The effects of a high carbohydrate and high fat diet and dietary interventions in the molecular tumourigenesis of colorectal cancer

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Abstract

Colorectal cancer (CRC) is the most commonly diagnosed cancer affecting both men and women in Australia. The Epidermal Growth Factor Receptor (EGFR) plays an important role in the development and progression of CRC. EGFR regulates a number of essential cellular processes including proliferation, migration, differentiation and survival however the overexpression of EGFR is a major driver of CRC tumourigenesis. Numerous studies have demonstrated that there is a strong association between a high carbohydrate and high fat diet (HCHF), EGFR expression and the tumourigenesis of CRC. One of the activators of EGFR signalling is A disintegrin and metalloprotease domain 17 (ADAM17) and this protein has also been implicated in high fat diet induced CRC. One of ADAM17's roles is the proteolytic cleavage of EGFR activating ligands including the transforming growth factor alpha (TGFα).

Curcumin has demonstrated promising anti-inflammatory, antineoplastic, antioxidant and chemopreventative properties in the colon and has been shown to suppress the growth of colon cancer cells in animal models and human cell culture. The effectiveness of curcumin in reducing the expression of CRC biomarkers caused by a HCHF diet has not yet been investigated but may prove to be an effective strategy for preventing CRC.

This study provided evidence that a HCHF diet significantly increases the mRNA expression of the CRC biomarkers *EGFR, ADAM17* and *TGFα* in the distal colon tissue obtained from rats fed a HCHF diet compared to those fed a normal corn starch diet. Furthermore, it demonstrated that the effects of these changes could be prevented by supplementing the HCHF diet with 100 mg/kg body mass of curcumin. A preliminary experiment also found that the molecular changes that occurred in response to the HCHF diet could be driven by the aberrant expression of epigenetic modifying enzymes. Understanding the mechanisms by which diet

regulates tumourigenic changes in the colon is vitally important as diet is modifiable and may be a crucial target for preventing high fat diet induced CRC or at least reducing risk.

Declaration

I hereby declare that the work presented in this document is of my own creation, except for where acknowledged. I also declare that this research is original and has previously not been submitted for assessment.

Signature of Candidate:  Date: 11/11/2016

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Endorsement:

Supervisor:  Date: 11/11/2016

Dr Eliza Whiteside

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I would like to dedicate this project to my grandmother who passed away during this year. Each and every day you inspired me to pursue my passion and for that I will be forever grateful.

In loving memory of

Jean Frances Payne

(1941-2016)

List of Abbreviations

ADAM10- a disintegrin and metalloprotease domain 10

ADAM17- a disintegrin and metalloprotease domain 17

APC- adenomatous polyposis coli

BRAF- v-Raf murine sarcoma viral oncogene homolog B

CIN- Chromosomal instability

CIMP-CpG island methylator phenotype

CRC- Colorectal cancer

CS- Corn starch

DNMT3a- DNA methyltransferase 3a

EGFR- Epidermal Growth Factor Receptor

EZMT2- euchromatic histone lysine methyltransferase 2

HAT- Histone acetyltransferase

HCHF- High carbohydrate, high fat

HDAC- Histone deacetylase

H8C8- High carbohydrate diet 8 weeks, Corn starch diet 8 weeks

HMT-Histone methyltransferase

HUVEC- human vascular endothelial cells

HVSMC- human vascular smooth muscle cells

MSI- microsatellite instability

RT-qPCR- real time reverse transcription quantitative polymerase chain reaction

TGFα-Transforming growth factor alpha

TP53- Tumour protein 53

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Glossary

Adenocarcinoma: a malignant tumour formed in the glandular structures in epithelial tissue

Angiogenesis: the process in which new blood vessels are formed

Biomarker: a naturally occurring molecule, gene or characteristic by which a particular pathological or physiological process or disease can be identified

Cytokine: A small protein released by cells that exerts particular effect on interactions between cells or on cell behaviour.

Dimierisation: a chemical reaction in which two identical molecular entities react to form one single dimer

Differentiation: process by which cells or tissue change from relatively generalised to highly specialised during development

Epigenetic: relating or arising from non-genetic influences on gene expression

Epimutation: A heritable change in gene expression that does not impact actual base pair sequence of DNA

Histone: any group of five small basic proteins occurring in the nucleus of eukaryotic cells that organise DNA strands into nucleosomes by forming molecular complexes around which DNA winds.

Homodimer: A protein made of paired identical polypeptides

Heterodimer: a protein comprised of two polypeptide chains differing in composition in the order, number or kind of amino acid residues

Hypermutation: process by which mutations are occurring at a higher frequency then expected

Hyperplastic: an abnormal increase in the number of cells in an organ or tissue with consequent enlargement

Knock-down/out: genetic technique that leads to gene becoming inoperable

Neoplastic: relating to neoplasm which is characterised as uncontrolled growth of abnormal tissue

Phosphorylation: The transferring of phosphoryl group from a donor to the recipient molecule

Polyp: a small clump of cells protruding from the mucous membrane of the colon

Polyphenol: a compound containing more than one phenolic hydroxyl group

Post- translational modification: processes occurring after translation of a polypeptide, that are often needed to generate a fully functional polypeptide. modifications include the addition or removal of functional groups such as acetyl, methyl or phosphate group to the n-terminal tail of histones

Proliferation: rapid multiplication of cells

Tumourigenesis: process of tumour formation

1.1 Cancer and tumourigenesis

Cancer is a disease characterised by the uncontrolled proliferation of abnormal cells which can invade into surrounding tissue layers and metastasise to distant tissues via the blood and lymphatic systems (Bardhan & Liu 2013). The proliferation of normal cells is tightly regulated via a balance between both growth promoting and growth supressing signals, so that proliferation only occurs when required (for example in tissue repair and growth) (Sa & Das 2008). Tumourigenesis is the process whereby cells accumulate DNA mutations or epimutations that stimulate growth promoting signals and/or inhibit growth suppressing signals even when cells have experienced DNA damage. These DNA mutations may be germline (occurring in all of the body's cells) mutations in genes that usually control the cell cycle such as Tumour Protein 53 *(TP53)* or DNA repair such as Breast Cancer 1 *(BRCA1)* or can be sporadic mutations in these genes arising only in the precursor primary tumour cells. Mutations alter the DNA sequence of the gene and can change the structure and/or function of the transcribed protein. Epimutations do not actually change the DNA sequence of genes but influence their expression by increasing or decreasing the methylation of their promoters. Epimutations that can drive tumourigenesis occur in genes that control growth factor signalling, DNA damage repair, cell migration and invasion. Over time, the accumulation of mutations and/or epigenetic mutations in tumourigenic cells further stimulates the tumour to degrade and migrate into surrounding tissue and in some instances, cells from the tumour can invade into blood and lymphatic vessels and spread to distant parts of the body forming secondary or metastatic tumours (Al-Sohaily et al. 2012; Lahtz & Pfeifer 2011).

1.2 Colorectal cancer development and progression

The incidence of colorectal cancer (CRC) is increasing globally with the highest incidence occurring in Australia (as illustrated in Figure 1) (Torre et al. 2015). CRC is now the most commonly diagnosed cancer affecting both men and women in Australia (Australian Institute of Health and Welfare, AIHW, 2015). In 2011 a total of 15,151 new cases were diagnosed in Australia and 17,070 new cases were projected to be diagnosed in 2016 (AIHW, 2015). Patients diagnosed with metastatic CRC currently have a five year survival rate of less than 15% (Pabla et al. 2015). CRC occurs in both hereditary and sporadic forms, with sporadic forms most strongly associated with lifestyle factors such as consuming a diet that is high in simple carbohydrates, high in animal fats and low in dietary fibre as well as physical inactivity (Schulz et al. 2014). A poor diet combined with physical inactivity can lead to changes in metabolism which has consequences at both a cellular and molecular level, potentially driving CRC tumourigenesis (Hagland et al. 2013). Understanding the mechanisms by which diet regulates tumourigenic changes in the colon is vitally important as diet is modifiable and may be a crucial target for preventing colorectal cancer or at least reducing the risk of it occurring.

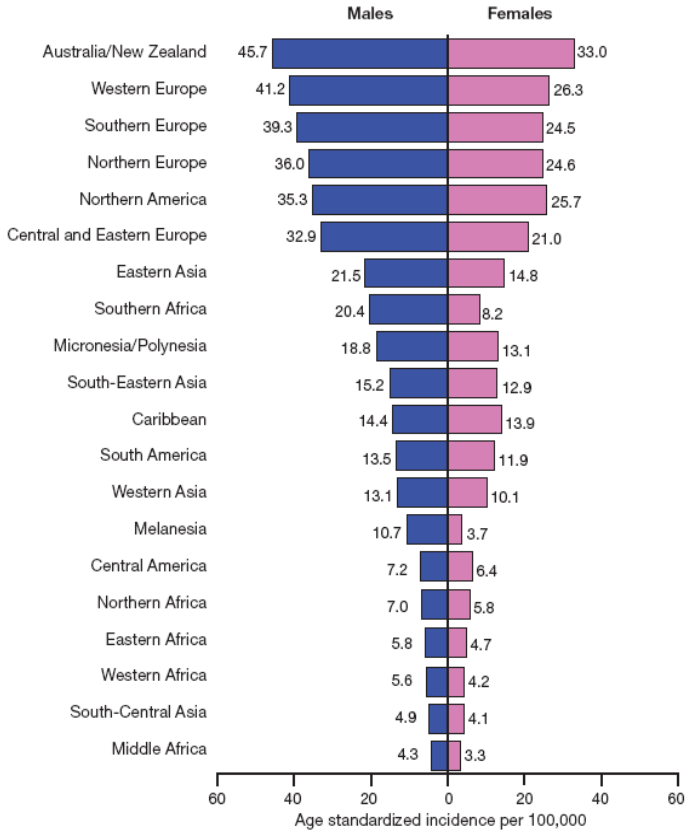

Figure 1: Global colon cancer statistics demonstrating the highest incidence in Australia and New Zealand (Torre et al. 2015).

CRC is caused by the accumulation of genetic and/or epigenetic mutations within a population of epithelial cells in the colon or rectum. This drives these cells to transform into an adenocarcinoma (Bardhan & Liu 2013). CRC usually progresses slowly with the disease typically arising from a precursor legion such as a benign polyp (hyperplastic polyp) within the epithelial lining of the colon or rectum. As mutations continue to occur there is an increased risk of the polyp transforming into a premalignant adenoma (tubular adenoma) which can then progress into malignant adenocarcinoma, which if untreated can metastasise to distant sites such as liver and lung tissue (as illustrated in Figure 2) (Bardhan & Liu 2013; Hagland et al. 2013). The development of genetic instability is a key driver of CRC tumourigenesis and the three main phenotypes/pathways in which it occurs are chromosomal instability (CIN), microsatellite instability (MSI) and the CpG island methylator phenotype (CIMP). The majority of sporadic CRC arises as a result of CIN. The CIN phenotype is characterised by genetic alterations such as insertions, inversions, deletions and rearrangements within the chromosome (Lengauer et al. 1997), all of which cause changes in gene expression. The exact mechanisms causing CIN are not fully understood, however genes believed to be implicated in the development of CIN are involved in cell cycle checkpoint, mitotic spindle check point, chromosome segregation and condensation and sister chromatid cohesion (Hagland et al. 2013). CIN tumours have been shown to display a number of commonly mutated genes implicated in the pathogenesis of CRC including adenomatous polyposis coli (*APC*), Kirsten Rat Sarcoma Viral Oncogene Homolog (*KRAS*) and *TP53* (Geigl et al. 2008). MSI accounts for approximately 15-20% of sporadic CRCs and are a result of epigenetic silencing of the *MLH1* gene and other genes involved in DNA mis-match repair and are commonly associated with the methylation of CpG islands. CIMP is characterised by changes in methylation patterns of promoter regions of a number of genes (Hagland et al. 2013). CpG islands are located in

promoter region of 50% of genes and therefore aberrant expression in these areas can have deleterious effects, with changes in methylation described as a major driver of tumourigenesis as it causes aberrant silencing or expression of genes that control tumour suppression, cell cycle activation and control, DNA repair and/or cell invasion (Kim & Deng 2007).

