± 0.1	7.2 ± 0.1	7.7 ± 0.1	7.8 ± 0.1*	6.9 ± 0.2**
Total SCFAs	µmol/g	13.4 ± 3.4	9.9 ± 1.5	48.7 ± 4.8***	16.2 ± 2.1	12.3 ± 2.3	36.9 ± 2.9***
Acetate	µmol/g	7.7 ± 2.2†	5.2 ± 1.0	15.1 ± 1.6†††	8.3 ± 1.2†	5.5 ±1.0	11.8 ± 1.2†††
Butyrate	µmol/g	2.9 ±0.5	3.0 ± 0.7	18.8 ± 1.9***	5.0 ± 0.7	4.3 ± 0.9	14.1 ± 1.1***
Propionate	µmol/g	2.8 ± 0.8	1.8 ± 0.2	14.8 ± 1.6***	2.9 ± 0.4	2.6 ± 0.4	10.9 ± 0.9***

* p<0.05; Faecal pH significantly different to HC+RS and HRM+RS
** p<0.01; Faecal pH significantly different to LC, HC, LRM, HRM
***p<0.00001; Faecal butyrate, propionate and total SCFA significantly different to LC, HC, LRM and HRM
††† p<0.00001; Faecal acetate significantly different to HC and HRM
†p<0.05; Faecal acetate significantly different to HC+RS
LC: Low Casein, HC: High Casein, HC+RS: High Casein + RS, LRM: Low Red Meat, HRM: High Red Meat, HRM+RS: High Red Meat + RS.

Table 4.3: Faecal analysis of phenols, p-cresols and ammonia

Variable	Unit	Experimental Diet Groups					
		LC	HC	HC+RS	LRM	HRM	HRM+RS
Phenol	µmol/g	1.8 ± 0.4	2.0 ± 0.7	0.9 ± 0.2	2.1 ± 0.2	5.2 ± 0.9**	1.9 ± 0.6
***p*-cresol**	µmol/g	11.8 ± 2.7	7.5 ± 2.0	12.5 ± 1.2	9.1 ± 0.9	22.3 ± 4.1*	12.2 ± 1.6
Ammonia	µmol/g	7.7 ± 3.4	13.5 ± 4.1	12.7 ± 4.5	9.7 ± 2.5	11.2 ± 3.6	11.1 ± 5.4

**p<0.01; Phenols significantly different to LC, HC, HC+RS, LRM, HRM+RS
* p<0.05; p-cresols significantly different to HC, LRM, HC+RS, HRM+RS
LC: Low Casein, HC: High Casein, HC+RS: High Casein + RS, LRM: Low Red Meat, HRM: High Red Meat, HRM+RS: High Red Meat + RS.

4.4.2. Immunohistochemical analysis

Evaluation of the colonic crypt height, apoptosis and proliferation rates in the distal colon are outlined in Table 4.4. There were no changes in colonic crypt height or apoptosis rates between any of the dietary treatments, nor were there any effects or interactions for each dietary component. Distal colon proliferation

was significantly lower in mice consuming a red meat diet with RS compared to a high casein diet with RS ($p<0.05$). Mice on red meat diets had significantly lower proliferation rates compared to casein diets ($p<0.05$), and there was a significant interaction of red meat combined with RS on lowering proliferation rates in the mouse colon ($p<0.01$). The level of protein in the diet had no significant effect on crypt height, apoptosis or proliferation in the mouse colon.

Table 4.4: Distal colonic crypt height, apoptosis and proliferation

Measure	**Unit**	**Experimental Diet Groups**						**Main Effects and Interactions**			
		LC	**HC**	**HC+RS**	**LRM**	**HRM**	**HRM +RS**	**PL**	**RM**	**RS**	**RM x RS**
Crypt Height	Cells/ crypt	17.7 ± 0.5	17.8 ± 0.5	18.9 ± 0.2	19.0 ± 0.9	18.2 ± 0.6	18.3 ± 0.4	0.532	0.508	0.278	0.316
Apoptosis	Cells/ crypt	0.02 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.684	0.701	0.416	0.684
Proliferation	Cells/ crypt	5.5 ± 0.2	5.4 ± 0.2	6.3 ± 0.4	5.6 ± 0.3	5.5 ± 0.3	5.0 ± 0.2*	0.646	<0.05	0.596	<0.01

* $p<0.05$; Proliferation is significantly different to HC+RS
PL, Protein Level; RM, Red Meat; RS, Resistant Starch.
LC: Low Casein, HC: High Casein, HC+RS: High Casein + RS, LRM: Low Red Meat, HRM: High Red Meat, HRM+RS: High Red Meat + RS.

The level of O^6MeG adducts in the distal colon of mice are presented in Table 4.5. Consumption of a high red meat diet with RS significantly increased the sum of staining intensity of O^6MeG compared to all other dietary treatments ($p<0.01$). A significant increase in O^6MeG adducts was observed after consumption of red meat ($p<0.0001$) as well as with RS intake ($p<0.01$). Furthermore, consuming red meat with RS was shown to have a significant interaction of increasing O^6MeG adducts ($p<0.02$). There was no effect of increasing protein intake on increasing O^6MeG adducts.

Table 4.5: Distal colonic O^6MeG DNA adduct staining

	Experimental Diet Groups						Main effects and Interactions (p-values)			
	LC	**HC**	**HC +RS**	**LRM**	**HRM**	**HRM +RS**	**PL**	**RM**	**RS**	**RM x RS**
Sum of O^6MeG Staining	23.9 ± 1.0	21.3 ± 0.6	23.4 ± 1.6	25.9 ± 1.9	24.7 ± 1.3	32.6 ± 2.2**	0.699	<0.0001	<0.01	<0.02

** p<0.01: O^6MeG DNA adduct staining is significantly different to all other dietary groups
PL, Protein Level; RM, Red Meat; RS, Resistant Starch.
LC: Low Casein, HC: High Casein, HC+RS: High Casein + RS, LRM: Low Red Meat, HRM: High Red Meat, HRM+RS: High Red Meat + RS.

4.4.3. Colorectal tumours

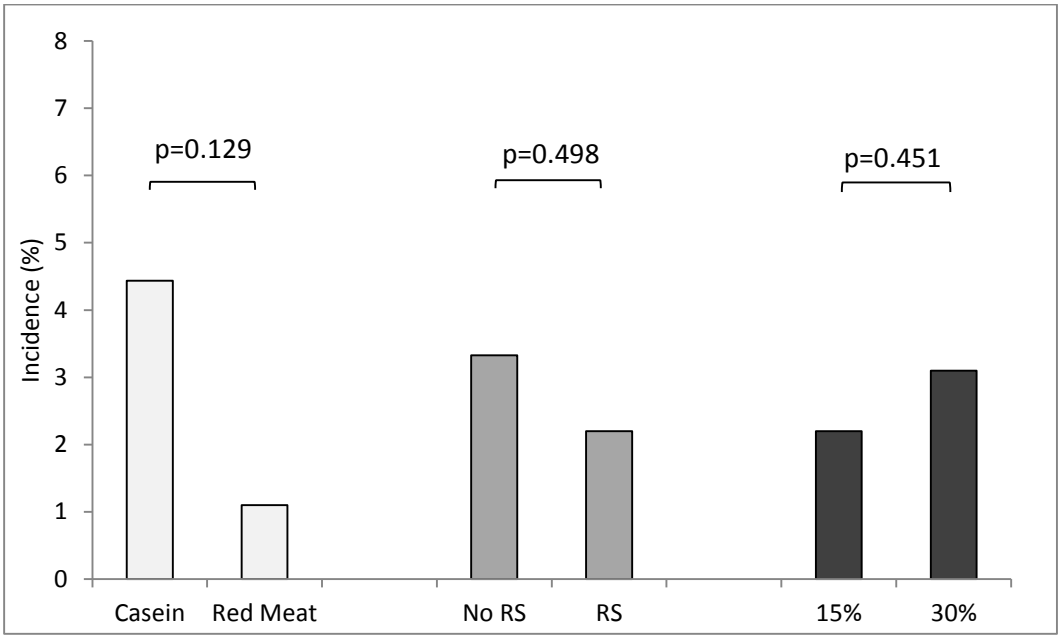
Overall there was a low incidence rate of colorectal tumours observed in all dietary treatment groups (Table 4.5). There were no significant effects of red meat intake, RS supplementation or protein level on colon tumour outcome (Figure 4.2.). There was a trend of decreasing colon tumours in mice consuming a red meat diet compared to a casein diet, however this was not statistically significant (p=0.129).

Table 4.6. Colon tumour incidence (%) for each dietary treatment

	Experimental Diet Groups					
	LC	**HC**	**HC+ RS**	**LRM**	**HRM**	**HRM+ RS**
Colon Tumour Incidence (%)	4.4	6.7	2.2	0	2.2	2.2

LC: Low Casein, HC: High Casein, HC+RS: High Casein + RS, LRM: Low Red Meat, HRM: High Red Meat, HRM+RS: High Red Meat + RS.

Figure 4.2: Colon tumour incidence (%)



Main effects of protein source (casein vs. red meat), RS supplementation (No RS vs. RS) and protein level (15% vs. 30%) on colon tumour incidence in the Western model of spontaneous CRC. Data are presented as a percentage of mice that harbour colorectal tumours (adenoma or adenocarcinoma). Crosstabs performed with Fisher's exact test.

4.5. Discussion

The results from this chapter showed long term red meat consumption could increase O^6MeG adducts in the colonic epithelial cells of mice, however, consumption of RS did not reduce DNA adduct formation. In fact, co-consumption of RS with red meat increased adducts significantly more than consuming either dietary component alone. Furthermore, red meat with RS also reduced cellular proliferation rates in the distal colon. There was no link between increased O^6MeG adducts and proliferation in the colon and reduced risk of colonic tumourigenesis in the current mouse model used. The results in this chapter suggest that consuming red meat, high protein or RS diets as a part of the Western diet model of spontaneous CRC is not associated with the formation of CRC.

Based on the evidence from a previous short term study in mice fed red meat (Winter et al. 2011), the main hypothesis of this chapter was O^6MeG adducts would increase after red meat consumption and that co-consumption with a source of RS would reduce these adducts. However, data presented here did not fully support this hypothesis. Long term feeding of a high red meat diet increased O^6MeG adducts and when supplemented with RS a substantially *higher* amount of O^6MeG adducts were recorded. This finding is unexpected and difficult to interpret. It is clear that RS can still ferment readily in the presence of red meat, evidenced by the increased SCFA content in the faeces (Table 4.2). One plausible theory is the interaction of RS metabolites (butyrate) in the presence of red meat metabolites (NOCs), enhancing the alkylating capacity of red meat and thereby increasing DNA O^6MeG adducts. Butyrate is a known histone deacetylase inhibitor, which causes histone acetylation and subsequently the normally compact chromatin structure unwinds, exposing the underlying DNA structures (Hinnebusch et al. 2002). Histone acetylation by butyrate can trigger radiation induced apoptosis and inhibit repair mechanisms such as homologous and non-homologous end joining (Purrucker et al. 2010, Koprinarova et al. 2011). However, the observed increase in DNA pro-mutagenic adducts by long term red meat consumption did not trigger an increase in apoptosis (Table 4.4.). The DNA within colon cells of mice consuming red meat with RS could be more exposed as a result of histone deacetylation by butyrate, and subsequently more susceptible to alkylation by red meat metabolites. More understanding of the interactive effects of butyrate and red meat metabolites on histone acetylation and DNA damage and repair are required to confirm this observation.

Although a significant increase in O^6MeG adducts were detected in the colon after long term red meat and RS intake, this had no effect on risk for developing neoplasms in the colon, nor did it trigger hyper-proliferation. In fact, the combination of red meat and RS inhibited cell proliferation in the distal colon. Short term consumption of red meat and RS in mice and humans has previously been shown to reduce proliferation of the colonic epithelia (Winter et al. 2011, Humphreys et al. 2014). The likely explanation being generation of butyrate, which has been shown to arrest the cell cycle after exposure to chemical alkylating agents (Le Leu et al. 2009). In Chapter 3 of this thesis, it was also shown that increases in pro-mutagenic adducts did not enhance tumourigenesis in the Western diet mouse model, even with an enhanced proliferative state after consumption of dietary haem. The combination of a red meat diet with RS does not induce sufficient O^6MeG adducts to trigger apoptosis, nor does it produce a hyper-proliferative state in the colon and it does not increase risk for CRC in the Western diet model. The chemical carcinogen azoxymethane does indeed reach a "threshold" for DNA O^6MeG adducts in the rodent colon and subsequently invasive tumours form in the distal colon (Wali et al. 1999, Nasuno et al. 2014). Therefore, the level of dietary induced O^6MeG adducts, as shown to be generated by red meat, are a good measure of exposure to dietary alkylating agents but do not appear to be associated with risk for colorectal oncogenesis. Using the O^6MeG adduct as a bio-marker for spontaneous CRC as a consequence of red meat exposure in the Western diet remains uncertain. Identifying novel bio-markers more closely linked with spontaneous CRC needs to be explored.

This chapter has confirmed previous evidence (Birkett et al. 1996, Geboes et al. 2006, Le Leu et al. 2007, Winter et al. 2011) that RS can increase

fermentation of beneficial compounds such as SCFA and decrease toxic protein metabolites after consumption of a high protein diet. Such properties of RS could play a role in protecting the colon from damage generated by protein fermentation and maintain a healthy colonic environment (Lin and Visek 1991). However, reduction in phenols, p-cresols and ammonia by RS consumption did not have any beneficial effect on tumour outcome in the current Western model of spontaneous CRC. Certainly, these products are known to be toxic but speculation remains as to whether they are mutagenic or carcinogenic (Le Leu and Young 2007). Protein fermentation into NOCs, which are suggested to be pro-mutagenic due to their ability to alkylate DNA (Bingham et al. 1996, Hughes et al. 2001) are likely candidates. However, NOCs were not measured in the current study due to tissue sampling, equipment availability and time constraints. Nonetheless, findings from this chapter suggest delivery of more protein to the colon via high protein diets and/or red meat does not increase risk for spontaneous tumour formation in the current mouse model.

In conclusion, fermentation of starch in the colon of mice lowers protein fermentation metabolites but this does not affect risk of CRC in the current model. High red meat diets increases O^6MeG adduct accumulation, which is enhanced by co-consumption with RS; however, this did not enhance sporadic colon tumour formation in the colon of mice consuming a typical Western diet. Alkyl adducts generated by dietary exposure may not have reached a threshold of DNA damage to trigger the oncogenesis pathway. Thus, the O^6MeG adduct does not appear to be a bio-marker for spontaneous CRC, but a measure of exposure to dietary alkylating agents. Use of the O^6MeG adduct as a bio-marker for red meat associated CRC remains speculative.

Chapter 5

Effects of red meat and butyrylated resistant starch on rectal O^6-Methyl-2-deoxyguanasine adducts and cell proliferation: A randomised clinical trial.

5.1. Introduction

Several mechanisms have been proposed to link red and processed meat consumption and CRC risk (Ferguson 2010). For red meat in particular it has been suggested that haem iron is a substantial contributor to CRC risk (Pierre et al. 2003, Ijssennagger et al. 2012). Red meat is fermented in the colon, altering the microbiota composition and generating potentially genotoxic metabolites that may play a role in oncogenesis (Hughes et al. 2000). These molecules include N-nitroso compounds (NOC), a complex mix of nitrite-derived products formed exogenously in processed meat itself or endogenously within the human gut via bacterial metabolism (Bingham et al. 2002, Hughes et al. 2002, Cross et al. 2003). NOCs are alkylating agents that generate DNA adducts in human colonocytes after high red meat consumption (Lewin et al. 2006). It has recently been shown that the pro-mutagenic adduct O^6-methyl-guanine (O^6MeG) is increased in rodent distal colon cells after consuming a diet high in red meat (Winter et al. 2011). The O^6MeG adduct is a known toxic and mutagenic base modification which, if unrepaired, can induce GC→AT transition mutations in oncogenes such as k-ras (Lees et al. 2004) as well as sister chromatid exchanges (Margison et al. 2002). These adducts are repaired quickly and efficiently by O^6-methylguanine‑DNA

methyltransferase (MGMT) protein in a suicide reaction where the alkyl group is cleaved from the DNA base (Povey et al. 2002). Although these adducts have been measured in rodent cells *in-situ* after exposure to high red meat diets, no such measures have been carried out within human colorectal tissues in response to red meat.

Dietary fibre is a heterogeneous group of compounds, principally indigestible carbohydrates of plant origin. Starches that escape digestion in the small intestine are termed resistant starches (RS) and are a source of dietary fibre. One possible mechanism for the reduction in CRC risk by fibre is production of short-chain fatty acids (SCFA) via fermentation of RS by the large bowel microbiota (Topping and Clifton 2001). One of the major SCFAs is butyrate, which is the preferred metabolic substrate for colonocytes (Donohoe et al. 2011) and can promote a normal cellular phenotype (Le Leu et al. 2009). *In vitro* studies with CRC cell lines have shown that butyrate induces apoptosis (Medina et al. 1997), reduces cell proliferation and promotes differentiation (Bartram et al. 1993). Animal experiments have shown that butyrate may reduce colorectal carcinogenesis by enhancing the apoptotic response to the alkylating carcinogen AOM (Le Leu et al. 2003, Clarke et al. 2012), which has been shown to substantially increase O^6MeG adducts and apoptosis in rat colon epithelial cells (Nyskohus et al. 2013).

Initial animal studies have shown that dietary RS is able to oppose colonocyte DNA strand breaks, telomere shortening and pro-mutagenic DNA adduct formation in rodents fed red meat (Toden et al. 2007, Winter et al. 2011, O'Callaghan et al. 2012). The protective effect correlated most closely with faecal butyrate levels, supporting a role for fermentation in risk modification. Acylated

starches (classified as RS4, chemically modified) in which the acyl group is linked to the starch framework by an ester bond, can deliver specific SCFA to the large bowel where bacterial esterases release the SCFA. Ingestion of butyrylated resistant starch (HAMSB) raises colonic butyrate levels in animals (Bajka et al. 2008, Clarke et al. 2008) and humans (Clarke et al. 2011, Humphreys et al. 2014). Raising colonic butyrate supply has the potential to improve bowel function and lower disease risk. RS is thought to be particularly effective as its fermentation generally favours butyrate production. Red meat and fibre (including RS) are generally consumed together as components of foods.

5.2. Aims and hypotheses

An integral part of this project was part of a human clinical trial investigating the effect of red meat intake on DNA adduct accumulation within the rectal tissue, and how dietary RS might modulate these adducts. This study was carried out with healthy individuals to determine whether high red meat consumption increased O^6MeG adducts in rectal epithelial cells and if concurrent consumption of butyrylated high amylose maize starch opposed this effect. It also investigated the effects of these diets on other measures of colonic health including cell proliferation and fermentation products in the lumen, since these might participate in generation of adducts.

The hypotheses of this chapter are that red meat consumption will result in enhanced adduct formation in rectal biopsy samples from human volunteers consuming a high red meat diet, but that co-consumption of red meat with RS will reduce formation of pro-mutagenic adducts generated by a high red meat diet. Also, red meat will increase fermentation of toxic metabolic products in the faeces

of healthy human volunteers, but addition of RS to the diet will reduce these products and increase fermentation of beneficial bacterial metabolites. This is because starch is a preferred substrate for the bacteria, with its presence diverting bacterial fermentation away from protein for energy production. The specific aim of this chapter is as follows:

1. To identify if feeding healthy humans a diet high in red meat will increase DNA O^6MeG adducts within the rectal epithelial tissue, and if co-consumption of a high red meat diet with HAMSB (butyrylated resistant starch) could modify adduct generation.
2. To determine if HAMSB supplementation of a high red meat diet could promote a more healthy colonic environment by reducing fermentation of red meat metabolites and enhancing short chain fatty acid production in faecal material from healthy human volunteers.

5.3 Materials and methods

5.3.1. Study design and participants

The study was a randomised crossover trial consisting of two intervention periods of four weeks each, preceded by a 4-wk run-in (baseline) and separated by a 4-wk (washout) period with all details of the study listed in Chapter 2.6 and Appendix H. In brief, twenty-three healthy volunteers participated in the trial. Ten volunteers completed the HRM intervention as first diet period; while 13 volunteers completed the HRM+HAMSB intervention first. During the entry (baseline) and washout periods, participants consumed their habitual diets. For the interventions, they were allocated randomly to a HRM diet or to a HRM diet supplemented with 40 g of butyrylated high amylose maize starch per day

(HRM+HAMSB diet). During HRM+HAMSB intervention participants were required to consume a total of two pre-packed 20 g sachets of HAMSB daily, one in the morning and one in the evening by mixing the powder into 250 mL reduced fat milk or orange juice. During the intervention periods the participants reduced their intake of their habitual diet to accommodate the extra 300g of red meat. Participants were instructed to maintain their usual diet during the study but to avoid consuming high levels of protein or fibre, or probiotic supplements, except those prescribed for the study.

Participants were also asked to avoid consuming, or record the use of, any medication that could interfere with bowel function (including antibiotics). Participants were monitored by a trial nurse (weekly) and dietician (at end of each 4-wk period) to ensure that diet and intervention guidelines were followed, and weight was kept stable. The study was approved by Flinders Clinical Research Ethics Committee (reference no. 155/09; Flinders Medical Centre, Bedford Park, SA, Australia) and all volunteers gave written informed consent.

5.*3.2. Sample collection and analysis*

Stool and rectal pinch biopsy specimens were obtained at the completion of the 4-wk entry period (baseline) and at the end of each intervention and the washout period. A complete faecal collection was conducted by all the participants for the last 48 hours of each dietary period, and samples were stored in portable freezers (-20˚C). At each visit to the Flinders Medical Centre clinic an experienced gastroenterologist collected 4 rectal mucosal biopsies with rigid forceps through sigmoidoscopic examination performed without bowel preparation or prior dietary restriction. Biopsies were formalin-fixed and

dehydrated through gradient alcohols and xylene before being embedded in paraffin wax (Chapter 2.6.2). Stool specimens were measured for SCFA concentration and NOC content as outlined in Chapter 2.6.3. Rectal epithelial tissue was analysed for O^6MeG adduct load and cellular proliferation as outlined in Chapter 2.6.4.

5.4. Results

5.4.1. Participants

Each participant was followed up for the four-month duration of the project. Twenty-five participants were assigned randomly with 12 allocated to the HRM dietary intervention first and 13 allocated to the HRM+HAMSB dietary intervention first. Two participants withdrew prior to commencement of the intervention diets; one due to unrelated medical problems and the other to intolerance of the first rectal biopsy. Approximately one third of participants reported increased flatulence on trial diets.

Only results arising from the respective baselines and the first arm of the dietary intervention (i.e. at week 4 of the intervention) are described below, as results of the second arm (cross-over) have been excluded due to carry-over effects (see Chapter 2.7.3). Participants maintained consistent body weight, with mean weights of 77.1 ± 6.4 kg and 82.8 ± 3.3 kg after the HRM and HRM+HAMSB interventions respectively. Overall 17 males and 6 females participated in the trial aged between 50-75 years.

Analysis of the dietary intake was carried out by Dr. Karen Humphreys at the Flinders Centre for Innovation in Cancer and has already been published (Humphreys et al. 2014). There was no difference between the diets for reported

intake of energy, total and saturated fat, total carbohydrates and sugar, starch or alcohol (Table 5.1). Compared with their respective baselines, protein intake was significantly increased by HRM intervention ($p<0.05$) and HRM+HAMSB ($p<0.01$). Fibre intake was decreased in the HRM group at week 4 compared to its baseline ($p<0.01$). Fibre intake was significantly lower in the HRM group compared to HRM+HAMSB group after 4 weeks ($p<0.01$).

Table 5.1: Dietary intake of study participants during each diet period, based on three-day weighed food records[1]

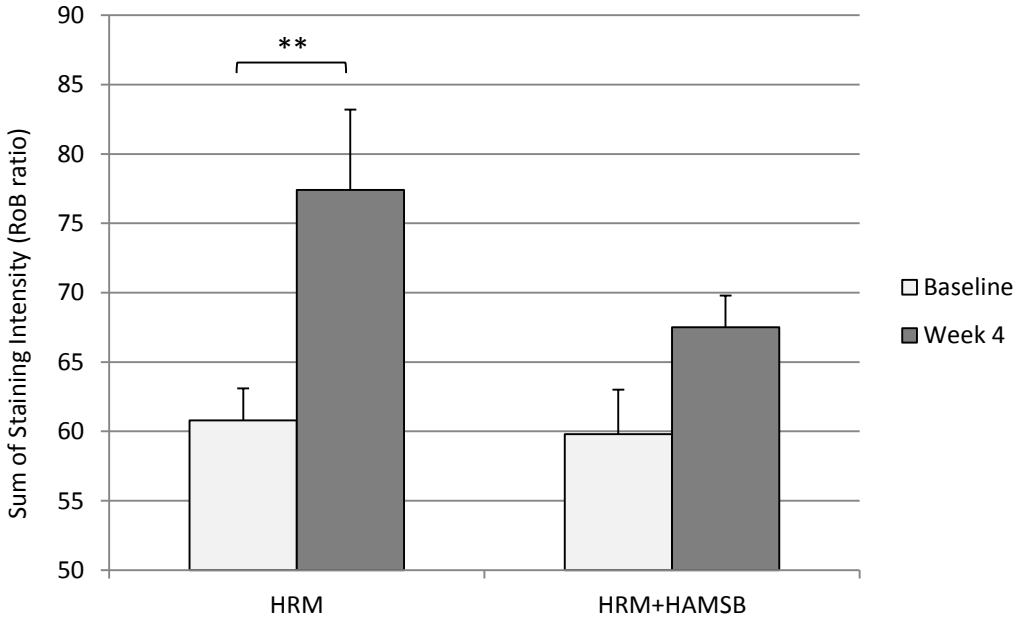
	HRM (n=10)			HRM+HAMSB (n=13)			P-value treatment difference[3]
	Baseline	**Week 4**	**Increment (% change)**	**Baseline**	**Week 4**	**Increment (% change)**	
Energy (kJ/d)	9169±72	9463±61	+294 (3%)	8578±421	9250±553	+672 (7%)	0.98
Protein (g/d)	101±11	124±5*	+23 (19%)	88±4	119±7**	+31 (26%)	0.81
Fat (g/d)	80±10	77±9	-3 (4%)	67±4	70±8	+3 (4%)	0.80
Saturated fat (g/d)	31±5	34±4	+3 (9%)	24±2	30±3	+6 (20%)	0.75
Carbohydrate (g/d)	221±28	222±20	+1 (1%)	244±17	256±16	+12 (5%)	0.36
Sugar (g/d)	112±16	121±10	+9 (7%)	120±12	129±9	+9 (7%)	0.78
Starch (g/d)	108±16	99±17	-9 (-9%)	122±9	125±9	+3 (2%)	0.21
Fibre (g/d)	24±2	19±2**	-5 (-26%)	28±3	29±3	+1 (3%)	0.01†
Alcohol (g/d)	21±6	22±8	+1 (5%)	11±2	7±2	-4 (-57%)	0.52

[1] All values are means ± SEMs. Different from baseline at week 4: * P<0.05; ** P<0.01; *** P<0.001 (linear mixed model). HRM, high red meat; HAMSB, butyrylated high amylose maize starch.
[2] HAMSB supplement contains 88% total carbohydrate, approximately 20% dietary fibre, 10% moisture, <1% total fat and <0.75% protein.
[3] P value for treatment difference at week 4: † P<0.05; †† P<0.01; ††† P<0.001 (linear mixed model).

5.4.2. Rectal epithelial measures

Rectal O^6MeG adduct load was increased at week 4 in the HRM group compared to its baseline ($p<0.01$); however addition of HAMSB to the HRM diet prevented this increase (Figure 5.1 and Table 5.2). Relative to their respective baselines the number of PCNA labelled cells in the rectal epithelium increased for both HRM ($p<0.001$) and HRM+HAMSB groups ($p<0.05$) but the number was lower following HRM+HAMSB than HRM ($p<0.05$) (Table 5.2). Representative sections of one individual from each treatment group showing the immunohistochemical staining of DNA O^6MeG adducts are shown in Figure 5.2.

Figure 5.1 Rectal DNA O^6MeG Adduct Load per crypt



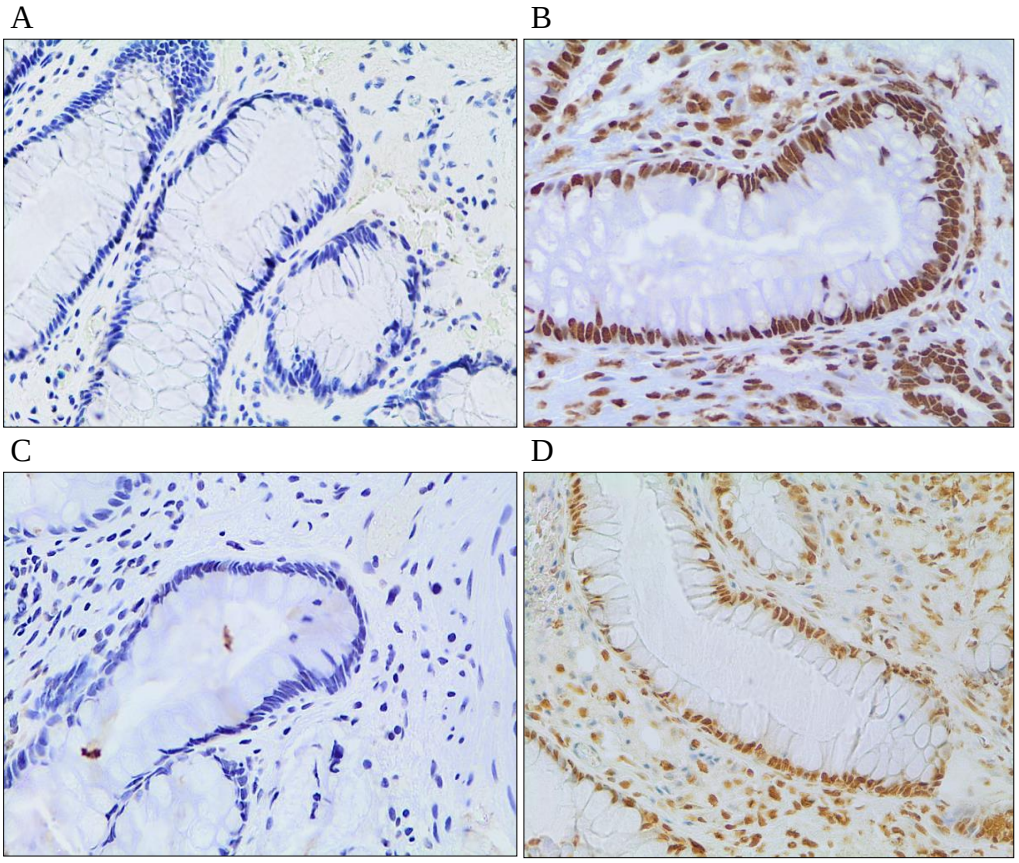
Sum of DNA O^6MeG adduct staining per colonic crypt in rectal biopsies taken at baseline and week 4. All values are means ± SEMs. Different from baseline at week 4: ** $p<0.01$; (linear mixed model).

Table 5.2: Effect of dietary intervention in first period on rectal biology

	HRM group (n=10)			HRM + HAMSB group (n=13)			P-value treatment difference[2]
	Baseline	week 4	Increment (%change)	Baseline	week 4	Increment (% change)	
O^6MeG	60.8 ± 2.3	77.4 ± 5.8**	16.6 (21.4%)	59.8 ± 3.2	67.5 ± 2.3	7.7 (11.4%)	0.14
PCNA	6.2 ± 0.3	9.9 ± 1.0***	3.8 (38%)	6.6 ± 0.3	8.6 ±0.7*	2.0 (23%)	0.05†

[1] All values are means ± SEMs. Different from baseline at week 4: * p<0.05; ** p<0.01; *** p<0.001 (linear mixed model). HRM, high red meat; HAMSB, butyrylated high amylose maize starch.
[2] P value for treatment difference at week 4: † P<0.05; (linear mixed model).

Figure 5.2: O^6MeG DNA adduct immunohistochemical staining in human rectal epithelial tissue



Light microscope images (20 x optical zoom) of human rectal crypts showing O^6MeG staining intensity at baseline and after 4wk intervention phase taken from one individual with a large change in adducts after red meat consumption (A). Representative section from HRM baseline group, (B) representative section from HRM 4 week treatment, (C) representative section from HRM+HAMSB baseline group, (D) representative section from HRM+HAMSB 4 week treatment

5.4.3. Faecal fermentation products

Results of stool analyses are presented in Table 5.3. Stool weights and pH did not differ significantly between treatments. Stool excretion of acetate, propionate, butyrate and total SCFA was higher in the HRM+HAMSB group at 4 weeks compared to its baseline ($p<0.05$) as was stool concentrations of acetate, propionate and total SCFA ($p<0.05$). Faecal *p*-cresol concentration was lower in the HRM+HAMSB group at 4 weeks compared to its baseline ($p<0.01$) and the HRM group ($p<0.05$). Branched chain fatty acids (BCFA), phenols, ammonia and NOC were unaffected by treatment.

