Effects of carrot consumption on intestinal cancer risk

By

Humphrey Garti

Thesis submitted for the Degree of **Doctor of Philosophy**

School of Agriculture, Food and Rural Development
Newcastle Upon Tyne

June, 2016

Abstract

Intestinal cancer is a leading cause of death, and epidemiological studies associate high intakes of fruits and vegetables to reduced risk of the disease.

This study investigated the effects of carrot consumption on intestinal tumourigenesis in the *Apc*$^{min/+}$mouse model, homologous to the human Familial Adenomatous Polyposis mutation. Mice were fed either standard RM3 mouse feed (control) or diets enriched with 20% freeze-dried carrot powder prepared from blanched carrots. In Experiment 1, both diets were fed as pellets, manufactured by SDS Diets by mixing, extruding and drying at 90-120^{o}C for 20-30 minutes, while in experiment 2; both diets were fed to the mice as unprocessed powder. Dams were fed either carrot enriched or control diets from two weeks prior to mating with *Apc*$^{min/+}$ sires and throughout pregnancy and lactation. At weaning, all offspring were randomised to either carrot enriched diet or control diets. At 15 weeks post-natal, intestinal tumour number, size and location were recorded alongside body and organ weights. Using tissue from tumours and normal intestinal segments from min and wild-type mice in experiment 2, Total RNA was isolated, reverse transcribed and Real-time PCR performed, to assess expression of 6 potentially cancer related genes: Retinoid X receptor beta, (*RXRb*); Retinoid acid receptor alpha, (*RARα*); *Cyclin D*; ββ-Carotene 15,15-monooxygenese-1, (*BCMO1*); Cyclooxygenase-2, (COX-2); and Metrilysin-7, (MMP7).

In experiment 1, consumption of carrot pellets post weaning increased total gut tumour number by 42% (P = 0.038) whereas in experiment 2, carrot powder feeding post weaning reduced the total gut tumour number by 21% (P = 0.037). The control diet tumour numbers did not differ between experiments and maternal/pre weaning diets did not affect tumour numbers in offspring significantly. Powdered diets did not affect expression of the measured genes.

So while powdered carrot as expected reduced cancer severity, pellets had the opposite effect. According to Duan and Barringer, (2012) ~~paper~~, using high temperature for drying of carrot causes formation of the volatile carcinogen furan, providing a possible explanation for this difference.

Declaration

I declare the content of this thesis is my own and has not been submitted anywhere for any other degree or qualification. Where work carried out by others is presented acknowledgement have been made in the text and/or within the acknowledgements below

Acknowledgement

I would like to thank my team of supervisors, Dr Kirsten Brandt, Georg Lietz and Jill McKay for their expert guidance and support throughout my study. In full gratitude I acknowledge the efforts you invested into reading through my submissions and giving me your valuable comments. It had been a very difficult period for me and I am particularly grateful for your patience and encouragement.

I would also like to thank Adele Kitching for her support and for putting at my disposal her expert knowledge that made this work possible. Many thanks to Sandra Hogg and Neil Hamilton for providing all the needed support and encouragement that made work in the animal house a delight.

I will forever cherish the technical contributions of the laboratory staff of the School of Agriculture, Food and Rural Development to my work. I am particularly grateful to Christopher Bulman for the support, encouragement and the inspiration he provided during sample collection and throughout the laboratory work.

My special thanks go to Anthony Oxley and Adam Clark for the technical advice and friendship not forgetting Dr Tahar Taybi of the School of Biology for exposing me to the technicalities of Real-Time PCR.

To my brothers and my wife I say thank you for being the source of inspiration and motivation. You will forever remain in my heart.

Abbreviations

AFAP	Attenuated Familial Adenomatous Polyposis
AOM -	Azoxymethane
AP-1 -	Activator protein-1
APC	Adenomatous polyposis coli
ATBC	Alpha-tocopherol beta-carotene cancer prevention study
ATM -	ataxia telangiectasia mutated
BCMO1	β, β 15, 15-monooxygenase
BUB	Bbudding uninhibited benzimidazole
CACNAIG,	Cav 3.1 T-type calcium channel gene
CAPE	Caffeic acid phenethyl ester
CARET	Beta-Carotene and Retinol Efficacy Trial
CAV	Campus for Ageing and Vitality
Cdk-	Cyclin dependent kinase
CDK4	Cyclin dependent kinase 4
CDK6	Cyclin dependent kinase 6
CDKN2A	Cyclin dependent kinase 2A
CHRPE	Congenital hypertrophy of retinal pigment epithelium
CIN –	Chromosomal instability
CK1-	Casein kinase 1
c-Myc	Cellular oncogenes v-Fos FBJ murine osteosarcoma viral oncogenes
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CpG	Cytosine, phosphoguanine
CRC	Colorectal cancer

DCC	Deleted In Colorectal Cancer
DMBA	7, 12-dimethyelbenz[a]anthracene
DMH	1,2-dimethylhydrazine
DNA	Deoxyribonucleic Acid
DNMT -	DNA-methyltransferase
EGFR	Epidermal growth factor receptor
EPIC	European Prospective Investigation in Cancer and Nutrition Study
ERK1/2	Extracellular regulated-kinase 1/kinase 2
FAP -	Familial adenomatous polyposis
GSK3	Glycogen synthase kinase 3
GSK-3β.	Glycogen synthase kinase-3beta
HMGR	hydrxy-3-methyglutaryl CoA reductase,
hMLH1,	Human MUtL homologue 1
hMSH2,	Human MUtL homologue 2
HNPCC	Hereditary nonpolyposis colorectal cancer
hPMS1	Human postmeoitic segregation increased 1
hPMS2	Human postmeoitic segregation increased 2
IGF2,	Insulin-like growth factor 2
IL-1-	Interleukin-1
K-RAS -	Kirsten-rRas
LEF	Lymphocyte enhancer factor –
LOH -	Loss of heterozygosity
MAP	Mitogen-activated proten
MDCS	Malemo Diet and Cancer Study
MeCP-1	Methyl-CpG-binding protein 1
MINT	Methylated in tumours
MLH1	MutL homologue 1

MMP-2	Matrix metalloproteinase-2
MMP7	Metrix metalloproteinase -7
MMP-9	Metrix metalloproteinase -9
MMR	Mismatch repair
mRNA	messenger ribonucleic acid
MSH2	MutS homologue 2
MSH3,	MutS homologue 3
MSH6	MutS homologue 6
MSI -	Microsatellite instability
MYC,	Mut Y homologue
NF-kB	Nuclear factor kappa B
PCNA -	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PMS1-	Prokaryotic MutL gene homologue 1
PMS2-	Prokaryotic MutL gene homologue2
pRb -	Retinoblastoma protein
RARbeta-	Retinoid-Acid-Receptor beta
RUNX3	Runt-Related Transcription Factor3
RXRalpha -	Retinoid-X-Receptor-alpha
SDS	Special Diet Services
TNM -	Tumour, Node and Metastasis
TPA -	12-0-tetradecanoylphorbol-13-acetate

Dedication

Dedicated to the memory of Catherine and Rose Garti

List of contents:

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

1 Chapter 1 Introduction

1.1 Carrots

Carrots (*Daucas carota* var. satirus) form a group of vegetables that have been widely studied because of their bioactive compound content. They belong to the Apiaceae family and are widely distributed in temperate regions (Zidorn *et al.*, 2005). They are known to contain a number of bioactive compounds such as polyphenols, carotenoids and falcarinol-type polyacetylenes with many functional characteristics (antifungal, antiallergenic, cytotoxic, anti-platelet aggregation and anti-inflammatory) that suggest cancer preventive effects (Brandt *et al.*, 2004). These plant metabolites, modulate gene expression, epigenetic regulation, cell signaling, inflammation, antioxidant function, detoxification and immune function (Andarwulan *et al.*, 2012).

1.1.1 Bioactive Compounds

Fruits and vegetables constitute the fundamental source of micronutrients, fibre, carotenoids and phytochemicals (Bernart *et al.*, 1996; de Andrade Júnior and Andrade, 2014) which traditionally are of no nutritional significance but exert a biological effect. The characteristics of these compounds suggest health promoting effects thus making them the subject of epidemiological, clinical and experimental investigations. Many of such studies have examined the protective effects of fruits and vegetables in cardiovascular diseases (CVDs) and cancers (Kris-Etherton *et al.*, 2002). A number of epidemiological studies consistently associated high intake of fruits and vegetables with reduced incidence of chronic diseases including cancer (Cook *et al.*, 2013; Singh, 2013).

These bioactive compounds show wide variations both in structure and function and are grouped as phenolic compounds , carotenoids and polyacetylenes (Kris-Etherton *et al.*, 2002).

1.1.2 Carotenoids

1.1.3 Beta- carotene

Beta-carotene, the most nutritionally active carotenoid constitutes about 15 – 30 % of the total serum carotenoids many of which are said to promote immune response, inhibition of mutagenesis, reduce induced nuclear damage and also provide protection against neoplastic

events in cells, tissues and whole animals. β-carotene prevents nuclear damage and has a chemo-preventive or chemo-protective function against cancer, especially skin cancer (Bendich and Olson, 1989; Krinsky, 1989). Prospective and retrospective epidemiological studies indicate an inverse relationship between serum β-carotene, associated with intake of fruits and vegetables high in carotenoids, and the risk of cancer development (Bendich and Olson, 1989). In spite of the evidence of anti-carcinogenic properties of β-carotene and other carotenoids, inconsistencies exist within the literature. Results of meta-analysis of data from alpha-tocopherol, beta-carotene lung cancer prevention study (ATBC) by Albanes *at al.,* (1996) suggest increased incidence of lung cancer with β-carotene (20mg) supplementation especially among heavy cigarette smokers and alcoholics whereas data from base line dietary and serum β-carotene concentration suggested protective effect. Furthermore, investigations into β-carotene and Vitamin A supplementation by Omenn *et al.* (1996) in Beta-Carotene and Retinol Efficacy Trial (CARET) also suggested that β-carotene and vitamin A enhanced the incidence of lung cancer. In the CARET study individuals received β-carotene and Vitamin A supplements together thus there was distinction between the individual effects of β-carotene and vitamin A. Beta-carotene was also reported to accelerate the development of 7,12-dimethyelbenz[a]anthracene (DMBA) 12-0-tetradecanoylphorbol-13-acetate (TPA) induced papillomas tumours in mice in a dose-dependent manner (Chen *et al.,* 1993).

The increased incidence of lung cancer associated with β-carotene supplementation was attributed to high blood β-carotene concentrations which was 17 fold higher than observed with dietary intake of β-carotene (Albanes *et al.,* 1996). On the other hand the inverse association between dietary intake of β-carotene and lung cancer was probably not due solely to β-carotene considering the many different compounds present in plant diet, but possibly other compounds, acting as individuals or synergistically.(Bendich and Olson, 1989) . A Chinese intervention study of more than 29,500 adults between the ages of 40 and 69 years suggested that β-carotene supplementation in combination with vitamin E and selenium showed significant reduction in mortality as a result of reduced rates of stomach cancer (Blot *et al.,* 1993). This study like the previous ones could not associate the observed risk reduction wholly to beta-carotene.

Anticancer activities of beta-carotene is effected through retinoic acid which inhibits tumour cell invasion and metastasis in a process mediated by retinoid X receptor and retinoic acid receptor (Dillard and Lane, 2007b; Pham *et al.,* 2013). A study involving the examination of colonic biopsy specimens taken from patients with adenoma polyps before and after β-carotene supplementation showed that β-carotene inhibits colonic crypt cell proliferation.(Cahill *et al.,*

1993). Animal model studies also suggest protective effects of β-carotene against colon cancer (Temple and Basu, 1987; Alabaster *et al.*, 1995). Alabaster *et al.* (1995), fed rats high fat diets containing either high or low fibre and varying amounts of beta-carotene supplements and reported that beta-carotene significantly reduced aberrant crypt foci and tumour development. Temple and Basu (1987), tested the effect of beta-carotene enriched diet on 1, 2-dimethylhydrazine induced intestinal tumourigenesis by varying amounts of beta-carotene in laboratory chow fed to female Swiss Webster mice. Feeding mice β-carotene significantly reduced adenomas and more significantly adenocarcinoma, suggesting that beta-carotene is most effective at preventing progression from adenoma to carcinoma. Though no clear mechanism of action was defined in these studies for β-carotene, it was believed that beta-carotene acted either by preventing oxidative damage of cell membrane, DNA and other cell constituents or by enforcing ‘ immune response system in the colonic tissues (Temple and Basu, 1987; Alabaster *et al.*, 1995). Bendich and Olson (1989), also proposed that the protective mechanism of β-carotene could be by preventing free radicals from lipid peroxidation reactions therefore inhibiting damage of membranes, enzymes and nucleic acids. Other carotenoids that have been implicated in cancer risk reduction include lutein, lycopene and β-cryptoxanthin.

1.1.4 Phenolic Compounds

Polyphenols form an important health enhancing functional group of plant metabolites. They are found in many different plant species and vary widely in structure from C_6 ring structure to highly polymerized forms such as tannins (Kris-Etherton *et al.*, 2002). The high structural diversification coupled with variation in quantity makes accurate determination of dietary intake difficult. Their concentrations in foods are affected by a number of factors such as plant species, variety, light, the degree of ripeness, processing and storage (Duthie *et al.*, 2000).

Phenolic acid, flavonoids, stilbenes and lignans are the most commonly occurring polyphenols in plants. Flavonoids are the most abundant, forming 60 % of the highest occurring polyphenols with phenolic acids forming 30 % of dietary polyphenols. The most common phenolic acids in plants are derived from hydroxybenzoic acid (gallic acid, vanillic acid, procatechnic acid and syringic acid) and from hydroxy cinnamic acid (p-coumaric acid, caffeic acid and ferulic acid) (Nichenametla *et al.*, 2006). They are associated with some plant properties such as colouring of the leaves and fruits to either attract or repel insects and also protect the plant against herbivores. They occur largely in fruits, vegetables, leaves, nuts, seeds, flowers and barks of

plants and are essential to the plant's physiology in diverse functions such as structure, pigmentation, pollination, pathogen and predator resistance and growth and development (Dewick, 2011). Besides their physiological functions in plants, polyphenols produce beneficial health effects such as modulating gene expression, epigenetic regulation, cell signaling, inflammation, antioxidant function, detoxification and immune function in human (Andarwulan *et al.*, 2012). It is believed that the disease prevention properties of fruits and vegetables are probably due to their polyphenol content and are associated with their antiviral, immunomodulatory, antibacterial, cytotoxic and antiproliferative properties (Rapta *et al.*, 1995; Rice-Evans *et al.*, 1996; Agullo *et al.*, 1997). Acting as antioxidants, phenolic compounds prevent oxidative mechanisms that may lead to degenerative illness by reducing or donating hydrogen to compounds scavenging free radicals and quenching singlet oxygen (Çalişkan and Aytekin Polat, 2011).

One of the most studied polyphenol groups, flavonoids, are hypothesized to be responsible for the protection against diseases such as cancer and cardiovascular diseases associated with fruits and vegetables (Prior and Cao, 2000). Several studies associate flavonoid consumption with reduced risk of certain types of cancer (Kris-Etherton et. al., 2002). Although some reports suggest *in vitro* mutagenicity of flavonoids, *in vivo* and other *in vitro* studies support their anticarcinogenic properties (Harborne, 1993).

A Finish study of 9,959 subjects, suggest dietary intake of flavonoids significantly reduced the risk of lung cancer (Knekt *et al.*, 1997). The study also observed a reduction in colorectal cancer risk though this was not significant. The effect of flavonoid intake was attributed to quercetin which formed about 95 % of the flavonoids intake in the Finnish population (Knekt *et al.*, 1997). Later, *in vitro* studies reported the induction of apoptosis in gastric cancer cells (BGC-823) (Wang *et al.* (2011) and in human breast cancer cells (MDA-MB-2) (Chien *et al.*, 2009) treated with quercetin.

The health promoting properties of flavonoids is linked to their antioxidant activities which may be exhibited in many different ways: either by breaking the oxidation chain, recycling antioxidants (e.g. tocopherol), and preventing lipid peroxidation or by donating a hydrogen atom to the tocopherol molecule. They may also chelate pro-oxidant metal ions (iron, copper) preventing them from forming free-radicals while maintaining their own free-radical scavenging abilities (Kris-Etherton *et. al.*, 2002).

Other phenolic compounds suggested to reduce cancer risk include curcumin (Kubota *et al.*, 2012) catechin (Epigallocatechin 3-Gallate) (Ju *et al.* (2005)) and isothiocyanate (sulforaphane) (Hu *et al.*, 2006).

Several *in vitro* investigations have investigated the antitumour characteristics of phenolic acids. Caffeic acid phenethyl ester (CAPE) was shown to significantly inhibit the invasion and metastasis of human fibrosarcoma cells (HT1080) by down regulating the expression of matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9) (Hwang et al., 2006). Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic endoenzymes that degrade extracellular matrix and facilitate tumour cell evasion and migration (Ellerbroek and Stack, 1999; Kleiner and Stetler-Stevenson, 1999).
By its cytotoxic effect, CAPE inhibited both *in vitro* and *in vivo* growth of tumour cells, angiogenesis, NF-kB 1 and 2 activity, induced apoptosis and caused cell cycle arrest at the S-phase in MDA-231 and MCF-7 human breast cell (Wu *et al.*, 2011). Deactivation of NF-kB resulted in increased apoptosis (Beg and Baltimore, 1996).

In addition to the supporting evidence from *in vitro* studies, some *in vivo* evidence supports the anti-carcinogenic activities of phenolic acids. In rat model studies by Tanaka *et al.* (1993), chlorogenic and ferulic acids reduced preneoplastic tongue lesions. Caffeic acid was also shown to prevent the development of tongue neoplasms(Tanaka *et al.*, 1993). CAPE, exhibiting its anti-proliferation properties reduced the development of azoxymethane induced aberrant crypts in rat colon (Borrelli *et al.*, 2002). CAPE and curcumin reduced intestinal tumour formation by 63 % in the $Apc^{min/+}$ mouse model, whereas CAPE reduced tumour formation at a dietary concentration of 0.15 %, curcumin was effective at 0.1 % (Mahmoud *et al.*, 2000).

Nagaoka *et al.* (2003), tested the effect of CAPE on lung cancer metastasis in female Balb/C mice. CAPE was orally administered to the mice before and after intravenous inoculation with cultured colon 26-L5 carcinoma cells. Results showed inhibition of metastasis by a reduction in lung tumour weight and number of nodules by 50 %, suggesting chemotherapeutic properties of CAPE. The observed effect was probably due to cytotoxicity, growth inhibition or inhibition of cancer cell invasion.

Whilst many studies suggest phenolic acids are anticarcinogenic, one reported carcinogenic properties. Lee *et al.* (2000b), observed that caffeic acid enhanced the development of stomach papillomas when fed to F344 rats over a two-year period in a multi-initiation model.

1.1.5 Polyacetylenes

Polyacetylenes are chemically reactive compounds that are widely distributed in many plant families including Apiaceae (carrot/celery/parsley) and Araliaceae (aralia) (Hansen and Boll, 1986; Christensen, 1992).

Carrots are exclusively the main source of polyacetylenes in human diet (Kobæk-Larsen *et al.*, 2005) and include, falcarinol, falcarindiol, falcarindiol-3 acetate, of which the most abundant and most biologically active is falcarinol (Hansen *et al.*, 2003)

Falcarinol type polyacetylenes (falcarinol, falcarindiol, falcarindiol-3 acetate) have been reported to have anti-inflammatory characteristics (Resch *et al.*, 2001). These characteristics enable them to modify prostaglandin metabolism by inhibition of 15-hydroxyprostaglandin dehydrogenase (prostaglandin catabolising enzyme) and also to prevent the action of lipoxygenases (Christensen and Brandt, 2006). They are also known to inhibit diacylglycerol acyltransferase, nitric oxide synthase, cholesteryl ester transfer protein and microsomal and mitochondrial enzymes (Zidan *et al.*, 2005). The activities of this enzyme are very significant in the growth and development of colorectal cancer. Increased expression of 15-hydroxyprostaglandin dehydrogenase reduces the level of prostaglandin E2 and the number of intestinal polyps in Apc^{min+} mice (Backlund *et al.*, 2005) Lipoxygenase like *COX-2* is involved in the metabolism of polysaturated fatty acid, arachidonic and linoleic acid, the products of which are closely linked with carcinogenesis. Whilst the metabolic products of 5-, 8- and 12-lipoxygenase are thought to be procarcinogenic those of 15-lipoxygenase-1 and -2 are said to be anticarcinogenic (Shureiqi and Lippman, 2001). Induced nitric oxide synthase is involved in the production of nitric oxide which is genotoxic, causing DNA deamination and single strand breaks in the DNA (Felley-Bosco, 1998). The concentration of nitric oxide synthase correlates positively with tumour angiogenesis (Cianchi *et al.*, 2003). According toAhn and Ohshima (2001), inhibition of nitric oxide synthase in $Apc^{min/+}$ mice significantly reduced the development of intestinal polyps.

Falcarinol-type polyacetylenes, exemplified by falcarinol, have biphasic properties promoting cell proliferation *in vitro* when applied within a low concentration range of 0.01 to 0.05 μgml^{-1} falcarinol, while inhibiting cell growth beyond 1 μgml^{-1} falcarinol with maximum inhibition of over 90 % at 10μgml^{-1} falcarinol (Hansen *et al.*, 2003). In separate experiments, Young *et al.*, (2007) and Purup *et al.*, (2009) supported earlier findings that C17 aliphatic polyacetylenes have a hormesis effect when tested in cultures of human intestinal epithelial (FHs 74 Int) and cancer cells (CaCO-2). The ability of falcarindiol to inhibit intestinal cell proliferation was

significantly enhanced when applied at low concentration in combination with falcarinol suggesting synergistic inhibitory effect (Purup *et al.*, 2009) .

In vivo studies involving falcarinol-type polyacetylenes confirm that falcarinol and some other plant metabolites hold the potential for the prevention and/or treatment of cancer in humans particularly colorectal cancer (Ju *et al.*, 2005; Kobæk-Larsen *et al.*, 2005; Purup *et al.*, 2009). Kobaek-Larsen *et al.*, (2005) investigated the effects of supplementing standard rat feed with purified falcarinol and freeze-dried carrot containing the equivalent falcarinol quantity on the development of azoxymethane (AOM) induced colon cancer in male BDIX rats. After 20 weeks of feeding, compared to mice fed a control diet those fed diets supplemented with both purified falcarinol and freeze-dried carrot had less (pre)-cancerous lesions. Mice have the ability to detect and reject nutritionally inadequate diet and select for diets that support normal growth especially those with the complements of indispensable amino acids (IAAs) (Gietzen *et al.*, 2007). Freeze dried carrot supplementation provided dietary concentration of 35 µgfalcarinol/g which was considered as physiologically relevant as that was the amount of freeze-dried carrot (10 %) chosen by the rats when given free access to both freeze dried carrot and laboratory chow. This translates into 370 g cooked carrot in human diet which is just below the recommended minimum daily intake of 400 g suggested to reduce the risk of cancer (World Health Organisation, 2004). According to Gundgaard *et al.* (2003), daily vegetable intake of 400 – 600 g reduce cancer incidence by 19 and 32 %.

1.2 Colorectal Cancer

1.2.1 Epidemiology of Colorectal Cancer

Colorectal cancer (CRC) is the third most common cancer in the world after lung and breast cancer. CRC accounts for 9.7 % of the global 14.1 million new cases of all cancers and is the third leading cause of cancer related death comprising 8.5% of 8.2 million deaths worldwide attributed to cancer in 2012. Colorectal cancer accounted for approximately 10 % and 9.2 % of e new cancer cases in men and women respectively in 2012, making it the third most diagnosed cancer in men and the second in women (Ferlay *et al.*, 2015).

There is global disparity in the incidence and mortality rates of the disease. Of the 1,233,700 new cases reported in 2008, the highest diagnosis of 29.4 % and 14.4 % occurred in Eastern Asia and North America respectively. The incidence in Western Europe was 11.2 % whilst it was 10.5 % in Central and Eastern Europe. Africa accounted for 2.8 % of the world total with

the highest and lowest diagnosis of 0.8 % and 0.2 % occurring in North and Middle Africa respectively. Except in South Africa and West Africa where estimated new cases decreased by 3 and 4 % respectively, there was a general increase in diagnosis from 2008 to 2012. Also apart from North America where estimated new cases decreased by 5.6 % and South Eastern Asia where diagnosis appeared stable there was general increased in estimated diagnosis in the Americas and Asia. Increases in new cases over Europe was generally low compared to other regions of the world with rates ranging between - 0.5 % for Western Europe to 3.7 % for Central and Eastern Europe (Ferlay *et al.*, 2010; Ferlay *et al.*, 2015). However, incidence rates are still high in the Czech Republic and Slovakia in Eastern Europe and Kuwait and Israel in Western Asia, areas hitherto considered as low risk areas. For example, males in the Czech Republic rank fourth in the world for CRC incidence with mortality to incident ratio of 0. 5. (Dusek et. al., 2015). These high rates have been attributed to the prevalence of risk factors such as physical inactivity, obesity, excessive alcohol intake and consumption of diets low in fruits and vegetables (Center *et al.*, 2009; Dusek et. al., 2015).

In spite of the estimated new cases and deaths, incidence and mortality rates have been on the decline in the developed countries over the past two decades (American Cancer, 2015). Whereas about 55 to 60 % of estimated CRC new cases were reported in developed countries, most of estimated mortality occurred in less developed countries. These declining trends in developed countries could be accounted for by decrease in the prevalence of risk factors coupled with increased screening tests (colonoscopy and sigmoidoscopy) and removal of precancerous adenoma (Center *et al.*, 2009; Edwards *et al.*, 2010).

Diagnosis of CRC cancer in individuals increases with age. More than 90 % of new cases were reported in people who are 50 years and above (American Cancer, 2015) with diagnosis over 40-fold in people aged 60 and 79 years and above compared to those under 45 years (Society, 2013).

Also affecting the incidence rate of colorectal cancer is hereditary and medical history of individuals. Persons with chronic inflammatory bowel disease (ulcerative colitis and Crohn disease) are at a higher risk of developing the disease (Janout and Kollárová, 2001). In addition people whose close relations had CRC are more likely to develop the disease especially, if these relatives were diagnosed of the disease below 60 years of age (Boardman *et al.*, 2007).

Incidence and mortality rates worldwide were lower in women than in males, a phenomenon attributed to menopausal hormone therapy involving the use of oestrogen and progestin

especially in the developed world (Johnson *et al.*, 2009). For instance in Europe 47 and 46 % of estimated new cases for 2008 and 2012 respectively were in women whilst in Asia the rate was 44.3 and 42.8 %. Of the estimated deaths in Europe for the same period 48 and 47.3 % occurred in women whilst 45.7 and 44.2 % respectively were in women (Ferlay *et al.*, 2010; Ferlay *et al.*, 2015).

Migration is another contributing factor. Migrants from countries with low-incidence rates to industrialised high incidence rate countries show higher incidence of the disease compared to their relations in their home countries as a result of adaptation to westernised life styles of the host nations to underscore the importance of environmental influence in colorectal cancer risk (Curado *et al.*, 2007). CRC incidence rate was also known to vary between ethnic groups within a population. For instance in Singapore, Chinese and Malay migrants showed different incidence rates (Lim *et al.*, 2003) and in Israel, the Jews differ in incidence rates from the non-Jew Arabs (Center *et al.*, 2009).

1.2.2 Aetiology of Colorectal Cancer

The development of CRC is a multistep process which is developed over decades through an accumulation of genetic alterations in tumour suppressor genes, oncogenes and mismatch repair genes in individuals (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996b; Xu *et al.*, 2012). It is suggested that mutation in *APC*, tumour suppressor gene and the loss of normal function is the initiating process of neoplastic transformation of normal epithelia cells to carcinoma(Kinzler and Vogelstein, 1996b). This dysregulates cell proliferation, and the progress of cellular transformation is enhanced by mutations of oncogenes (Fearon and Vogelstein, 1990) (Fig. 1.1)

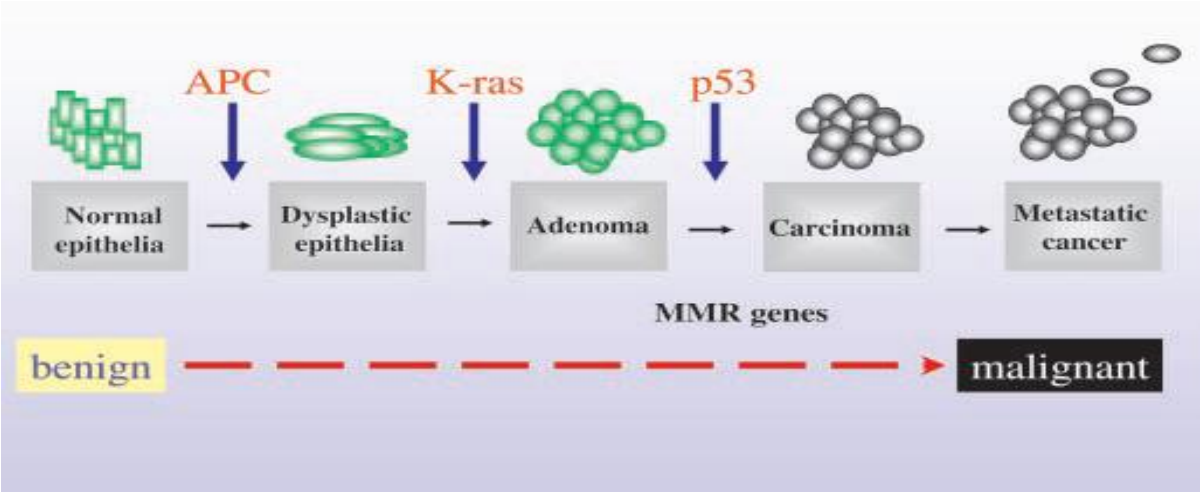


Figure 1. 1 A model of genetic alterations from adenoma to carcinoma in Colorectal cancer development (Smith et al., 2002).

Whereas K-RAS is activated by allelic mutation, *DCC* and *P53* are activated after mutation of the allelic pair (Fearon and Vogelstein, 1990). Other genes believed to be involved in the process are mismatch repair genes (hMSH2, hMLH1, hPMS1, hPMS1 or hPMS2) (Gryfe *et al.*, 1997). These genes enhance the rate of tumourigenesis in hereditary non-polyposis colorectal cancer patients whilst in familial adenomatous polyposis patients, tumour initiation is enhanced by mutation in *APC* gene (Lynch *et al.*, 1996). Although the genetic alterations of tumourigenesis occur in a preferred order, the biological characteristics of the tumour is determined by the accumulated alterations and not order of mutational events (Fearon and Vogelstein, 1990).

Colorectal cancer may be classified into stages according to the severity of the disease thus making the stage of diagnosis critical for the survival of the patient. For example, five year survival for Duke's stage A (Table 1.1) is over 90 % but it is 5 % for Duke's stage D (Albert de la Chappelle, 2004).

Other systems of tumour classification include Astler-Coller's (Table 1.2) modification of Duke's classification and the Tumour, Node and Metastasis (TNM) (Table 1.3).

Table 1. 1 Duke's Stages of Colorectal Cancer

Stage	Definition
A	Carcinoma limited to walls of rectum
B	Carcinoma spreads to extra rectal tissue but no nodal involvement
C	Lymph nodes involved
D	Distant metastasis

Source: (Zinkin, 1983)

Table 1. 2 Astler-Coller

Stage	Definition
A	Tumours invade through the muscularis mucosa into the submucosa
B1	Tumours invade into the muscularis propria
B2	Tumours completely penetrate the smooth muscle layer into the serosa
C	Tumours encompass any degree of invasion but are defined by regional lymph node involvement
C1	Tumours invade the muscularis propria with fewer than four positive nodes
C2	Tumours completely penetrate the smooth muscle layer into the serosa with four or more involved nodes
D	Lesions with distant metastases

John Hopkins Colon cancer Centre

Table 1. 3: Tumour, Node, Metastasis (TNM) Staging System for Colorectal cancer

T1	Tumour invades submucosa	
T2	Tumour invades muscularis propria	
T3	Tumour spreads through the muscularis into subserosa or in to the pericolic ornperirectal tissue	
T4	Tumour invades other organs or structures, and/or perforates	
Nodes		
N0	No regional lymph node metastasis	
N1	Metastasis in 1 to 3	
N2	Metastasis on 4 or more regional lymph nodes	
M0	No distance metastasis	
M1	Distance metastasis present	
Stage Groupings		
Stage	T1 N0 M0: T2 N0 M0	Cancer spread but still in inner lining
Stage II	T3 N0 M0: T4 N0 M0	Cancer spreads to organs near colon or rectum but not in lymph nodes
Stage III	Any T, N1-2, M0	Cancer spread to lymph nodes only
Stage IV	T, Any N, M1	Cancer spread through lymph to body, liver and lung (metastasis)

Source: http://www.healthcommunities.com/colon-cancer/staging.shtml#TNM (02/07/2015)

1.2.3 Hereditary colorectal cancer

Colorectal can be categorised as sporadic or familial. Familial CRC is reported to be responsible for between 15-30 % (De la Chapelle, 2004; Rustgi, 2007).

Hereditary nonpolyposis colorectal cancer (HNPCC) forms about 3 – 4 % of familial CRC, while about 1 % of CRC cases are associated with familial adenomatous polyposis (FAP) and less than 1 % are associated with a number of conditions such as hamartomatous polyposis syndrome, MYH-associated polyposis (MAP) and hyperplastic polyposis (Rustgi, 2007; Shen *et al.*, 2007).

1.2.4 Hereditary Non-polyposis Colorectal Cancer (HNPCC)

Hereditary non-polyposis colorectal cancer (HNPCC) – Lynch Syndrome, is an autosomal dominant disease defined by the development of colorectal cancer at an early age and is associated with extra colonic tumours (endometrium, ovaries, stomach, small bowel, hepatobiliary epithelium, ureopithelia epithelium and brain tumours) and microsatellite instability (Umar *et al.*, 2004; de la Chapelle, 2004). It is usually right sided and largely proximal colorectal cancer (Lynch and de la Chapelle, 2003). It is the most common hereditary disorder and has an early onset of about 45 years (Lynch and Lynch, 2000). The disease is caused by mutations in DNA mismatch repair (MMR) genes [(*MutL homologue 1* (*MLH1), MutS homologue (MSH2 MSH3, MSH6*) *), (Prokaryotic MutL gene homologue (PMS1 and PMS2),),*] and carriers of germline mutations in these genes have 80 % , 60% and 20 % life time risk of developing colorectal, endometrial and other forms of cancer respectively (De la Chapelle, 2004; Rustgi, 2007). Even though individuals may carry germline mutations in the mismatch repair genes, loss of heterozygosity is necessary for the development of the disease and occurs by somatic mutation either by *MLH1*, CpG island methylation or by deletion (Hemminki *et al.*, 1994; Liu *et al.*, 1995; Rustgi, 2007). In HNPCC mutations in *MSHS6* have lower penetrance (proportion of individuals carrying a mutation and show phenotypic features associated with the mutation) relative to mutations in *MLH1, MSH2 (*Miyaki *et al., 1997). MSH2* mutations are associated with higher-penetrance predisposition to extra colonic cancer. About 60 – 80% of germline mutations characterizing HNPCC could be associated with *MLH1, MSH2* and *MSH6* genes (Rustgi, 2007). Microsatellite instability involving *MLH1* methylation alone accounts for nearly 15% of all colorectal (Umar *et al.*, 2004).

1.2.5 Familial Adenomatous Polyposis (FAP)

FAP is an autosomal dominant disease characterized by the development of thousands of adenomatous polyps in the large intestine during the second and third decades of life of affected individuals. The disease is linked to germline mutations on chromosome 5q21 in the *APC* gene (Galiatsatos and Foulkes, 2006). According to Knudson's 'two hit' hypothesis, an inherited germline mutation of one allele is not enough to cause the disease suggesting that a second 'hit' somatic mutation of the wild-type is the rate-limiting step to FAP (Green *et al.*, 1998).

FAP accounts for about 1 % of the total colorectal cancer burden, affects 1 in 10,000 individuals and has close to 100 % penetrance (Lal and Gallinger, 2000). Individuals affected by FAP are

characterized with loss of heterozygosity (LOH) and development of large number of colorectal adenomas at an average age of forty years (Nugent *et al.,* 1994; Segditsas and Tomlinson, 2006). The disease is also associated by extra colonic manifestations, including upper gastrointestinal tract polyps, retinal lesions (congenital hypertrophy of retinal pigment epithelium – CHRPE), desmoids tumours, and brain osteoma. These extra colonic phenotypic features distinguish FAP patients based on the region of mutation in the *APC* gene (Kinzler and Vogelstein, 1996). Individuals carrying mutations between codons 169–1,600 are generally associated with the development of develop 1000s or more adenomas. Whilst retinal lesions (CHRPE) are features associated with mutations between codon 463 – 1387 mutations between codons 1,403 and 1,578 referred to as Gardner's syndrome lead to osteomas, skin fribromas and epidermoid cysts (Olschwang *et al.,* 1993; Kinzler and Vogelstein, 1996b; De la Chapelle, 2004). Mutations occurring before codon 157 are associated with the development of relatively fewer adenomas at a later age, an attenuated form of FAP (AFAP) (de la Chapelle (1999), Kinzler and Vogelstein (1996).

1.2.6 Genomic Instability

Genomic instability is fundamental to carcinogenesis and promotes accelerated genetic alteration for tumour formation (Matsubara, 2012). It is caused by a) mutation and/or altered methylation of regulatory genes (name genes) in the DNA repair system. Genomic instability may be divided into 2 groups, each with a distinct developmental pathway, chromosomal instability (CIN) and microsatellite instability (MSI).

1.2.7 Microsatellite Instability and Colorectal Cancer

Microsatellites are repeats of simple DNA base pairs that occur abundantly and randomly throughout the human genome. It may consist of approximately 10 – 50 repeats of 1 – 6 bps that are normally relatively stable (Weber, 1990; Wheeler *et al.,* 2000). These repeat sequences are usually transmitted without any alterations through the process of mitosis and meiosis (Thibodeau *et al.,* 1993). Cytosine and adenine dinucleotide repeats are the most abundant microsatellite and are widely distributed in the human genome (Thibodeau *et al.,* 1993). Microsatellite instability (MSI) thus refers to the situation where the base pair repeat sequence (microsatellite) undergoes frequent change in length due to either deletion or insertion of repeated units (Wheeler *et al.,* 2000; Söreide *et al.,* 2006). In colorectal cancer MSI is

characterized by accumulation of germline mutations and hypermethylation in DNA mismatch repair (MMR) genes (*MLH1, MSH2, MSH6, PMS1 and PMS2*) and transforming growth factor β-type II receptor and *BAX?* (Lynch and de la Chapelle, 1999; Matsubara, 2012) These mutations and hypermethylation result in the loss of DNA mismatch repair function (Sinicrope and Sargent, 2012) The errors most often encountered in microsatellite instability are base mismatches and insertion/deletions in DNA coding regions. This results in frameshift mutations and subsequent protein truncations and loss of MMR function (Sinicope and Sargent, 2012).

Tumours in this pathway have diploid DNA and occur most in the right colon. About 15 % and 90 % of sporadic colorectal cancer and HNPCC colorectal cancer respectively are caused by MSI (Umar *et al.*, 2004; Söreide *et al.*, 2006).

In HNPCC, MSI is caused by germline mutations in one of three MMR genes (*MLH1, MSH2*, and *MSH6*) (Grady, 2004; Soreide *et al.,* 2006). These germline predisposing mutations associated with *MLH1* and *MSH2* are either by missense, deletions or insertion. One wild-type allele is enough to maintain MMR activity. HNPCC kindreds exhibit loss of heterozygosity at the gene loci either due to germline mutation or somatic mutation of the remaining wild-type allele (Hemminki *et al.*, 1994). This makes the lifetime risk of developing colorectal cancer in children of HNPCC parents more than 80 % while the risk is about 6 % in general population (Schoen, 2002; Replication, 2003).

In sporadic colorectal cancers, MSI is due to somatic inactivation of MMR genes, predominantly *MLH1* (suggesting that *MLH1* is more susceptible to somatic alterations). This is achieved by hypermethylation (epigenetic silencing) of CpG islands of the promoter region of *MLH1* (Kane *et al.*, 1997; Thibodeau *et al.*, 1998).

1.2.8 Chromosomal Instability (CIN)

The second pathway, chromosomal instability (aneuploidy) (CIN), describes the frequent changes in the number and structure of the chromosome of cancer cells over time (Negrini *et al.*, 2010a) and is characterized by systematic mutations or deletions of *K-RAS*, *APC*, *P53* and *DCC*. CIN is the most common genomic instability (Negrini *et al.*, 2010b) and occurs in about 85 % colon tumours (Grady, 2004).

Tumours associated with CIN possess aneuploid DNA, are left sided and invasive (De la Chapelle and Lynch, 1999 and Matsubara, 2012). CIN is linked to inactivation of regulatory

genes such as *TP53*, ataxia telangiectasia mutated (ATM) and ATM-related genes, and human homologue of budding uninhibited benzimidazole (BUB) at the mitotic-spindle check points during DNA replication. The protein products of these genes are known to be involved in sister-chromatid separation at mitosis and induce cycle arrest at metaphase-anaphase when activated. The loss of normal function of these genes affects effective regulation of DNA replications which is necessary for maintenance of structural consistency) (Cahill *et al.*, 1998; Grady, 2004; Negrini *et al.*, 2010a).

1.2.9 Epigenetics and Colorectal Cancer

Epigenetics refers to heritable chemical modifications of cytosine bases and histone proteins of the genome that do not affect the basic DNA sequence. These modifications are covalent methylation of cytosine and occurs predominantly in the CpG (cytosine , phosphoguanine) dinucleotides of the DNA and post-translational modifications of histone such as acetylation, methylation, phosphorylation and ubiquitination (Bernstein *et al.*, 2007). CpG Island is a short segment of DNA with a high concentration of cytosine-phosphoguanine nucleotides and is usually located in the promoter region (region of first exon) of the gene where DNA transcription is initiated. The cytosine bases in this region are often methylated, blocking transcription and therefore determine the expression/silencing of a gene. The addition of methyl group to cytosine is mediated by the enzyme, DNA-methyltransferase (DNMT) to form 5-methylcytosine. Half of mammalian genes have CpG islands located in the promoter region and about 70 % of these CpG islands are unmethylated in normal cells. Methylation is important in fundamental biological processes such as imprinting, cell differentiation and silencing of the X chromosome throughout the lifecycle of a female mammal (Feinberg and Tycko, 2004; Jones and Baylin, 2007). According to Knudson's model, epigenetics provides an alternative pathway of inactivation of tumour-suppressor genes in carcinogenesis (Kondo and Issa, 2004). Aberrant DNA methylation patterns exhibited by cancer cells are usually global hypomethylation and concurrent hypermethylation of gene-specific promoter regions (Kanai and Hirohashi, 2007; Matsubara, 2012).

Gene inactivation/silencing mediated by CpG methylation may be explained by two mechanisms:

1. Transcription repression is by methyl-CpG binding protein (MeCP-1) which limits access of transcription factors to promoter sites. Inactivation is due to exclusion of transcription factors

by MeCP-1 which are attached to the promoters in methylated-CpG Island (Boyes and Bird, 1992b). Gene silencing is affected by the density of methylation. In high density methylation a stable interaction with the MeCP-1 is formed (Boyes and Bird, 1992a) leading to permanent silencing as in the X chromosome of the female mammal(Goel and Boland, 2012).

2. The inhibition of the ability of transcription factors to bind to the methylated promoter sites for transcriptional activation (Kando and Issa, 2004).Methyl groups are covalently attached to the promoter sites of the CpG islands in a reaction catalysed by DNA-methyltransferase to prevent any interaction between the transcription factors and their DNA binding sites (Goel and Boland, 2012).

Hypermethylation of CpG islands is associated with the transcriptional silencing of tumour suppressor genes (Jones and Takai, 2001) and is related to the microsatellite instability (MSI) pathway (mutated pathway) in colorectal carcinogenesis (Curtin *et al.*, 2011; Matsubara, 2012). Tumours with widespread CpG island methylation constitute a subset of colorectal cancers referred to as CpG island methylator phenotype (CIMP) and are distinguished by promoter CpG island methylation of *MLH1*. Other genes affected in CIMP include *CDKN2A* (p16), *BRAF, KRAS, MINT (methylated in tumours)* (Hughes *et al.*, 2012; Matsubara, 2012). It was suggested that CIMP + occur most in the elderly, women, right colon and in the proximal colon position (Toyota *et al.*, 2000; Hawkins *et al.*, 2002). CIMP has further been classified as positive (CIMP+) or negative (CIMP-) depending on panel of markers adopted for their classification. These panels vary among authors leading to the classification of 24 % to 51 % of colorectal cancers as CIMP+. The most used markers however, include *HLM1, p16, MINT1*, *MINT2* and *MINT* 31(Toyota et. al., 1999). Weisenberger *et al.* (2006), addressed the inadequacy associated with the earlier panel of markers by proposing *CACNAIG, IGF2, NEUROGI, RUNX3, SOCSI* as alternative markers. With these panels all CRC with *BRAF* mutation are defined as *CIMP+*.

1.3 Tumour Suppressor Genes

1.3.1 Adenomatous polyposis coli (APC)

The *APC* gene encodes a multifunctional protein, 312 kDa in size with several proteins binding sites which enables it to regulate many processes including intercellular adhesion, and Wnt-signaling pathway (Leslie *et al.*, 2002; Aoki and Taketo, 2007). *APC* consists of 15 exons and is located on chromosome 5q21-q22. Most of the coding sequence occur in exon 15 and is often

the target of mutations (Béroud and Soussi, 1996; Brenner and Duggan, 2004). This is mutation cluster region (MCR) and contains over 90 % of mutations found in sporadic and familial cancers (Kinzler and Vogelstein, 1996a). Adenomatous polyposis coli (*APC*) gene functions as the "gate keeper" to colon tumorigenesis and its inactivation is considered the initiation of CRC (Smith *et al.*, 1993; Swamy *et al.*, 2006). Most of the tumours in colorectal cancer (FAP, HNPCC) carry mutations in *APC* gene, a negative regulator of the Wnt/β-catenin pathway (Liu *et al.*, 1996) The majority of these mutations (insertions and deletions) affect the C-terminal of the protein leading to stabilization of β-catenin and its accumulation in the nucleus where it binds to transcription factor T-cell factor/lymphocyte and subsequently activate transcription of *c-MYC, Cyclin D1, MMP7* and other Wnt target oncogenes (Sierra *et al.*, 2006).