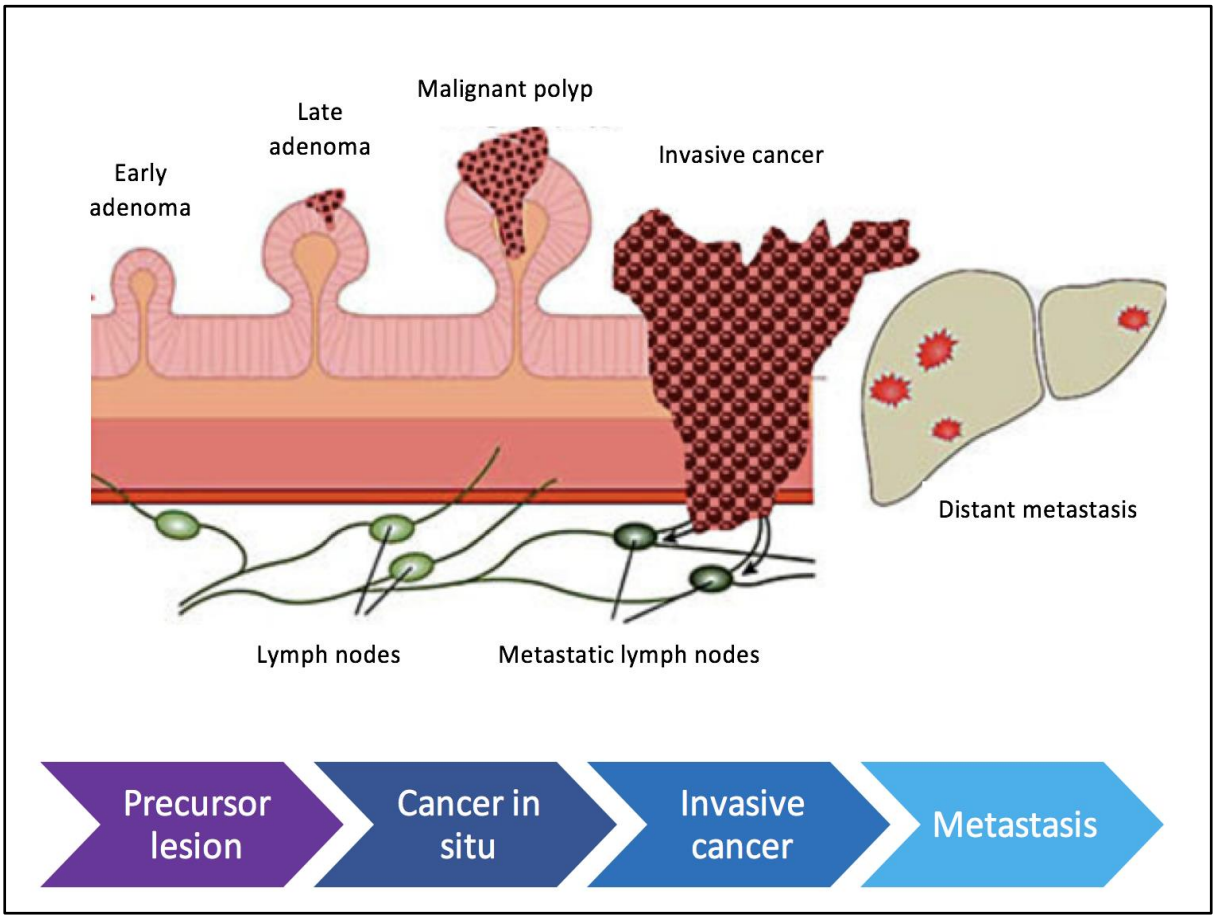

Figure 2: Development and progression of CRC from precursor lesion to distant metastasis (Hagland et al. 2013).

1.3 Proximal versus distal colon cancer

Until early this decade, CRC was treated as one disease, however recently it has become apparent that tumours arising in the proximal or right side colon commonly display a

molecular profile with mutations and epigenetic modifications that are different to those seen in distal or left side colon tumours (outlined in Figure 3). The progression of CRC is also site-specific with proximal tumours disseminating more commonly to abdominal viscera and lymph nodes whereas distal tumours commonly metastasise to the liver and chest wall (Missiaglia et al. 2014). Proximal tumours more commonly arise through hypermutation (the state in which a genetic mutation will occur at a higher rate than expected), MSI or CIMP pathway and a large proportion of them contain BRAF-mutations and lead to activation of the mitogen-activated protein kinase (MAPK) pathway (Missiaglia et al. 2014). This is a key signalling pathway involved in the regulation of normal cell proliferation, survival and differentiation (Roberts & Der 2007) as well as driving CRC tumourigenesis. Proximal tumours are reported to have a worse overall survival with a survival after relapse rate of only 1.3 years (Loupakis et al. 2015; Missiaglia et al. 2014). Distal tumours are commonly associated with the CIN pathway and overexpression of epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2). Only approximately 13% of distal tumours are associated with BRAF mutations (Missiaglia et al. 2014). These tumours are associated with a more favourable outcome in terms of overall survival, progression-free survival and survival after relapse of 2.7 years (non-BRAF mutations) although distal tumours with BRAF mutations have the worst survival at 0.8 years (Loupakis et al. 2015). Recent research has shown that CRC is a heterogenic disease and therefore individual tumour mutation and gene expression profiles should be used to categorise patients into groups where they receive treatment approaches that will be most beneficial to the patient (Missiaglia et al. 2014).

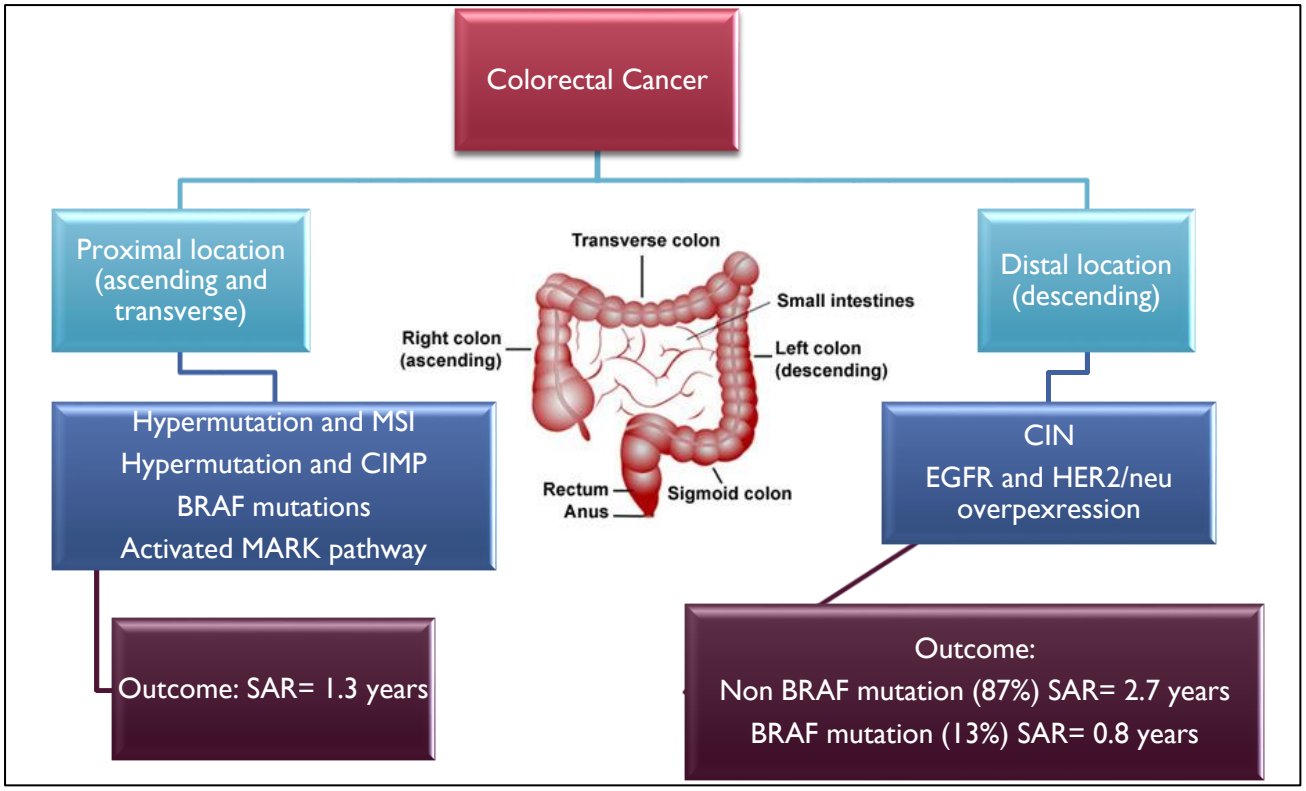

Figure 3: Biological characteristics of proximal and distal tumours and their associated survival after relapse outcomes (Missiaglia et al. 2014).

1.4 Colorectal cancer and diet

Over the past decade, both epidemiological and molecular research studies have demonstrated a strong association between the so-called Western diet characterised by the increased consumption of foods that are typically high in animal fats particularly from red meat, protein and simple and low fibre carbohydrates with the incidence and development of CRC (Dougherty et al. 2009; Lee et al. 2008; Lipkin et al. 1999). A landmark study published in 1968 comparing the incidence of CRC amongst Japanese-American emigrants and Japanese living in America demonstrated considerably higher rates of CRC in the emigrants after two generations of living in the United States (Haenszel & Kurihara 1968). The authors of this study

suggested that this pattern was a consequence of the emigrants changing from their traditional Japanese diet that is low in simple carbohydrates and low in fat to the Western diet and lifestyle, typified by a high simple carbohydrate and high fat diet and lower levels of physical activity (Haenszel & Kurihara 1968). More recent studies in human populations have also demonstrated that a diet high in animal fats increases the risk of CRC (Aleksandrova et al. 2014). Other studies have suggested that it is the obesity that arises from a high fat diet rather than the high fat diet itself that stimulates the development of CRC (Li et al. 2014; Liu et al. 2012).

A number of rodent models have been used to investigate the effects of the Western diet in the development of CRC. These models stimulate CRC tumourigenesis using chemical carcinogens or genetic engineering approaches (Fujisawa et al. 2008; Qi et al. 2015) and then measure the effects of the high fat diet. A recent review comparing various rodent models by Panchal and Brown (2010) reported that a diet high in simple carbohydrates as well as fat is more representative of the Western diet compared to a high fat diet alone and therefore recommended that this diet can better mimic Western diet-related diseases in rodents (Panchal & Brown 2010).

1.5 Molecular pathways in CRC tumourigenesis

The dysregulation of a number of molecular pathways has been implicated in CRC tumourigenesis, however the impact of a short-term diet high in both simple carbohydrates and animal fat on CRC development, in particular the CRC tumourigenesis biomarkers

epidermal growth factor receptor (EGFR), a disintegrin and metalloprotease domain 17 (ADAM17) and transforming growth factor alpha (TGFα) has not been published.

1.5.1 The role of EGFR

It is well-established that EGFR plays a pivotal role in the development and progression of CRC. Since the discovery of EGF in 1962 and the subsequent identification of its receptor by Cohen et al (1962), numerous studies have reported its involvement in CRC. The overexpression of EGFR is a major driver of CRC, with overexpression of EGFR associated with up to 85% of CRC cases and generally accompanied by a poorer prognosis (Ellina et al. 2014). Overexpression of EGFR leads to increased cell proliferation and cell survival, both of which are hallmark characteristics of cancer.

EGFR is a 170kDa transmembrane protein that harbours intrinsic protein tyrosine kinase activity. EGFR along with the human epidermal growth factor receptor 2 (HER2), human epidermal growth factor receptor 3 (HER3 or ErbB3) and human epidermal growth factor receptor 4 (HER4 or ErbB4) comprise the human epidermal growth factor receptor family of type 1 protein tyrosine kinases (Ellina et al. 2014). EGFR consists of two cysteine-rich domains that contain ligand binding regions on the extracellular domain. A single alpha-helical transmembrane domain connects the ligand-binding region to the intracellular receptor, which is comprised of three domains. The first domain acts as a site feedback attenuation by protein kinase C (PKC) and extracellular signal regulated kinases (ERK)-MAPK, the second domain is a tyrosine kinase domain and the third domain is a carboxyl-terminal tail. EGFR is found on all epithelial and stromal cells and is also expressed on glial and smooth cells (Pabla,

et al. 2015). Activation of EGFR is initiated by the binding of one or more of the seven peptide growth factors including EGF, TGF-α, heparin binding EGF-like growth factor (HB-EGF), amphiregulin, betacellulin, epiregulin and epigen (Schneider & Wolf 2009). Aberrant expression of EGFR and the prime natural ligands of EGF and TGF-α and have been implicated in driving CRC tumourigenesis (Ellina et al. 2014). The binding of ligand to the EGFR results in receptor dimerisation which leads to the formation of homodimers (both EGFR) and heterodimers (EGFR and HER2, HER3 or HER4) and triggers the intracellular activation of tyrosine kinase. The phosphorylation of intracellular pathways then occurs and induces the activation of multiple signal transduction pathways. The two major intracellular pathways that are activated by EGFR are the MAPK pathway and the phosphatidylinositol 3-kinase (PI3K) protein kinase B (AKT) pathway (see Figure 4). Both of these pathways lead to the subsequent activation of numerous transcription factors that stimulate cellular responses such as proliferation, migration, differentiation and survival (Ellina et al. 2014).