Table 5.3: Effect of dietary intervention in first period on stool biochemistry[1]

	HRM group (n=10)			HRM + HAMSB group (n=13)			P-value for treatment difference[2]
	Baseline	**week 4**	**Increment (% change)**	**Baseline**	**week 4**	**Increment (% change)**	
Faecal output (g/48 h)	266.4±42	264.7±46.7	-1.7(-0.6)	226.8±50.1	293.6±36	66.8(22.8)	0.61
Faecal pH	7.1±0.1	7.2±0.1	0.1(1.4)	7.2±0.1	7.2±0.1	0(0)	0.63
Total SCFA (µmol/g)	86.5±10.1	91.6±10.2	5.1(5.6)	73.2±8.3	89.3±7.7*	16.1(18)	0.53
Acetate (µmol/g)	50.8±6.2	54±5.4	3.2(5.9)	39.7±4.2	48.9±4.5*	9.2(18.8)	0.81
Propionate (µmol/g)	14.4±2	14.9±2.2	0.5(3.4)	13±1.6	16.9±1.5*	3.9(23.1)	0.13
Butyrate (µmol/g)	14.1±2	14.7±2.3	0.6(4.1)	12.9±2.4	16.1±2.5	3.2(19.9)	0.26
BCFA (µmol/g)	7.2±0.8	8.2±0.9	1(12.2)	7.6±0.9	7.3±0.7	-0.3(-4.1)	0.33
Total SCFA (mmol/48 h)	25.8±6.4	26.7±6.5	0.9(3.4)	19.3±5.1	26.3±4.1*	7(26.6)	0.48
Acetate (mmol/48 h)	15.2±3.9	15.7±3.7	0.5(3.2)	10.3±2.6	14.4±2.3*	4.1(28.5)	0.61
Propionate (mmol/48 h)	4.4±1.1	4.4±1.1	0(0)	3.7±1.1	5.2±0.9*	1.5(28.8)	0.27
Butyrate (mmol/48 h)	4.3±1.1	4.3±1.1	0(0)	3.6±1.2	4.7±1*	1.1(23.4)	0.28
Phenol (µg/g)	1.2±0.3	0.7±0.1	-0.5(-71.4)	1.3±0.4	1.6±1	0.3(18.8)	0.63
p-Cresol (µg/g)	65.9±11.7	68.8±9.8	2.9(4.2)	78.1±8.1	51.6±10.1**	-26.5(-51.4)	0.02†
Ammonia (µmol/g)	20.4±2	16.1±1.5	-4.3(-26.7)	17±1.6	15.5±1	-1.5(-9.7)	0.93
NOC (ng/mL)	516±112	406.5±72	-109.5 (-26.9)	481.9±126.4	388.1±51.7	-93.8(-24.2)	0.96

[1] All values are means ± SEMs. Different from baseline at week 4: * P<0.05; ** P<0.01; (linear mixed model). ; HRM, high red meat; HAMSB, butyrylated high amylose maize starch; SCFA, short chain fatty acids; BCFA, branched chain fatty acids; NOC, N-nitroso compounds.

[2] P value for treatment difference at week 4. 4: † P<0.05; (linear mixed model).

5.4. Discussion

Previously, it has been reported that feeding a diet high in red meat to rodents can increase the level of the pro-mutagenic DNA adduct O^6MeG in the colon whereas co-consumption of RS can prevent their accumulation (Winter et al. 2011). This study has now shown that when healthy humans consumed their normal habitual diet containing an additional 300g/day of red meat over a 4 week period, there was increased formation of the O^6MeG in the rectal epithelium. This increase in adduct formation might account, in part, for the increased risk of CRC associated with consuming high levels of red meat.

Studies in rodents have shown a positive correlation between cumulative O^6MeG levels and tumour load (Povey 2000). This association is also supported in humans by the prevalence of higher O^6MeG levels in DNA isolated from the distal region of the colon, where most sporadic CRC occur (Povey et al. 2000). The current study is the first to report on the effect of feeding a HRM diet to humans on the most common alkyl DNA adduct O^6MeG in rectal epithelial tissue. In a randomized crossover study comparing high red meat, vegetarian and high red meat/high fibre diets, an increase in O^6-carboxymethylguanine adducts was observed in exfoliated colonic epithelial cells isolated from the feces of healthy volunteers consuming a high red meat diet (Lewin et al. 2006). However, the relevance of DNA adducts in exfoliated cells to the *in situ* epithelial adduct load is unclear. The findings here show that such adducts do form in cells residing within the crypt and thus have the potential to form mutations that might progress to cancer.

The present study also confirms that dietary resistant starch in the form of HAMSB taken together with a glass of milk or orange juice can protect against red meat-induced colorectal DNA alkyl adducts in humans. This is consistent with epidemiological evidence that dietary fibre consumption reduces the risk of CRC. The current study and previous work in rodents (Toden et al. 2006, Winter et al. 2011, Conlon et al. 2012, Winter et al. 2014) all point towards SCFAs, particularly butyrate, to be key mediators in preventing meat-induced DNA adducts and DNA strand breaks in colonic mucosa. Butyrate is the preferred metabolic substrate for colonocytes and has strong anti-tumorigenic properties *in vivo* (Le Leu et al. 2002, Clarke et al. 2012). In the present study, ingestion of HAMSB in combination with HRM was also able to generate a favourable colonic environment by increased levels of SCFAs and a reduction in the toxic protein fermentation product *p*-cresol. This elevation of faecal butyrate with HAMSB confirms previous studies in humans (Clarke et al. 2011), and therefore has the potential to improve colonic health and offer protection against CRC. Consumption of a blend of types 2 and 3 RS in a recent human trial failed to reduce tumor incidence in Lynch syndrome patients (Mathers et al. 2012). The relatively low daily intake of RS used in that study (15g of RS obtained from 30g Novelose 240 and Novelose 330 per day) may have been insufficient to raise SCFA levels in stool, which were not measured. At least 20 g of RS per day may be needed to increase stool levels of SCFA (Topping et al. 2009, Worthley et al. 2009).

A plausible explanation for the observed increase in O^6MeG adducts and proliferation with the high red meat intervention is the presence of dietary haem. Red meat is rich in haem, with the vast majority passing into the large bowel

(Young et al. 1989). Presence of haem can catalyse the conversion of nitrogen containing substrates into potential carcinogenic NOCs within the colonic lumen (Cross et al. 2003, Joosen et al. 2009). Dietary haem increases the production of reactive oxygen species causing cellular toxicity and pro-mutagenic lesions (Glei et al. 2006). This oxidative capacity of haem can stimulate epithelial proliferation within the colonic epithelial cells (de Vogel et al. 2008, Ijssennagger et al. 2012), and promote formation of pre-cancerous lesions in chemical carcinogenic rodent models of CRC (Pierre et al. 2003).In Chapter 3 of this thesis, dietary haem could increase cell proliferation and stimulate accumulation of O^6MeG adducts in the colon of mice after prolonged feeding in the Western model of CRC. Therefore, accumulation of pro-mutagenic alkyl lesions and increases in epithelial proliferation, by increasing exposure to haem from red meat, could be one potential mechanism for triggering the oncogenesis pathway in colorectal cells.

In this study we anticipated that NOCs would increase in stool of participants consuming the HRM diet and that this would explain a higher O^6MeG adduct load. High dietary haem and red meat have previously been associated with increased faecal NOCs in humans (Hughes et al. 2001, Lunn et al. 2007). There is a dose response relationship between increasing red meat intake and faecal NOC, suggesting low faecal NOC (374 µg/kg) at low red meat intake (60 g/d) and a 4-5-fold increase in faecal NOC with increased red meat intake above 240 g/d (Hughes et al. 2001). Lewin et al. (2006) also observed an increase in faecal NOCs in volunteers fed 420 g/d of red meat in comparison to a vegetarian diet and showed NOCs are likely involved in the generation of alkyl adducts. However, Cross et al (2006) showed no effect of red meat or haem consumption on faecal NOC concentration, nor any correlation between total faecal NOCs and

subsequent DNA damage. They cited large individual variation of DNA damage (measured by strand breaks in the comet assay) as a likely reason for the lack of consistency between NOC generation and colonic DNA damage. In addition, human MGMT activity (for DNA alkyl repair) in normal and tumour tissue has a high inter-individual variability, particularly when combined with factors such as smoking, age and medication use (Margison et al. 2003). The results presented in this chapter showed an increase in O^6MeG adduct load with consumption of at least 300 g/d of red meat, but there was no correlation with faecal NOCs. This suggests that measuring O^6MeG adducts in rectal epithelial cells as a consequence of NOC exposure after red meat consumption may not be a reliable indicator for apparent risk of oncogenesis. This could be explained by inter-individual variations of response to NOCs as well as difference in the ability to repair DNA alkyl adducts by MGMT between individuals.

In summary, these findings show that high dietary red meat intake increases pro-mutagenic DNA adducts and epithelial cell proliferation. Conversely, raising faecal butyrate levels with HAMSB reduced O^6MeG adduct load. These findings might explain the increased risk for CRC associated with high red meat consumption and could point to a beneficial effect of butyrate-generating RS. This is in line with previously published short term mouse studies showing that red meat can increase DNA adducts but that RS can reduce adduct formation (Winter et al. 2011). However, the results from this chapter do not agree with results from Chapter 4 of this thesis where long term red meat and RS feeding in mice actually enhanced DNA adduct accumulation, rather than protected from it. It could also be true for humans that long term, chronic exposure to red meat with RS could enhance DNA alkylation in the colonic

epithelium. Further long term intervention trials with red meat in humans are needed to better understand how chronic exposure to red meat in the colon might interact with the epithelia, and how this might change risk factors of colorectal oncogenesis.

Chapter 6

Role of red meat and resistant starch in pro-mutagenic adduct formation, thymic lymphoma and intestinal tumourigenesis in *Msh2* deficient mice

6.1. Introduction

The mechanisms by which red meat might initiate or promote CRC are still being debated. Such processes include: oxidative stress and hyper-proliferation by haem (Santarelli et al. 2010); saturated fat promoting tumour formation via secondary bile acid production and lipid peroxidation (Reddy 1992); protein fermentation by luminal microbiota generating toxic products (Le Leu and Young 2007); carcinogen formation with high temperature cooking of red meat (Zheng and Lee 2009); and lastly, endogenous and exogenous production of alkylating NOCs that incorporate alkyl adducts in the DNA (Winter et al. 2011). Rodent models incorporating chemically derived alkylating agents can generate large amounts of DNA O^6MeG adducts in colonic epithelial cells, and are directly linked with an increase in colorectal tumours (Povey et al. 2002, Jackson et al. 2003). The first line of defence against these lesions involves the MGMT repair protein sequestering the O^6MeG adduct in a suicide reaction. Without MGMT repair the DNA polymerase complex recognises unrepaired O^6MeG adducts incorrectly, resulting in GC→AT transition mutations, after which other repair complexes including MMR are recruited (Margison et al.

2002). A high red meat diet in humans is demonstrated to generate significant NOC's in the faecal contents, and this correlates with alkyl adduct formation within exfoliated rectal epithelial cells (Lewin et al. 2006). In rodents, short term consumption of dietary red meat can increase colonic O^6MeG adducts compared to a non-meat protein source (Winter et al. 2011). Production of known pro-mutagenic alkyl adducts in the colon by red meat consumption may play a defining role in oncogenesis of the colon and might act as a biomarker for red meat intake, and the associated cancer risk.

Resistant starch (RS) is the component of starch delivered to the colon and fermented by the resident bacteria. It is thought that RS may protect against cancer through mechanisms associated with metabolic products of anaerobic bacterial fermentation in the colon (Young and Le Leu 2004). Studies in rodents have shown associations between SCFA production by RS and protection against hyper-proliferation, pre-neoplastic markers and CRC (Bauer-Marinovic et al. 2006, Le Leu et al. 2007, Le Leu et al. 2009). In addition, rodents fed high dietary protein display increased colonic DNA strand breaks, particularly with red meat consumption, but RS could protect against their formation (Toden et al. 2003, Toden et al. 2006, Toden et al. 2007). More recently, RS has been identified as an inhibitor of red meat-induced pro-mutagenic O^6MeG adducts in the mouse, an effect that correlated with faecal butyrate levels (Winter et al. 2011). In human trials, RS has shown to reduce colonic proliferation in the colon of CRC patients (Dronamraju et al. 2009) and in healthy individuals consuming a high red meat diet (Humphreys et al. 2014). However, due to inadequate RS intake the effects on epithelial kinetics and adenoma prevention in human trials are somewhat conflicting (van Gorkom et al. 2002, Burn et al. 2008). RS demonstrates potential

for reducing the risk of CRC associated with a Western lifestyle, particularly when consuming a high red meat diet. To date, the combination of red meat and RS has not been studied regarding CRC risk at later time points of the oncogenesis pathway, such as pre-cancerous lesions and tumours in rodents or humans.

A majority of studies to date use the carcinogen AOM, an alkylating agent that generates high levels of pro-mutagenic adducts in the distal colon. Therefore, chemical carcinogenic exposure in rodent models of CRC is not an ideal representative of natural dietary carcinogenic exposures as humans are rarely exposed to this artificial chemical. An alternative model for studying dietary effects on CRC are genetically modified rodents that already exhibit an increased susceptibility to CRC. Knockout mice deficient in the *Msh2* protein lack MMR capability, and as a consequence generate more colonic tumours when exposed to AOM (Mladenova et al. 2011). Although several genetic mouse models of cancer prevention with nutritional compounds have been used, the *Msh2* knockout has not been studied extensively (Tammariello and Milner 2010). In the human setting, loss of MMR underlies hereditary non-polyposis CRC (HNPCC), or Lynch syndrome, a common familial inherited form of CRC. HNPCC patients demonstrate increased rates of CRC compared to the normal population as well as earlier onset of disease (Lynch et al. 1991). In particular, carriers of the *Msh2* mutation and have a higher age-specific cumulative risk for CRC of 48% by age 70, compared to the other common mutations *MLH1* (41%) and *Msh6* (12%) (Bonadona et al. 2011). The *Msh2* knockout mouse represents a relevant and potentially valuable model of study, particularly in relation to red meat-induced adducts and tumour outcome.

6.2. Aims and hypotheses

It is known that red meat consumption is a risk factor for colorectal oncogenesis, and that RS has the capacity to reduce that risk. Consequently, this chapter aimed to determine if feeding red meat to mice deficient in MMR capacity would increase O^6MeG DNA adducts and risk for colonic neoplasia. It also determined if feeding RS could regulate these effects. Therefore, the hypotheses for this chapter are that red meat feeding in wild type mice will increase DNA O^6MeG adducts, pre-neoplastic lesions and colorectal tumours in the colon of *Msh2* deficient mice but that addition of RS to a red meat diet fed to *Msh2* deficient mice will reduce these damaging effects in the colon. The specific aims are as follows:

1. Determine if feeding a high red meat diet for 6 months can increase DNA O^6MeG adducts in the colon of *Msh2* deficient mice and whether RS can modulate the red meat induced adducts.
2. To determine the risk of developing pre-neoplastic and neoplastic lesions in the colon of *Msh2* deficient mice with consumption of a high red meat diet for 6 months, and if co-consumption with RS can protect against these changes.
3. To identify if there is an association between DNA O^6MeG adduct accumulation in the colon of *Msh2* deficient mice and risk of developing CRC.

6.3. Materials and methods

6.3.1. Animals and study design

Male and female *Msh2*$^{-/-}$ mice initially bred on a 129/OLA background (de Wind et al. 1998) but then backcrossed with C57Bl6J for multiple generations, and their wild type litter mates, were imported from Australian BioResources (Moss Vale, New South Wales). A total of 100 *Msh2*$^{-/-}$ and 60 wild type mice were used in dietary experiments. All mice were placed into cages according to gender, with a maximum of 5 per cage. They were randomly divided into dietary groups and the feeding experiment was strictly under controlled conditions of 22±2°C (SD), 80±10% humidity, and 12 h light/dark cycle. Mice were fed *ad libitum* and weighed once weekly throughout the entire experiment. Duration of dietary treatment was designed for 6 months, or until euthanasia attributed to illness. Due to the unexpected high rate of early lymphoma and small intestinal tumour development in several mice, an additional 36 *Msh2*$^{-/-}$ mice were included for analysis of cancer and pre-cancerous end points. Research with animals was conducted according to the Australian code for the care and use of animals for scientific purposes. The Flinders University of South Australia Animal Welfare Committee approved all experimental procedures (ethics approval number 809/12).

Diets were based on the American Institute of Nutrition (AIN) diet AIN-76 with some modifications (Table 6.1). A total of 4 dietary interventions were investigated: Control, red meat, resistant starch (RS) and red meat + RS. Details of the dietary groups are explained in Chapter 2.3. All dry ingredients were mixed thoroughly before liquid ingredients (oil, lard) were combined. Approximately 500-800ml of distilled water was blended into the diet mixture before being compressed into pellets. Final pelleted food was stored in air-tight containers at -20°C and replaced in the cage feeders every 1-2 days.

Table 6.1: Composition of experimental diets (g/100g of diet)

	Control	**Resistant Starch**	**Red Meat**	**Red Meat + Resistant Starch**
Casein	20	20	-	-
Red Meat[a]	-	-	25	25
Corn starch	41.72	31.64	39.25	29.17
Hi-Maize™	-	10	-	10
Sucrose	10.95	10.95	10.95	10.95
Sunflower Seed oil	17.66	17.74	17.68	17.76
Lard[a]	2.55	2.55	-	-
α-cellulose	2	2	2	2
L-cysteine	0.3	0.3	0.3	0.3
Choline	0.17	0.17	0.17	0.17
Minerals[b]	3.5	3.5	3.5	3.5
Vitamins[b]	1	1	1	1
Dl-Methionine	0.15	0.15	0.15	0.15
Total	100	100	100	100

[a]Red meat dry powder was added with it's own moisture content
[b]Lard was added to the control diets to balance for saturated fat content of the red meat
[c]AIN-76 standard vitamin and mineral mixtures (see appendix B)

6.3.2. Specimen collection, storage and tissue preparation

At the end of the 6 month feeding experiment, or when signs of deteriorating illness were evident, mice were humanely euthanized by CO_2 asphyxiation and cervical dislocation. The entire length of the colon was resected, faecal contents removed, and cut open longitudinally before being affixed to Hi-bond™ membrane paper. Colons were submerged in a 10% buffered formalin solution containing 3.6% formaldehyde for 24 hours and transferred to 70% ethanol for storage. The small intestine was examined and any suspected tumours were removed placed into 10% buffered formalin solution and transferred to 70% ethanol at 24 hours. Diagnosis of lymphoma included but not limited to: significant weight loss (10-15%), deterioration of overall mouse health before

death/euthanasia, rapid shallow breathing, enlarged thymus, spleen or liver, and/or lung tumour.

6.3.3. Small intestinal tumour histology

Suspected small intestinal tumours were processed through gradient alcohols and xylene before being embedded in paraffin wax (Chapter 2.5.1). A 5µm section was taken and rehydrated in gradient alcohols before staining with H&E for visualisation under light microscope. Tumours were classified as adenomas or adenocarcinomas.

6.3.4. Analysis of colonic aberrant crypt foci (ACF)

Excess mucus and debris were washed from the colon with 0.9% solution of saline and gently wiped clean with a cotton tip before incubation in 0.4% solution of methylene blue diluted with 0.9% saline solution for approximately1-2 minutes. Visualisation of ACF was performed under a dissecting microscope and identified as having crypts with large openings, unusual shape and stained darker than the normal surrounding crypts. ACF were classified as small (≤3 ACs), large (≥4 ACs) and total ACF.

6.3.5. Immunohistochemical (IHC) quantification of proliferation, O^6MeG adducts and MGMT repair.

After ACF analysis, 0.5 cm of colorectal tissue was cut from the distal colon and another 0.5cm from the proximal portion where the "herringbone" pattern of the proximal colon meets the flatter, middle colon. Both small intestinal tumours and the colonic tissue segments were dehydrated through gradient alcohols and xylene before embedded in paraffin wax (Chapter 2.5.1). Distal and

proximal sections of 4µm were used to quantify cell proliferation using an antibody against the nuclear proliferating antigen Ki-67 (AbCam SP6; Cat# ab16667) and O^6MeG DNA adducts using the primary monoclonal antibody (clone EM 2-3; Squarix Biotechnology). Methods for IHC protocols are described Chapter 2.5.4 and 2.5.5. Slides were coded and a total of 20 intact colonic crypt columns were counted blind by a single observer.

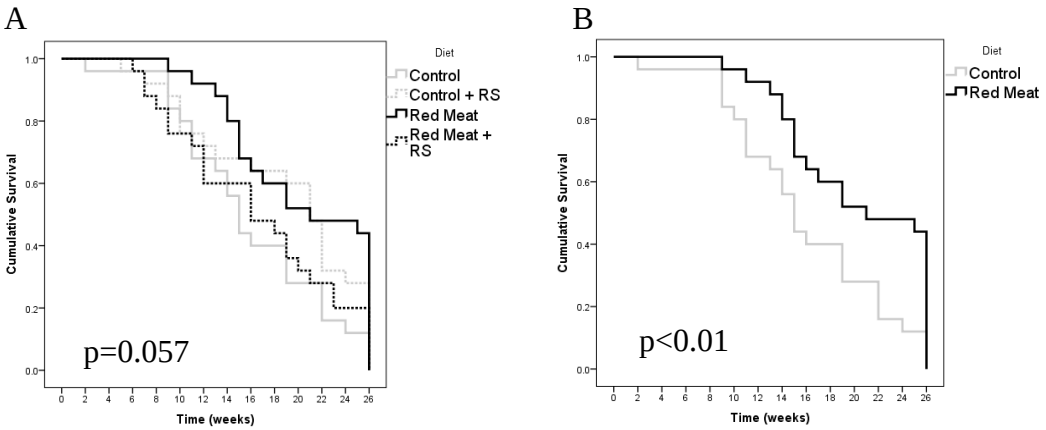
Quantification of MGMT protein was performed with a mouse monoclonal antibody (SPM287; Cat# ab54306) diluted 4-fold with PBS (pH 7.4) in combination with a Mouse-on-Mouse (MoM) polymer linking kit (Covance) as described in Chapter 2.5.5. Proliferation was measured as positive ki-67 cells per crypt column. O^6MeG and MGMT were calculated as the sum of pixel intensity (red over blue (RoB) ratio) measured by computer image analysis (detailed in Chapter 2.5.5) Slides were coded and a total of 20 intact colonic crypt columns were counted blind by a single observer.

6.4. Results

6.4.1. Bodyweights, Msh2$^{-/-}$ survival and tumour analysis

Final body weights of both *Msh2*$^{-/-}$ and wild type mice were not significantly affected by dietary treatment, however *Msh2*$^{-/-}$ mice had significantly lower bodyweights (35.51 ± 1.15) compared to wild type mice (40.61 ± 1.12) ($p<0.002$). Comparison of overall survival for *Msh2*$^{-/-}$ mice between all 4 diet groups did not quite reach statistical significance ($p=0.057$; Figure 6.1.A). However, when comparing mice that were consuming a control diet vs. a red meat diet, there was a statistically significant improvement in survival for those mice consuming the red meat diet ($p<0.01$; Figure 6.1.B).

Figure 6.1: *Msh2*$^{-/-}$ survival curves



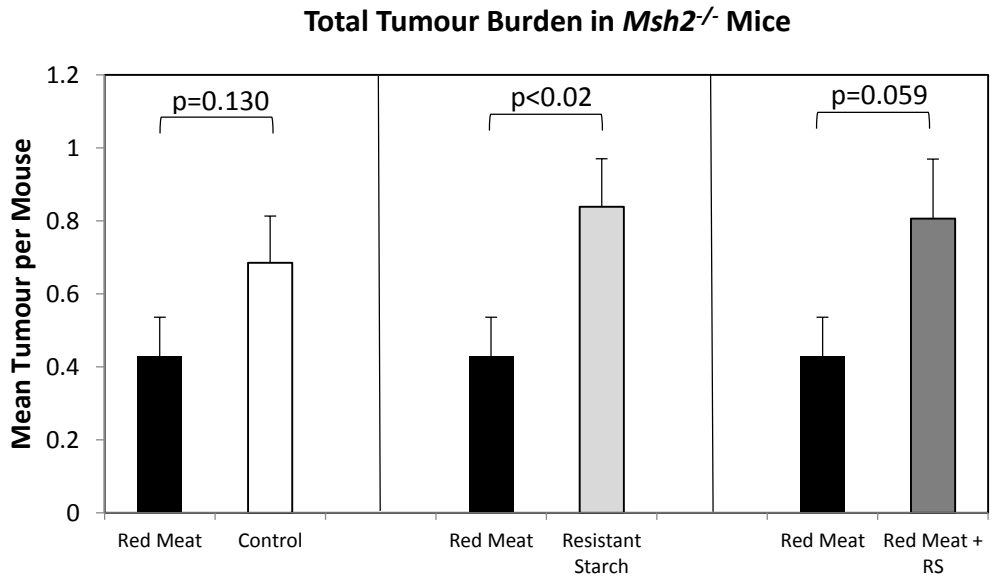
Cumulative survival of Msh2$^{-/-}$ mice over 6 month dietary treatment (26 weeks) presented as Kaplan-Meyer curves showing comparisons of all dietary treatments (A) and control vs. red meat diet (B).

Loss of *Msh2* was associated with an increase in mean ACF per mouse from no observed ACF at all in the wild type mice to 0.18 ± 0.05 ($p<0.014$) in Msh2$^{-/-}$ mice; however, these are very low numbers of ACF compared to what is seen in carcinogen models using agents such as AOM . There were no significant dietary effects of red meat or RS consumption on small, large or total ACF. Overall, very few ACF were observed in any of the dietary groups (Table 6.2). *Msh2*$^{-/-}$ mice consuming a red meat diet had lower rates of both small intestinal (SI) tumours and lymphoma, although not reaching significance (Table 6.2). Analysis of mean tumour burden per mouse by combining SI tumours and lymphoma (Figure 6.2) showed a significant reduction in tumours with red meat compared to RS ($p<0.02$), as well as a trend towards a decrease with red meat vs. control ($p=0.13$) and red meat vs. red meat + RS ($p=0.059$). No colorectal tumours (adenomas or adenocarcinomas) were observed in any mice.

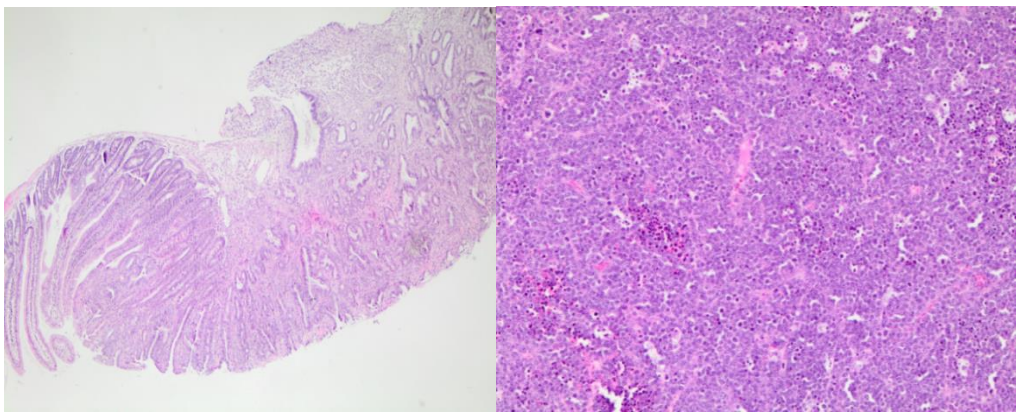
Table 6.2: Incidence (%) of colonic ACF, small intestinal (SI) tumours and lymphoma in *Msh2*$^{-/-}$ mice.

	Experimental Diets				Dietary Effects (p value)	
	Control (n=35)	**RS (n=31)**	**Red Meat (n=28)**	**Red Meat + RS (n=31)**	**RS Effect**	**Red Meat Effect**
SI Tumours	20.6	30	10.7	22.6	0.194	0.412
Lymphoma	38.2	33.3	28.6	35.5	0.625	0.520
Small ACF	11.42	12.90	14.28	16.12	0.754	0.620
Large ACF	0	3.225	7.142	3.225	0.987	0.286
Total ACF	11.42	19.35	21.42	19.35	0.486	0.592

Figure 6.2: Total tumour burden in *Msh2*$^{-/-}$ mice and H&E staining of small intestinal adenocarcinoma and thymic lymphoma



B C



Total tumour burden combining small intestinal tumours and lymphoma (A). Analyses carried out using independent samples t-test and data represented as mean ± SE. Representative images of H&E staining of normal appearing epithelium and small intestinal adenocarcinoma (B) and a thymic lymphoma (C).

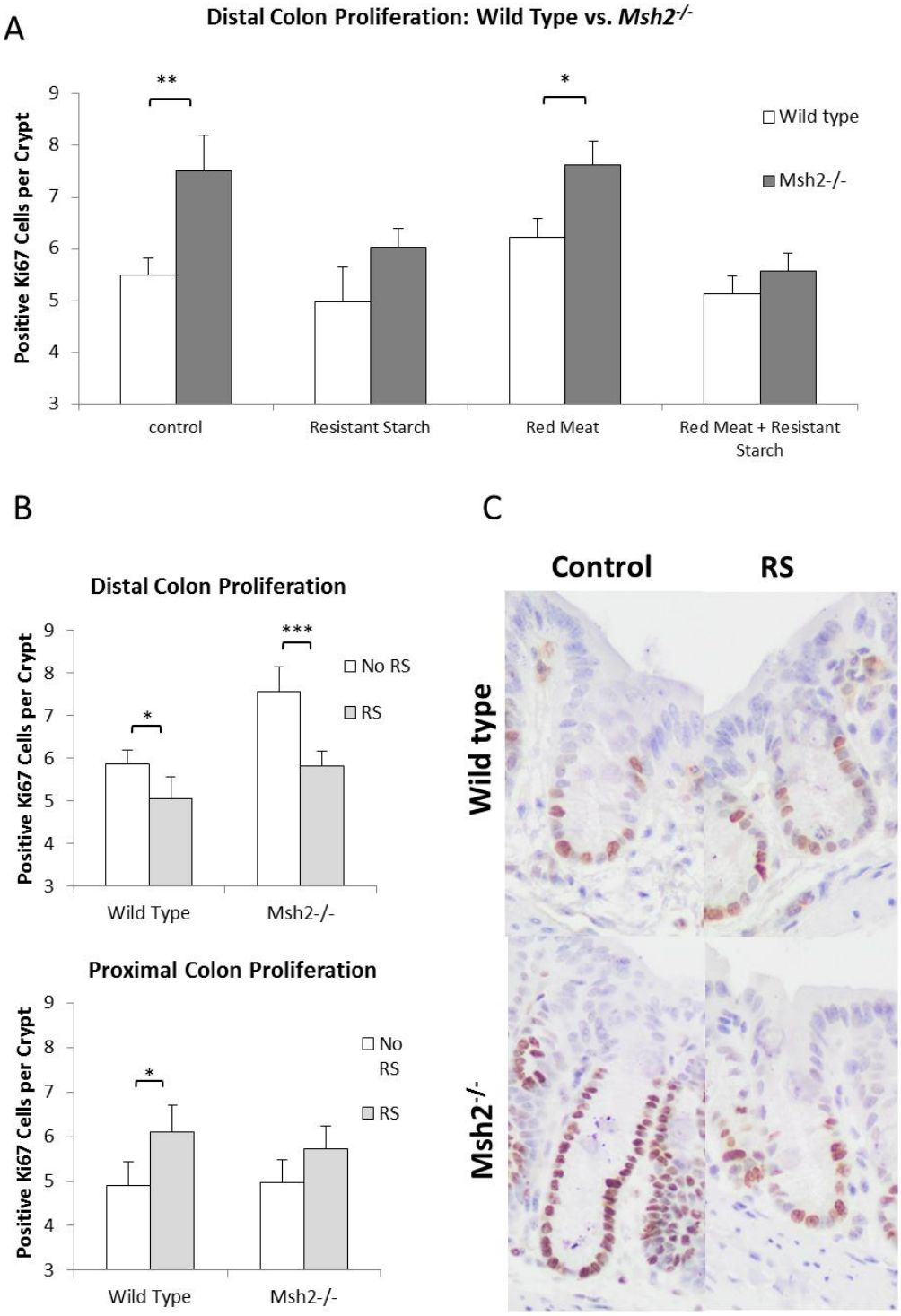
6.4.2. Colonic cellular proliferation

Proliferation rates in the distal colon for wild type and *Msh2*$^{-/-}$ mice are shown in Figure 6.3A. The level of positive Ki-67 cells per crypt was significantly higher in *Msh2*$^{-/-}$ mice consuming control or red meat diets compared to their wild type litter mates ($p<0.01$ and $p<0.05$, respectively). Hyper-proliferation in *Msh2*$^{-/-}$ mice was supressed by addition of RS to the diet ($p<0.001$), and RS significantly decreased proliferation in wild type mice ($p<0.05$) (Figure 3B). However, this effect of RS was not seen in the proximal colon (Figure 3B). In contrast, RS significantly increased proliferation in the proximal colon compared to diets without RS for wild type mice ($p<0.05$), although this did not reach significance in *Msh2*$^{-/-}$ mice ($p=0.163$).