The functional *APC g*ene antagonizes the Wnt/β-catenin pathway (Giles *et al.,* 2003) through the formation of a 'destruction complex' with, axin, glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) in the absence of Wnt/β-catenin signaling (Munemitsu *et al.*, (1995). This 'destructive complex' targets β-catenin for proteasome degradation by phosphorylating its N-terminal.

This regulatory role is realised by three different mechanisms:

1. *APC* protein at the nuclear level binds to β-catenin (i.e. nuclear β-catenin) and prevents its interaction with transcription factor T-cell factor/ lymphocyte enhancer factor (TCF/LEF)to reduce the expression of β-catenin dependent genes Neufeld *et al.* (2000) and Hood and Silver (1999),

2. The nuclear *APC protein*-β-catenin complex formed is exported into the cytoplasm where the β-catenin is degraded (Henderson, 2000; Neufeld *et al.*, 2000; Rosin-Arbesfeld *et al.*, 2000).

The involvement of wild type APC in regulation of cell proliferation further suggests its role as a tumour suppressor gene. APC protein restricts cell cycle progression from G1 to S phase by interacting with CDK proteins. This restriction is however blocked or downregulated by over expression of CDK2 alongside cyclin E depending on the levels of their expression. In cells overexpressing APC protein, the activity of CDK2 is down regulated (Baeg *et al.,* 1995). The pathway of this restriction was different from that of p53 and retinoblastoma protein.

APC protein possess active β-catenin binding site which enables it to bind to and export β-catenin from the nucleus to the cytoplasm for destruction or attached to E-cadherin and to adherens junctions where it functions in cell adhesion. This reduces free β-catenin in the nucleus

and limits its transcriptional activation of the nuclear target genes (Bienz, 2002; Rosin-Arbesfeld *et al.*, 2003).

1.3.2 Kirsten Ras (K-ras)

An important oncogene in colorectal cancer tumorigenesis is the *Kirsten Ras* (*K-ras*). *K-ras* is activated by allelic mutation at codon 12 or 13 and explains the 90% *K–ras* mutations observed in colorectal cancer (Gryfe *et al.*, 1997). Ras proteins belong to the guanosine triphosphatases family of proteins that regulate a number of signalling pathways including cell proliferation, differentiation, angiogenesis and apoptosis (Boguski and McCormick, 1993). Mutated *K–ras* genes have been identified in about 13 – 58 % of aberrant crypt foci presumed as precursors of colorectal cancer (Pretlow *et al.*, 1993; Smith *et al.*, 1994; Yamashita *et al.*, 1995). Thus a mutation of the *Kras* proto-oncogene is considered an early event in adenoma development (Kinzler and Vogelstein, 1996; Gryfe *et al.*, 1997). It is suggested that mutations in *K-ras* gene is necessary for transformation of adenomatous polyps formed in second and third decades in FAP patients to adenocarcinomas in later life.

K-ras mutations are usually in the gene structure and results in alterations of particular amino acids which affects the regulation of the protein. There is therefore continuous signaling for cell growth through activated signal transduction pathway and individuals are exposed to high risks of specific cancers when these mutations are carried in the heterozygote state (Levine *et al.*, 1994).

1.3.3 Deleted In Colorectal Cancer

Deleted in colorectal cancer (*DCC*) is a large gene situated on chromosome 18q21. It belongs to a family of dependence receptors and functions as a tumour suppressor gene by enhancing apoptosis in the absence of its ligand netrin-1.((Mehlen *et al.*, 1998; Krimpenfort *et al.*, 2012) It is deleted in about 70 % colorectal cancers (Mehlen and Guenebeaud (2010). Lost or reduced expression of the gene is associated with most advanced CRCs (Mehlen and Fearon, 2004; Krimpenfort *et al.*, 2012). It is a late cancer gatekeeper gene and its deletion is not necessary for primary tumour development. In normal function *DCC* protein undergoes caspase cleavage at the intracellular domain to induce apoptosis. In the presence of netrin-1, DCC enhances cell differentiation and migration whiles its apoptotic properties are inhibited. In normal intestinal

epithelium at the base of the crypt where netrin-1 is high in concentration there is rapid cell proliferation but at the apex there is programmed cell death where *DCC* is high. The loss of *DCC* products promotes aggression and metastases of p53 deficient cells thus favouring the survival of cells. The *DCC* as a tumour suppressor gene provides a safe guard mechanism by enhancing apoptosis in the absence of its ligand (netrin 1) (Castets *et al.*, 2011; Krimpenfort *et al.*, 2012).

1.3.4 P53

This is a tumour suppressor gene was located on chromosome 17p and is associated with a number of sporadic and inherited forms of human cancer (Levine *et al.*, 1991). In normal function, the P53 protein is involved in spindle checkpoint control, identifying DNA damage, causes cell cycle arrest for DNA repair processes, induction of apoptosis and elimination of cells with genetic abnormalities (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992; Lowe *et al.*, 1993; Cross *et al.*, 1995). In response to DNA damage *P53* protein induces the transcription of p21, Cdk inhibitor which inhibits cell cycle progression by binding to PCNA (proliferating cell nuclear antigen) and also by inhibiting Cdk-cyclin complexes. The P53 protein is activated by a number of stimuli such as DNA damage, heat shock, metabolic changes, hypoxia and cytokines (Steele *et al.*, 1998). The tumour suppression role of P53 is evidenced by the fact that about 60% of human cancers and 75 – 80% of colon carcinomas carry mutations in the P53 gene through missense (point mutation) and deletion leading to complete loss of wild-type alleles (Levine *et al.*, 1991; Levine *et al.*, 1994). Most of the mutations occur between codons 120 and 290 (DNA-binding domain) resulting in the loss of transcription factor functionality of the of the P53 protein.(Levine *et al.*, 1994; Steele *et al.*, 1998). In mice, a complete loss of wild-type P53 alleles leads to the development of cancers within 9 months of birth. (Donehower *et al.* (1992) and Levine *et al*., (1994)), Many mutant P53 alleles behave in a dominant manner, promoting growth and transformation of cells carrying wild-type P53 alleles. Wild-type P53 protein has a short life-span not exceeding 30 mins while the mutant form are more stable and accumulate to reach concentration far above the level seen of wild-type proteins (Weinberg, 1991). Cells with mutant *P53* genes are resistant to apoptosis when exposed to DNA damaging stimuli such as chemotherapeutic drugs used in cancer treatment, oxygen and growth factor deficiencies (Lodish *et al.*, 2000)

It was suggested that mutations in *P53* are relatively late events in colorectal tumourigenesis and coincide with the transition from adenomas to invasive carcinomas (Xu *et al.*, 2012). Thus even though *P53* is involved in colorectal cancer unlike *APC* it does not initiate the process (Kinzler and Vogelstein, 1996).

1.3.5 Cyclin D1

Cyclin D1 is one of the prominent regulators of cell proliferation that characterises cancer development and it is encoded by *CCND1* gene, whose expression is modified in cancer cells either by chromosome translocation or gene amplification (Balcerczak *et al.*, 2005). Cyclin D1 affects cell proliferation at G1 to S phase of the cell cycle by modulating the activities of retinoblastoma protein (pRb) (Fu *et al.*, 2004; Hulit *et al.*, 2004). Retinoblastoma protein restricts cell cycle progression when it binds to transcription factor, EF2 and prevents transcription of genes whose transcription products are required for progression (Sherr, 2000). Cyclin D1 is activated through phosphorylation by *CDK4* and *CDK6* and the complex formed phosphorylates pRb causing it to release EF2 to initiate DNA replication(Donnellan and Chetty, 1998). Besides colorectal cancer, the expression of cyclin D1 is dyregulated in most human cancers including breast, bladder, oesophageal, and endometrial and it is an early event in carcinogenesis (Zhang *et al.*, 1997; Donnellan and Chetty, 1998). Amplification of cyclin D1 is induced in different cells by different growth factors such as epithelial growth factor and IGF-1 and IGF 11, amino acids, retinoic acids and peroxisome proliferator-activated receptor gamma (PPAR)γ ligand and gastrointestinal hormones (Fu *et al.*, 2004). It is suggested that cyclin D1 enhances cell proliferation as oncogene with its normal protein and does not undergo any mutational transformation (Rosenberg *et al.*, 1993). The high level of cyclin D1 observed in cancer is due in part to amplification and largely to post-translational dysregulation (Alao, 2007). Cyclin D1 is a downstream target for β-catenin in the Wnt signalling or β-catenin/LEF (lymphoid enhancing factor)/T-cell factor pathway and the transcriptional activation by β-catenin could be a contributor to the high concentration (Shtutman *et al.*, 1999). It was suggested that very high levels of cyclin D1 inhibit DNA replication by binding to PCNA and cdk2 and its degradation is therefore necessary for DNA synthesis (Fukami-Kobayashi and Mitsui, 1999).

Cyclin D1 is degraded through phosphorylation by glycogen synthase kinase-3β (*GSK-3β*). *GSK-3β* phosphorylates cyclin D1 and causes its translocation to the cytoplasm and subsequent destruction in ubiquitin-proteasome system (Shtutman *et al.*, 1999; Alao, 2007)

Hulit *et al.,* (2004), investigated the involvement of cyclin D1 on colonic epithelial cell differentiation and tumour number in Apc^{Min} mice by mating Apc^{Min} mice with cyclin D1-/- mice. The results of this study suggest decreased cell proliferation and increased differentiation accompanied by reduced tumour number in Apc^{Min} mice heterozygous or nullizygous for cyclin D1, an indication that downregulating cyclin D1 expression offer protection against CRC. Silibinin, a nontoxic chempopreventive agent was shown to decrease size and number of intestinal polyp in $Apc^{Min/+}$ mice alongside reduction in the expression of cyclin D1, c-Myc and beta-catenin in the polyps (Rajamanickam *et al.,* 2009). The results showed a positive correlation between expression of cyclin D1 in intestinal polyps and polyp size and number. Thus suggesting increased expression of cyclin D1 is associated with polyp proliferation. Studies have also shown that *Cyclin D1* deficient mice are resistant to development of mammary carcinoma induced by neu and ras oncogene, an indication of the critical involvement of cyclin D1 in cancer (Sutherland and Musgrove, 2004).

1.3.6 Cyclooxygenase-2 (COX-2)

Prostaglandin G/H synthases (*COX-1* and *COX-2*) are the main enzymes that catalyse the synthesis of prostaglandins from arachidonic acid. Whereas cyclooxygenase-1 (*COX-1*) is constitutively expressed, cyclooxygenase-2 (*COX-2*) is induced by growth factors including epidermal growth factor and tumour growth factor α(TGF-α) and inflammatory cytokines such as interleukin-1 (IL-1), and interferon gamma (INF-γ) at the sites of inflammation (Brown and DuBois, 2005; Palozza *et al.*, 2005). The level of *COX-1* remained unchanged in intestinal tumour cells and is involved in the synthesis of prostacyclin (cytoprotective prostaglandin) and PGE2, associated with the protection of gastric mucosa (Williams *et al.*, 1999). About 85 % human colorectal adenocarcinomas show high level expression of both the protein and *mRNA* of *COX-2* but are undetected in normal intestinal tissue (Tsujii *et al.*, 1997). It was also shown to be highly expressed in azoxymethane induced polyps in the colon of rodents (DuBois *et al.*, 1996), in human colon tumours (Eberhart *et al.*, 1994; Soslow *et al.*, 2000) and in colon cancer cells (HCA-7) treated with tumour growth factor-α and also in rat intestinal epithelial cells (Palozza *et al.*, 2005). It was suggested, expression of *COX-2* enhances tumourigenesis by

inhibition of apoptosis, promoting angiogenesis and tumour invasiveness (Richter *et al.*, 2001; Sinicrope and Gill, 2004), enhanced expression of anti-apoptotic protein (Bcl-2) and decreased levels of pro-apoptotic *Bax* and *Bcl*-x) (Liu *et al.*, 2001).

A number of colorectal cancer chemopreventive studies have shown that inhibiting *COX-2* with non-steroidal antinflammatory drugs (NSAID) reduced colorectal cancer related death by 40-50 %. *In vitro* application and dietary administration of celecoxib (selective *Cyclooxygenase-2* inhibitor, a nonsteroidal anti-inflammatory drug (NSAID)) and atorvastatin (hydrxy-3-methyglutaryl CoA reductase, (HMGR)) inhibitor in combination to colon cancer cells (HT-29) (Malisetty *et al.*, 2002) and $Apc^{min/+}$ mice (Swamy *et al.*, 2006) respectively, showed induction of apoptosis and suppression of tumourigenesis. Rajamanickam *et al.* (2009), treated $Apc^{Min/+}$ mice with silibinin and showed reduced ($P < 0.001$) expression of *COX-2 in the* intestinal polyps alongside a reduction ($P < 0.001$) in polyp number and size. Also a decreased expression of *COX-2* and PGE-2 were observed by Rajamanickam *et al.* (2010) and is accompanied by reduced cell proliferation and inhibition of angiogenesis, two important processes of carcinogenesis. Oshima *et al.* (1996), showed that intestinal polyps number and size in $Apc^{\Delta 716}$ could be by significantly enhanced by increasing the expression of COX-2.

COX-2 production may involve different mechanisms along different pathways such hypoxia/NF-kB vascular endothelial system or APC/Wnt pathway (Brown and DuBois, 2005) and the fact that it occurs in almost all cancers make it important biomarker in cancer chemopreventive investigation.

1.3.7 RXRalpha and RARbeta

These are members of steroid/thyroid hormone superfamily of nuclear receptor known to mediate the anti-tumour activities of carotenoids (Allenby *et al.*, 1993; Lee *et al.*, 2000a; Pham *et al.*, 2013). They modulate many biological processes (may function) as heterodimer or may exert their regulatory effects as separate entities along different pathways. Different isoforms (α, β, γ) of these receptors exist and affect a wide variety of genes (Allenby *et al.*, 1993). Retinoid, its natural and synthetic derivatives are associated with the modulation of a cell proliferation and differentiation, two critical processes in tumourigenesis (Dillard and Lane, 2007b; Pham *et al.*, 2013). These regulatory functions are mediated by *RXR* and *RAR*. These receptors have N-terminal that contains ligand-independent activation function-1(AF-1) and C-

terminal with ligand-dependent activation-function-2 (AF-2) and are known to inhibit or promote DNA transcription in the absence or presence of their respectively ligands (Xiao *et al.*, 2003). The transcription repression is achieved by retinoid receptors acting as transcription factors through recruitments of histone deacetylases which prevent opening of chromatins for attachment of transcription factors (Freemantle *et al.*, 2003). The concentrations of retinoid receptor proteins are modulated by degradation upon binding to their respective ligand and effecting transcription (Osburn *et al.*, 2001). *RXR* is known to provide a third alternative pathway (retinoid-X-receptor (*RXR*) dependent degradation) by which the concentration of beta-catenin in the cytoplasm is regulated. Dysregulation in beta-catenin degradation pathways is common and underlie the development of many cancers (Dillard and Lane, 2007b). *In vitro study* by Dillard and Lane (2007b), showed that retinol induced β-catenin protein destruction in the cytoplasm of three human colon cancer cells (HCT-116, WiDr, SW620) by proteasomal degradation mediated by *RXR*. Retinol enhanced the expression of *RXR* and induced dimerization between *RXR* and beta-catenin in the nucleus (Lu *et al.*, 2005). The dimer then translocates into the cytoplasm where the beta-catenin is destroyed. This reduced the expression of *mRNAs* of β-catenin/TCF/LEF target genes (*c-Myc, Cyclin D1*) that are associated with cell proliferation.

Of the *RAR* isoforms, *RARb* is considered the most important because of its role in the maintenance of epithelial homeostasis and its reduced or loss of expression is found in many human tumours (Gebert *et al.*, 1991; Tanaka *et al.*, 2012). It was suggested *RARb* induces sensitivity to retinol in retinoic acid resistant colon cancer cells and promotes apoptosis in malignant cells (Lee *et al.*, 2000a). Also a significant clinical response of premalignant oral lesions to retinol *was observed in patients* with increased expression *RARb* (Lotan *et al.*, 1995). The expression of *RARb* is affected by the tissue concentration of vitamin A. In Vitamin A deficient rat models, *RARb* was lowly expressed in many organ but these was reversed with increased retinoic acid (Verma *et al.*, 1992). This relates positively to the suggestion by de The *et al.* (1990) that retinoic acid modulates the transcription of its own receptor. Studies have shown that *RARβ* inhibits epidermal growth factor receptor (*EGFR*), activator protein-1 (AP-1), reduces *COX-2* activity and prevents phosphorylation of ERK1/2 in cancers (Song *et. al.*, 2009; Sun *et al.*, 2012). It was suggested that *RAR* inhibit cancer promoting activities of beta-catenin at the transcription level by binding to LEF/TCF transcription factors to prevent transcription of beta-catenin target genes (Cyclin D1, C-Myc etc) and subsequently decrease cell proliferation (Easwaran *et al.*, 1999).

These observations suggest nuclear retinoic receptors could serve as intermediate biomarker in molecular studies involving diet plant.

1.3.8 Matrix metalloproteinase (MMP7) and β, β 15,15-monooxygenase (BCMO1)

β,β-carotene 15,15'-monooxygenase (*BCMO1*,also known as *BCDO1*) is an enzyme involved in the central cleavage pathway of β-carotene and other pro vitamin A carotenoids metabolism and converts β-carotene to retinaldehyde which may be converted to retinol or retinoic acid (Olson and Hayaishi, 1965). However, the activity of *BCMO1* is influenced by quantity and quality of protein eaten (Lietz *et al.*, 2010). Mice fed protein deficient diet showed reduced *BCMO1* enzyme activity in the intestine than those fed protein rich diet. Also vitamin A deficient rats showed increased *BCMO1* activity when fed diet containing higher amount of protein compared to those fed diet with reduced protein content (Parvin and Sivakumar, 2000; Hosotani and Kitagawa, 2005). The intestine is the main site for the conversion β-carotene to retinaldehyde though it is also produced in the liver, kidney and testis (Takitani *et al.*, 2011). Retinaldehyde, a product of *BCMO1* activity prevents tumour cell invasion and metastasis by inhibiting the formation of *RXR/PPARα* which modulates fatty acid oxidation and creation of oxidative stress leading DNA damage (Pyper *et al.*, 2010; Leclerc *et al.*, 2013). Cells treated with β-carotene showed increased activity *BCMO1* and increased concentration of retinoic acid alongside a reduction in expression of *MMP7* in an *in vitro* study to suggest that the anti-tumour property of β-carotene was mediated by *BCMO1* and retinoic acid (Leclerc *et al.*, 2013).

MMPs are zinc-dependent proteolytic endoenzymes that degrade extracellular matrix and facilitate tumour cell evasion and migration (Ellerbroek and Stack, 1999; Kleiner and Stetler-Stevenson, 1999). The high incidence of these enzymes in cells is linked to disease stage (Zucker and Vacirca, 2004). Of all MMPs associated with invasion and progression of tumours, *MMP-7* is considered the most important because of its small size and unique structure(Gaire *et al.*, 1994). It is reportedly involved in the invasion and metastasis in gastrointestinal cancer and overexpressed in many primary tumours and in 80% colorectal cancers (Zeng *et al.*, 2002; Pham *et al.*, 2013) suggesting, its expression is an early event in tumourigenesis (Wilson *et al.*, 1997; Zucker and Vacirca, 2004). In a study involving *MMP-7* deficient Min/+ mice Wilson *et al.* (1997) showed that *MMP-7* is necessary not only for tumour multiplicity but also for tumour growth. *MMP-7* is a downstream target of β-catenin (Brabletz *et al.*, 1999) and it is overexpressed by the tumour cells in CRC (Zucker and Vacirca, 2004)

1.4 Fruits and Vegetables and Health

Fruits and vegetables form very important component of a healthy human's diet and are known rich sources of nutritive factors many of which are vital for normal metabolic functions of the body. According to World Health Organization (2005), less than 400g daily intake of fruits and vegetable constitute one of the top 10 risk factors of global death and was estimated to have caused 1.7 million deaths globally, 14 % of which was attributed to gastrointestinal cancer, 11 % to ischaemic heart diseases and 9 % to stroke.

A critical review of relationships between fruits and vegetable intake and the risk of chronic diseases supports World Health Organization (2005) held health position on increased intake of fruits and vegetables to reducing CVD, diabetes, stroke, and cancer among others (Hung *et al.*, 2004; Boeing *et al.*, 2012; Wang *et al.*, 2014).

1.4.1 Fruits and Vegetables and Colorectal Cancer Risk

Many of the health benefits of fruits and vegetables are associated with their vitamins and minerals, phytochemicals and dietary fibre contents (Boeing *et al.*, 2012; Slavin and Lloyd, 2012).

Meta-analysis of data from the Dutch cohort studies on diet and cancer with over 120,800 (age group, 55-69) participants suggest protective effects of fruits and vegetables vary with types of fruits and vegetables, cancer site and sexes (Voorrips *et al.*, 2000). No significant statistical association between colon cancer and total fruits or total vegetables was observed whilst inverse association was observed in women who took fruits and vegetables together. Of the vegetables investigated Brassica and cooked leafy vegetables had greater protective effect in men than in women. This effect was stronger in the distal than in the proximal colon (Voorrips *et al.*, 2000). Also, meta-analysis by Nomura *et al.* (2008) showed a significant reduction in CRC cancer risk reduction in men with fruits and vegetables consumption which was more pronounced in the colon than rectum. This prospective study which included 215,000 participants and over 1.4 million person observation years, corroborated the findings of (McCullough *et al.*, 2003; Park *et al.*, 2007). Park *et al.*, (2007) investigated data from a 5 year follow-up study of 488,043 retired individuals (NIH-AARP National Institute of Health-American Association of Retired Persons) and suggests a strong inverse association between colorectal cancer and green leafy vegetable consumption in men but not in women. Significantly, the study also found, risk of CRC was high in men with very low intake of fruits and vegetables and decreased with

increased intake. A similar inverse association for colon cancer was reported by McCullough *et al.,* (2003). Pooled meta-analysis of data from 14 prospective studies of 756, 217 men and women and a follow-up period of 6 – 20 years showed fruit and vegetable intake decreased risk of developing cancer in terminal colon (Koushik *et al.*, 2007).

Other prospective studies however, suggest no association between fruits and vegetable intake and CRC risk in men and women. This includes studies by Michels *et al.* (2000), which examined data from the Nurses' Health Study and Health Professionals' Follow-up Study. Similarly Tsubono *et al.* (2005) reported no association among Japanese men and women. On the other hand, analysis of data from eight years follow up Alpha-tocopherol, beta-carotene cancer prevention study suggested consumption of cruciferous vegetables related positively to the risk of colorectal cancer incidence (Pietinen *et al.*, 1999).

Despite the controversies of epidemiological studies many *in vitro* and animal experiments, involving total fruit and vegetable or isolated phytochemicals produced biologically plausible evidence to suggest protection against CRC. Feeding Black/6 mice individual vegetables or mixed vegetables diet suggest modulation of expression of genes associated with CRC (van Breda *et al.*, 2005a), whilst a ten week dietary administration of sulforaphane, one of the main isothiocyantes in cruciferous vegetables such as broccoli, was shown to reduce intestinal tumour number and size in $Apc^{Min/+}$ mice (Shen *et al.*, 2007). *In vitro* investigation revealed retinoic acid decreased concentration of nuclear beta-catenin, an important cancer biomarker which enhances transcription of genes such as *Cyclin D1, C-Myc* and Matrix metalloproteinase (*MMP7*) involved in cell proliferation in intestinal tumour development (Dillard and Lane, 2007a).

1.4.2 Dietary Fibre

The role of dietary fibre in colorectal cancer risk has been inconsistent. Whereas most case-control studies support the hypothesis that dietary fibre intake decreased risk of CRC, prospective studies have been inconsistent. Meta-analysis of 13 case control studies suggested increased intake of fibre decreased colorectal cancer risk in both men and women (Howe *et al.*, 1992). Ecological analysis of data from case control studies over seven countries also showed a strong inverse association between increase intakes of fibre and colorectal cancer risk. This study suggests increase intake of 10 g/day of fibre was associated with 33 % reduction in risk

(Jansen *et al.*, 1999). Increased intake of dietary fibre was also shown to decrease colorectal cancer risk in a nested case control study of colorectal cancer patients (Dahm *et al.*, 2010).

Data from prospective cohort studies of Nurses' Health Study and from Swedish population indicate, dietary fibre intake offered no protection against CRC (Fuchs *et al.*, 1999; Terry *et al.*, 2001). Similarly, analysis of data from 10 to 16 years prospective study of US health workers showed no significant effect of dietary fibre on colorectal cancer risk (Michels *et al.*, 2000). However, in a systematic review, Aune *et al.* (2011) reported inverse association between dietary fibre intake and CRC risk in a dose response analysis of meta-analysis of data from a large number of prospective cohort studies. Fibre from fruits was shown to be more protective against colorectal cancer than fibre from other food sources (Papas *et al.*, 2004). Bingham *et al.* (2003), reported increased intake of dietary fibre reduced risk of colon cancer from European Prospective Investigation in Cancer and Nutrition Study (EPIC). A report from the same study in 2005 but with 656 additional new cases suggests a stronger protective effect of fibre against CRC (Bingham *et al.*, 2005). Evidence from Malemo Diet and Cancer Study (MDCS), Sweden suggests the protective effects of fibre against CRC are influenced by the cancer site, source of fibre and gender of affected persons. High intake of fruits and berries reduced colon cancer risk in women and high intakes of fibre-rich cereal may be protective against metastasis (Vulcan *et al.*, 2015), whilst EPIC study report suggests cereal fibre reduced risk of cancer in the rectum rather than fruit and vegetables (Murphy *et al.*, 2012).

It was proposed that the protective ability of fibre against colorectal cancer may be due to mechanisms that are limited to the colon (Vulcan *et al.*, 2015). These include decrease in stool colonic transit time, absorption of moisture, and increase in stool bulk, dilute potential carcinogens, and induced anaerobic fermentation of fibre by colonic microbacteria. Bacteria fermentation produces short-chain fatty acids which reduce pH and prevents conversion of primary bile acids to secondary bile acids. The primary bile acids inhibit cell proliferation and induce differentiation and apoptosis (Young *et al.*, 2005).

1.5 Developmental Origin of Adult Diseases

Barker hypothesis suggests that the environmental factors to which an individual is exposed during intrauterine and neonatal life modulate disease risk during adulthood. These factors include nutritional factors (McMillen and Robinson, 2005), psychological or physiological

stressors (Lazinski *et al.*, 2008) and changes in endocrine signalling between mother and fetus (Seckl, 2004).

These factors cause development of survival adaptations/adaptive response which may involve modulation of cell proliferation and differentiation to maintain normal function of essential organs (Langley-Evans and McMullen, 2010; Mathew and Ayyar, 2012). These adaptations may produce irreversible structural and functional modifications of the body/organs with the capacity to modulate response to the environment in later life (Langley-Evans, 2006). The degree of the modifications achieved is determined by the nature and timing or developmental stage of exposure to the stimuli or insult (Langley-Evans, 2000). The effect of timing or stage of development of fetus at which it is exposed to stimuli was observed in the Dutch famine during the World War II. Infants who were subjected to nutrient deficiency during early gestation had normal weight but developed coronary heart disease, atherogenic lipid profile, obesity and altered blood clotting, whilst exposure during mid and late gestation produced low-birth weight, glucose intolerance and micro albuminuria (Roseboom *et al.*, 2001). For example people with low-birth weight have reduced number of glomeruli in the kidney and increased flow of blood through the glomeruli later in life cause hyper filtration and subsequently glomerulosclerosis (Luyckx and Brenner, 2005). There is also loss of glomeruli with age. These together results in increased blood pressure with further loss of glomeruli which is self-perpetuating (Barker, 2004a). A similar observation of reduced number of nephron was observed in mice (Hoppe *et al.*, 2007) and rats (Woods *et al.*, 2004) exposed to low-maternal protein diet.

Reports suggest that individuals with low-birth weight develop in later life diseases such as, coronary heart disease, hypertension, obesity, type II diabetes and metabolic syndrome ((Barker, 2004b). Low birth weight has been associated with inadequate nutrition thus the development of the diseases are the results of adaptive response to nutritionally challenged environment (Mathew and Ayyar, 2012). According to Langley and Jackson (1994), rats exposed to low-protein diet *in utero* develop high blood pressure in adulthood. This effect of low-protein diet is influenced by the type of carbohydrates in the diet. As suggested by Langley-Evans (2000), high blood pressure occurs in rats if the low-protein diet they were exposed to *in utero* contained complex carbohydrates but absent if the diet contained largely glucose.

The ‘Thrifty hypothesis, has it that, under conditions of nutritional deprivation the fetus channels nutrients to the development of vital organs (brain) at the expense of non- essential

organs (beta cell od islet) (Mathew and Ayyar, 2012). This is underlined by the concept of hormonal programming which suggests metabolic and endocrine adaptations in the fetus aimed at energy preservation.

The mechanism by which the fetus maintains normal function of the brain for instance in nutrient deficient environment is to divert blood and nutrient supply to the brain whilst directing insulin and growth hormone resistance to the periphery (Mathew and Ayyar, 2012). Insulin resistance enables the maintenance of glucose concentration in the blood for the developing brain (Barker, 2004). The resulting phenotype therefore has survival advantage in a nutritionally poor environment postpartum. However in a postpartum nutrient rich environment, the phenotype is disadvantaged by enhanced metabolic efficiency (Neel, 1962).Such individuals are associated with low-birth weight or thin body at birth with most exhibiting good catch-up growth early postnatal and are at risk of developing cardiovascular diseases, type II diabetes and polycystic ovarian syndrome (Forsen *et al.*, 1997; Osmond and Barker, 2000).

The association between nutrition, early life and disease in later life may be explained by epigenetic modifications of gene expression through DNA methylation or histone acetylation (Razin, 1998). These modifications which have been shown to occur during prenatal and early postnatal stages of development are associated with long-term anatomical and physiological implications in later life (Razin, 1998; McMullen *et al.*, 2004). The process is mediated by nutrition through the availability of possible methyl donors such as amino acids (eg., methionine), folic acids, vitamin B6 and B12 in diet or by affecting the activities of DNA methyl transferase (enzymes involved in DNA methylation) (Niculescu and Zeisel, 2002; Waterland and Jirtle, 2003; Young *et al.*, 2004). In animals, effects of dietary factors on DNA methylation vary with site or organ and nature of methylation (McKay and Mathers, 2011). For example in rats increased intake of dietary Selenium increased DNA methylation in the colon and not in the liver whilst folate deficient diet decreased methylation of *p53* gene and not genomic DNA (McKay and Mathers, 2011). This suggests that adequate maternal nutrition during early development is a prerequisite for healthy adult life.

Results from investigation by Langley-Evans *et al.* (2005) indicate that the expression of a number of genes in the kidney and genes related to appetite in the hypothalamus is modulated by maternal protein restriction during gestation. Also preconception maternal folate, B2, B6, and B12 supplementation affected intestinal tumour number in adult APC1638N mice offspring

(Ciappio *et al.*, 2011). Similarly, maternal folic acid supplementation was shown to decrease azoxymethane induced adenocarcinoma in rats (Sie *et al.*, 2011).

In addition to genetic and nutritional factors *in utero* and during early postnatal life influencing the health status in adulthood, studies have shown that, individual's life styles such as moderate to heavy smoking, and excessive alcohol consumption also play a critical role. The interplay of these factors in the development of adulthood diseases is summarised by Conceptual health pendulum model for cardiovascular and cancer risks (Mathers, 2002).

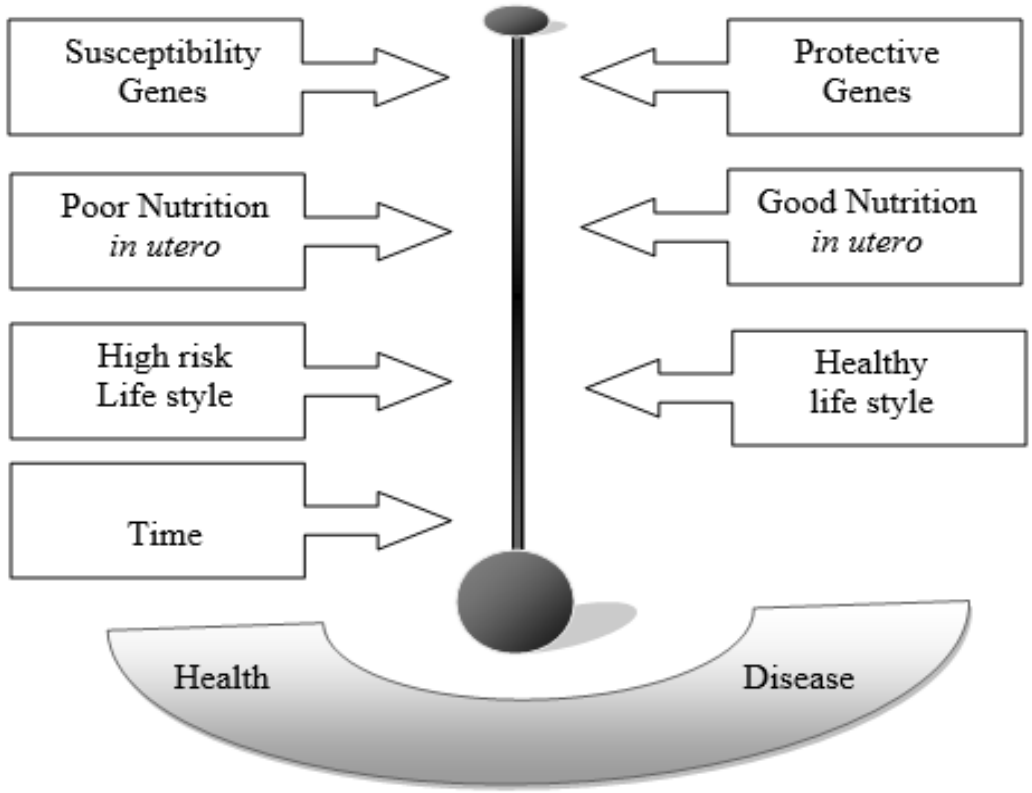


Figure 1. 2 The Health Pendulum (Mathers 2002). Conceptual model of health determining factors

1.6 Colorectal cancer model

The development of CRC is a multistep process and it develops over decades through accumulation of genetic alterations in tumour suppressor genes, oncogenes and mismatch repair genes in individuals (Fearon and Vogelstein, 1990; Kinzler and Vogelstein, 1996). The long period over which the disease develops coupled with the knowledge of defined steps of changes in colonic epithelium provide molecular targets for therapeutic interventions (Wong *et al.*, 2007). A number of models have been developed to facilitate chemoprevention trials.

1.6.1 Cell line model

Human colorectal cancer cells such as HCT-116 p53 wild type, HCT-116 null (HCT-116 p53-/-), HT-29 and SW-620 extracted from tumour tissues of colorectal cancer patients provide cell line models for *in vitro* investigations of colorectal carcinogenesis in human (Nautiyal *et al.*, 2011). Cell lines produce large number of live cells in relatively short time and at a much reduced cost whilst enabling the researcher control over the experimental factors (Ahmed *et al.*, 2013). Using cell line in *in vitro* studies revealed certain characteristics (aggregation, migration and invasion) of tumour cells (Young *et al.*, 2013). It was also possible to determine the effects and mechanisms of action of cancer preventing agents on tumour cells such as antiplatelet aggregation (Alanko *et al.*, 1994) inhibition of cell proliferation (Hansen *et al.*, 2003) and apoptosis of tumour cells (Purup et al., 2009). Cell line models however have limitations; colorectal cancer develops over long periods and the processes of tumour initiation, and growth are not easily followed in a given cell culture (Johnson and Fleet, 2013). Cell lines grow in a medium devoid of complex interactions in the microenviroment which may affect homeostasis of normal colon cell, eg. oxygen levels between cells (Young *et al.*, 2013). Further more it was not possible to determine effects of tumour development on human immune systems and angiogenesis *in vitro* (Johnson and Fleet, 2013; Young *et al.*, 2013). The transfer of results of cell line investigations to humans may be limited by ethical considerations (Johnson anf Fleet, 2013).

1.6.2 Animal Model

The deficiencies associated with of cell lines provide the opportunity for the development and use of animal models to investigate possible effects of chemopreventive agents and to test hypothesis from cell models in the complex mammalian physiology of the colon (Johnson and Fleet, 2013).

1.6.3 Xenograft

This model permits investiagtion of cancer cell invasion and metastasis (Young *et al.*, 2013). A number of methods are available for this and involves the injection of tumour cells or cell suspension directly into the intestine (orthotopic injection), into the portal vein or subcutaneously or injected intraperitoneally (Karim and Huso, 2013; Young *et al.*, 2013). The cells may also be implanted surgically to sites of interest (Young *et al.*, 2013). To prevent rejection of tumour, the recipient rodent or mice are either nude (lack the ability to produce T-

lymphocyte) or severely combined immune deficiency (SCID) mice (lack both B and T-lymphocytes) (Karim and Huso, 2013; Young *et al.*, 2013).

The model has the advantage of using cells or tumour tissues from sites that in parallel represent the relevant human cancer and grow in a microenvironment with complex cellular interactions (Karim and Huso, 2013). Orthotopic implantation enables monitoring of metastasis by imaging.(Karim and Huso, 2013) A major disadvantage with xenograft is the use of immunocompromised mice or rodents which my reduce the extent of complex interactions between host and tumour (Young *et al.*, 2013).

1.6.4 Carcinigen-induced Model

One of the models for investigating the potency of colorectal cancer chemopreventive agents is the 1,2-dimethylhydrazine (DMH)/Azoxymethane (AOM) model of chemically induced CRC (Wong *et al.*, 2007). These chemicals are employed to induce colon tumour in rats and mice. In rats intraperitoneal injection of AOM, once a week for two weeks is enough to cause tumour development in a manner similar to humans (Reddy, 2000; Femia and Caderni, 2008) whilst mice require repeated injection of lower doses for the development of colonic tumours (Reddy and Maeura, 1984). With this model, tumours are developed mainly in the colon and the progression from abberant crypt foci (ACF) through adenomas and to carcinomas are similar to human (Wong *et al.*, 2007). However, the chemicals are carcinogenic to the experimentor and are excreted by breathing (Wong et al., 2007). Also the tumours rarely show mutations in *Apc* or *p53* genes which are commonly found in human. (Wong *et al.*, 2007).

1.6.5 Genetically modified model

One of the animal models widely used in the colorectal cancer research is $Apc^{Min/+}$ (Min, multiple intesinal neoplasia) mouse. The $Apc^{min/+}$ mouse carries a dominant germ line heterozygous mutation at codon 850 of the mouse homologue of human *APC* gene (Moser *et al.*, 1990). This mutation causes $Apc^{min/+}$ mice to develop multiple intestinal adenomas, predominantly in the small intestine and die of intestinal obstruction and/or severe anaemia, often not surviving beyond 120 to 150 days (Su *et al.*, 1992; Moser et al., 1990). This models colorectal tumourigenesis in humans, where *APC* mutations are common. $Apc^{min/+}$ mice therefore provide a very unique model for both mechanistic and chemopreventive investigation

of colorectal cancer and has been used frequently by many researchers (Ju *et al.,* 2005; Shen *et al.,* 2007; Fini *et al.,* 2011). However the relevance of this model to the study of colorectal cancer has been subjected to criticism on the bases of differences in location of tumours between $Apc^{Min/+}$ mice and human and also *K-ras* and *p53* mutations are not detected in Min mice (Wong *et al.,* 2007).

1.7 Research Hypothesis

This research was aimed at determining the effects of carrot consumption on intestinal cancer. Largely, however, studies have been centred on isolated or purified bioactive compounds (Mahmoud *et al.,* 2000; Kállay *et al.,* 2002; Swamy *et al.,* 2006). Investigations involving the use of vegetable diet in animal models is limited (Rijnkels and Alink, 1998; van Breda *et al.,* 2005b) and not much is known of the downstream molecular effects. Furthermore, the effects of vegetable diet consumption during specific windows of time, i.e. *in utero* and post weaning, on intestinal tumourigenesis is unknown. In addition the significant reduction in intestinal tumour number by preconception maternal supplementation of folic acid in APC1638N offspring mice (Ciappio *et al.,* 2011) coupled with intestinal tumour number reduction in $Apc^{Min/+}$ mice by post weaning dietary intake of plant secondary metabolites (Shen *et al.,* 2007; Ju *et al.,* 2005) suggest the availability of opportunity to influence intestinal tumourigenesis either *in utero* or post weaning with a diet rich in these compounds.

I therefore hypothesis that

Carrot consumption in utero and/or post weaning reduce intestinal tumourigenesis in $Apc^{Min/+}$ mouse.

To thoroughly address the hypotheses, the following questions were considered;

- Does carrot consumption *in utero* affect intestinal tumourigenesis in later life?
- Does carrot consumption post-weaning affect intestinal tumourigenesis?
- How is intestinal tumourigenesis affected if individuals exposed to carrot diet *in utero* are fed non carrot diet post-weaning?
- What is the effect on intestinal tumourigenesis if individual exposed non carrot diet *in utero* is fed carrot diet post-weaning?

- What are the effects of the diet treatments in this study on the expression of cancer related biomarkers (Retinoid X receptor beta, (*RXRb*); Retinoid acid receptor alpha, (*RARα*); *Cyclin D*; ββ-Carotene 15,15-monooxygenese-1, (*BCMO1*); Cyclooxygenase-2, (COX-2); and Metrilysin-7, (MMP7)

The design necessary to address these questions is explained in detail in materials and methods section.

Following the results of study one where consumption of heat processed carrot pellet increased intestinal tumour number significantly in the $Apc^{Min/+}$ mice, contradicting many epidemiological and animal experimental investigations, a second hypothesis was proposed to investigate the findings of study one.

Second Research Hypothesis:

Consumption of carrot powder in utero post weaning decreased intestinal tumourigenesis $Apc^{Min/+}$ mouse

This hypothesis was investigated on the principles of the first hypothesis but with carrot powder and without heat processing.

2 Chapter 2 Material and Methods

2.1 Section A

2.1.1 General Animal Housing and Husbandry

The study was undertaken with the approval of Newcastle University Ethics Committee within the limits defined by The United Kingdom Home Office Animals (Scientific Procedures) Act 1986 under the Project Licence, PPL60/4294 and Personal Licence No. 10265C1BE.

Six heterozygous male Min mice (C57BL/6J-*Apc*$^{min/+}$) were purchased from Jackson Laboratory (Bar Harbor, USA) and fifteen wild-type (C57BL/6J) female mice purchased from the Charles River Laboratories at the age of five weeks and a breeding colony was established in the animal facility at the Campus for Ageing and Vitality (CAV), Newcastle University. They were housed under standard laboratory conditions of 21 ± 2ºC, 55 % ± 10 % humidity and 12 hour light/dark cycles. They were acclimatised for two weeks on standard Rats and Rodents diet pelleted (RM3 E) (control diet) produced by Special Diet Services ((SDS), Essex, CM8 3AD, UK.), after which they were randomised to either control or experimental diets for two weeks prior to mating (see individual study descriptions for details of diets) in order to capture all periods of intrauterine development. Mating trios consisted of two wild-type females and a min male. The dams remained on their respective diet throughout mating, gestation and lactation alongside the offspring. The females were removed to a separate cage and their diet increased to 10 g/day on the presence of a vaginal plug. Two weeks post-partum the diet for the dams in each cage was increased to 20 g/day in recognition of increased nutritional needs of the young offspring. At a mean age of 28 days ± 3 StDev), offspring were randomly weaned to control or experimental diets, with an equal number of each sex allocated to each diet. Each cage contained an average of six mice and allocated diet and water were available *ad libitum.* Offspring were weighed at weaning and on a weekly basis (in the morning of every seventh day before feeding) thereafter and weights were recorded. All mice were examined on daily bases for any sign of ill health such as weight loss, anaemia and intestinal obstruction throughout the study. Food intake was measured twice over seven days, at the age of six and nine weeks. Food contained in food hoppers was weighed at the start and end of these periods without refilling. Food intake per mouse was calculated as the mean of the total amount of food consumed divided by the number of animals per cage. The mice were caged on the bases of sex, with mixed genotypes in each cage, therefore the effect of genotype on food intake was not be determined.

2.1.2 Mouse Identification and Genotyping

For the purposes of identifying individual mice in each cage, animals were ear notched at weaning. For a given cage notches were positioned at unique ear locations for each mouse. Mice were anaesthetised with gaseous isoflourane and transferred into their cages following the notch procedure. Ear notch samples were placed in labelled well plates, sealed and sent to Transnetylx Inc. (Cordova, Tennessee, USA) for genotyping for the presence of the Min mutation.

2.1.3 Source of Carrot

Carrots for the study were organic variety Nairobi carrot baton (6 x 6 x 40) mm obtained from Hartley's, York, UK. These were blanched, cut into batons, and freeze dried by European Freeze Dry Limited, Preston, UK at below 50^{o}C. The freeze dried batons were packed in sealed aluminium bags and stored at -20^{o}C until required.

2.2 Section B

2.2.1 Study One

Animal housing and husbandry is described above (section A). A summary of the study design is outlined in Figure 2.1 below. Dams were randomised to either pelleted control diet RM3 (E) (control pellet) or carrot enriched pelleted (carrot pellet) experimental diets. At weaning, offspring were further randomised to either control pellet or carrot pellet experimental diets resulting in adult offspring exposed to one of the following 4 *in utero*-post weaning dietary regimes; carrot pellet-carrot pellet; carrot pellet-control pellet; control pellet-control pellet; control pellet-carrot pellet.

2.2.2 Control and Experimental Pelleted diets

Diets were manufactured by Special Diet Services (SDS) with standard professional equipment to specification and dried at a temperature range of 90^{o}C to 120^{o}C. Control pellet diet (RM 3 E) was as the manufacturer's usual specifications (see Appendix). The carrot pellet diet consisted of 20 % powdered freeze dried carrot and 80 % Rats and Rodents powder (RM3 P) mixed and subsequently pelleted. The choice of 20 % carrot for this study was based on the evidence of preliminary results from the study by Saleh *et al.* (2013) where it was shown to reduce intestinal tumour number in *Apc*$^{Min/+}$ mice at age 12 weeks without any significant

difference in mean bodyweight gain between treatment groups or produced any observable toxicity effect. Mice have the ability to detect and reject nutritionally inadequate diet and select for diets that support normal growth especially those with the complements of indispensable amino acids (IAAs) (Gietzen *et al.*, 2007). The twenty percent carrot may be considered physiologically relevant since this was the amount chosen by the mice when given free access to freeze dried carrot and laboratory chow (Saleh *et al.*, 2013). Twenty percent carrot component of human diet translates into consumption of 740 g carrot/day which may be relevant in reducing the risk of developing diseases. Disease specific recommendation by WHO suggests that a minimum daily intake 400 g of total fruits and vegetables will reduce the risk of developing cancer (World Health Organization, 2005).