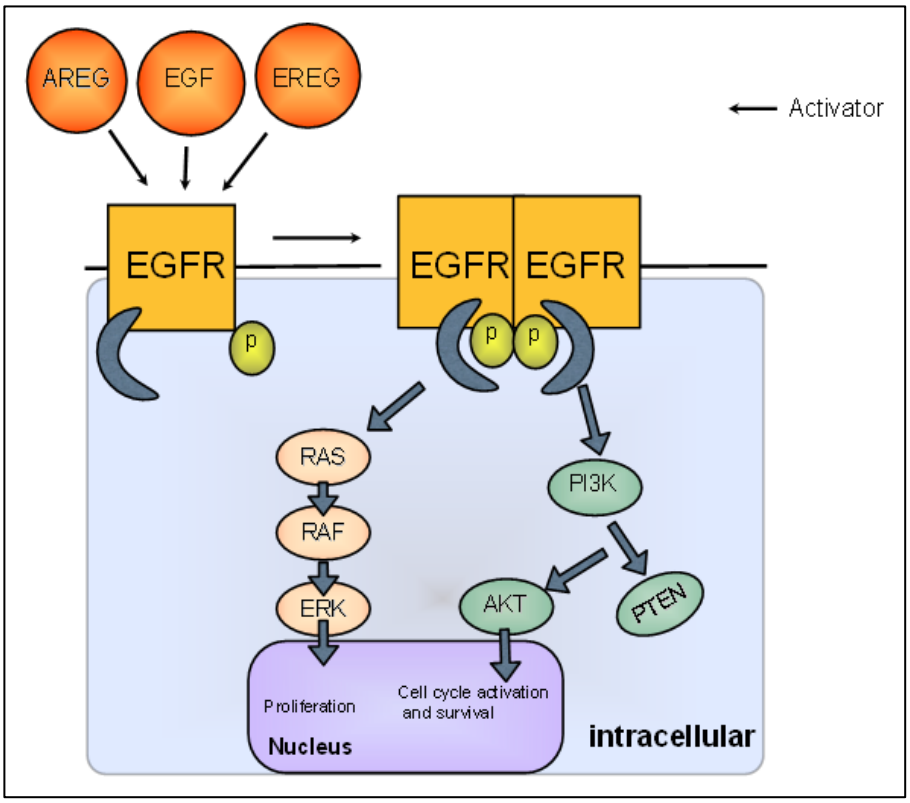

Figure 4: Signalling pathways initiated by the epidermal growth factor receptor (Yarom & Jonker 2011).

EGFR signalling is also strongly implicated in the diet-induced tumourigenesis of CRC. *EGFR* knock-out studies conducted by Dougherty et al (2009) demonstrated that EGFR is required for dietary fat- induced tumour promotion. They compared rats with the wild type EGFR ($EGFR^{wt}$) with rats with a genetically engineered waved-2 EGFR mutation ($EGFR^{wa2}$), which caused a loss of 90% of EGFR function. The results of this study demonstrated that 31% of the $EGFR^{wt}$ mice fed a 20% fat diet developed CRC compared to 11.5% of $EGFR^{wt}$ mice fed a 5% fat diet, however the EGFR ^{wa2} rats that were fed the same 20% fat diet as $EGFR^{wt}$ had significantly lower tumour incidence (Dougherty et al. 2009). Although there is compelling evidence that EGFR plays a major role in the development of CRC in response to a high fat diet, it still remains unclear as to what is driving the increase in EGFR signalling.

1.5.2 The role of ADAM17

A number of studies have highlighted the involvement of ADAM17 in the activation of EGFR and consequently the tumourigenesis of CRC. ADAM17, also commonly referred to as the tumour necrosis factor alpha (TNF-α) converting enzyme (TACE), is one of a number of ADAMs which are transmembrane proteins that cleave membrane-anchored proteins that control the release of soluble factors that activate key processes in cells such as proliferation and apoptosis (Nakagawa et al. 2009). An important function of ADAM17 is the activation of EGFR through the process of ectodomain shedding. Ectodomain shedding is the proteolytic removal of the extracellular domain of transmembrane proteins that are EGFR-activating ligands. ADAM17 regulates the release of growth factors and their receptors, cytokines, cell adhesion molecules and ectoenzymes and is implicated as an integrator of diverse signals that can potentially influence the control of tumourigenesis. A study by Blanchot-Jossic et al (2005) was the first to report that *ADAM17* mRNA was upregulated in 90% of the human CRCs that they investigated compared to adjacent normal tissue. The study also demonstrated that *EGFR* was overexpressed in 77% of these tumours, therefore suggesting that the overexpression of both ADAM17 and EGFR may be inter-related. This concept is supported by the role in which ADAM17 plays in the activation of EGFR through the shedding of its ligands (Blanchot-Jossic et al. 2005). ADAM17 knockout studies by Nakagawa et al (2009) also support this hypothesis as ADAM17-deficient mice experienced perinatal death and demonstrated widespread epithelial defects, a similar phenotype to EGFR-deficient mice. Furthermore, the ADAM17-deficient mice also demonstrated diminished expression of EGFR compared to ADAM17 wildtype litter mates (Nakagawa et al. 2009). Mutusfi et al (2016) has demonstrated the importance of ADAM17 in CRC tumourigenesis, with knockout studies involving the

targeted deletion of ADAM17 from colon epithelial cells leading to a significant reduction in tumour development (Mustafi et al. 2016).

A recent study has also demonstrated a link between a diet high in fats and the overexpression of ADAM17. Matsui et al (2014) compared ADAM17-overexpressing transgenic (Tg) mice and wild type mice fed a high fat (60%) diet to reveal that overexpression of ADAM17 in the Tg mice led to an increase in TNF and macrophage-related cytokines and chemokines which are indicative of the inflammatory response that precedes obesity (Matsui et al. 2014). Another recent study by Mustafi et al (2016) has shown that a high fat diet causes an increase in the expression of the ligand CXCL12 which upregulates expression of its receptor, CXCR4. The up-regulation of CXCR4 has a flow on effect that results in the up-regulation of ADAM17, which leads to an increase in TGFα. As TGFα is one of the EGFR ligands, its up-regulation ultimately leads to overexpression of EGFR, which as already mentioned, is a major driver of CRC (Mustafi et al. 2016).

Shimoda et al (2016) proposed that ADAM17 is the rate limiting factor controlling colon epithelial cell proliferation and differentiation by shedding diverse EGFR ligands which once bound to EGFR initiate a downstream signalling cascade that results in the stimulation of cellular proliferation and differentiation (Shimoda et al. 2016).

1.6 Epigenetic modifications in CRC

The role of epigenetic modification of the genome in CRC tumourigenesis has received a substantial amount of attention over the past two decades with recent research suggesting

that it plays a pivotal role. Epigenetic modifications of tumour suppressor or oncogenes have been strongly associated with pathogenesis of the CRC, with recent studies on the CRC epigenome demonstrating that these cells are riddled with aberrant gene methylation (Bardhan & Liu 2013). There are several mechanisms by which the epigenome is modified but the most common are DNA methylation and histone modification. These two major epigenetic mechanisms play a vital role in the development of CRC and other cancers. These processes are not independent, instead they work together to control gene expression and therefore tumourigenesis of CRC.

1.6.1 DNA methylation

DNA methylation is the process by which a methyl group is added to the 5' position of cytosine to produce 5-methyl cytosine leading to methylation of CpG sites. This reaction is catalysed by a group of enzymes called DNA methyltransferase (DNMTs: DNMT1, DNMT3a and DNMT3b). DNA methylation is an essential process required for mammalian cell survival demonstrated by the finding that DNMT mutations result in embryonic lethality (Li et al. 1992). Aberrant methylation patterns have been strongly linked to CRC tumourigenesis, specifically the hypomethylation of oncogenes and hypermethylation of promoter regions of tumour suppressor genes such as those involved in DNA repair. Hypermethylation causes gene silencing and genome instability as well as altered apoptotic mechanisms, DNA repair and cell cycle. (Bardhan & Liu 2013). DNA hypomethylation is involved in CRC tumourigenesis through the loss of methylation of the CpG sites of important genes required to maintain genomic stability (Bardhan & Liu 2013).

1.6.2 Histone modification

Chromatin is comprised of DNA that is wound around histone proteins and the state of the chromatin in a cell controls gene expression. A chromatin conformation that is opened/relaxed allows transcription factors to gain access to bind to promoter regions to activate the transcription of genes. However, when the chromatin is in a condensed or closed state, it limits the accessibility of transcription factors to bind to promoter regions and therefore represses the activation of gene transcription (as illustrated in Figure 5). The chromatin is constantly alternating between a relaxed and condensed state (Bardhan & Liu 2013). Post-translational modifications to the histone proteins in chromatin include acetylation, deacetylation, methylation and phosphorylation and these modifications can change the chromatin confirmation and therefore gene transcription.

The process of histone acetylation is controlled by two types of enzymes: histone acetyltransferases (HATs) and histone deactylases (HDACs) which regulate the addition or deletion of an acetyl group to the lysine residues on the N-terminal tails of histones (Bardhan & Liu 2013). The addition of an acetyl group neutralises the lysine's positive charge and causes the histones and DNA to repel against each other resulting in weakened interactions and to some extent interactions between neighbouring histones (Roth et al. 2001). These changes alter the structure of chromatin which leads to a more open and relaxed formation allowing gene transcription to occur (Roth et al. 2001). The function of HDACs is opposite to HATs and involves the removal of the acetyl group from the N-terminal of core histones which re-instates the positive charge. This enables a stronger association between the histones and DNA resulting in a condensed chromatin and as a result transcription factors are denied access to the promoters of genes therefore gene expression is blocked (Bardhan & Liu 2013). HATs

and HDACS are considered transcriptional co-activators and co-suppressors. Imbalances between HATs and HDACs plays an important role in the inactivation of transcriptional factors resulting in the epigenetic silencing of tumour suppressors therefore contributing to CRC tumourigenesis.

Histone methylation is another essential post-translational modification of histones and helps to regulate gene expression via the addition or removal of methyl groups to lysine and arginine residues on the N-terminal tails. The balance between histone methylation and demethylation is controlled by two groups of enzymes named histone methyltransferases (HMTases) and histone demethylases (HDMs) (Bardhan & Liu 2013). A disruption in histone methylation homeostasis can lead to tumourigenic changes. Overexpression of the HMTase Euchromatic Histone Lysine Methyltransferase 2 (*Ezmt2*) has been associated with CRC and other cancers as it is directly involved in cell proliferation (Cho et al. 2011; Gonzalez et al. 2009; Natarajan et al. 2010; Yu et al. 2007).

Histone phosphorylation involves the addition of a phosphate group to serine residues (Cohen et al. 2011), relaxing the binding of DNA to histones and allowing transcription of genes within the affected region (Dou & Gorovsky 2002). Histone phosphorylation has also been associated with a number of vital cellular processes such as transcriptional activation, mitosis, meiosis, DNA damage repair and apoptosis and therefore aberrant expression of histone phosphorylation enzymes has been linked to tumourigenesis (Cohen et al. 2011).