6.4.3. Colonic O^{6}MeG adducts

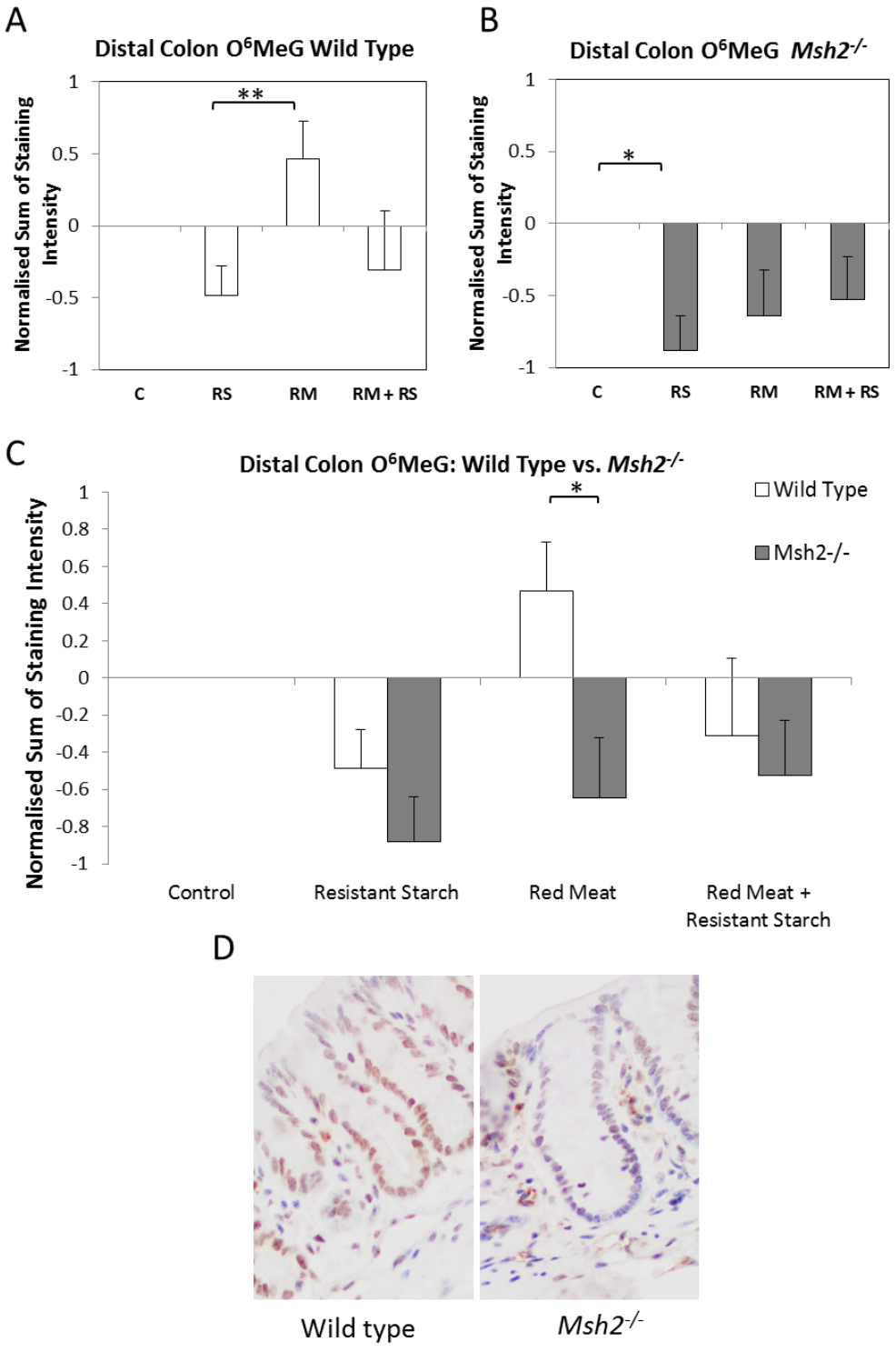
Results of O^{6}MeG DNA adducts normalised to control are presented in Figure 6.4A-C. For wild type mice, red meat generated significantly more O^{6}MeG adducts in the distal colon compared to mice consuming RS diet ($p<0.01$). In *Msh2*$^{-/-}$ mice, RS protected against O^{6}MeG adducts compared to control ($p<0.05$), but not against red meat or red meat + RS. Loss of *Msh2* did not enhance red meat induced O^{6}MeG adducts (Figure 4D). In fact, O^{6}MeG adducts significantly decreased in *Msh2*$^{-/-}$ mice compared to wild type mice consuming red meat diets ($p<0.05$). No significant changes in O^{6}MeG adduct accumulation were observed in the proximal colon (Appendix J).

Figure 6.3: Cellular proliferation in the distal and proximal colon



Comparison of proliferation rates by independent samples t-test between wild type and Msh2$^{-/-}$ mice for each dietary intervention in the distal colon (A). Univariate analysis of the main effects with RS supplementation of the diet on proximal colon and distal colon proliferation (B). Representative images showing the hyper-proliferative state of distal colon in Msh2$^{-/-}$ mice and its reduction with RS supplementation (C). Data are represented as means ± SE (* $p<0.05$; ** $p<0.01$; *** $p<0.0001$).

Figure 6.4: DNA O^6MeG Adduct immunohistochemical staining in the distal colon

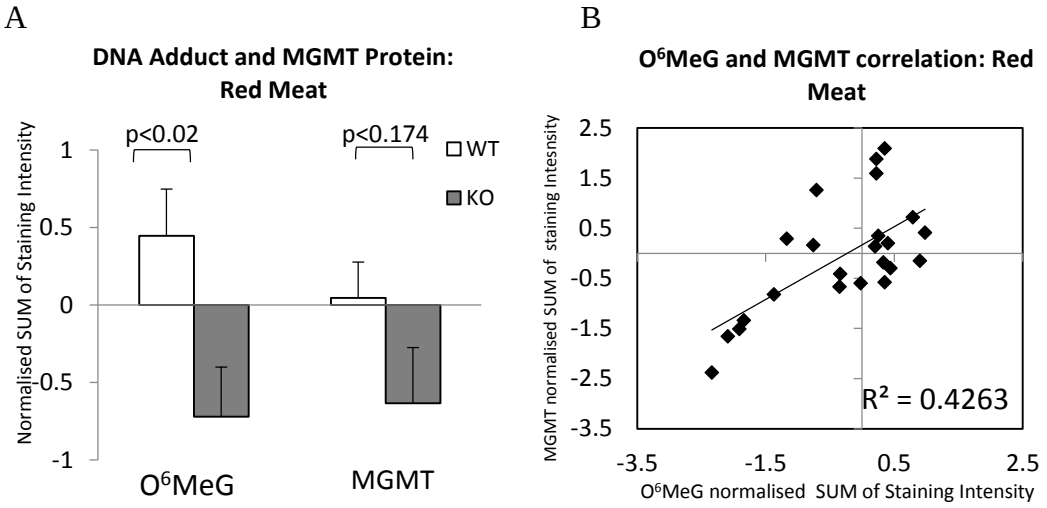


Sum of normalised O^6MeG DNA adduct staining intensity in distal colon cells for wild type mice (A), $Msh2^{-/-}$ mice (B) and comparison of the genotypes for each dietary treatment (C). Data represented as mean ± SE. (*$p<0.05$; **$p<0.01$). Representative images of nuclear DNA O^6MeG adduct staining of the distal colon in $Msh2^{-/-}$ and wild type mice consuming a red meat diet (D).

6.4.3. MGMT repair

To determine if changes in MGMT repair might be the mechanism by which *Msh2*$^{-/-}$ displays altered DNA adducts in mice consuming red meat, MGMT protein expression was measured in the distal colon. Similar to O^6MeG DNA adduct observations, *Msh2*$^{-/-}$ mice displayed less MGMT protein expression in the distal colon compared to wild type mice, although not significantly ($p=0.174$; figure 6.5.A). There was a strong positive correlation ($R^2 = 0.4263$) of O^6MeG DNA adduct accumulation and MGMT protein expression in the distal colon ($p<0.001$; figure 6.5.B). Thus enhanced MGMT protein expression did not appear responsible for the reduction of DNA adducts generated with a red meat diet.

Figure 6.5: MGMT and DNA O^6MeG adduct correlation in the distal colon after red meat consumption



Distal colon SUM of staining intensity for DNA adducts and MGMT in mice consuming a red meat diet in both wild type and Msh2$^{-/-}$ mice (A) and Pearson's bivariate correlation analysis for adducts and repair in both genotypes (B). Data displayed as mean (± SE).

6.5. Discussion

This chapter has shown for the first time that consumption of red meat can reduce the risk of development of lymphoma and small intestinal tumours in MMR deficient mice and that MMR deficiency has a protective effect on the formation of DNA pro-mutagenic alkyl adduct caused by red meat in the distal colon. It has also confirmed previous data showing that red meat consumption in normal mice increases alkyl adducts but that RS consumption can protect against formation of these lesions in the distal colon. Loss of *Msh2* capacity increased cell proliferation and spontaneous ACF formation in the colon. These results also show that a diet high in RS can impede *Msh2*$^{-/-}$ associated hyper-proliferation of the distal colon and return the epithelium to a normal proliferative state. Yet, none of the observed changes with the dietary interventions had a significant effect on formation of ACF or colonic tumours within the 6-month duration of this experiment.

Contrary to the original hypothesis, red meat consumption in an *Msh2* deficient mouse did not enhance O^6MeG DNA adducts and was not a risk factor for ACF formation. Red meat consumption actually reduced O^6MeG adducts in *Msh2* deficient mice compared to wild type mice. What this means for CRC risk cannot be assessed since no animals developed CRC, certainly not within the 6-month duration of the experiment. Since MMR capacity is deficient in these mice it seemed possible that red meat consumption might produce an adaptive response of MGMT repair to account for the loss of Msh2, thereby reducing adduct load. However, when MGMT protein expression was measured, a significant positive correlation between MGMT and O^6MeG was observed. This suggests *Msh2*$^{-/-}$ mice do not have enhanced MGMT protein expression in response to red meat

consumption, and does not explain the reduction in O^6MeG adducts in $Msh2^{-/-}$ mice consuming red meat. It is important to note that the antibody used in the IHC analysis measures both active and inactive forms of MGMT. Therefore, it is possible red meat could still be enhancing the activity of MGMT and thus reducing adduct load, even though changes in MGMT protein expression were not identified. Due to inadequate tissue sampling, MGMT activity was not measured but future analysis in *Msh2* deficient mice is justified.

Although there were detected changes in DNA adduct accumulation, and these correlated with MGMT repair in mice consuming red meat, no effect on colonic proliferation or pre-cancerous lesions were identified. In humans, carriers of the *MGMT* Ile143Val polymorphism (which dampens MGMT activity) show a significant interaction of red meat and processed red meat intake (over 56g/day) and increased risk of CRC (Loh et al. 2010). This suggests that accumulation of red meat induced alkyl adducts due to ineffective MGMT activity might be important for development of CRC in MMR proficient individuals. However, carriers of MMR gene defect do not display the same risk of CRC after consumption of a high red meat diet (Botma et al. 2013, Parr et al. 2013). Parr et al (2013) found an increased risk of CRC within a sporadic CRC population consuming red meat ranging from <4 times a week to >7 times a week (p=0.08), but no such risk amongst the HNPCC population (p=0.26). Meat consumption over 7 times a week in the study by Parr et al (2013) demonstrated an OR for CRC of 4.1 (95% CI 0.7-23) for the sporadic CRC cases and only 0.4 (95% CI 0.1-2.2) for the HNPCC CRC cases. These data, together with our results in *Msh2*$^{-/-}$ mice, suggest that the effect of red meat on colonic epithelial cells with an MMR deficient background is not the same as in the general population. There appears

to be an undetermined component of red meat, or apparent change in repair mechanism induced by red meat intake, that might protect against alkyl adduct formation as a result of MMR loss. The significance of the DNA O^6MeG adduct acting as a biomarker for exposure of red meat on a normal genetic background, and not necessarily as a biomarker of CRC risk, seems to be likely. However, the model used here may not have been entirely adequate to confirm this theory.

Unexpectedly, a reduction in small intestinal tumours and lymphoma in MMR deficient mice consuming a red meat diet was observed, which resulted in a significantly enhanced survival rate in these mice. *Msh2*$^{-/-}$ mice develop intestinal tumours similar to HNPCC in older mice but spontaneous lymphomas occur at an earlier age (1/3 survival at 19.5 weeks) (de Wind et al. 1998). It was hypothesised that red meat might act as an initiating "carcinogen" (since it has been shown to increase adduct formation (Winter et al. 2011)), and that colonic neoplasms would develop (similar to chemical alkylation administration with AOM) as the MMR defect would make them more susceptible to the effects of red meat. Our results show that the *Msh2*$^{-/-}$ mouse is not a good model in determining CRC risk as a result of red meat consumption. It is possible that heterozygous *Msh2* mice may be more informative. In fact, HNPCC patients are heterozygous carriers of a MMR gene defect and do not normally develop cancers in childhood. In contrast, children inheriting a homozygous MMR gene defect from families of affected HNPCC patients (MMR-deficiency syndrome) have a distinct pathology of gastrointestinal cancers, hematologic malignancies and neurological tumours all occurring in early childhood (Bandipalliam 2005). In addition to MMR, *Msh2* is also important for somatic intrachromosomal recombination (Hooker et al. 2004), an important process in protection against tumours via a properly functioning

immune system. The role of red meat consumption and its protective effect on malignancies associated with homozygous MMR deficiency is noteworthy. Whether red meat may be enhancing the immune system via other pathways within *Msh2*$^{-/-}$mice, thereby reducing the risk of malignancies as a result of defective immune responses is unknown. More studies elucidating the effects of red meat on tumourigenesis and the immune system in a MMR deficient background is needed to validate these findings.

The finding from this chapter support previous data (Le Leu et al. 2007, Le Leu et al. 2010, Winter et al. 2011) emphasising how RS can reduce proliferation and DNA alkyl adducts in the distal colon of wild type rodents. We have also shown that functioning *Msh2* protects against hyper-proliferation and ACF formation, and that RS consumption protects against colonic hyper-proliferation caused by the genetic defect of MMR. Previous research has shown that fermentation of RS in the rodent colon, both with and without red meat protein, generates significantly high levels of short chain fatty acids (SCFA) in the colonic lumen, including butyrate (Toden et al. 2006, Toden et al. 2007, Winter et al. 2011). Anti-neoplastic properties identified *in vitro* and *in vivo* highlight butyrate's ability to increase removal of highly damaged colon cells via apoptosis, as well as reducing cellular proliferation of the distal colon to allow for repair processes (Hague et al. 1995, Emenaker et al. 2001, Le Leu et al. 2003). These effects of butyrate imply that it is responsible for reducing ACF and colon tumours in genetically normal rodents (Perrin et al. 2001, Le Leu et al. 2007) although no such reduction was evident in non-carcinogenic models of spontaneous CRC (see Chapter 3 & 4) (Winter et al. 2014). Reduction of hyper-proliferation by RS was not seen in the proximal colon, and this could be due to

the rapid formation of SCFAs once RS reaches the proximal colon from the caecum, with a steady decline in SCFAs further along the colon (Topping and Clifton 2001). Butyrate is the primary energy source for colorectal cells and such a rise in SCFAs in this region of the colon might explain the increase in proliferation, and not necessarily a link to increased risk for cancer. Our ACF findings in *Msh2*$^{-/-}$ mice support data from the CAPP2 trial of HNPCC patients where RS consumption did not reduce CRC risk, although doses of RS in that study were arguably low at approximately 15g of RS (in the 30g/d of high amylose maize starch administered) (Burn et al. 2008, Mathers et al. 2012). Maintaining colonic proliferation via RS fermentation may not reduce ACF formation induced by loss of MMR. In the current study, *Msh2*$^{-/-}$ mice did not survive long enough for a substantial amount of ACF to form and longer intervention times may be required. RS may protect against CRC in chemical carcinogenic rodent models, but its chemo-protective capacity against cancer generated by a Western diet remains questionable.

Contrary to the hypotheses, this study has revealed an unexpected protective effect of MMR deficiency on colon O^6MeG adduct formation, lymphoma and small intestinal cancer as a result of red meat consumption. However, it did not show a link between colonic pro-mutagenic adducts and red meat consumption, or any associated risk for CRC. Although RS supressed hyper-proliferation of the distal colon as a result of MMR deficiency, no link between lowered proliferation rates and ACF formation was identified. Consequently, the CRC risk associated with changes in colonic proliferation by RS fermentation in the context of MMR remain questionable. Variations in epithelial markers after red meat and RS consumption on a normal genetic background do not behave in a

similar fashion with MMR deficient capacity. These findings highlight the potential implications for dietary guidelines given to individuals at high risk of developing CRC, particularly those with an inherited genetic defect in MMR.

Chapter 7

The effect of red meat and green tea intake on epithelial kinetics and pro-mutagenic DNA adduct formation in the colon of methyl-guanine-methyl-transferase deficient mice

7.1. Introduction

Red meat has now been established as a significant risk for developing CRC by the World Cancer Research Fund . However, there is still no consensus on the exact mechanism(s) by which red meat exerts is damaging effects on colonic epithelial cells. One plausible mechanism is the generation of lesions on the DNA strand by exposure to alkylating agents from the diet, particularly by red meat exposure in the colon (Lewin et al. 2006, Winter et al. 2011, Humphreys et al. 2014). The alkylating adduct O^6-methyl-2-deoxyguanasine (O^6MeG) is pro-mutagenic and known to generate GC→AT transition mutations. The DNA polymerase complex integrates the wrong base after 2 rounds of replication where the unrepaired adduct is attached (Margison et al. 2002). The O^6MeG adduct can be quickly and effectively removed from the DNA without any interruption of DNA replication by the enzyme O^6-methylguanine DNA-methyltransferase (MGMT)(Povey et al. 2002). The lesion is removed in a single suicide reaction, with no need for further downstream repair mechanisms. Inadequate repair or a saturation of MGMT due to very high levels of alkyl lesions can lead to induction of apoptosis (Nyskohus et al. 2013), most likely via the mismatch repair (MMR) pathway (Bugni et al. 2009) or base excision repair

(BER) pathway (Wirtz et al. 2010). Experiments in mice without functional *MGMT* are sensitive to the lethal killing effects of chemical alkylating carcinogens and generate significantly more aberrant crypt foci (ACF) and tumours compared to normal mice (Glassner et al. 1999, Bugni et al. 2009, Wirtz et al. 2010). Furthermore, humans who have genetic polymorphisms in the *MGMT* gene show a significant interaction of red meat intake and increased CRC risk (Loh et al. 2010). *MGMT* deficient mice could be a potentially useful model in identifying the risk of red meat induced O^6MeG adducts and initiation of colorectal oncogenesis.

Green tea is a common beverage in East Asian countries, particularly Japan. Evidence from rodent and cell culture studies shows the capability of green tea as a preventative agent against CRC, although human studies are somewhat conflicting (Fujiki et al. 2012, Butt et al. 2013). Several mechanisms of action have been identified both *in vivo* and *in vitro* including anti-inflammatory (Barnett et al. 2013, Sadik 2013), anti-oxidant (Dashwood et al. 1999), anti-proliferative (Du et al. 2012) and pro-apoptotic (Xiao et al. 2008, Du et al. 2012) processes, with the active constituent being polyphenol compounds, mainly (-)-epicatechin-3-gallate (EGCG) . *In vivo*, green tea consumption has been shown to reduce ACF and tumour burden after exposure to chemically derived alkylating carcinogens (Ju et al. 2003, Hu et al. 2013) and heterocyclic amines (HCA) (Dashwood et al. 1999, Xu and Dashwood 1999). Protection against HCA induced CRC is linked to a reduction of bulky DNA adducts, which are thought to be "quenched" by the phenolic compounds found in green tea (Xu and Dashwood 1999, Schut and Yao 2000). However, several epidemiological reviews and meta-analysis of human data suggest there is very weak evidence that green tea can protect against CRC (Arab and Il'yasova 2003, Sun et al. 2006, Wang et al. 2012, Wang et al. 2012). Recently a large prospective study in

Japan (The JPHC Study**)** with over 140 000 participants found that green tea had no significant effect on CRC at 5 and 10 year follow up after diagnosis, but other food components including fish and soy could reduce CRC risk (Tsugane and Sawada 2014). Trying to identify a link between CRC and green tea consumption in humans is difficult, partly due to heterogeneity amongst populations, differences in bioactivity of the green tea used, genetic diversity of the populations being studied, as well as the possibility that sex hormones might also influence the effectiveness of green tea use on CRC prevention (Wang et al. 2012). Overall, green tea shows promise as a natural compound that might slow or prevent chemically induced CRC via several different mechanisms.

There is some evidence suggesting green tea could protect against the alkylating capacity of red meat. Phenolic compounds in green tea can act to inhibit N-nitroso compounds (NOCs) formation both *in vitro* and in humans (Wu et al. 1993). Excretion of NOCs in the urine of human volunteers consuming nitrate in the drinking water was significantly decreased after consumption green tea (Stich 1992, Vermeer et al. 1999). However, higher intakes of green tea (up to 8 cups or 4g equivalent) actually increased NOC formation, suggesting that high doses of phenolic compounds might catalyse nitrosation in the gut, thereby increasing NOC output (Vermeer et al. 1999). To date, one study has examined red meat in combination with tea consumption (but not green tea), measuring NOCs excretion in the faeces from human volunteers. Hughes et al (2002) found that supplementing a 420g/day red meat diet with 3g/day of freeze dried tea extract could reduce excretion of nitrite in the faeces compared to a red meat only or red meat with vegetable diet, but did not reduce total faecal NOC excretion (Hughes et al. 2002). There have been no studies in rodents identifying the effects of red meat in combination with green

tea. Preliminary data in rodents shows 0.5% green tea in a standard diet can up-regulate MGMT activity in the colon (Hu et al. unpublished). It might be plausible that green tea could protect against alkylating effects of red meat not only by sequestering NOCs but also by supressing DNA alkylating adducts via enhancing MGMT activity.

7.2. Aims and hypotheses

Red meat and the chemical carcinogen AOM can induce alkyl adducts in normal mice, with AOM adducts being directly linked to colorectal oncogenesis in rodents. Green tea is thought to protect against genetic damage via up-regulation of *MGMT* and apoptosis in response to alkylating carcinogens. The hypotheses are that red meat consumption and azoxymethane exposure will increase O^6MeG adduct formation in the colon of mice and that loss of MGMT activity will enhance this adduct accumulation further. Also, that green tea consumption will protect against adduct accumulation (by red meat and AOM) and enhance the acute apoptotic response to AOM in the colon of mice, as a direct consequence of *MGMT* regulation by green tea.

This chapter aims to:

1. Identify if green tea alone, or in combination with red meat, could protect against O^6MeG adduct formation and/or enhance the acute apoptotic response to an alkylating carcinogen in the colon;

2. Determine if green tea alone or in combination with red meat can lower O^6MeG adducts in the colon cells via regulation of *MGMT*.

7.3. Materials and methods

7.3.1. Animals and diets

48 male and 48 female c57bl6/J mice at 4-5 weeks of age and of similar weight were purchased from Adelaide University in South Australia. 96 male and female *MGMT*$^{-/-}$ mice were purchased from School of Medicine Animal Facility at Flinders University at 4-5 weeks of age. All experiments were carried out at School of Medicine Animal Facility, Flinders University. Mice were acclimatised on a standard rodent chow for a period of one week. After recording bodyweights all mice were placed into cages according to gender, with a maximum of 5 per cage. They were randomly divided into 4 dietary groups (n=12 males and n=12 females per diet) and the feeding experiment was strictly under controlled conditions of 22±2°C (SD), 80±10% humidity, and 12 h light/dark cycle. Mice were fed ad libitum and weighed once weekly throughout the experiment. The Flinders University of South Australia Animal Welfare Committee approved all experimental procedures (ethics approval number 809/12) and were carried out according to the Australian code for the care and use of animals for scientific purposes. Diets were based on the American Institute of Nutrition (AIN) diet AIN-76 with some modifications (Table 7.1). A total of 4 dietary interventions were investigated: Control, red meat, green tea and red meat + green tea, and were prepared as detailed in Chapter 2.3.

Table 7.1: Composition of experimental diets (g/100g of diet)

Ingredients	Control (C)	Green Tea (GT)	Red Meat (RM)	Red Meat + Green Tea (RM+GT)
Casein	20	20	0	0
Red Meat	0	0	25	25
Green Tea[b]	0	0.5	0	0.5
Corn starch	40.94	40.44	38.98	38.48
Sugar	10.95	10.95	10.95	10.95
Sunflower Seed oil	18	18	18	18
Lard[c]	3.04	3.04	0	0
α-cellulose	2	2	2	2
L-cysteine	0.3	0.3	0.3	0.3
Choline	0.12	0.12	0.12	0.12
Minerals[d]	3.5	3.5	3.5	3.5
Vitamins[d]	1	1	1	1
DL-Methionine	0.15	0.15	0.15	0.15

[a]Red Meat dry powder was added with its own moisture content
[b]Polyphenon 60 was used as the extract source from green tea
[c]Lard was added to the control diets to balance for saturated fat content of the red meat
[d]AIN-76 standard vitamin and mineral mixtures (Appendix B)

7.3.2. Experimental procedures and specimen collection

After 4 weeks of dietary intervention, half of the mice from each diet group (n=6 males and n=6 females) were injected with a 2mg/ml solution of azoxymethane (AOM) diluted in 0.9% saline to give a final dose of 10mg/kg bodyweight. The other half of the mice (n=6 males and n=6 females) received vehicle treatment only. After 6 hours all mice were euthanised via carbon dioxide asphyxiation and cervical dislocation. Approximately 10mm of distal colorectal tissue was resected, cleared of excess faecal material and placed into 10% buffered formalin solution containing 3.6% formaldehyde for 24 hours and transferred to 70% ethanol for storage. Samples were then cut into 5mm cross section pieces before being processed for immunohistochemical analyses as outlined in Chapter 2.5.1. Evaluation of apoptosis, Ki67 and DNA O^6MeG adducts were quantified in the distal colorectal tissue as outlined in Chapter 2.5.3, 2.5.4 and 2.5.5, respectively. All slides were randomly

coded and counted by a single observer. For details of statistical analyses refer to Chapter 2.7

7.4 Results

7.4.1. General observations

All mice gained weight at a steady rate over the 4 weeks of dietary interventions with the exception of one female *MGMT*$^{-/-}$ mouse which did not thrive and was euthanised after one week. As expected, final bodyweights for female mice were lower than their male counterparts ($p<0.001$) and *MGMT*$^{-/-}$ mice weighed less than wild type mice ($p<0.001$) (Table 7.2), which is a typical phenotypic trait of *MGMT* null mice (Tsuzuki et al. 1996). Final bodyweights of mice consuming green tea diets were significantly lower compared to mice on non-green tea diets (Table 7.2; $p<0.00001$). Significant 2-way and 3-way interactions were observed for green tea consumption, gender and genotype on final bodyweights (Table 7.3). Female mice consuming green tea and deficient in *MGMT* weighed significantly less than their wild type male counterparts consuming non-green tea diets ($p<0.001$).

7.4.2. Colonic O^6MeG adducts

Treatment with AOM resulted in a significant increase in DNA O^6MeG adduct accumulation in the distal colon (Table 7.2; $p<0.00001$). Dietary exposure to red meat also generated significant O^6MeG adducts in the distal colon compared to diets without red meat ($p<0.05$), however, green tea supplementation did not reduce adduct load either alone or in combination with red meat (Table 7.2) . As anticipated *MGMT*$^{-/-}$ mice had significantly more O^6MeG adducts compared to wild type mice ($p<0.001$), and treatment with AOM in *MGMT*$^{-/-}$ mice resulted in significantly more

adducts compared to wild type mice that were also treated with AOM (Figure 7.1A). There was a trend for female mice to have more O^6MeG adducts than their male counterparts ($p=0.06$). In particular, wild type female mice had more adducts than male wild type mice, and loss of *MGMT* only generated significant increases of colonic adducts in male *MGMT*$^{-/-}$ mice, with no change in overall adduct load of female *MGMT*$^{-/-}$ mice ($p<0.05$; Figure 7.1B).

7.4.3. The acute apoptotic response to AOM

AOM treatment significantly enhanced the acute apoptotic response in distal colonocytes compared to mice without treatment ($p<0.00001$). Somewhat unexpectedly, *MGMT*$^{-/-}$ mice showed less apoptotic cells compared to wild type mice ($p<0.01$), especially in response to AOM treatment ($p<0.00001$; exposure ($p<0.00001$; Figure 7.2B). Green tea also supressed the acute apoptotic response after AOM in the distal colon (Figure 7.2B; $p<0.05$). Furthermore, female mice showed higher rates of apoptosis compared to males ($p<0.05$), particularly after AOM treatment ($p<0.00001$; Figure 7.2C). There was no effect of red meat consumption on apoptosis, either with or without AOM treatment, and there was no interaction of red meat with green tea on apoptosis.

Table 7.2: Bodyweight and distal colon epithelial measurements according to AOM treatment, green tea or red meat consumption, gender and *MGMT* status

	Treatment Effect		**Green Tea Effect**		**Red Meat Effect**		**Gender Effect**		**Genotype Effect**	
	Vehicle	*AOM*	*No GT*	*GT*	*No RM*	*RM*	*Male*	*Female*	*WT*	*MGMT*$^{-/-}$
Bodyweight	23.90 ± 0.49	25.0 ± 0.46	26.89	21.94	24.57 ± 0.53	24.28 ± 0.40	25.58 ± 0.40	23.27 ± 0.52	25.26 ± 0.53	23.59 ± 0.41
p value	<0.05		<0.00001		0.660		<0.0001		<0.001	
O^{6}MeG Staining Intensity	0.30 ± 0.14	2.07 ± 0.14	1.17 ± 0.14	1.22 ± 0.19	0.97 ± 0.17	1.43 ± 0.16	1.01 ± 0.18	1.39 ± 0.15	0.88 ± 0.14	1.53 ± 0.19
p value	<0.00001		0.597		<0.05		0.06		<0.001	
Apoptosis per Crypt	0.05 ± 0.01	0.65 ± 0.04	0.44 ± 0.05	0.26 ± 0.03	0.33 ± 0.04	0.37 ± 0.05	0.26 ± 0.03	0.44 ± 0.05	0.43 ± 0.05	0.27 ± 0.03
p value	<0.00001		<0.01		0.512		<0.01		<0.01	
Positive ki-67 per crypt	6.02 ± 0.13	6.35 ± 0.19	5.88 ± 0.14	6.51 ± 0.18	6.17 ± 0.16	6.21 ± 0.17	6.09 ± 0.15	6.28 ± 0.18	6.16 ± 0.17	6.21 ± 0.16
p value	0.165		<0.01		0.867		0.443		0.851	
Crypt Height	21.29 ± 0.15	20.82 ± 0.14	21.12 ± 0.16	21.03 ± 0.12	21.03 ± 0.15	21.57 ± 0.14	20.97 ± 0.14	21.14 ± 0.16	20.47 ± 0.12	21.64 ± 0.15
p value	<0.05		0.491		0.837		0.361		<0.00001	

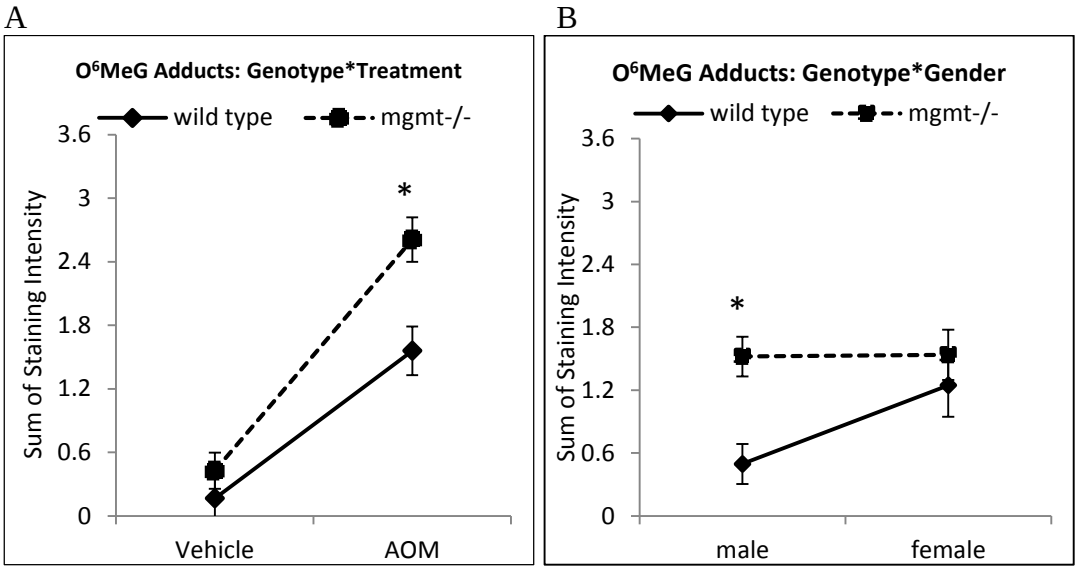
Univariate analysis of Variance test of between-subject effects for DNA O^{6}MeG adducts, apoptosis and proliferation. Significant values are highlighted.