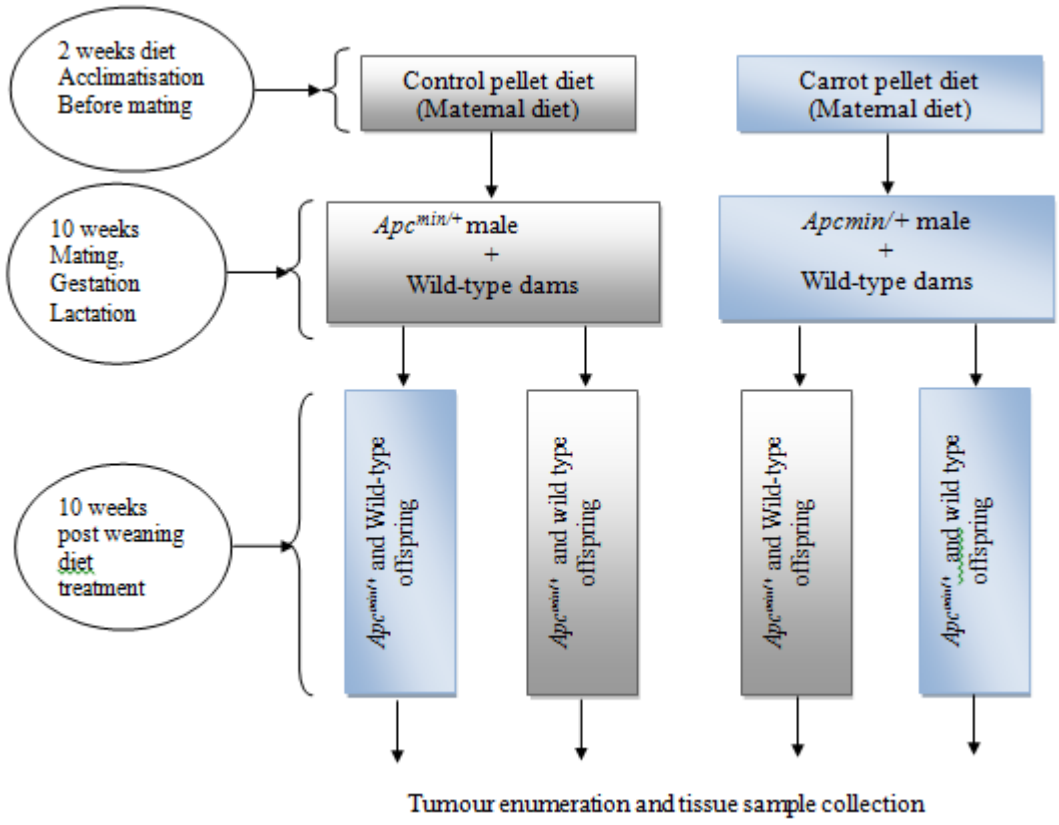


Figure 2. 1: Summary of experimental protocol to evaluate the effects carrot pellet on intestinal tumourigenesis in *Apc*$^{min/+}$ mice

2.2.3 Sample size

Sample size was determined using General Full factorial Design (Sample and Power Size) of Minitab 17 Inc. Software. It was estimated that 23 mice in each dietary group will allow for detection of differences in dietary effects in group means in a statistical model at a statistical power of 80 %. This was determined based on 40 % small intestinal tumour inhibitory effect of 0.08 % (-)-epigallocatechin-3-gallate, the main catechin in green tea in intestine of *Apc*$^{Min/+}$

mice at a standard deviation of 15.5 and treatment effect of 13.1 compared to the control (Ju et al., 2005). See appendix for power curve.

2.2.4 Necropsy

Mice were killed at 15 weeks (mean age of 110 days ± 0.497 days) by cervical dislocation to prevent death from intestinal tumour complications. *Apc*$^{Min/+}$ mice develop multiple intestinal tumours largely in the small intestine and die of intestinal obstruction and /or severe anaemia, often not surviving beyond 120 to 150 days (Su et al., 1992; Moser *et al*., 1990). It was also expected to enhance tumour number for observation of differences in treatment effect if there was any. Salah *et al.,* (2013) recorded an average of three and two tumours per control diet fed female and male *Apc*$^{Min/}$ mice respectively killed at 12weeks. On the other hand *Apc*$^{Min/+}$ mice killed at 15 weeks developed mean number of 51.5 intestinal polyps per mouse (Shen *et al* 2007). Similarly, Mahmoud *et al.,* (2000) at age 14 weeks observed an average of 48.8 ± 4.3 and 34.3 ± 3.8 in male and female Min mice respectively.

2.3 Study Two

Animal housing and husbandry was as described above (Section A). A summary of the study design is outlined in Figure 2.2 below. Dams were randomised to either control powder (RM3 (P)) diet or carrot powder experimental diets. At weaning, offspring were further randomised to either control powder, carrot powder or stored carrot pellet experimental diets resulting in adult offspring exposed to one of the following 5 *in utero*-post weaning dietary regimes; Control powder-control powder, control powder-carrot powder, control powder –stored carrot pellet, carrot powder-control powder and carrot powder-carrot powder.

2.3.1 Control and Experimental diets

The control powder and stored carrot pellet diets were manufactured by Special Diet Services (SDS) as the manufacturer's usual specification (See Appendix.). The carrot powder diet was made of 20 % powdered freeze dried carrot and 80 % RM3 (P). The carrot powder was produced by milling freeze dried carrot baton in a sample mill (Tecator Cyclo Tec 1093, Sweden). These were weighed using Myweigh i2600 electronic scale (capacity 2600 x 0.1 g) and mixed in Hobart (HSM30, UK) food mixer at low speed until a visually uniform mixture was obtained. The diet was then store at -20^{o}C and removed from the freezer at least 12 hours prior to administration to ensure sufficient defrosting at room temperature in an air tight container. The

stored carrot pellet used in this study was the same carrot pellet used in study 1 that was stored at -20^{o}C for nine months.

Proximate Analysis

Macro nutrient composition of the diets was determined (see Appendix 2 for procedures). Also nutrient composition (macro and micro nutrient composition) determined by SDS on control and carrot pellets are available in Appendix 1.

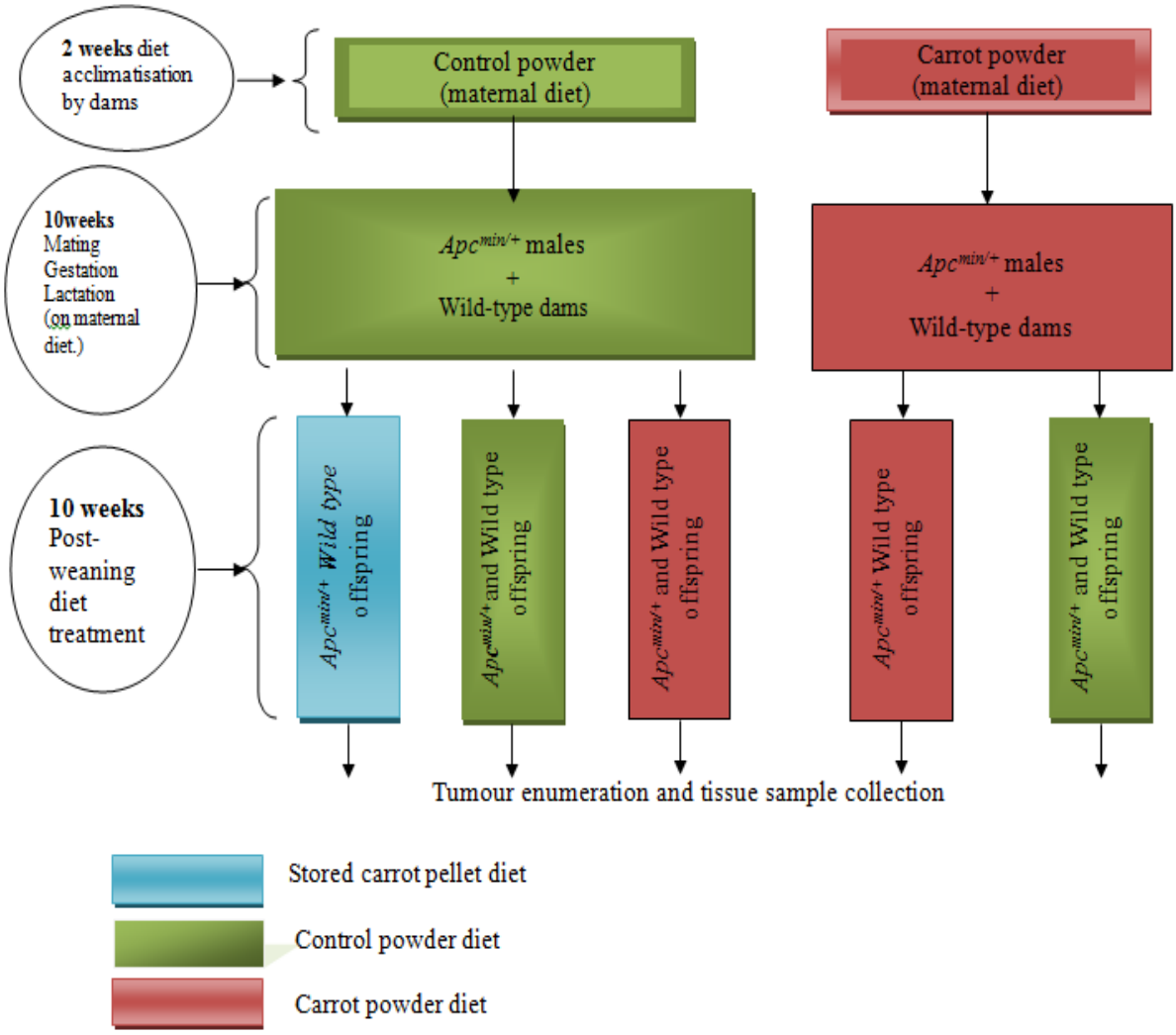


Figure 2. 2: Summary of experimental protocol to evaluate the effects carrot powder on intestinal tumourigenesis in *Apc*$^{min/+}$ mice

2.3.2 Necropsy

Mice on this study, according to the initial study design were to be killed at age15 weeks but for reasons beyond the control of the researcher, they were killed at different time points, starting from age 13 weeks to age 15 weeks.

2.4 Section C

2.4.1 Data and tissue sample collection

This was done according to the procedure of McKay *et al*., (2008)

2.4.2 Data collection

All weight measurements during sample collection were taken with the Mettler AJ150 Weighing Balance (Toledo Ltd, Switzerland)

2.4.3 Tissue sample collection

Animals were killed by cervical dislocation, and body weights recorded immediately after death. Organs (liver, heart, right and left kidney, spleen), were removed, weighed and snap frozen in liquid nitrogen and stored at -80^{o}C.

2.4.4 Intestinal sampling

The intestine was removed from the pyloric sphincter of the stomach to the rectal end and mesenteric fat removed. This was then dissected into small intestinal, caecal and colonic sections.

2.4.5 Small intestine

The length of the small intestine was measured with minimum stretching and the weight when full was recorded. It was then transversely divided in to proximal and terminal parts which were sprayed with Phosphate Buffered Saline (PBS). Each section was opened longitudinally and food and faecal materials gently removed. The two sections were weighed together and recorded as weight of empty small intestine.

2.4.6 Colon

The colon was minimally stretched and the length measured. The weight when full was also recorded. It was sprayed with PBS, opened longitudinally and faecal materials gently scrapped off. The weight was taken and recorded as weight of empty colon.

2.4.7 Tumour Mass/ Volume

Tumour diameter was visually estimated in millimetre and tumour volume calculated as Volume (mm^3) = $4/3\ \pi\ (R/2)^2$, where R = estimated tumour diameter (mm) (Cai *et al.*, 2010).

2.4.8 Tumour and tissue sampling

The segments of the intestine (proximal, terminal and colon) were carefully examined under the dissecting microscope and visible tumours recorded in terms of location, size and number. From each of the three sections of the intestine, mucosa and tumour samples were taken. For normal tissue samples, one centimetre of seemingly normal tissue sections were cut from the midpoint of each of the three intestinal segments and immersed in RNA*later*. From the three intestinal segments mucosa samples were gently scrapped with glass microscope coverslip into RNA*later*. In addition visible tumour samples were excised using a pair of scissors and immersed in RNA*later*. All samples preserved in RNA*later* were kept at 4^{o}C overnight before storing at -20^{o}C for gene expression studies.

2.5 Section D

2.5.1 Molecular studies

2.5.2 Extraction of RNA from intestinal tissue tumour samples

Total RNA was extracted from seemingly normal intestinal tissue and intestinal tumour samples preserved in RNA*later* using RNeasy Plus Mini Kit from Qiagen according to the manufacturer's instructions. Between 23 and 30 mg of tissue was homogenised in 600 µl of Buffer RLT with aid of Tissue Ruptor probes (Qiagen). The lysate was centrifuge at maximum speed for 3 minutes in Eppendorf Centrifuge 5430R (Eppendorf AG 2231, Hamburg, Germany) and the supernatant carefully transferred into gDNA Eliminator spin column in a 2 ml collection tube and centrifuged at 10,000g for 30secs.

Six hundred microliters of 70 % ethanol was added to the filtrate, mixed thoroughly and pipetted into RNeasy spin column in a 2 ml collection tube and centrifuged at 10,000 g for 15 seconds. The residue/filter was washed with 700 µl Buffer RWI and centrifuge at 10,000 g for 15 seconds. The residue is again washed twice consecutively with 500µl RPE Buffer (containing absolute ethanol in the ratio of 4 to1 (v/v) (ethanol to Buffer RPE)) and centrifuged at 10,000 g for 15seconds and 2 minutes respectively. There after it was centrifuged at full speed for 1 minute to remove any traces of alcohol. The purified RNA was then eluted with 50 µl RNase-free water into 1.5 ml collection tube and preserved at -80^{o}C until required. The purity and yield of isolated RNA was determined with the Nanodrop ND-1000 Spectrophotometer. The ratio of absorbance at 260/280 for all the isolates ranged between 2.0-2.14 suggesting good quality RNA.

2.5.3 Reverse Transcription

One microgram (1µg) DNase free RNA was reverse transcribed to generate cDNA in a 20 µl reaction volume using QuantiTect® Reverse Transcription Kit (Qiagen) as described by the manufacturer using Eppendorf Master Cycler Gradient (Eppendorf AG 22331 Humburg, Germany). Any traces of genomic DNA (gDNA) in the isolated RNA was removed in a 14ul gDNA elimination reaction consisting of 2 µl of gDNA Wipeout Buffer, 1 µg of RNA and RNase free water and incubated at 42^{o}C for 2 minutes. The reverse transcription was carried out by incubating a 20 µl reaction volume containing 14 µl gDNA elimination reaction, 1µl Reverse-transcriptase, 4 µl Quantiscript RT Buffer and 1ul RT Primer Mix for 15 minutes at 42^{o}C. cDNA transcription included a no-template control (NTC) (no RNA included in reaction mix) and no-enzyme control (NEC) (Reverse Transcriptase was excluded from the reaction) which served as contamination control. The cDNA produced was made up to 200µl with RNase and DNase-free water and aliquoted in 50 µl volumes for storage at -80^{o}C.

2.5.4 Real time PCR amplification

Real-time PCR was performed on six target genes of interest, *Retinoid X receptor alpha (RXRα), β, β-carotene-monooxygenase (BCMO1), Retinoid acid receptor-beta (RARβ), Cyclin D1, Cyclooxygenase-2 (COX-2), and Matrix metalloproteinase/Matrilysin (MMP7) and β-Actin* which served as the Reference gene. Transcript levels of the genes of interest were measured on a CFX-96 Thermal Cycler with Real-Time Detection System (Bio-Rad) in a total reaction volume of 20 µl using 1.0µl transcript specific primers (Eurofins, UK), 10 µl

QuantiTect SYBR green mix (Qiagen Cat. No. 204145), 4 µl 1:20 diluted cDNA and 4.0 µl water.

The amplification conditions were denaturation at 94ºC for 15seconds followed by 40 cycles of annealing for 30 seconds at differing temperatures for individual genes (Table 2.1), extension at 72ºC for 30 seconds and a melting curve.

Table 2. 1: Primer details and annealing temperatures

Gene	Oligo-nucleotide Primer sequence		Annealing temperature
Cyclin D1	Forward	5'-ATGGAACACCAGCTCCTGTG-3'	60ºC
	Reverse	5'-ACCTCCAGCATCCAGGTGGC-3'	
RXRa	Forward	5'-CTTTGACAGGGTGCTAACAGAGC-3'	60ºC
	Reverse	5'- ACGCTTCTAGTGACGCATACACC-3	
BCMO1	Forward	5'-TCTGAGTTCGGAACCATGGC-3'	60ºC
	Reverse	5'-GTGTGAGACAAGTAGGAGAAAGCT-3'	
RARb	Forward	5'-GCCTGCAGAAGTGCTTTGAAGT-3'	56.5ºC
	Reverse	5'-GCTTTCCGGATCTTCTCAGTGA-3'	
MMP7	Forward	5'-GATGAGGACGCAGGAGTGAAC-3'	56.5ºC
	Reverse	5'-GAAGAGTGACTCAGACCCAGAGAGT-3'	
COX-2	Forward	5'-GCATTCGCCCAGCACTT-3'	52.5ºC
	Reverse	5'-AGACCAGGCACCGACCAAAGA-3'	
B-Actin	Forward	5'-TAGACTTCGAGCAGGAGATGGC-3'	60ºC
	Reverse	5'-CCACAGGATTCCATACCCAAGA-3'	

An Internal control (IC) sample, to compare data between individual runs, was prepared by combining cDNA reverse transcribed from RNA obtained from mice on various dietary groups, includes males and females of both min and wild-type. An IC-NEC was also prepared. A third

control group was no template controls (NTC) which were contained all reaction components but included water instead of cDNA to test for the presence of contamination control during Real-Time PCR. For each sample to be tested the amplification reactions were carried out in duplicate, with single NEC amplification reactions alongside them to test for contamination at the level of cDNA synthesis. All other control reactions (IC, IC-NEC and NTC) were in triplicate.

2.5.5 Assay Optimisation

IC cDNA described above was used to optimise PCRs for each primer set in Eppendorf Master Cycler Gradient (Eppendorf AG 22331 Hamburg). (See Appendix 3 for Standard curves) PCR was performed at different annealing temperatures using gene specific oligonucleotide primers and QuantiTect® SYBR® Green with the following PCR amplifications conditions; denaturation at 94ºC for 15seconds followed by 40cycles of annealing for 30 seconds between 51ºC and 61.8ºC, followed by extension at 72ºC for 30 seconds and a final melting curve step. For each oligonucleotide primer, annealing temperature gradient between 51ºC and 61.8ºC was employed. The reactions were carried out in duplicate. The amplicons were subjected to gel electrophoresis using standard 2 % agarose in IX TBE and stained with 1.5µl Ethidium Bromide. The stained images were viewed with UV light in Syngene, Multi Genius Bio Imaging system (Division of Synoptic LTD). Observation of a single band of required product length suggests primer specificity and the corresponding annealing temperature is selected and used in the next step (Table 2.1).

Serial dilutions were produced from the IC cDNA sample and real-time PCR performed using QuantiTect® SYBR® Green, gene specific oligonucleotide primer, 4ul of each dilution and DNase-RNase-free water in a reaction volume of 20 µl using CFX-96 Thermal Cycler with Real-Time Detection System (Bio-Rad Laboratories Ltd) in line with the manufacturer's protocol. Each reaction was carried out in duplicate and standard curves generated by plotting the threshold cycles (CT) values against the log of the dilution factor in the range of 1:5 to 1:500. Table 5 A below presents PCR amplification information of target genes are presented

Amplification efficiency (E) and percentage efficiency (% E) of the assay were therefore calculated using the formula:

$$E = 10^{-1/slope}$$

E= EFFICIENCY

% E = (E-1) X 100 %

Expression of target gene is calculated with the formula

2^CT

Where CT = $\frac{(\%ER)^{\wedge CtR}}{(\%ET)^{\wedge CtT}}$

%ER=efficiency of reference gene; CtR= mean ct of reference gene

%ET= efficiency of target gene; CtT= mean ct of target gene

Table 2. 2: PCR Amplification information of target gene and reference gene

Target Gene	Amplicon Size (bp)	Slope of standard curve	% Efficiency	R^2
BCMO1	77	-2.88	122.47	0.999
RARbeta	151	-3.15	107.85	0.986
RXRalpha	173	-3.37	98.02	0.992
Cyclin D1	200	-3.12	109.27	0.992
COX-2	192	-3.41	96.58	0.99
BetaActin[1]	151	-3.33	99.84	0.998
MMP7	78	-342	96.18	0.99

[1] Reference gene

2.6 Statistical Analysis

ANOVA (General Linear Model and Fit General Linear Model) of Minitab 17 was employed for the analysis to determine the effects of maternal and post weaning diets, sex, genotype and their interactions on growth, tumour number and volume, body weight, and internal organ dimensions of mice in a factorial design. Statistical details are outlined on the results pages.

Values presented by tables and graphs were LSM (Least square means) considered to be most appropriate estimates of means of effects of diet treatment

after the adjustments of any inequalities in sex and genotype distribution to dietary regime.

Before analyses all data were tested for normal distribution by examining the skewness and kurtosis using descriptive statistics and probability plots. Where data was not normally distributed it was transformed to reduce skewness and Kurtosis using Box Cox transformation Tool of Minitab 17 Statistical Software. This provides the best transformation for a given data set and is defined by value of lambda as follows;(Osbourne, 2002)

0 = log transformation

0.5 = square root transformation

1 = no transformation required

2 = square transformation

-1 = reciprocal transformation

2.7 Differences between current research and the research of Saleh et al., (2013)

Saleh *et. al.,* (2013) investigated the effects carrot consumption on intestinal tumour tumorigenesis in *Apc*$^{Min/+}$ mice. In their study carrot pellets were fed to breeding trio prior to mating, through gestation and lactation. Weaned pups were continued on their respective dams' diet until killed at age 12 weeks.

In the current study, carrot feeding started at gestational age zero by randomising breeding trio to experimental diet (carrot pellet) or control pellet prior to mating, through gestation and lactation. However, unlike Saleh *et al.,* (2013), weaned offspring were randomised to either control pellet or experimental diet to create four in utero-post weaning dietary groups strictly adhered to until killed at 15 weeks. This study therefore examined the effects of maternal and post weaning carrot diet on intestinal tumourigenesis.in adult offspring, a complete departure from the study of Saleh *et al.,* (2013) and to my knowledge is the first of its kind.

The similarity between the current study and that of Saleh *et al*., (2013) is the use of carrot pellet diet consisting of 20 % carrot powder and 80 % RM3 (P). However, the pellet used by Saleh *et al.,* (2013) was handmade and therefore lacked consistency in terms of size and texture and hardness. Also drying was at room temperature as such required much time to dry creating

opportunity for growth of mould. These were conditions that informed the mode of experimental diet production for the current study. Pellet for the current study were manufactured in the industry with professional equipment to specification and dried at a predefined temperature range of 90 – 120^{o}C.

The second part of the current study investigated effects of carrot powder consumption *in utero* and post weaning and also effects of stored carrot pellet post weaning on intestinal tumourigenesis.

3 Chapter 3 Results for Study One

3.1 Proximate Composition

Proximate data was analysed using ANOVA, General Linear Model (GLM) with carrot powder, control powder and stored carrot pellet as factors. Data was not transformed. Results are presented as LSM ± SEM. Tukey's test was employed to locate significant difference between means where necessary. Data on micro nutrient composition of carrot pellet and control diet are presented in appendix 1.

There were significant differences in macronutrient composition of the experimental diets however the total energy content based on the total macro nutrient composition of the diets was not statistically different suggesting the diets were isocaloric (Table 3.1). See Appendix 2 for analytical procedures.

Table 3. 1: Nutrient composition of experimental diet

Sample	Carrot powder	Control powder	Stored carrot pellet	P-Value
Parameter (%)	Mean ± StDev	Mean ± StDev	Mean ± StDev	
Oil (%)	2.84 ± 0.259^{b}	3.05 ± 0.122^{b}	4.69 ± 0.344^{a}	< 0.001
Fibre (%)	6.26 ± 0.166^{a}	3.98 ± 0.058^{b}	6.72 ± 0.31^{a}	< 0.001
Ash (%)	6.5 ± 0.025^{b}	6.9 ± 0.004^{a}	6.93 ± 0.026^{a}	< 0.001
Moisture (%)	10.16 ± 0.07^{b}	10.58 ± 0.052^{a}	9.05 ± 0.081^{c}	< 0.001
Protein (%)	15.58 ± 0.893^{b}	19.48 ± 0.361^{a}	18.7 ± 0.651^{a}	< 0.001
NFE (%)	58.61 ± 1.111^{a}	56.03 ± 0.288^{b}	50.87 ± 4.68^{c}	< 0.001
Energy (mj/kg)	13.69 ± 0.111^{a}	13.99 ± 0.025^{a}	13.59 ± 0.967^{a}	0.069

Means that do not share a letter in a row are significantly different.

3.2 Effects of diet on pregnancy outcome

Effects of maternal diets, control pellet and carrot pellet on pregnancy outcome was determined by comparing the litter size, sex distribution, and genotypes of offspring of dams across the diets using ANOVA (GLM). All weaned offspring were included in the analysis. Data was presented as LSM ± SEM.

Eight dams were randomised to control pellet and seven to carrot pellet diets. A total of 96 offspring were weaned, 46 were born to dams on carrot pellet and 50 to dams on control pellet. Maternal diet had no significant effect on pregnancy outcome except on the number of wild

type females that was significantly (P = 0.02) higher in litter of wild type dams fed carrot pellet (Table 3.2).

Table 3. 2: Maternal diet and pregnancy outcome

	Maternal diet		P value
	Carrot pellet (n = 7)	Control pellet (n = 8)	
	Mean± SEM	Mean ± SEM	
Litter size	6.57 ± 0.409	6.25 ± 0.382	0.576
Total male	2.71 ± 0.322	3.5 ± 0.301	0.098
Total female	3.86 ± 0.424	2.75 ± 0.397	0.079
Total Min	3.57 ±0.625	3.38 ±0.585	0.822
Total Wild type	3.00 ±0.501	2.88 ±0.469	0.858
Min males	1.57 ± 0.435	1.75 ± 0.407	0.769
Min females	2.0 ± 0.39	1.63 ± 0.365	0.495
Wild type male	1.14 ± 0.397	1.75 ± 0.372	0.285
Wild type female	1.86 ± 0.203	1.13 ± 0.189	**0.02**

3.3 Dietary Groups of animals on study

A total of 96 mice (47males and 49 females) were randomised at weaning to control or carrot pellet diets to form four in utero post weaning dietary groups. One female *Apc*$^{Min/+}$ mouse of carrot pellet-control pellet dietary group died during the course of the study. Chi-square analysis suggested that there was no difference between the number males and females (P = 0.15) (Table 3.3).

Table 3. 3: Dietary Groups of Experimental Animals Stratified by Sex and Genotype

Control pellet-control pellet				Control pellet-carrot pellet				Carrot pellet-control pellet				Carrot pellet-carrot pellet			
Wild type		Min		Wild type		Min		Wild type		Min		Wild type		Min	
Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
9	5	7	6	5	4	7	7	5	6	4	6	3	7	7	8

3.4 Growth Data Analysis

Effects of maternal diet, post weaning diet, sex and genotype on growth were analysed in a factorial design using ANOVA (GLM). Weaning weight was subtracted from weekly weights for statistical analysis. To determine the effects of sex and genotype on growth data for both sexes and genotypes were put together and genotype added as covariate looking at effects of sex whilst sex was added as covariate looking at effects of genotype. As sex and genotype significantly affected growth they were added as covariate to investigate effects of maternal diet and post weaning diet on growth. The effects of maternal and post weaning diets on growth were analysed for Min and Wild type separately and also with combined data for the genotypes. Effects of maternal diet on weaning weight was investigated with maternal diet, sex and genotype as factors alongside their interactions with litter size as covariate. Results are presented as Least Square Means (LSM) ± SEM. All data were uniformly distributed and therefore not transformed.

3.4.1 Weaning weight

Maternal diet had no significant effect on mean weaning weight of offspring (Table.3.4). Male offspring were heavier than females by 6.5 % ($P < 0.001$) whiles wild type mice were heavier than *Apc*$^{Min/+}$ mice by 4.4 % ($P = 0.023$) at weaning. Neither of the interactions between the main factors had any significant effect on weaning weight (Table 3.4).

Table 3. 4: Effects of Maternal diet, Sex and Genotype on weaning weights of experimental mice

	Maternal diet			Sex			Genotype			Probability effects of main factor interactions			
	Control pellet (n = 50)	Carrot pellet (n = 46)	P value	Female (n = 49)	Male (n = 47)	P value	*Apc*$^{Min/+}$ (n = 52)	Wild type (n = 44)	P value	Maternal diet*Sex	Maternal diet* Genotype	Sex * Genotype	Maternal diet*Sex* Genotype
	Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM					
Weaning weight	13.7 ± 0.333	12.80 ± 0.349	0.084	12.39 ± 0.335	14.11 ± 0.345	< 0.001	12.67 ± 0.321	13.83 ± 0.357	0.023	0.639	0.874	0.231	0.646

Weaning weight data was Box Cox transformed for P value

3.4.2 Effects of Sex and Genotype on Growth of Mice

As expected males were heavier ($P < 0.001$) than females at death at age 15 weeks (Fig.3.1). Sex, maternal diet and post weaning diet interactions had no significant effect on mean weight gain post weaning at death. Wild type mice were also heavier ($P < 0.003$) than Min mice at death at age 15 weeks (Fig. 3.2). Genotype, maternal diet and post weaning diet interactions had no significant effect on mean weight gain post weaning.

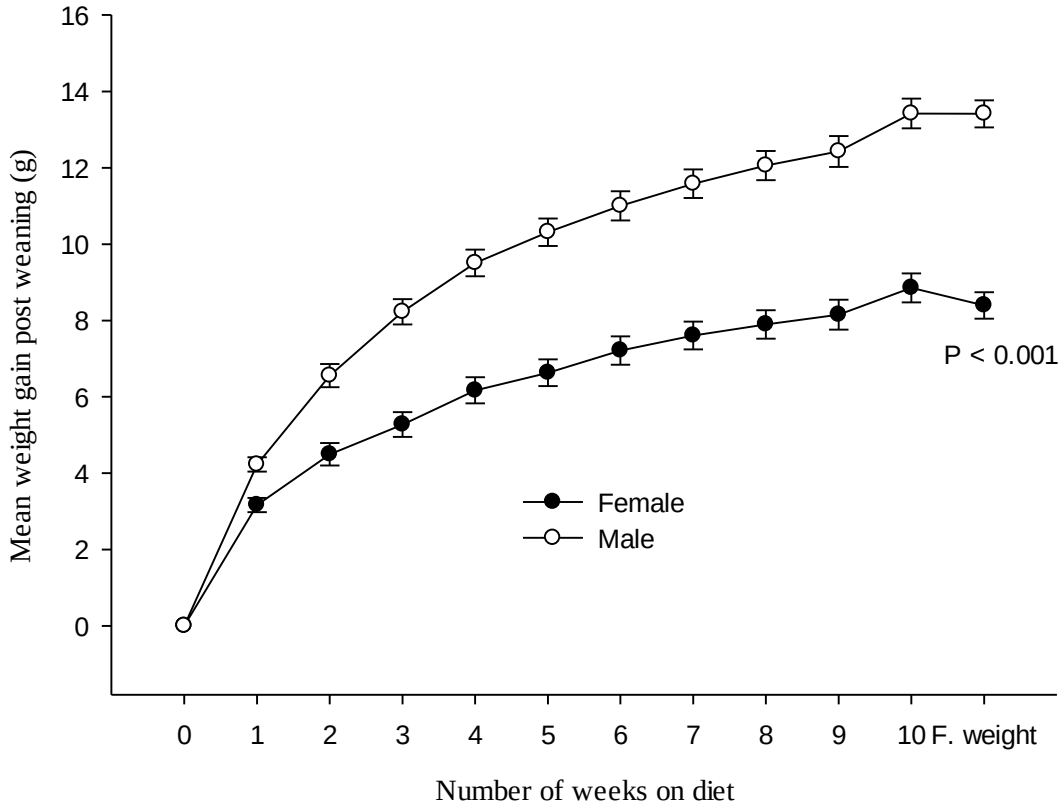


Figure 3. 1: Growth curves of mean weight gain post weaning for male and female mice from all dietary groups. Genotype was added as covariate in looking at effects sex on growth (Female n = 48; male n = 47). Weaning weight was subtracted from weekly weights for statistical analysis. Data were uniformly distributed and therefore not transformed.

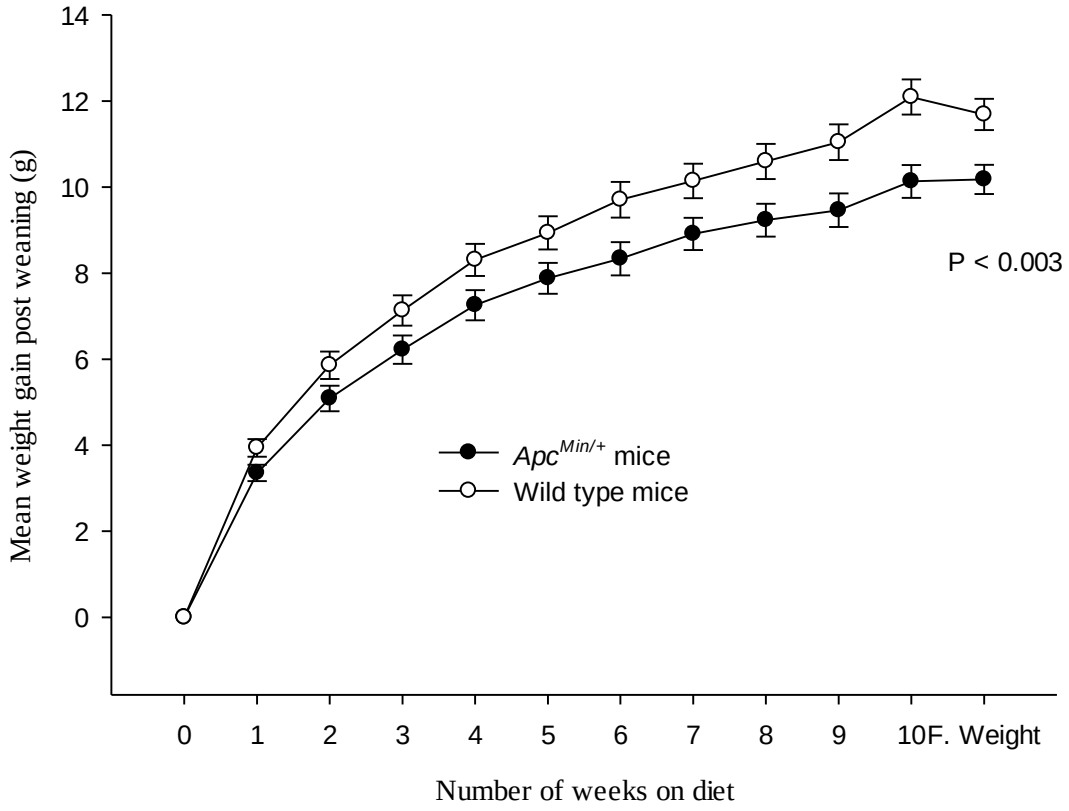


Figure 3. 2: Growth curves of mean weight gain post weaning for Min and Wild type mice from all dietary groups. Sex was added as covariate in looking at effect of genotype on growth (*Apc*$^{Min/+}$ n = 51; Wild type n = 44). Weaning weight was subtracted from weekly weights for statistical analysis. Data were uniformly distributed and therefore not transformed.

3.4.3 Effects of Maternal and Post weaning diet on Growth of Mice

As sex and genotype significantly influenced growth they were added as covariate to investigate effects of maternal diet and post weaning diet on growth measured as mean weight gain post weaning.

Curves present effects of maternal and post weaning diet interactions on growth. At death at age 15 weeks, maternal and post weaning diet interactions had no statistically significant (P = 0.957) effect on mean weight gain post weaning by Min mice (Fig. 3.3). Neither maternal (P = 0.562) nor post weaning (P = 0.386) diet had any significant effect on mean weight gain post weaning (data not shown).

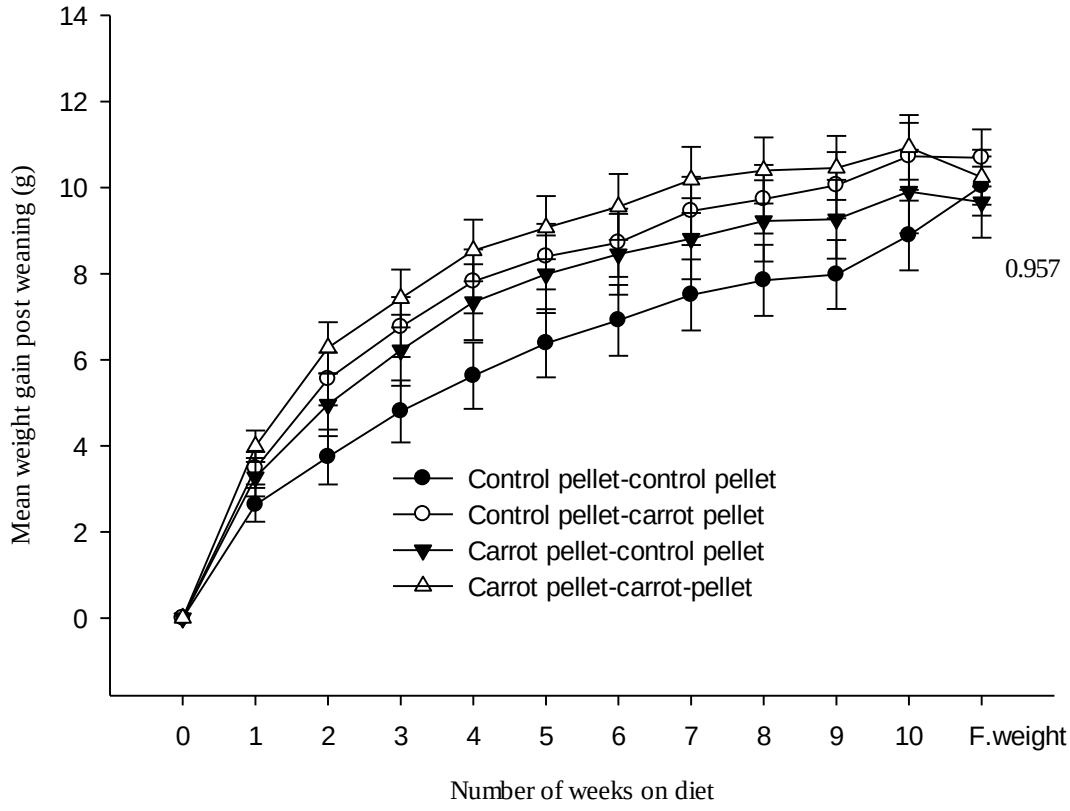


Figure 3. 3: Growth curves of mean weight gain post weaning for Min mice exposed to different dietary regimes. Sex was added as covariate looking at the effects of the diets on growth, (Control pellet-control pellet n = 13; Control pellet-carrot pellet n = 14; Carrot pellet-control pellet n = 9; Carrot pellet-carrot pellet n = 15). Weaning weight was subtracted from weekly weights for statistical analysis. Data were uniformly distributed and therefore not transformed.

Curves present effects of maternal and post weaning diet interactions on growth. Interactions of maternal and post weaning diets had no statistically significant effect on mean weight gain post weaning by wild type mice at death at age 15 weeks (P = 0.099) (Fig. 3.4). Wild type mice fed carrot pellet post weaning had 6.4 % (P = 0.04) higher mean weight gain compared to control pellet fed at death at age 15 weeks. Maternal diet had no statistically significant effect (P = 0.712). Data not shown.

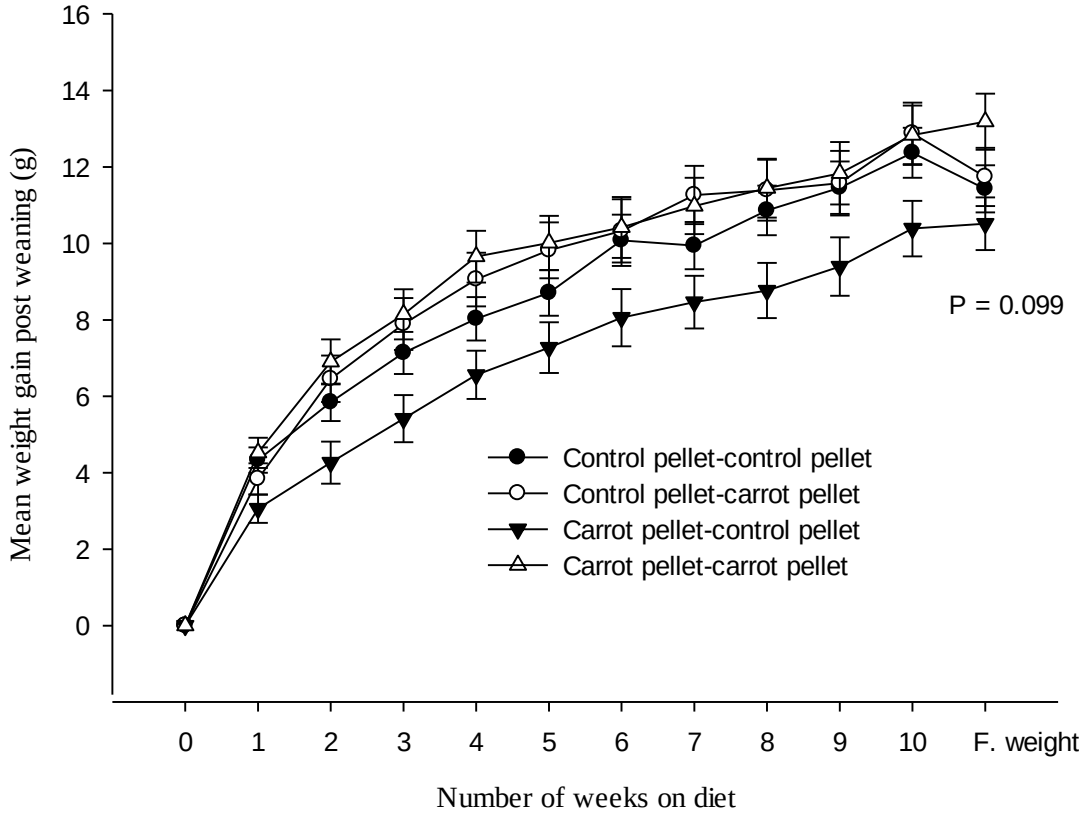


Figure 3. 4: Growth curves of mean weight gain post weaning for wild type mice exposed to different dietary regimes. Sex was added as covariate looking at the effects of diet on growth. (Control pellet-control pellet n = 14; control pellet-carrot pellet n = 9; carrot pellet-control pellet n = 11; carrot pellet-carrot pellet n = 10) Weaning weight was subtracted from weekly weights for statistical analysis. Data were uniformly distributed and therefore not transformed.

Curves present effects of maternal and post weaning diet interactions on growth. At death at age 15weeks interactions of maternal and post weaning diet had no significant effect (P = 0.321) on mean weight gain post weaning by mice (both Min and Wild type) exposed to different dietary regimes ((Fig.3.5). Mice fed carrot pellet post weaning had 4.7 % (P = 0.041) higher mean weight gain than those fed control pellet at death at age 15 weeks. Maternal diet had no statistically significant effect (P = 0.677) (data not shown).

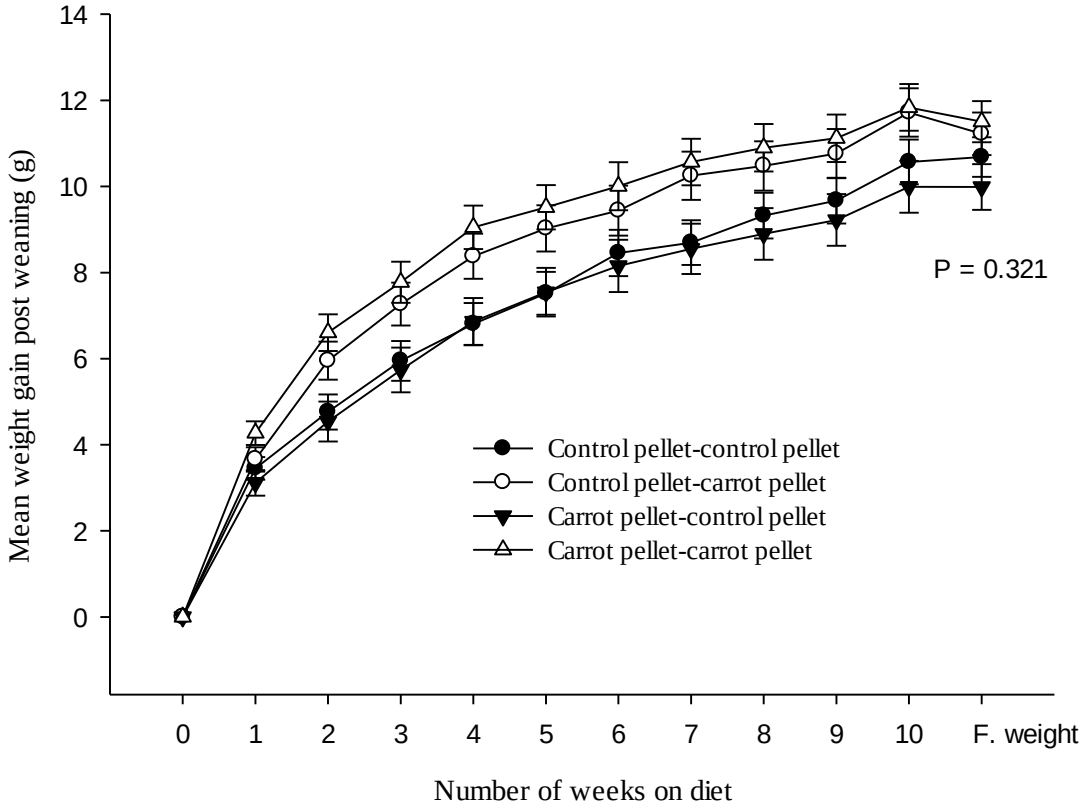


Figure 3. 5: Growth curves of mean weight gain post weaning for mice (Min and Wild type) exposed to different dietary regimes. Sex was added as covariate looking at the effects of diet on growth, (Control pellet-control pellet n = 27; Control pellet-carrot pellet n = 23; Carrot pellet-control pellet n = 20; Carrot pellet-carrot pellet n = 25). Weaning weight was subtracted from weekly weights for statistical analysis. Data were uniformly distributed and therefore not transformed.