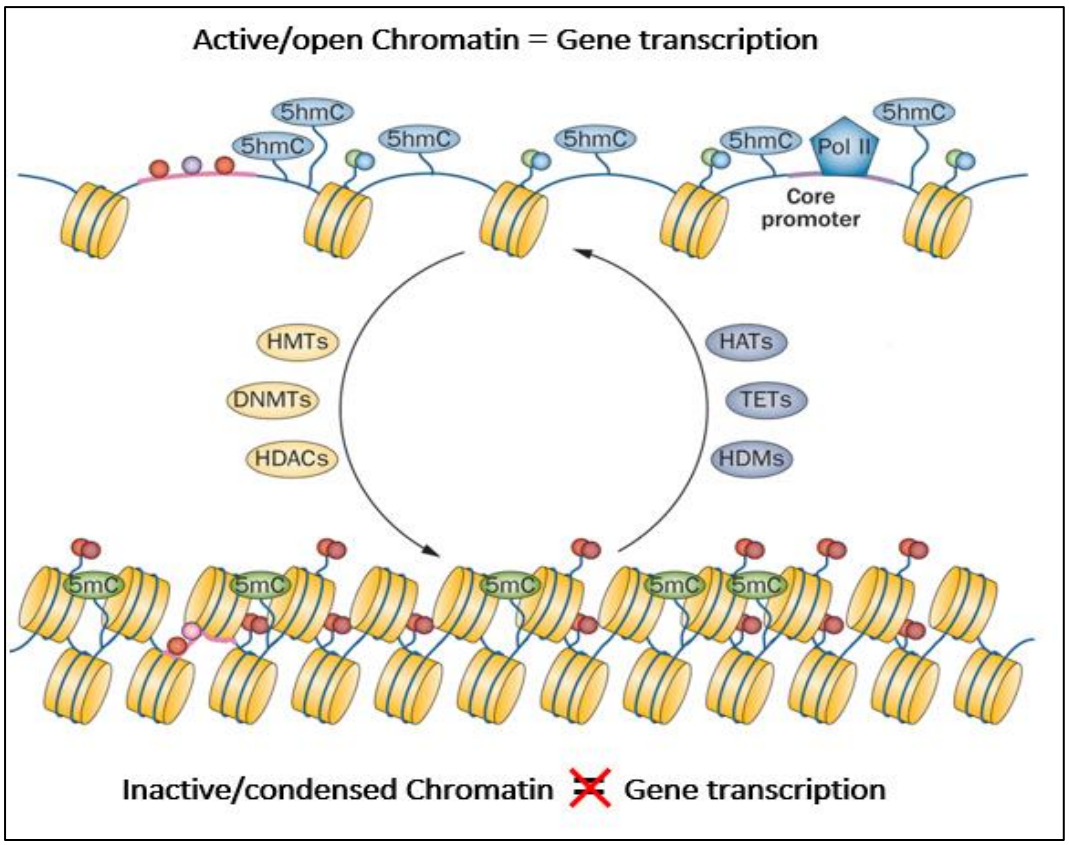

Figure 5: Conformation of chromatin controls gene expression as it regulates accessibility of transcriptional factors to bind to promoter regions to initiate gene transcription (modified from Greco & Condorelli 2015).

1.7 Dietary interventions as potential anti-CRC agents

Curcumin is a polyphenol, extracted from the rhizomes of *Curcuma longa* (turmeric) plant and has long been considered an important therapeutic agent in both Indian and Chinese medicine, which may account for the lower incidence of CRC in these countries. Curcumin is relatively inexpensive to produce and has shown promising pharmacological potential demonstrating anti-inflammatory, antineoplastic, antioxidant and chemopreventative properties (Hardy & Tollefsbol 2011). Numerous studies have also demonstrated low toxicity of curcumin with a number of dose escalation clinical trials establishing the safety of curcumin even at high doses of 12 grams per day (Patel et al. 2010). Cancer cells that have been treated

with curcumin have demonstrated characteristics similar to apoptotic cells, with curcumin interfering with a number of cell signalling pathways, such as EGFR, resulting in cell death (Starok et al. 2015).

In CRC studies using animal models, curcumin has been shown to supress tumour activity through downregulation of EGFR expression resulting in the inhibition of growth in neoplastic (abnormal) cells (Chen & Xu 2005; Chen et al. 2006). Curcumin exerts its effects on EGFR via two pathways, the first is the reduction in intrinsic kinase activity and the second is the EGF-mediated phosphorylation of the receptor which prevents the binding of ligands to the receptor. Curcumin not only targets EGFR but also the HER2 resulting in decreased tyrosine kinase activity of this receptor as well, which is important as activation of EGFR commonly involves formation of heterodimers with HER2 (Patel et al. 2010). Curcumin also further impacts *EGFR* and *HER2* expression as it inhibits transcription of these genes by inhibiting early growth response protein gene 1 (*Egr-1*) (Chen et al. 2006; Singh et al. 1996), which is a nuclear phosphoprotein that binds to GC-rich sequences within promotor regions of genes involved in the regulation of other gene expression, including *EGFR* (Chen et al. 2006).

Curcumin has also been shown to target another key hallmark of cancer, angiogenesis, that is the formation of new blood vessels. The migration, proliferation and differentiation of human vascular endothelial cells (HUVEC) promotes angiogenesis (Patel et al. 2008) and curcumin has been found to inhibit the proliferation of HUVEC and human vascular smooth muscle cells (HVSMC) causing the suppression of vascular tubule formation (Singh et al. 1996). Curcumin has also demonstrated an ability to sensitise cancer cells to chemotherapy treatment. Recent clinical trials administering curcumin in combination with cytotoxic chemotherapy agents 5-fluorouracil and oxaplatin resulted in a greater apoptotic response and growth inhibition of

cancer cells when compared to chemotherapy treatment alone (Patel et al. 2008). Unlike many other therapeutic agents involved in cancer treatment, curcumin appears to only target cancer cells whilst sparing its cytotoxic effects on normal healthy cells (Aggarwal et al. 2006), further adding to its potential as a therapeutic agent for treating not only CRC but many other cancers. The effect of curcumin on actually preventing high fat diet-induced CRC has not been published.

Using an established male Wistar rat obesity model (Panchal & Brown, 2010), this study has investigated the effects of a diet high in simple carbohydrates and animal fat (HCHF) on the gene expression of CRC tumourigenic markers, *ADAM17*, *EGFR* and *TGFα* and epigenetic modifying enzymes in both the proximal and distal colon. It has also investigated the effect of curcumin in preventing any of these molecular changes due to the HCHF diet. The hypothesis was that curcumin could prevent the CRC gene expression changes in the HCHF rats to that exhibited in the colons of rats fed a normal corn starch diet.

The aims of this study were to investigate:

1. The expression of CRC biomarkers *EGFR, ADAM17* and *TGFα* in the proximal colon and distal colon of rats fed either a HCHF diet or a corn starch diet for 16 weeks or a HCHF diet for eight weeks followed by a corn starch diet for another eight weeks;
2. The effects of supplementing 100 mg/ kg mass of curcumin on the expression of CRC biomarkers in the colons of rats fed a HCHF diet or a corn starch diet for 16 weeks; and
3. The difference in expression of epigenetic modifying enzymes in the proximal and distal colons of rats fed a HCHF diet or a corn starch diet for 16 weeks.

2.1 High fat and high carbohydrate rat model

Colon tissue samples were collected from a total of fifteen male Wistar rats that were being used for other experiments under USQ ethics approval (15REA008) and in accordance with the re-use of animal tissues principle of the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition (2013). Twelve rats were generously provided by Jessica Pahl and Ryan Du Preez after these researchers had removed tissue for their experiments. Three of these rats had been on a control standard corn starch diet (CS), three on a high carbohydrate and high fat diet (HCHF), three on the control corn starch diet supplemented with 100 mg/kg body mass of curcumin (C-100) and three on the high carbohydrate and high fat diet supplemented with 100 mg/kg body mass of curcumin (H-100). The additional three rats had been fed a HCHF diet for the first eight weeks and then changed to a CS diet for the final eight weeks of their treatment and these rats were generously provided by Dr Sunil Panchal and Dr Stephen Wanyonyi after they had removed tissue for their experiments. A total of five different diets were investigated: CS (control standard diet), HCHF (treatment diet), CS diet combined with dietary intervention curcumin at 100 mg/ kg body mass (control for the effect of curcumin), HCHF with curcumin at 100 mg/ kg body mass (treatment with intervention diet) and finally HCHF administered for eight weeks and then reversed back to CS diet for remaining eight weeks (H8C8) (the dietary compositions are described in Table 1). Rats were eight weeks old when they were started on the diet protocol, with interventions introduced after eight weeks. Rats remained on protocol for a total of 16 weeks and

euthanised via an intraperitoneal injection of pentobarbitone sodium (Lethabarb, Virbac) at a dose of 100 mg/kg. Prior to euthanising, each rat was weighed and their mass recorded.

Table 1: Diet protocols and sample size

CS diet (7% fat): 570 g of cornstarch, 155 g of powdered rat food (Specialty Feeds, Glen Forest, Western Australia), 25 g of Hubble, Mendel, and Wakeman salt Mixture and 250 g of water per kilogram of diet; n=3
HCHF diet (24% fat): 175 g of fructose, 395 g of sweetened condensed milk, 200 g of beef tallow, 155 g of powdered rat food, 25 g of Hubble, Mendel and Wakeman salt mixture, and 50 g of water per kilogram of diet. Drinking water supplemented with 25% fructose; n=3
Control curcumin diet: CS diet 8 weeks, CS (as above) +100 mg/kg body mass curcumin 8 weeks; n=3
HCHF curcumin diet: HCHF diet (as above) 8 weeks, HCHF (as above) + 100 mg/kg body mass curcumin 8 weeks; n=3
H8C8 diet: HCHF diet 8 weeks, CS diet 8 weeks; n=3

16 weeks

2.2: Tissue dissection

Tissue samples were collected on four separate occasions over a two week period. Following euthanasia, a section of proximal and distal colon approximately 2.5 cm in length was dissected from each rat and immediately placed in liquid nitrogen. Approximately half of the sample was transferred into a 1.5 mL microfuge tube for protein extraction as outlined in 2.7. A needle punch biopsy was performed on the remaining sample and these tissue fragments

(representative of different locations in the colon) were transferred into a 1.5 mL microfuge tube and homogenised using a 25 gauge needle and 1 mL syringe in 600 µl of RLT lysis buffer (Qiagen). Samples were stored at -80°C prior to RNA extraction.

2.3 RNA extraction and quantification

Total RNA was extracted from tissue samples following the manufacturer's protocol from a commercially available RNA extraction kit (Qiagen RNAeasy Mini kit). The exact components of the buffers are commercial in confidence. Briefly, 700 µl of each sample was transferred onto Qiagen QIAshredder columns (Qiagen) and centrifuged at 12 k rpm for one minute. Samples were then transferred onto spin columns and centrifuged for a further 15 seconds at 10 k rpm. 700 µl of RW1 wash buffer (Qiagen) was then added to each column and then centrifuged again at the same speed and length of time (15 seconds at 10 k rpm). Following this step, 500 µl of RPE extraction buffer (Qiagen) was added and column was centrifuged for 15 seconds at 10 k rpm. Another 500 µl of RPE buffer was added to the column which was then centrifuged for two minutes at 10 k rpm. The column was then placed in a new 1.5 mL collection tube and 40 µl of RNase free water was added directly to the membrane and then centrifuged for one minute to elute the RNA. The concentration of RNA was then determined using a Nano Drop (Thermo Scientific).

2.4 cDNA synthesis

Complementary DNA (cDNA) synthesis was performed using the Bio-Rad iScript cDNA synthesis kit, according to the manufacturer's instructions on 1 µg of RNA to produce a total

volume of 20 µl cDNA. The kit included both random hexamer primers and oligo(dT) primers. The incubation conditions used for cDNA synthesis included 25°C for five minutes, 46°C for 20 minutes and 95°C for one minute. The cDNA produced was then used as the template for real time reverse transcription polymerase chain reaction (RT-PCR).

2.5 Real time reverse transcription polymerase chain reaction (RT-qPCR)

2.5.1 EGFR pathway analysis

PCR master mixes were prepared for each PrimePCR assay (Bio-Rad) EGFR, ADAM17, TGFα and beta actin by combining primers, SYBR Green master mix (Bio-Rad) and RNase-free water according to the Bio-Rad real time RT-PCR protocol. A total of 9 µl of the appropriate master mix was aliquoted into each well. One µl of cDNA from each control or treatment group or RNase-free water (no template control) was added to the master mix and reactions were performed in triplicate. The plate was then subjected to 40 cycles of PCR using a Bio-Rad CFX-384 Touch Thermocycler. Cycle thresholds (Cts) for each cDNA/primer combination were determined by the Bio-Rad CFX Manager and Qbase PLUS software.

The primer sequences for the PrimePCR assays (Bio-Rad) were not provided by the supplier however the chromosome locations of the amplicons are listed in Table 2. Each assay was validated by Bio-Rad and this data is available on the web site (see http://www.bio-rad.com/en-au/prime-pcr-assays/assay/qrnocid0056984-primepcr-sybr-green-assay-actb-rat). The house-keeping gene beta actin was designed to be intron-spanning so that the melt curve analysis could be used to ensure that there was no genomic DNA contamination. Genomic contamination would be indicated by two peaks.

Table 2: Information on the Bio-Rad PrimePCR assays used for SYBR Green real time gene expression analysis.