Table 7.3: Statistical outcomes of 2- way and 3- way interactions for bodyweight, O^6MeG DNA adduct staining intensity and apoptosis according to AOM treatment, green tea or red meat consumption, gender and *MGMT* status

Bodyweight	**2-way**	**Treatment* Genotype**	**Treatment* Gender**	**Treatment* Green Tea**	**Gender* Genotype**	**Green Tea* Genotype**	**Green Tea* Gender**
	p value	<0.05	0.327	0.053	<0.01	<0.00001	0.053
	3-way	**Treatment*Gender* Genotype**	**Treatment*Green Tea* Genotype**		**Treatment*Green Tea* Gender**		**Gender*Genotype* Green Tea**
	p value	0.086	0.162		0.251		<0.001
O^6MeG DNA Adducts	**2-way**	**Treatment* Genotype**	**Treatment* Gender**	**Treatment* Red Meat**	**Gender* Genotype**	**Red Meat* Genotype**	**Red Meat* Gender**
	p value	<0.05	*0.477*	*0.174*	<0.05	*0.581*	*0.194*
	3-way	**Treatment*Gender*Genotype**					
	p value	*0.153*					
Apoptosis	**2-way**	**Treatment* Genotype**	**Treatment* Gender**	**Treatment* Green Tea**	**Gender* Genotype**	**Green Tea* Genotype**	**Green Tea* Gender**
	p value	<0.00001	<0.00001	<0.00001	*0.504*	*0.746*	*0.651*
	3-way	**Treatment*Gender* Genotype**		**Treatment*Green Tea* Genotype**		**Treatment*Green Tea* Gender**	
	p value	*0.610*		*0.469*		*0.383*	

Step wise Univariate analysis of Variance test of between-subject interactions for DNA O^6MeG adducts and apoptosis. Significant values are highlighted.

Figure 7.1: Interaction analysis of O^6MeG adducts in the colon

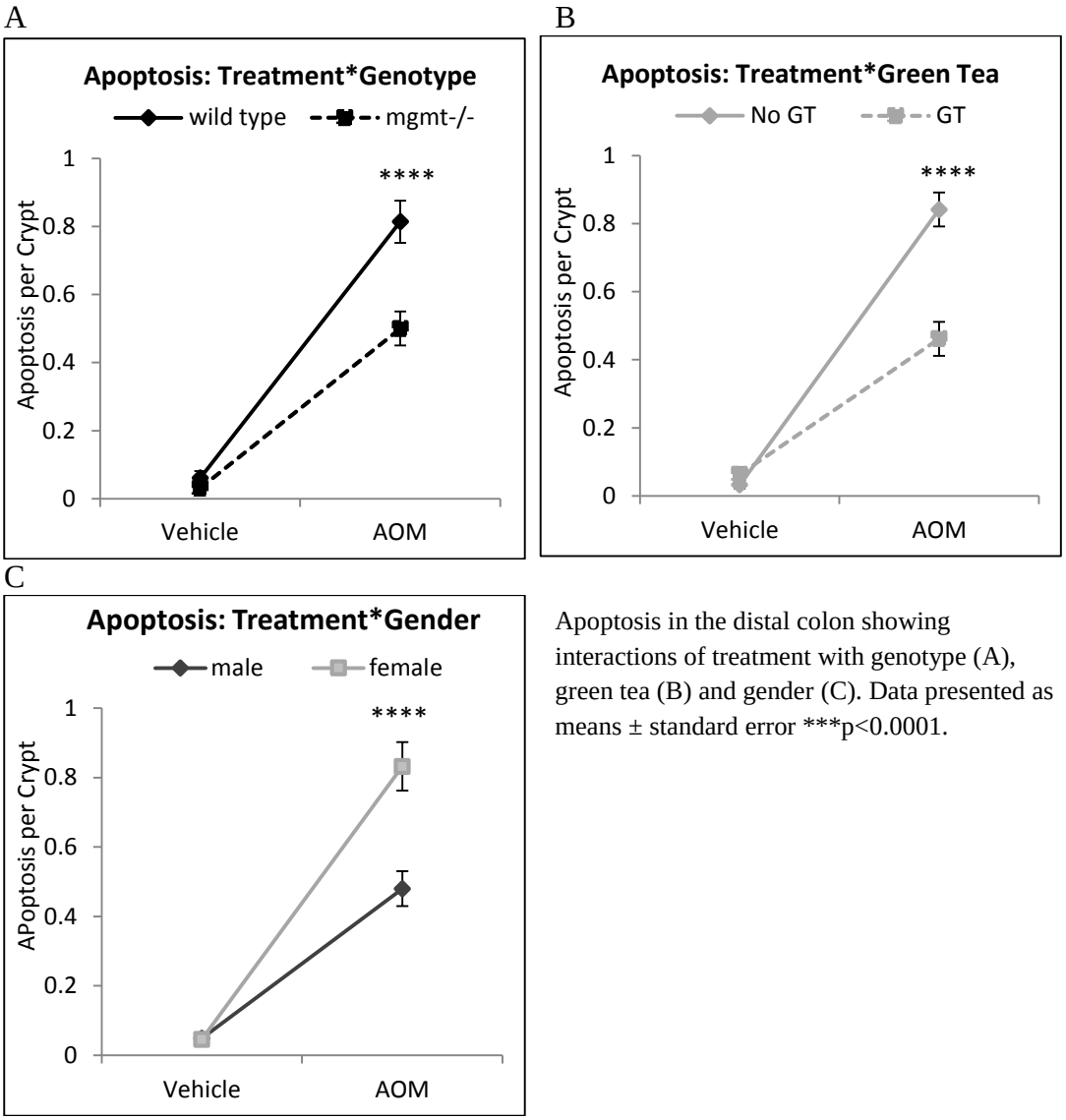


O^6MeG adduct accumulation in the distal colon showing interactions for genotype and treatment (A) as well as genotype and gender (B). Data presented as means ± standard error *p<0.05

7.4.4. Crypt height and cell proliferation

Crypt heights in the distal colon significantly reduced 6 hours after AOM treatment compared to mice that did not receive any AOM (Table 7.2; $p<0.05$), which can largely be attributed to the high rate of apoptosis. Loss of MGMT activity generated significant increases in crypt height compared to wild type mice ($p<0.00001$), this was not attributed to enhanced proliferation in *MGMT*$^{-/-}$ mice ($p=0.851$) and likely was a consequence of less cells undergoing apoptosis in response to AOM. Green tea consumption generated a significant increase in proliferative cells compared to diets without green tea ($p<0.01$), although this did not result in a larger cell mass even with a supressed apoptotic response to AOM (Table 7.2). There were no significant 2- or 3-way interactions of cell proliferation or crypt height as a result of AOM treatment or dietary exposures (Table 7.3).

Figure 7.2: Interactions analysis of apoptosis in the distal colon



Apoptosis in the distal colon showing interactions of treatment with genotype (A), green tea (B) and gender (C). Data presented as means ± standard error ***p<0.0001.

7.5. Discussion

Contrary to the original hypotheses, green tea did not protect against red meat-induced or AOM-induced adduct formation in the distal colon, nor did it enhance the acute apoptotic response to AOM. Indeed, green tea supressed apoptosis after AOM exposure and enhanced proliferation of colorectal cells. However, this study does confirm the hypothesis that red meat, AOM exposure and *MGMT* deficiency all enhance the formation of O^6MeG adducts in distal colon cells.

Although there is a lot of evidence showing green tea has an abundance of anti-cancer properties, there is also suggestion green tea can promote colorectal oncogenesis. The findings described in this chapter showed that 0.5% of green tea in the diet as polyphenol extract can supress apoptosis and enhance proliferation of distal colon cells. Others have shown that similar doses of green tea (0.5%-1%) can increase inflammatory markers and enhance chemically induced and colitis-associated colorectal carcinoma in rodents, whereas low doses (0.01-0.1%) can reduce inflammatory markers and protect against subsequent CRC development (Hirose et al. 2001, Kim et al. 2010). ECGC from green tea can also enhance reactive oxygen species in the HT29 CRC cell line (Kim et al. 2005), increase cox-2 expression in macrophage cells (Park et al. 2001) and increase oxidative DNA adducts in leukaemia cells (Furukawa et al. 2003), all of which can lead to changes in apoptosis and proliferation. These data suggest green tea at 0.5% or more in the diet, with or without red meat, could potentially increase the risk of oncogenesis by creating an imbalance of cell turnover in the colonic epithelium. Therefore, longer term tumour studies would need to be undertaken to better understand how varying doses of green tea, with or without red meat, might affect CRC risk.

The results in this chapter showed that loss of *MGMT* can increase O^6MeG adduct staining in colorectal cells and supress the acute apoptotic response to an alkylating carcinogen 6 hours after exposure. MGMT directly repairs O^6MeG adducts quickly and efficiently (Margison et al. 2002) with saturation of the enzyme occurring within 2 hours after AOM exposure (Nyskohus et al. 2013), with the alkylated form of MGMT inactivated and targeted for ubiquitous degradation (Povey et al. 2002). Previous evidence suggests that by 24 and 48 hours post AOM, *MGMT* deficient mice display much higher apoptosis rates in the distal colon compared to

wild type mice, due to mismatch repair (MMR) and base excision repair (BER) triggered apoptosis (Bugni et al. 2009, Wirtz et al. 2010). Therefore, the anti-apoptotic nature of *MGMT*$^{-/-}$ mice shown here was not anticipated. The dose of AOM used was the same in all studies at 10mg/kg of bodyweight, the different response to AOM at earlier time points without *MGMT* functioning is difficult to interpret. Although alkyl repair is its main responsibility, the MGMT protein has been suggested to play a role as a transcription regulator of estrogen-induced proliferation in breast cancer cell lines (Teo et al. 2001). This evidence was later disputed by Bungi et al (2007) who showed there was no physiological interaction of MGMT with the estrogen receptor. In the current study, loss of *MGMT* significantly enhanced O^6MeG adduct formation at 6 hours post AOM but significantly supressed the apoptotic response. This indicates that MGMT, or alkylated-MGMT, may directly affect the acute apoptotic response to AOM (independent of O^6MeG accumulation). It is unclear how MGMT would regulate apoptosis and more studies at various time points post AOM exposure are required to validate these findings.

Female mice displayed a greater apoptosis response to AOM than male mice. A likely reason for these changes is the influence of estrogen on the epithelial cells. *In vitro* estrogen exposure can induce apoptosis in COLO-205 colon cells, which was linked to suppression of the anti-apoptotic protein bcl-2 (Wilkins et al. 2010). Ovariectomised female rats given oestradiol had higher rates of apoptosis 12 hours after AOM exposure (Armstrong et al. 2011). Female rats have significantly less ACF formation than male rats several weeks after exposure to chemical carcinogens (Ochiai et al. 1996, Weige et al. 2009), and colorectal tumours are significantly reduced with estrogen treatment, even without a functioning estrogen receptor (Guo et al. 2004). Human data also provide evidence that females have lower rates of CRC

than men, and have a substantially decreased risk after prolonged hormone replacement therapy post-menopause (Calle et al. 1995). The data presented here support previous evidence that female mice have an enhanced apoptotic response to carcinogens. This pro-apoptotic nature of estrogen in women could be partly responsible for their lower CRC rates compared to men.

This chapter highlights the inability of 0.5% green tea to act as a protective food component against alkylating carcinogens in the colon, whether of chemical or dietary sources. It also presents evidence that MGMT may have other regulatory effects on the acute apoptotic response to carcinogens other than alkyl repair. Red meat can generate alkyl adducts, but not at doses high enough to induce apoptosis, suggesting these lesions might be a biomarker of NOC exposure in the colon and not necessarily a biomarker of oncogenesis. However, it is important to note that red meat is a "chronic" inducer of these adducts, whereas AOM is an "acute" inducer. Therefore, the cell has sufficient time to develop inherent responses to red meat-induced DNA adducts, which might explain why we don't see the same effect for red meat as we do for AOM exposure. It might also suggest that other factors of red meat including saturated fat, mutagen formation and/or higher intakes of red meat over longer periods of time might be required to drive CRC, and not simply exposure to NOCs alone. The complex interactions of genetic, dietary and gender factors on cellular responses within the colon highlight the multifaceted nature of CRC and the various ways dietary components can either initiate or protect against genetic damage, which might lead to genomic instability.

Chapter 8

Cellular responses to azoxymethane in c57Bl6/J and O^6-methylguanine DNA-methyltransferase (*MGMT*) deficient mice

8.1. Introduction

Use of the chemical carcinogen AOM in rodent models of CRC has been studied for many years. AOM is an alkylating agent that is hydroxlyated in the liver to methylazoxymethanol (MAM) (Fiala 1977), a highly reactive and potent alkylating agent (Riggs 1965). This alkylating potential of AOM is responsible for the initiation of colorectal carcinogenesis. The pro-mutagenic adduct O^6MeG is one form of alkylating adduct that is generated in rodent colonic epithelial cells in response to AOM. This pro-mutagenic alkyl lesion is repaired directly by MGMT in a rapid reaction that does not interrupt the DNA replication fork (Povey et al. 2002). Unrepaired lesions can lead to induction of apoptosis after MGMT activity is overwhelmed in response to AOM exposure (Nyskohus et al. 2013). Apoptosis occurs via the mismatch repair (MMR) or base excision repair (BER) pathway, which can remove un-repaired lesions after 2 rounds of cell cycles (post 48 hours) (Margison et al. 2002). However, if these lesions still persist they can form GC→AT transition mutations in proto-oncogenes such as *K-ras* and trigger the oncogenesis pathway (Jackson et al. 1997).

The MGMT repair protein is an importance defence mechanism for colorectal cells against CRC. Inhibition of MGMT activity in mice after treatment with benzylguanine (a chemical that binds active MGMT) can significantly increase tumour formation in mice exposed to AOM (Wali et al. 1999). Similarly, genetically modified mice lacking in *MGMT* are susceptible to ACF and tumours of the colon after exposure to AOM (Bugni et al. 2009, Wirtz et al. 2010), and are particularly sensitive to the lethality of alkylating agents (Glassner et al. 1999). This lethality is rescued by loss of MMR genes such as *MLH1*. The *MGMT/MLH1* double knockout mouse enables survival after AOM treatment but as a consequence they develop substantially more colorectal tumours than *MGMT* or *MLH1* single knockouts alone (Kawate et al. 1998). With loss of MGMT function, highly damaged cells can be removed from the system thorough MMR or BER induced apoptosis once the cell recognises unrepaired alkyl lesions. Indeed, at 24 and 48 post AOM exposure $MGMT^{-/-}$ mice have significantly higher rates of apoptosis compared to wild type mice (Bugni et al. 2009). However, in Chapter 7 of this thesis it was suggested MGMT could potentially be regulating apoptosis directly in response to AOM at the earlier time point of 6 hours. It is possible that the acute apoptotic response to AOM may be different compared to wild type mice due to the potentially pro-apoptotic nature of the MGMT protein.

In rats, MGMT activity is saturated as early as 2 hours post AOM, with peak adduct load within 6 hours post AOM (Nyskohus et al. 2013) and the peak apoptotic response shortly after at 8 hours (Nyskohus et al. 2013, Nasuno et al. 2014). Suppression of the acute apoptotic response has been linked to a suppression of colorectal tumour formation in rats (Nasuno et al. 2014) and mice in AOM-induced CRC models (Hu et al. 2008). The innate cellular response to AOM exposure over

time has been studied in detail within the rat colorectal epithelium (Nyskohus et al. 2013) but there is no detail of the response in mice. Indeed, mice and rats have different rates of tumours in the colon after exposure to AOM. The incidence and multiplicity of tumours in rats after a total dose of 30mg/kg of AOM is approximately 0.73 tumours per rat (Le Leu et al. 2007, Le Leu et al. 2010). In mice, the same dose only gives 0.57 tumours per mouse (Hu et al. 2008). When the total dose of AOM in mice is doubled to 60mg/kg, 0.8 tumours per mouse was observed (Hu et al. 2005), which is similar to the 0.73 tumours observed per rat given 30mg/kg of AOM. This highlights the importance of understanding how colon cells respond to exposure of alkylating carcinogens within different rodent models. This becomes particularly crucial when choosing rodent models for studying the mechanism of chemo-preventative agents in colorectal oncogenesis.

8.2. Aims and hypotheses

Apoptosis and DNA adduct accumulation have been shown to peak around 6-8 hours post AOM in rats with MGMT activity recovering a week after exposure. Wild type mice also present with high levels of DNA alkylation around 6 hours post AOM. *MGMT* deficient mice have previously been shown to suppress apoptosis at 6 hours post AOM (Chapter 7) but enhance apoptosis 24-48 hours post AOM (Bugni et al. 2009), with effects at later time-points unknown. We aimed to examine the time course of DNA adduct formation and apoptotic response after AOM exposure in normal and *MGMT* deficient mice, to understand how mice respond to alkylating carcinogen-induced DNA damage. The hypotheses tested here are that early time points post AOM (4-8 hours), *MGMT* deficient mice will have a lowered apoptotic response to AOM but more O^6MeG adducts compared to wild type mice, whereas at later time points (24-48 hours) *MGMT* deficient mice will have more apoptosis and

higher O^6MeG adducts compared to wild type mice. Also, peak apoptosis will occur at 8 hours post AOM for wild type mice and 48 hours for *MGMT*$^{-/-}$ mice. The specific aims of this chapter are:

1. To establish the role of MGMT activity in colonic cellular DNA damage responses of apoptosis, proliferation and O^6MeG adducts at early and late time points after exposure to the alkylating carcinogen AOM.
2. Identify the peak DNA damage responses of apoptosis, proliferation and O^6MeG adducts in wild type and *MGMT* deficient mice
3. Examine the pattern of DNA adduct formation along the crypt axis to asses cellular responses to AOM in mouse proliferative and more differentiated cells

8.3. Materials and methods

8.3.1. Animals, study design and azoxymethane treatment

A total of 72 male and 72 female c57Bl6/J mice at 4 weeks of age and of similar weight were purchased from the School of Medicine Animal Facility at Flinders University. They were acclimatised on standard rodent chow for 2 weeks, followed by 2 weeks on standard AIN76 diet formula (Appendix B and Chapter 2.3) for a further 2 weeks. Bodyweights were recorded weekly and immediately prior to AOM injections. A total of 6 time-points post AOM exposure were chosen to study the acute apoptotic responses at 4 and 8 hours, as well as the later apoptotic responses at 24, 48 and 72 hours. The chemical carcinogen AOM was purchased from Sigma and diluted in a 0.9% solution of saline to a concentration of 2mg/ml. Each mouse received a single i.p. injection to the abdomen at a final dose of 10mg/kg of bodyweight, except for the 0 hour time-point where the mice received vehicle

treatment only. All injections were carried out between the hours of 8-10am on any given day.

8.3.2. Immunohistochemical analysis of the distal colon

At each time point, 6 males and 6 females were euthanised by carbon dioxide asphyxiation and cervical dislocation. A 10mm segment of distal colorectal tissue was resected and processed as outlined in section 2.5.1. Cellular proliferation of the colon was assayed using a monoclonal Anti-Ki67 marker (AbCam SP6) in combination with a biotin labelled detection kit (Signet) as described in Chapter 2.5.4. Positive Ki67 cells were labelled with DAB chromagen and counted as positive cells per crypt. Apoptosis was analysed (Chapter 2.5.3) under a light microscope by Harris Haematoxylin stain and counted as positive cells per crypt displaying morphological characteristics indicative of apoptosis including cell shrinkage, condensed chromatin and appearance of apoptotic bodies within the nucleus. O^6MeG adduct staining was quantified using a monoclonal antibody against the O^6MeG lesion (clone EM 2-3; Squarix Biotechnology) in combination with a polymer linking kit and computer image analysis program, as detailed in section 2.5.5. All slides were first randomly coded and counted blind before de-coding for statistical analysis of the data (Chapter 2.7).

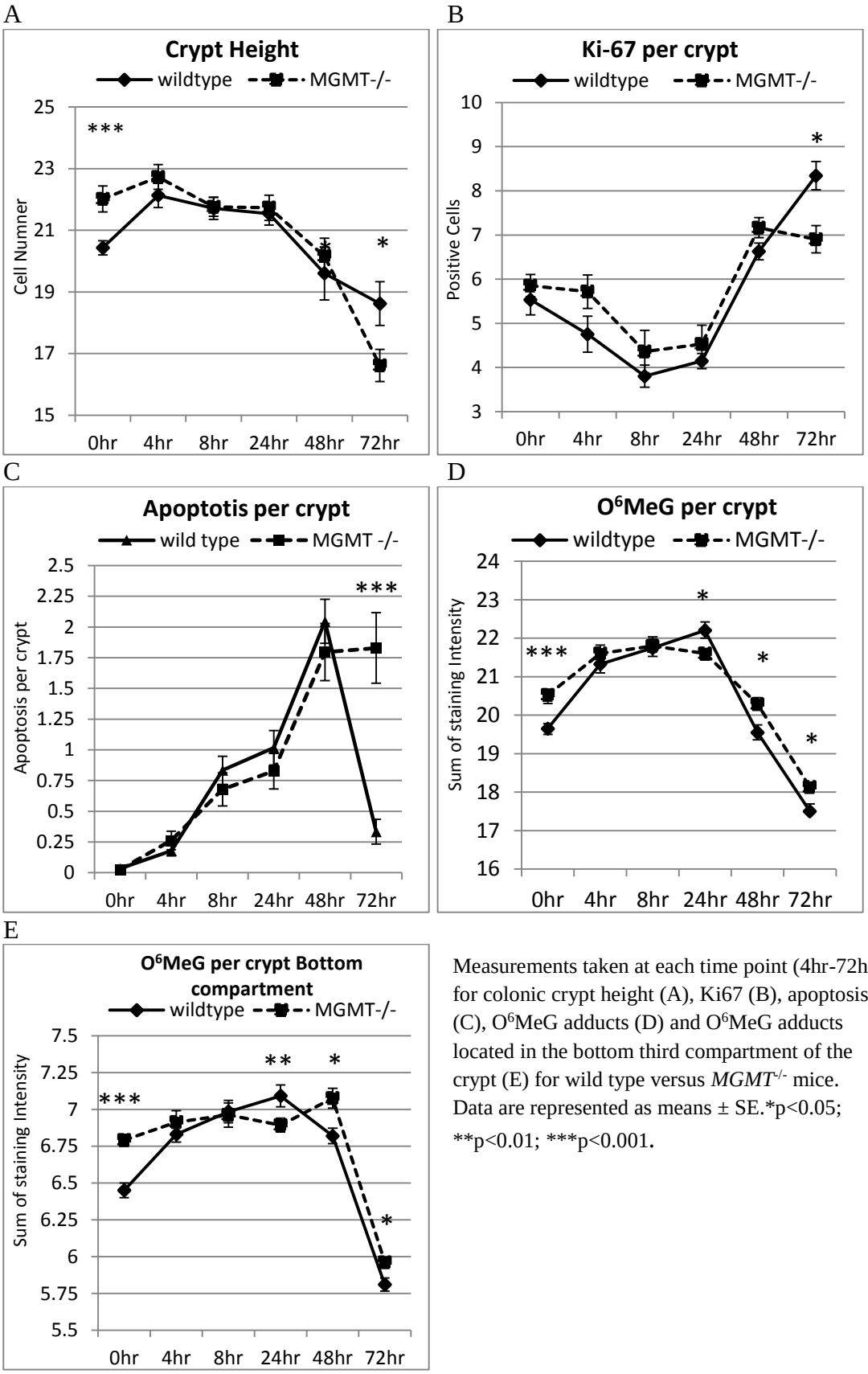
8.4. Results

8.4.1. Wild type versus $MGMT^{-/-}$ responses to AOM over time

Data for crypt height, cell proliferation, apoptosis and DNA O^6MeG adducts for wild type versus *$MGMT^{-/-}$* mice are presented in Figure 8.1 A-E. Before AOM exposure, *$MGMT^{-/-}$* mice have more cells per crypt compared to normal mice ($p<0.0001$), but

the proliferation rates were no different (Figure 8.1A and 8.1B). Over time, the drop in crypt height and proliferation after AOM exposure was similar at every time point except 72 hours where *MGMT*$^{-/-}$ mice had significantly less cell per crypt than wild type mice and a lower rate of proliferation ($p<0.05$). The acute apoptotic response to AOM was not significantly different in *MGMT*$^{-/-}$ mice compared to wild type mice (Figure 8.1C). In both mouse strains, apoptosis peaked at 48 hours. However, at 72 hours apoptosis in wild type mice dropped substantially but *MGMT*$^{-/-}$ mice retained peak apoptosis rates ($p<0.0001$). Before AOM treatment adduct levels (Figure 8.1 D&E) were significantly higher in *MGMT*$^{-/-}$ mice compared to their wild type counterparts ($p<0.0001$). DNA O^6MeG adduct accumulation was slightly dampened in *MGMT*$^{-/-}$ mice 4-24hours post AOM and was significantly lower than wild type mice at 24 hours post AOM ($p<0.05$). However, at 48 hours O^6MeG adducts began to drop sharply in wild type mice. A drop in adducts for *MGMT*$^{-/-}$ mice was also observed, although levels were still higher in *MGMT*$^{-/-}$ mice compared to normal mice at both 48 and 72 hours post AOM ($p<0.05$). Changes in adduct load over time within the base crypt compartment was very similar to the whole crypt changes (Figure 8.1E), with the dampened effect of adduct load more prominent in the stem cell region at 24 hours in *MGMT*$^{-/-}$ mice compared to wild type mice ($p<0.01$).

Figure 8.1: Colonic epithelial responses to AOM over time in wild type and *MGMT* deficient mice

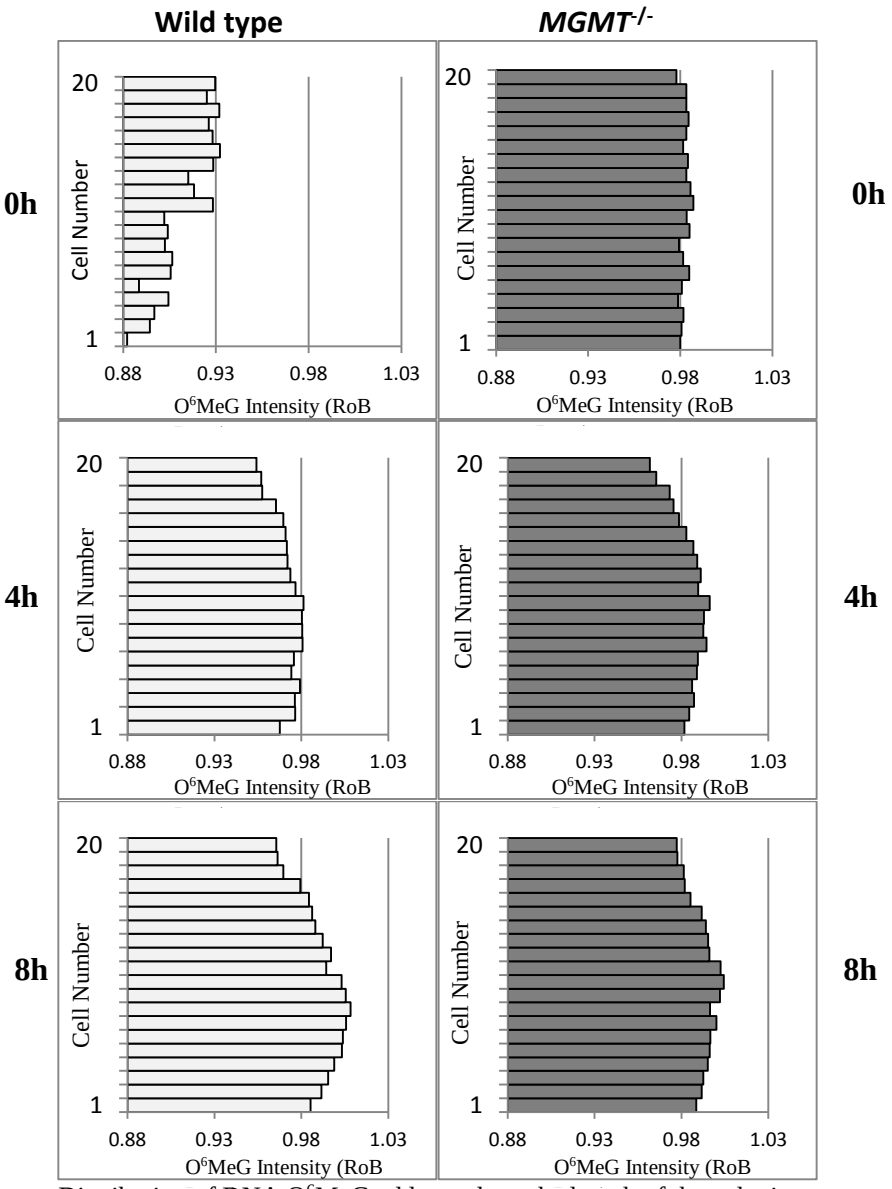


Measurements taken at each time point (4hr-72hr) for colonic crypt height (A), Ki67 (B), apoptosis (C), O^6MeG adducts (D) and O^6MeG adducts located in the bottom third compartment of the crypt (E) for wild type versus *MGMT*$^{-/-}$ mice. Data are represented as means ± SE.*p<0.05; **p<0.01; ***p<0.001.