3.5 Effects of Maternal and Post weaning diets, Sex and Genotype on Body weight and Internal Organ dimensions of mice at death

Effects of maternal diet, post weaning diet and sex and their interactions on body weight and internal organ dimension of mice at death at age 15 weeks were analysed in a factorial design using ANOVA (LSM). SI length, body, colon, right and left kidneys and spleen weights ~~and~~ were Box Cox transformed for P values. Means were calculated as LSM ± SEM. Results are presented in tables 3.5, 3.6, and 3.7.

3.5.1 Effects of Sex and Genotype on Body weight and Organ dimensions

As expected, male mice were significantly heavier, (27.62 g ± 0.287 SEM) ($P < 0.001$) than their female counterparts (21 g ± 0.28 SEM) at time of death at 15 weeks (Table 3.5). Apart from weights of terminal SI ($P = 0.075$) and colon ($P = 0.087$) all organs weighed were significantly heavier in males than females. The length of SI ($P < 0.001$) and colon ($P = 0.008$) were also longer in males than in females.

Also at time of death as expected, Wild type mice had higher mean body weight (25.67 ± 0.294) ($P < 0.001$) than Min mice (23.06 ± 0.275 SEM) (Table 3.5). Significantly heavier in the Wild types were liver ($P = 0.026$), right and left kidneys ($P < 0.001$) and the heart ($P < 0.001$). The weights of terminal SI, colon and spleen were higher ($P < 0.001$) in Min mice than in Wild types. Weights of the liver and right kidney were significantly affected by sex and genotype interaction (Table 3.5).

Table 3. 5: Effects of sex and genotype on body and organ dimensions of mice exposed to different dietary regime at death at age 15 weeks

	Sex			Genotype			Probability effects of Sex * Genotype
	Female (n = 48)	Male (n = 47)	P value	*Apc*$^{Min/+}$ (n = 51)	Wild type (n = 44)	P value	
	Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM		
Body weight (g)	20.99 ± 0.28	27.62 ± 0.287	< **0.001**	23.06 ± 0.275	25.67 ± 0.294	< **0.001**	0.175
Full colon weight (g)	0.41 ± 0.012	0.47 ± 0.12	< **0.001**	0.45 ± 0.011	0.43 ± 0.012	0.212	0.144
Proximal SI weight (g)	0.67 ± 0.01	0.75 ± 0.01	< **0.001**	0.72 ± 0.01	0.7 ± 0.01	0.136	0.581
Terminal SI weight (g)	0.58 ± 0.01	0.6 ± 0.01	0.075	0.63 ± 0.01	0.55 ± 0.01	< **0.001**	0.671
SI length (cm)	29.18 ± 0.304	31.23 ± 0.312	< **0.001**	30.17 ± 0.292	30.31 ± 0.312	0.936	0.183
Colon weight (g)	0.23 ± 0.006	0.25 ± 0.006	0.087	0.26 ± 0.006	0.22 ± 0.006	< **0.001**	0.233
Colon length (cm)	6.14 ± 0.071	6.41 ± 0.073	**0.008**	6.35 ± 0.068	6.24 ± 0.073	0.253	0.965
Caecum weight (g)	0.56 ± 0.014	0.71 ± 0.014	< **0.001**	0.63 ± 0.014	0.62 ± 0.014	0.453	0.194
Liver weight (g)	1.08 ± 0.029	1.51 ± 0.03	< **0.001**	1.25 ± 0.029	1.34 ± 0.031	**0.026**	**0.039**
Right kidney weight (g)	0.14 ± 0.003	0.18 ± 0.003	< **0.001**	0.14 ± 0.003	0.17 ± 0.003	< **0.001**	**0.017**
Left kidney weight(g)	0.14 ± 0.002	0.17 ± 0.002	< **0.001**	0.14 ± 0.002	0.16 ± 0.002	< **0.001**	0.067
Spleen weight (g)	0.19 ± 0.014	0.21 ± 0.014	**0.029**	0.29 ± 0.013	0.09 ± 0.014	< **0.001**	0.484
Heart weight (g)	0.19 ± 0.006	0.23 ± 0.006	< **0.001**	0.2 ± 0.006	0.23 ± 0.006	< **0.001**	0.153

3.5.2 Effects of Maternal and Post weaning diet on Body and Organ Dimensions of Mice at Death

At time of death mean body weight of adult offspring born to dams fed control pellet (24.8 g ± 0.272 SEM) ($P = 0.011$) was higher than offspring born to carrot pellet fed dams (23.8 g ± 0.288 SEM) (Table 3.6). Carrot pellet consumption by dams decreased mean weight of the heart ($P = 0.005$) (Table 3.6).

Feeding carrot pellet diet to mice post weaning increased mean weights of proximal SI, terminal SI and caecum ($P < 0.001$) but decreased that of the heart ($P = 0.01$) at time of death. Maternal and post weaning diet interactions affected the weight of heart ($P = 0.047$) (Table 3.6).

Table 3. 6 Effects of Maternal and Post weaning diets on body and internal organ dimensions of mice at death at 15 weeks

	Maternal diet			Post weaning diet			Probability effects of Maternal diet * Post weaning diet
	Control pellet (n = 50)	Carrot pellet (n=45)	P value	Control pellet (n = 47)	Carrot pellet (n = 48)	P value	
	Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM		
Body weight (g)	24.80 ± 0.272	23.76 ± 0.288	**0.011**	24.66 ± 0.284	23.91 ± 0.278	0.089	0.314
Full colon weight (g)	0.45 ± 0.011	0.44 ± 0.012	0.663	0.44 ± 0.012	0.45 ± 0.012	0.583	0.32
Proximal SI weight (g)	0.70 ± 0.01	0.71 ± 0.010	0.724	0.67 ± 0.01	0.74 ± 0.01	< **0.001**	0.732
Terminal SI weight (g)	0.59 ± 0.01	0.59 ± 0.010	0.823	0.57 ± 0.01	0.62 ± 0.01	< **0.001**	0.763
SI length (cm)	30.46 ± 0.296	29.9 ± 0.313	0.181	29.99 ± 0.308	30.36 ± 0.302	0.433	0.158
Colon weight (g)	0.23 ± 0.006	0.25 ± 0.006	0.155	0.24 ± 0.006	0.24 ± 0.006	0.622	0.087
Colon length (cm)	6.31 ± 0.07	6.25 ± 0.074	0.582	6.26 ± 0.073	6.30 ± 0.071	0.68	0.298
Caecum weight (g)	0.62 ± 0.013	0.63 ± 0.014	0.609	0.56 ± 0.014	0.70 ± 0.014	< **0.001**	0.737
Liver weight (g)	1.31 ± 0.029	1.27 ± 0.031	0.337	1.28 ± 0.03	1.3 ± 0.03	0.696	0.464
Right kidney (g)	0.16 ± 0.003	0.16 ± 0.003	0.497	0.16 ± 0.003	0.16 ± 0.003	0.317	0.427
Left kidney (g)	0.15 ± 0.002	0.15 ± 0.002	0.247	0.15 ± 0.002	0.15 ± 0.002	0.796	0.408
Spleen	0.19 ± 0.013	0.20 ± 0.014	0.063	0.2 ± 0.014	0.2 ± 0.013	0.621	0.341
Heart	0.22 ± 0.006	0.20 ± 0.006	**0.005**	0.22 ± 0.006	0.2 ± 0.006	**0.01**	**0.047**

Table 3. 7: Probability effects of interactions of Maternal and Post weaning diets, Sex and Genotype on Body and organ Dimensions Study

	Maternal diet * Sex * Genotype	Post weaning diet* Sex * Genotype	Maternal diet * Post weaning diet Sex * Genotype
Body weight (g)	0.842	0.813	0.283
Full colon weight (g)	0.743	0.237	0.951
Proximal SI weight (g)	0.691	0.358	0.546
Terminal SI weight (g)	0.067	0.72	0.469
SI length (cm)	0.718	0.573	0.533
Colon weight (g)	0.853	0.162	0.771
Colon length (cm)	0.251	0.744	0.367
Caecum weight (g)	0.906	0.44	0.301
Liver weight (g)	0.852	0.804	0.267
Right kidney (g)	0.33	0.456	0.743
Left kidney (g)	0.533	0.168	0.288
Spleen weight (g)	0.457	0.526	0.428
Heart weight (g)	0.283	0.397	0.701

3.6 Tumour Data Analysis

Tumour number in the three sections of the intestine of the Min mice was compared in One-Way ANOVA. Effects of maternal diet, post weaning diet, sex and their interactions on tumour number and volume were analysed as a factorial design using ANOVA, GLM. Data from proximal SI, terminal SI, colon and total gut were Box Cox transformed for P values. The main factor effects are presented in figures 3.7 - 3.12. Effects of main factor interactions are presented in table 3.8.

3.6.1 Tumour Distribution in Intestine of Min mice

There were significant ($P < 0.001$) differences in distribution of tumour in gut of Min mice at time of death. Most of the tumour occurred in the terminal SI (31.4 ± 1.49 SEM). Tumour number in proximal and colon were 11.3 ± 1.49 SEM and 1.8 ± 1.49 SEM in the colon (Fig.3.6). Mean tumour number per mouse for the dietary regimes ranged from 43.2 ± 5.5 SEM (carrot pellet-control pellet) to 53.3 ± 4.9 SEM (carrot pellet-carrot pellet). Mean tumour number per mouse for carrot pellet-carrot pellet was higher than that control pellet-control pellet by 12.5 ($P = 0.002$)

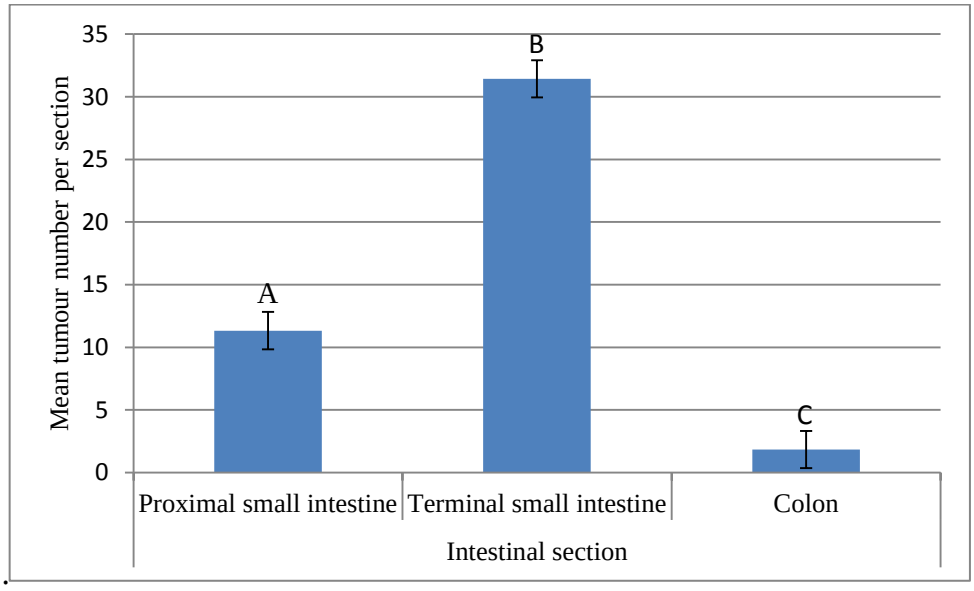


Figure 3. 6: Tumour distribution in the intestine of min mice (n = 51) Error bars represent SEM, Means with different letters are significantly different $P < 0.001$

3.6.2 Effects of Sex on Tumour Number and Volume

Sex had no statistically significant effect on intestinal tumour number (Fig. 3.7) and volume (Fig. 3.8) in min mice.

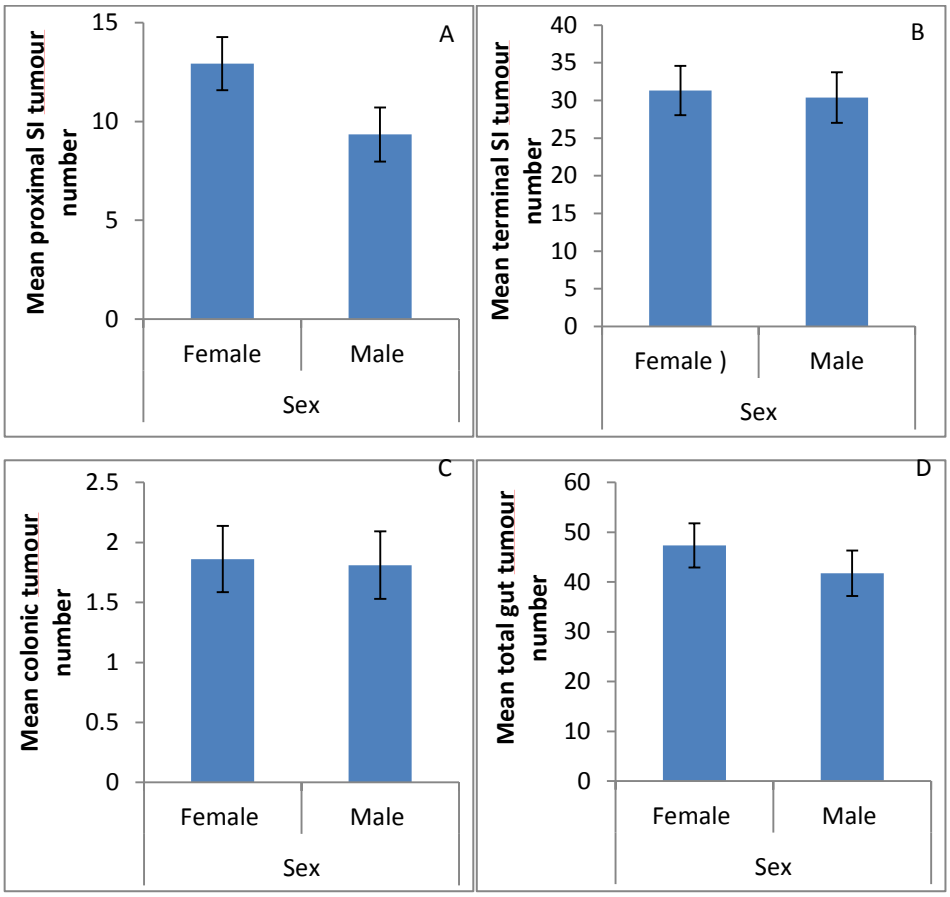


Figure 3. 7: Effects of sex on (A) proximal small intestine (B) terminal small intestine (C) colon and (D) total gut intestinal tumour number in *Apc*$^{min/+}$ mice. (Male n = 25, female n =26) Error bars represent standard error of means (SEM)

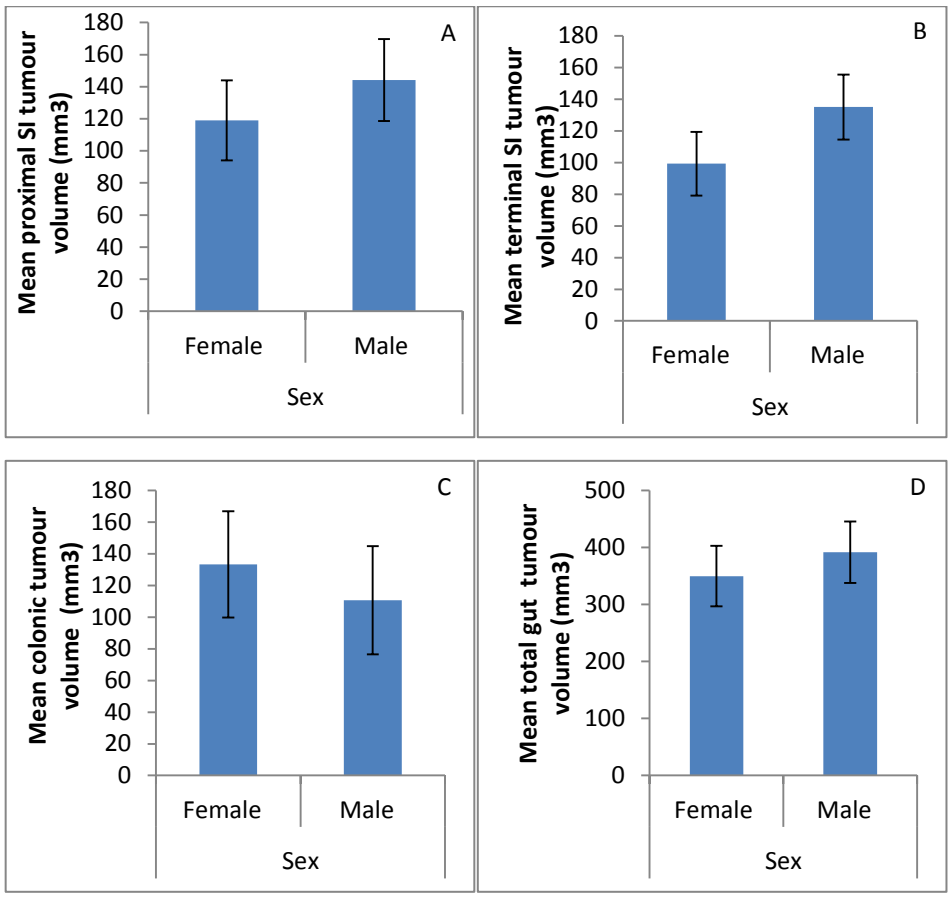


Figure 3. 8: Effects of sex on tumour volume in (A) proximal SI (B) terminal SI(C) colon and (D) total gut intestinal tumour volume in *Apc*$^{min/+}$ mice. (Female n = 26; males n = 25) Error bars represent SEM.

3.6.3 Effects of Maternal diet on Tumour Number and Volume

Maternal diet had no significant influence on tumour number (Fig.3.9) and volume (Fig. 3.10) in adult Min mice offspring at death at age 15 weeks.

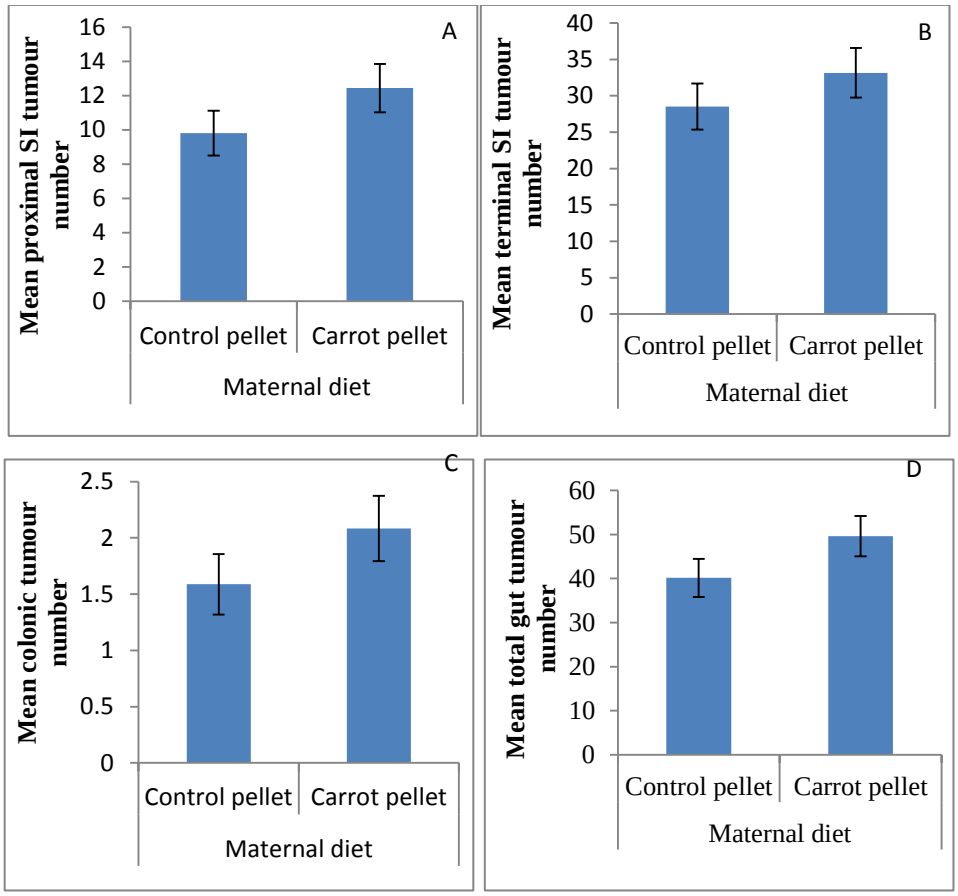


Figure 3. 9: Effects of maternal diet on tumour number in (A) proximal SI (B) terminal SI (C) colon and (D) total gut intestinal tumour number in *Apc*$^{min/+}$ mice (Control pellet n = 27, carrot pellet n = 24).Error bars represent standard error of means (SEM).

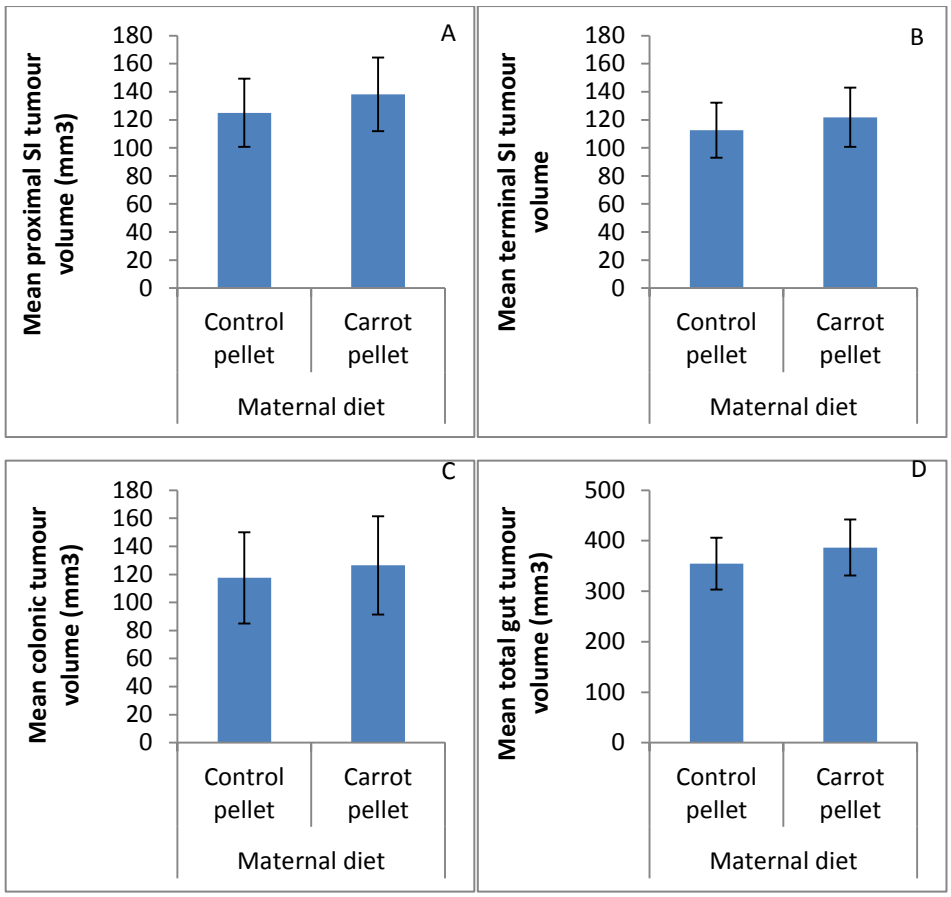


Figure 3. 10: Effects of maternal diets on (A) proximal SI (B) terminal SI (C) colon and (D) total gut intestinal tumour volume in *Apc*$^{min/+}$ mice. (SEM) Control pellet n = 27, carrot pellet n = 24) Error bars represent (SEM).

3.6.4 Effects of Post weaning diet on Tumour Number and Volume

Carrot pellet consumption by offspring post weaning significantly increased mean tumour number (P = 0.044) in terminal small intestine (Fig. 3.11 B) and mean total gut tumour number (P = 0.038) (Fig. 3.11 D). Post weaning diet had no significant effect on tumour volume in the intestine of Min mice at death (Fig.3.12).

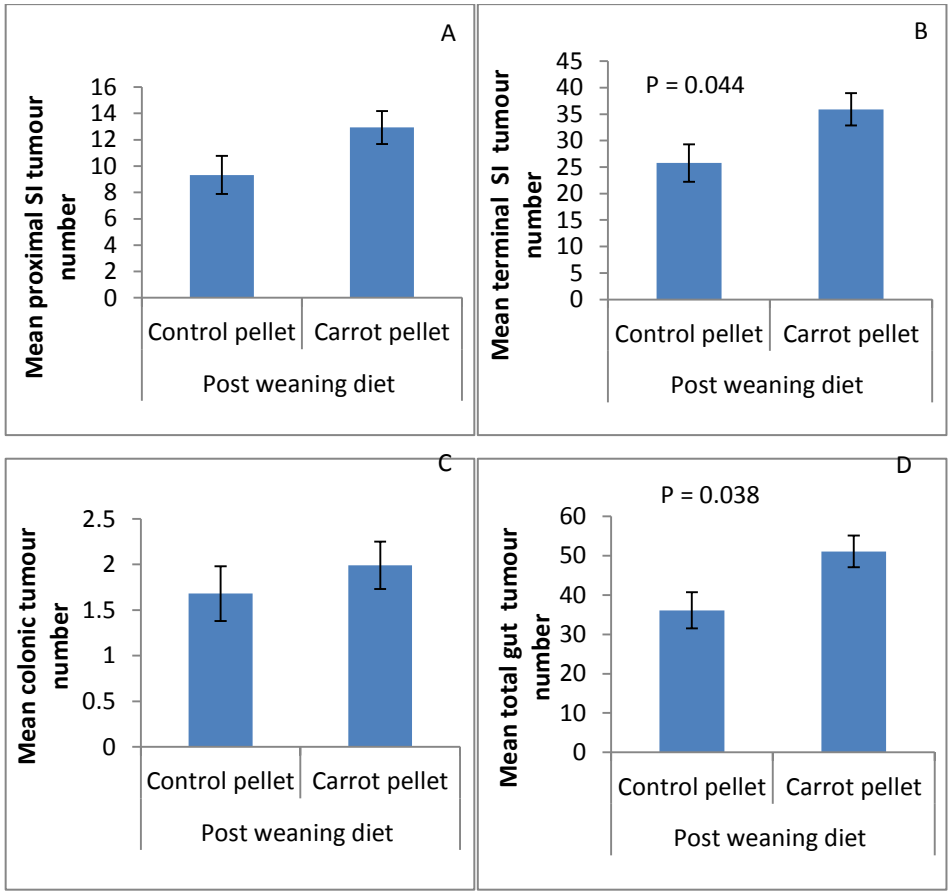


Figure 3. 11: Effects of post weaning diet on (A) proximal SI (B) terminal SI (C) colon and (D) total gut intestinal tumour number in *Apc*$^{min/+}$ mice (Control pellet n = 22, carrot pellet n = 29) Error bars represent SEM.

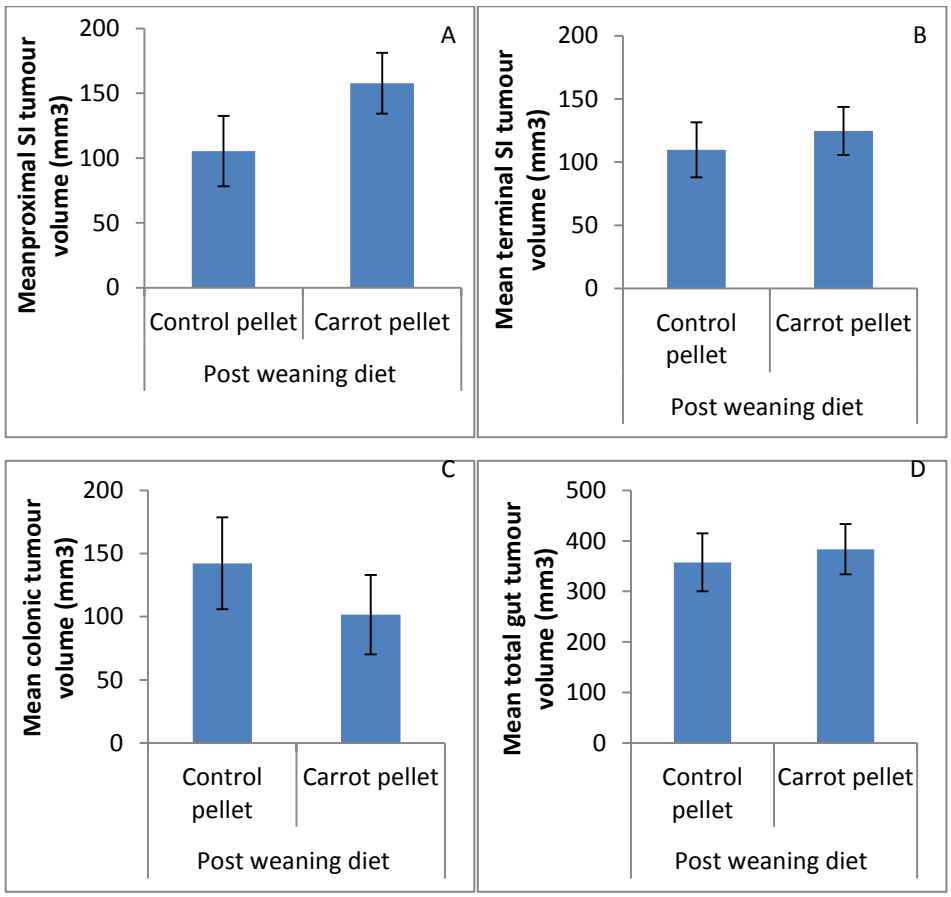


Figure 3. 12: Effects of post weaning diet on tumour volume in (A) proximal SI (B) terminal SI (C)colon and (D) total gut tumour volume in *Apc*$^{min/+}$ mice (Control pellet n = 22, carrot pellet n = 29) Error bars represent SEM.

3.6.5 Effects of Interaction of Maternal and Post weaning diet and Sex on Tumour Number and Volume

No statistically significant interaction was observed between sex, maternal and post weaning diets for tumour number and volume at death in the intestine of Min mice (Table 3.7).

Table 3. 8: Effects of interactions of sex, maternal and post weaning diets on intestinal tumour number and volume (mm^3) in Min mice at time of death at age 15 weeks (CP = control pellet, CPP = carrot pellet, Fem = female).

	Maternal diet * Post weaning diet					Maternal diet * Sex					Post weaning diet *Sex				
	CP-CP (n = 13)	CP- CPP (n = 14)	CPP-CP (n = 9)	CPP-CPP (n = 15)	P value	CP-Fem (n = 13)	CP-Male (n = 14)	CPP-Fem (n = 13)	CPP-male (n = 11)	P value	CP-Fem (n = 11)	CP-Male (n = 11)	CPP-Fem (n = 15)	CPP-Male (n = 14)	P value
	Mean					Mean					Mean				
Proximal SI Tumour Number	8.23	11.29	10.08	14.44	0.674	10.44	9.07	15.14	9.38	0.228	10.28	8.02	15.29	10.43	0.487
Terminal SI Tumour Number	21.64	35.36	31.10	36.72	0.364	30.93	26.07	32.04	35.79	0.377	25.10	27.64	37.87	34.21	0.667
Colonic Tumour Number	1.33	1.86	2.13	2.12	0.591	1.55	1.64	2.19	2.05	0.753	1.83	1.63	1.90	2.07	0.56
Total Gut Tumour Number	31.2	48.5	43.3	53.28	0.574	42.9	36.79	49.36	47.21	0.906	37.22	37.29	55.06	46.71	0.612
Proximal SI Tumour volume (mm^3)	110.9	140.2	96.9	175.4	0.157	127.4	123.8	107.3	165	0.620	84.4	123.5	150.3	165.3	0.736
Terminal SI Tumour volume (mm^3)	115.2	109.9	98.5	139.1	0.717	96.8	128.3	98.9	138.7	0.588	82.4	131.3	113.3	135.7	0.831
Colonic Tumour volume (mm^3)	158.4	78.5	118	123.6	0.714	134	102.9	129	112.6	0.843	186.1	90.3	76.9	125.2	0.258
Total gut tumour volume (mm^3)	384.5	328.6	313.4	438.2	0.335	358.2	354.9	335.2	416.4	0.769	352.8	345.1	340.6	426.2	0.697

Maternal diet*post weaning diet* sex had no statistically significant effects on any of the tumour parameters evaluated, data not shown.

4 Chapter 4 Results for Study Two

4.1 Effects of Maternal diet on Pregnancy Outcome

Twenty one and sixteen dams were randomised to carrot and control powder respectively. Litter size, sex distribution and genotype of offspring were compared between dams across diet using ANOVA. All offspring that reached weaning age were included in the analysis. One hundred and fourteen offspring born to dams on carrot powder and 100 to control powder fed dams were weaned. Two wild type male offspring born to carrot powder fed dams and randomised to carrot powder diet post weaning, died a week after weaning. This was probably due to either struggle for food or ear punch sampling for genotype analysis.

Maternal diet had no significant effect on pregnancy outcome except total mean number of Min offspring born to dams fed control powder which was significantly (P = 0.015) higher compared to the number born to carrot powder fed dams. The mean number of males and females born to dams in each dietary group did not differ; control pellet fed dams (P = 0.827, males, n = 3.2 ± 0.38 SEM, females, n = 3.1 ± 0.42 SEM), carrot pellet fed dams (P = 0.256. males n = 3 ±0.41 SEM, females n = 2.4 ± 0.27 SEM) (Table 4.1)

Table 4. 1: Maternal diet and pregnancy outcome of dams

	Maternal diet		
	Carrot powder (n =21)	Control powder (n = 16)	P value
	Mean ± SEM	Mean ± SEM	
Litter size	5.4 ± 0.44	6.3 ± 0.51	0.231
Total male	3.0 ± 0.38	3.2 ± 0.44	0.748
Total female	2.4 ± 0.32	3.1 ± 0.36	0.198
Total Min	1.9 ± 0.31	3.1 ± 0.36	**0.015**
Total wild type	3.5 ± 0.31	3.1 ± 0.36	0.411
Min males	1.0 ± 0.24	1.7 ± 0.28	0.071
Min females	0.9 ± 0.24	1.4 ± 0.27	0.151
Wild type male	2.0 ± 0.30	1.5 ± 0.34	0.280
Wild type female	1.5 ± 0.22	1.6 ± 0.26	0.769

4.2 Animal Distribution on Study

Two hundred and fourteen (214) weaned pups were randomised onto the five dietary groups at weaning. Two wild type males from carrot powder fed dams and randomised to carrot powder post weaning died a week after weaning, reducing the number of wild type males in carrot powder-carrot powder to 15 from 17 (Table 4.3). The death was suspected to be due to genotyping. There was no significant difference in the distribution of animal on the study, (P = 0.53) (Table 4.2).

Table 4. 2: Dietary Groups of Experimental Animals stratified by Sex and Genotype (F= female; M = male).

Control powder – control powder				Control powder – carrot powder				Carrot powder – control powder				Carrot powder – carrot powder				Control powder – stored carrot pellet			
Wild type		*Apc*$^{Min/+}$		Wild type		*Apc*$^{Min/+}$		Wild type		*Apc*$^{Min/+}$		Wild type		*Apc*$^{Min/+}$		Wild type		*Apc*$^{Min/+}$	
M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F
6	10	12	4	6	11	9	13	25	22	8	9	17	10	13	11	12	5	6	5

4.3 Growth Data Analysis

Growth was followed to seven weeks of post weaning diet exposure after which animals were killed at different time points. As all mice had at least seven weeks of post weaning dietary exposure growth analysis was limited to seven weeks to remove effects of variations in number of days on diet. Effects of maternal diet, post weaning diet, sex and genotype were analysed in a factorial design using ANOVA (GLM). To determine the effects of sex and genotype on growth data for both sexes and genotypes were put together and genotype added as covariate looking at effects of sex whilst sex was added as covariate looking at effects of genotype. As sex and genotype significantly affected growth they were added as covariate to investigate effects of maternal diet and post weaning diet on growth. Weaning weight was subtracted from weekly weights for statistical analysis. Results are presented as Least Square Means (LSM). Error bars represent ± SEM. Factors for weaning weight analysis were maternal diet, sex and genotype with litter size as covariate.

4.3.1 Weaning weight

Mean weaning weight of offspring born to carrot powder fed dams was significantly lower by 7.73 % ($P < 0.001$) compared to that of offspring born to control powder fed dams. Females had 5.3 % ($P < 0.001$) lower mean weaning weight compared to the males. Probability effects of maternal diet interaction sex and sex interaction genotype were $P = 0.052$ and $P = 0.056$ respectively (Table 4.3). Weaning weight data was not uniformly distributed and was therefore transformed for P values.

Table 4. 3: Effects of Maternal diet, Sex and Genotype on weaning weights of mice

	Maternal diet			Sex			Genotype			Probability effects of main factor interactions			
	Control powder (n = 100)	Carrot powder (n = 114)	P value	Female (n = 100)	Male (n =114)	P value	*Apc*$^{Min/+}$ (n = 90)	Wild type (n = 124)	P value	Maternal diet*Sex	Maternal diet* Genotype	Sex * Genotype	Maternal diet*Sex* Genotype
	Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM					
Weaning weight	16.86 ± 0.214	14.44 ± 0.21	< 0.001	14.82 ± 0.213	16.48 ± 0.203	< 0.001	15.58 ± 0.223	15.73 ± 0.192	0.618	0.052	0.941	0.056	0.913

4.3.2 Effects of Sex and Genotype on Growth of Mice

Growth of mice was significantly influenced by sex and genotype. At seven weeks of post weaning diet exposure, males had as expected, higher ($P < 0.001$) mean weight gain post weaning compared to the females (Fig. 4.1) Wild type mice also had higher mean weight gain post weaning ($P = 0.002$) (Fig. 4.2) than Min mice. Interactions of sex maternal diet (sex * maternal diet) and sex post weaning diet (sex * post weaning diet) had no significant effects on growth.

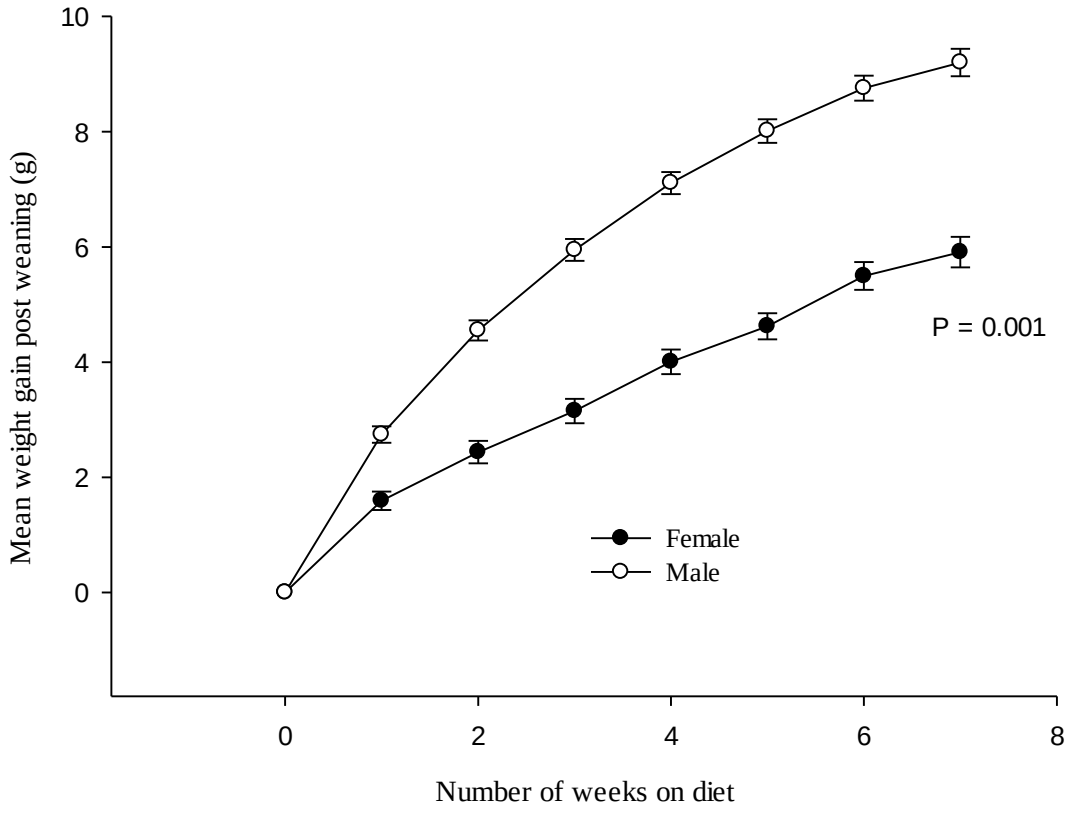


Figure 4. 1: Growth curves of mean weight gain post weaning for males and females from all dietary regimes. Weaning weight was subtracted from weekly weights for statistical analysis. Genotype was added as covariate looking at effects of sex on growth. (Females n = 100; males n = 112). Data were uniformly distributed and therefore not transformed. Error bars represent ± SEM.

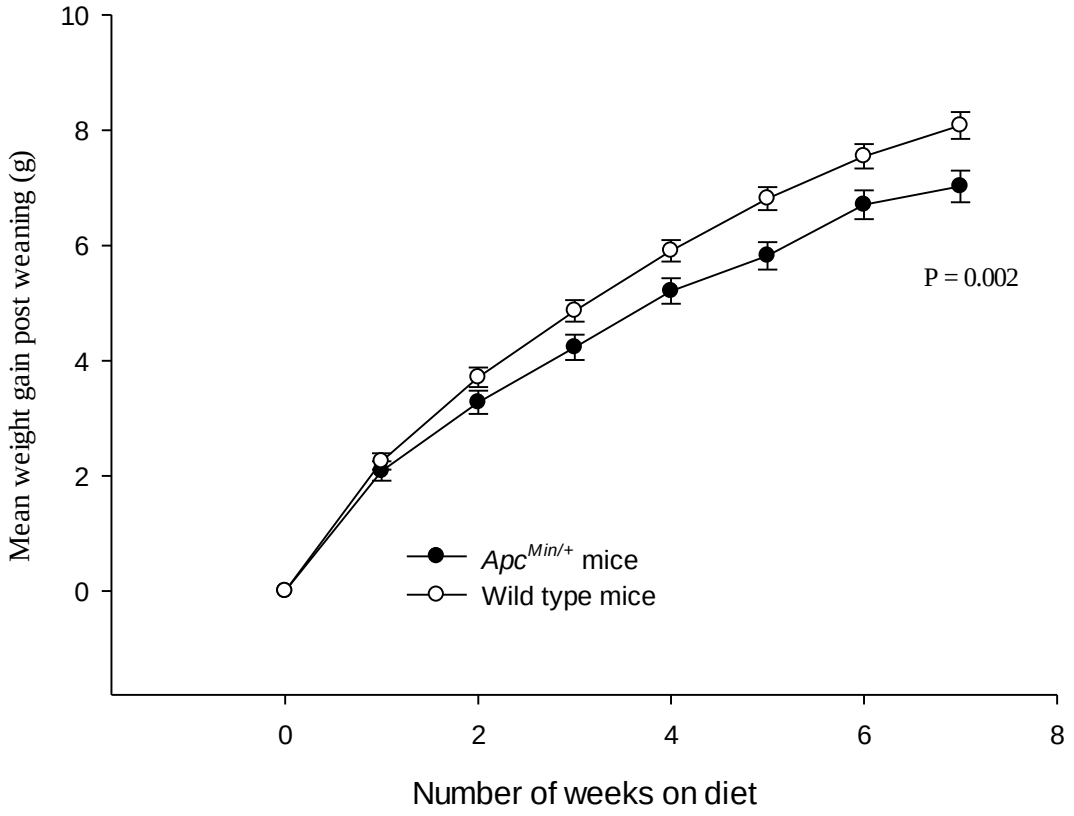


Figure 4. 2: Growth curves of mean weight gain post weaning for Min and wild type mice from all dietary regimes. Weaning weight was subtracted from weekly weights for statistical analysis Sex was added as covariate looking at effects of genotype on growth. (*Apc*$^{Min/+}$ mice n = 90; Wild type mice n = 122). Data were uniformly distributed and therefore not transformed Error bars represent ± SEM.

4.3.3 Effects of Maternal and Post weaning diet on Growth of Mice

As sex and genotype significantly influenced growth they were added as covariates looking at effects maternal and post weaning diets. Mice fed carrot pellet post weaning were born to dams fed control powder they were excluded to investigate effect of maternal diet on growth.

Maternal diet significantly influenced mean weight gain post weaning by offspring. Mice born to carrot powder fed dams were 9 % (P = 0.011) heavier compared to those from control powder fed dams (Fig. 4.3).