Primer Target	Unique Assay ID	Chromosome Location
EGFR	qRnoCID0008508	14:100442581-10044463
ADAM17	qRnoCID0004148	15:53225651-53226718
TGFα	qRnoCID0006255	4:182534498-182603831
Beta actin	qRnoCID0056984	12:15745866-15746909

2.5.2 Epigenetic modification real time PCR analysis

Primers for eighty-four genes which encode for enzymes involved in epigenetic modification were pre-dried on a 384 well PrimePCR pathway epigenetic chromatin modification enzyme analysis plate (Bio-Rad). The functions of the genes analysed included DNA methylation and histone acetylation, methylation and phosphorylation. Five housekeeping genes were also included for normalisation of expression. The PCR mastermixes were prepared consisting of cDNA pooled from three rat colons (either distal CS, distal HCHF, proximal CS or proximal HCHF), SYBR green and RNase free water, then 9.5 µl of each mastermix was added into each well. The plate was then subjected to 40 cycles of PCR using a Bio-Rad CFX-384 Touch. Threshold cycles (CTs) for each cDNA/primer combination were determined by the Bio-Rad CFX Manager and Qbase PLUS software.

2.6 Relative gene expression (CT) analysis

Threshold cycle (CT) values obtained from the Qbase PLUS software were used to calculate changes in expression using the delta delta (ΔΔ) CT data analysis method. For each target, ΔCT value was calculated by normalising the expression of the target gene (such as EGFR) with the reference or housekeeping gene (beta actin). Then ΔΔCT was calculated by using the formula

ΔCT (treatment/HCHF/intervention cDNA) – ΔCT (control/CS cDNA). Finally, the fold change in target gene expression was calculated using this formula $2^{-\Delta\Delta CT}$ (Rao et al. 2013).

2.7 Protein Extraction

During tissue extraction approximately half of the sample was dissected into small sections and placed in a 1.5 mL microfuge tube containing 1 mL of radioimmunoprecipitation (RIPA) lysis buffer (0.2% Triton X-100; 0.1% sodium deoxycolate; 0.1% SDS; 1M NaCl; 1M Tris supplemented with 1% Thermo Scientific Halt protease and phosphatase inhibitors). Samples were homogenised using short bursts of sonication. To prevent the denaturation of protein, samples were placed on ice intermittently during sonication. Once homogenised, samples were then placed on ice on an orbital shaker for two hours. Samples were then centrifuged for 20 mins at 12,000 X g at 4°C and supernatants were collected. The concentration of protein samples were then determined by performing a BCA assay (Thermo Scientific) following the manufacturer's instructions.

2.8 Polyacrylamide gel electrophoresis (PAGE)

Due to the size of the EGFR protein, an 8% bisacrylamide SDS separating and 4% stacking gel was used to analyse this protein. Five µL of 5x Laemmli SDS loading buffer was added to the protein samples and a positive control of EGFR-containing lysate before they were heated to 95°C, cooled and then loaded into the gel. Electrophoresis was performed for one hour at 160 volts.

2.9 Western immunoblot

After PAGE, proteins were transferred onto nitrocellulose membranes using a transblot apparatus (Thermo Scientific) overnight at 10 volts, and 4°C. Membranes where then incubated in blocking buffer (5% skim milk powder in tris-buffered saline, 0.1% Tween 20; TBST buffer) for one hour at room temperature on an orbital shaker. Membranes were then incubated with a monoclonal mouse anti-human EGFR antibody (1:1000 dilution; Precision Antibodies) in blocking buffer overnight at 4°C. Membranes were then washed and incubated in an anti-mouse horse radish peroxidase (HRP) conjugated secondary antibody (1:10000 dilution) in blocking buffer. Bands were detected using a Pierce enhanced chemiluminescence (ECL) detection reagent (Thermo Scientific) and imaged by a Fusion Fx digital imager.

2.10 Statistical analysis of real time RT-PCR results

Fold changes in the gene expression for each target in the colons of rats fed a HCHF diet and interventions compared to those fed a CS diet were derived from the $2^{-\Delta\Delta CT}$ calculations. The statistical significance of these fold changes was analysed using one way ANOVA and Tukey Post Hoc test (SPSS, IBM).

3.1: Effect of diet on body mass

As expected, after 16 weeks on a HCHF diet, rats were significantly heavier than rats fed a CS diet (p value = 0.002) and rats in the H8C8 diet group (p value = 0.009), suggesting that reverting back to a CS diet can reduce body mass after only eight weeks. Interestingly no significant difference was seen between the HCHF diet and the HCHF diet with curcumin intervention (H-100 mg curcumin) or the CS diet and the CS diet with curcumin intervention (C-100 mg curcumin) suggesting that curcumin doesn't affect body mass.

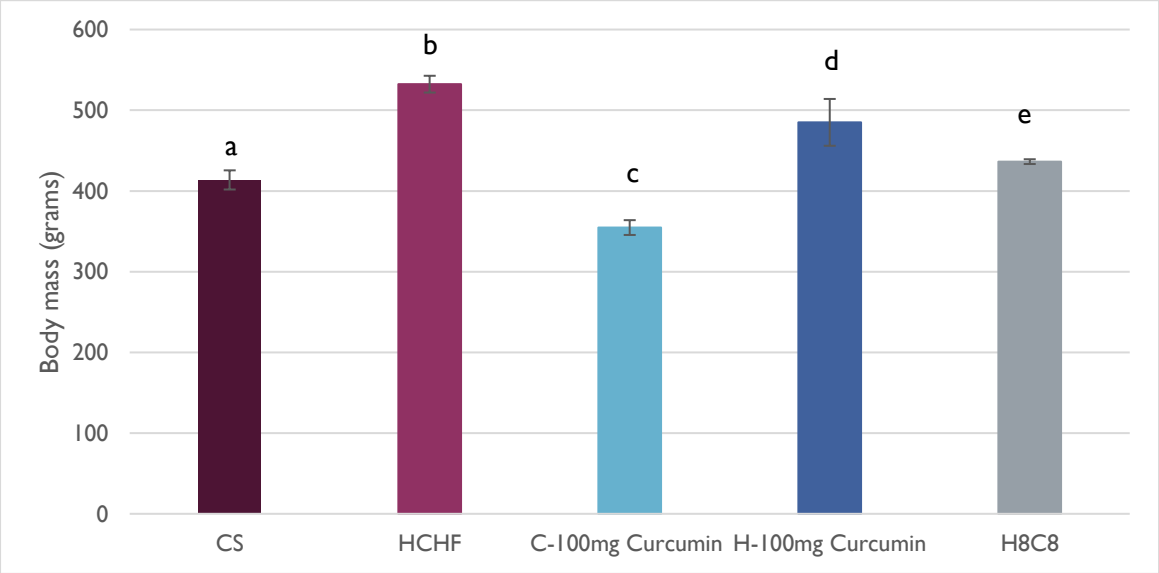

Figure 6: Final body mass was significantly higher in HCHF compared to CS (a vs b p-value = 0.002). Curcumin did not significantly change the body mass of either CS or HCHF rats (p-value> 0.05). H8C8 significantly reduced body mass compared to HCHF (b vs e p-value= 0.009). No significant difference between control curcumin and H8C8 compared to CS (a vs c p-value=0.11, a vs e p-value = 0.84).

3.2: HCHF diet upregulates the expression of EGFR, ADAM17 and TGFα

In order to determine whether a HCHF diet could upregulate the expression of the CRC biomarkers *EGFR, ADAM17* and *TGFα* gene expression fold change was determined using real

time RT-PCR, CT analysis and $2^{-\Delta\Delta CT}$. Figures 7 - 9 demonstrate that the HCHF diet significantly increased the expression of *EGFR, ADAM17* and *TGFα* and in the distal colon of rats, therefore suggesting that HCHF diet upregulates the expression of CRC biomarkers in distal colon after only 16 weeks. Unlike the distal colon, no statistically significant difference was observed for any of the CRC biomarkers in the proximal colon although there was a trend towards an increase in CRC biomarker expression (Figures 10 – 12).

3.3 Dietary interventions prevent the high expression of CRC biomarkers due to the HCHF diet

3.3.1 Curcumin

Curcumin was hypothesised to be an effective therapeutic agent for preventing HCHF diet induced CRC biomarker gene expression. As demonstrated in Figure 7, there was a ten-fold decrease in the expression of *EGFR* in the distal colon in response to the addition of curcumin to the HCHF diet. The addition of curcumin to the HCHF diet also led to a three-fold reduction in the expression of *ADAM17* in the distal colon (Figure 8). Interestingly curcumin did not appear to have the same effect on *EGFR* and *ADAM17* in the proximal colon, with no significant changes in expression observed between HCHF and H-100mg curcumin groups (Figures 10 and 11). Curcumin also did not appear to alter the expression of *TGFα* in proximal or distal colon (illustrated in Figures 9 and 12). No significant difference was seen between CS group and C-100mg curcumin group for any of the targets suggesting that curcumin doesn't lead to tumourigenic changes in the colon of rats fed a normal diet (Figures 7 – 12).

3.3.2 H8C8

To evaluate the effects of reverting from a HCHF diet back to a normal CS diet on the expression of the CRC biomarkers, rats were fed a HCHF diet for eight weeks and the changed to CS diet for the final eight weeks of the protocol. *EGFR, ADAM17* and *TGFα* expression was significantly decreased in the distal colons in H8C8 group compared to HCHF group (Figures 7, 8 and 9) suggesting that reverting back to a normal diet after eight weeks on a HCHF diet is effective in reversing tumourigenic changes caused by HCHF diet in the distal colon.

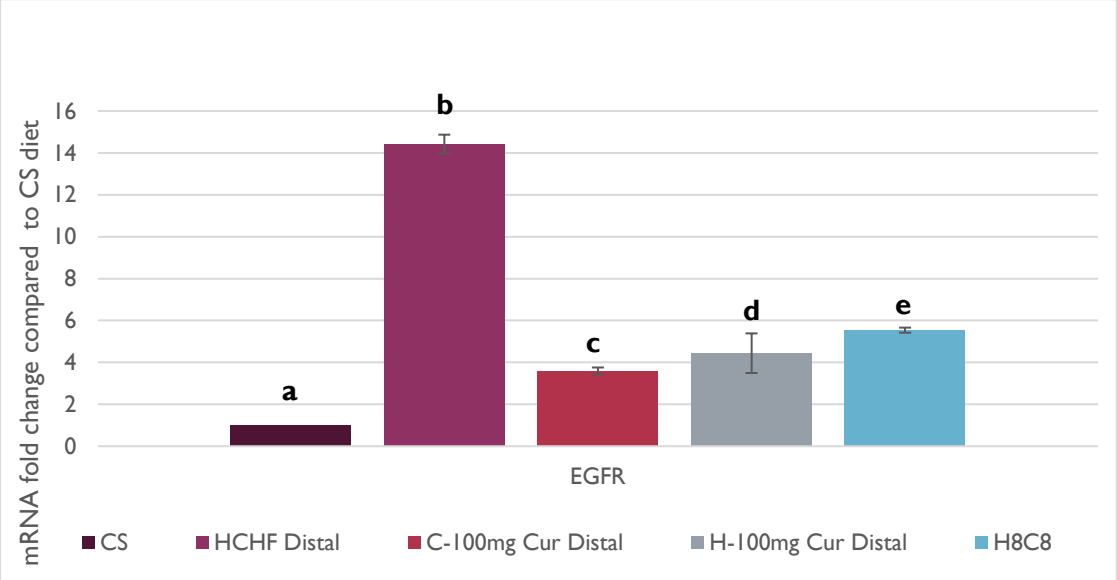

Figure 7: mRNA fold change expression of distal colon EGFR compared to CS HCHF diet significantly increased EGFR expression in distal colon (a vs b p-value=0.001). Curcumin significantly reduced expression of EGFR compared to HCHF (b vs c p-value=0.001, b vs d p-value=0.001). H8C8 also significantly reduced EGFR expression compared to HCHF (b vs e p-value=0.001). No significant increase in expression of EGFR between control and treatments (p-value>0.05).