8.4.2. Distribution of O^6MeG adducts along the crypt

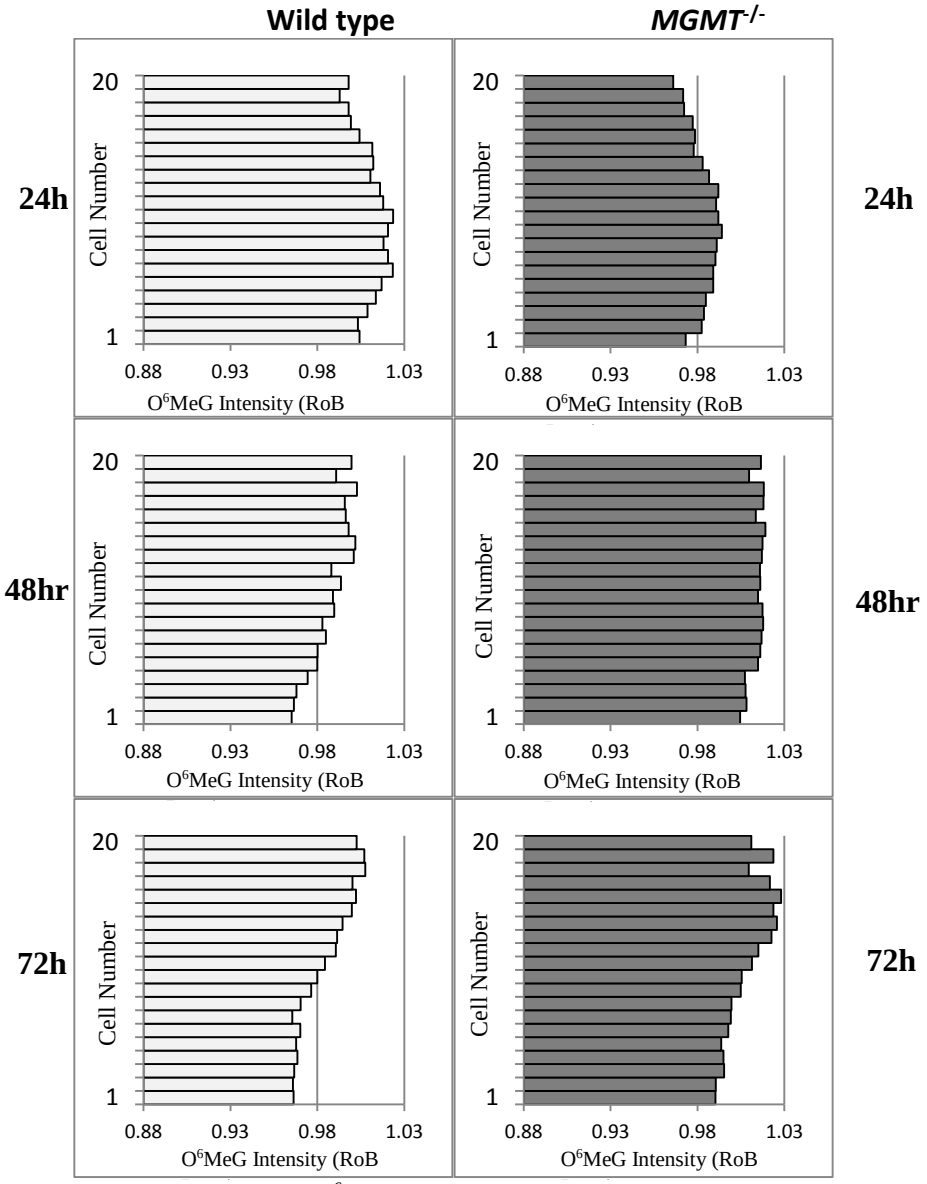
The distribution of adducts along the length of the crypt after AOM exposure over 0-8hour is presented in Figure 8.2 and over 24-72 hours in Figure 8.3. In normal mice at baseline (0 hour), adduct intensity increased from the base of the crypt (cell number 1) to the top of the crypt (cell number 20), whereas *MGMT* deficient mice had equal amounts of staining along the entire crypt length. As indicated previously in Figure 8.1, *MGMT*$^{-/-}$ mice had more adducts than wild type mice at baseline, and this was evident across the entire crypt (Figure 8.2). At 4 hours post AOM exposure, O^6MeG DNA adducts generated rapidly in all cell compartments for wild type mice, particularly at the base of the crypt, and this pattern continued until the adducts peaked at 24 hours. From 48 to 72 hours in wild type mice, adducts cleared from the base of the crypt as the cells regenerate and propagate toward the lumen. In *MGMT*$^{-/-}$ mice the distribution of adducts 4 hours after AOM is very similar to wild type mice, but diverges at 48 hours where a rise in adduct staining was observed in all cells along the crypt not just at the luminal surface. There is little clearance of adducts in the base compartment of *MGMT*$^{-/-}$ mice at 48 hours. At 72 hours post AOM adduct levels are begin to recover at the bottom of the crypt, although levels are still higher than wild type mice.

Figure 8.2: Distribution of O^{6}MeG Adducts along the length of the colonic crypt at 0, 4 and 8 hours post AOM



Distribution of DNA O^{6}MeG adducts along the length of the colonic crypt in wild type and *MGMT*$^{-/-}$ mice at 0, 4 and 8 hours.

Figure 8.3: Distribution of O^{6}MeG Adducts along the length of the colonic crypt at 24, 48 and 72 hours post AOM



Distribution of DNA O^{6}MeG adducts along the length of the colonic crypt in wild type and *MGMT*$^{-/-}$ mice at 24, 48 and 72 hours.

8.5. Discussion

Contrary to the hypothesis and in disagreement with the established literature, *MGMT* knockout mice displayed a similar apoptotic response to AOM to that of the WT mice between 0 and 48 hours post AOM. Also, after 72 hours *MGMT* deficiency enhanced the apoptotic response whereas apoptosis in wild type mice began to return to levels seen at baseline. Furthermore, the peak

apoptosis in wild type mice occurred at 48 hours, much later than the 8 hours that was expected based on what has been seen in rat studies.

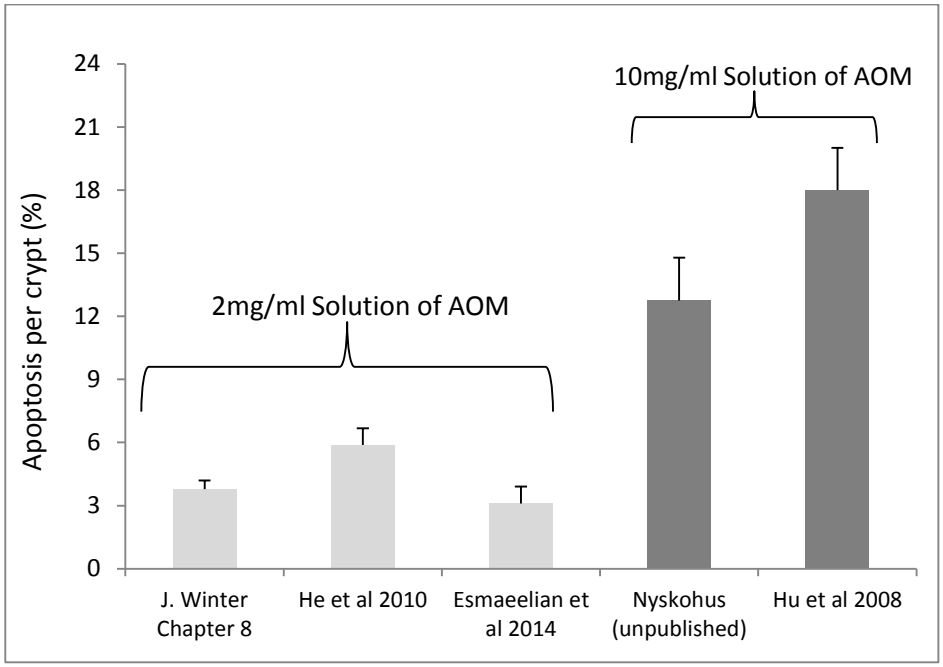
In Chapter 7 of this thesis it was shown that loss of *MGMT* could dampen the apoptotic response 6 hours post AOM. This finding has not been confirmed here, where apoptosis was not different to wild type mice between 0 and 48 hours post AOM. The difference might be explained, at least in part, by the different diets (red meat and green tea) that the *MGMT*$^{-/-}$ mice consumed in Chapter 7, and this might have some influence on the apoptotic response that was observed. In the current study, all mice were consuming a control AIN 76 diet to avoid any influence of diet on the outcome. Therefore, the current study presents a better assessment of how colon cells respond to AOM and suggests the acute apoptotic response to AOM does not require functioning *MGMT* in mice. Previously it has been suggested that the MGMT protein may regulate proliferation and therefore have the ability to regulate other cellular processes besides DNA alkyl repair (Teo et al. 2001), however, others have since disputed this claim (Bugni et al. 2007). In this chapter we found loss of *MGMT* did not change proliferation rates, nor did it change the acute apoptotic response. Therefore, the data presented in this chapter supports the notion that MGMT repairs O^6MeG adducts in the colon but does not directly affect proliferation or apoptosis.

The current study has shown that lack of MGMT repair can enhance the apoptotic response to AOM, but only after 72 hours (3 cell cycles), likely as a result of initiated MMR or BER. Previously it has been shown that *MGMT* deficiency enhances the apoptotic response to AOM as early as 24 and 48 hours (Bugni et al. 2009, Wirtz et al. 2010). The authors of these studies suggest that MMR and BER are responsible for the enhanced apoptosis at these time points.

However, if the enhanced apoptosis was due to unrepaired O^6MeG, MMR or BER induced apoptosis should not be occurring until after 48 hours post AOM, as it takes at least 2 cell cycles for the mismatched base to be recognised (Margison et al. 2002). One major discrepancy between the study in this chapter and these 2 published studies (Bugni et al. 2009, Wirtz et al. 2010) is the level of colonic apoptosis in wild type mice as well as *MGMT*$^{-/-}$ mice. The authors of those studies indicated there was almost zero apoptosis at 24 and 48 hours post AOM, whereas in the current chapter 48 hours saw peak levels of apoptosis, at 2 apoptotic cells per crypt – a significant discrepancy between studies. The final dose of AOM per kg of bodyweight used was the same in all studies, including in the current chapter, at 10mg/kg and does not explain the difference in apoptosis. However, the reason for such a large discrepancy could be several fold: 1) origin of the mouse strains used; 2) different suppliers and batches of alkylating agent being administered; 3) method of detection of apoptosis, and; 4) inter-laboratory variations. However, even taking these into consideration, the differences in apoptosis are quite substantial.

Upon further investigation, it was found that within our own laboratory (using different batches of AOM from 2 different suppliers) considerable differences in apoptosis are observed, depending on the concentration of AOM solution being used to inject the mice at the same final dose of 10mg/kg of bodyweight (Figure 8.4). Wild type mice receiving AOM diluted to a solution containing 10mg/ml can have colonic apoptosis of up to 20% (Hu et al. 2008) and 12% (Nyskohus, unpublished data) per crypt,6-8 hours after AOM treatment. Whereas mice receiving a 2mg/ml solution of AOM (as described in the current chapter) have approximately 3-5% apoptosis per crypt 6-8 hours post AOM

Figure 8.4: Apoptosis rates in the colon at 6-8h post AOM at 10mg/kg bodyweight for 5 different studies in the same laboratory



exposure (He et al. , Esmaeelian et al. 2014), a considerably lower rate of apoptosis. This suggests that the apoptotic response to alkylating carcinogens is highly dependent on the concentration of the carcinogen solution being injected into the mouse, even if the final dose per kg of bodyweight is identical. After searching the published literature extensively, no published studies reported the concentration of AOM solution administered, with only the final dose being reported. Future studies using the AOM mouse model would need to consider the concentration of the AOM administration when designing studies and analysing responses to AOM in the colon. Such differences in the acute apoptotic response could potentially have major implications on colon tumour outcome in the AOM model of CRC.

Previously, *MGMT* deficiency has shown to enhance the formation of adducts at 6 hours post AOM (Chapter 7). Therefore, it was anticipated that

MGMT deficiency would enhance the apoptotic response to AOM at each time points post AOM in this chapter. *MGMT* knockout mice did show a level of basal O^6MeG adducts higher than wild type mice. However, at 24 hours *MGMT* knockout mice had less adducts compared to wild type mice, although not sufficiently different to trigger an enhanced apoptotic response. The O^6MeG adduct load, and their distribution in the colonic crypts, over the 48 hours was not any different to wild type mice. Previously shown in rats, MGMT activity is exhausted within only 2 hours post AOM, with apoptosis being triggered in line with the peak alkylation (Nyskohus et al. 2013). In the current study it was only after 72 hours when a significant increase in adducts and subsequent apoptotic removal was observed in *MGMT*$^{-/-}$ mice, with cells still not returning to base line apoptotic levels. This enhanced apoptosis at 72 hours in *MGMT* deficient mice corresponds with the MMR and BER responding to unrepaired adducts, which only happens after at least 2 cell cycles (Margison et al. 2002). This supports previous data confirming that MMR and BER are up-regulated in *MGMT* deficient mice when exposed to alkylating carcinogens (Bugni et al. 2009, Wirtz et al. 2010). Longer time points are needed to determine when the colonic crypts have stabilised beyond 72 hours.

Other repair pathways of the cell are important factors in the apoptosis response to alkylating carcinogens, including the tumour suppressor gene *p53* (Hu et al. 2008), the base excision repair intermediate *aag* (Wirtz et al. 2010) and other proteins involved in MMR including *Msh6* (Bugni et al. 2009). Although MGMT represents an integral part of the response to alkylating carcinogens, there are several other pathways recruited that play a significant role in removal of DNA damage via the acute response to alkylating carcinogens. From the data

presented here, it is likely MGMT plays an important role in O^6MeG repair without exposure to high doses of AOM, where AOM induced adducts are not overwhelming the cell's MGMT repair capabilities. In response to AOM, MGMT is important for clearing O^6MeG adducts in the first 3 days post AOM, however, other cellular response mechanisms (likely MMR) can remove such unrepaired adducts caused by *MGMT* deficiency by triggering apoptosis.

In conclusion, the MGMT protein does not regulate the acute apoptosis response to AOM. Mice show different apoptotic responses to the carcinogen AOM than do rats, a likely explanation for the different rates of tumours observed in rat and mouse models of AOM. The peak apoptotic response to AOM in mice occurs at 48 hours, but would likely depend on the concentration of the carcinogenic agent being administered. Future studies using AOM as the initiating carcinogen in rodent CRC models should report AOM concentration administered, as this may have direct consequences on colon tumourigenesis.

Chapter 9

Discussions and conclusions

DNA alkylation of colonic epithelial cells has been proposed as a key step in initiating colorectal oncogenesis after exposure to exogenous alkylating agents. Evidence shows that high red meat diets can cause significant DNA alkylation of the colonic epithelial cells and this could be associated to increasing CRC risk associated with red meat consumption (Lewin et al. 2006, Winter et al. 2011, Vanden Bussche et al. 2014). Furthermore, consumption of dietary RS and green tea are implicated in chemo-prevention of CRC, likely through physical and physiological interactions taking place within the colonic environment (Clarke et al. 2008, Xiao et al. 2008, Le Leu et al. 2010, Du et al. 2012).

The central aim of this thesis was to determine whether dietary-induced DNA adducts by red meat consumption act as bio-markers for risk of CRC. The studies aimed to validate and extend beyond existing studies that have demonstrated red meat, and haem from red meat, as an inducer of pro-mutagenic adducts. RS and green tea were used in combination with red meat to ascertain any protective role they might have against such pro-mutagenic formation in the colon. The risk of developing CRC with high red meat and dietary haem from red meat consumption was also explored, with RS evaluated as a potentially protective CRC agent. The hypotheses of this thesis were that red meat and haem from red meat would increase DNA adducts, but that RS and green tea consumption would reduce red meat-induced DNA adducts. Also, that red meat and haem from red meat would increase

risk for developing CRC, but RS could reduce such risk posed by red meat consumption. This thesis also extrapolated pre-clinical studies of red meat and RS consumption into the human setting, to determine if dietary induced DNA alkyl adducts were also generated in human colorectal tissue after red meat feeding, and if RS could protect against such lesions. The following sections endeavour to address the questions and hypotheses posed in more detail, to bring together all the studies and give an overall perspective about dietary induced DNA adducts and CRC risk. The next section will also identify any new questions that have arisen as a consequence of the work carried out and list further research needed to address any gaps in the knowledge.

9.1 Haem and resistant starch in the Western diet mouse model.

The hypotheses addressed in this chapter were that consumption of dietary haem will increase DNA adduct accumulation in the distal colon, but that RS consumption with or without haem will reduce DNA adducts. Also, that dietary haem will increase the incidence of colonic tumours in the mouse but RS intake will reduce colon tumours both with and without haem consumption. The specific aims were as follows:

1. To ascertain if consumption of haem, as hemin chloride, will enhance accumulation of DNA O^6MeG and DNA 8-oxo adducts in the colon of mice over time, and if co-consumption with RS will reduce adduct formation in the colon.
2. Identify if dietary haem, as hemin chloride, will increase spontaneous tumour formation in the colon of mice after 18 months consuming a Western diet, and if co-consumption of haem with RS will protect against tumour formation.

3. To determine if accumulation of colonic DNA adducts are associated with spontaneous tumour formation in the colon of mice

This study identified dietary haem, consumed chronically in the diet, as an agent that can increase DNA adducts considered to be pro-mutagenic, increase cell proliferation and reduce apoptosis rates in the colon of mice. However, co-consumption of haem with RS did not reduce DNA adduct formation. RS was able to promote beneficial bacterial fermentation and reduce toxic protein fermentation in the colon of rodents over short periods, but SCFA production was not sustained over longer periods in combination with haem intake. All these changes observed had no influence on CRC risk in the animal model tested. Therefore, haem at 0.2µmol/g was not linked to increased risk of CRC, nor did RS protect. Furthermore, dietary accumulation of pro-mutagenic DNA adducts were not associated with increased risk of CRC, noting though that the agent inducing the adducts (haem) was administered chronically.

Certainly the results from this chapter have identified that adducts generated by chronic, long term haem consumption (at low levels) are not linked to CRC risk. It possible that haem may need to be present as a component of red meat and not simply as an additive of hemin chloride, as haem can catalyse the formation of more NOCs when consuming a high red meat diet (Vanden Bussche et al. 2014). This could be tested by feeding mice a Western diet with hemin chloride and feeding another group high red meat with added hemin chloride. Secondly, the result in this chapter that haem-induced DNA adducts do not increases risk of cancer could also suggest that adducts have not reached a “threshold” of DNA damage to initiate cancer. This would be simple to test by repeating the Western model of spontaneous CRC and feeding increasing doses of haem, from 0.2µmol/g and above and measure

DNA adducts and the outcome of colon tumours in each group. Further to this, the time for DNA adducts to initiate and progress to pre-cancerous lesions and cancer after exposure to haem may not have been adequate, and longer feeding times beyond 18 months would be required to answer this question. Lastly, it could be suggested that the current Western diet model was not suitable for testing the hypothesis and it may be that other rodent models might be implemented. Mice with a targeted *APC* gene knock out in the colon tissue only, termed the *CDC;Apc* mice, develop intestinal tumours spontaneously within a few months and are predominately located in the distal colon rather than the small intestine (Adachi et al. 2014). Such a model might pose as a more suitable candidate in measuring tumour outcome in response to DNA adducts generated by red meat and its associated components, such as haem.

9.2 Red meat and resistant starch in the Western diet mouse model

The hypotheses addressed in this chapter were that high protein diets, particularly a high red meat diet, would increase the risk for developing CRC by generating DNA O^6MeG adducts. Also, that a high fibre diet with a large amount of RS might be protective by compromising red meat-induced DNA adducts. The specific aims of this chapter were as follows:

1. Determine if long term feeding of high protein diets and more specifically a red meat protein source could increase O^6MeG adducts in the colonic epithelial cells of mice, and if RS consumption could lower these adducts.
2. Determine if the increased O^6MeG adducts from consuming red meat and high protein diets is associated with an increased risk of formation of colon cancer,

and if resistant starch could reduce the risk of CRC associated with high protein and/or red meat diets

High red meat diets (consumed over a period of time) increased O^6MeG adduct accumulation, which is *enhanced* by co-consumption with RS; however, this did not lead to an increase in sporadic colon tumour formation in the colon of mice consuming a typical Western diet. Fermentation of starch in the colon of mice lowers protein fermentation metabolites but this also does not affect risk of CRC in the current model in the context of red meat consumption.

In line with results from the previous chapter, this study identified that putatively-mutagenic DNA adducts generated in a chronic manner after long term consumption of red meat are not linked with risk of CRC. In this model, dietary induced adducts generated by red meat consumption are not produced at a "threshold" sufficient to link red meat consumption with spontaneous CRC. As mentioned previously, it might be postulated that the time taken for colon cancer to form has not been adequate, and longer studies taken beyond 18 months feeding in this model would be required to answer this. Also, other candidate mouse models such as the CDC;Apc model mentioned previously might be more suitable for testing the effects of red meat and RS on colorectal oncogenesis, as CDC;Apc mice develop spontaneous tumours restricted to the large intestine (Adachi et al. 2014). In addition to this, it could also be suggested that other components of red meat might be more strongly associated with CRC risk, such as carcinogen formation after cooking meat at high temperatures, which has been linked to increased risk of CRC in humans (Rohrmann et al. 2009). The current study controlled for carcinogen formation by cooking the red meat at low temperatures for short periods of time. To determine if high temperature cooking was responsible for increasing CRC risk, another group of

mice consuming red meat cooked to the point of charring and comparing this to a non-charred red meat group could clarify this argument. Lastly, red meat in the form of processed red meat may be more closely associated with risk of CRC than unprocessed red meat (Santarelli et al. 2010). Processing red meat by nitrite-curing can enhance oxidative stress and increase DNA alkyl adducts *in vitro* (Van Hecke et al. 2014). Therefore, it would also be beneficial to include a "processed red meat" group in the current study design to identify if nitrite treatment can enhance DNA adduct formation *in vivo* and determine if CRC risk is affected.

The other key finding was that combining red meat with RS significantly enhanced DNA O^6MeG adducts, although other markers of protein fermentation and cell proliferation were significantly lowered. It was noted in the discussion of Chapter 4 that butyrate is a histone deacetylase inhibitor, which allows for the DNA chromatin structure to be more available for repair processes, but that this may also enhance DNA alkylation after red meat exposure in the colon. Firstly, levels of NOCs in the faeces would need to be confirmed in mice consuming a red meat and RS diet, to identify if RS affects NOC generation. Secondly, assuming NOC generation in mice with red meat and RS consumption is the same (or lower), the next question to ask is does increasing butyrate delivery to the colon in the presence of the same levels of red meat enhance DNA adduct generation over the long term? This could be answered simply by feeding a group of mice with a butyrylated RS called HAMSB (as used in the human study in Chapter 5) in combination with red meat and comparing this to a non-butyrylated RS in combination with red meat. It is known that HAMSB can deliver significantly more butyrate to the colon compared to a non- butyrylated RS and this has significant effects on the cellular kinetics in the colon and can prevent AOM-induced CRC in rats (Bajka et al. 2006, Clarke et al.

2008). Furthermore, correlating levels of histone acetylation in the colon with DNA O^6MeG adducts, could give further insight into how RS might enhance red meat induced O^6MeG adducts via increased butyrate fermentation, and determine whether this has any effect on CRC risk.

9.3 Red meat and resistant starch in randomised human trial

The hypotheses addressed in this chapter were that red meat consumption would result in enhanced adduct formation in rectal biopsy samples from human volunteers consuming a high red meat diet, but that co-consumption of RS with red meat would reduce formation of pro-mutagenic adducts generated by a high red meat diet. Also, red meat would increase fermentation of toxic metabolic products in the faeces of healthy human volunteers, but addition of RS to the diet would reduce these products and increase fermentation of beneficial bacterial metabolites. The specific aims of this chapter were as follows:

1. To identify if feeding healthy humans a diet high in red meat will increase DNA O^6MeG adducts within the rectal epithelial tissue, and if co-consumption of a high red meat diet with HAMSB (butyrylated resistant starch) could modify adduct generation.
2. To determine if HAMSB supplementation of a high red meat diet could promote a more healthy colonic environment by reducing fermentation of red meat metabolites and enhancing short chain fatty acid production in faecal material from healthy human volunteers.

This study has shown for the first time that when healthy humans consumed their normal diet containing an additional 300g of red meat per day over a 4 week period, there was increased formation of the O^6MeG in the rectal epithelium. This

increase in adduct formation was attenuated by the addition of a butyrylated RS to a high red meat diet.

Changes in DNA alkylation by red meat and RS consumption observed in this trial has confirmed previous evidence in mouse studies that red meat can enhance O^6MeG adducts, but that RS can protect against them in the short term (Winter et al. 2011). Thus, the findings data presented here also support epidemiological evidence that red meat can increase risk for CRC, but dietary fibre can protect (WCRF/AICR 2011). However, in light of the evidence just reported in Chapter 4 of this thesis showing that long term consumption of red meat and RS can enhance O^6MeG formation but does not affect CRC risk, it is uncertain whether measuring these particular adducts in the human colon actually equates to an increase in risk of cancer. It is also uncertain whether the same effects seen in the mouse colon over the long term will also be observed in the human colorectal tissue after long term chronic consumption of red meat with RS. It is known that long term consumption (up to 4 years) of 15g of RS a day in a high risk population does not lower their risk for developing CRC (Burn et al. 2008, Mathers et al. 2012).Future human trials comprising of long term high red meat consumption with long term RS consumption, ideally in a high risk population, would give insight into how RS might change CRC risk associated with red meat consumption. Furthermore, by measuring changes in DNA O^6MeG adducts in the colon tissue of this study group would give a clearer picture of how dietary induced DNA adducts might influence CRC risk in humans and determine their potential as a bio-marker for risk of CRC in individuals who consume high red meat diets. However, considering the potential risk to human health by consuming high red meat diets for extended periods of time, this particular trial would likely be difficult to implement due to ethical considerations. This

highlights the importance in finding a more ideal pre-clinical model to study the effect of long term red meat and RS consumption and their effects on colon health and oncogenesis.

9.4 Red meat and resistant starch intake with *Msh2* deficient mice

The hypotheses addressed in this chapter were that red meat feeding in wild type mice will increase DNA O^6MeG adducts, pre-neoplastic lesions and colorectal tumours in the colon of *Msh2* deficient mice but that addition of RS to a red meat diet fed to *Msh2* deficient mice will reduce these damaging effects in the colon. The specific aims are as follows:

1. Determine if feeding a high red meat diet for 6 months can increase DNA O^6MeG adducts in the colon of *Msh2* deficient mice and whether RS can modulate the red meat induced adducts.
2. To determine the risk of developing pre-neoplastic and neoplastic lesions in the colon of *Msh2* deficient mice with consumption of a high red meat diet for 6 months, and if co-consumption with RS can protect against these changes.
3. To identify if there is an association between DNA O^6MeG adduct accumulation in the colon of *Msh2* deficient mice and risk of developing CRC.

Contrary to the hypotheses, this study has revealed an unexpected protective effect of MMR deficiency on colon O^6MeG adduct formation, lymphoma and small intestinal cancer as a result of red meat consumption. There was no identifiable link between colonic pro-mutagenic adducts and red meat consumption, or any associated risk for CRC. Although RS supressed hyper-proliferation of the distal colon as a result of MMR deficiency, no link between lowered proliferation rates and ACF formation was identified.

This chapter adds to the limited knowledge of red meat and RS consumption in mice with a genetic modification that poses a higher susceptibility to intestinal tumourigenesis and lymphogenesis. However, why red meat protects against DNA alkyl adducts in these mice is uncertain, being both interesting and counterintuitive. Firstly, considering that this study and the outcome have not been studied or reported before, more trials would have to be undertaken to confirm the finding. Second, it might be postulated that *Msh2*$^{-/-}$ mice might actually have a different bacteria profile after consumption of red meat and therefore have different alkylating capabilities in the colon. This could be tested by measuring NOC content in the faeces and analysing bacterial populations related to red meat fermentation in the colon to determine any difference between the normal mice and *Msh2* deficient mice. Thirdly, it might be that the MGMT activity of Msh2 deficient mice is significantly enhanced due to the inadequate MMR capability. The MGMT assay is relatively simple; however, it requires large tissue sample sizes and therefore it difficult to carry out in mouse tumour studies where available colon tissue samples are limited.

The unexpected protection of red meat against small intestinal (SI) and thymic lymphoma in *Msh2*$^{-/-}$ mice needs to be validated in future studies. It was postulated in Chapter 6 discussions that the immune system may be enhanced by red meat consumption, thereby lowering the adduct accumulation and overall tumour burden, particularly lymphoma. In light of this finding, it would be valuable to measure if markers of the immune system are affected (e.g. CD cells) by red meat consumption on an *Msh2* deficient background. Further to this, it would also be beneficial to determine if red meat can protect against tumourigenesis in other pre-clinical models that are immuno-compromised. It should be noted that the basal

protein intake in these animals is as per the international standard and is not perceived in any way to be deficient.

There are also questions raised in this chapter about the use of *Msh2*$^{-/-}$ mice in determining risk of dietary exposures on colorectal oncogenesis. Certainly, the *Msh2*$^{-/-}$ mice showed a high mortality rate over the course of the study, which means that insufficient mice survived long enough to develop colonic neoplasms, and therefore no interpretation of the dietary interventions on CRC risk could be carried out. Further to this point, the *Msh2*$^{-/-}$ mouse might not best represent the human familial form of MMR inherited CRC, as HNPCC individuals are heterozygous carriers of the mutated MMR alleles. Homozygous individuals inheriting gene defects from affected HNPCC subjects (MMR-deficiency syndrome) have a distinct pathology of gastrointestinal cancers, hematologic malignancies and neurological tumours all occurring in early childhood (Bandipalliam 2005). The latter elements only of the syndrome are represented in the *Msh2*$^{-/-}$ knockout mouse. Therefore, it is suggested that future studies determining any CRC risk associated with dietary interventions of red meat and RS would be carried out in *Msh2*$^{+/-}$ mice. Although it might take longer for these mice to progress to colon tumours as an end point, it might give a better representation of the human progression of CRC in HNPCC patients.

9.5 Red meat and green tea in *MGMT* deficient mice

The hypotheses addressed in this chapter were that red meat consumption and azoxymethane exposure will increase O^6MeG adduct formation in the colon of mice and that loss of MGMT activity will enhance this adduct accumulation further. Also, that green tea consumption will protect against adduct accumulation (by red meat

and AOM) and enhance the acute apoptotic response to AOM in the colon of mice, as a direct consequence of *MGMT* regulation by green tea.

This chapter aims to:

1. Identify if green tea alone, or in combination with red meat, could protect against O^6MeG adduct formation and/or enhance the acute apoptotic response to an alkylating carcinogen in the colon;

2. Determine if green tea alone or in combination with red meat can lower O^6MeG adducts in the colon cells via regulation of *MGMT*.

This chapter highlights that green tea does not affect adduct formation in the colon, whether of chemical or dietary sources, despite its capacity to regulate *MGMT*. Rather, green tea consumption at 0.5% of the diet showed an enhanced proliferative state and reduced acute apoptotic response (AAR) to the carcinogen AOM. This chapter also presents evidence that MGMT may have other regulatory effects on the acute apoptotic response to carcinogens other than alkyl repair – this was later disputed in data from Chapter 8 of this thesis. Red meat and loss of MGMT function could generate DNA alkyl adducts, but not at doses high enough to induce apoptosis.

The most striking finding was that green tea could stimulate proliferation and was unable to efficiently remove highly damaged cells via apoptosis, in response to a high dose of alkylating carcinogen in wild type mice. As discussed in Chapter 7, this effect may be due to green tea having distinct influences in the colon at a wide range of doses (0.01 - 1%). Doses of green tea at 0.5% - 1% can increase inflammatory markers and enhance chemically induced and colitis-associated colorectal carcinoma in rodents, whereas low doses (0.01%-0.1%) can reduce inflammatory markers and

protect against subsequent CRC development (Hirose et al. 2001, Kim et al. 2010). Therefore, green tea may actually be pro-inflammatory at higher doses, a potential reason why an increase in proliferation was observed in the current study at 0.5%. Feeding a range of green tea concentrations from 0.01 – 1% to mice and repeating the acute AOM design would determine if green tea can have pro- and anti-proliferative, inflammatory and apoptotic responses in colonocytes. Extending this same dosing regimen into a tumour study would identify if such changes observed with low-to-high green tea concentrations in the diet would have a significant effect on CRC risk. This could have significant consequences for consumption of green tea in humans, particularly where higher daily doses of green tea could actually be doing harm rather than protecting against colonic damage.

Females were shown to be more "sensitive" to AOM exposure than their male counterparts, demonstrating a significantly higher apoptotic response to AOM 6 hours after exposure. This is in agreement with many other published data showing that female rodents have higher apoptosis rates after carcinogen exposure (Armstrong et al. 2011), likely due to the estrogen hormone. Subsequently, female rodents have lower pre-cancerous lesions and tumour rates than male rodents (Ochiai et al. 1996, Guo et al. 2004, Weige et al. 2009). In humans, females and females undergoing hormone replacement therapy (HRT) have lower CRC rates compared to men and compared to women who do not undergo HRT after menopause (Calle et al. 1995). These facts highlight the importance of selecting *both* genders when performing chemoprevention trials in animal models of CRC. Most studies use one gender or the other (mostly males) to avoid such variables in the results. However, typically the central aim of pre-clinical studies is to translate any meaningful findings into the human situation. Thus, including males and females in the study design of

AOM rodent models, and likely many other rodent models of disease, should be considered fundamental.

The last key finding to consider here is that red meat and loss of *MGMT* could enhance DNA O^6MeG adduct accumulation, as was expected and stated in the hypotheses. However, the slight but significant change in adducts did not exceed the threshold of damage needed to induce apoptosis, as was the case for AOM exposure where the adducts generated were substantially higher than red meat alone. In the context of a normal, healthy colonocyte, red meat exposure and *MGMT* loss do not trigger sufficient DNA adducts to overwhelm the cells ability to repair such lesions. Certainly, other repair processes such as BER and MMR can intervene to repair alkyl adducts (Bugni et al. 2009, Wirtz et al. 2010). Therefore, whether these changes in DNA adducts via red meat or loss of *MGMT* are "mutagenic" or "carcinogenic' is questionable, as cells may not exposed to sufficient levels of damage to initiate mutations. However, it is known that certain polymorphisms of *MGMT* in the human population demonstrate an increased risk of CRC when consuming a high red meat diet (Loh et al. 2010). Determining the risk of mutational load and subsequent CRC by red meat-induced DNA O^6MeG adducts on an *MGMT* deficient background could be answered by feeding *MGMT* knockout mice and wild type mice a red meat diet for extended periods of time and compare this to a control diet. Such a study in *MGMT* knockout mice would fill the gap in the knowledge of how dietary induced DNA adducts might initiate or promote CRC.