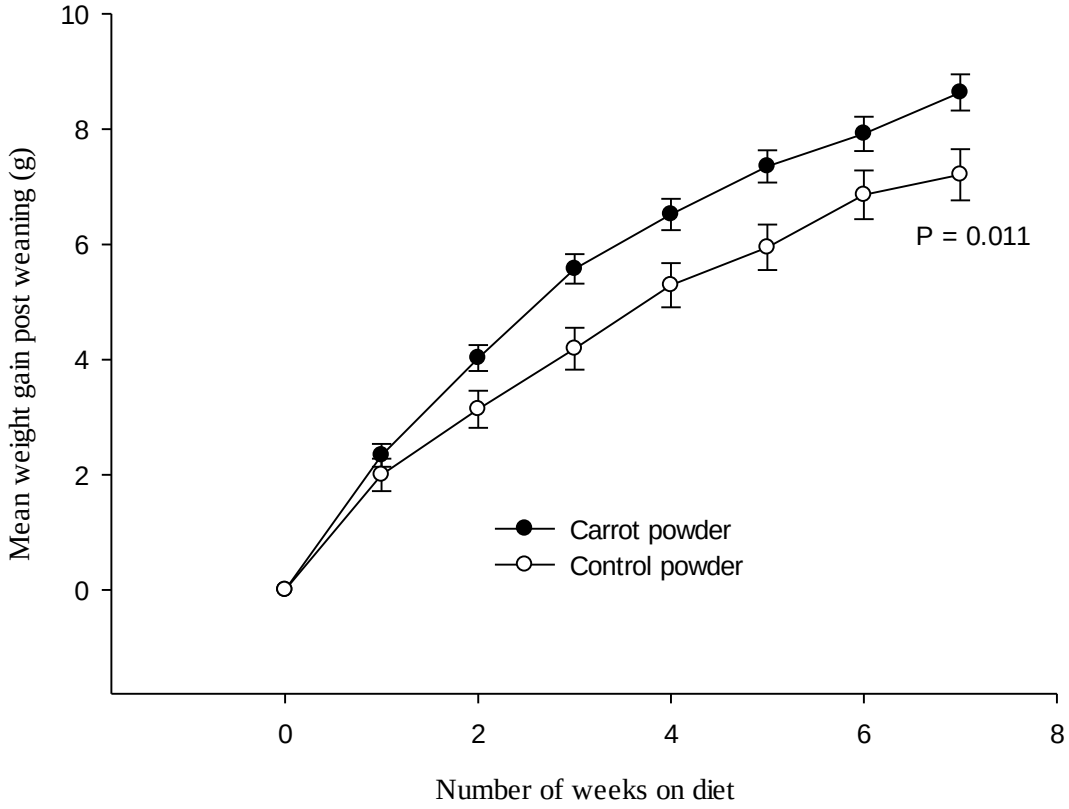


Figure 4. 3: Growth curves of mean weight gain post weaning for mice exposed to maternal carrot powder diet and fed control powder post weaning. Weaning weight was subtracted from weekly weights for statistical analysis Means were calculated as Least Square Means (Control powder n = 32; carrot powder n = 63) Error bars represent ± SEM. Data were uniformly distributed and therefore not transformed.

Feeding mice carrot powder post weaning had no significant effect on mean weight gain post weaning compared to control powder (P = 0.187) at death. (Fig. 4.4)

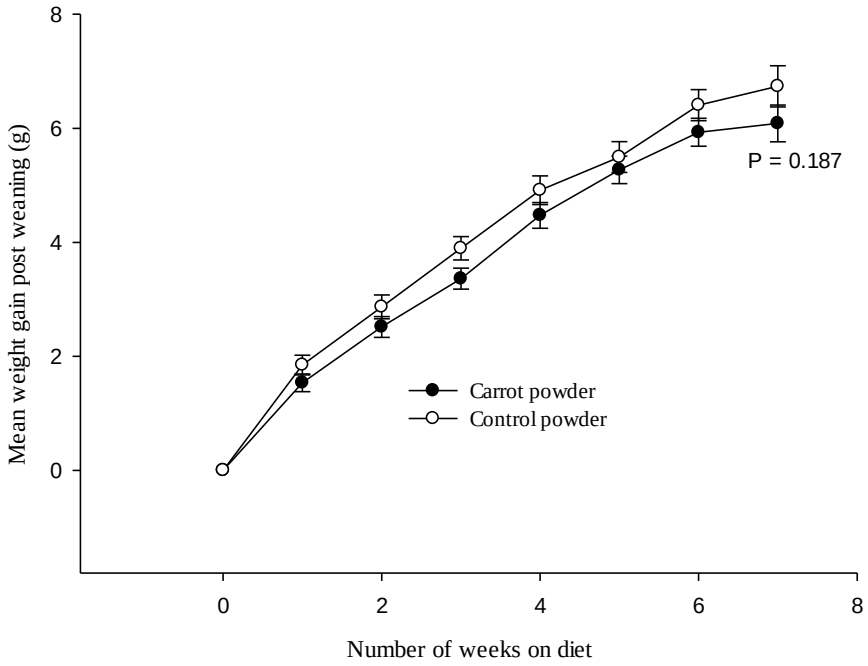


Figure 4. 4: Growth curves of mean weight gain post weaning for mice fed carrot powder post weaning but born to dams fed control powder. AS sex and genotype had significant effects on growth they were added as covariate.in evaluating effects of post weaning diet Weaning weight was subtracted from weekly weights for statistical analysis. Means were calculated as LSM (Control powder n = 32, carrot powder n = 40). Error bars represent ± SEM. Data was Box Cox transformed for P value.

Stored carrot pellet consumption post weaning by mice had no statistically significant effect on mean weight gain post weaning at death (Fig. 4.5).

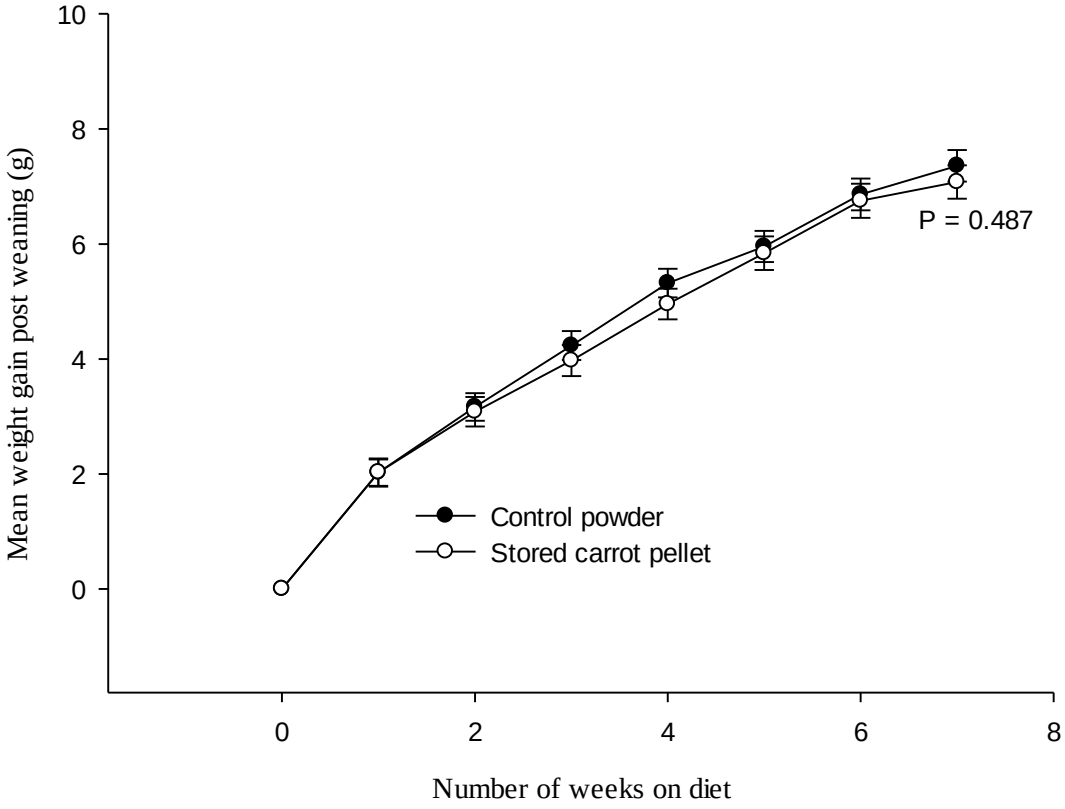


Figure 4. 5: Growth curves of mean weight gain post weaning of mice fed carrot pellet post weaning. These mice were born to dams fed control powder. As sex and genotype had significant effects on growth they were added as covariate.in evaluating effects of post weaning diet. Weaning weight was subtracted from weekly weights for statistical analysis. Means were calculated as LSM (Control powder n = 32, stored carrot powder n = 28) Error bars represent ± SEM. Data was not transformed.

As all mice fed stored carrot pellet diet post weaning were born to control pellet fed dams, they were excluded in order to investigate effects of maternal and post weaning diet interactions. There were no statistically significant (P = 0.187) difference in mean weight gain post weaning by mice from different post weaning dietary regimes (Fig. 4.6).

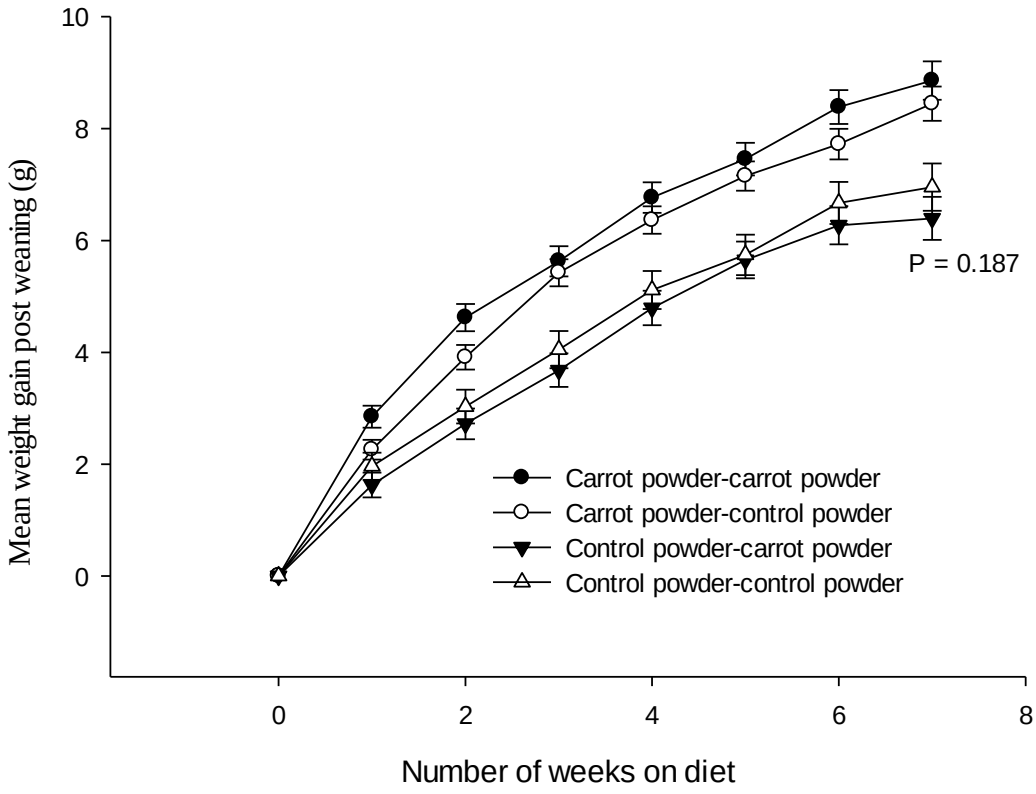


Figure 4. 6: Growth curves of mean weight gain post weaning for mice exposed to different dietary regimes excluding mice fed stored carrot pellet post weaning. As sex and genotype had significant effects on growth they were added as covariate.in evaluating effects of treatment diets on weight gain post weaning. Weaning weight was subtracted from the weekly weights for statistical analysis. Means were calculated as LSM. (Carrot powder-carrot powder n = 49; carrot powder-control powder n = 63; control powder-carrot powder n = 40; control powder-control powder n = 32) Error bars represent SEM. Data was not transformed.

4.4 Effects of Maternal and Post weaning diets, Sex and Genotype on Body weight and Internal Organ dimensions of mice at death

The effects were determined in a factorial design using ANOVA GLM with diet (maternal diet-post weaning diet), sex and genotype as main factors. Maternal diet and post weaning diet were treated as diet dietary regime because dams were not fed stored carrot pellet and all mice fed stored carrot pellet were born to dams fed control powder As mice were killed at different time points, days on diet was added as covariate looking at effects of the factors and their interactions. Means were calculated as LSM ± SEM. Body, full SI, colon, caecum, liver, right kidney, left kidney and spleen weights and colon length were Box Cox transformed for P values as data were not uniformly distributed. Results are presented in Tables 4.4, 4.5 and 4.6.

4.4.1 Effects of Sex and Genotype on Body weight and Organ Dimensions of Mice at death

As expected males were (26.9 g ± 0.168 SEM) ($P < 0.001$) heavier compared to the females (21.5 g ± 0.184 SEM) at the time of death. With the exception of the spleen ($P = 0.911$) all organs measured were significantly heavier in the males than the female counterparts. SI and colon were longer in males ($P < 0.001$) than in females at death (Table 4.4).

Wild type mice (25.1 g ± 0.166 SEM) at death were heavier than Min mice (23.3 g ± 0.189 SEM) ($P < 0.001$). Also heavier in the wild types were, the right ($P < 0.005$) and left ($P < 0.001$) kidneys and the liver ($P < 0.001$). Weights of the full colon, colon, terminal SI ($P < 0.001$), and spleen ($P < 0.001$) were higher in Min mice compared to wild type mice. SI and colon were longer in Wild types ($P < 0.001$) than in the Min mice (Table 4.4).

Full SI weight was significantly affected by genotype-sex interaction

Table 4. 4: Effects of sex and genotype on body and organ dimensions of mice at death

	Genotype			Sex			Genotype*Sex
	ApcMin/+ (n = 90)	Wild type (n = 122)	P value	Female (n = 100)	Male (n= 112)	P value	
	Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM		
Body weight (g)	23.29 ± 0.189	25.09 ± 0.166	**< 0.001**	21.46 ± 0.184	26.92 ± 0.168	< 0.001	**0.08**
Full SI weight (g)	1.54 ± 0.014	1.51 ± 0.012	0.476	1.50 ± 0.013	1.55 ± 0.012	0.007	**0.024**
Full colon weight (g)	0.47 ± 0.011	0.43 ± 0.009	**0.004**	0.43 ± 0.010	0.47 ± 0.009	0.003	0.495
Proximal SI weight (g)	0.66 ± 0.009	0.65 ± 0.008	0.666	0.63 ± 0.009	0.68 ± 0.008	< 0.001	0.669
Terminal SI weight (g)	0.58 ± 0.009	0.51 ± 0.008	**< 0.001**	0.53 ± 0.009	0.56 ± 0.008	0.008	0.700
SI length (cm)	28.96 ± 0.306	31.97 ± 0.269	**< 0.001**	29.60 ± 0.298	31.32 ± 0.272	< 0.001	0.624
Colon weight (g)	0.26 ± 0.006	0.22 ± 0.005	**< 0.001**	0.23 ± 0.006	0.25 ± 0.005	0.002	0.084
Colon length (cm)	6.17 ± 0.081	6.8 ± 0.071	**< 0.001**	6.29 ± 0.079	6.68 ± 0.072	< 0.001	0.920
Caecum weight (g)	0.61 ± 0.012	0.62 ± 0.010	0.594	0.54 ± 0.011	0.69 ± 0.010	< 0.001	0.876
Liver weight (g)	1.27 ± 0.020	1.35 ± 0.017	**< 0.001**	1.13 ± 0.019	1.49 ± 0.018	< 0.001	0.331
Right kidney (g)	0.17 ± 0.003	0.18 ± 0.003	**0.005**	0.15 ± 0.003	0.2 ± 0.003	< 0.001	0.999
Left kidney (g)	0.16 ± 0.002	0.18 ± 0.002	**< 0.001**	0.15 ± 0.002	0.19 ± 0.002	< 0.001	0.082
Spleen weight (g)	0.19 ± 0.007	0.1 ± 0.006	**< 0.001**	0.14 ± 0.007	0.14 ± 0.006	0.612	0.394
Heart weight (g)	0.2 ± 0.005	0.21 ± 0.004	0.086	0.18 ± 0.005	0.23 ± 0.004	< 0.001	0.114

4.4.2 Effects of Maternal and Post weaning dietary group on Body Weight and Organ Dimensions of Mice at Death

The dietary groups to which the mice were exposed had significant effect on mean body weight ($P = 0.003$) at the time of death (Table.4.5.). Mean body weights ranged from 23.7 g ± 0.233 SEM to 24.9 g ± 0.314 SEM for carrot powder-carrot powder and control powder-control powder respectively. The first diet of each dietary regime refers to maternal diet and the second to post weaning diet. Except the weights of the liver, right kidney and spleen, parameters of all internal organs evaluated were significantly affected by the dietary regimes with P values ranging between 0.001 and 0.037 (Table 4.5). The weights of the proximal and terminal SI varied from 0.6 g ± 0.011 SEM and 0.5 g ± 0.012 SEM to 0.9 g ± 0.013 SEM and 0.6 g ± 0.013 SEM ($P = 0.006$ and $P = 0.037$) respectively for carrot powder-control powder to control powder-carrot powder. The SI ($P = 0.004$) and colon ($P < 0.001$) lengths were also affected by the dietary regimes. The SI was longer in mice fed carrot powder-control powder compared to the other dietary groups while colon length was higher in those fed control powder-carrot powder (6.8 cm ± 0.115 SEM) and control powder carrot pellet (6.8 cm ± 0.141 SEM). Exposing mice to control powder-carrot pellet and control powder carrot powder dietary regimes significantly increased their caecum weights compared to other dietary regimes. Feeding mice carrot powder-control powder produced no change in weight of heart compared to those fed control dietary regime whiles weight of the heart was the same in those fed carrot powder-carrot powder and control powder-carrot powder. (Table 4.5). With exceptions of SI and right kidney, it could be suggested that the weights of organs measured and the length of the colon at time of death were determined by the post weaning diet of a given dietary regime.

No statistically significant effect of interactions between dietary regimes and sex was observed for any of the measured organs and body except for the liver ($P = 0.005$). Diet and genotype interaction significantly affected the mean weights of full SI, proximal and terminal SI, full colon, colon and the left kidney. Diet, sex and genotype interaction also affected the mean weights of body, full colon, colon, proximal SI weights and mean colon length (Table 4.6.)

Table 4. 5: Effects of maternal and post weaning dietary groups and their interactions with sex and genotype on body and organ dimensions of mice at death

	Dietary Groups					
	Carrot powder - carrot powder (n = 49)	Carrot powder - control powder (N = 63)	Control powder – carrot powder (n = 40)	Control powder - control powder (n = 32)	Control powder - carrot pellet (n = 28)	P value
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM	
Body weight (g)	23.65 ± 0.233^{b}	24.61 ± 0.235^{a}	23.97 ± 0.268^{ab}	24.89 ± 0.314^{a}	23.82 ± 0.329^{ab}	**0.003**
Full SI weight (g)	1.58 ± 0.017^{a}	1.44 ± 0.017^{b}	1.59 ± 0.019^{a}	1.45 ± 0.023^{b}	1.58 ± 0.024^{a}	**< 0.001**
Full colon weight (g)	0.44 ± 0.013^{b}	0.42 ± 0.013^{b}	0.47 ± 0.015^{b}	0.41 ± 0.018^{b}	0.53 ± 0.018^{a}	**< 0.001**
Proximal SI weight (g)	0.67 ± 0.011^{a}	0.63 ± 0.011^{b}	0.68 ± 0.013^{a}	0.64 ± 0.015^{ab}	0.66 ± 0.016^{ab}	**0.006**
Terminal SI weight (g)	0.56 ± 0.012^{ab}	0.52 ± 0.012^{b}	0.57 ± 0.013^{a}	0.54 ± 0.016^{ab}	0.55 ± 0.016^{ab}	**0.037**
SI length (cm)	30.50 ± 0.377^{ab}	31.15 ± 0.381^{a}	30.86 ± 0.433^{a}	28.79 ± 0.509^{b}	31.00 ± 0.532^{a}	**0.004**
Colon weight (g)	0.25 ± 0.007^{b}	0.22 ± 0.007^{a}	0.24 ± 0.008^{b}	0.23 ± 0.01^{ab}	0.25 ± 0.010^{b}	**< 0.001**
Colon length (cm)	6.43 ± 0.100^{ab}	6.17 ± 0.101^{b}	6.82 ± 0.115^{a}	6.23 ± 0.135^{b}	6.78 ± 0.141^{a}	**< 0.001**
Caecum weight (g)	0.64 ± 0.014^{b}	0.56 ± 0.014^{a}	0.67 ± 0.016^{b}	0.54 ± 0.019^{a}	0.68 ± 0.02^{b}	**< 0.001**
Liver weight (g)	1.31 ± 0.024	1.33 ± 0.025	1.3 ± 0.028	1.32 ± 0.033	1.29 ± 0.035	0.865
Right kidney (g)	0.16 ± 0.004	0.18 ± 0.004	0.18 ± 0.005	0.18 ± 0.006	0.17 ± 0.006	0.085
Left kidney (g)	0.17 ± 0.003^{b}	0.18 ± 0.003^{a}	0.16 ± 0.003^{b}	0.18 ± 0.003^{a}	0.16 ± 0.004^{b}	**< 0.001**
Spleen weight (g)	0.14 ± 0.008	0.15 ± 0.008	0.12 ± 0.01	0.16 ± 0.011	0.13 ± 0.012	0.153
Heart weight (g)	0.2 ± 0.006^{b}	0.22 ± 0.006^{ab}	0.2 ± 0.007^{ab}	0.22 ± 0.008^{a}	0.19 ± 0.009^{b}	**0.007b**

The first of each dietary group refers to maternal diet and the second to post weaning diet

Table 4. 6: Probability effects of interactions of Diet (Maternal-Post weaning), Sex and Genotype on Body and organ Dimensions Study

	Diet* Genotype	Diet* Sex	Diet * Sex * Genotype
Body weight (g)	0.087	0.295	**0.042**
Full SI weight (g)	< **0.001**	0.474	0.862
Full colon weight (g)	**0.004**	0.510	**0.013**
Proximal SI weight (g)	**0.025**	0.425	**0.12**
Terminal SI weight (g)	**0.033**	0.463	0.461
SI length (cm)	0.333	0.822	0.538
Colon weight (g)	**0.014**	0.633	< **0.001**
Colon length (cm)	0.789	0.980	**0.049**
Caecum weight (g)	0.257	0.292	0.446
Liver weight (g)	0.808	**0.005**	0.705
Right kidney weight (g)	0.117	0.210	0.509
Left kidney weight (g)	**0.051**	0.062	0.37
Spleen weight (g)	0.080	0.543	0.218
Heart weight (g)	0.240	0.084	0.137

4.5 Tumour Data Analysis

Tumour number in the three sections of the intestine of the Min mice was compared in One-Way ANOVA Tumour data (number and volume) was analysed as a factorial design using ANOVA, GLM with maternal diet, post weaning diet and sex as main factors alongside their interactions. As mice were killed at different time points, days on diet was added as covariate to control for the effects of age differences on tumour number and volume. Data from proximal SI, terminal SI, colon and total gut were Box Cox transformed for P values. Data analysis was done in two forms. The first analysis included data from stored carrot pellet diet fed to offspring post weaning. Results of main factor effects for this analysis are presented in figure 4.8 – 4.13. Effects of significant interactions (post weaning diet and sex) for terminal SI and total gut tumour number are shown in figures 4.14 and 4.15 respectively. No significant interaction was observed for maternal diet interactions with sex, data not presented. Maternal diet post weaning diet interactions could not be estimated because mothers were not fed stored carrot pellet.

The second part of tumour data analysis evaluated effects of carrot powder. As all mice fed stored carrot pellet post weaning were born to dams fed control powder, they were removed from the analysis to investigate effects of maternal and post weaning diets interactions. Results of significant main effects and interactions are presented in 4.16 to 4.20 and Table 4.6 respectively.

4.5.1 Tumour Distribution in Intestine of Min mice

Most tumours were found in the terminal SI (22.7 ± 0.848 SEM) ($P < 0.001$) compared to proximal SI (4.4 ± 0.849 SEM) and colon (2.3 ± 0.848 SEM) at time of death (Fig. 4.7).

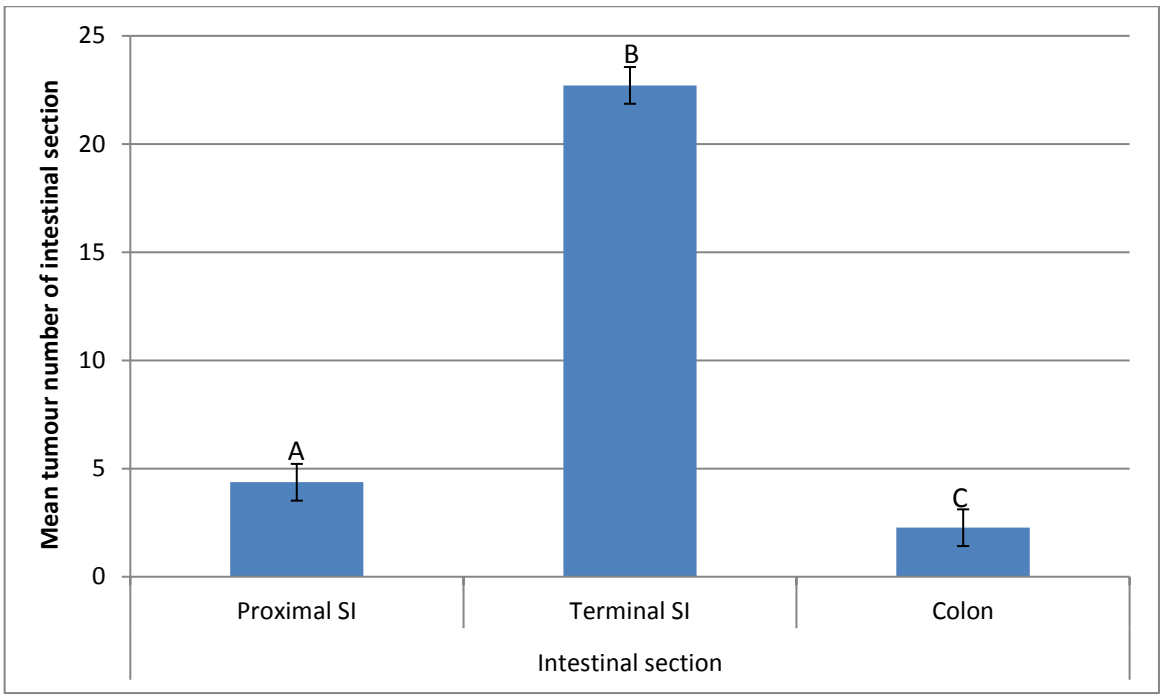


Figure 4. 7: Tumour distributions in intestine of min mice. (n = 90) Days on diet was added as a covariate. Error bars represent SEM**.** Means with different letters are significantly different, $P < 0.001$.

4.5.2 *Effects of Sex and Diet on Tumour Number and Volume in Min mice*

Tumour data (number and volume) was analysed as a factorial design using ANOVA, GLM with maternal diet, post weaning diet and sex as main factors alongside their interactions. As mice were killed at different time points days on diet was added as covariate to control for the effects of age difference on tumour number and volume. Data from proximal SI, terminal SI, colon and total gut were Box Cox transformed for P values. Data analysis was done in two forms. The first analysis included data from carrot pellet diet fed to offspring post weaning. Results of main factor effects for this analysis are presented in figure 4.8 – 4.13. Effects of significant interactions (post weaning diet and sex) for terminal SI and total gut tumour number are shown in figures 4.14 and 4.15 respectively. No significant interaction was observed for maternal diet interactions with sex, data not presented. Maternal diet post weaning diet interactions could not be estimated because mothers were not fed stored carrot pellet.

The second part of tumour data analysis evaluated effects of carrot powder. As all mice fed carrot pellet post weaning were born to dams fed control powder, they were removed from the analysis to investigate effects of all interactions. Significant main effects and interactions are presented in 4.16 to 4.20 and Table 4.6 respectively.

4.5.3 Effects of Sex on Tumour Number and Volume

There was no significant difference in tumour number and volume between male and female mice (Figs 4.8 and 4.9) except in the colon where female had significantly reduced tumour number compared to males (P = 0.033) (Fig. 4.8 C).

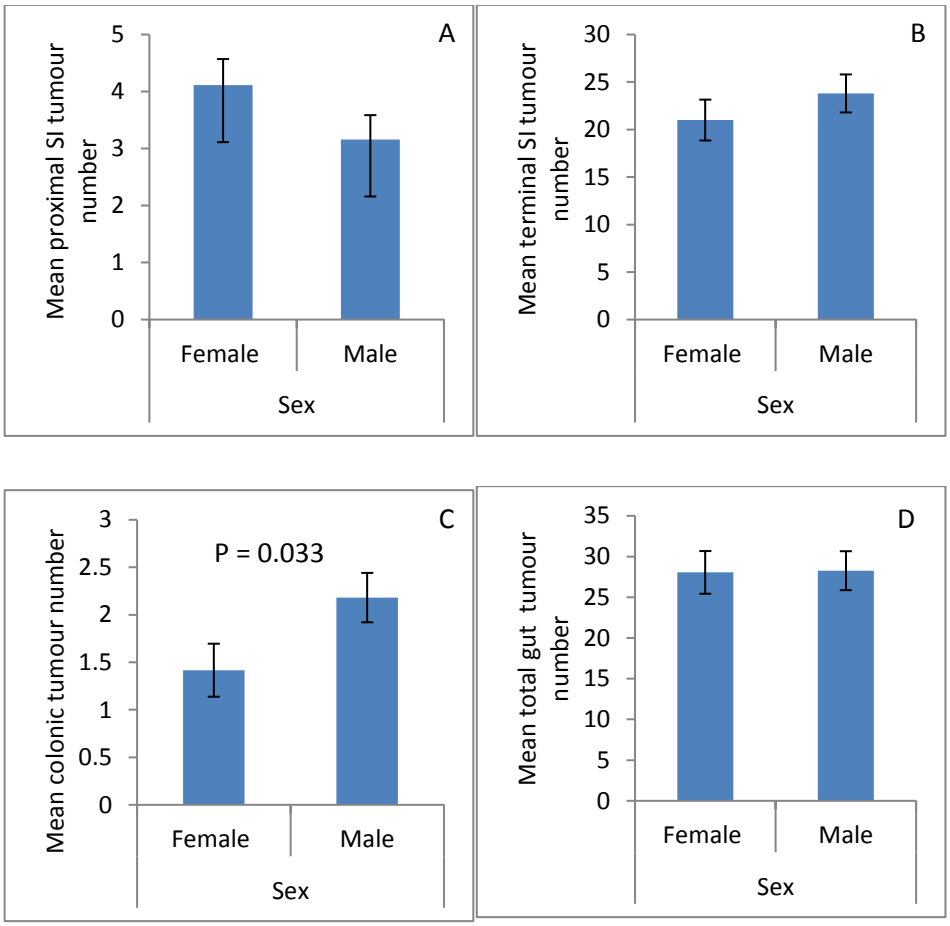


Figure 4. 8: Effects of sex on tumour number in (A) proximal SI (B) terminal SI (C) colon and (D) total gut of min mice (Male n = 48, female n = 42) Days on diet was added as covariate. Error bars represent SEM.

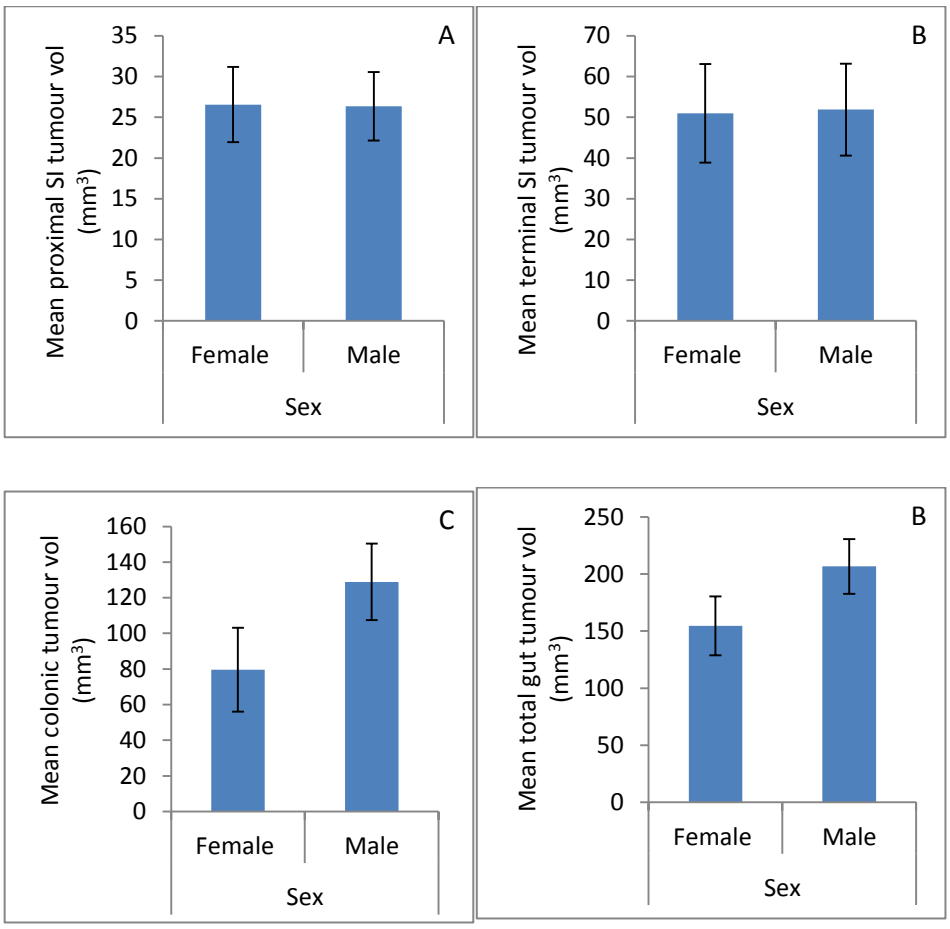


Figure 4. 9: Effects of sex on tumour volume in (A) proximal SI (B) terminal SI (C) colon and (D) total gut of min mice (Male n = 48, female n = 42) Error bars represent SEM. Days on diet was added as covariate.

4.5.4 Effects Maternal diet on Tumour Number and Volume

Maternal diet type had no significant effect on intestinal tumour number and volume in adult offspring at death (Fig. 4.10 and 4.11).

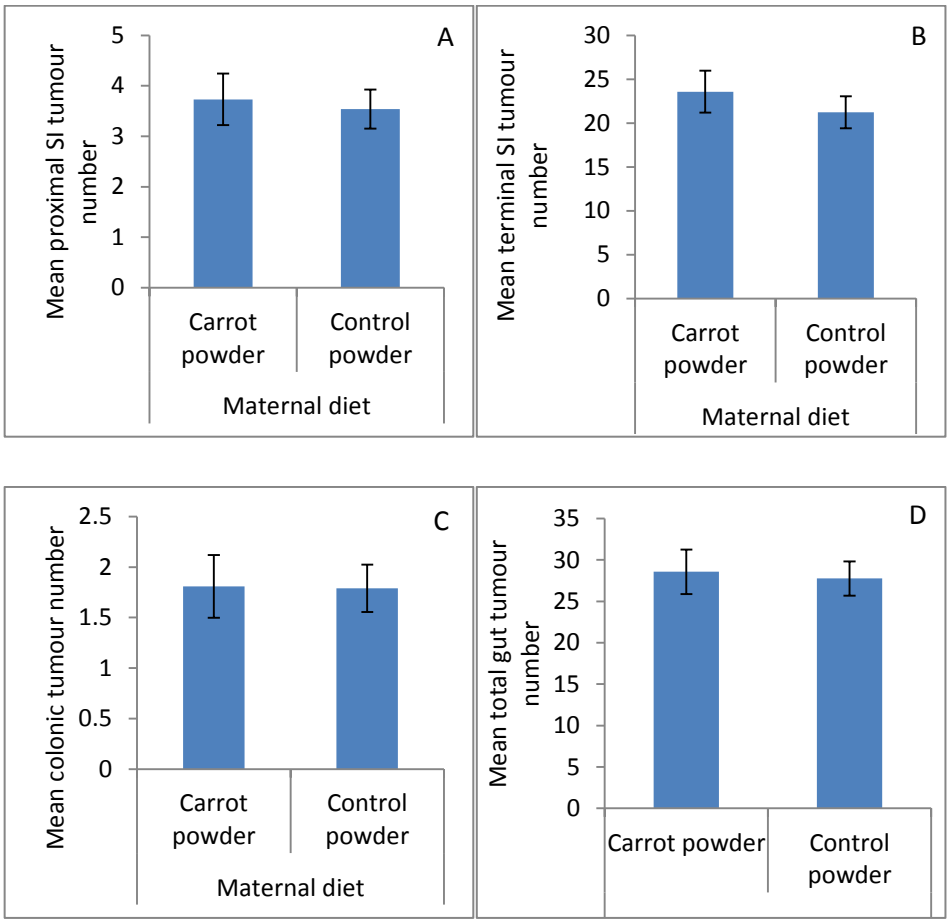


Figure 4. 10: Effects of maternal diet on tumour number in (A) proximal SI (B) terminal SI (C) colon (D) total gut of min mice (Control powder n = 50, carrot powder n = 40) Error bars represent standard error of means (SEM). Days on diet was added as covariate.

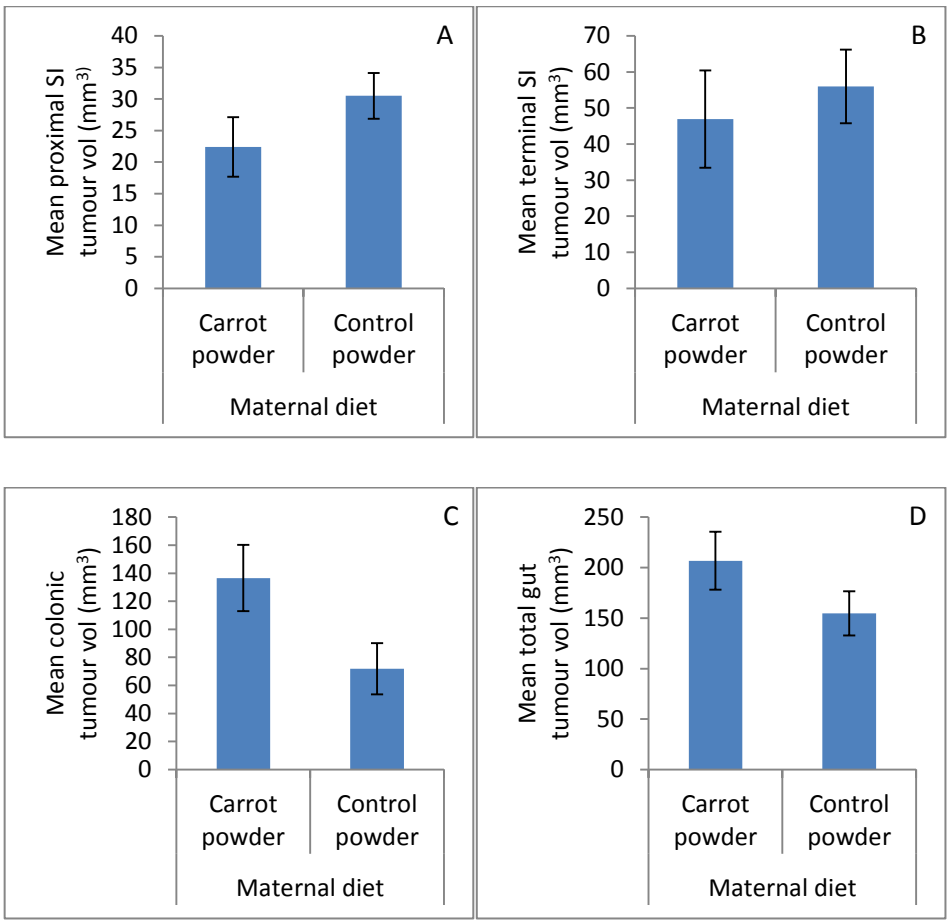


Figure 4. 11: Effects of maternal diet on tumour volume in (A) proximal SI (B) terminal SI (C) colon and (D) total gut of min mice (Control powder n = 50, carrot powder n = 40) Error bars represent standard error of means (SEM) Days on diet was added as covariate.

4.5.5 Effects of Post weaning diet on Tumour Number and Volume

Feeding carrot powder and stored carrot pellet from weaning had no significant effect on tumour number (Fig. 4.12) or volume (Fig. 4.13)

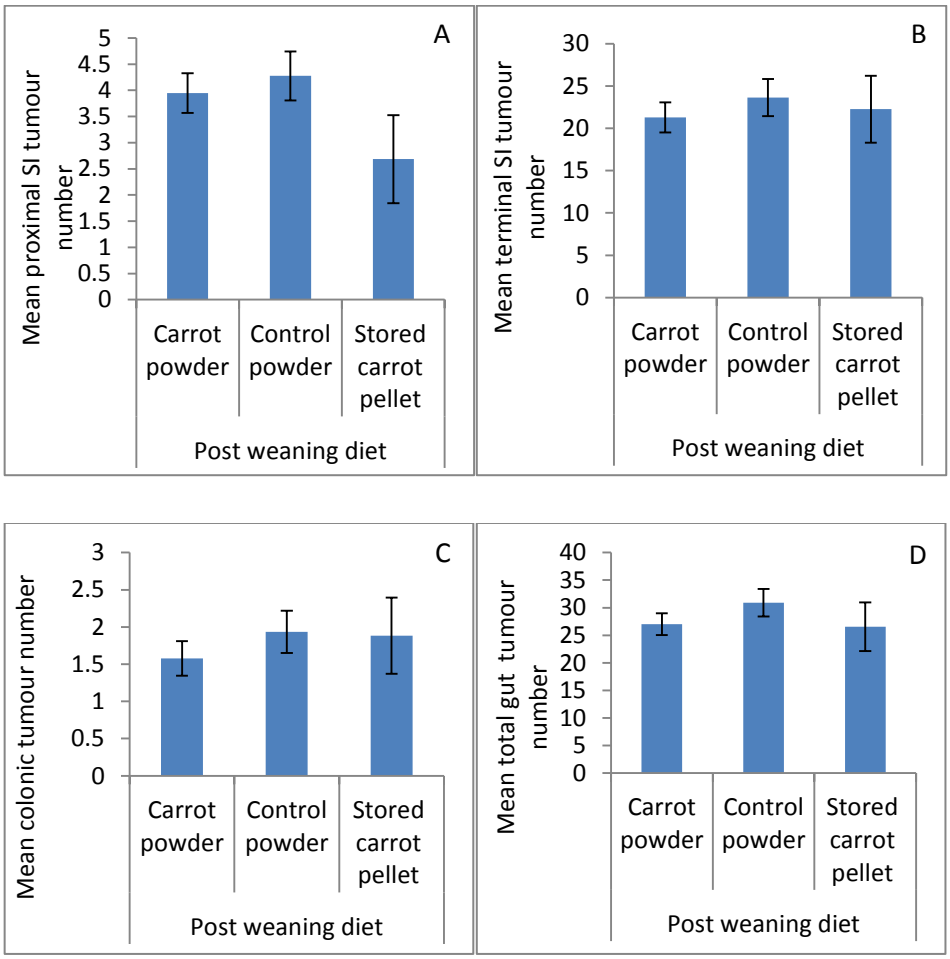


Figure 4. 12: Effects of post weaning diets on tumour number in (A) proximal SI (B) terminal SI (C) colon (D) total gut of min mice (Control powder n = 32, carrot powder n = 47, stored carrot pellet n = 11). Error bars represent standard error of means (SEM) Days on diet was added as covariate.

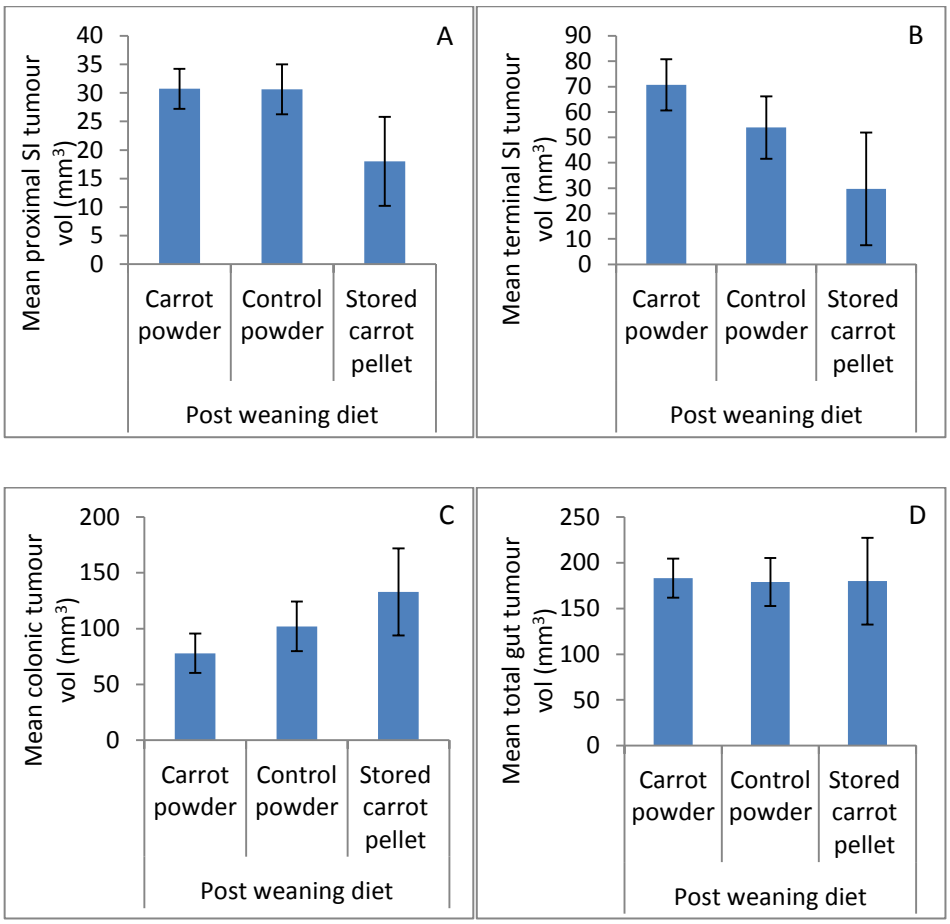


Figure 4. 13: Effects of post weaning diets on tumour volume in (A) proximal SI (B) terminal SI (C) colon (D) total gut of min mice (Control powder n = 32, carrot powder n = 47, stored carrot pellet n = 11) Error bars represent standard error of means (SEM) Days on diet was added as covariate.

There was a statistically significant interaction between post weaning diet and sex for tumour number in terminal small intestine (P = 0.044). Carrot powder diet consumption post weaning by female mice, reduced tumour number in terminal small intestine significantly (P = 0.027) compared to control powder (Fig. 4.14). No significant (P = 0.375) effect was observed in male mice (data not shown).