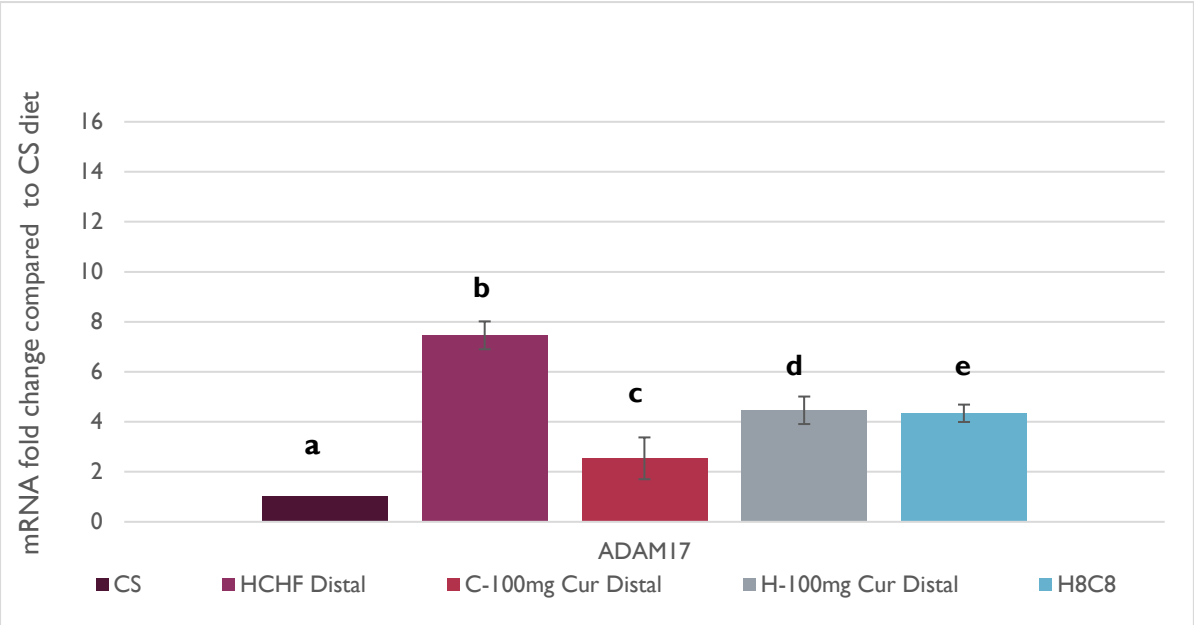

Figure 8: mRNA fold change expression of distal colon ADAM17 compared to CS. HCHF diet significantly increased ADAM17 expression in distal colon (p-value= 0.005). Curcumin significantly reduced expression of ADAM17 compared to HCHF (b vs d p-value=0.004).H8C8 also significantly reduced ADAM17 expression compared to HCHF (b vs e p-value=0.035). No significant increase in expression of ADAM17 between control and treatments (p-value>0.05).

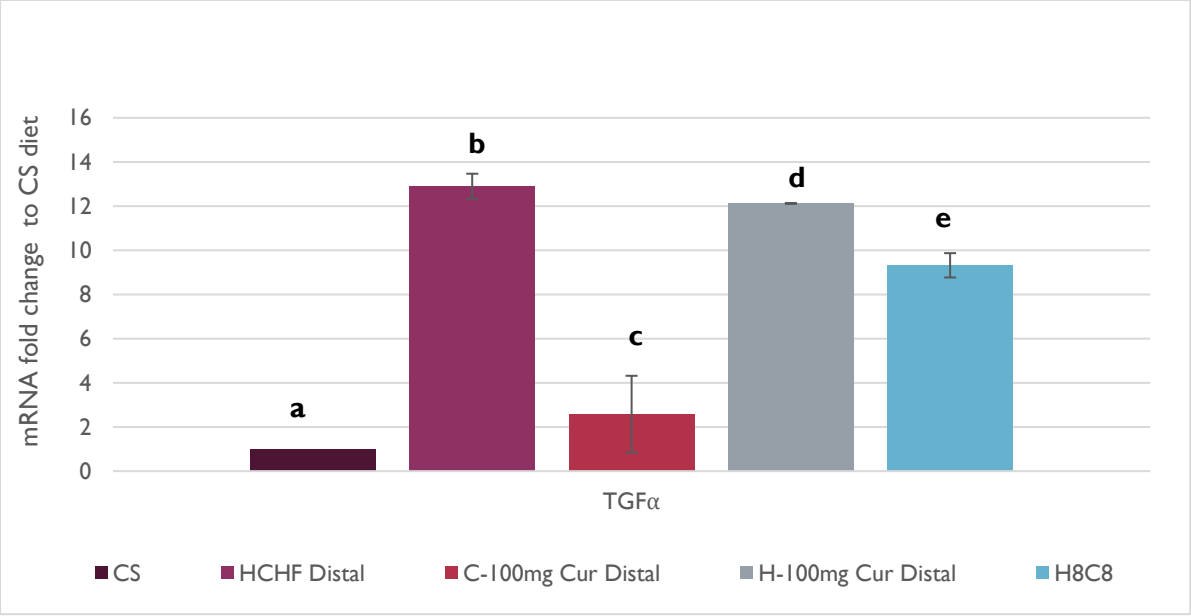

Figure 9: mRNA fold change expression of distal colon TGFα compared to CS. HCHF diet significantly increased TGFα expression in distal colon (a vs b p-value= 0.039). Curcumin did not significantly reduce expression of TGFα compared to HCHF (b vs d p-value=0.6) .H8C8 also significantly reduced TGFα expression compared to HCHF (b vs e p-value=0.035). No significant increase in expression of TGFα between control and treatments (a vs c p-value= 0.9 and a vs e p-value=0.8).

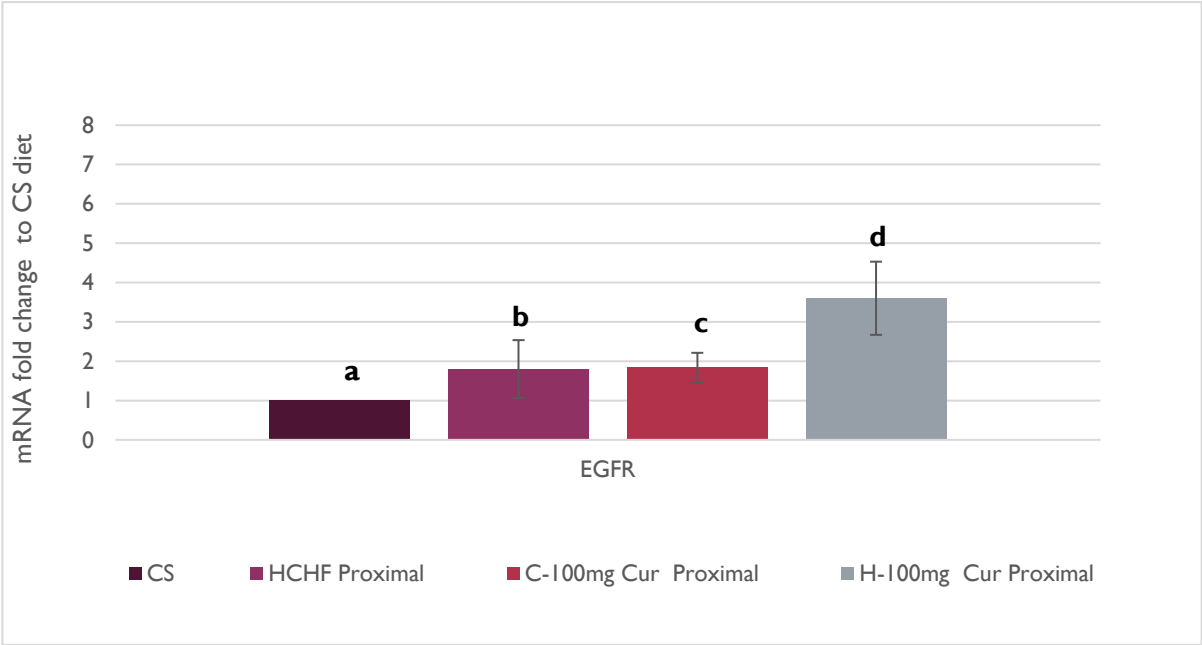

Figure 10: mRNA fold change expression of proximal colon EGFR compared to CS. One way ANOVA p-value=0.5698, Tukey post hoc: no significant difference between any test samples all p-value> 0.05.

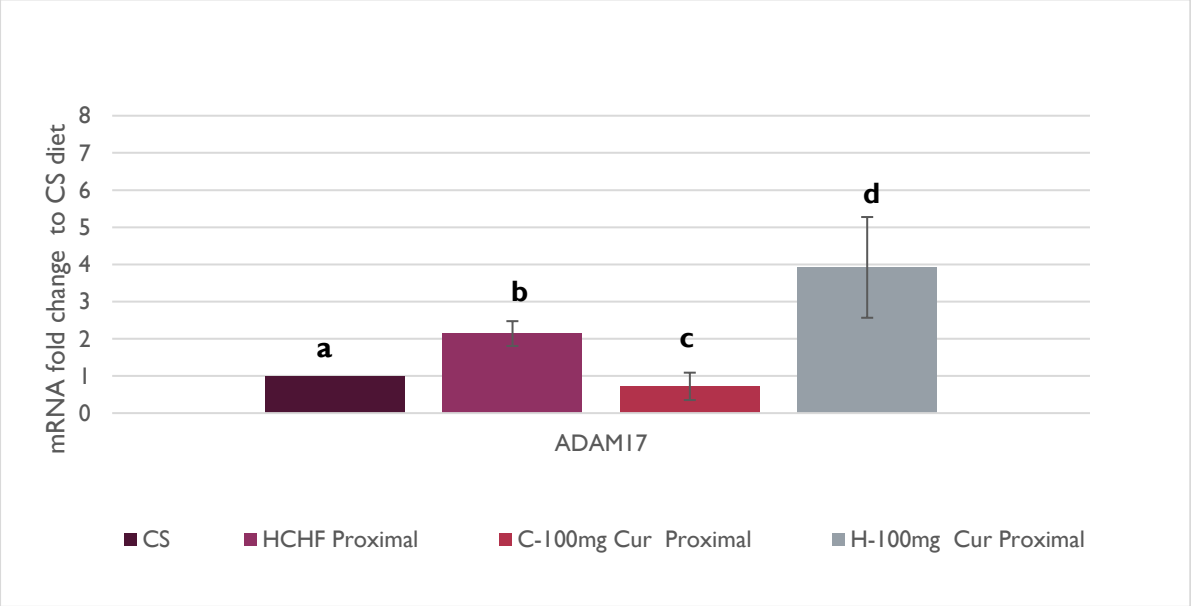

Figure 11: mRNA fold change expression of proximal colon ADAM17 compared to CS. One way ANOVA p-value=0.4796, Tukey post hoc: no significant difference between any test samples all p-value> 0.05.

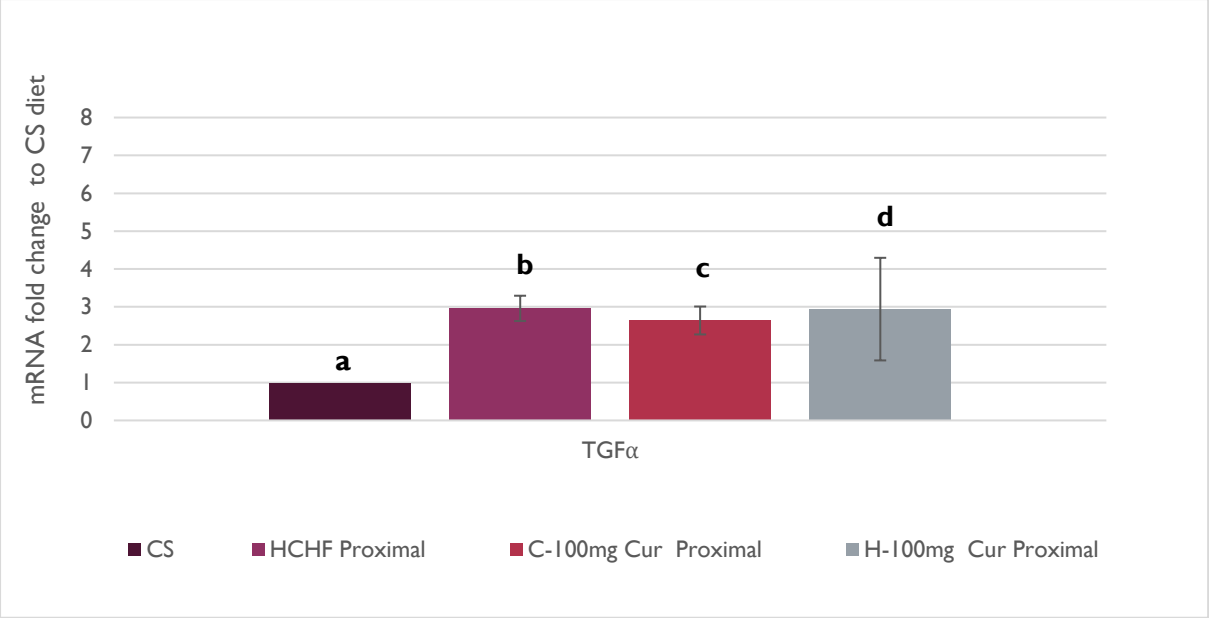

Figure 12: mRNA fold change expression of proximal colon TGFα compared to CS One way ANOVA p-value=0.7463, Tukey post hoc: no significant difference between any test samples all p-value> 0.05.5.