9.6 Cellular response to AOM in *MGMT* deficient mice

The hypotheses tested here were that early time points post AOM (4-8 hours) when administered to *MGMT* deficient mice will have a lowered apoptotic response

to AOM but more O^6MeG adducts compared to wild type mice, whereas at later time points (24-48 hours) *MGMT* deficient mice will have more apoptosis and higher O^6MeG adducts compared to wild type mice. Also, peak apoptosis will occur at 8 hours post AOM for wild type mice and 48 hours for *MGMT*$^{-/-}$ mice. The specific aims of this chapter are:

1. To establish the role of MGMT activity in colonic cellular DNA damage responses of apoptosis, proliferation and O^6MeG adducts at early and late time points after exposure to the alkylating carcinogen AOM.
2. Identify the peak DNA damage responses of apoptosis, proliferation and O^6MeG adducts in wild type and *MGMT* deficient mice
3. Examine the pattern of DNA adduct formation along the crypt axis to asses cellular responses to AOM in mouse proliferative and more differentiated cells

The MGMT protein does not regulate the acute apoptosis response to AOM. Mice show different apoptotic responses to the carcinogen AOM than do rats, with the peak apoptotic response to AOM in mice occurring at 48 hours post AOM exposure. The apoptotic response to AOM will likely be dependent on the concentration of the AOM solution being administered to the rodent.

In contrast to the finding from Chapter 7 where *MGMT* loss appeared to play a part in the *acute* apoptotic response to AOM, it was shown here that there is no difference between wild type mice and *MGMT* knockout mice at 0–48 hours post AOM. This key finding is in direct disagreement with published literature that observed a significantly higher apoptotic response in *MGMT* deficient mice 24 and 48 hours after AOM, where the authors state induction of MMR and BER as the likely explanation (Bugni et al. 2009). The current chapter extended beyond the 48

hour time-point used in Bugni et al (2009) to 72 hours, where *MGMT*$^{-/-}$ mice displayed significantly higher rates of apoptosis compare to wild type mice. According to other well cited literature, O^{6}MeG adducts are not repaired by MMR and BER until after 2 rounds of the cell cycle (Margison et al. 2002), and therefore the results presented in this chapter better represent apoptosis as a result of unrepaired adducts in *MGMT* deficient mice. As pointed out in Chapter 8 discussion section, there are several reasons why differences in apoptosis would be seen in different experiments and different laboratories, with the key factor likely to be the concentration of the AOM solution used. Future experiments using varying concentrations of AOM solutions, both in wild type and *MGMT* deficient mice would probably clear this uncertainty between the data. Furthermore, since the *MGMT*$^{-/-}$ mice have not returned to "baseline" levels, longer time points should be included to determine how long it takes for the cells to stabilise after AOM exposure in mice.

Another key finding from this chapter was that mice display a peak apoptotic response to AOM at 48 hours post AOM, rather than the 6-8 hours hypothesised because previous studies in rats show that apoptosis peaks around 8 hours, with O^{6}MeG adducts also peaking around 6-8 hours (Nyskohus et al. 2013, Nasuno et al. 2014). It may be that AOM metabolising enzymes in the liver might be present at different activity levels in rats compared to mice, or certainly MGMT activity capacity might be different in rats compared to mice thus adducts and therefore apoptotic responses might vary. Indeed, mice and rats show different susceptibility to form tumours and ACF in the colon following AOM exposure. The incidence and multiplicity of tumours in rats after a total dose of 30mg/kg of AOM is approximately 0.73 tumours per rat (Le Leu et al. 2007, Le Leu et al. 2010). In mice, the same dose only gives 0.57 tumours per mouse (Hu et al. 2008). When the total

dose of AOM in mice is doubled to 60mg/kg, 0.8 tumours per mouse was observed (Hu et al. 2005), more similar to the 0.73 tumours observed per rat given 30mg/kg of AOM. In addition, clinical observations of rodents in our laboratory experiments demonstrate that mice are unpredictable in their response to AOM, particularly where high doses of AOM can induce acute toxicity leading to severe ill health and death within the first 24-48 hours after exposure, whereas no such effects are reported in rats. There are pros and cons of using both models, for example different food costs for dietary intervention studies, purchase and maintenance cost of mice versus rats and volume of tissue sampling g available to use in laboratory experiments differ quite substantially. This highlights the importance of animal choice when using models of CRC for chemo-preventative studies.

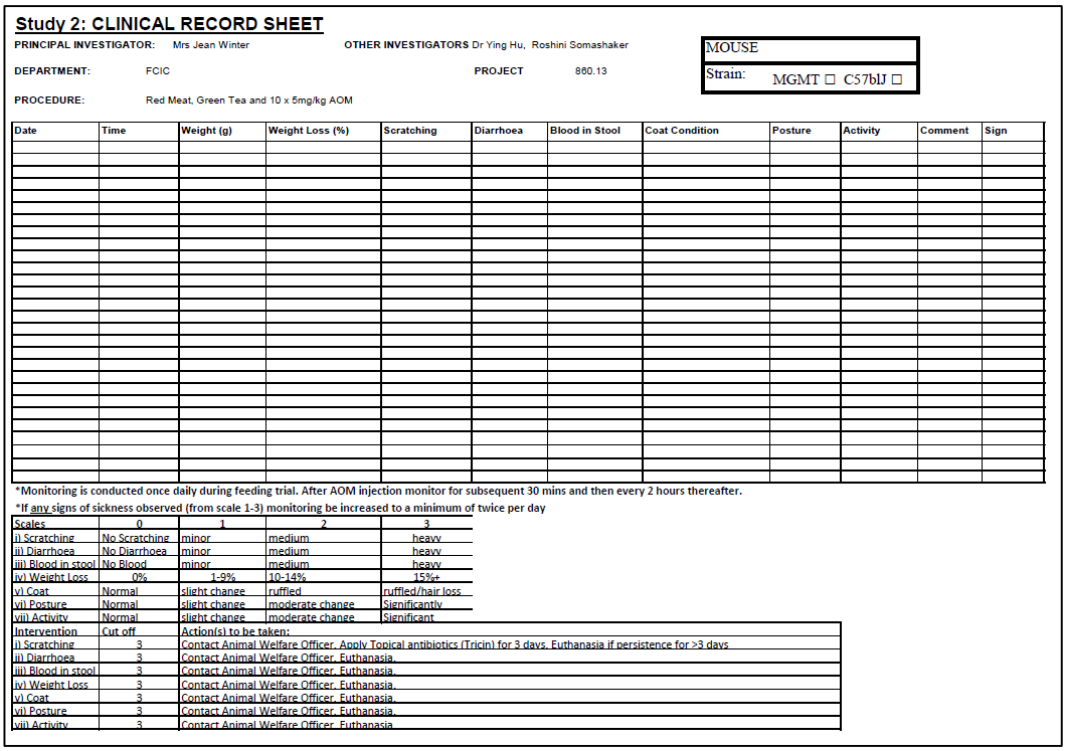
9.7 Overall conclusion

The thesis presented here has added to the knowledge of how dietary compounds can influence the colonic environment and that this can also be dependent on the underlying genetic make-up. Furthermore, this project has extended limited knowledge on dietary-induced DNA adducts and their association with colon cancer formation, particularly in terms of red meat and RS consumption. In conclusion, chronic consumption of a high red meat diet can generate DNA lesions in colonic epithelial cells and RS consumption can ameliorate this affect in the short term, but this does not lead to consequent changes in risk after long term consumption in the chosen mouse models. Consequently, dietary-induced DNA O^6MeG and 8-oxo adducts could perhaps be described as a marker for exposure to alkylating and oxidative agents in the diet, including red meat and its associated components such as haem, and not necessarily described as a bio-marker for CRC risk.

Appendices

Appendix A: Clinical monitoring sheet for laboratory mice

Figure A: Example of a clinical monitoring sheet used to observe the health of animals in the dietary experiments



Study 2: CLINICAL RECORD SHEET

PRINCIPAL INVESTIGATOR: Mrs Jean Winter **OTHER INVESTIGATORS** Dr Ying Hu, Roshini Somashaker

DEPARTMENT: FCIC **PROJECT** 860.13

PROCEDURE: Red Meat, Green Tea and 10 x 5mg/kg AOM

MOUSE
Strain: MGMT ☐ C57blJ ☐

Date	Time	Weight (g)	Weight Loss (%)	Scratching	Diarrhoea	Blood in Stool	Coat Condition	Posture	Activity	Comment	Sign

*Monitoring is conducted once daily during feeding trial. After AOM injection monitor for subsequent 30 mins and then every 2 hours thereafter.

*If any signs of sickness observed (from scale 1-3) monitoring be increased to a minimum of twice per day

Scales	0	1	2	3
i) Scratching	No Scratching	minor	medium	heavy
ii) Diarrhoea	No Diarrhoea	minor	medium	heavy
iii) Blood in stool	No Blood	minor	medium	heavy
iv) Weight Loss	0%	1-9%	10-14%	15%+
v) Coat	Normal	slight change	ruffled	ruffled/hair loss
vi) Posture	Normal	slight change	moderate change	Significantly
vii) Activity	Normal	slight change	moderate change	Significant

Intervention	Cut off	Action(s) to be taken:
i) Scratching	3	Contact Animal Welfare Officer. Apply Topical antibiotics (Tricin) for 3 days. Euthanasia if persistence for >3 days
ii) Diarrhoea	3	Contact Animal Welfare Officer. Euthanasia.
iii) Blood in stool	3	Contact Animal Welfare Officer. Euthanasia.
iv) Weight Loss	3	Contact Animal Welfare Officer. Euthanasia.
v) Coat	3	Contact Animal Welfare Officer. Euthanasia.
vi) Posture	3	Contact Animal Welfare Officer. Euthanasia.
vii) Activity	3	Contact Animal Welfare Officer. Euthanasia.

Appendix B: Standard diet and Western diet components

Table B.1: Vitamin mixes for the standard AIN-76 and the Western diet

Vitamin	AIN-76 Diet (g)	Western Diet (g)
Nicotinic acid	3	3
Ca pantothenate	1.6	1.6
Pyridoxine HCL	0.7	0.7
Thiamine HCL	0.6	0.6
Riboflavin	0.6	0.6
Folic acid	0.2	0.023
D – biotin	0.02	0.02
Vitamin B-12*	500µl	500µl
Vitamin E#	5	5
Vitamin D_3	0.25	0.0275
Vitamin A	0.8	0.8
Vitamin K1^	0.075	0.075
Sugar	986.655	987
Total	**1000**	**1000**

*B12, 100mg B12 in 10ml H_2O
#Stock Vitamin E is 1000iU/g
^Vitamin K, 1g Vitamin K in 50ml sun flower seed oil.

Table B.2: Mineral mixes for standard AIN-76 and the Western diet

Mineral	AIN-76 Diet (g)	Western Diet (g)
$CaHPO_4.2H_2O$*	316	62.5
Na_2HPO_4	-	330
NaCl	74	-
$K_3C_6H_5O_7.H_2O$	220	220
K_2SO_4	52	52
MgO	24	24
$MnSO_4H_20$	5.01	5.01
Ferric citrate	6	6
$ZnSO_4.7H_2O$	5.7	5.7
$CuSO_4.5H_2O$	0.606	0.606
KlO_3	0.01	0.01
Na_2SeO_3	0.007	0.007
$CrK(SO_4)_2.12H_2O$	1.39	1.39
Sugar	300g	293
Total	**1000**	**1000**

*****Calcium is added at half the levels in typical AIN76 mineral mix: an adequate level of calcium for maintenance of growth but not significant levels to interfere with haem adsorption in the gut.

Appendix C: Fat Extraction method for red meat fat analysis

1. Weigh 1g of dried, powdered red meat and place into 50ml polypyrene tubes with lid on. Record the weight of the tube and meat powder (weight a).
2. Add 5ml solution of chloroform/methanol (1:1, v/v) and mix thoroughly with a vortex.
3. Leave tube standing upright with the lid off and let stand for 48 hours in a fume hood at room temperature.
4. Place the lid back on and re-weigh the tube and meat powder (weight b).
5. Calculate the % of fat as follows:

   (weight a – weight b) x 100%

Appendix D: Solutions and Buffers

Acetone Solution
- 4.5ml distilled H_2O (dH_2O)
- 20ml Acetone
- 0.5ml of 1M Hydrochloric Acid
- Use immediately

SCFA/phenols Internal Standard (IS)
- 800ml dH_2O
- 120µl Heptanoic acid
- 50mg o-Cresol
- Adjust pH to 7.0 with 1M Sodium Hydroxide or 1M hydrochloric acid as needed
- Make up to 1000ml with dH_2O
- Store at 4°C

3% H_2O_2
- 100ml of dH_2O
- 100ml of Absolute Ethanol
- 6ml of 30% H_2O_2
- Use immediately

0.01M Citrate buffer
- 1000ml of dH_2O
- 2.94g of tri sodium citrate dehydrate
- Adjust pH to 6.5 with 1M Hydrochloric acid
- Store at room temperature

0.01M Phosphate buffered saline (PBS)
- 1000ml of dH_2O
- 1 packet of phosphate buffered saline 'ready to use' powder (Sigma; P-3813)
- Check pH is at ~7.4
- Store at room temperature

RNase Treatment
- 20µl of 4mg/ml RNase A (Fermentas, EN0531)
- 5µl of 10unit/ml RNase T (Fermentas, EN0541)
- 1000µl of PBS (pH 7.4)
- Use immediately

Alkali Treatment
- 1500µl of cold 70mM NaOH/140mM NaCl solution
- 1000µl cold Methanol
- Use immediately

Acid Ethanol Dip

- 160ml 70% Ethanol
- 75mL Water
- 2.5mL 1M HCl

Ammonia Water

- 250mL Water
- 3 Drops Ammonia

Appendix E: Calculation of haem content in red meat sample

Content of haematin µg/g of red meat = (Average absorbance read from triplicate samples at 640nm X 6800)/5g of red meat

= (0.4077 X 6800)/5

= 555 µg/g

The calculation of haem iron content from haematin in the sample of cooked and dried red meat (as beef) was calculated as follows:

HFe = calculated content of haematin µg/g of red meat X (AW of Iron/MW of haematin)

HFe = 555 µg/g X (55.847g/mol / 634.503g/mol)

HFe = 48.85 µg/g

A typical high red meat diet of 30% total weight contains 40.9 grams of red meat per 100 grams of diet. Therefore, in a high red meat diet the HFe content was calculated as follows:

48.85µg/g / 40.9g/100g = 1.19µg of HFe per 100g of diet.

Therefore, concentration of haemin per 100 g of diet is calculated as follows:

= 1.19µg per 100g of diet / MW of HFe

= 1.19µg per 100g of diet / 634.503g/mol

= 0.000001875 mol per 100g (of diet)

= 0.1875 µmol/g.

This number was rounded up to **0.2µmol/g** of haemin in the total diet for the final diet preparations prepared in Chapter 3.

Appendix F: Tissue processing and embedding

Tissue processing:

1. Use embedding cassette and biopsy pads and label the cassette.
2. Place biopsy pad in the cassette. Place the tissue on the biopsy pad, cover tissue with another biopsy pad and seal the embedding cassette lid.
3. Place cassette in steel basket of Microm Spin Tissue Processor and submerge in 70% ethanol (station C1)
4. Fill other stations (C2-C12) with ethanol, xylene and wax as follows:

   C2-70%
   C3-70%
   C4-70%
   C5-85%
   C6-90%
   C7-100%
   C8-100%
   C9-Xylene
   C10-Xylene
   C11-Tissue-Tek Paraffin Wax (60^{o}C)
   C12-Tissue-Tek Paraffin Wax (60^{o}C)

5. Turn Microm Processor on and start program 1 (takes approximately 16 hours to complete program)
6. When complete remove basket and cassettes ready for embedding

Embedding:

1. Transfer cassettes from Tissue Processor to wax dispenser when wax has melted.
2. Ensuring tweezers and steel mould are warm, fill the mould with wax from the dispenser and place on the warm plate
3. Orientate the tissue into position using tweezers, the open lumen should be facing down toward the mould base to create a final transverse cross section of the tissue.
4. Holding the tissue in place move the mould across to the cold plate
5. Release the tissue when it is set within the cooling wax.
6. Place the lid on top of the steel mould and add more wax to fill.
7. Leave to cool on the large cold platform for 10-15 minutes.
8. Store in a cool, dry place

Appendix G: Tissue staining protocols

Rehydration of tissue:

1. Microscope slides with cut tissue sections are placed into a 60°C drying oven for 30 minutes
2. Transfer slides to HistoChoice® Clearing Agent (Sigma; H2779) x2 for 10 minutes each.
3. Slides are transferred through decreasing concentration of ethanol solutions as follows:

- 100% x2 at 2 minutes each
- 95% x2 at 2 minutes each
- 70% x2 at 2 minutes each
- 50% x2 at 2 minutes each
- dH_2O x1 at 1 minute

Haematoxylin stain:

1. Rehydrate sections in ethanol (see above)
2. Shake off excess water and place in JJ Harris Haematoxylin (Fronine Laboratories) for 3 minutes
3. Rinse in distilled water until it runs clear
4. Acid Ethanol dip 3 times
5. Rinse in distilled water
6. Ammonia water dip until stain turns blue (approximately 3 times)
7. Rinse in distilled water then dehydrate sections in ethanol washes:
    a. 50% x 2 (1 min with intermittent shaking)
    b. 70% x 2 “
    c. 95% x 2 “
    d. 100% x 2 “
8. Place into HistoChoice® clearing agent x2 (1 min each with shaking)
9. Dry slides and place coverslip with Gurr DePex mounting medium

TUNEL Staining – Mouse (FraGEL DNA Kit) following manufacturer’s instructions:

1. Rehydrate sections (as above)

2. Circle tissue with PAP Pen
3. Permeability of Tissue
    - Mix 10µl of Proteinase K (2mg/ml) in 1000µl of 10mM Tris pH 8 (room temp 20 mins)
    - Rinse in PBS
4. Positive Control
    - 1µg/µl DNase I in (1XPBS/1mM MgSO4) (room temp for 20 mins)
    - Rinse in PBS
5. Endogenous Enzyme Block
    - 3% H_2O_2 in 50% Ethanol (see Appendix D) at room temp. 5 mins
    - Rinse PBS
6. Labelling
    - Mix 200µl of 5X TdT equilibration buffer with 800µl of dH2O (room temp 20mins)
7. (Prepare next step while waiting)
    - Working TdT Labelling Reaction Mixture (*WTmix*):
    1. Vortex TdT Reaction mix and pulse spin TdT Enzyme
    2. Mix 570µl TdT Reaction Mix with 30µl TdT Enzyme
    3. PLACE ON ICE
    - Shake excess equilibration buffer off slides and place *WT mix* on slides, cover with parafilm (37ºC 1.5hours)
8. Stop labelling Reaction
    - Rinse in PBS
    - Cover in Stop Solution (room temp 5 mins)
    - Rinse in PBS
9. Visualisation
    - Incubate with Blocking Buffer (room temp. 10 mins)
    - Mix 20µl of 50X Conjugate with 980µl of blocking buffer and apply to slides (room temp. 30mins)
    - Rinse slides with PBS
    - Apply DAB by dissolving 1 DAB tablet, 1 H2O2/Urea tablet in 1 ml tap H_2O. (room temp 10-15 mins)
    - Rinse in H_2O
10. Counterstain

- Stain with JJ Harris Haematoxylin (3 mins room temp)
- Rinse in H_2O, 3 dunks acid ethanol solution
- Rinse in H_2O, 3 dunks ammonia water, rinse in H_2O
- Dehydrate sections and coverslip (see above)

Appendix H: Human trial study design:

Figure H: Detail of human randomised trial (Chapter 5).

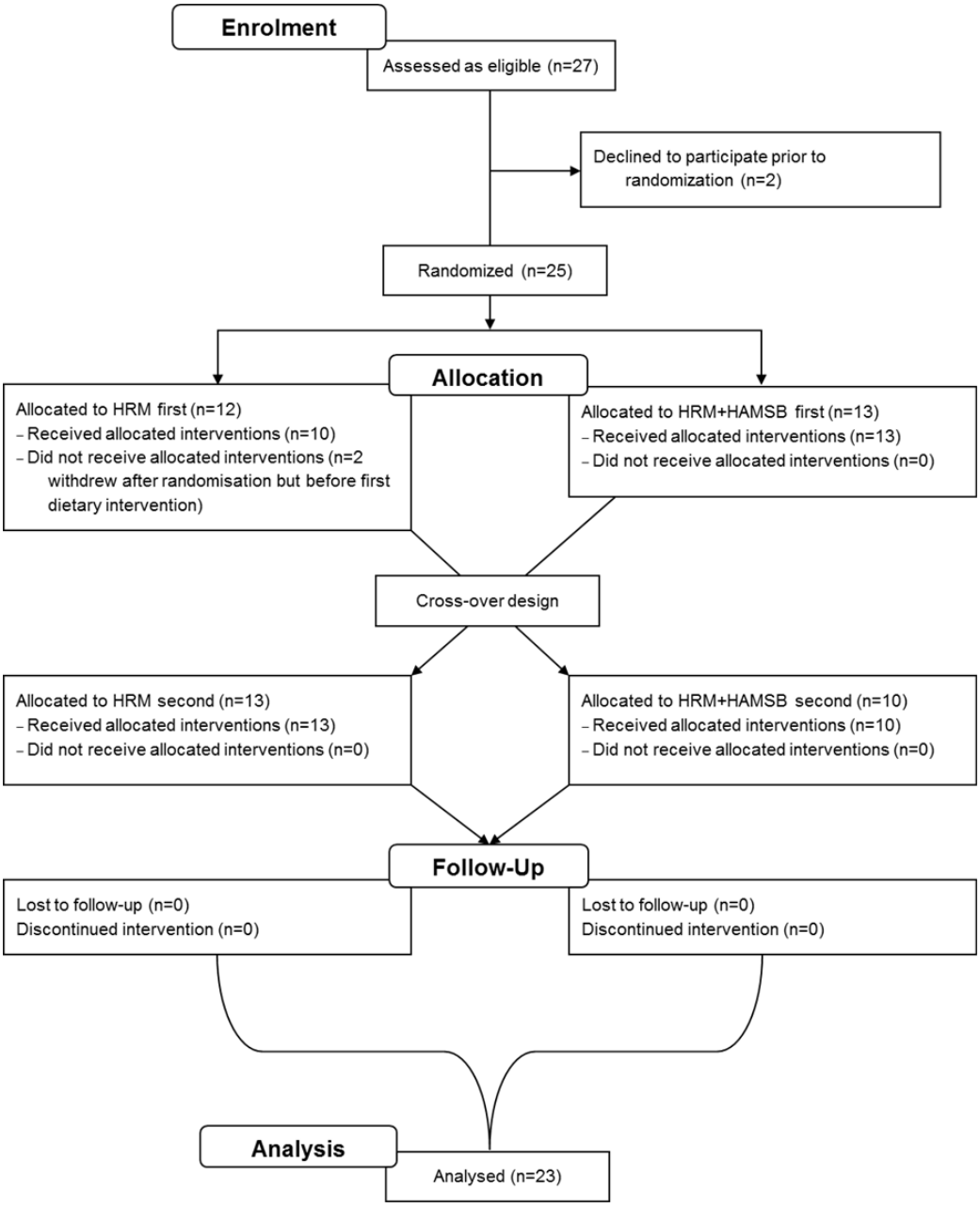
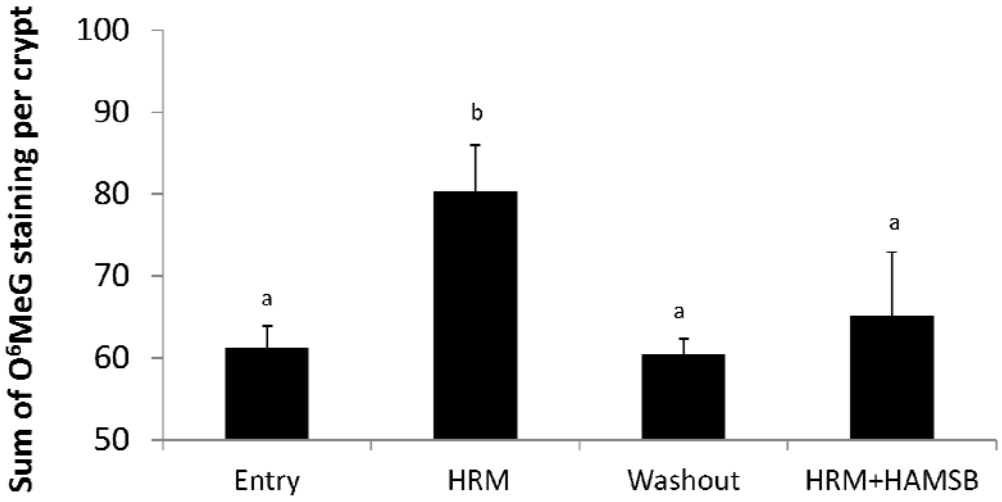


Figure adapted from Humphreys et al (2014) page 789.

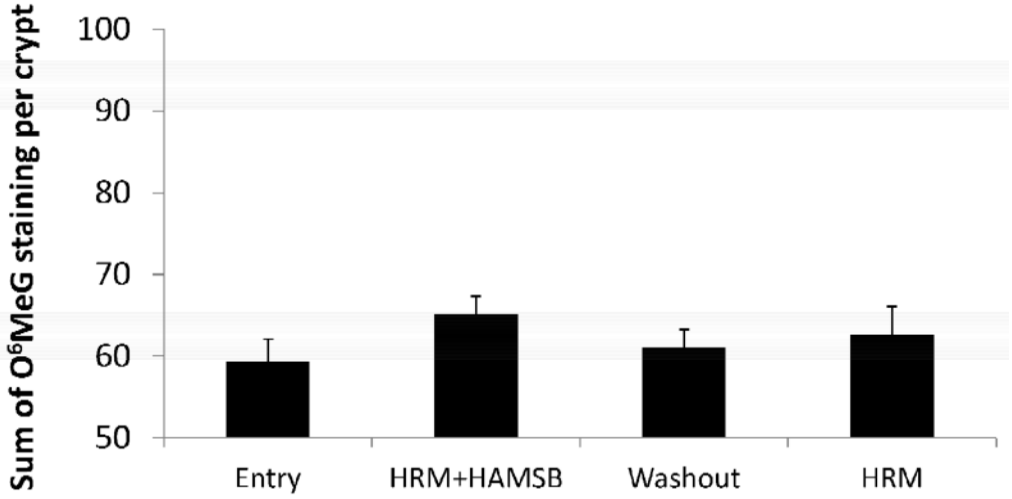
Appendix I: Carry over effect of DNA adducts:

Figure I: DNA O^6MeG adducts in human rectal epithelial cells

A



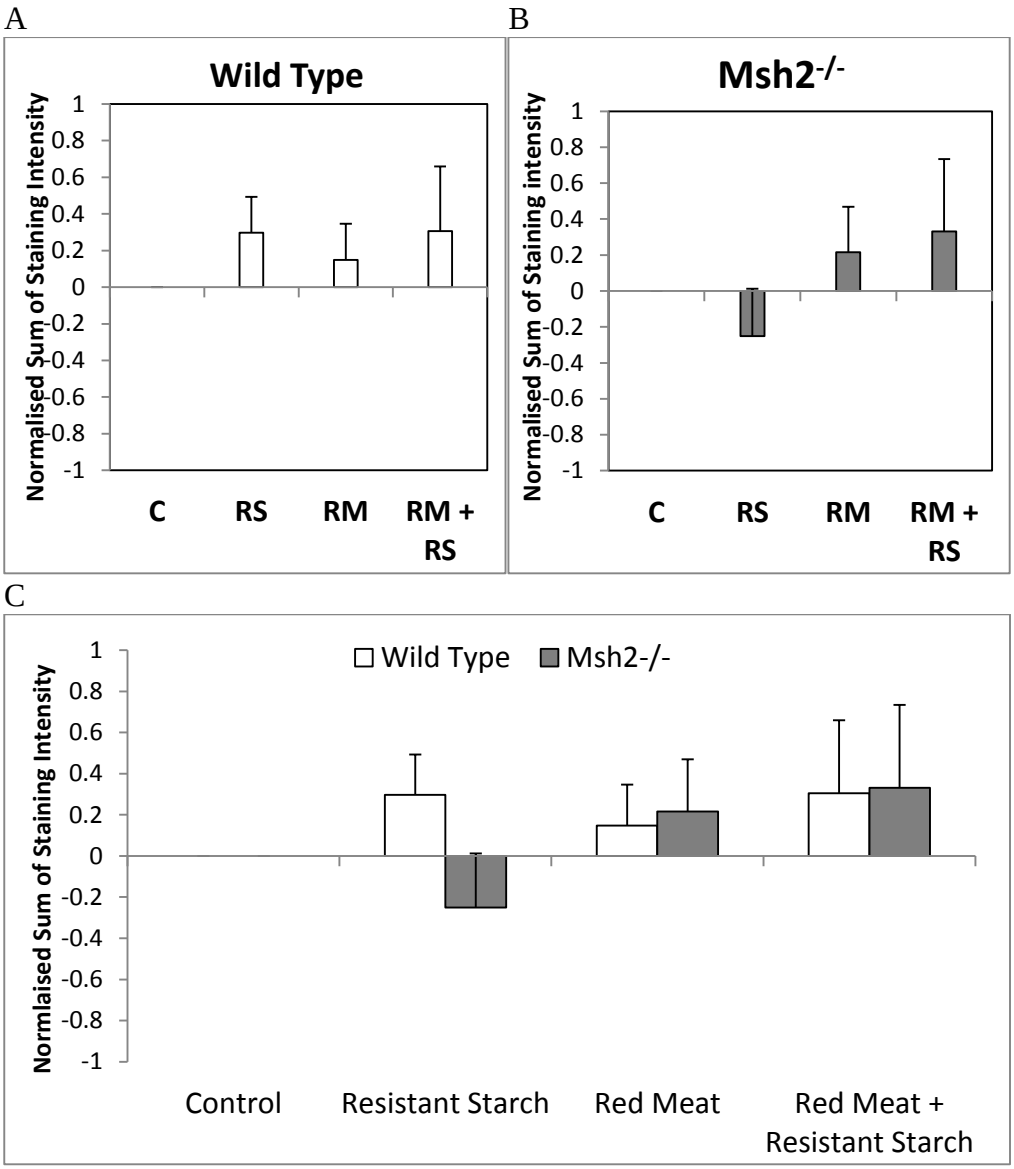
B



Effect of diet and diet order on O^6MeG adduct load in the rectal crypts. (A) Effect when individuals consumed the HRM treatment after entry. (B) Effect when the individuals consumed the HRM+HAMSB intervention after entry. Values with different superscript letters are significantly different, $p<0.05$. Analysed by Linear Mixed-Effects Model; HRM: high red meat; HRM+HAMSB: high red meat and butyrylated high amylose maize starch

Appendix J: Proximal colon O^6MeG adducts in *Msh2* knockout and wild type mice

Figure J: O^6MeG adducts in the proximal colon



Normalised sum of O^6MeG DNA adduct staining intensity in proximal colon cells for wild type mice (A), $Msh2^{-/-}$ mice (B) and comparison of the genotypes for each dietary treatment (C). Data represented as mean ± SE. No significant differences were identified.

Appendix K: Publications arising from this thesis:

1) Winter et al (2013)

Mol. Nutr. Food Res. 2013, *00*, 1–9 DOI 10.1002/mnfr.201300430

RESEARCH ARTICLE

Accumulation of promutagenic DNA adducts in the mouse distal colon after consumption of heme does not induce colonic neoplasms in the western diet model of spontaneous colorectal cancer

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Received: June 14, 2013
Revised: August 1, 2013
Accepted: August 7, 2013

Scope: Red meat is considered a risk factor for colorectal cancer (CRC). Heme is considered to promote colonic hyperproliferation and cell damage. Resistant starch (RS) is a food that ferments in the colon with studies demonstrating protective effects against CRC. By utilizing the western diet model of spontaneous CRC, we determined if feeding heme (as hemin chloride) equivalent to a high red meat diet would increase colonic DNA adducts and CRC and whether RS could abrogate such effects.