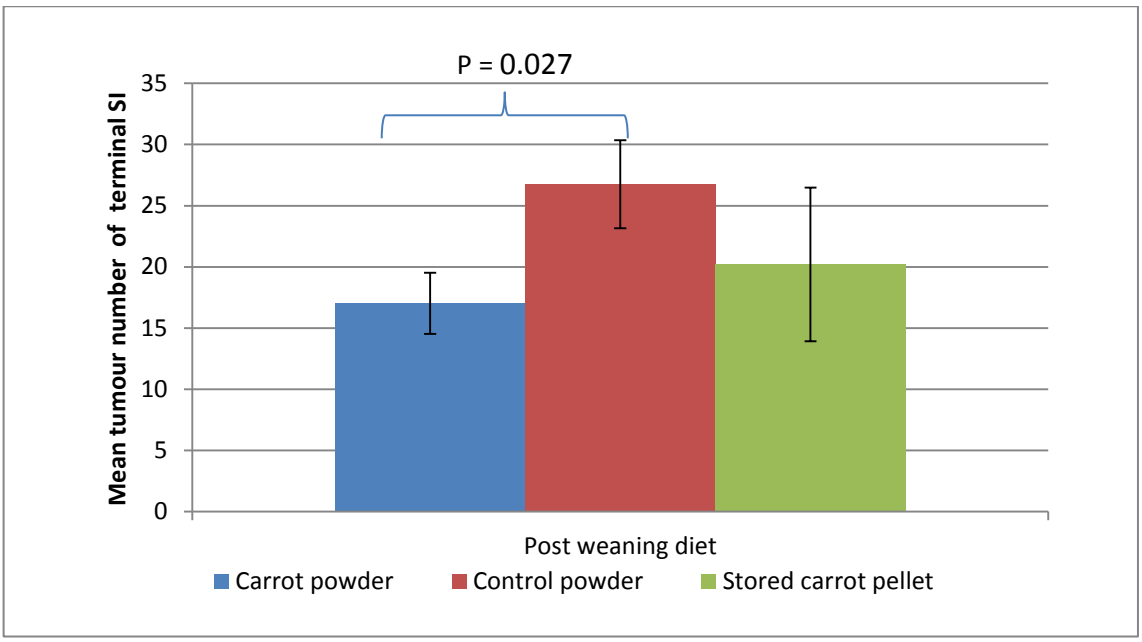


Figure 4. 14: Effects of post weaning diet on terminal intestinal tumour number in female Min mice (Female: carrot powder n = 25, control powder n = 12, stored carrot pellet n = 5) Error bars represent SEM. Bars that do share letter are significant P = 0.027

Also there was an interaction (P = 0.054) between post weaning diet and sex with total gut tumour number. Though the interaction was near significance a further probe reveals that in female mice, consumption of carrot powder diet post weaning decreased significantly (P = 0.039) mean total gut tumour number (Fig. 4.15). No significant effects of post weaning diets were observed in the males (Data not shown). The effects of post weaning diet on total gut tumour in the female mice appeared to be drawn from the dietary effect in the terminal SI.

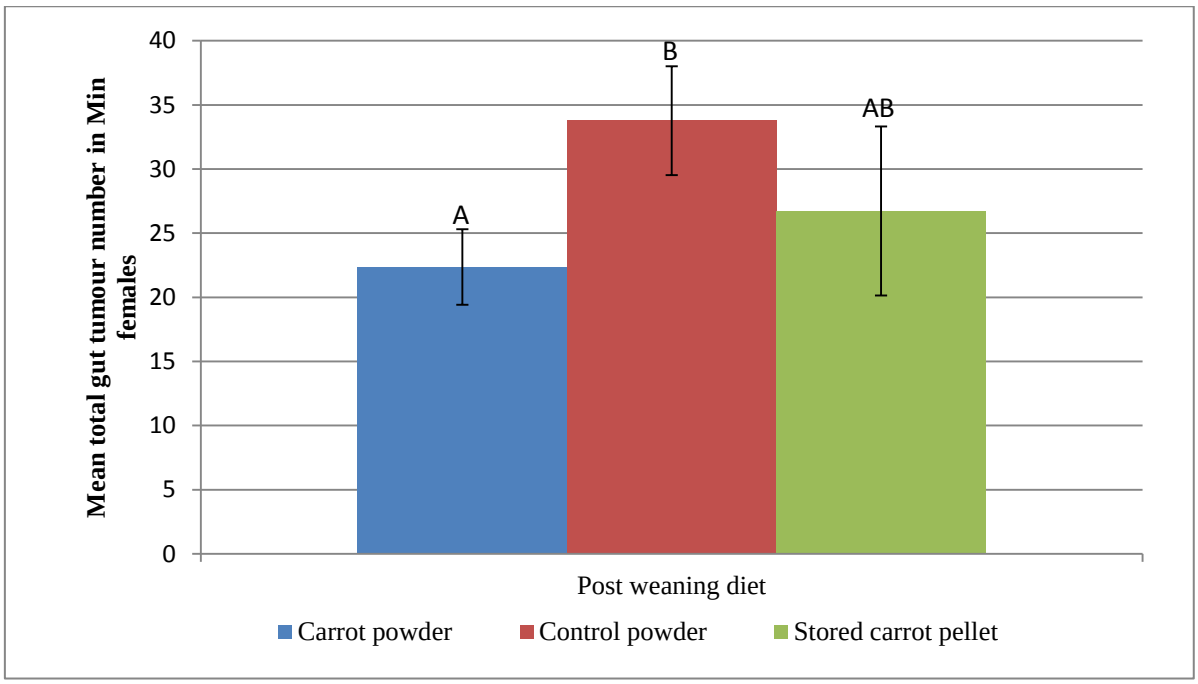


Figure 4. 15: Effects of post weaning diet on total gut tumour number in female mice at death (Female: Carrot powder n = 25, control powder n = 12, stored carrot pellet n = 5) Error bars represent SEM. Bars that do not share letter are significantly different. P = 0.039

4.5.6 Effects of Carrot powder on Tumour Number

As all mice fed the stored carrot pellet diet post weaning were born to control powder fed dams, these mice were removed to investigate carrot powder and interaction between maternal and post weaning diets.

Consumption of carrot powder post weaning decreased terminal small intestinal tumour number (P = 0.046) (Fig. 4.16) in Min mice and total gut tumour number (P = 0.044) (Fig. 4.17).

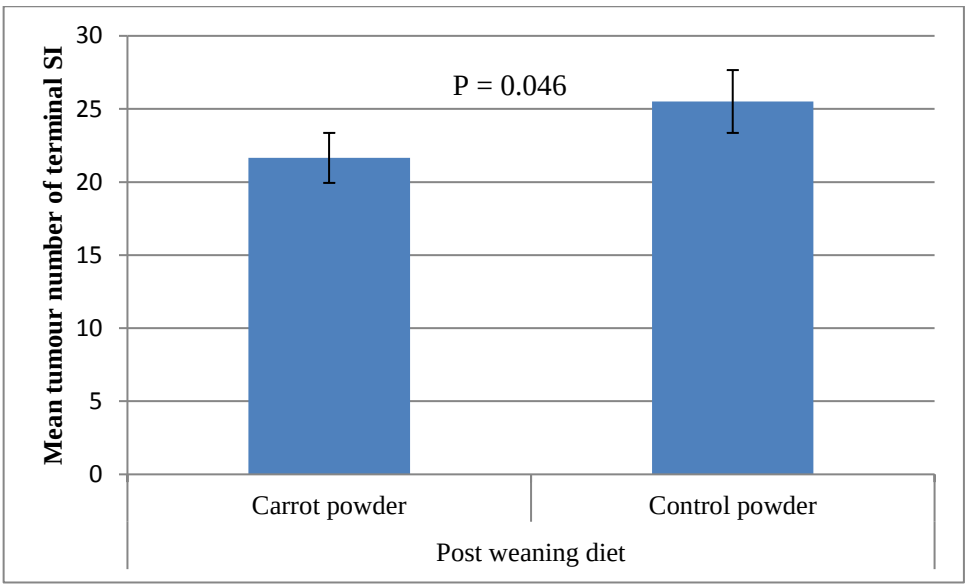


Figure 4. 16:. Effects of post weaning diet on terminal Si tumour number (Carrot n = 47, control powder n = 32) Error bars represent SEM

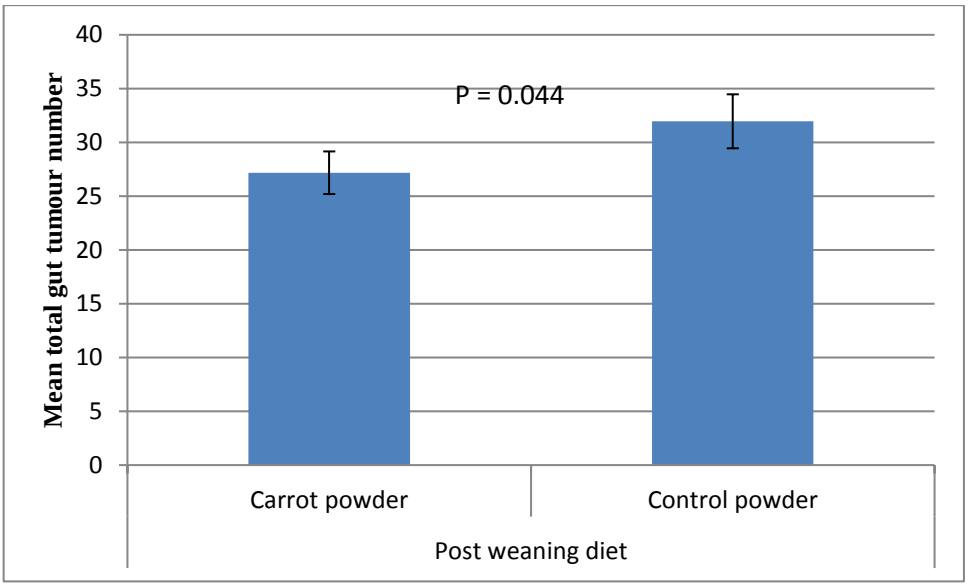


Figure 4. 17: Effects of post weaning diet on total gut tumour number using sex as covariate (Carrot powder n = 47, control powder n = 32) Error bars represent SEM

4.5.7 Effects of Interactions of Maternal and Post weaning diet and Sex on Tumour Number and Volume

There was a significant interaction between maternal and post weaning diets (P = 0.017) for colonic tumour number (Table 4.6). Offspring born to control powder fed dams and then randomised to carrot powder post weaning had fewer tumours in the colon than offspring from control powder dietary regime (Fig 4.18). A significant interaction between post weaning diet

and sex (P = 0.007) for terminal SI tumour number was observed. Female offspring fed carrot powder post weaning had reduced tumour number in terminal SI compared to control powder fed females (Table 4.6, Fig 4.19).

A significant interaction between post weaning diet and sex (P = 0.01) was also observed for the total gut tumour number. Again, females fed carrot powder post weaning had significantly lower total gut tumour number compared to control powder fed females. (Table 4.6 Fig 4.20).

Table 4. 7: Effects of interactions of sex, maternal and post weaning diets on intestinal tumour number and volume in min mice. (Diet: CP = control powder, CPP = carrot powder; Sex: Fem = female) (Control powder-control powder n = 16, carrot powder-carrot powder n = 24, carrot powder-control powder n = 16, control powder carrot powder n = 23) Days on diet was added as covariate

	Maternal diet * Post weaning diet					Maternal diet * Sex					Post weaning diet * Sex				
	CP-CP (n = 16)	CP-CPP (n=23)	CPP-CP (n=16)	CPP-CPP (n=24)	P value	CP-Fem (n=13)	CP-Male (n=14)	CPP-Fem (n=13)	CPP-male (n=11)	P value	CP-Fem (n=11)	CP-Male (n=11)	CPP-Fem (n=15)	CPP-Male (n=14)	P value
	Mean					Mean					Mean				
Proximal SI tumour number	4.13	4.12	4.69	3.81	0.644	4.77	3.48	4.87	3.64	0.979	5.41	3.41	4.22	3.71	0.315
Terminal SI tumour number	28.20	19.40	22.81	23.91	0.175	24.61	22.99	21.74	24.98	0.335	28.91	22.11	17.44	25.86	**0.007**
Colonic tumour number	2.69	1.23	1.38	1.87	**0.017**	1.83	2.08	1.14	2.11	0.331	1.87	2.19	1.10	2.00	0.524
Total gut tumour number	35.10	24.79	28.82	29.56	0.172	31.26	28.63	27.76	30.62	0.351	36.29	27.63	22.73	31.62	**0.010**
Proximal SI tumour volume (mm^3)	35.30	34.37	25.94	27.11	0.784	36.34	33.33	28.54	24.52	0.97	31.46	29.79	33.42	28.06	0.843
Terminal SI tumour volume (mm^3)	59.70	82.10	54.40	63.00	0.876	70.00	71.80	61.80	55.60	0.951	67.80	46.30	64.00	81.00	0.139
Colonic tumour volume (mm^3)	93.40	34.30	116.70	122.00	0.079	69.20	58.50	60.20	178.50	0.187	85.60	124.50	43.80	112.50	0.961
Total gut tumour volume (mm^3)	188.50	150.70	197.10	212.10	0.438	175.60	163.60	150.60	258.60	0.151	184.90	200.60	141.30	221.60	0.417

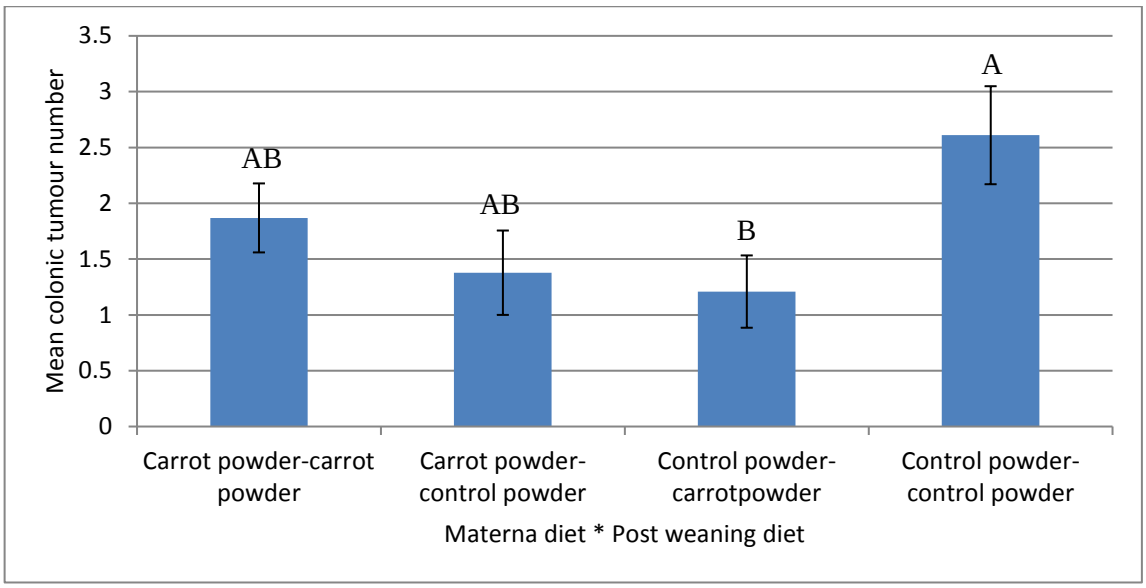


Figure 4. 18: Interactive effects of maternal and post weaning diets on colonic tumour number in Min mice (Control powder-control powder n = 16, control powder-carrot powder n = 23, carrot powder-control powder n = 16, carrot powder-carrot powder n = 24). Error bars represent SEM. Means that do not share a letter are significantly different, P = 0.017. First two words refer to maternal diet and the next two refer to post weaning diet.

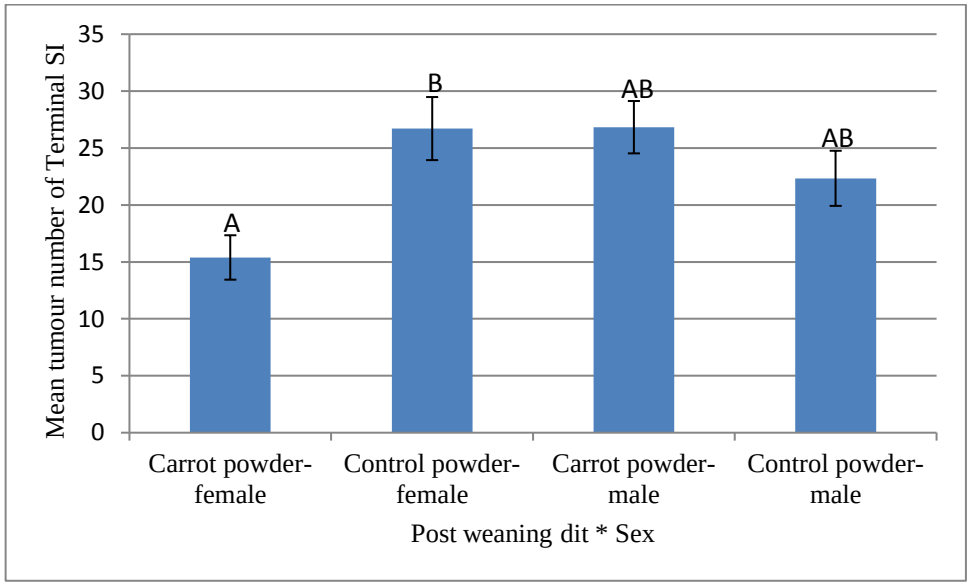


Figure 4. 19: Effects of post weaning diet on tumour number in terminal SI of male and female Min mice. Data was not transformed Effects of post weaning diets were separately analysed. (Control powder –Female n = 12, control powder-male n = 20, carrot powder-female n = 25, carrot powder-Male n = 22) Error bars represent SEM. Means that do not share a letter are significantly different at P = 0.002.

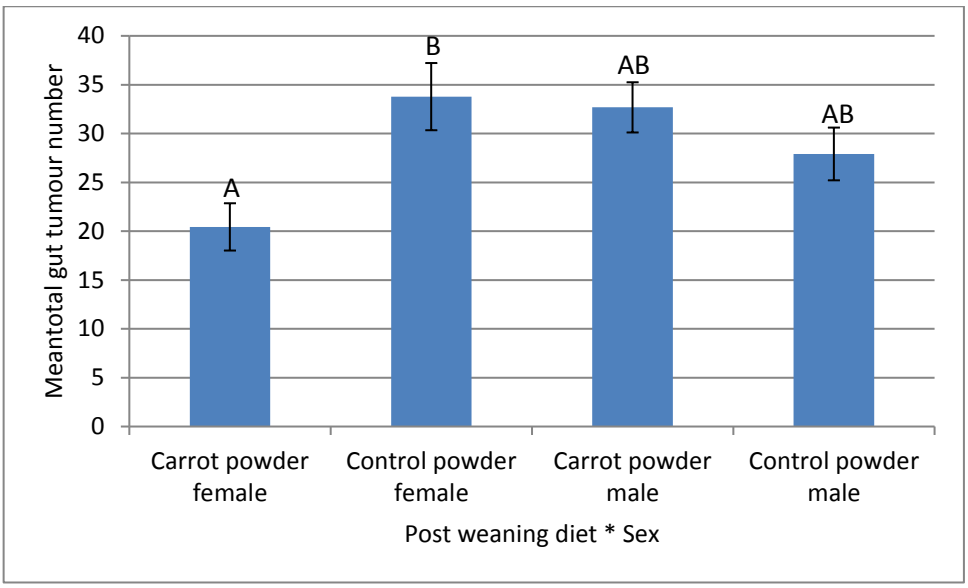


Figure 4. 20: Effects of post weaning diet on mean total gut tumour number in male and female mice. (Carrot powder-Female n = 25, carrot powder-male n = 22, control powder-female n =12, control powder-male n = 20) Error bars represent SEM. Means that do not share a letter are significantly different at P = 0.003.

5 Chapter 5 Results: Gene expression studies

5.1 Genes expression in Min and Wild type tissue

5.2 Data analysis

Effects of maternal and post weaning diets and sex and their interactions on gene expressions was analysed in a factorial design using ANOVA GLM separately for Min and Wild type mice. Data was presented as LSM ± SEM. Stability of reference gene was analysed for dietary regimes, genotype and tissue type in a One-way ANOVA using Tukey's comparison Test and results presented as Mean ± StDev.

5.2.1 Stability of Reference Gene

There was no significant variation in reference gene expression between the dietary groups, Min and Wild type mice and tumour and intestinal tissues (Table .5.1)

Neither maternal nor post weaning diets had any significant effect on expression of *mRNA* of target gene in tumour (Table 5.2) and normal (Table 5.3) terminal SI (TSI) tissue of $Apc^{Min/+}$ mice.

Sex significantly affected expression of *Cyclin D1* in tumour tissues. The expression was higher in females (P = 0.035) than in males (Table 5.2). The expression of *MMP7* in normal TSI tissues of the Min mice was higher in males (P = 0.043) than in females (Table 5.3). No statistically significant effects of maternal diet, post weaning diet and sex interactions on the expression of any of the target genes was observed.

Table 5. 1: Effects of dietary regime, genotype and tissue type on expression of reference gene in analytical groups

	Dietary regimen					Genotype			Tissue type		
	Carrot powder Carrot powder (n = 36)	Carrot powder-control powder (n = 28)	Control powder carrot powder (n = 36)	Control powder control powder (n = 36)	P value	*Apc*$^{Min/+}$ (n = 88)	Wild type (n = 48)	P value	Tumour (n = 44)	Normal Intestine (n = 92)	P value
	Mean ± StDev	Mean ± StDev	Mean ± StDev	Mean ± StDev		Mean ± StDev	Mean ± StDev		Mean ± StDev	Mean ± StDev	
CT	16.24 ± 0.670	16.05 ± 0.679	16.48 ± 0.770	16.11 ± 0.725	0.091	16.27 ± 0.688	16.15 ± 0.782	0.387	16.12 ± 0.727	16.28 ± 0.717	0.218

Analysis was by One-Way ANOVA. And comparison was by Tukey's Test. CT = Threshold cycle

5.2.2 Target Gene expression

Table 5. 2: Effects of sex, maternal and post weaning diets on expression of *mRNA* of target gene in terminal small intestinal tumour of Min mice at death

mRNA of target gene	Maternal diet			Post weaning diet			Sex			Probability effects		
	Carrot powder (n = 10)	Control powder (n = 12)	P value	Carrot powder (n = 11)	Control powder (n = 11)	P value	Female (n = 10)	Male (n = 12)	P value	Maternal * Post weaning	Maternal * Sex	Post weaning diet*Sex
	Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM				
RXRalpha	3.02±0.049	3.03 ± 0.042	0.929	3.05 ± 0.044	3 ± 0.048	0.451	2.99 ± 0.049	3.06 ± 0.042	0.285	0.887	0.665	0.931
Cyclin D1	1.09 ± 0.004	1.09 ± 0.003	0.428	1.09 ± 0.003	1.09 ± 0.004	0.42	1.1 ± 0.004	1.08 ± 0.003	**0.035**	0.446	0.785	0.471
BCMO1	1 ± 0.000	1 ± 0.000	0.428	1 ± .000	1 ±0.000	0.786	1 ± 0.000	1 ± 0.000	0.135	0.067	0.754	0.752
RARbeta	1.09 ± 0.004	1.08 ± 0.004	0.759	1.08 ± 0.004	1.09 ± 0.004	0.763	1.08 ± 0.004	1.08 ± 0.004	0.2	0.446	0.921	0.43
MMP7	4.45 ± 0.048	4.52 ± 0.042	0.297	4.49 ± 0.043	4.48 ± 0.048	0.792	4.42 ± 0.048	4.55 ± 0.042	0.07	0.295	0.895	0.118
COX-2	6.22 ± 0.4	6.48 ± 0.346	0.591	6.48 ± 0.357	6.22 ± 0.394	0.965	6.24 ± 0.4	6.46 ± 0.346	0.518	0.992	0.649	0.419

Maternal diet * Post weaning diet * Sex was not statistically significant

Table 5. 3: Effects of sex, maternal and post weaning diets on expression of target genes in normal terminal small intestinal tissue of Min mice at death

mRNA of target gene	Maternal diet			Post weaning diet			Sex			Probability effects		
	Carrot powder (n = 10)	Control Powder (n = 12)	P value	Carrot powder (n = 11)	Control powder (n = 11)	P value	Female (n = 10)	Male n = 12)	P value	Maternal * Post weaning diet	Maternal *Sex	Post weaning diet*Sex
	Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM				
RXRalpha	3.06 ± 0.05	3.1 ± 0.046	0.599	3.09 ± 0.048	3.07 ± 0.0525	0.796	3.06 ± 0.053	3.1 ± 0.046	0.548	0.48	0.875	0.755
Cyclin D1	1.08 ± 0.004	1.08 ± 0.003	0.574	1.08 ± 0.003	1.08 ± 0.0035	0.224	1.08 ± 0.004	1.08 ± 0.003	0.404	0.419	0.992	0.99
BCMO1	1.00 ± 0.000	1.00 ± 0.000	0.444	1 ± 0.000	1 ± 0.00019	0.673	1 ± 0.000	1 ± 0.000	0.413	0.921	0.412	0.841
RARbeta	1.08 ± 0.006	1.07 ± 0.005	0.523	1.08 ± 0.005	1.08 ± 0.006	0.703	1.08 ± 0.006	1.07 ± 0.005	0.517	0.943	0.984	0.831
MMP7	4.66 ± 0.064	4.69 ± 0.055	0.7	4.66 ± 0.0567	4.68 ± 0.063	0.835	4.58 ± 0.063	4.76 ± 0.055	**0.043**	0.91	0.295	0.989
COX-2	6.7 ± 0.429	7.13 ± 0.371	0.453	7.09 ± 0.383	6.73 ± 0.423	0.533	6.65 ± 0.429	7.18 ± 0.371	0.358	0.877	0.579	0.699

Maternal diet * Post weaning diet * Sex was not statistically significant (data not shown).

Feeding dams carrot powder significantly decreased expression of *MMP7 mRNA* in TSI of 15 weeks old wild type offspring (Table 5.4)

MMP7 mRNA was also expressed more in TSI of males (P = 0.007) than females. Significant interaction between maternal diet and sex (P= 0.028) was observed for *MMP7 mRNA* expression in TSI of wild type mice (Table 5.4, Fig. 5.1). Post weaning diet and sex interactions were observed for expressions of *mRNA of Cyclin D1 (*P = 0.002) (Table 5.4, Fig.5.2), *MMP7 (*P = 0.028) (Table 5.4, Fig. 5.3) *and BCMO1* (P= 0.025) (Table 5.4, Fig 5.4).

Table 5. 4: Effects of sex, maternal and post weaning diets on expression of *mRNA* of target genes in terminal small intestinal tissue of Wild type mice at time. Data for MMP7 was Box Cox transformed for P value.

mRNA of target gene	Maternal diet			Post weaning diet			Sex			Probability effects		
	Carrot powder (n= 12	Control powder (n= 12)	P value	Carrot powder (n= 12)	Control powder (n = 12)	P value	Female (n = 12)	Male (n = 12)	P value	Maternal * Post weaning	Maternal Diet * Sex	Post weaning diet*Sex
	Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM		Mean ± SEM	Mean ± SEM				
RXRalpha	3.05 ± 0.034	3.05 ± 0.034	0.863	3.06 ± 0.034	3.04 ± 0.034	0.609	3.02 ± 0.034	3.08 ± 0.034	0.187	0.443	0.894	0.173
Cyclin D1	1.08 ± 0.003	1.08 ± 0.003	0.865	1.08 ± 0.003	1.08 ± 0.003	0.402	1.08 ± 0.003	1.08 ± 0.003	0.241	0.072	0.862	**0.002**
BCMO1	1.00± 0.000	1.00 ± 0.000	0.274	1.00 ± 0.000	1.00 ± 0.000	0.377	1.00 ± 0.000	1.00 ± 0.000	0.208	0.061	0.915	**0.025**
RARbeta	1.08 ± 0.004	1.08 ± 0.004	0.608	1.08 ± 0.004	1.08 ± 0.004	0.716	1.08 ± 0.004	1.08 ± 0.004	0.598	0.615	0.96	0.138
MMP7	4.43 ± 0.047	4.65 ± 0.045	**0.003**	4.6 ± 0.047	4.48 ± 0.045	0.086	4.44 ± 0.047	4.64 ± 0.045	**0.007**	0.692	**0.028**	**0.027**
COX-2	6.69 ± 0.294	6.77 ± 0.294	0.835	6.77 ± 0.294	6.69 ± 0.294	0.829	6.53 ± 0.294	6.93 ± 0.294	0.325	0.237	0.82	0.096

Maternal diet * Post weaning diet * Sex was not statistically significant (data not shown).

5.2.3 *Interactions of Maternal and Post weaning diet in Terminal SI Tissue of Wild type Mice*

Wild type males born to carrot powder fed dams had low level expression of *MMP7 mRNA* (P = 0.01) compared to female offspring born to carrot powder fed dams (Fig.5. 1).

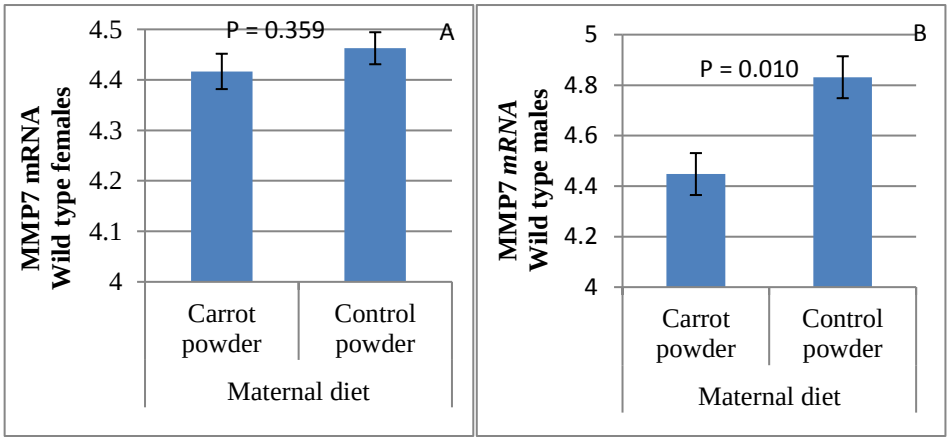


Figure 5. 1: Effects of maternal diet on expression of *MMP7 mRNA* in terminal SI of (A) female (B) male wild type mice (Carrot powder n = 6, control powder n = 6) Error bars represent SEM

Post weaning carrot powder consumption reduced *Cyclin D1 mRNA* expression in wild type males (P = 0.023) but enhanced it in females (P = 0.076) (Fig. 5. 2).

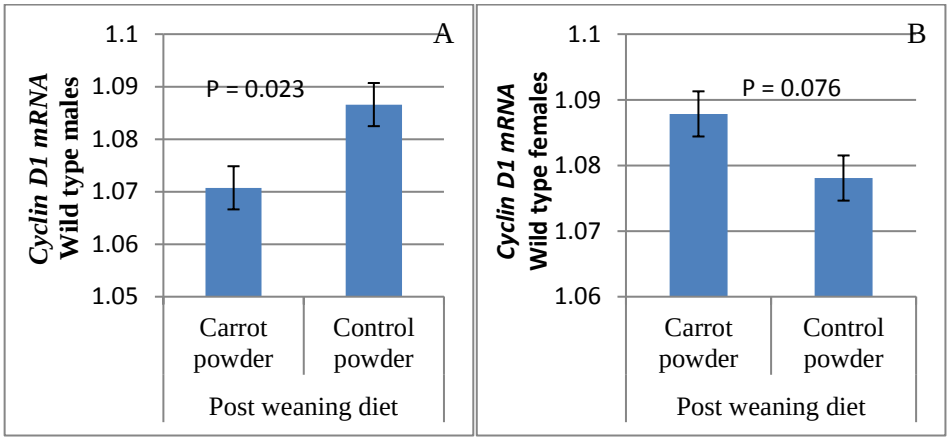


Figure 5. 2: Effects of post weaning diet on expression of *Cyclin D1 mRNA* in terminal SI (A) male (B) female wild type mice (Carrot powder n = 6; control powder n = 6) Error bars represent SEM

Post weaning consumption of carrot powder significantly increased level of expressed of *MMP7 mRNA* in wild type males (P = 0.039) compared to control powder. (Fig. 5.3).

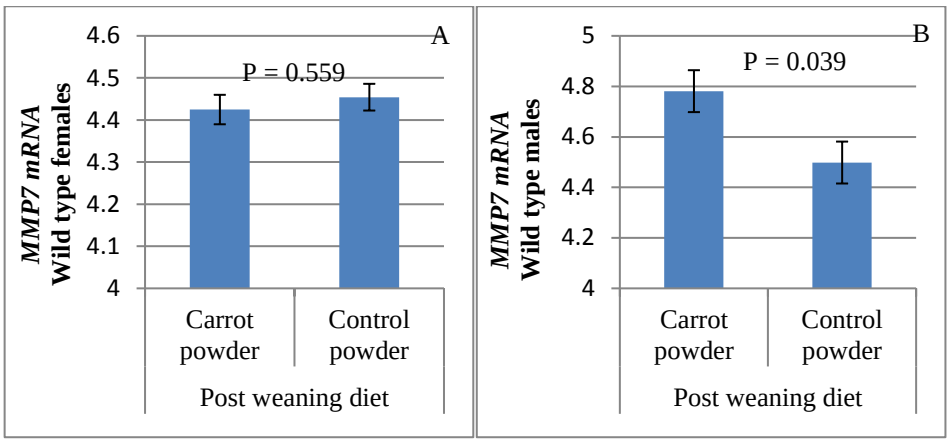


Figure 5. 3: Effects of post weaning diet on expression of *MMP7 mRNA* in terminal SI of (A) female (B) male wild type mice (Carrot powder n = 6; control powder n = 6) Error bars represent SEM.

Though a significant interaction between post weaning diet and sex was noticed for *BCMO1 mRNA* expression, it only approaches significance in males (P = 0.069) when the effect in the sexes was investigated (Fig. 5.4).

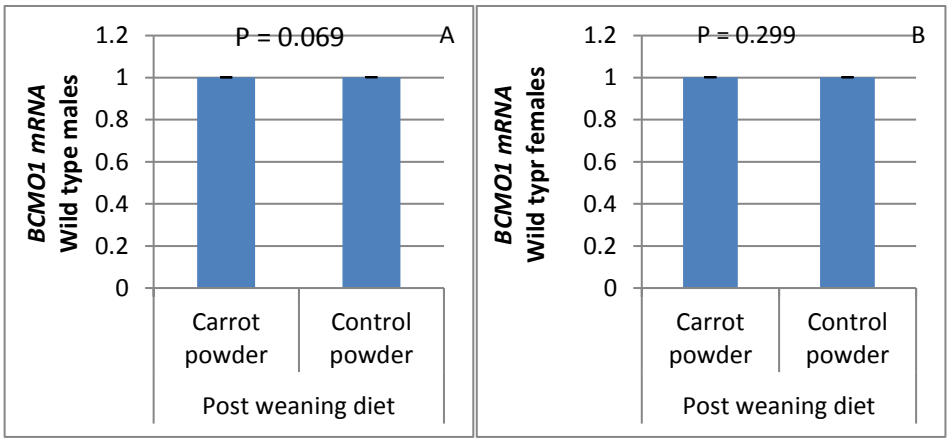


Figure 5. 4: Effects of post weaning diet on expression of *BCMO1 mRNA* in terminal SI of (A) male (B) female wild type mice (Carrot powder n = 6; control powder n = 6). Error bars represent standard error of means (SEM)

Target gene *mRNA* levels of expression in normal and tumour tissues in the terminal SI of the Min mice were compared in a Two-Sample T-Test

Results suggest, expression of *Cyclin D1 mRNA* was higher in tumours (P = 0.004) than in normal TSI tissue whilst *MMP7 mRNA* was higher in normal (P = 0.003) than in tumour tissues (Table 5.5).

Expressions of target gene *mRNA* in normal tissue of Min and Wild type mice (genotype effect) were compared a One-Way ANOVA.

MMP7 mRNA expression was higher in Min (P = 0.055) compared to Wild type mice (Table 5.6).

Table 5. 5: Differences in expression of *mRNA* of target genes in tumour and normal terminal SI tissue samples of Min mice at death

mRNA of target genes	Terminal small intestinal tissue type		P value
	Tumour (n = 22)	Normal (n = 22)	
	Mean ± SEM	Mean ± SEM	
RXRalpha	3.03 ± 0.027	3.07 ± 0.029	0.237
Cyclin D1	1.09 ± 0.002	1.08 ± 0.002	**0.004**
BCMO1	1.00 ± 0.000	1.00 ± 0.000	0.771
RARbeta	1.08 ± 0.003	1.08 ± 0.003	0.104
MMP7	4.51 ± 0.031	4.68 ± 0.041	**0.003**
COX-2	6.41 ± 0.22	7 ± 0.24	0.077

Table 5. 6: Effects of genotype on expression of target genes in normal terminal small intestinal tissues of Min and Wild type mice at death

mRNA of target genes	Min (n = 22)	Wild type (n= 24)	P value
	Mean ± StDev	Mean ± StDev	
RXRalpha	3.07 ± 0.137	3.05 ± 0.113	0.531
Cyclin D1	1.08 ± 0.01	1.08 ± 0.011	0.346
BCMO1	1.00 ± 0.001	1.00 ± 0.000	0.741
RARbeta	1.08 ± 0.015	1.08 ± 0.014	0.549
MMP7	4.69 ± 0.192	4.54 ± 0.246	**0.055**
COX-2	7.02 ± 0.114	6.73 ± 0.983	0.39

6 Chapter 6 General Discussion

6.1 Weight measurements

As expected gender and genotype had significant effect on growth throughout study one and study two. Distinguishing males from females and wild type from *Apc*$^{Min/+}$ mice regarding weight gain post weaning, indicates that the weight measurements for both studies were well executed and therefore would be sufficiently robust to detect any of the effects of diet on growth discussed under each of the two studies.

6.2 Effects of maternal and post weaning diet on Body weight and Organ Dimensions of mice at Death

Nutrition is important non-genetic factor that affects the growth and body composition (Corva and Medrano, 2000) and nutritional status of mothers prior to conception and during conception may influence physical characteristics of the offspring in later life (Mortensen *et al.*, 2010).

Maternal carrot consumption in study one reduced mean body weight in adult offspring by 2 % at time of death. The results contradict findings of other studies which suggest maternal nutrient deficiency (depleted folate) had no significant effect of mean body weight of adult offspring (McKay *et al.*, 2008). Most individuals who suffer nutritional insult in utero and have low birth weights or thin bodies show rapid catch-up growth early post-natal (Osmond and Barker, 2000). The reduction in body weight with maternal diet could be due to nutritional insult *in utero* probably as a result of poor digestion and absorption of carrot diet not only by mothers but also by offspring post weaning. Birth weight was not investigated on this study but if individual with low birth weight existed and were continued on carrot diet post weaning then the possibility of reduced body weight could occur as suggested by dietary regimes in study 2. Offspring fed carrot diet post weaning irrespective of maternal diet had 2 % lower mean body weight compared to those fed control diet to suggest the significance of post weaning diet in adult life. In addition data from this study showed that at weaning offspring born to carrot powder fed dams were 7.7 % lower in mean body weight compared to control. This was in contrast to the results obtained by Saleh *et al.*, 2013 where 20 % carrot consumption in utero and post weaning produced no difference in body weight. Similarly no significant effect on body weight was observed when rats were fed a diet containing 10 % freeze dried carrot (Kobæk-Larsen *et al.*, 2005). Also feeding mice a diet containing 40 % freeze dried conventionally grown carrots

produced no significant difference in body weight compared to the control (Jensen *et al.*, 2012). In line with the current results were findings of a study that investigated effects of Western diet and annurca apple juice on intestinal tumourigenesis in $Apc^{Min/+}$ mice (Fini *et al.*, 2011). Rao *et al.* (2000), observed differences in body weight at time of death when $Apc^{Min/+}$ mice were fed a diet containing 1, 4-phenylene *bis* (methylene) (p-XSC) compared to control. These differences could be attributed to intestinal tumour and associated complications including anaemia and cachexia (Swamy *et al.*, 2006; Fini *et al.*, 2011). $Apc^{Min/+}$ mice were shown to develop multiple intestinal tumours largely in the small intestine and die of intestinal obstruction and /or severe anaemia, often not surviving beyond 120 to 150 days (Moser *et al.*, 1990; Su *et al.*, 1992; Heyer *et al.*, 1999).

Findings of study2 suggest dietary regimes to which mice were exposed (in utero-post weaning) had significant effects on mean body weight and dimensions of internal organs measured

The observed differences in dimensions of internal organs may be the results of nutritional insults during early developmental stages. Studies have shown that in adequate nutrient supply may lead to survival adaptations in a fetus with the capacity to modulate response to the environment in later life (Gluckman *et al.*, 2005). Those adaptive modifications are not limited to intrauterine stages of development but even through neonatal stages (Gluckman *et al.*, 2005). Such adaptations may involve restriction of nutrients to organs the fetus might consider non-essential leading to irreversible structural and functional modifications of affected organs (Langley-Evans and McMullen, 2010). Certain organs may therefore develop more than others in what may be described as selective trade off or disproportionate growth (Bateson *et al.*, 2004). Studies suggest that the small intestine is the main site for digestion and absorption of nutrients and its structural organisation could significantly influence the performance and nutrient utilisation of an offspring (Duarte *et al.*, 2013). The increase in SI length could be adaptation to increase the internal surface area to enhance nutrient absorption capacity. Enhanced intestinal dimensions were reported in many studies, Duarte *et al.* (2013), reported increased weight and length of small intestine alongside enhanced structure of villi in bovine fetus of diet restricted mothers. McKay *et al.*, (2008) reported longer colon in offspring of folic acid restricted Black/6 dams.

Also the significant increase in weight of the intestine may be explained by the high tumour number, especially the terminal and proximal SI where more than 70 % of tumour occurred in both studies. As observed in study one carrot pellet consumption post weaning enhanced tumour

number in the proximal and terminal SI by 39 %. Weights of these sections of the intestine also increased (P <0.001) by 11 and 10 % respectively compared to the control

This is in conformity with observed tumour distribution in many chemoprevention studies using *Apc*$^{Min/+}$ mouse model (Ju *et al.*, 2005; Mohammed *et al.*, 2011).

Summary

- Carrot diet significantly decreased body weight
- Carrot diet consumption post weaning significantly increased the weights of proximal and terminal SI and the caecum
- Carrot diet enhanced length of proximal and terminal SI and colon

6.3 Tumour distribution

In both studies irrespective of the dietary regime, most tumours occurred in the terminal SI compared with the proximal SI and the colon. A similar trend of tumour distribution was reported in several studies (Hu *et al.*, 2006; Cai *et al.*, 2010; Fini *et al.*, 2011), making it convenient to compare the finding with other studies.

6.3.1 Effects of Maternal and Post Weaning Diets on Tumour Number and Volume

6.3.2 Effect of post-weaning diet on tumour number

Post weaning consumption of carrot pellet increased total gut tumour number by 41.5 % (P = 0.038) and terminal SI tumour number by 39.4 % (P = 0.044) in fifteen week old adult *Apc*$^{Min/+}$ offspring in study one, while post weaning consumption of carrot powder and stored carrot pellet reduced total gut tumour number by 14.96 (P = 0.037) and 14.11 % and terminal SI tumour number by 15.5 % (P = 0.044) and 5.8 % respectively, compared to the control powder. These results appear to contradict each other, however findings of similar studies show that similar discrepancies have been reported previously. Feeding *Apc*$^{Min/+}$ mice a diet containing 20 % carrot powder post weaning significantly decreased intestinal tumour number (Saleh *et al,*. 2013). Kobæk-Larsen *et al.* (2005) found that an experimental diet with 10 % carrot powder showed a reduction in number of chemically induced colon polyps in of BDIX male rats.. Dietary supplementation of sulforaphane, an isothiocyanate commonly consumed in

cruciferous vegetables was also shown to decrease number of polyps in intestine of *Apc*$^{Min/+}$ mice (Hu *et al.*, 2006). Further experimental evidence suggest a reduction in number of intestinal polyps in *Apc*$^{Min/+}$ mice with separate dietary administration of sulforaphane and dibenzoylmethane and in combination by 48 %, 50% and 57 % respectively (Shen *et al.*, 2007). The description of the diet preparation in these papers did not specify whether or not the diets were dried at high temperatures. In addition a number of other experimental animal studies involving fruits and vegetables and purified or semi purified phytochemical in *Apc*$^{Mn/+}$ mice suggest protective effect (Myzak *et al.*, 2006; Rajamanickam and Agarwal, 2008; Murphy *et al.*, 2011). However, in agreement with study one, van Kranen *et al.* (1998) reported an increase in SI tumour number by 20 % in male and 12 % in female *Apc*$^{Min/+}$ mice fed pelleted vegetable fruit mixture. Also in agreement was the findings of Alink *et al.* (1993) that addition of vegetables and fruits into baked or heated human diets enhanced 1,2-dimethylhydrazine induced colon tumours in rats. However this result is contrary to many epidemiological observations which suggest reduction in colorectal cancer risk with increased intake of fruits and vegetables (Aune *et al.*, 2011). The above-mentioned studies where carrot feeding increased cancer severity in animal models has one factor in common: the experimental diets were pelleted, while studies using powdered or fresh diets generally reduced tumour numbers or other measures of cancer. A possible reason for the observed high tumour number with consumption of carrot pellet diet post weaning in study one could therefore be the presence of furan, a volatile carcinogen. Furan is a low boiling cyclic ether identified by FDA as a possible human carcinogen detected in many heat treated canned foods (Food and Drug, 2004; Duan and Barringer, 2012). It is produced when carrots at moisture content of 10 % and below are exposed to temperatures of 93°C or higher (Duan and Barringer, 2012). As moisture content falls below 40 %, furan concentration begins change and increases with increasing temperature and decreasing moisture. The production increases exponentially at 4-7 % moisture and between 100 and 133°C. The carrot pellet used in this study was dried between 80-120°C to attain moisture content of about 10 %, which may have resulted in the presence of furan in the diet. In the present study, the relevance of furan was not noticed initially, until it was too late to test for it. However if furan was present in the pellets in study one, it may be responsible for the observed increase in tumours in carrot pellet fed mice. When the same pellets were tested in study two, after one year of storage, the detrimental effect on tumour number had disappeared. This observation reemphasised the possible effect of furan which is likely to have evaporated during storage and therefore may plausibly explain the difference in the effects of carrot pellet and stored carrot pellet feeding on tumorigenesis observed between study one and study two.