3.4 Dietary induced epigenetic changes

Analysis of diet-induced epigenetic modifications was conducted by measuring the expression of 84 key enzymes known to stimulate epigenetic changes. As illustrated in Figures 13 and 14, the expression of epigenetic modification enzyme genes in the distal and proximal colons from HCHF rats differed from the expression pattern seen in the CS rat colons. In the distal colon of HCHF rats, there was an increase in the genes that code for enzymes involved in DNA methylation (*Dnmt3a*), histone phosphorylation (*AURKB*) and histone methylation (*Ehmt2*) and a decrease in the expression of histone deacetylases (*HDAC1* and *HDAC7*) and another histone methylation enzyme gene (*Symd3*) (Figure 13). Analysis of the proximal tissue showed an increase in the expression of genes that code for enzymes associated with histone acetylation (*CIITA*) and a decrease in the expression of genes involved in histone methylation (*Suv39h1, Suv39h2, Suv420h2 and Ezh2*) (Figure 14).

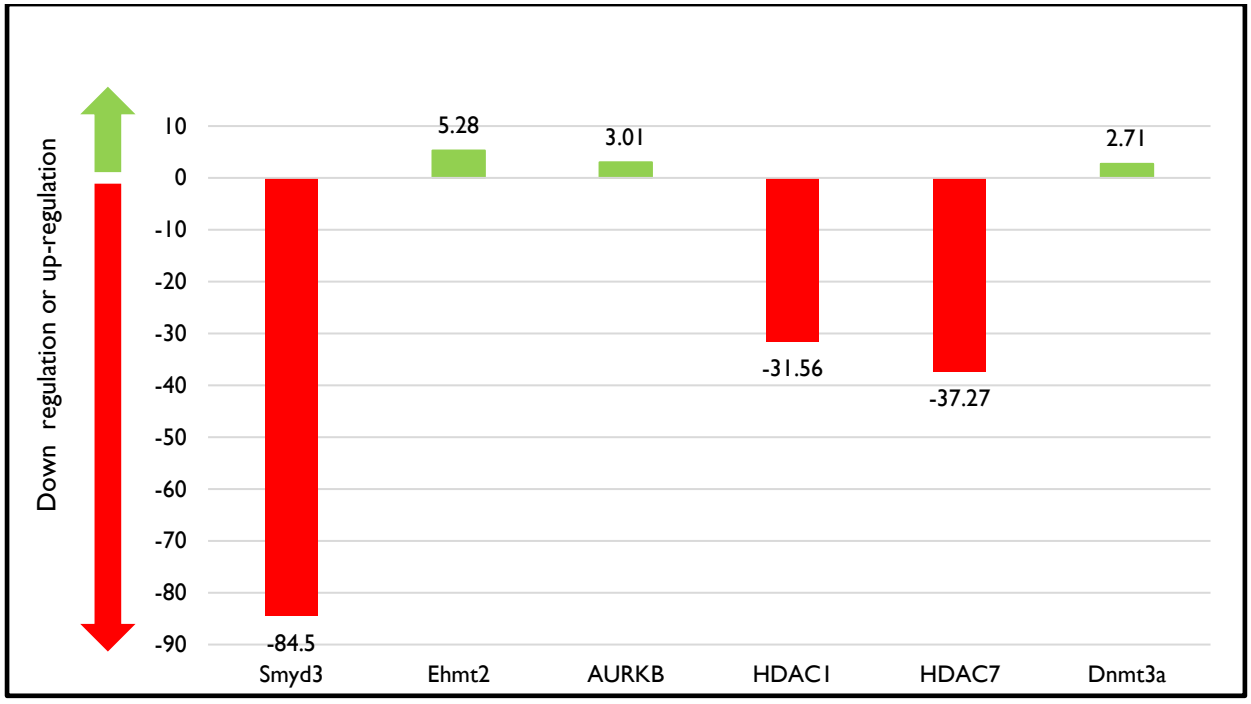

Figure 13: Fold –changes in mRNA expression of epigenetic modification enzyme genes in the distal colons of rats in response to HCHF diet (compared to corn starch diet). Samples representative of pooled cDNA from three rats in each group.

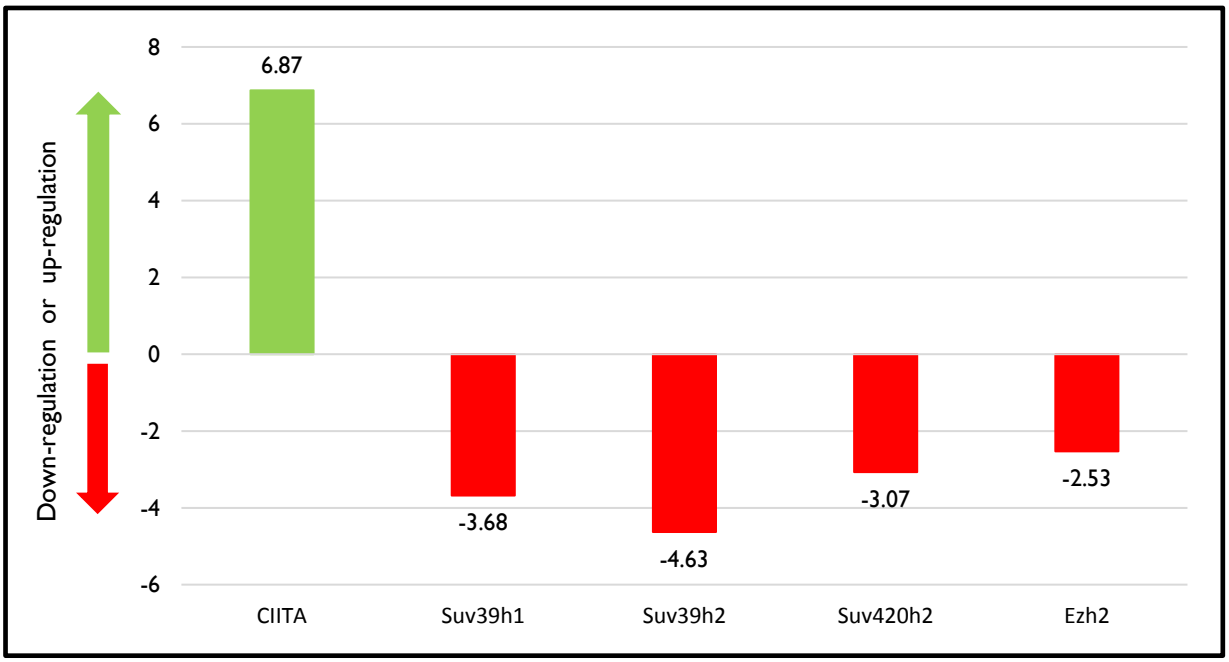

Figure 14: Fold –changes in the mRNA expression of epigenetic modification enzyme genes in the proximal colons of rats in response to HCHF diet (compared to corn starch diet). Samples representative of pooled cDNA from three rats in each group.

3.5 Dietary induced changes in the protein levels of EGFR

Western immunoblot was used to determine whether the changes in gene expression revealed by real time RT-PCR were also occurring at the protein level. Protein lysates were prepared from the colons of rats fed the CS, HCHF and intervention diets and protein levels of EGFR and beta-actin were investigated. Due to time constraints, a successful result was not achieved and further optimisation is required.

The impact of colorectal cancer is a global concern and more effective strategies for preventing the disease are urgently required. Increased rates of sporadic CRC have been strongly linked to the Western diet and lifestyle, that is consuming a diet that is high in simple carbohydrates, high in animal fats and low in dietary fibre as well as physical inactivity (Schulz et al. 2014). As diet is a modifiable risk factor for CRC tumourigenesis this study sought to determine whether the short term consumption of a high animal fat and high simple carbohydrate diet could stimulate the molecular pathways that commonly drive sporadic CRC. It also investigated whether dietary interventions of either curcumin or reverting back to a balanced diet could protect the colon from these molecular changes. A rat model was used to demonstrate that the Western diet can significantly increase the mRNA expression of *EGFR, ADAM17* and *TGFα* which are established molecular drivers of CRC. This effect was only statistically significant in the distal colon which is in congruence with the finding that *EGFR* overexpression is more common in colon cancers located in the distal colon. Although curcumin has been previously investigated as a treatment for established colon cancer, according to the published literature this is the first study to demonstrate that curcumin could have a protective effect in normal colon tissue by preventing the up-regulation of *EGFR* and *ADAM17*. This study also provided preliminary evidence that a diet high in animal fats and simple carbohydrates could be driving gene expression changes via epigenetic modifications.

The overexpression of *EGFR* has been reported to occur in up to 85% of sporadic, that is, non-hereditary CRC cases (Ellina et al. 2014). There is also a significantly higher frequency of *EGFR* over-expression and amplified *EGFR* signalling in tumours located in the distal colon

compared to tumours located in the proximal colon (Missiaglia et al. 2014). This study also demonstrated that *EGFR* expression was significantly higher in the distal colon compared to the proximal colon of rats fed the HCHF diet.

Genetically modified mouse models designed to induce CRC tumourigenesis have been primarily used to investigate the role of EGFR and diet. Dougherty et al (2009) demonstrated the relationship between EGFR and high fat diet-induced CRC, using a mouse model that had been genetically manipulated to lose 90% of its EGFR function. These EGFR knockdown mice displayed a significantly lower CRC tumour incidence and number of CRC tumours per animal compared to those with normal functioning EGFR despite being fed the same high fat diet for 40 weeks (Dougherty et al. 2009). This study utilised colon samples from a genetically unmodified (wildtype) rodent model administered a HCHF diet (more representative of the Western diet than high fat alone) for a time period of 16 weeks. The study demonstrated a significant fold change increase in the distal colon mRNA expression of *EGFR*. According to the literature, this is the first study to demonstrate a significant increase in *EGFR* mRNA expression in the colon in response to HCHF diet in a genetically normal rat. These findings further support the role of EGFR in driving high fat diet-induced CRC development in the distal colon.

The mRNA expression of both *ADAM17* and *TGFα* was also significantly increased in the distal colons of rats fed a HCHF diet compared to rats fed a corn starch diet. ADAM17 is a more recent target that has been associated with CRC tumourigenesis. It's normal role in the colon is to regulate colonic epithelial cell proliferation and differentiation through the proteolytic shedding of EGFR pro-ligands such as TGFα (Shimoda et al. 2016). Once released from the cell membrane, TGFα binds to EGFR to initiate downstream signalling cascades leading to

increased cellular proliferation and differentiation (Shimoda et al. 2016). Blanchet-Jossic et al (2005) reported the up-regulation of *ADAM17* mRNA in 90% of CRC tumours investigated along with the up-regulation of *EGFR* mRNA in 77% of these tumours (Blanchet-Jossic et al. 2005). More evidence to support the role of ADAM17 in diet-induced CRC tumourigenesis was published recently by Mustafi et al (2016) who reported that a high fat diet administered for 40 weeks significantly up-regulated the mRNA expression of *ADAM17* in CRC tumours in a mouse model. Furthermore, the targeted deletion of *ADAM17* within the colonic epithelial cells significantly decreased tumour incidence, further supporting a role for ADAM17 in diet-induced CRC (Mustafi et al. 2016).

It had previously been reported that a high fat diet (20% fat) for 40 weeks increased *TGFα* mRNA expression in CRC (Dougherty et al. 2009; Mustafi et al. 2016), however the present study demonstrated that HCHF diet increased *TGFα* mRNA after only 16 weeks. This suggests that tumourigenic changes are potentially occurring in a much shorter time period than previously reported. As ADAM17 can shed TGFα from its membrane-bound precursor proTGFα it can be speculated that the increase in ADAM17 increased the bioavailability of TGFα which when bound to EGFR triggers downstream signalling cascades and further up-regulation of *TGFα* gene expression.

Curcumin as a dietary intervention has shown therapeutic potential as an anti-inflammatory, antineoplastic, antioxidant and chemopreventative agent (Patel et al. 2008). The effects of curcumin on *EGFR* expression in CRC cells has been investigated in both animal models and human cell culture. Multiple studies have demonstrated that curcumin inhibits CRC through a dual mode of action that targets both the inhibition of receptor tyrosine activity and suppression of gene expression involved in the activation and transcription of EGFR (Chen &

Xu 2005; Chen et al. 2006; Starok et al. 2015). Despite its extensive investigation as a CRC treatment, there was no published study that demonstrated curcumin to have a protective effect against diet-induced up-regulated *EGFR* expression in the colon.