Methods and results: Four groups of mice: control, heme, RS and heme + RS were fed diets for 1 or 18 months. Colons were analyzed for apoptosis, proliferation, DNA adducts "8-hydroxy-2-deoxyguanosine" and "O^6-methyl-2-deoxyguanosine" (O^6MeG), and neoplasms. In the short term, heme increased cell proliferation ($p < 0.05$). Changes from 1 to 18 months showed increased cell proliferation ($p < 0.01$) and 8-hydroxy-2-deoxyguanosine adducts ($p < 0.05$) in all groups, but only heme-fed mice showed reduced apoptosis ($p < 0.01$) and increased O^6MeG adducts ($p < 0.01$). The incidence of colon neoplasms was not different between any interventions.

Conclusion: We identified heme to increase proliferation in the short term, inhibit apoptosis over the long term, and increase O^6MeG adducts in the colon over time although these changes did not affect colonic neoplasms within this mouse model.

Keywords:
Heme / 8-Hydroxy-2-deoxyguanosine / O^6-Methyl-2-deoxyguanosine / Red meat / Resistant starch

1 Introduction

Colorectal cancer (CRC) is a major burden on public health in developed countries with high incidence and mortality rates globally [1]. Although the development of CRC can be attributed partly to familial inherent mutations, a major driving force behind CRC formation is related to lifestyle factors, in particular dietary choices [2]. The consumption of red meat has been identified as a risk factor for developing CRC by the World Cancer Research Fund [3]. However, the mechanisms by which red meat might initiate oncogenesis in the colon are not clearly known.

Increased DNA alkyl adducts in the colon via excess endogenous *N*-nitrosation is one mechanism that is thought to play a role in colorectal oncogenesis, although little is known about DNA adduct formation in the colon in response to red meat consumption [4, 5]. Our recent studies have shown that red meat can enhance the production of the promutagenic adduct O^6-methyl-2-deoxyguanosine (O^6MeG) after feeding of high red meat diets to mice, without chemical carcinogenic intervention [4]. The O^6MeG adduct is a known mutagenic lesion in both animals and humans resulting from

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Abbreviations: **ACF**, aberrant crypt foci; **CRC**, colorectal cancer; **NOC**, *N*-nitroso compound; **O^6MeG**, O^6-methyl-2-deoxyguanosine; **8-oxo**, 8-hydroxy-2-deoxyguanosine; **RS**, resistant starch

exposure to alkylating agents and is repaired by the enzyme O^6-methylguanine-DNA methyltransferase. Persistence of unrepaired O^6MeG adduct changes the chemical bonds in the DNA backbone and thus the DNA polymerase reads the G as an A after one round of replication. If this mismatch is not repaired, further changes in the DNA backbone lead to the polymerase incorporating a T instead of a C, therefore a GC:AT transition mutation arises following a second round of replication [6]. This transition mutation has been identified in several genes including the *K-ras* gene, where mutation of this gene is a known mechanism of human oncogene activation in CRC [6].

The DNA oxidative adduct 8-hydroxy-2-deoxyguanosine (8-oxo) has been implicated in development of cancer for several years [7]. 8-Oxo is a promutagenic adduct that if not repaired or removed from the cell can incorporate base insertions into the DNA, with G → T transversions being the most abundant [8]. The major repair pathway for this DNA adduct is via base excision repair, with the DNA glycosylase enzyme OGG1 having an affinity for excising the 8-oxo adduct from the DNA [9]. Patients with CRC and inflammatory bowel disease show higher levels of 8-oxo measured in plasma [10, 11] and CRC patients show higher levels of 8-oxo DNA adducts measured in colon tissue compared to patients without CRC [12, 13]. 8-Oxo adducts are clearly linked with carcinogenesis although the significance of the 8-oxo adduct and consumption of red meat or heme in CRC has not been studied extensively.

Heme iron in red meat has been implicated in development of CRC in humans [14]. There are many different proteins regulating heme iron absorption in the small intestine and although it is readily bioavailable, there is a limitation to its absorption, therefore it is easily able to reach the colon [15, 16]. Various animal studies have shown that diets with heme concentrations above average human consumption levels (0.25–1.5 μmol/g) [17] contribute to cell toxicity, hyper-proliferation, reduced apoptosis, and increased rates of CRC [16–22]. A diet incorporating low levels of heme (at 0.25 μmol/g) has shown a reduction in total number of aberrant crypt foci (ACF) per colon, although the ACF size was larger with heme diets compared to a control diet [18]. The intake of heme from a 30% red meat diet as beef would equate to an average concentration of 0.14 μmol/g [19], indicating that 0.25 μmol/g of heme more closely resembles that of a high red meat intake in humans. Besides causing damage to the colon cells directly, heme-iron from red meat also triggers the formation of potentially damaging DNA alkylating *N*-nitroso compounds (NOCs) either via increasing bacterial nitrate reductase activity or via reacting with nitrite or nitric oxide in the lumen [20]. In humans, consumption of high levels of red meat has been directly related to increased alkyl adducts in the colon, and this was correlated to increased fecal NOC output [21]. There are strong indications that heme contributes to development of CRC. However to date, there have been no rodent studies conducted examining a heme diet at levels relevant to human consumption to identify its long-term effects on CRC risk without utilizing chemical carcinogens to induce cancer to date. However, to directly assess the carcinogenic potential of dietary heme without utilizing chemical carcinogens or other spontaneous models, large numbers of animals per group are required to detect any possible effect on carcinogenicity.

On the opposing side, a dietary component that has been implicated in protection against CRC is resistant starch (RS), the component of starch that is undigested in the small intestine and fermented rapidly in the colon. It is thought that the preferential fermentation of carbohydrate over protein, when RS is incorporated into high protein diets, leads to a reduction in DNA changes that might initiate CRC [4, 22]. This fermentation increases the production of SCFAs [23], in particular butyrate, which is important for large bowel function and is the major energy source for colon epithelial cells. RS has also been shown to reduce production of toxic protein fermentation products [4, 24]. In vivo animal models demonstrate the ability of butyrate to increase the acute apoptotic response to a chemical carcinogen and reduce cellular proliferation to allow for repair or removal of highly damaged cells [25]. Studying the effect of RS in human subjects is complex due to varying RS sources and maintaining adequate dosing, thus it is difficult to directly pinpoint the protective effect RS has against CRC in humans [23, 24, 30–33]. Daily intake of fiber sources in the above human studies were mostly over 30 g, but actual RS intake would differ due to varying RS content in different RS food sources. This suggests that the dose of RS might not have been achieved in some of these studies to have a direct effect on the colon and higher doses of RS should be aimed for.

As heme is considered to be oncogenic, either directly or indirectly, and RS has the capacity to reduce oncogenic DNA lesions in the colon, we investigated whether feeding heme (at human-relevant amounts) to mice as part of the western diet model of spontaneous CRC for short or long periods of time could induce prooncogenic adducts or colonic neoplasms, and if epithelial events relevant to formation of DNA lesions were affected. We also aimed to find out if adding RS at levels appropriate for human dietary intake could manipulate these parameters.

2 Materials and methods

2.1 Animals and diets

A total of 225 eight-wk-old wild-type c57bl/J male mice were obtained from the Animal Resource Centre, Perth, Western Australia. The Flinders University of South Australia Animal Welfare Committee approved all experimental procedures (ethics approval number 809/12). Animals were randomly placed. Four mice per cage were placed and divided into dietary groups under controlled conditions of 22 ± 2°C (SD), 80 ± 10% humidity, and 12 h light/dark cycle. Animals had free access to food and water and were weighed once weekly

Table 1. Composition of experimental diets in g/100 g

Ingredient	Control	Control + RS	Heme	Heme + RS
Casein	17.6	17.6	17.6	17.6
Corn starch	10	–	10	–
Hi-Maize™	–	10	–	10
Sucrose	45.48	45.48	45.48	45.48
Sunflower Seed oil[b)]	16.8	16.8	16.8	16.8
Lard	3.2	3.2	3.2	3.2
α-Cellulose	2	2	2	2
L-Cysteine	0.3	0.3	0.3	0.3
Choline	0.12	0.12	0.12	0.12
Minerals[a)]	3.5	3.5	3.5	3.5
Vitamins[a)]	1	1	1	1
DL-Methionine	0.15	0.15	0.15	0.15
Hemin[c)]	–	–	0.01304	0.01304

a) AIN-76 vitamin and mineral mixtures with modified calcium at 0.5 mg/g, phosphorus at 3.6 mg/g, folic acid at 0.23 mg/g, and vitamin D3 at 0.11 IU/g.
b) Sunflower seed oil contained no added antioxidants.
c) Hemin is at 0.2 μmol/g of total diet.

throughout the study period. A total of 60 mice consumed diets for 4 wk (short term) and 160 mice were on diets for 18 months (long term).

The experimental diets were based on the American Institute of Nutrition (AIN) diet AIN-76 with modified amounts of vitamins and minerals for rats and mice adapted from the western diet model of spontaneous CRC created by Newmark et al. [26, 27] (Table 1). Our experimental diets were a modification of the AIN76 diet to give 15% total protein using casein w/w and 15% protein with 10% high amylose maize starch. High amylose maize starch was supplied by the National Starch and Chemical Company, Bridgewater, NJ, and contains approximately 50% RS [28], therefore a total of 5% RS was added to the diet. The amount of heme used in this study is at a final concentration of 0.2 μmol/g (supplied as hemin chloride, Sigma catalog #51280). This heme concentration resembles the heme content from a high red meat diet [4, 19]. Final diet preparations were placed into air-sealed containers, stored at 4°C with fresh food in the mouse cages replaced daily.

2.2 Fecal analysis

After 3 wk (short term) or 6 months (long term) on experimental diets, fresh fecal samples were collected from each mouse. For measurement of fecal pH, feces were placed into three times the w/v of cold saline, vortexed until emulsified, and pH was measured using a glass-embodied electrode (Eutech Instruments). Another fresh fecal sample was placed into three times the w/v of an SCFA/phenols internal standard (800 mL Milli Q water, 120 μL heptanoic acid, 50 mg of *o*-cresol, pH 7.0). Duplicate 100 μL aliquots were used to measure SCFAs (butyrate, acetate, and propionate) and phenols and *p*-cresols. For SCFAs, 100 μL was injected into an Agilent Technologies 6890N Network Gas Chromatograph System fitted with a Zebron ZB-FFAP column (0.53 mm × 30 mm) and measured as described previously [29]. For phenols and *p*-cresol, another 100 μL of distillate was injected into a Shimadzu LC-10AD HPLC machine with RF-10AXL fluorescence detector set at excitation 284 nm, emission 310 nm, and measured as described previously [30].

2.3 Tissue sample collection

All colon samples were fixed with 10% buffered formalin solution containing 3.6% formaldehyde for 24 h, then transferred to 70% ethanol for processing. On completion of short-term experimental diets, 1 cm of distal colon was used for analysis. After the long-term feeding experiment, the entire colon was cut longitudinally for analysis of tumors under a dissecting microscope before a 1 cm distal colon segment was taken for analysis and all tumors were collected and processed for H&E staining for histological confirmation. Distal colonic tissue and tumors were processed through gradient alcohols and xylene, embedded in paraffin wax before sectioning on a microtome.

2.4 Cell proliferation and apoptosis measurement

Proliferative activity of distal colonic epithelial cells was measured using an antibody specific for the nuclear proliferating antigen ki-67 (rat-anti-mouse clone TEC-3, Dako, USA) in combination with an immunohistochemistry detection method in paraffin-embedded sections, as used previously [31]. Slides were visualized under light microscopy by brown nuclear staining and proliferation was assessed as the ki-67-positive cells per crypt column length. Apoptosis was measured using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay kit (Trevigen, USA) in paraffin-embedded sections, following the manufactures instructions. Slides were visualized under light microscopy by brown nuclear staining and apoptosis in the distal colon was assessed as the number of positive apoptotic nuclei per crypt column length. All slides were independently and randomly coded.

2.5 DNA adduct quantification

DNA alkylation was quantified using an antibody specific for the DNA adduct O^6MeG (Clone EM 2–3, Squarix Biotechnology, Germany) and the level of oxidative adducts was measured using an antibody specific for the DNA adduct 8-oxo (Trevigen) combined with a mouse-on-mouse polymer horseradish peroxidase detection system (Covance Laboratories) on paraffin-embedded distal colon sections. The primary antibody concentration for O^6MeG was 1:1000 (in PBS) and for 8-oxo, the primary antibody was applied at 1:2000

Table 2. Short-term study measures of body weight and fermentation[a)]

Variable	Unit	Experimental Diet Groups			
		Control ($n = 12$)	Control + RS ($n = 12$)	Heme ($n = 12$)	Heme + RS ($n = 12$)
Body weight	g	28.5 ± 0.4	28.4 ± 0.5	29.3 ± 0.9	28.6 ± 0.5
Fecal pH	-	7.8 ± 0.2	7.1 ± 0.2[b)]	7.6 ± 0.1	7.0 ± 0.1[b)]
Total SCFAs	μmol/g	45.6 ± 11.3	109.9 ± 10.4[b)]	55.6 ± 12.2	117.3 ± 16.9[b)]
Butyrate	μmol/g	1.8 ± 0.8	19.2 ± 3.2[b)]	4.9 ± 0.9	20.4 ± 3.1[b)]
Acetate	μmol/g	36.1 ± 8.7	61.2 ± 6.6[b)]	42.0 ± 9.8	68.1 ± 10.3[b)]
Propionate	μmol/g	7.6 ± 1.9	29.6 ± 2.5[b)]	8.7 ± 1.8	28.8 ± 4.1[b)]
Phenol	μg/g	7.2 ± 1.6	0.8 ± 0.6[b)]	7.3 ± 1.7	0.2 ± 0.1[b)]
p-cresol	μg/g	21.7 ± 2.5	11.8 ± 1.6[b)]	21.5 ± 3.3	9.3 ± 1.1[b)]

a) Data represented as means ± SE.
b) Control + RS and Heme + RS is significantly different to Control and Heme.

(in PBS). Immunohistochemical staining procedures and computer image analysis of stained slides were carried out as previously described [4]. Total sum of adduct formation was calculated along the crypt length for each mouse and averaged for each dietary treatment. All slides were independently and randomly coded so that dietary groups were not known to the counter.

2.6 Statistical methods

Values are represented as means and standard error. Tumor outcomes are expressed as incidence (proportion of mice that develop neoplasia). General linear model univariate analysis was used to determine the main effect of RS addition and heme on all outcomes measured. Independent sample *t*-tests were performed on each variable to determine the difference between short-term and long-term feeding of each diet on DNA markers and cell kinetics. Colon tumors were analyzed by Crosstabs Fisher's Exact test. The null hypothesis was rejected at the 0.05 level for all statistical tests carried out using SPSS version 19 (IBM).

3 Results

3.1 Body weight and fecal analysis

In the short term, no significant differences in body weight (g) were observed after consuming the experimental diets for 4 wk; although after long-term feeding, mice consuming dietary heme had significantly reduced body weights compared to mice on control diet ($p < 0.01$) (Table 2). After both short- and long-term feeding, addition of RS to the diet significantly lowered fecal pH compared to those diets without RS ($p < 0.0001$) and markedly increased fecal total SCFAs concentration ($p < 0.0001$) including acetate ($p < 0.006$), propionate ($p < 0.0001$), and butyrate ($p < 0.0001$) (Table 2). RS was also able to reduce concentration of fecal *p*-cresol ($p < 0.0001$) and phenol ($p < 0.0001$) in the short term (Table 2). Heme addition had no significant effect on fecal fermentation of SCFAs, phenol, or *p*-cresols compared to the control after long-term feeding (Table 3). Although when heme was combined with RS in the long-term experiment, there were significantly reduced concentrations of acetate ($p < 0.001$), propionate ($p < 0.05$), butyrate ($p < 0.05$), and total SCFAs

Table 3. Long-term study measures of body weight and fermentation[a)]

Variable	Unit	Experimental diet groups			
		Control ($n = 40$)	Control + RS ($n = 40$)	Heme ($n = 40$)	Heme + RS ($n = 40$)
Body weight	g	58.3 ± 2.1	52.6 ± 1.8	48.0 ± 2.1[b)]	51.7 ± 1.4
Fecal pH	-	7.7 ± 0.2	7.1 ± 0.2[b)]	7.5 ± 0.1[e)]	6.9 ± 0.1[b),d)]
Total SCFAs	μmol/g	25.3 ± 3.8[c)]	52.4 ± 5.1[b)]	22.3 ± 3.1[c)]	19.0 ± 2.6[c)]
Butyrate	μmol/g	4.9 ± 1.5[c)]	18.8 ± 1.9[b)]	6.8 ± 1.3[c)]	6.2 ± 0.7[c)]
Acetate	μmol/g	17.7 ± 2.8	15.1 ± 1.6	8.4 ± 1.0[b)]	7.1 ± 1.3[b),c)]
Propionate	μmol/g	2.8 ± 0.3[c)]	14.8 ± 1.6[b)]	4.5 ± 0.7[c)]	3.5 ± 0.5[c)]
Phenol	μg/g	2.0 ± 0.7	0.9 ± 0.1	1.8 ± 0.2	1.2 ± 0.4
p-cresol	μg/g	7.5 ± 2.1	11.1 ± 1.7	7.8 ± 0.7	6.7 ± 1.1

a) Data represented as mean ± SE.
b) Significantly different to Control.
c) Significantly different to Control + RS.
d) Significantly different to Heme.
e) Significantly different to Heme + RS.

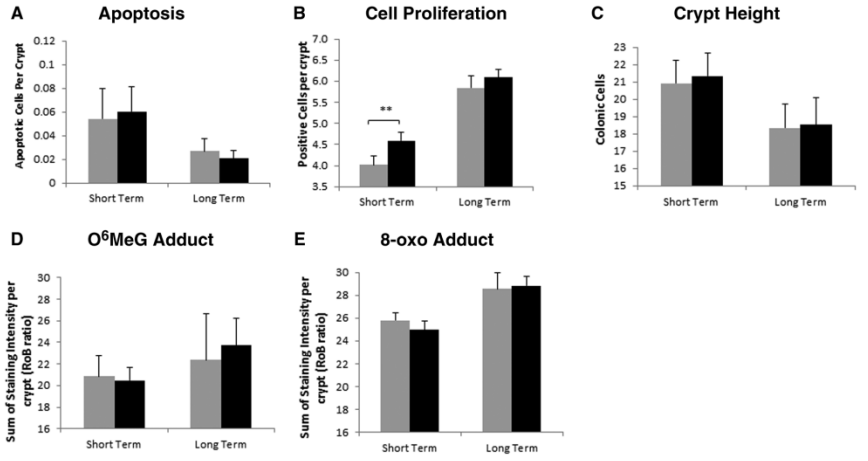

Figure 1. Effect of dietary heme on apoptosis (A), cell proliferation (B), colonic crypt height (C), O^6MeG adduct (D), and 8-oxo adduct (E) load in the distal colon after short-term and long-term experiments. Diets without heme represented by gray bars, diets with heme represented by black bars. Data are represented as means ± SE. $^{**}p < 0.01$

($p < 0.01$) (Table 3) compared to the control + RS diet. After long-term feeding, diets containing RS showed significantly reduced fermentation of phenols but not *p*-cresols compared to diets without RS ($p < 0.05$).

3.2 Short-term effects of heme and RS

In the short term, heme at 0.2 μmol/g in the diet increased cell proliferation (Fig. 1B) as measured by positive ki-67 cells in the colon compared to diets without heme ($p < 0.05$). RS significantly increased crypt height in the colon compared to diets without RS ($p < 0.05$) but it did not increase the proportion of ki-67-positive cells (Fig. 2B and C). There were no significant effects of heme or RS on apoptotic cell counts in colonic epithelial cells (Figs. 1A and 2A). There were no significant differences after 4 wk of feeding either in the O^6MeG adduct (Figs. 1D and 2D) or the 8-oxo adduct (Figs. 1E and 2E) between heme and control diets or after the addition of RS to the diet.

3.3 Long-term effects of heme and RS

After 18 months on experimental diets, those mice that were fed heme showed no changes in proliferation, crypt height, apoptosis, or DNA adduct levels compared to mice fed a heme-free diet (Fig. 1A–E). Diets containing RS increased colonic crypt height ($p < 0.01$) as well as cell proliferation ($p < 0.01$) compared to diets without RS (Fig. 2B and C). However, RS did not affect apoptosis or DNA adducts (Fig. 2C–E). Although it appears heme lowered neoplasm incidence in the colon, there were no statistically significant differences in colon neoplasm incidence for any of the dietary interventions (Table 4).

3.4 Diet-related changes over time

To ascertain if diets led to changes over time, we compared short- and long-term feeding of heme and RS (Fig. 3). Dietary heme significantly lowered rates of apoptosis in older mice compared to their younger counterparts (Fig. 3A, $p < 0.01$). Cell proliferation increased and crypt height decreased with age (Fig. 3B and C) for all dietary treatments ($p < 0.01$). Diets that contained heme ($p < 0.01$) or heme with RS ($p < 0.001$) showed a significant increase in O^6MeG DNA adduct accumulation over time; whereas diets without heme (control and control + RS) showed no significant difference between the two time points (Fig. 3D). 8-Oxo adducts in the colon accumulated over time (Fig. 3E) with all four dietary interventions (control: $p < 0.05$, control + RS: $p < 0.001$, heme: $p < 0.01$, and heme + RS: $p < 0.000001$).

4 Discussion

We have identified dietary heme at a human relevant amount to increase proliferation in colon cells of mice in the short

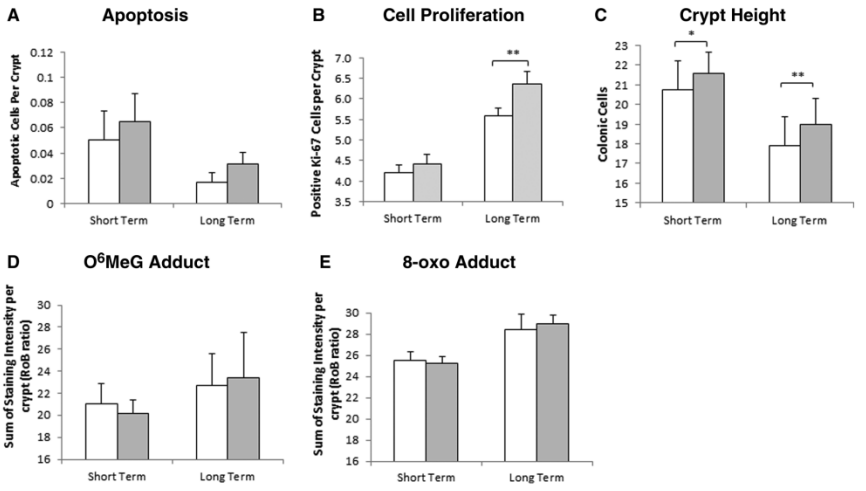

Figure 2. Effect of dietary RS on apoptosis (A), cell proliferation (B), colonic crypt height (C), O^6MeG adduct (D), and 8-oxo adduct (E) in the distal colon after short-term and long-term experiments. Diets without RS represented as white bars, diets with RS represented as gray bars. Data are represented as means ± SE. $*p < 0.05$, $**p < 0.01$

Table 4. Main effects of dietary interventions on colon neoplasm incidence

Heme Effect	Incidence (%)	RS effect	Incidence (%)
No Heme	4.45	No RS	3.35
Heme	0	RS	1.1
p value[a)]	0.134	*p* value[a)]	0.523

a) Cross tabulation performed for colon cancer using Fishers exact test.

term, inhibit colonic apoptosis over the long term, and contribute to accumulation of O^6MeG promutagenic adducts in the colon over time. Interestingly, these adverse changes invoked by this level of dietary heme did not increase rates of colon neoplasms within the western diet model of spontaneous CRC in the mouse.

There is evidence that increased heme intake from red meat will catalyze the production of more NOCs in the colonic lumen, leading to increased risk in developing colon cancer [20]. Heme-induced increases in NOC can lead to increased alkylating adducts in the colon, as NOC are potent initiators of such alkylating adducts [21]. We observed an accumulation of O^6MeG adducts over time with heme consumption, however this did not accelerate colorectal oncogenesis under the conditions of the experiment. It may be possible that as mice age, they become more susceptible to accumulation of DNA adducts in the presence of heme, possibly due to the cells being more inefficient at repairing such adducts with increasing age. Measurement of DNA repair of oncogenic adducts might be warranted in future studies to determine if the aging process effects DNA repair mechanisms and therefore accumulation of DNA lesions with consumption of high red meat diets.

Heme alone may catalyze the production of NOCs but red meat and particularly processed meat [32] contain NOCs that might further contribute to alkylating DNA lesions of the colon. This could be one reason why heme (at the studied dose) alone did not influence rates of CRC. Clearly, the alkyl adducts generated were not sufficient to initiate or promote CRC. Whether this was due to failure to reach a sufficient number or whether it was in the context of a low-risk setting for CRC, given that no other mutagen was given or that there was no high-risk factor present, we cannot be certain. This might be further explored by feeding higher amounts of heme but such would be above what is reasonably encountered in the human diet. It is likely that NOC generation in mice fed with heme diets would be minimal as NO and NO_2 (required for NOC generation) were not added to the diet or water, and rodent saliva does not recycle NO_2 as human saliva does [33]. Due to sampling limitations, NOCs were not measured in the current study, but this would be warranted in future studies examining dietary heme as a cancer-causing agent. It might also be that the duration of the current study may need

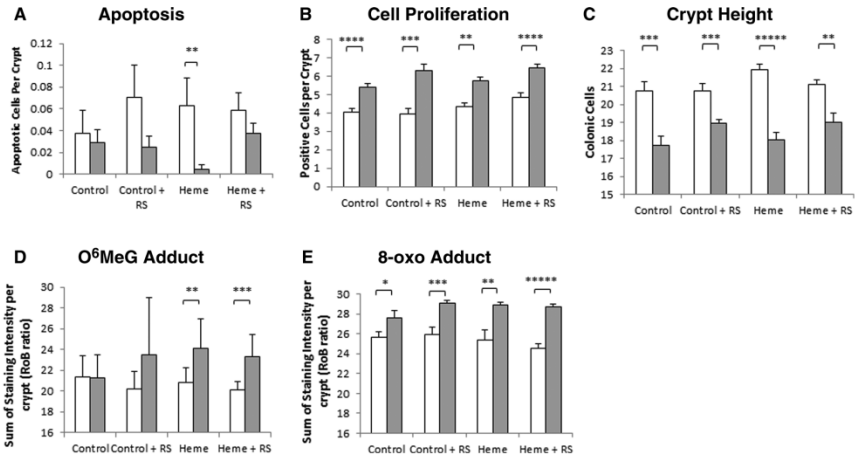

Figure 3. Changes in the distal colon over time from 4 wk (white bars) to 18 months (gray bars) for apoptosis (A), cell proliferation (B), colonic crypt height (C), O^6MeG adduct (D), and 8-oxo adduct (E). Data are represented by means ± SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.000001$, *****$p < 0.000001$.

to be extended beyond 18 months to see if there is further accumulation of O^6MeG adducts and whether this increases risk for CRC. The diets, we based our experiments on, were adapted from Newmark et al. [26, 27] where end-points of 18 and 24 months had significant increases in CRC compared to normal diets. However, due to a significant weight loss in heme-fed mice (Table 3), as well as some early losses of mice in all dietary interventions resulting from the low nutritional quality of the westernized diet, 18 months was used as the cutoff point in our study to avoid further loss of mice.

A high level of dietary heme has been previously shown by other researchers to increase cell proliferation and decrease apoptosis in the colon of rats, possibly as a result of increased oxidative stress and a loss of cell feedback between damaged cells and proliferating cells [34]. We also observed increased cell proliferative activity in the short term and a reduction of apoptosis over the long term in the current study, but this did not equate to a higher CRC risk. More recently, Ijssennagger et al. [35] showed that 0.2 μmol of heme was sufficient enough to induce acute oxidative stress and delayed cytotoxic stress in the lumen of mice after only 2–14 days of heme consumption, with a dramatic increase in proliferating colon cells. In a study where heme was used at a similar concentration of 0.25 μmol/g, ACF size increased; however, there was a statistically significant reduction in number of azoxymethane-induced ACFs per colon (after 100 days) compared to the control diet (92 ± 16 compared to 125 ± 25) [18]. There are some distinct differences in this study including different base diets used and the treatment with azoxymethane to drive the CRC pathway, whereas we used only heme as the potential carcinogenic agent. This indicates that heme may increase the risk of developing CRC at higher doses but not at lower doses more relevant to human consumption over a longer period of time, and could explain why we saw no significant effect on CRC in the current study. According to two studies [17, 19], the level of heme-iron in cooked red meat as beef is on average 2.63 mg/100 g of meat. This equates to an average concentration of heme in a 30% red meat diet to be 0.14 μmol/g [19]. Most studies have used much higher doses, up to ten times this amount of heme, ranging from 0.25 up to 1.5 μmol/g of diet [18, 34, 36], which is quite unrealistic in the context of human consumption. The concentration of heme in our study at 0.2 μmol is very similar to that of the high red meat diet of 30%, representing a maximum intake of heme achieved in humans. Although heme may disrupt homeostasis of the cell by increasing proliferation and decreasing apoptosis, this had no influence on initiation or promotion of CRC. The effect of heme in the form of hemin on colon carcinogenesis remains questionable, with levels of heme present in a relatively normal diet not affecting CRC risk in the western diet model of spontaneous CRC. However, more studies in other animal models that also include a more detailed dose evaluation are required to confirm heme's effect on colorectal carcinogenesis.

Our findings on the effects of dietary RS support previous literature that this food source acts to reduce toxic protein fermentation and acts as a promoter of SCFA production, particularly the anticancer compound butyrate. There is a firm

belief that the preferential fermentation of RS in the colon over protein leads to increased production of chemoprotective compounds and reduction of potentially toxic compounds in the lumen [24]. Rodent models consistently show fermentation of RS and production of SCFAs is linked to decreased rates of DNA lesions in colon cells, ACFs, and tumors in chemical carcinogenic models of CRC [22, 25, 37]. We have shown previously that RS is able to reduce DNA adducts induced by high red meat diets, but not high casein diets, without the use of chemical carcinogens, and this was related to increased SCFA and decreased protein fermentation [4]. The levels of heme used here did not induce detectable changes in DNA adducts between individual diets (both alkylating and oxidative) in the short term to detect a protective effect of RS despite increased SCFA production. Although control + RS diet appeared to increase O^6MeG adducts over time, there was a large variation between the mice and therefore this did not reach statistical significance. Also, over the long term, addition of RS was not able to reduce the observed increase of adducts induced by heme and one reason for this could be the decrease in SCFA production by RS in the presence of heme after long periods. It is likely that the bacterial population in the colon has shifted with consumption of heme over longer periods, favoring a move away from beneficial bacteria fermentation provided by an RS diet. Previous studies have confirmed that consumption of dietary heme does create shifts in populations of bacteria in the colonic lumen [38]. Due to the small fecal sample size of the mice, it was not possible to measure bacterial profile but this would be warranted in future studies to identify the interaction of heme with other dietary agents.

We have identified dietary heme as an agent that can increase DNA promutagenic adducts in the colon of mice, increase cell proliferation, and reduce apoptosis rates in the colon. We also confirm that RS can promote beneficial bacterial fermentation and reduce toxic protein fermentation in the colon of rodents over short periods, but SCFA production was not sustained over longer periods in combination with heme intake. All these changes observed had no influence on CRC risk when heme was studied at a human relevant amount with the western diet model of spontaneous CRC. The level of heme in a typical western diet may not be sufficient to initiate or promote CRC alone. This suggests that heme may act with other components of red and processed meat, such as NOCs, contributing to DNA damaging events in the colon that might lead to colorectal carcinogenesis.

Funding for this project was provided by the National Health and Medical Research Council of Australia (Project number 535079). We would like to acknowledge the Royal Society of Edinburgh for funding a visit for Dr. Silvia Gratz from UK to Australia to carry out work associated with this project.

The authors have declared no conflict of interest.

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Cancer Prevention Research

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Cancer Prev Res 2014;7:786-795.