Furan may be measured in carrots by selected ion flow tube mass spectrometry (SIFT-MS) method described by (Duan and Barringer, 2012)

Combining all this information, the study's results support the epidemiological observations that consumption of raw or boiled carrots reduce the growth of already formed tumours, while no benefit was observed during the tumour initiation period (in utero and pre-weaning). For humans the implication is that carrot consumption may reduce the risk or severity of cancer, and is likely to benefit patients with already diagnosed cancer as well as healthy adults.

6.3.3 Effect of maternal diet on tumour number and volume

In these studies, maternal diet had no significant effect on intestinal tumour number and volume in *Apc*$^{Min/+}$ mice. No significant effect was observed when effect of dietary curcumin on intestinal tumourigenesis in *Apc*$^{Min/+}$ mice was investigated *in utero* and through lactation (Perkins *et al.*, 2003a). McKay *et al.* (2008b), also suggested that maternal folate depletion (0.4mg/ folic acid/kg diet) did not affect intestinal tumourigenesis significantly compared to normal folate (2mg/kg). However, results from (Ciappio *et al.*, 2011) showed that preconception maternal supplementation of folic acid (8m g/kg), B2 (24 mg/kg), B6 (28 mg/kg) and B12 (200 ug/kg) significantly reduced intestinal tumours in APC1638N offspring mice compared to offspring from control and deficient mothers. Additionally, maternal folic acid supplementation reduced by 64% azoxymethane induced adenocarcinoma in rats (Sie *et al.*, 2011). A possible explanation for this observation could be that the effects of maternal diet may not be sufficiently powerful to overcome the genetic predisposition of *Apc*$^{Min/+}$ mice to develop intestinal neoplasia and thus diet may not be preventative against tumour initiation. Alternatively, the concentration of the carrot components of maternal diet used in these studies may not have been sufficient to influence tumour development in the foetus or suckling pups and higher consumptions may be required to observe any beneficial effect in the offspring. Indeed, blood level concentrations of curcumin were very low when fed to mothers (Ireson *et al.*, 2001) and not much was available either from maternal blood or breast milk for chemopreventive efficacy in *Apc*$^{Min/+}$ offspring (Perkins *et al.*, 2003b).

6.3.4 Interactions of Gender with Other Factors

Study two suggested males had more tumours in terminal SI (6.3 %), colon (21.2 %, P = 0.03) and total gut than females. Whilst females had 13.1% more tumours in the proximal SI than males, males had 3.5 % more tumours in the total SI than females. In study one, females had 16.1 % and 6.7 % more tumours in the proximal SI and total gut tumour number respectively than males. In the two studies the females had more tumours in the proximal SI than males. Hormonal differences between the sexes may account for the observed disparity in tumour number between the sexes. A study by Amos-Landgraf *et al.* (2014), showed that females developed 5.6 % more tumours in the small intestine than males whilst males developed 20.9 % more tumours in the colon than females, a finding that was in agreement with this study. Amos-Landgraf *et al.* (2014), investigated the effects of testosterone on tumourigenesis and suggested male hormone testosterone was more important in promoting tumour development than the inhibition from female hormone. Other studies attributed the observed differences between the sexes to the female hormone, oestrogen which has significant influence on permeability of the intestinal epithelium in women (Vulcan *et al.*, 2015). Low levels of oestrogen in matured/menopausal females enhance permeability of the colon to much substance which may result in cancer (Vulcan *et al.*, 2015). This was consistent with suggestions of Weyant *et al.* (2001) that 17β-oestradiol, a potent ovarian and placental oestrogen protects female mice against intestinal tumourigenesis. Another study suggested that females had 27.2 % more tumours in SI than males with no significant difference in colonic tumour number between the sexes (McKay *et al.*, 2008a). Also no difference in total tumour number was observed when $Apc^{Min/+}$ males and females were treated with different concentrations of green tea polyphenols. Hao *et al.* (2007), reported a larger variation in the reduction of tumour volumes after treatment in females than males, with reported reductions varying between 24.4-71 % in females, and 14 -39.9 % in males. The current, study two, observed that the effects of sex on tumour volume was also seen on increased tumour number, for example, 0.9 %, 23.7 % and 14.4 % higher tumour volume in terminal SI, colon and total gut compared to females and 6.3, 21.2 %, and 0.4 % higher tumour numbers than females respectively.

A post weaning diet * sex interaction (P = 0.044) was observed for tumour number in the terminal SI whereby females fed carrot powder post weaning had reduced tumour number in terminal SI compared to their male counterparts. They also had 36 % (P= 0.027) less tumours in the terminal section compared to the control powder fed females. A similar interaction (P = 0.006) was again observed when analysis was done after removal of stored carrot pellets. These

data suggests carrot powder may be better anticarcinogen in females than in males if administered post weaning. This brings into focus the suggestion by Vulcan *et al.* (2015) that development of tumours in females is influenced by the permeability of the intestinal epithelium which is controlled by female hormone, oestrogen. These mice were relatively young (15 weeks old) and the level of oestrogen may be high enough to regulate effectively the permeability of the intestinal epithelium to compounds that could enhance tumour development and growth. It may also be possible that components of carrot powder interacted with female hormone or some other compound to reduce permeability thus reducing the entry of potential carcinogen. Another interaction, post weaning diet and sex ($P = 0.054$) was observed for total gut tumour number in which females fed carrot powder post weaning had lesser tumours than males and lesser ($P = 0.039$) than females fed control powder. This observation was probably driven by tumour number in the terminal SI

There was maternal * post weaning diet interaction ($P = 0.023$) for colonic tumour number whereby offspring exposed to maternal control powder but fed carrot powder post weaning had fewer colonic tumour number (54.3 %) compared to offspring exposed to control powder dietary regime.

This interaction suggests carrot powder may have the potential of reducing intestinal cancer risk if administered to females post weaning.

Summary

- Post weaning consumption of carrot pellet significantly increased the total gut tumour number and terminal SI tumour.
- Post weaning consumption of carrot powder decreased total gut and terminal SI tumour number.
- Post weaning consumption of stored carrot pellet reduced total gut and terminal SI tumour number.
- Carrot consumption either raw or boiled reduced the growth of already formed tumours but has no effect on tumour initiation.
- Maternal diet had no significant effect on tumour number and volume.

6.4 Effects of Maternal and post weaning Diet on the Expression Target gene

The present study investigated the effects of maternal and post weaning diet on the expression of genes in the tumour and normal terminal SI of $Apc^{Min/+}$ and wild type mice. Gene investigated were *Retinoid X receptor alpha (RXRα), β, β-carotene-monooxygenase (BCMO1/BCO1), Retinoid acid receptor-beta (RARβ), Cyclin D1, Cyclooxygenase-2 (COX-2), and Matrix metalloproteinase/Matrilysin (MMP7)*

Neither maternal nor post weaning diet had any significant effect on the expression of target genes in the tumour and normal intestinal tissues of the Min mice. In Min mice *MMP7 mRNA* expression was lower in tumour tissue of females by 1.4% (P = 0.07) compared to males and is also generally lower in tumour tissue by 1.8 % (p = 0.055) than in normal tissue. In the wild type mice maternal consumption of carrot powder decreased expression of *MMP7* by 2.4 % (P = 0.003) whilst post weaning carrot powder enhanced expression (P = 0.086). A similar trend was observed in the tumour and normal tissue of Min mice, though statistically insignificant. These results suggest that carrot had a modulating effect on *MMP7 mRNA* expression.

There was a significant interaction effect (P = 0.028) between maternal diet and sex on one hand and post weaning diet and sex on the other hand on expression of *MMP7 mRNA* in Wild type mice. Whilst maternal carrot diet reduced expression of *MMP7 mRNA* in the male offspring (P = 0.01), post weaning carrot powder upregulated the expression in same suggesting antagonistic effects of the dietary regime in males in relation to *MMP7mRNA.* Both diets had no significant effect in female offspring. Relating these results to the observed effects in the Min suggest maternal diet has the potential of reducing intestinal tumour risk in male offspring. *MMP7* is a downstream target of β-catenin and is involved in digestion of extracellular matrix and therefore promotes tumour cell invasion and metastasis (Curran and Murray, 2000). It is known to be involved in tumour initiation and growth (Brabletz *et al.*, 1999) and over expressed in most colorectal cancers (Zeng *et al.*, 2002; Pham *et al.*, 2013) Downregulating its expression will enhance intestinal cancer risk reduction. It was therefore possible that the reduced tumour number associated with carrot powder consumption was as a result of reduced expression of *MMP7 mRNA.*

The absence of significant modulating effect of carrot powder in Min mice on expression of some of the biomarkers in the current study could be due to low concentration of active components of carrot required for effect either as a result of poor digestion and absorption by mothers and /or offspring due to tumours in the intestine or the carrot component of diet was

low. Carotenoids are poorly absorbed in the intestine of mice and rats and to achieve a significant modulating effects in short term studies higher doses are required (Sharoni *et al.,* 2004). van Breda *et al.* (2005b), investigated the effects of vegetable diet on the expression of cancer biomarkers in mice and observed significant effects only with diet containing 40 % vegetable. Anticancer activities of carotenoids are mediated by retinoic acid acting to activate *RARs* and *RXRs,* two critical receptors whose expressions are regulated at the transcription level by retinoic acid (Ribot *et al.*, 2004; Pham *et al.*, 2013). The expression of these receptors (*RXR* and *RAR*) both in vivo and in vitro were shown to require a concentration of retinoic acid above normal physiological concentration in tissues of rodents (Ribot *et al.*, 2004, Bonet et al, 1997). Considering the suggestion that retinoic acid modulates the activities of *BCO1/BCMO1* and *MMP7* (Leclerc *et al.*, 2013), *Cyclin D1* (Fu *et al.*, 2004), *RXRα* and *RARβ* (Bonet *et al.*, 1997; Ribot *et al.*, 2004), it was possible that the apparent lack of significant differences in the expression of the study biomarkers was due to the concentration of retinoic acid which was too low to overcome the genetic predisposition of the mice to development tumours and/or too low for any chemopreventive action post weaning. It could also be said that the reduction in tumour number observed was due mechanisms that did not involve some of the investigated biomarkers.

Min females had increased expression of *Cyclin D1* by 0.5 % (P = 0.035) in the tumour tissue compared to the males in the current study. *Cyclin D1* is associated with regulation of cell proliferation by restricting cell cycle progression at the G1 phase. This regulation occur by modulating the expression of retinoblastoma protein (pRb) (Fu *et al.*, 2004) which in normal function binds to transcription factor, EF2 to prevent transcription of genes whose transcription products are required for cell cycle progression (Sherr, 2000). Activated *Cyclin D1* causes the release EF2 in tumour cells which then initiates DNA replication (Donnellan and Chetty, 1998). At very high levels of expression cyclin D1 inhibits DNA replication, thus reducing cell proliferation (Fukami-Kobayashi and Mitsui, 1999). This may account for the reduced tumour number especially in the terminal SI of female mice fed carrot powder post weaning. Tumour number in the terminal section of females fed carrot powder post weaning was 22.2 % (P = 0.027) lower than control powder fed and 8.1 % lower than in stored carrot pellet fed mice. Results of the current study showed that in the Wild type mice carrot powder consumption post weaning enhanced expression of *Cyclin D1 mRNA* in females by 0.5% (P= 0.076) but down regulated the expression in males by 0.8 % (P = 0.023) compared to the controls.

Summary

MMP7 mRNA expression was lower in tumour of females than in males

Maternal and post weaning carrot had antagonistic effects on expression of *MMP7 mRNA* in male mice. Maternal carrot decreased expression whilst post weaning carrot diet increased expression.

The downregulation of *MMP7* associated with maternal carrot consumption in male offspring suggest a putative sex-specific protection against intestinal tumourigenesis.

Post weaning carrot powder consumption reduced tumour number in females by enhancing expression of *Cyclin D1*.

7 Chapter 7 Conclusion and future work

7.1 Conclusion

This study has demonstrated that consumption of freeze-dried blanched carrot powder post weaning reduced intestinal tumour number in *Apc*$^{Min/+}$ mice, whilst consumption of the same carrot material shortly after it was incorporated into pellets and dried at high temperatures enhanced tumourigenesis.

Maternal consumption of carrot powder diet prior to gestation and during gestation and lactation had no effect on intestinal tumourigenesis *in Apc*$^{Min/+}$ offspring, and a trend for increased tumorigenesis after maternal consumption of high-temperature treated pellets was not significant.

Consumption of carrot powder by females post weaning reduced the risk of tumourigenesis more than in males.

The implication of these findings for human is that consumption of carrots cooked without removal of water (boiled, steamed, fried, roasted etc.) may reduce the risk or severity of cancer, and is likely to benefit patients with already diagnosed cancer as well as healthy adults. However it raises potential concerns about the safety of vegetable products produced by high-temperature drying.

7.2 Future work

In this study furan was suspected to be responsible for the increased tumour number observed with feeding *Apc*$^{Min/+}$ mice carrot pellets dried at a high temperature. This was noticed long after the effect when furan might have evaporated from the diet. It will therefore be necessary to repeat the experiment with similarly processed carrot diet and test for the presence of compounds formed during the drying process to confirm or otherwise the presence of furan or other possible carcinogens.

Also, carrot diet seems to have no significant effect on the expression of target genes possibly because of the low dose of carrot in the diet. In the next experiment it might be important to increase the dose to either 30 % or 40 %.

Furthermore, if possible a study should be designed to investigate post weaning diet (carrot) and sex interaction which in the present study suggest a beneficial effect in females.

Appendices

Appendix 1 Diet formulation and specification

Special Diets Services

DIET FORMULATION AND SPECIFICATION DATA

BASIC DIET INFORMATION:

Code:	824099
Name:	RM3 (P) + 20% Carrot (P)
Date:	15/04/2013

CALCULATED ANALYSIS:

		FRESH	10% H2O
TOTAL	%	100.00	100.42
MOISTURE	%	10.38	10.00
CRUDE OIL	%	4.51	4.53
CRUDE PROTEIN	%	21.03	21.12
CRUDE FIBRE	%	3.38	3.39
ASH	%	6.89	6.92
NFE	%	45.82	46.01
PECTIN	%	1.30	1.31
HEMICELLULOSE	%	8.80	8.84
CELLULOSE	%	3.51	3.52
LIGNIN	%	0.98	0.98
STARCH	%	19.43	19.51
SUGAR	%	4.07	4.09
GROSS ENERGY	MJ/kg	13.13	13.19
DIGESTIBLE ENERGY	MJ/kg	10.48	10.52
METABOLISABLE ENERGY	MJ/kg	9.46	9.50
AF ENERGY	kcal/kg	2592.73	2603.72
C14 1 MYRISTOLEIC	%	0.00	0.00
C16 1 PALMITOLEIC	%	0.02	0.02
C18 1 W9 OLEIC	%	0.71	0.71
C18 2 W6 LINOLEIC	%	1.80	1.81
C18 3 W3 LINOLENIC	%	0.23	0.23
C20 4 W6 ARICHIDONIC	%	0.08	0.08
C22 5 W3 CLUPANODONIC	%	0.00	0.00
C12:0 LAURIC	%	0.01	0.01
C14:0 MYRISTIC	%	0.02	0.02
C16:0 PALMITIC	%	0.41	0.41
C18:0 STEARIC	%	0.10	0.10
ARGININE	%	1.22	1.23
LYSINE	%	1.22	1.23
S LYS	%	0.18	0.18
METHIONINE	%	0.35	0.35
S METH	%	0.03	0.03
CYSTINE	%	0.35	0.35
S CYST	%	0.00	0.00
TRYPTOPHAN	%	0.25	0.25
S TRYPT	%	0.00	0.00
HISTIDINE	%	0.49	0.49

		FRESH	10% H2O
CL	%	0.35	0.35
S CL	%	0.31	0.31
K	%	0.79	0.79
S K	%	0.00	0.00
MG	%	0.21	0.21
S MG	%	0.04	0.04
FE	mg/kg	205.17	206.04
S FE	mg/kg	118.91	119.41
CU	mg/kg	19.79	19.87
S CU	mg/kg	8.75	8.79
MN	mg/kg	93.64	94.04
S MN	mg/kg	52.70	52.92
ZN	mg/kg	43.87	44.06
S ZN	mg/kg	8.64	8.68
CO	µg/kg	716.84	719.88
S CO	µg/kg	661.25	664.05
I	µg/kg	863.12	866.78
S I	µg/kg	793.75	797.12
SE	µg/kg	372.82	374.40
S SE	µg/kg	200.00	200.85
F	mg/kg	7.53	7.56
VIT A	iu/kg	20314.27	20400.41
S VIT A	iu/kg	19375.00	19457.15
VIT D3	iu/kg	2900.00	2912.30
S VIT D3	iu/kg	2900.00	2912.30
VIT E	iu/kg	101.56	101.99
S VIT E	iu/kg	89.25	89.63
VIT B1 THI	mg/kg	20.69	20.78
S VIT B1	mg/kg	15.80	15.87
VIT B2 RIB	mg/kg	8.14	8.17
S VIT B2	mg/kg	6.20	6.23
VIT B6 PYR	mg/kg	14.72	14.78
S VIT B6	mg/kg	12.02	12.07
VIT B12 CY	µg/kg	17.93	18.01
S VIT B12	µg/kg	17.75	17.83
VIT C ASCO	mg/kg	0.00	0.00
S VIT C	mg/kg	0.00	0.00
VIT K MENE	mg/kg	4.24	4.26

THREONINE	%	0.76	0.76
S THREO	%	0.00	0.00
ISOLEUCINE	%	0.86	0.86
LEUCINE	%	1.48	1.49
PHENYLALAN	%	0.99	0.99
VALINE	%	0.97	0.97
TYROSINE	%	0.66	0.66
TAURINE	%	0.00	0.00
GLYCINE	%	0.82	0.82
ASPARTIC A	%	1.71	1.72
GLUTAMIC A	%	3.93	3.95
PROLINE	%	1.30	1.31
SERINE	%	1.02	1.02
HYD PROLIN	%	0.00	0.00
HYD LYSINE	%	0.00	0.00
ALANINE	%	0.90	0.90
CA	%	1.22	1.23
S CA	%	1.11	1.11
TOTAL P	%	0.74	0.74
S PHOS	%	0.28	0.28
PHYTATE P	%	0.23	0.23
AVAIL P	%	0.50	0.50
NA	%	0.22	0.22
S NA	%	0.19	0.19

S VIT K	mg/kg	3.99	4.01
FOLIC ACID	mg/kg	1.05	1.05
S FOLIC	mg/kg	0.48	0.48
NICOTINIC	mg/kg	74.74	75.06
S NICOTIN	mg/kg	19.11	19.19
PANTOTHENI	mg/kg	37.76	37.92
S PANTOTH	mg/kg	24.22	24.32
CHOLINE	mg/kg	1483.80	1490.09
S CHOLINE	mg/kg	426.01	427.82
INOSITOL	mg/kg	1210.00	1215.13
S INOSITOL	mg/kg	0.00	0.00
BIOTIN	µg/kg	275.12	276.29
S BIOTIN	µg/kg	0.00	0.00

INGREDIENTS:

NAME
WHEATFEED BULK
FD CARROT BATONS (Cust. Prov)
DEHULLED EXTRACTED TOASTED SOYA
BARLEY
WHEAT
YEAST
CALCIUM CARBONATE
HYDROLYSED WHEAT GLUTEN
FULL FAT SOYA
SOYA OIL
POTATO PROTEIN
DICALCIUM PHOSPHATE DIHYDRATE
MAIZE GLUTEN MEAL
L-LYSINE HCl
VITAMIN & MINERAL MIX

Rat and Mouse No.3 Breeding

Pelleted

SUITABLE SPECIES AND APPLICATIONS

Rats and mice for breeding, lactation, and growth of young stock.

BENEFITS

- High nutrient levels promote excellent breeding performances and fast growth rates in young stock.

FEEDING GUIDE

Ad-lib feeding is recommended.

AVAILABLE AS

Diet	Form	Product Code
Standard		
RM3 (P)	*9.5mm Pelleted*	*801700*

- All diets are available irradiated and are available in a range of packaging.
- All Standard diets are available with full analysis on request.

INGREDIENTS

Wheat, Wheatfeed, De-hulled Extracted Toasted Soya, Barley, Macro Minerals, Yeast, Potato Protein, Hydrolised Wheat Gluten, Full Fat Soya, Soya Oil, Maize Gluten Meal, Dextrose Monohydrate, Vitamins, Micro Minerals, Amino Acids.

Calculated Analysis

NUTRIENTS		Total	Supp (9)
Proximate Analysis			
Moisture (1)	%	10.00	
Crude Oil	%	4.20	
Crude Protein	%	22.45	
Crude Fibre	%	4.42	
Ash	%	8.05	
Nitrogen Free Extract	%	50.40	
Digestibility Co-Efficients (7)			
Digestible Crude Oil	%	3.81	
Digestible Crude Protein	%	20.18	
Carbohydrates, Fibre and Non Starch Polysaccharides (NSP)			
Total Dietary Fibre	%	16.15	
Pectin	%	1.53	
Hemicellulose	%	9.61	
Cellulose	%	4.13	
Lignin	%	1.54	
Starch	%	33.88	
Sugar	%	4.37	
Energy (5)			
Gross Energy	MJ/kg	15.11	
Digestible Energy (15)	MJ/kg	12.22	
Metabolisable Energy (15)	MJ/kg	11.18	
Atwater Fuel Energy (AFE) (8)	MJ/kg	13.76	
AFE from Oil	%	11.48	
AFE from Protein	%	27.28	
AFE from Carbohydrate	%	61.24	
Fatty Acids			
Saturated Fatty Acids			
C12:0 Lauric	%	0.05	
C14:0 Myristic	%	0.17	
C16:0 Palmitic	%	0.37	
C18:0 Stearic	%	0.11	
Monounsaturated Fatty Acids			
C14:1 Myristoleic	%	0.01	
C16:1 Palmitoleic	%	0.09	
C18:1 Oleic	%	1.01	
Polyunsaturated Fatty Acids			
C18:2(ω6) Linoleic	%	1.26	
C18:3(ω3) Linolenic	%	0.17	
C20:4(ω6) Arachidonic	%	0.12	
C22:5(ω3) Clupanodonic	%		
Amino Acids			
Arginine	%	1.42	
Lysine (6)	%	1.34	0.18
Methionine	%	0.37	0.03
Cystine	%	0.35	
Tryptophan	%	0.27	
Histidine	%	0.55	
Threonine	%	0.88	
Isoleucine	%	0.98	
Leucine	%	1.87	
Phenylalanine	%	1.23	
Valine	%	1.15	
Tyrosine	%	0.87	
Taurine	%		
Glycine	%	1.85	
Aspartic Acid	%	1.40	

NUTRIENTS		Total	Supp (9)
Glutamic Acid	%	4.39	
Proline	%	1.56	
Serine	%	1.01	
Hydroxyproline	%		
Hydroxylysine	%		
Alanine	%	0.27	
Macro Minerals			
Calcium	%	1.24	1.11
Total Phosphorus	%	0.83	0.28
Phytate Phosphorus	%	0.26	
Available Phosphorus	%	0.56	0.28
Sodium	%	0.24	0.19
Chloride	%	0.36	0.31
Potassium	%	0.81	
Magnesium	%	0.29	0.04
Micro Minerals			
Iron	mg/kg	163.44	82.50
Copper	mg/kg	20.53	8.75
Manganese	mg/kg	102.71	52.70
Zinc	mg/kg	48.67	8.64
Cobalt	µg/kg	604.44	525.00
Iodine	µg/kg	867.77	775.00
Selenium	µg/kg	388.94	200.00
Fluorine	mg/kg	8.67	
Vitamins			
β-Carotene (2)	mg/kg	1.67	
Retinol (2)	µg/kg	6670.95	5812.50
Vitamin A (2)	iu/kg	22213.06	19375.00
Cholecalciferol (3)	µg/kg	73.70	72.50
Vitamin D (3)	iu/kg	2948.01	2900.00
α-Tocopherol (4)	mg/kg	100.90	81.14
Vitamin E (4)	iu/kg	111.02	89.25
Vitamin B_1 (Thiamine)	mg/kg	28.39	19.11
Vitamin B_2 (Riboflavin)	mg/kg	10.28	7.60
Vitamin B_6 (Pyridoxine)	mg/kg	18.87	14.45
Vitamin B_{12} (Cyanocobalamine)	µg/kg	19.23	17.75
Vitamin C (Ascorbic Acid)	mg/kg	1.33	
Vitamin K (Menadione)	mg/kg	4.14	3.72
Folic Acid (Vitamin B_9)	mg/kg	2.99	0.49
Nicotinic Acid (Vitamin PP) (6)	mg/kg	85.74	19.11
Pantothenic Acid (Vitamin $B_{3/5}$)	mg/kg	40.79	23.80
Choline (Vitamin $B_{4/7}$)	mg/kg	1422.37	366.60
Inositol	mg/kg	1839.97	
Biotin (Vitamin H) (6)	µg/kg	316.66	

Notes

1. All values are calculated using a moisture basis of 10%.
   Typical moisture levels will range between 9.5 - 11.5%.
2. a. Vitamin A includes Retinol and the Retinol equivalents of β-carotene.
   b. Retinol includes the Retinol equivalents of β-Carotene.
   c. 0.48 µg Retinol = 1 µg β-carotene = 1.6 iu Vitamin A activity
   d. 1 µg Retinol = 3.33* iu Vitamin A activity
   e. 1 iu Vitamin A = 0.3 µg Retinol = 0.6 µg β-carotene
   f. The standard analysis for Vitamin A does not detect β-carotene
3. 1µg Cholecalciferol (D_3) = 40.0 iu Vitamin D
4. 1 mg all-rac-α-tocopherol = 1.1 iu Vitamin E activity
   1 mg all-rac-α-tocopherol acetate = 1.0 iu Vitamin E activity
5. 1 MJ = 239.23 Kcalories = 239.23 Calories = 239,230 calories
6. These nutrients coming from natural raw materials such as cereals may have low availabilities due to the interactions with other compounds.
7. Based on in-vitro digestibility analysis.
8. AF Energy = Atwater Fuel Energy = ((CO%/100)*9000)+ ((CF%/100)*4000)+((NFE%/100)*4000)/239.23
9. Supplemented nutrients from manufactured and mined sources.
15. Calculated.

Appendix 2 Proximate composition

Dry Matter |Method (DM, AOAC Official method934.01)

Apparatus

Aluminium foils and porcelain crucible

Analytical weighing scale (Mettler AJ150, Toledo Ltd, Switzerland)
A desiccator

Sample mill (Tecator Cyclotec 1093, Sweden.)

Oven drier

Procedure

Pelleted feed samples were finely ground to pass through 1mm mesh sieve in a mill (Tecator Cyclotec 1093, Sweden). About 0.5 g of each sample was weighed into a pre washed and oven dried and weighed porcelain crucible. Porcelain and sample was then dried in a hot air oven at 105^{o}C for 24 hours. Porcelain with dried samples were removed from oven, cooled in a desiccator and weighed. Dry matter content of sample was calculated as follows:

Equation:

C : Wt of crucible(g)

CS_0 : Wt of crucible with fresh sample (g)

S0 : Wt of fresh sample (g), $S_0 = CS_0 - C$

CS1 : Wt of crucible with dry sample (g)

S1 : Wt of dried sample (g), $S_1 = CS_1 - C$

$$\text{DM (gDM/kg fresh sample)} = \frac{S_1}{S_0} \times 100$$

Ash Determination

Apparatus

Furnace (Carbolite, AAF11/18, England)

Analytical weighing scale (Mettler AJ150, Toledo Ltd, Switzerland)

Desiccator

Procedure:

Sample from DM analysis were ignited in a furnace and the temperature allowed rise slowly to 550^{o}C for 5 hours. Sample was removed cooled in a desiccator and weighed. Ash content of samples were expressed in percentages.

Equation:

C : Wt of crucible(g)

CS_0 : Wt of crucible with dried sample (g)

S0 : Wt of dried sample (g), S = CS-C

CS1 : Wt of crucible with ash (g)

S1 : Wt of ash (g), A = CA – C

$$\text{Ash (\%)} = \frac{A}{S} \times 100$$

Ether Extract (EE, AOAC official Method 920.39)

Apparatus:

A set of soxhlet extractor (thimble, flask, soxhlet extractor, condenser, heating mantle)

Analytical weighing scale

Condenser

Reagents

Petroleum ether 40 – 60^{o}C (solvent)

Procedure:

Adequate petroleum ether was measured into pre-oven dried round bottom flask. About 1.5 g of dried finely milled sample was weighed into a thimble and plugged with cotton wool at the top. Thimble with sample was then placed in an extractor and fitted to the flask. Extractor and flask were set on a heating mantle and connected to a condenser. The flask was heated until the solvent boiled gently to start extraction. This was continued for 6 hours. The flask was removed and residual solvent containing oil was oven dried at 60oC overnight. The flask with oil was cooled in a desiccator and weighed.

Equation:

T : Wt of thimble (g)

F : Wt of flask (g)

TS : Weight of thimble with dry sample (g)

S : Wight of dried sample (g), S = TS – T

FE : Weight of flask with ether extract

E : Wt of ether extract (g), E = FE –F

$$EE\ (g/kg\ DM) = \frac{E}{S} \times 100$$

Crude Protein (CP),

Apparatus

Elementar Vario Macro Cube (Germany). This machine determines Nitrogen, carbon and sulphur in three-in-one process for a similar sample.

Analytical scale

Thin foil cup

Procedure:

About 0.1 g of dried ground sample was weighed into a pre-tarred tin foil cup. This was carefully folded and squashed into pellet to expel air using a tool provided by Elementar. Carbon and nitrogen determination was done in a CN mode using combustion, post combustion and reduction tube in the furnace of the analyser. The sample was dropped into at combustion tube at 930oC via a carousel and ball valve. Oxygen was used to burn the sample and this was carried off in helium the combustion and reduction tubes, which were also heated, to detectors within the analyser. For sulphur analysis, the combustion and reduction tubes were at 1150oc and 850oC respectively. Before each run a set of standards were run to ensure that the analyser was working correctly. Standards were also run half way through as ample run as well. To check that the analyzer has performed correctly, a daily factor figure is worked out after each run and this should fall between 0.9 and 1.1. Runs that did not meet these criteria were discarded. Each element was analysed separately and a percentage figure was then obtained. Cp content was calculated by multiplying N content with 6.25.

Equation:

Fc : Wt of foil cup (g)

FcS : Wt of foil cup with sample (g)

S : Wt of sample

Np : Content in percent (%)

N : N in gram (g), N = Np/100 X100

CP (g/kg DM) = 6.25 X N

Acid detergent fibre (ADF) (van Soest, 1990),

Apparatus:

100 ml tubes fit to the rack on digestion chamber

A setoff digestion chamber (Gerhardt Kjeldaterm, Germany)

Sintered glass crucibles (porosity no. 1). These were pre washed, ashed at 550oC for 3 hours, cooled in desiccator, weighed and placed back into the desiccator until required

A set of Buchner flask and vacuum pump

Glass rod stirrer

Reagents:

Acid detergent solution (AD); Add 20 g cetyl trimethylammonium bromide (CTAB, Technical grade) to 1 L 0.5 M H2SO4 (added 27.7 ml H2SO4 (95 – 98%) to 972.3 ml H2O)

Acetone

Procedure:

About 0.5 g of dried ground samples were weighed into digestion tubes and 50 ml AD added. The tubes were placed into a set of digestion chamber and the temperature set at 120oC. The temperature was reduced if foaming became rapid to avoid spill over. This was allowed to boil for 1 hour. The tubes were then removed, swirled and the content of each filtered in a pre-weighed sintered glass crucible using light vacuum suction. The fibre residue in the crucible was washed by filling two thirds of crucible with hot water (90 – 100^{o}C). This was stirred, allowed to soak for a few minutes and drained using vacuum suction. The sides of the crucible were washed twice with hot water. The residue was again washed with acetone. The stirring

rod was also washed into the crucible and acetone drained off using suction pump. The crucible and fibre residue were oven dried at 100^{o}C overnight, cooled in the desiccator and weighed.

Calculation of fibre content:

Equation:

F : Wt of tube (g)

FS : Wt of tube with dried sample (g)

S : Wt of dried sample (g)

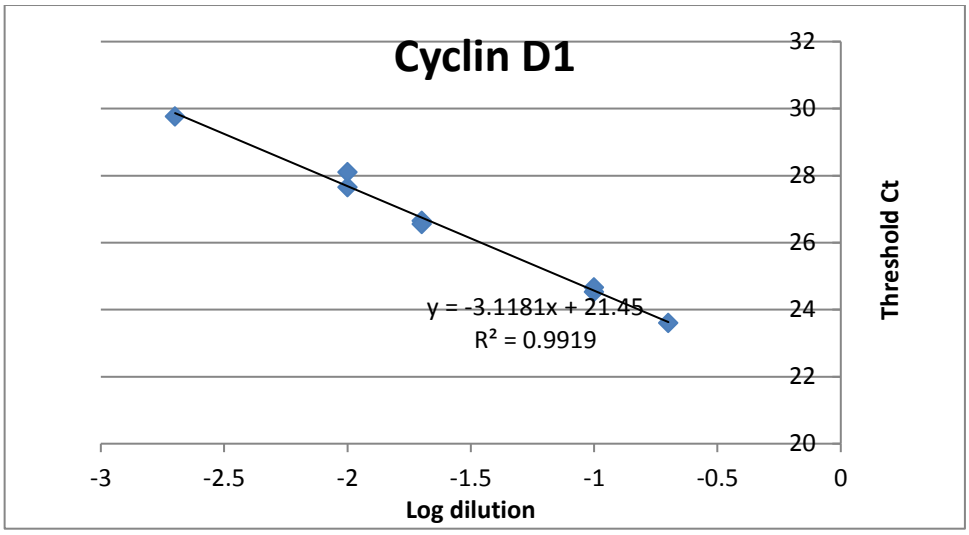
C : Wt of sintered glass crucible (g)

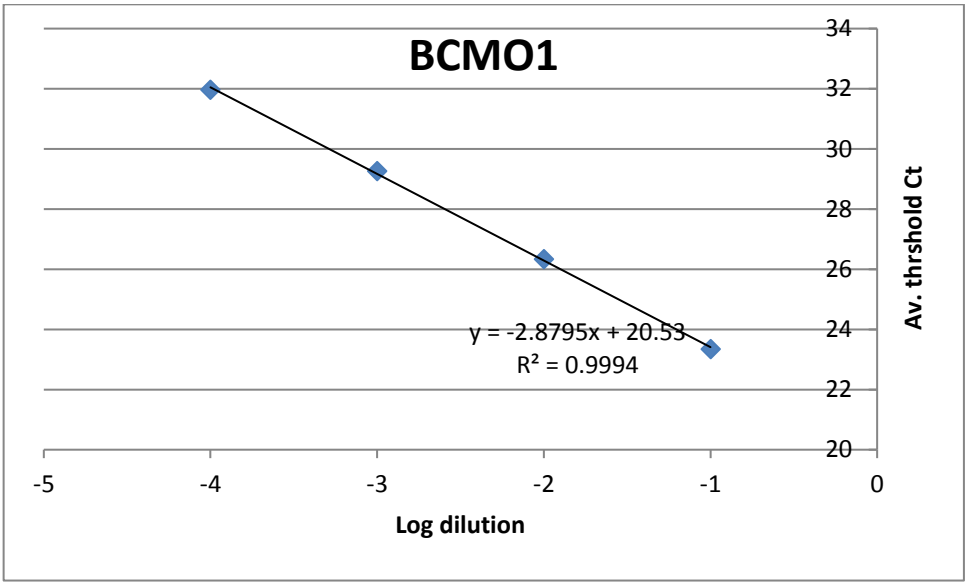
CR : Wt of sintered glass crucible with dried fibre residue (g)

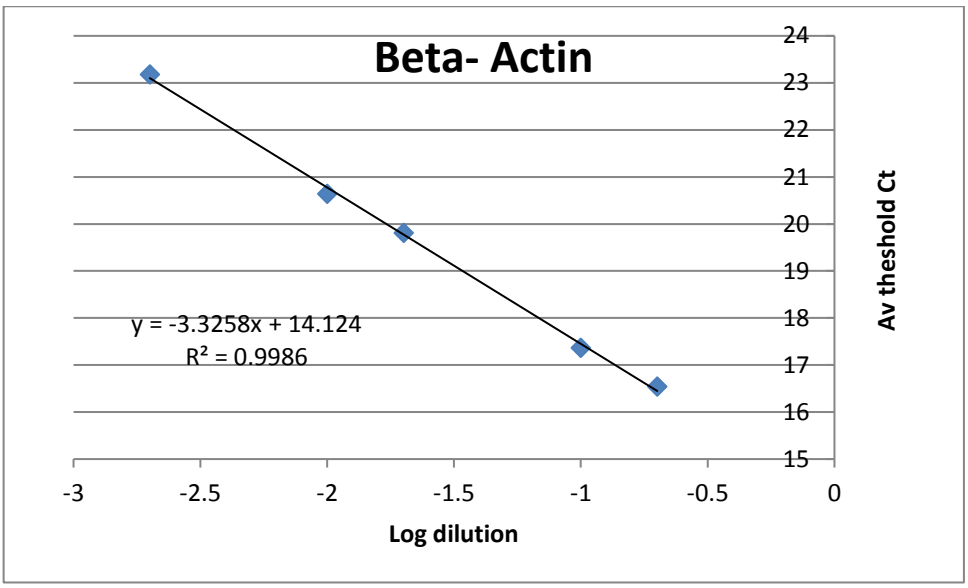
R : Wt of dried fibre residue (g); R = CR – C

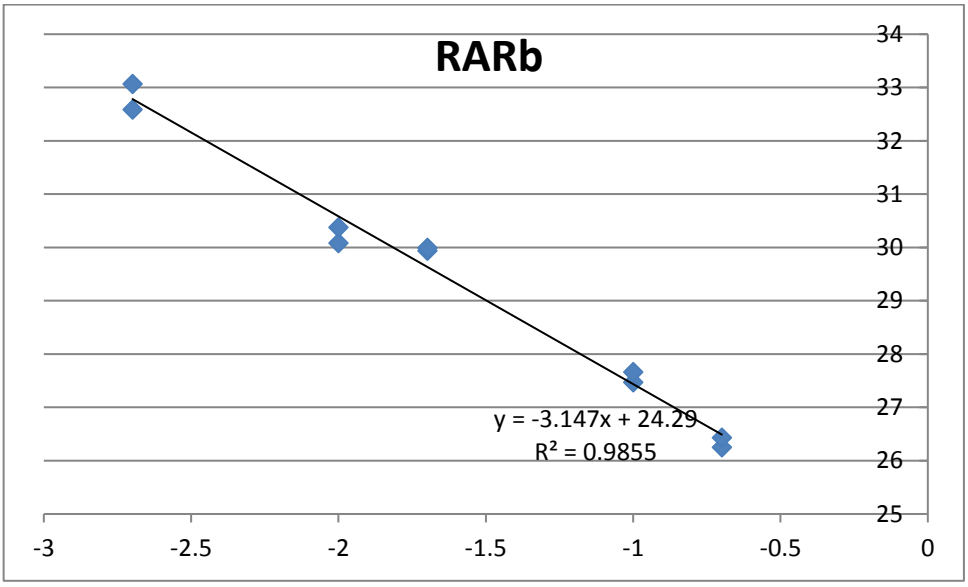
$$ADF\ (\%) = \frac{R - A}{S} \times 100$$

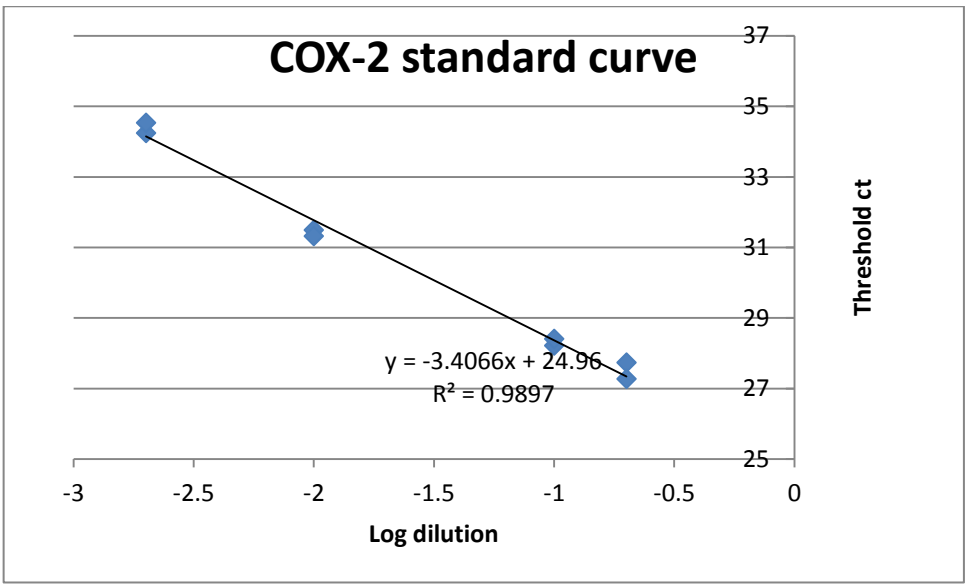
Appendix 3 Standard curves for Real Time PCR optimisation

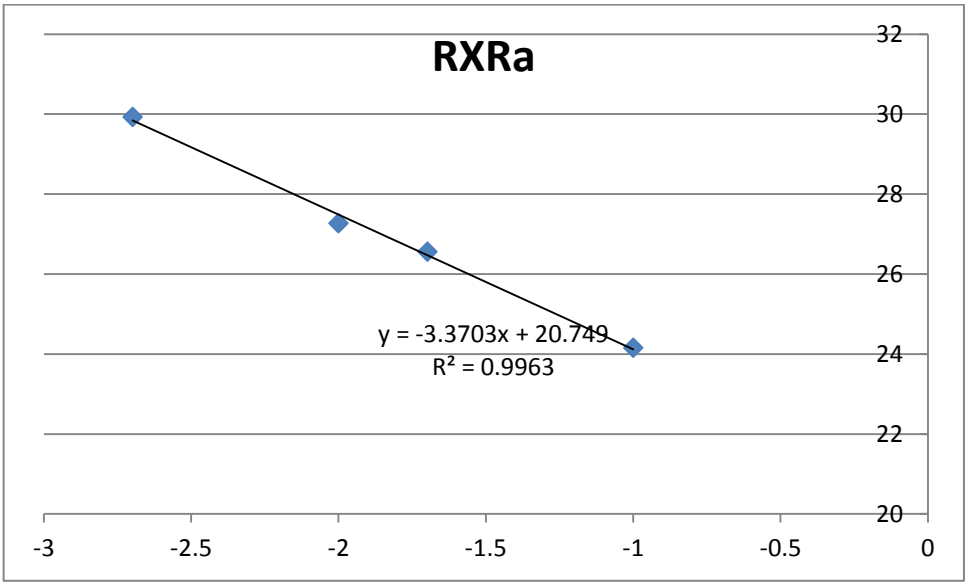


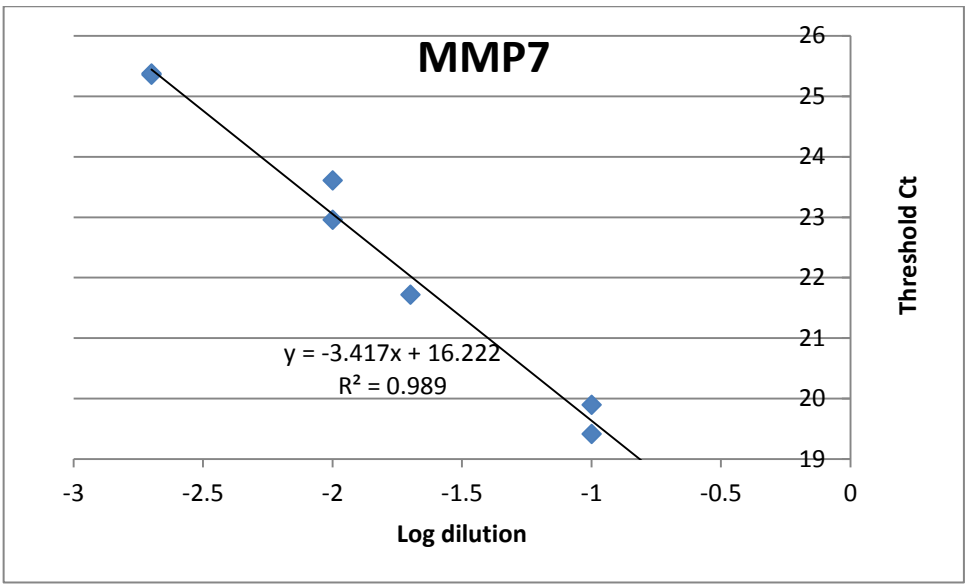



Beta- Actin
y = -3.3258x + 14.124
R² = 0.9986
Av theshold Ct
Log dilution
24
23
22
21
20
19
18
17
16
15
-3
-2.5
-2
-1.5
-1
-0.5
0

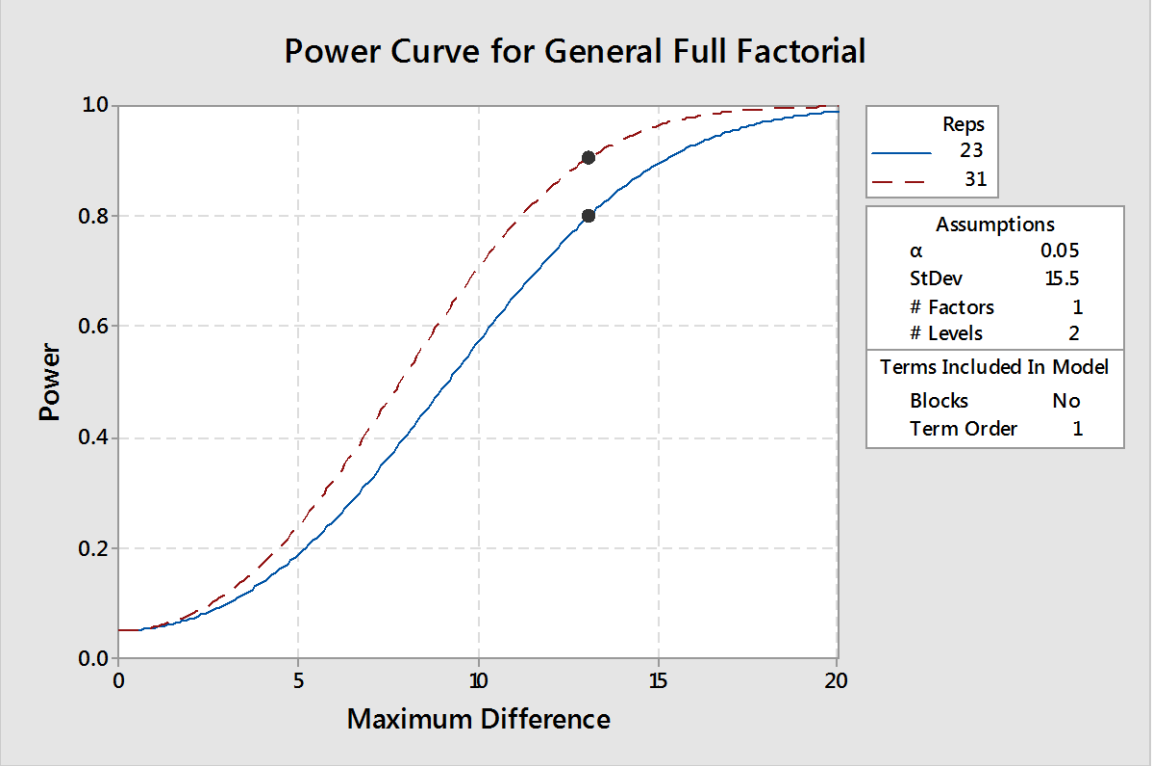

RARb
y = -3.147x + 24.29
R² = 0.9855
34
33
32
31
30
29
28
27
26
25
-3
-2.5
-2
-1.5
-1
-0.5
0


COX-2 standard curve
37
35
33
31
29
27
25
Threshold ct
y = -3.4066x + 24.96
R² = 0.9897
-3
-2.5
-2
-1.5
-1
-0.5
0
Log dilution


RXRa
32
30
28
26
24
22
20
y = -3.3703x + 20.749
R² = 0.9963
-3
-2.5
-2
-1.5
-1
-0.5
0


MMP7
26
25
24
23
22
21
20
19
Threshold Ct
y = -3.417x + 16.222
R² = 0.989
-3
-2.5
-2
-1.5
-1
-0.5
0
Log dilution

Appendix 4 Sample size determination plot



References

Agullo, G., Gamet-Payrastre, L., Manenti, S., Viala, C., Rémésy, C., Chap, H. and Payrastre, B. (1997) 'Relationship between flavonoid structure and inhibition of phosphatidylinositol 3-kinase: a comparison with tyrosine kinase and protein kinase C inhibition', *Biochemical Pharmacology*, 53(11), pp. 1649-1657.