Results from this study demonstrated that supplementing the HCHF diet with 100 mg/kg body mass of curcumin prevented the increase in mRNA expression of *EGFR* induced by the HCHF diet. Interestingly there was no effect on *TGFα* mRNA expression, suggesting that *TGFα* expression may be resistant to the effects of curcumin. Overexpression of *TGFα* has been associated with resistance to anti-EGFR therapy, with the overexpression of *TGFα* shown to induce EGFR-MET interactions. MET, also known as Hepatocyte Growth Factor Receptor, is a cell membrane tyrosine kinase receptor and aberrant expression is linked to a number of cancers including CRC (Cecchi et al. 2012; Di Renzo et al. 1995). Dysregulation of MET signalling has been implicated as one of the main pathways in which growth inhibition by anti-EGFR drugs can be bypassed. EGFR ligands such as TGFα can transactivate MET by inducing the formation of heterodimers with members from the EGFR family in the absence of MET specific ligands. As a result, anti-EGFR induced growth inhibition is overcome by activating both EGFR and MET signalling cascades, which stimulates numerous cellular processes involved in cancer progression. Down-regulation of *TGFα* expression using siRNAs has been shown to re-sensitise colon cancer cells to anti-EGFR therapy and therefore combining anti-EGFR drugs with TGFα or MET inhibitors could be considered to improve treatment response (Troiani et al. 2013).

Results from this study also demonstrated that curcumin was effective in preventing the increase in mRNA expression of *ADAM17* caused by the HCHF diet in the distal colon. This is the first study to investigate the effects of curcumin on the expression of *ADAM17* in the colon

and therefore the mechanism by which this occurs is unknown. Another mechanism that may explain why curcumin prevented the HCHF-stimulated increase in mRNA expression of *ADAM17* and *EGFR* but not *TGFα* may be that curcumin is exerting its effect through the inhibition of Nuclear Factor kappa B (NF-κB). NF-κB has been shown to play an important role in both the maturation of ADAM17 and activation of EGFR but with no evidence of its involvement in TGF a functionality. In order for NF-κB activation to occur, proteolytic degradation of *IkBa* is required. A study by Pendurthi et al (1997) demonstrated that curcumin prevents the degradation of *IkBa* and therefore prevents the subsequent activation of NF-κB (Pendurthi et al. 1997).

NF-κB has been identified as a key regulator of ADAM17, as it is required for the maturation of ADAM17 from its pro-form and subsequent activation. Inhibition studies using NF-κB inhibitor demonstrated that inhibition of this protein resulted in suppression of ADAM17) expression (Takamune et al. 2008). Multiple studies have suggested that EGFR and NF-κB work together to produce an environment that promotes tumour development and progression via sustained cell proliferation, survival and invasion (Biswas and Iglehart, 2006; Habib et al. 2001 and Sordella et el. 2004). The activation of EGFR and NF-κB are inter-related with growth factors such as EGF linked to activation of NF-κB when bound to EGFR. EGF activates NF-κB via the *I*k*B* kinase complex. Targeted genes involved in activation of NF-κB such as *KIAA1199* and *I*k*B*α and *I*k*B*β have been shown to display feedback mechanisms that impact on EGFR-dependent signaling pathways in a number of cancer types. In cervical cancer KIAA1199 (which is activated by NF-κB) has been shown to reduce cell death by stimulating EGFR phosphorylation activating downstream singling cascades that promote cell survival and also stimulates EGF-dependent epithelial mesenchymal transition thereby promoting

migration and invasion (Shostak et al. 2014). In head and neck cancers knockdown of *I*k*B*α and/or *I*k*B*β both of which are required for NF-κB activation, resulted in significantly reduced levels of *EGFR* mRNA and protein expression (Nottingham et al. 2014).

Based on current knowledge and the results of the experiments presented here it is plausible to hypothesise that curcumin is preventing the increase in *ADAM17* and *EGFR* mRNA in response to HCHF diet through the suppression of NF-κB activation. Currently there is no known association between NF-κB and *TGFα* expression and therefore it is expected that inhibition of NF-κB would not affect the expression of *TGFα,* which is supported by the results of this experiment.

Due to its promising response, further investigation is required to determine how curcumin is exerting its inhibitory effect on *ADAM17* mRNA expression. In addition to its function in activating EGFR and numerous EGFR ligands, ADAM17 has been implicated in CRC pathogenesis as a stimulator of tumour migration and angiogenesis both of which have been classified as hallmark characteristics of CRC (Hanahan and Weinberg, 2000). Inhibition of ADAM17 leads to a reduction in the formation of HUVEC blood vessel-like structures (Göőz et al. 2009). Göőz et al (2009) also proposed that ADAM17 stimulates blood vessel formation through the activation of matrix metalloprotease 2 (MMP-2) (Göőz et al. 2009). Previous studies have demonstrated that curcumin has an inhibitory effect on the formation of new blood vessels using HUVEC and HVSMC models and therefore inhibits angiogenesis, which is an essential requirement for tumourigenesis (Patel et al. 2010; Singh et al. 1996).

Curcumin has been extensively studied in a number of research and clinical studies and proven to be a safe compound (Cheng et al. 2001; Lao et al. 2006; Shoba et al. 1998) therefore adding to its attractiveness as a preventative agent to reduce risk of diet-induced CRC. Based

on these findings and the literature demonstrating the safety of curcumin, there is potential for curcumin to be an effective preventative agent to reduce HCHF diet-induced overexpression of *EGFR* and *ADAM17*.

In addition to investigating the effects of the HCHF diet on CRC biomarkers, this study also explored the effects of HCHF diet on the mRNA expression of epigenetic modifying enzymes in the distal and proximal colon. The distal colon of HCHF rats showed an increase in the expression of the histone phosphorylation enzyme, *AURKB,* the histone methyltransferase enzyme *Ehm2* and the DNA methylation enzyme *Dmnt3a*. Previous studies exploring the epigenetic modifications that can occur in the colon in response to a HCHF diet have also shown an increase in these targets (Cho et al. 2011; Robertson et al. 1999; Weis et al. 2015). The aberrant expression of these enzymes has been implicated in carcinogenesis (Cho et al. 2011; Robertson et al. 1999; Weis et al. 2015). The dysregulation of AURKB is associated with mitotic abnormality and errors in chromosomal formation resulting in instability (Fu et al. 2007; Ota et al. 2002). This instability can potentially lead to tumourigenic changes in the colon via the CIN pathway, which is the most common molecular pathway in sporadic CRC (Hagland et al. 2013). Ehmt2 stimulates cellular proliferation and when this mRNA was inhibited via small interfering RNAs it led to a decrease in bladder cancer cell growth and caused apoptotic cell death (Cho et al. 2011). In the present study, *Ehmt2* increased in response to the HCHF diet which suggests that this could be a potential mechanism by which diet drives CRC tumourigenesis. Increased expression of *Dnmt3a* has also been associated with widespread regional hypermethylation resulting in the suppression of tumour suppressor genes (such as *APC*) enabling tumourigenic changes to occur within the colon (Weis et al. 2015). *Dnmt3a* is highly expressed in CRC and the targeted deletion of this gene

can inhibit tumour growth (Weis et al. 2015). Dnmt3a is involved in a number of cellular mechanisms implicated in tumourigenesis including cell growth, cell proliferation, cell survival and inhibition of cell death (Robertson et al. 1999; Weis et al. 2015). The present study has shown that the HCHF diet increased the expression of *Dnmt3a*, therefore suggesting that one of the multiple pathways of HCHF diet-induced CRC, might arise through the increased expression of *Dnmt3a* in the distal colon resulting in the suppression and inactivation of a number of tumour suppressor genes which would usually control tumourigenic changes in the colon.

In the distal colon, a reduction in the expression of *Symd3* and *HDACs 1 and 7* was also observed. Symd3 is a histone methyltransferase and is referred to as a proto-oncogene as it has a functional role in the activation of transcriptional regulators associated with cell proliferation, epithelial-mesenchymal transition and various other oncogenic pathways. Overexpression of *Symd3* has been implicated as a major driver of CRC and other cancers (Sarris et al. 2016). Increased expression of HDACs is also commonly associated with CRC and linked to a worse survival compared to tumours with normal expression of HDACs. As a result of its function, up-regulation of HDACs has been shown to cause an increase in transcriptional silencing of tumour suppressors and therefore increases risk of developing CRC (Bardhan & Liu 2013). Due to their important functions in CRC development it was expected that both *Symd3* and *HDAC* expression would be increased in response to the HCHF diet, however the results showed that these genes were substantially downregulated. This finding requires further investigation to determine whether this is an anomaly or whether the increased expression of these genes is not involved in diet-induced CRC.

In the proximal colon, the HCHF diet led to the up-regulation of the histone acetylation enzyme *CIITA* gene expression and down-regulation in the expression of several histone methylation transferases including *Suv39h1, Suv39h2, Suv420h2* and *Ezh2*. The latter group of enzymes are involved in transcription repression, gene silencing, cell migration and invasion (Ozdag et al. 2006; Yokoyama et al. 2014). CRC is typically associated with overexpression of *Suv39h1, Suv420h2* and *Ezh2*, however results from this study have demonstrated that HCHF diet leads to reduced expression of these genes compared to the corn starch diet. Further investigation is required as this finding only investigated pooled samples from three rats in each group. The finding that *Suv39h2* was down-regulated in response to the HCHF diet was supported by a published study by Peters et al (2001). The *Suv39h2* knock-down mouse experienced chromosomal instabilities that resulted in an increased risk of developing CRC tumours demonstrating that *Suv39h2* has a protective role against CRC tumourigenesis (Peters et al. 2001). The present study showed *Suv39h2* to be down-regulated in response to the HCHF diet, suggesting a possible link between HCHF diet, down-regulated *Suv39h2* expression and CRC.

One of the limitations in the study was the relatively short length of time in which the rats were exposed to the diets and treatments when compared to human diets, where the majority of humans have been exposed to HCHF diet throughout their entire lifetime or at least their adult life.

The promising results obtained in this study warrants further investigation, especially the use of curcumin to prevent the increased expression of *EGFR* and *ADAM17* and potentially the risk of developing CRC due to a diet that is high in simple carbohydrates and high in animal fat. Moving forward, further investigation into the relationship between HCHF diet and other

CRC biomarker expression is required and future studies should determine whether the increase observed in mRNA levels is translatable to an increase in protein levels. To understand the long term effectiveness of curcumin, experimental studies will need to be carried out over a longer period of time. Furthermore, investigation is required into the effectiveness of curcumin in ameliorating the expression of other potential biomarkers involved in the EGFR/ADAM17 axis, including other EGFR ligands and ADAM10. The translation of this research into human patients is the ultimate future study where the effects of adding curcumin to the diets of individuals at a high risk of CRC would be explored.

In conclusion, this study has provided preliminary evidence that a diet high in simple carbohydrates and animal fats can significantly upregulate the expression of *EGFR, ADAM17* and *TGFα* in the distal colon after only 16 weeks. As EGFR has been implicated as a major driver of CRC tumourigenesis, and increased mRNA expression of *ADAM17* and *TGFα* are also common in many CRC tumours, this study proposes that the mechanism of diet-induced CRC in the distal colon could be via the up-regulation of these genes. The study has also demonstrated that HCHF diet has the potential to cause epigenetic changes via expression changes in epigenetic modifying genes in both the distal and proximal colon that may potentially drive CRC tumourigenesis independently of *EGFR, ADAM17* and *TGFα*.

With the continuing increase in CRC incidence coupled with the wide-spread obesity epidemic, the global incidence of CRC is projected to increase exponentially with more than 2.2 million new cases and 1.1 million deaths by 2030 (Arnold et al. 2016). With these alarming statistics, new cost effective and safe therapeutic approaches are urgently needed to help prevent Western diet induced CRC or at least reduce the risk. Curcumin is an inexpensive and safe dietary compound which we demonstrated to prevent the increased expression of biomarkers implicated in HCHF induced CRC development and progression.

Chapter 6: References

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Supplementary Table 1: Body mass

Sample	CS	HCHF	C-100	H-100	H8C8
Rat1	412	550	346	515	442
rat 2	435	514	345	427	432
rat 3	394	533	373	513	435
Average	413.67	532.33	354.67	485	436.33
Standard error	11.865	10.398	9.1712	29.006	2.9627