Cancer Prevention Research

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Dietary Manipulation of Oncogenic MicroRNA Expression in Human Rectal Mucosa: A Randomized Trial

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Abstract

High red meat (HRM) intake is associated with increased colorectal cancer risk, while resistant starch is probably protective. Resistant starch fermentation produces butyrate, which can alter microRNA (miRNA) levels in colorectal cancer cells *in vitro*; effects of red meat and resistant starch on miRNA expression *in vivo* were unknown. This study examined whether a HRM diet altered miRNA expression in rectal mucosa tissue of healthy volunteers, and if supplementation with butyrylated resistant starch (HRM+HAMSB) modified this response. In a randomized cross-over design, 23 volunteers undertook four 4-week dietary interventions; an HRM diet (300 g/day lean red meat) and an HRM+HAMSB diet (HRM with 40 g/day butyrylated high amylose maize starch), preceded by an entry diet and separated by a washout. Fecal butyrate increased with the HRM+HAMSB diet. Levels of oncogenic mature miRNAs, including miR17–92 cluster miRNAs and miR21, increased in the rectal mucosa with the HRM diet, whereas the HRM+HAMSB diet restored miR17–92 miRNAs, but not miR21, to baseline levels. Elevated miR17–92 and miR21 in the HRM diet corresponded with increased cell proliferation, and a decrease in miR17–92 target gene transcript levels, including *CDKN1A*. The oncogenic miR17–92 cluster is differentially regulated by dietary factors that increase or decrease risk for colorectal cancer, and this may explain, at least in part, the respective risk profiles of HRM and resistant starch. These findings support increased resistant starch consumption as a means of reducing risk associated with an HRM diet. *Cancer Prev Res; 7(8); 786–95. ©2014 AACR.*

Introduction

The majority of colorectal cancers occur sporadically, with development influenced by environmental and lifestyle factors, including diet (1). Systematic reviews of cohort and case–control studies have found high red meat (HRM) or processed meat intake to be a convincing risk factor (1, 2), with intake of more than 500 g of cooked red meat per week significantly increasing colorectal cancer risk (1). Plausible mechanisms include inducing DNA strand breaks and enhancing promutagenic DNA adduct formation (3, 4). HRM consumption has also been linked to gut microbiome changes and inflammation (5, 6). In contrast, dietary fiber probably protects against colorectal cancer, with systematic review evidence identifying a dose–response relationship, and 10% decreased risk per 10 g fiber intake per day (1). Interventional studies provide less conclusive evidence, and longer-term trials and higher fiber levels may be needed to reproduce effects from observational studies (7).

One protective mechanism for fiber is the production of fermentation products, particularly the short-chain fatty acid (SCFA) butyrate (1). Butyrate is a histone deacetylase inhibitor, with antitumorigenic effects (8–12). Aberrant microRNA (miRNA) expression contributes to colorectal cancer development (13–15), with miRNAs such as miR21 and the miR17–92 cluster of miRNAs often increased in colorectal cancers and possessing oncogenic properties (16, 17). We have shown that butyrate can modulate miRNA expression in colorectal cancer cells *in vitro* (18). The miR17–92 cluster, comprising miR17, miR18a, miR19a, miR20a, miR19b, and miR92a, was significantly decreased with butyrate. This decrease may be partially responsible for the antiproliferative effects of butyrate, with addition of miR17–92 mimics reversing this and increasing proliferation; miR19a and miR19b in particular were key promoters of proliferation (18). Through epigenetic mechanisms, butyrate may be able to reverse the miRNA dysregulation observed in colorectal cancer (18).

Higher colonic butyrate levels can be achieved with resistant starch supplementation compared with other fiber sources (19). Resistant starch can also be acetylated with butyrate; butyrylated high amylose maize starch (HAMSB)

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doi: 10.1158/1940-6207.CAPR-14-0053

can deliver esterified butyrate to the human colon, leading to increased fecal butyrate compared with standard high amylose maize starch ($P < 0.0001$; refs. 20 and 21). In rodents, resistant starch supplementation to an HRM diet increased colonic butyrate, altered gut microbiota, decreased inflammation, and attenuated red meat-induced DNA damage (3, 5, 22). HAMSB was more effective than standard amylose maize starch in lowering genetic damage (23). One human trial has suggested inconclusively that fiber may modify DNA adduct formation in the context of HRM consumption (4); however, to date no other human trials have examined the combined effects of red meat and resistant starch. There has been no previous examination *in vivo* of the effects of these substances on miRNA expression in colorectal cells.

This human trial aimed to determine if consumption of a diet high in lean red meat altered miRNA expression in rectal mucosa tissue, and if supplementation with resistant starch could protect against this dysregulation by increasing butyrate levels in the colorectum. In a randomized cross-over design, markers of colorectal cancer risk were measured in healthy human volunteers who undertook 2 4-week intervention diets, an HRM diet, and an HRM diet supplemented with butyrylated resistant starch (HRM+HAMSB; StarPlus, National Starch and Food Innovation, Bridgewater, NJ). It was hypothesized that regulation of miRNA expression may partially explain some of the chemo-protective effects of resistant starch and the increased colorectal cancer risk associated with HRM intake.

Materials and Methods

Subjects

The randomized, controlled cross-over trial was approved by the Flinders Clinical Research Ethics Committee and Clinical Drug Trials Committee, and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12609000306213). Sample size was based on the anticipated effect on a primary outcome (fermentation products). A group size of $n = 20$ gave 80% power to detect a 20% change with 95% probability. Twenty-five were recruited to allow for dropouts. A computer-generated randomization sequence was implemented by a trial nurse, to determine which intervention diet was received first. Because of the nature of the interventions, participants were not blinded. Healthy volunteers ages 50 to 75 years, with no active bowel disease, and able to provide informed consent were eligible for inclusion. Exclusion criteria included evidence of active bowel disease or malabsorption, intolerance to high-fiber foods, perceived contraindication to consumption of the high protein diet, previous bowel surgery (excluding polypectomy), ingestion of regular laxatives or probiotic complimentary medicines, and antibiotic therapy in the previous 4 weeks. Patients were recruited by advertisement or invitation from their physician at the Flinders Medical Centre gastroenterology outpatient clinics, and written informed consent was obtained. Participants could withdraw at any time.

Study design

Dietary interventions were explained during clinic visits. The study consisted of 2 intervention periods of 4 weeks each, preceded by a 4-week run-in (entry) period and separated by a 4-week washout period. Volunteers were randomized to an HRM diet (300 g raw weight of lean red meat per day) or HRM+HAMSB diet (300 g raw weight of lean red meat per day with 40 g of butyrylated high amylose maize starch per day (StarPlus, which is 50%–60% resistant starch; ref. 24). Volunteers received the alternative diet for the second intervention. Meat was supplied in frozen packs of lean mince, beef strips, or lamb strips. HAMSB was supplied as prepacked 20 g sachets, with 2 to be consumed daily by mixing into 250 mL reduced fat milk or orange juice. During the HRM diet, participants were also required to consume 2 serves of reduced fat milk or orange juice per day, to match the HRM+HAMSB diet. The level of red meat used is tolerated well, with studies often using 400 g of red meat per day (4). Intervention studies have shown that 40 g per day of butyrylated resistant starch significantly raises colonic butyrate concentrations (21). Participants were to maintain their usual diet during the study but to avoid additional high-protein, fiber, or probiotic supplements, and any medication that could interfere with bowel function. Participants were monitored by a trial nurse and dietitian, to ensure intervention guidelines were followed, and weight was kept stable.

Sample collection and analysis

The effects of the dietary interventions on colonic fermentation product formation and on epithelial consequences were examined as primary outcomes. SCFA were measured from fecal samples, and miRNA expression changes, target gene levels, and cell proliferation were measured from rectal mucosa samples. Fecal and rectal pinch biopsy specimens were obtained at the end of each 4-week dietary period. Details of medical history and medications, weight, bowel health, and adverse events were collected throughout the study. Composition of the participants' diets and compliance with the interventions was assessed using weighed food diaries, completed by participants for the last 3 days of each 4-week dietary period. Food diaries were entered into Foodworks Professional 7 nutritional calculation software (Xyris Software) by a dietitian, to calculate energy and macronutrient intake based on Australian food composition tables and food manufacturers' data.

Fecal collection was conducted by participants for the last 24 hours of each dietary period. Samples were homogenized in 3 volumes of internal standard solution (1.68 mmol heptanoic acid/L) and centrifuged. The supernatant was vacuum distilled, and 0.2 μL of each distillate was loaded onto a Zebron ZB-FFAP gas chromatography (GC) column (Phenomenex) within an Agilent 6890N Network GC system (Agilent). Concentrations of acetate, butyrate, propionate, and total SCFAs were reported.

Participants undertook anal examination by an experienced gastroenterologist at the end of each dietary period,

and 4 pinch rectal biopsies were taken with forceps through sigmoidoscopic examination performed without bowel preparation. Two biopsies of <0.5 cm in any dimension were stored in RNAlater (Ambion). Additional biopsies were formalin fixed. Biopsies stored in RNAlater were used for quantitation of miRNA and target gene mRNA levels. Each biopsy was homogenized in 0.5 mL TRizol Reagent (Ambion), and total RNA extracted according to the manufacturer's instructions. RNA was quantified using a Nanodrop-8000 spectrophotometer (Nanodrop Technologies).

miRNA expression analysis was conducted by relative quantitation real-time RT-PCR using TaqMan miRNA assays (Applied Biosystems). cDNA was synthesized from 20 ng total RNA using miRNA-specific primers, and real-time PCR was carried out using triplicate 10 μL reactions for each biological replicate including 1 μL of reverse transcription product, 0.5 μL miRNA-specific primer, and probe assay mix, and 1 × TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems; assay IDs: miR17: 000393, miR18a: 002422, miR19a: 000395, miR20a: 000580, miR19b: 000396, miR92a: 000430, miR16: 000391, miR21: 000397). Results were normalized relative to the endogenous small nuclear RNA gene RNU6B (assay ID: 001093) using Qgene (25). For mRNA expression analysis, real-time RT-PCR was conducted using TaqMan Gene Expression assays (Applied Biosystems). cDNA was synthesized from 0.6 μg total RNA using M-MLV Reverse Transcriptase, RNase H minus (Promega), and random hexamer primers. Real-time PCR was carried out using triplicate 10 μL reactions including 2 μL of RT product, 0.5 μL mRNA-specific gene expression assay mix, and 1× TaqMan gene expression master mix (Applied Biosystems; assay IDs: CDKN1A: Hs00355782_m1, PTEN: Hs02621230_s1, BCL2L11: Hs00708019_s1). Results were normalized relative to endogenous controls ACTB (β-actin; assay ID: Hs99999903_m1) and B2M (β-2-microglobulin; assay ID: Hs00984230_m1) using QbasePlus (Biogazelle).

To assess the proliferative activity and distribution of proliferating cells in the colonic crypts, the proliferating cell nuclear antigen (PCNA) assay was performed using standard immunohistochemical procedures (11). Deparaffinized rectal biopsy sections were rehydrated in a graded series of ethanol from 100% to 50% and then distilled water. Primary mouse monoclonal antibody (PC10; Santa Cruz Biotechnology) was placed on slides (1:500 dilution) and incubated overnight, followed by incubations with a Murine Ultra-Streptavidin HRP Detection Kit (Covance Laboratories). Visualization was performed using 3,3′-diaminobenzidine chromagen and substrate buffer (Covance Laboratories), with slides counterstained with hematoxylin. PCNA-positive cells were identified in 20 randomly chosen intact crypts by an independent observer who was blinded to the treatment groups.

Statistical analysis

Data were presented as mean ± standard error of the mean (SEM), with graphs prepared using GraphPad Prism 6 (GraphPad Software Inc.). Statistical analyses were performed in IBM SPSS Statistics 22 (IBM SPSS Inc.) using the repeated measures general linear model, with statistical significance for paired comparisons obtained using Sidak multiple comparisons test. For each variable, data were assessed for carry-over and period effects. For variables with no significant carry-over or period effects, data from both intervention periods were combined and the repeated measures analysis was used. Secondary analyses were performed for outcomes where there was a significant effect of treatment order, with the group that consumed the HRM diet first analyzed independently from the group that consumed the HRM+HAMSB diet first. An adjusted P value < 0.05 was considered significant.

Results

Demographic data and dietary intake

Recruitment commenced in July 2009, with participants followed-up for the 4-month duration of the interventions. Data collection was completed by September 2010. Twenty-five participants were randomly assigned, with 12 allocated to the HRM dietary intervention first, and 13 allocated to the HRM+HAMSB dietary intervention first (Fig. 1). Two participants withdrew before commencing the interventions; 1 because of unrelated medical problems, and 1 because of intolerance of the first rectal biopsy. At study completion, 23 participants had received both intervention diets (17 males and 6 females, ages 50–75 years), and data from these participants were analyzed. There were no major complications, and all 23 volunteers tolerated sample collection and the interventions, except 1 volunteer who usually had a vegetarian diet and found 300 g red meat per day difficult to tolerate. Approximately one third of volunteers reported increased flatulence on trial diets; it was unclear whether this could be linked to increased red meat or resistant starch intake. Participants maintained consistent body weight (mean of 80 kg after each diet; $P > 0.05$). Weighed food diaries showed that compared with the entry diet, protein intake was significantly increased in the HRM diet ($P < 0.0001$) and in the HRM+HAMSB diet ($P = 0.001$); intake was similar in the HRM and HRM+HAMSB diets ($P = 0.33$). Fiber and starch intake were decreased in the HRM diet compared with the entry ($P < 0.0001$ and $P = 0.02$, respectively) and HRM+HAMSB diets ($P < 0.0001$ and $P = 0.02$, respectively).

Fecal SCFA levels

There was a significant increase in acetate ($P = 0.002$), propionate ($P = 0.0006$), butyrate ($P = 0.005$), and total SCFA ($P = 0.0008$) in fecal samples for the HRM+HAMSB diet compared with the entry diet, and a significant increase in propionate ($P = 0.02$) and butyrate ($P = 0.04$) for the HRM+HAMSB diet compared with the HRM diet (Fig. 2). There was no significant difference between the entry and HRM diets for any of the SCFAs measured ($P > 0.05$), and between the entry and washout diets for any of the SCFAs measured ($P > 0.05$).

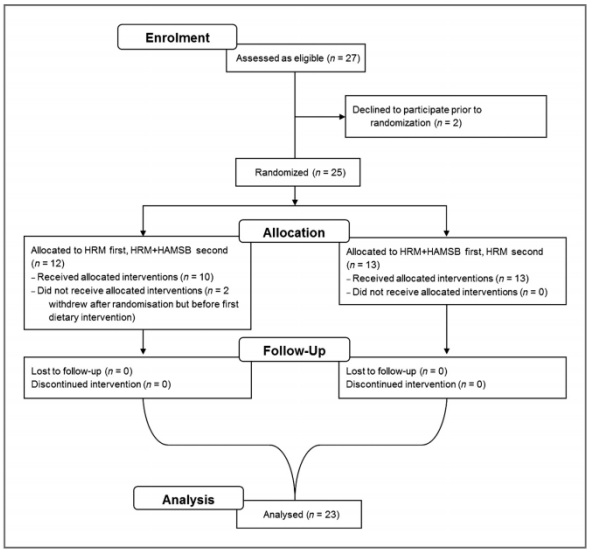

Figure 1. CONSORT diagram of participant flow for the HRM and resistant starch trial. HRM, red meat diet; HRM+HAMSB, red meat and resistant starch diet.

miRNA expression changes

miR17–92 cluster miRNA levels were examined in the rectal biopsy specimens, as these were altered with butyrate treatment in previous studies *in vitro* (18). Two miRNAs that were not altered by butyrate *in vitro* were examined for comparison (18); miR21, an oncogenic miRNA, and miR16, a miRNA that is generally abundantly and ubiquitously expressed in normal tissue (26). Levels of miR17–92 cluster miRNAs increased with the HRM diet compared with the entry diet, but not with the HRM+HAMSB diet. The increased expression of miRNAs within the miR17–92 cluster with the HRM diet versus the entry diet was significant for miR19a ($P = 0.04$) and miR19b ($P = 0.007$), and approaching significance for miR20a ($P = 0.08$; Fig. 3A). This rise in miR17–92 miRNA levels with the HRM diet alone was approximately 30% (Fig. 3B). Conversely, in the HRM+HAMSB diet, the miR17–92 cluster miRNA levels were lower than with the HRM diet alone (approximately 20%), which was significant for miR17 ($P = 0.005$), miR19a ($P = 0.04$), miR20a ($P = 0.003$), miR19b ($P = 0.02$), and miR92a ($P = 0.02$). There was no significant difference between the entry and HRM+HAMSB diet for any of the miR17–92 cluster miRNAs ($P > 0.05$), and no significant difference between the entry and washout diets for any of the miR17–92 miRNAs ($P > 0.05$). miR16 seemed stably expressed regardless of the intervention ($P > 0.05$ for all comparisons; Fig. 3C). There was a significant increase in miR21 with the HRM diet compared with the entry diet ($P = 0.03$), and a trend toward an increase with the HRM+HAMSB compared with the entry diet ($P > 0.05$; Fig. 3C). There was no significant difference between the HRM and HRM+HAMSB diets for miR21 ($P > 0.05$); thus, HRM seemed to alter miR21 levels, but resistant starch supplementation had little protective effect.

miR17–92 target gene changes

miR17–92 cluster miRNAs target genes that are important in cell-cycle control, including the cell-cycle inhibitor *CDKN1A* (target of miRs 17 and 20a) and the proapoptotic genes *PTEN* (target of miRs 17, 19a, 19b, and 20a) and *BCL2L11* (target of miRs 17, 18a, 20a, and 92a; refs. 27–30). The influence of the diet-induced changes in miR17–92 cluster miRNA levels on these target genes was investigated in the rectal biopsy samples. There was a trend toward decreased *CDKN1A*, *PTEN*, and *BCL2L11* mRNA levels with the HRM diet compared with the entry diet, which was statistically significant for *CDKN1A* ($P = 0.04$; Fig. 4A). For *PTEN* and *BCL2L11*, the HRM+HAMSB diet was not significantly different from the entry diet ($P > 0.05$) or HRM diet ($P > 0.05$); for *CDKN1A* there seemed to be decreased mRNA levels with the HRM+HAMSB diet compared with the entry diet ($P = 0.02$). *CDKN1A* and *BCL2L11* mRNA

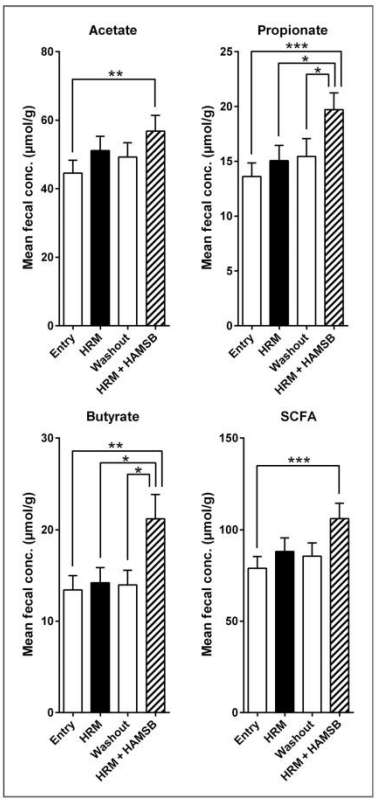

Figure 2. Fecal butyrate, acetate, propionate, and total SCFA levels of participants in the HRM and resistant starch trial. Fecal samples collected at the end of each 4-week diet (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). The mean ± SEM of the 23 participants is shown for each diet. Entry, entry diet; HRM, red meat diet; HRM+HAMSB, red meat and resistant starch diet.

levels seemed lower with the washout diet than with the entry diet; however, this was not statistically significant ($P > 0.05$).

Cell proliferation

A PCNA assay was used as a proliferation marker in the fixed rectal biopsies. The HRM diet increased proliferation compared with the entry diet ($P = 0.02$; Fig. 4B). Proliferation with the HRM+HAMSB diet seemed intermediate between the HRM diet and the entry diet, and not significantly different from either the entry diet ($P > 0.05$) or HRM diet ($P > 0.05$). Proliferation with the washout diet was significantly higher than with the entry diet ($P = 0.02$). There was a significant effect of treatment order; consuming the HRM diet first produced significantly higher proliferation compared with consumption of the HRM+HAMSB diet first ($P = 0.04$), and compared with consumption of the HRM diet as the second treatment ($P < 0.01$; Fig. 4C).

Discussion

This randomized cross-over trial is the first to examine the effects of an HRM diet and butyrylated resistant starch supplementation on miRNA expression in rectal mucosa of healthy volunteers. In a novel finding, HRM intake was shown to alter miRNA levels in rectal mucosa tissue, whereas HAMSB could mitigate some of these changes. HRM intake significantly increased rectal mucosa levels of miR17–92 cluster miRNAs and miR21, which are both elevated in colorectal cancer (16, 17). The miR17–92 cluster has been designated oncomir-1 (31), and can promote proliferation and angiogenesis, inhibit differentiation, and sustain cell survival (29, 30). Elevated miR17–92 levels have been associated with invasion and metastasis of colorectal cancer cells (32), and poorer survival (33). miR19a and miR19b in particular are key oncogenic determinants (29, 30), and both were significantly elevated with the HRM diet compared with the entry diet. miR21 has similarly been classed as oncogenic, and can also induce tumorigenesis, invasion, and metastasis (34–36). Elevated miR21 in colorectal cancer has been linked to poorer survival and therapeutic outcomes (37).

Although HRM intake increases colorectal cancer risk, butyrylated resistant starch can potentially ameliorate some of these effects. Rodent studies have shown that resistant starch can raise colonic butyrate levels, alter gut microbiota abundance, reduce adenocarcinoma formation in response to a carcinogen, and attenuate red meat-induced DNA damage (5, 11, 22, 38). In this human study, supplementation with butyrylated resistant starch significantly raised fecal butyrate levels. The study identified a novel mechanism by which resistant starch can be beneficial for bowel health, with some of the miRNAs that were elevated in rectal tissue with the HRM diet alone reduced and restored to baseline levels with resistant starch supplementation. In particular, miR17–92 miRNA levels were significantly lower when the HRM diet was supplemented with resistant starch. miR21 and miR16 levels remained similar in the HRM diets irrespective of resistant starch supplementation. The persistent elevation of miR21 with red meat intake warrants further investigation to determine any impact on colorectal cancer risk.

As miRNAs can simultaneously target hundreds of mRNAs, even small expression changes can have important cellular effects (39). miR17–92 and miR21 promote

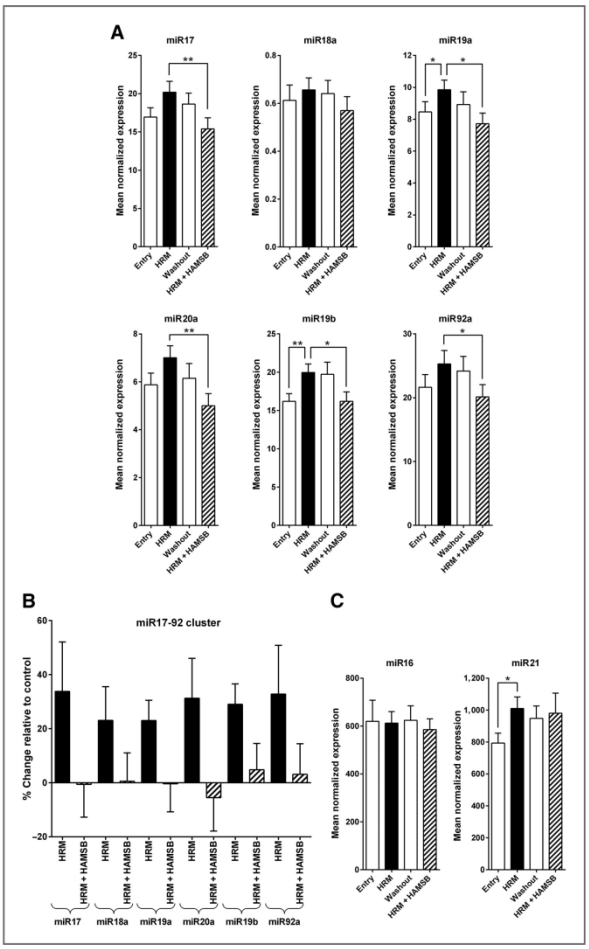

Figure 3. miR17–92, miR16, and miR21 levels in rectal biopsies from participants in the HRM and resistant starch trial. Rectal biopsies collected at the end of each 4-week diet (*, $P < 0.05$; **, $P < 0.01$). Expression measured by real-time RT-PCR and normalized to RNU6B levels. A, miR17–92 miRNA levels shown for each diet. B, summary of miR17–92 levels for the intervention diets, presented as percent change from entry diet. C, miR16 and miR21 levels shown for each diet. The mean ± SEM of the 23 participants is shown for each diet. Entry, entry diet; HRM, red meat diet; HRM+HAMSB, red meat and resistant starch diet.

proliferation (18, 29, 30, 34, 35), and examination of target gene expression and cell proliferation provided preliminary evidence about the possible influence of detected miRNA changes on cellular function. The increased miR17–92 miRNA levels with the HRM diet were associated with a decrease in mRNA levels of target genes, particularly the cell-cycle inhibitor *CDKN1A*. Through target gene regulation, the increase in miR17–92 miRNAs and miR21 with the HRM diet may contribute to the corresponding increase in cell proliferation. Resistant starch supplementation seemed

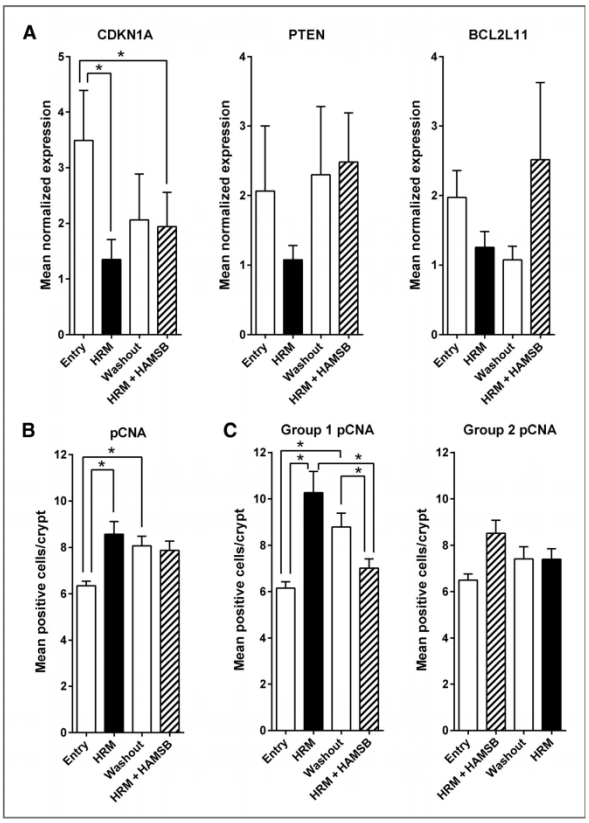

Figure 4. Gene expression and cell proliferation in rectal biopsies from participants in the HRM and resistant starch trial. Rectal biopsies collected at the end of each 4-week diet (*, $P < 0.05$). A, *CDKN1A*, *PTEN*, and *BCL2L11* mRNA levels shown for each diet. mRNA levels measured by real-time RT-PCR and normalized to *ACTB* and *B2M* levels. B, cell proliferation shown for each diet, measured by PCNA assay. C, cell proliferation by treatment group (group 1 had HRM diet first, group 2 had HRM+HAMSB diet first). The mean ± SEM of the 23 participants is shown for each diet. Entry, entry diet; HRM, red meat diet; HRM+HAMSB, red meat and resistant starch diet.

unable to completely restore proliferation to baseline, which could be associated with the miR21 levels that remained elevated compared with the entry diet. Other regulatory factors, including other miRNAs, may also be involved. It should be noted that the length of the washout period may have been insufficient for these outcomes; proliferation after the washout was significantly higher than after the entry diet, for example, indicating a potential carry-over effect. High variability in mRNA levels also limits the ability to draw firm conclusions from these data, and a larger sample size may have been needed for this outcome.

Another study limitation is the identification of correlations that do not necessarily have cause-and-effect relationships; for instance, it is unclear what component of red meat is increasing miR17–92 and miR21 levels. Heme iron, for example, has been associated with altered gene expression and hyperproliferation of colonic epithelium (40), and heme can also play a role in miRNA processing (41); however, any dietary heme uptake is likely limited to surface epithelial cells (40). High fat or high cholesterol diets can also alter miRNA expression in liver cells (42). It is also not conclusively determined what aspect of the resistant starch is protective. As butyrylated resistant starch was used, this is

likely to have directly administered further butyrate to the colon; butyrylated resistant starch can also be more effective in reducing carcinogen damage than standard resistant starch (23, 43). Offering support for the hypothesis that the miRNA changes with resistant starch supplementation may be due to increased butyrate production, is the *in vivo* replication of *in vitro* findings from previous work where the miR17–92 cluster but not miR21 or miR16 responded to butyrate treatment (18).

An important difference between this study and previous *in vitro* work was that it was performed in volunteers with normal rectal mucosa, rather than in colorectal cancer cells. Butyrate is a preferred energy source for normal colonic epithelium and assists in normal proliferation; whereas alternative fuel sources are preferred in colorectal cancer cells, and butyrate instead can inhibit proliferation and induce differentiation or apoptosis (9, 44, 45). Observations in carcinogen-treated rats showed that the colon cells responded to high butyrate levels in a manner more similar to cancer cells, with decreased proliferation and enhanced apoptosis (11, 38). In this study, there was a similar regulation of the miR17–92 cluster by butyrate in healthy rectal cells *in vivo*, as previously shown in colorectal cancer cells *in vitro*. This was observed in the context of HRM, with resistant starch restoring miRNA levels to those of the entry diet. Although there was no significant carry-over effect at the end of the washout period for any miRNA measured, for participants who had the HRM diet first, it is possible that residual red meat effects at the start of the washout period may have reduced the extent to which the resistant starch decreased miR17–92 miRNA levels, potentially leading to an underrepresentation of the degree of attenuation.

This study presents the first evidence in humans that HRM and butyrylated resistant starch have opposing effects on miRNA levels in rectal mucosa. Several studies have examined the effect of dietary components in other *in vivo* models. Examination of the miRNA response in rats fed diets containing corn or fish oil with pectin or cellulose and injected with a carcinogen or saline control particularly demonstrated a novel role for fish oil in protecting the colon from carcinogen-induced miRNA dysregulation, rather than a role for fiber (46, 47). Shah and colleagues (47) did however demonstrate that various dietary combinations and carcinogen exposure modulated a number of miRNAs, including miR17–92 cluster miRNAs and miR21 (47).

The oncogenic miR17–92 cluster was shown to be differentially regulated by dietary factors that increase or decrease colorectal cancer risk, and this may explain, at least in part, the respective risk profiles of HRM and resistant starch. Although the HRM diet increased miR17–92 cluster miRNA levels in rectal mucosa, with downstream consequences, addition of butyrylated resistant starch to the HRM diet restored miR17–92 levels to baseline. Although the red meat intake during the trial may exceed levels consumed by many in the general population, red meat intake in developed countries is substantial. Total meat consumption in the United States, European Union (EU), and the developed world has continued to increase from 1961 to 2003; nearly doubling in the EU and increasing 1.5-fold in the United States (48). In the United States, per capita total loss-adjusted meat consumption in 2004 was 154 g per day (48). The quantity of resistant starch used in the trial could be realistically applied to the general population. Long-term resistant starch supplementation in select populations has been shown to be feasible (49), and there has been a recent expansion in commercially available foods with increased resistant starch content (50). The findings in this study support increased resistant starch consumption as a means of reducing risk associated with an HRM diet.

Disclosure of Potential Conflicts of Interest

N.A. Kennedy has received speakers' bureau honoraria from MSD and has provided expert testimony (support to attend meetings) for Ferring, Abbvie, Shire, Warner Chilcott, and Norgine. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: M.Z. Michael, R.K. Le Leu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.J. Humphreys, G.P. Young, N.A. Kennedy, M.Z. Michael, R.K. Le Leu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.J. Humphreys, G.P. Young, R.K. Le Leu
Writing, review, and/or revision of the manuscript: K.J. Humphreys, M.A. Conlon, G.P. Young, D.L. Topping, Y. Hu, J. Winter, A.R. Bird, L. Cobiac, N.A. Kennedy, M.Z. Michael, R.K. Le Leu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.J. Humphreys, J. Winter
Study supervision: G.P. Young, D.L. Topping, Y. Hu, M.Z. Michael

Acknowledgments

The authors thank the volunteers for taking part in this study, the clinical trial nurses Libby Bambacas and Jane Upton for their help in coordinating the volunteers and sample collection, Dr. Julie Clarke for arranging the supply of butyrylated starch and its preclinical evaluation.

Grant Support

This work was supported by the National Health and Medical Research Council of Australia (Project no. 535079) and the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Preventative Health Flagship (grants to G. Young, R. Le Leu, D. Topping, A. Bird, M. Conlon), and the Flinders Medical Centre Foundation (grant to M. Michael).

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Received February 16, 2014; revised April 14, 2014; accepted May 12, 2014; published online August 4, 2014.

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