Ahmed, D., Eide, P.W., Eilertsen, I.A., Danielsen, S.A., Eknæs, M., Hektoen, M., Lind, G.E. and Lothe, R.A. (2013) 'Epigenetic and genetic features of 24 colon cancer cell lines', *Oncogenesis*, 2(9), p. e71.

Ahn, B. and Ohshima, H. (2001) 'Suppression of intestinal polyposis in Apc(Min/+) mice by inhibiting nitric oxide production', *Cancer Res*, 61(23), pp. 8357-60.

Alabaster, O., Tang, Z., Frost, A. and Shivapurkar, N. (1995) 'Effect of β-carotene and wheat bran fiber on colonic aberrant crypt and tumor formation in rats exposed to azoxymethane and high dietary fat', *Carcinogenesis*, 16(1), pp. 127-132.

Alanko, J., Kurahashi, Y., Yoshimoto, T., Yamamoto, S. and Baba, K. (1994) 'Panaxynol, a polyacetylene compound isolated from oriental medicines, inhibits mammalian lipoxygenases', *Biochemical Pharmacology*, 48(10), pp. 1979-1981.

Alao, J.P. (2007) 'The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention', *Molecular cancer*, 6(1), p. 24.

Albanes, D., Heinonen, O.P., Taylor, P.R., Virtamo, J., Edwards, B.K., Rautalahti, M., Hartman, A.M., Palmgren, J., Freedman, L.S. and Haapakoski, J. (1996) 'α-Tocopherol and β-carotene supplements and lung cancer incidence in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study: effects of base-line characteristics and study compliance', *Journal of the National Cancer Institute*, 88(21), pp. 1560-1570.

Alink, G.M., Kuiper, H.A., Hollanders, V.M.H. and Koeman, J.H. (1993) 'Effect of heat processing and of vegetables and fruit in human diets on 1, 2-dimethylhydrazine-induced colon carcinogenesis in rats', *Carcinogenesis*, 14(3), pp. 519-524.

Allenby, G., Bocquel, M.-T., Saunders, M., Kazmer, S., Speck, J., Rosenberger, M., Lovey, A., Kastner, P., Grippo, J.F. and Chambon, P. (1993) 'Retinoic acid receptors and retinoid X

receptors: interactions with endogenous retinoic acids', *Proceedings of the National Academy of Sciences*, 90(1), pp. 30-34.

American Cancer, S. (2015) 'American Cancer Society: Cancer Facts and Figures 2015'. American Cancer Society Atlanta, GA.

Amos-Landgraf, J.M., Heijmans, J., Wielenga, M.C.B., Dunkin, E., Krentz, K.J., Clipson, L., Ederveen, A.G., Groothuis, P.G., Mosselman, S. and Muncan, V. (2014) 'Sex disparity in colonic adenomagenesis involves promotion by male hormones, not protection by female hormones', *Proceedings of the National Academy of Sciences*, 111(46), pp. 16514-16519.

Andarwulan, N., Kurniasih, D., Apriady, R.A., Rahmat, H., Roto, A.V. and Bolling, B.W. (2012) 'Polyphenols, carotenoids, and ascorbic acid in underutilized medicinal vegetables', *Journal of Functional Foods*.

Aoki, K. and Taketo, M.M. (2007) 'Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene', *Journal of cell science*, 120(19), pp. 3327-3335.

Aune, D., Lau, R., Chan, D.S.M., Vieira, R., Greenwood, D.C., Kampman, E. and Norat, T. (2011) 'Nonlinear reduction in risk for colorectal cancer by fruit and vegetable intake based on meta-analysis of prospective studies', *Gastroenterology*, 141(1), pp. 106-118.

Backlund, M.G., Mann, J.R., Holla, V.R., Buchanan, F.G., Tai, H.-H., Musiek, E.S., Milne, G.L., Katkuri, S. and DuBois, R.N. (2005) '15-Hydroxyprostaglandin dehydrogenase is down-regulated in colorectal cancer', *Journal of Biological Chemistry*, 280(5), pp. 3217-3223.

Baeg, G.H., Matsumine, A., Kuroda, T., Bhattacharjee, R.N., Miyashiro, I., Toyoshima, K. and Akiyama, T. (1995) 'The tumour suppressor gene product APC blocks cell cycle progression from G0/G1 to S phase', *The EMBO journal*, 14(22), p. 5618.

Balcerczak, E., Pasz-Walczak, G., Kumor, P., Panczyk, M., Kordek, R., Wierzbicki, R. and Mirowski, M. (2005) 'Cyclin D1 protein and CCND1 gene expression in colorectal cancer', *European Journal of Surgical Oncology (EJSO)*, 31(7), pp. 721-726.

Barker, D.J.P. (2004a) 'The developmental origins of adult disease', *Journal of the American College of Nutrition*, 23(sup6), pp. 588S-595S.

Barker, D.J.P. (2004b) 'The developmental origins of well-being', *PHILOSOPHICAL TRANSACTIONS-ROYAL SOCIETY OF LONDON SERIES B BIOLOGICAL SCIENCES*, pp. 1359-1366.

Bateson, P., Barker, D., Clutton-Brock, T., Deb, D., D'Udine, B., Foley, R.A., Gluckman, P., Godfrey, K., Kirkwood, T. and Lahr, M.M. (2004) 'Developmental plasticity and human health', *Nature*, 430(6998), pp. 419-421.

Beg, A.A. and Baltimore, D. (1996) 'An essential role for NF-kappaB in preventing TNF-alpha-induced cell death', *Science (New York, NY)*, 274(5288), p. 782.

Bendich, A. and Olson, J.A. (1989) 'Biological actions of carotenoids', *The FASEB journal*, 3(8), pp. 1927-1932.

Bernart, M.W., Cardellina, J.H., Balaschak, M.S., Alexander, M.R., Shoemaker, R.H. and Boyd, M.R. (1996) 'Cytotoxic falcarinol oxylipins from Dendropanax arboreus', *Journal of natural products*, 59(8), pp. 748-753.

Bernstein, B.E., Meissner, A. and Lander, E.S. (2007) 'The mammalian epigenome', *Cell*, 128(4), pp. 669-681.

Béroud, C. and Soussi, T. (1996) 'APC gene: database of germline and somatic mutations in human tumors and cell lines', *Nucleic acids research*, 24(1), pp. 121-124.

Bienz, M. (2002) 'The subcellular destinations of APC proteins', *Nature Reviews Molecular Cell Biology*, 3(5), pp. 328-338.

Bingham, S.A., Day, N.E., Luben, R., Ferrari, P., Slimani, N., Norat, T., Clavel-Chapelon, F., Kesse, E., Nieters, A. and Boeing, H. (2003) 'European Prospective Investigation into Cancer and Nutrition. Dietary fibre in food and protection against colorectal cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC): an observational study', *Lancet*, 361(9368), pp. 1496-1501.

Bingham, S.A., Norat, T., Moskal, A., Ferrari, P., Slimani, N., Clavel-Chapelon, F., Kesse, E., Nieters, A., Boeing, H. and Tjønneland, A. (2005) 'Is the association with fiber from foods in colorectal cancer confounded by folate intake?', *Cancer Epidemiology Biomarkers & Prevention*, 14(6), pp. 1552-1556.

Blot, W.J., Li, J.-Y., Taylor, P.R., Guo, W., Dawsey, S., Wang, G.-Q., Yang, C.S., Zheng, S.-F., Gail, M. and Li, G.-Y. (1993) 'Nutrition intervention trials in Linxian, China: supplementation with specific vitamin/mineral combinations, cancer incidence, and disease-specific mortality in the general population', *Journal of the National Cancer Institute*, 85(18), pp. 1483-1491.

Boardman, L.A., Morlan, B.W., Rabe, K.G., Petersen, G.M., Lindor, N.M., Nigon, S.K., Goldberg, J. and Gallinger, S. (2007) 'Colorectal cancer risks in relatives of young-onset cases: is risk the same across all first-degree relatives?', *Clinical Gastroenterology and Hepatology*, 5(10), pp. 1195-1198.

Boeing, H., Bechthold, A., Bub, A., Ellinger, S., Haller, D., Kroke, A., Leschik-Bonnet, E., Müller, M.J., Oberritter, H. and Schulze, M. (2012) 'Critical review: vegetables and fruit in the prevention of chronic diseases', *European journal of nutrition*, 51(6), pp. 637-663.

Boguski, M.S. and McCormick, F. (1993) 'Proteins regulating Ras and its relatives'.

Bonet, M.L., Puigserver, P., Serra, F., Ribot, J., Vázquez, F., Picó, C. and Palou, A. (1997) 'Retinoic acid modulates retinoid X receptor α and retinoic acid receptor α levels of cultured brown adipocytes', *FEBS letters*, 406(1), pp. 196-200.

Borrelli, F., Izzo, A.A., Di Carlo, G., Maffia, P., Russo, A., Maiello, F.M., Capasso, F. and Mascolo, N. (2002) 'Effect of a propolis extract and caffeic acid phenethyl ester on formation of aberrant crypt foci and tumors in the rat colon', *Fitoterapia*, 73, pp. S38-S43.

Boyes, J. and Bird, A. (1992a) 'Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein', *Embo j*, 11(1), pp. 327-33.

Boyes, J. and Bird, A. (1992b) 'Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein', *The EMBO journal*, 11(1), p. 327.

Brabletz, T., Jung, A., Dag, S., Hlubek, F. and Kirchner, T. (1999) 'β-Catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer', *The American journal of pathology*, 155(4), pp. 1033-1038.

Brandt, K., Christensen, L.P., Hansen-Moller, J., Hansen, S.L., Haraldsdottir, J., Jespersen, L., Purup, S., Kharazmi, A., Barkholt, V. and Frokiaer, H. (2004) 'Health promoting compounds in vegetables and fruits:: A systematic approach for identifying plant components with impact on human health', *Trends in food science & technology*, 15(7-8), pp. 384-393.

Brenner, C. and Duggan, D. (2004) *Oncogenomics: molecular approaches to cancer*. John Wiley & Sons.

Brown, J.R. and DuBois, R.N. (2005) 'COX-2: a molecular target for colorectal cancer prevention', *Journal of Clinical Oncology*, 23(12), pp. 2840-2855.

Cahill, D.P., Lengauer, C., Yu, J., Riggins, G.J., Willson, J.K., Markowitz, S.D., Kinzler, K.W. and Vogelstein, B. (1998) 'Mutations of mitotic checkpoint genes in human cancers', *Nature*, 392(6673), pp. 300-3.

Cahill, R.J., O'Sullivan, K.R., Mathias, P.M., Beattie, S., Hamilton, H. and O'Morain, C. (1993) 'Effects of vitamin antioxidant supplementation on cell kinetics of patients with adenomatous polyps', *Gut*, 34(7), pp. 963-7.

Cai, H., Marczylo, T.H., Teller, N., Brown, K., Steward, W.P., Marko, D. and Gescher, A.J. (2010) 'Anthocyanin-rich red grape extract impedes adenoma development in the ApcMin mouse: Pharmacodynamic changes and anthocyanin levels in the murine biophase', *European Journal of Cancer*, 46(4), pp. 811-817.

Çalişkan, O. and Aytekin Polat, A. (2011) 'Phytochemical and antioxidant properties of selected fig (< i> Ficus carica</i> L.) accessions from the eastern Mediterranean region of Turkey', *Scientia Horticulturae*, 128(4), pp. 473-478.

Castets, M., Broutier, L., Molin, Y., Brevet, M., Chazot, G., Gadot, N., Paquet, A., Mazelin, L., Jarrosson-Wuilleme, L. and Scoazec, J.Y. (2011) 'DCC constrains tumour progression via its dependence receptor activity', *Nature*.

Center, M.M., Jemal, A. and Ward, E. (2009) 'International trends in colorectal cancer incidence rates', *Cancer Epidemiology Biomarkers & Prevention*, 18(6), pp. 1688-1694.

Chen, L.C., Sly, L., Jones, C.S., Tarone, R. and De Luca, L.M. (1993) 'Differential effects of dietary β-carotene on papilloma and carcinoma formation induced by an initiation-promotion protocol in SENCAR mouse skin', *Carcinogenesis*, 14(4), pp. 713-717.

Chien, S.Y., Wu, Y.C., Chung, J.G., Yang, J.S., Lu, H.F., Tsou, M.F., Wood, W.G., Kuo, S.J. and Chen, D.R. (2009) 'Quercetin-induced apoptosis acts through mitochondrial-and caspase-3-dependent pathways in human breast cancer MDA-MB-231 cells', *Human & experimental toxicology*, 28(8), pp. 493-503.

Christensen, L.P. (1992) 'Acetylenes and related compounds in Anthemideae', *Phytochemistry*, 31(1), pp. 7-49.

Cianchi, F., Cortesini, C., Fantappiè, O., Messerini, L., Schiavone, N., Vannacci, A., Nistri, S., Sardi, I., Baroni, G. and Marzocca, C. (2003) 'Inducible nitric oxide synthase expression in human colorectal cancer: correlation with tumor angiogenesis', *The American journal of pathology*, 162(3), pp. 793-801.

Ciappio, E.D., Liu, Z., Brooks, R.S., Mason, J.B., Bronson, R.T. and Crott, J.W. (2011) 'Maternal B vitamin supplementation from preconception through weaning suppresses intestinal tumorigenesis in Apc1638N mouse offspring', *Gut*, p. gut. 2011.240291.

Cook, L.T., O'Reilly, G.A., Goran, M.I., Weigensberg, M.J., Spruijt-Metz, D. and Davis, J.N. (2013) 'Vegetable consumption linked to decreased hepatic fat deposition in overweight Latino youth', *The FASEB Journal*, 27(1_MeetingAbstracts), p. 112.3.

Corva, P.M. and Medrano, J.F. (2000) 'Diet effects on weight gain and body composition in high growth (hg/hg) mice', *Physiological genomics*, 3(1), pp. 17-23.

Cross, S.M., Sanchez, C.A., Morgan, C.A., Schimke, M.K., Ramel, S., Idzerda, R.L., Raskind, W.H. and Reid, B.J. (1995) 'A p53-dependent mouse spindle checkpoint', *Science*, 267(5202), pp. 1353-1356.

Curado, M.-P., Edwards, B., Shin, H.R., Storm, H., Ferlay, J., Heanue, M. and Boyle, P. (2007) *Cancer incidence in five continents, Volume IX*. IARC Press, International Agency for Research on Cancer.

Curran, S. and Murray, G.I. (2000) 'Matrix metalloproteinases: molecular aspects of their roles in tumour invasion and metastasis', *Eur J Cancer*, 36(13 Spec No), pp. 1621-30.

Curtin, K., Slattery, M.L. and Samowitz, W.S. (2011) 'CpG island methylation in colorectal cancer: past, present and future', *Pathology research international*, 2011.

Dahm, C.C., Keogh, R.H., Spencer, E.A., Greenwood, D.C., Key, T.J., Fentiman, I.S., Shipley, M.J., Brunner, E.J., Cade, J.E. and Burley, V.J. (2010) 'Dietary fiber and colorectal cancer risk: a nested case–control study using food diaries', *Journal of the National Cancer Institute*.

de Andrade Júnior, M.C. and Andrade, J.S. (2014) 'Amazonian Fruits: An Overview of Nutrients, Calories and Use in Metabolic Disorders', *Food and Nutrition Sciences*, 2014.

De la Chapelle, A. (2004) 'Genetic predisposition to colorectal cancer', *Nature Reviews Cancer*, 4(10), pp. 769-780.

de The, H., del Mar Vivanco-Ruiz, M., Tiollais, P., Stunnenberg, H. and Dejean, A. (1990) 'Identification of a retinoic acid responsive element in the retinoic acid receptor & beta; gene', *Nature*, 343(6254), pp. 177-180.

Dewick, P.M. (2011) *Medicinal natural products: a biosynthetic approach*. Wiley.

Dillard, A.C. and Lane, M.A. (2007a) 'Retinol decreases beta-catenin protein levels in retinoic acid-resistant colon cancer cell lines', *Mol Carcinog*, 46(4), pp. 315-29.

Dillard, A.C. and Lane, M.A. (2007b) 'Retinol increases β-catenin-RXRα binding leading to the increased proteasomal degradation of β-catenin and RXRα', *Nutrition and cancer*, 60(1), pp. 97-108.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery Jr, C.A., Butel, J.S. and Bradley, A. (1992) 'Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours', *Nature*, 356(6366), p. 215.

Donnellan, R. and Chetty, R. (1998) 'Cyclin D1 and human neoplasia', *Molecular Pathology*, 51(1), p. 1.

Duan, H. and Barringer, S.A. (2012) 'CHANGES IN FURAN AND OTHER VOLATILE COMPOUNDS IN SLICED CARROT DURING AIR-DRYING', *Journal of Food Processing and Preservation*, 36(1), pp. 46-54.

Duarte, M.S., Gionbelli, M.P., Paulino, P.V.R., Serão, N.V.L., Martins, T.S., Tótaro, P.I.S., Neves, C.A., Valadares Filho, S.C., Dodson, M.V. and Zhu, M. (2013) 'Effects of maternal nutrition on development of gastrointestinal tract of bovine fetus at different stages of gestation', *Livestock Science*, 153(1), pp. 60-65.

DuBois, R.N., Radhika, A., Reddy, B.S. and Entingh, A.J. (1996) 'Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors', *Gastroenterology*, 110(4), pp. 1259-1262.

Dusek et. al. (2015) 'Epidemiology of colorectal acncer: international comparison', (4th European Colorectal Cancer Days, BRNO2015-Prevention and Screening : 29-30 2015 Brno, Czeck Republic

).

Duthie, G.G., Duthie, S.J. and Kyle, J.A.M. (2000) 'Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants', *Nutrition Research Reviews*, 13(1), p. 79.

Easwaran, V., Pishvaian, M. and Byers, S. (1999) 'Cross-regulation of β-catenin–LEF/TCF and retinoid signaling pathways', *Current biology*, 9(23), pp. 1415-1419.

Eberhart, C.E., Coffey, R.J., Radhika, A., Giardiello, F.M., Ferrenbach, S. and DuBois, R.N. (1994) 'Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas', *Gastroenterology-Orlando*, 107(4), pp. 1183-1188.

Edwards, B.K., Ward, E., Kohler, B.A., Eheman, C., Zauber, A.G., Anderson, R.N., Jemal, A., Schymura, M.J., Lansdorp-Vogelaar, I., Seeff, L.C., van Ballegooijen, M., Goede, S.L. and Ries, L.A. (2010) 'Annual report to the nation on the status of cancer, 1975-2006, featuring colorectal cancer trends and impact of interventions (risk factors, screening, and treatment) to reduce future rates', *Cancer*, 116(3), pp. 544-73.

Ellerbroek, S.M. and Stack, M.S. (1999) 'Membrane associated matrix metalloproteinases in metastasis', *Bioessays*, 21(11), pp. 940-949.

Fearon, E.R. and Vogelstein, B. (1990) 'A genetic model for colorectal tumorigenesis', *Cell*, 61(5), pp. 759-767.

Feinberg, A.P. and Tycko, B. (2004) 'The history of cancer epigenetics', *Nature Reviews Cancer*, 4(2), pp. 143-153.

Felley-Bosco, E. (1998) 'Role of nitric oxide in genotoxicity: implication for carcinogenesis', *Cancer and Metastasis Reviews*, 17(1), pp. 25-37.

Femia, A.P. and Caderni, G. (2008) 'Rodent models of colon carcinogenesis for the study of chemopreventive activity of natural products', *Planta medica*, 74(13), pp. 1602-1607.

Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C. and Parkin, D.M. (2010) 'Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008', *International Journal of Cancer*, 127(12), pp. 2893-2917.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D. and Bray, F. (2015) 'Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012', *International Journal of Cancer*, 136(5), pp. E359-E386.

Fini, L., Piazzi, G., Daoud, Y., Selgrad, M., Maegawa, S., Garcia, M., Fogliano, V., Romano, M., Graziani, G. and Vitaglione, P. (2011) 'Chemoprevention of intestinal polyps in ApcMin/+ mice fed with western or balanced diets by drinking annurca apple polyphenol extract', *Cancer Prevention Research*, 4(6), pp. 907-915.

Food, U.S. and Drug, A. (2004) 'Exploratory data on furan in food', *Washington, DC: FDA (May 7, 2004, updated June 7, 2004, June 15, 2005 and October 27, 2006, available at: http://www. cfscan. fda. gov/about~ dms/furandat. htm1).*

Forsen, T., Eriksson, J.G., Tuomilehto, J., Teramo, K., Osmond, C. and Barker, D.J.P. (1997) 'Mother's weight in pregnancy and coronary heart disease in a cohort of Finnish men: follow up study', *Bmj*, 315(7112), pp. 837-840.

Freemantle, S.J., Spinella, M.J. and Dmitrovsky, E. (2003) 'Retinoids in cancer therapy and chemoprevention: promise meets resistance', *Oncogene*, 22(47), pp. 7305-7315.

Fu, M., Wang, C., Li, Z., Sakamaki, T. and Pestell, R.G. (2004) 'Minireview: Cyclin D1: normal and abnormal functions', *Endocrinology*, 145(12), pp. 5439-5447.

Fuchs, C.S., Giovannucci, E.L., Colditz, G.A., Hunter, D.J., Stampfer, M.J., Rosner, B., Speizer, F.E. and Willett, W.C. (1999) 'Dietary fiber and the risk of colorectal cancer and adenoma in women', *New England Journal of Medicine*, 340(3), pp. 169-176.

Fukami-Kobayashi, J. and Mitsui, Y. (1999) 'Cyclin D1 inhibits cell proliferation through binding to PCNA and cdk2', *Experimental cell research*, 246(2), pp. 338-347.

Gaire, M., Magbanua, Z., McDonnell, S., McNeil, L., Lovett, D.H. and Matrisian, L.M. (1994) 'Structure and expression of the human gene for the matrix metalloproteinase matrilysin', *Journal of Biological Chemistry*, 269(3), pp. 2032-2040.

Galiatsatos, P. and Foulkes, W.D. (2006) 'Familial adenomatous polyposis', *The American journal of gastroenterology*, 101(2), pp. 385-398.

Gebert, J.F., Moghal, N., Frangioni, J.V., Sugarbaker, D.J. and Neel, B.G. (1991) 'High frequency of retinoic acid receptor beta abnormalities in human lung cancer', *Oncogene*, 6(10), pp. 1859-68.

Gietzen, D.W., Hao, S. and Anthony, T.G. (2007) 'Mechanisms of food intake repression in indispensable amino acid deficiency', *Annu. Rev. Nutr.*, 27, pp. 63-78.

Gluckman, P.D., Hanson, M.A. and Spencer, H.G. (2005) 'Predictive adaptive responses and human evolution', *Trends in ecology & evolution*, 20(10), pp. 527-533.

Goel, A. and Boland, C.R. (2012) 'Epigenetics of Colorectal Cancer', *Gastroenterology*, 143(6), pp. 1442-1460.e1.

Grady, W.M. (2004) 'Genomic instability and colon cancer', *Cancer and Metastasis Reviews*, 23(1), pp. 11-27.

Green, S.E., Bradburn, D.M., Varma, J.S. and Burn, J. (1998) 'Hereditary non-polyposis colorectal cancer', *International journal of colorectal disease*, 13(1), pp. 3-12.

Gryfe, R., Bapat, B., Gallinger, S., Swallow, C., Redston, M. and Couture, J. (1997) 'Molecular biology of colorectal cancer', *Current problems in cancer*, 21(5), pp. 233-299.

Gundgaard, J., Nielsen, J.N., Olsen, J. and Sørensen, J. (2003) 'Increased intake of fruit and vegetables: estimation of impact in terms of life expectancy and healthcare costs†', *Public health nutrition*, 6(01), pp. 25-30.

Hansen, L. and Boll, P.M. (1986) 'Polyacetylenes in Araliaceae: their chemistry, biosynthesis and biological significance', *Phytochemistry*, 25(2), pp. 285-293.

Hansen, S.L., Purup, S. and Christensen, L.P. (2003) 'Bioactivity of falcarinol and the influenceof processing and storage on its content in carrots (Daucus carota L)', *Journal of the Science of Food and Agriculture*, 83(10), pp. 1010-1017.

Hao, X., Bose, M., Lambert, J.D., Ju, J., Lu, G., Lee, M.-J., Park, S., Husain, A., Wang, S. and Sun, Y. (2007) 'Inhibition of intestinal tumorigenesis in Apc Min/+ mice by green tea polyphenols (Polyphenon E) and individual catechins', *Nutrition and cancer*, 59(1), pp. 62-69.

Harborne, J.B. (1993) *The flavonoids advances in research since 1986*. Chapman & Hall/CRC.

Hawkins, N., Norrie, M., Cheong, K., Mokany, E., Ku, S.L., Meagher, A., O'Connor, T. and Ward, R. (2002) 'CpG island methylation in sporadic colorectal cancers and its relationship to microsatellite instability', *Gastroenterology*, 122(5), pp. 1376-1387.

Hemminki, A., Peltomäki, P., Mecklin, J.P., Järvinen, H., Salovaara, R., Nyström-Lahti, M., de la Chapelle, A. and Aaltonen, L.A. (1994) 'Loss of the wild type MLH1 gene is a feature of hereditary nonpolyposis colorectal cancer', *Nature genetics*, 8(4), pp. 405-410.

Henderson, B.R. (2000) 'Nuclear-cytoplasmic shuttling of APC regulates beta-catenin subcellular localization and turnover', *Nature cell biology*, 2(9), pp. 653-660.

Heyer, J., Yang, K., Lipkin, M., Edelmann, W. and Kucherlapati, R. (1999) 'Mouse models for colorectal cancer', *Oncogene*, 18(38), pp. 5325-5333.

Hood, J.K. and Silver, P.A. (1999) 'In or out? Regulating nuclear transport', *Current opinion in cell biology*, 11(2), pp. 241-247.

Hoppe, C.C., Evans, R.G., Bertram, J.F. and Moritz, K.M. (2007) 'Effects of dietary protein restriction on nephron number in the mouse', *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, 292(5), pp. R1768-R1774.

Hosotani and Kitagawa (2005) 'Effects of dietary protein, fat and beta-carotene levels on beta-carotene absorption in rats', *International journal for vitamin and nutrition research*, 75(4), pp. 274-280.

Howe, G.R., Benito, E., Castelleto, R., Cornée, J., Estève, J., Gallagher, R.P., Iscovich, J.M., Deng-ao, J., Kaaks, R. and Kune, G.A. (1992) 'Dietary intake of fiber and decreased risk of cancers of the colon and rectum: evidence from the combined analysis of 13 case-control studies', *Journal of the National Cancer Institute*, 84(24), pp. 1887-1896.

Hu, R., Khor, T.O., Shen, G., Jeong, W.S., Hebbar, V., Chen, C., Xu, C., Reddy, B., Chada, K. and Kong, A.N.T. (2006) 'Cancer chemoprevention of intestinal polyposis in ApcMin/+ mice by sulforaphane, a natural product derived from cruciferous vegetable', *Carcinogenesis*, 27(10), pp. 2038-2046.

Hughes, L.A.E., Khalid-de Bakker, C.A.J., Smits, K.M., van den Brandt, P.A., Jonkers, D., Ahuja, N., Herman, J.G., Weijenberg, M.P. and van Engeland, M. (2012) 'The CpG island methylator phenotype in colorectal cancer: progress and problems', *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, 1825(1), pp. 77-85.

Hulit, J., Wang, C., Li, Z., Albanese, C., Rao, M., Di Vizio, D., Shah, S., Byers, S.W., Mahmood, R. and Augenlicht, L.H. (2004) 'Cyclin D1 genetic heterozygosity regulates colonic epithelial cell differentiation and tumor number in ApcMin mice', *Molecular and cellular biology*, 24(17), pp. 7598-7611.

Hung, H.-C., Joshipura, K.J., Jiang, R., Hu, F.B., Hunter, D., Smith-Warner, S.A., Colditz, G.A., Rosner, B., Spiegelman, D. and Willett, W.C. (2004) 'Fruit and vegetable intake and risk of major chronic disease', *Journal of the National Cancer Institute*, 96(21), pp. 1577-1584.

Hwang, H.J., Park, H.J., Chung, H.J., Min, H.Y., Park, E.J., Hong, J.Y. and Lee, S.K. (2006) 'Inhibitory effects of caffeic acid phenethyl ester on cancer cell metastasis mediated by the down-regulation of matrix metalloproteinase expression in human HT1080 fibrosarcoma cells', *J Nutr Biochem*, 17(5), pp. 356-62.

Ireson, C., Orr, S., Jones, D.J., Verschoyle, R., Lim, C.K., Luo, J.L., Howells, L., Plummer, S., Jukes, R., Williams, M., Steward, W.P. and Gescher, A. (2001) 'Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and

evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production', *Cancer Res*, 61(3), pp. 1058-64.

Janout, V. and Kollárová, H. (2001) 'Epidemiology of colorectal cancer', *ACTA-UNIVERSITATIS PALACKIANAE OLOMUCENSIS FACULTATIS MEDICAE*, pp. 5-10.

Jansen, M.C.J.F., Bueno-de-Mesquita, H.B., Buzina, R., Fidanza, F., Menotti, A., Blackburn, H., Nissinen, A.M., Kok, F.J. and Kromhout, D. (1999) 'Dietary fiber and plant foods in relation to colorectal cancer mortality: the Seven Countries Study', *International journal of cancer*, 81(2), pp. 174-179.

Jensen, M.M., Jørgensen, H., Halekoh, U., Watzl, B., Thorup-Kristensen, K. and Lauridsen, C. (2012) 'Health biomarkers in a rat model after intake of organically grown carrots', *Journal of the Science of Food and Agriculture*, 92(15), pp. 2936-2943.

Johnson, J.R., Lacey, J.V., Lazovich, D., Geller, M.A., Schairer, C., Schatzkin, A. and Flood, A. (2009) 'Menopausal hormone therapy and risk of colorectal cancer', *Cancer Epidemiology Biomarkers & Prevention*, 18(1), pp. 196-203.

Johnson, M. and Pace, R.D. (2010) 'Sweet potato leaves: properties and synergistic interactions that promote health and prevent disease', *Nutrition Reviews*, 68(10), pp. 604-615.

Johnson, R.L. and Fleet, J.C. (2013) 'Animal models of colorectal cancer', *Cancer and Metastasis Reviews*, 32(1-2), pp. 39-61.

Jones, P.A. and Baylin, S.B. (2007) 'The epigenomics of cancer', *Cell*, 128(4), pp. 683-692.

Jones, P.A. and Takai, D. (2001) 'The role of DNA methylation in mammalian epigenetics', *Science*, 293(5532), pp. 1068-1070.

Ju, J., Hong, J., Zhou, J., Pan, Z., Bose, M., Liao, J., Yang, G., Liu, Y.Y., Hou, Z. and Lin, Y. (2005) 'Inhibition of intestinal tumorigenesis in Apcmin/+ mice by (−)-epigallocatechin-3-gallate, the major catechin in green tea', *Cancer research*, 65(22), p. 10623.

Kállay, E., Adlercreutz, H., Farhan, H., Lechner, D., Bajna, E., Gerdenitsch, W., Campbell, M. and Cross, H.S. (2002) 'Phytoestrogens regulate vitamin D metabolism in the mouse colon: relevance for colon tumor prevention and therapy', *The Journal of nutrition*, 132(11), pp. 3490S-3493S.

Kanai, Y. and Hirohashi, S. (2007) 'Alterations of DNA methylation associated with abnormalities of DNA methyltransferases in human cancers during transition from a precancerous to a malignant state', *Carcinogenesis*, 28(12), pp. 2434-2442.

Kane, M.F., Loda, M., Gaida, G.M., Lipman, J., Mishra, R., Goldman, H., Jessup, J.M. and Kolodner, R. (1997) 'Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines', *Cancer research*, 57(5), pp. 808-811.

Karim, B.O. and Huso, D.L. (2013) 'Mouse models for colorectal cancer', *Am J Cancer Res*, 3(3), pp. 240-250.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B. and Craig, R.W. (1991) 'Participation of p53 protein in the cellular response to DNA damage', *Cancer research*, 51(23 Part 1), pp. 6304-6311.

Kinzler, K.W. and Vogelstein, B. (1996a) 'Lessons from hereditary colorectal cancer', *Cell*, 87(2), pp. 159-170.

Kinzler, K.W. and Vogelstein, B. (1996b) 'Lessons from Hereditary Review Colorectal Cancer', *Cell*, 87, pp. 159-170.

Kleiner, D.E. and Stetler-Stevenson, W.G. (1999) 'Matrix metalloproteinases and metastasis', *Cancer chemotherapy and pharmacology*, 43(1), pp. S42-S51.

Knekt, P., Järvinen, R., Seppänen, R., Heliövaara, M., Teppo, L., Pukkala, E. and Aromaa, A. (1997) 'Dietary flavonoids and the risk of lung cancer and other malignant neoplasms', *American Journal of Epidemiology*, 146(3), pp. 223-230.

Kobæk-Larsen, M., Christensen, L.P., Vach, W., Ritskes-Hoitinga, J. and Brandt, K. (2005) 'Inhibitory effects of feeding with carrots or (-)-falcarinol on development of azoxymethane-induced preneoplastic lesions in the rat colon', *Journal of agricultural and food chemistry*, 53(5), pp. 1823-1827.

Kondo, Y. and Issa, J.P.J. (2004) 'Epigenetic changes in colorectal cancer', *Cancer and Metastasis Reviews*, 23(1), pp. 29-39.

Koushik, A., Hunter, D.J., Spiegelman, D., Beeson, W.L., van Den Brandt, P.A., Buring, J.E., Calle, E.E., Cho, E., Fraser, G.E. and Freudenheim, J.L. (2007) 'Fruits, vegetables, and colon cancer risk in a pooled analysis of 14 cohort studies', *Journal of the National Cancer Institute*, 99(19), pp. 1471-1483.

Krimpenfort, P., Song, J.Y., Proost, N., Zevenhoven, J., Jonkers, J. and Berns, A. (2012) 'Deleted in colorectal carcinoma suppresses metastasis in p53-deficient mammary tumours', *Nature*, 482(7386), pp. 538-541.

Krinsky, N.I. (1989) 'Antioxidant functions of carotenoids', *Free Radical Biology and Medicine*, 7(6), pp. 617-635.

Kris-Etherton, P.M., Hecker, K.D., Bonanome, A., Coval, S.M., Binkoski, A.E., Hilpert, K.F., Griel, A.E. and Etherton, T.D. (2002) 'Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer', *The American journal of medicine*, 113, p. 71S.

Kubota, M., Shimizu, M., Sakai, H., Yasuda, Y., Terakura, D., Baba, A., Ohno, T., Tsurumi, H., Tanaka, T. and Moriwaki, H. (2012) 'Preventive effects of curcumin on the development of azoxymethane-induced colonic preneoplastic lesions in male C57BL/KsJ-db/db obese mice', *Nutrition and cancer*, 64(1), pp. 72-79.

Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V. and Kastan, M.B. (1992) 'Wild-type p53 is a cell cycle checkpoint determinant following irradiation', *Proceedings of the National Academy of Sciences*, 89(16), pp. 7491-7495.

Lal, G. and Gallinger, S. (2000) *Seminars in surgical oncology*. Wiley Online Library.

Langley-Evans, S.C. (2000) 'Critical differences between two low protein diet protocols in the programming of hypertension in the rat', *International journal of food sciences and nutrition*, 51(1), pp. 11-17.

Langley-Evans, S.C. (2006) 'Developmental programming of health and disease', *Proceedings of the Nutrition Society*, 65(1), pp. 97-105.

Langley-Evans, S.C. and McMullen, S. (2010) 'Developmental origins of adult disease', *Medical principles and practice*, 19(2), pp. 87-98.

Langley-Evans, S.C., Bellinger, L. and McMullen, S. (2005) 'Animal models of programming: early life influences on appetite and feeding behaviour', *Maternal & child nutrition*, 1(3), pp. 142-148.

Langley, S.C. and Jackson, A.A. (1994) 'Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets', *Clinical science*, 86(2), pp. 217-222.

Lazinski, M.J., Shea, A.K. and Steiner, M. (2008) 'Effects of maternal prenatal stress on offspring development: a commentary', *Archives of women's mental health*, 11(5-6), pp. 363-375.

Leclerc, D., Cao, Y., Deng, L., Mikael, L.G., Wu, Q. and Rozen, R. (2013) 'Differential gene expression and methylation in the retinoid/PPARA pathway and of tumor suppressors may modify intestinal tumorigenesis induced by low folate in mice', *Mol Nutr Food Res*, 57(4), pp. 686-97.

Lee, M.-O., Han, S.-Y., Jiang, S., Park, J.H. and Kim, S.J. (2000a) 'Differential effects of retinoic acid on growth and apoptosis in human colon cancer cell lines associated with the induction of retinoic acid receptor β', *Biochemical pharmacology*, 59(5), pp. 485-496.

Lee, Y.J., Liao, P.H., Chen, W.K. and Yang, C.C. (2000b) 'Preferential cytotoxicity of caffeic acid phenethyl ester analogues on oral cancer cells', *Cancer letters*, 153(1), pp. 51-56.

Leslie, A., Carey, F.A., Pratt, N.R. and Steele, R.J.C. (2002) 'The colorectal adenoma–carcinoma sequence', *British Journal of Surgery*, 89(7), pp. 845-860.

Levine, A.J., Momand, J. and Finlay, C.A. (1991) 'The p53 tumour suppressor gene', *Nature*, 351(6326), p. 453.

Levine, A.J., Perry, M.E., Chang, A., Silver, A., Dittmer, D., Wu, M. and Welsh, D. (1994) 'The 1993 Walter Hubert Lecture: the role of the p53 tumour-suppressor gene in tumorigenesis', *British journal of cancer*, 69(3), p. 409.

Lietz, G., Lange, J. and Rimbach, G. (2010) 'Molecular and dietary regulation of β, β-carotene 15, 15′-monooxygenase 1 (BCMO1)', *Archives of biochemistry and biophysics*, 502(1), pp. 8-16.

Lim, G.C.C., Yahaya, H. and Lim, T.O. (2003) 'The first report of the national cancer registry cancer incidence in Malaysia', *Kuala Lumpur: Ministry of Health*, pp. 57-58.

Liu, B., Nicolaides, N.C., Markowitz, S., Willson, J.K.V., Parsons, R.E., Jen, J., Papadopolous, N., Peltomäki, P., de la Chapelle, A. and Hamilton, S.R. (1995) 'Mismatch repair gene defects in sporadic colorectal cancers with microsatellite instability', *Nature genetics*, 9(1), pp. 48-55.

Liu, B., Parsons, R., Papadopoulos, N., Nicolaides, N.C., Lynch, H.T., Watson, P., Jass, J.R., Dunlop, M., Wyllie, A. and Peltomäki, P. (1996) 'Analysis of mismatch repair genes in hereditary non–polyposis colorectal cancer patients', *Nature medicine*, 2(2), pp. 169-174.

Liu, C.H., Chang, S.-H., Narko, K., Trifan, O.C., Wu, M.-T., Smith, E., Haudenschild, C., Lane, T.F. and Hla, T. (2001) 'Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice', *Journal of Biological Chemistry*, 276(21), pp. 18563-18569.

Lodish, H.F., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D. and Darnell, J. (2000) *Molecular cell biology*. WH Freeman New York.

Lotan, R., Xu, X.-C., Lippman, S.M., Ro, J.Y., Lee, J.S., Lee, J.J. and Hong, W.K. (1995) 'Suppression of Retinoic Acid Receptor–β in Premalignant Oral Lesions and Its Up-Regulation by Isotretinoin', *New England Journal of Medicine*, 332(21), pp. 1405-1411.

Lowe, S.W., Ruley, H.E., Jacks, T. and Housman, D.E. (1993) 'p53-dependent apoptosis modulates the cytotoxicity of anticancer agents', *Cell*, 74(6), p. 957.

Lu, D., Cottam, H.B., Corr, M. and Carson, D.A. (2005) 'Repression of beta-catenin function in malignant cells by nonsteroidal antiinflammatory drugs', *Proc Natl Acad Sci U S A*, 102(51), pp. 18567-71.

Luyckx, V.A. and Brenner, B.M. (2005) 'Low birth weight, nephron number, and kidney disease', *Kidney Int Suppl*, (97), pp. S68-77.

Lynch, H.T. and de la Chapelle, A. (1999) 'Genetic susceptibility to non-polyposis colorectal cancer', *Journal of medical genetics*, 36(11), pp. 801-818.

Lynch, H.T. and de la Chapelle, A. (2003) 'Hereditary colorectal cancer', *N Engl J Med*, 348(10), pp. 919-932.

Lynch, H.T., Smyrk, T. and Lynch, J.F. (1996) 'Overview of natural history, pathology, molecular genetics and management of HNPCC (Lynch syndrome)', *International journal of cancer*, 69(1), pp. 38-43.

Mahmoud, N.N., Carothers, A.M., Grunberger, D., Bilinski, R.T., Churchill, M.R., Martucci, C., Newmark, H.L. and Bertagnolli, M.M. (2000) 'Plant phenolics decrease intestinal tumors in an animal model of familial adenomatous polyposis', *Carcinogenesis*, 21(5), p. 921.

Malisetty, V.S., Cooma, I. and Reddy, B.S. (2002) 'Caspase-3-activity, and apoptosis induction by a combination of HMG-Coa reductase inhibitor and COX-2 inhibitors: a novel approach in developing effective chemopreventive regimens', *Int. J. Oncol*, 20, pp. 753-759.

Mathers, J.C. (2002) 'Pulses and carcinogenesis: potential for the prevention of colon, breast and other cancers', *British Journal of Nutrition*, 88(S3), pp. 273-279.

Mathew, V. and Ayyar, S.V. (2012) 'Developmental origins of adult diseases', *Indian journal of endocrinology and metabolism*, 16(4), p. 532.

Matsubara, N. (2012) 'Epigenetic regulation and colorectal cancer', *Diseases of the Colon & Rectum*, 55(1), p. 96.

McCullough, M.L., Robertson, A.S., Chao, A., Jacobs, E.J., Stampfer, M.J., Jacobs, D.R., Diver, W.R., Calle, E.E. and Thun, M.J. (2003) 'A prospective study of whole grains, fruits, vegetables and colon cancer risk', *Cancer Causes & Control*, 14(10), pp. 959-970.

McKay, J.A. and Mathers, J.C. (2011) 'Diet induced epigenetic changes and their implications for health', *Acta Physiologica*, 202(2), pp. 103-118.

McKay, J.A., Williams, E.A. and Mathers, J.C. (2008a) 'Gender-specific modulation of tumorigenesis by folic acid supply in the Apc mouse during early neonatal life', *Br J Nutr*, 99(3), pp. 550-8.

McKay, J.A., Williams, E.A. and Mathers, J.C. (2008b) 'Gender-specific modulation of tumorigenesis by folic acid supply in the Apc+/Min mouse during early neonatal life', *British journal of nutrition*, 99(03), pp. 550-558.

McMillen, I.C. and Robinson, J.S. (2005) 'Developmental origins of the metabolic syndrome: prediction, plasticity, and programming', *Physiol Rev*, 85(2), pp. 571-633.

McMullen, S., Gardner, D.S. and Langley-Evans, S.C. (2004) 'Prenatal programming of angiotensin II type 2 receptor expression in the rat', *British journal of nutrition*, 91(01), pp. 133-140.

Mehlen, P. and Fearon, E.R. (2004) 'Role of the dependence receptor DCC in colorectal cancer pathogenesis', *Journal of clinical oncology*, 22(16), pp. 3420-3428.

Mehlen, P., Rabizadeh, S., Snipas, S.J., Assa-Munt, N., Salvesen, G.S. and Bredesen, D.E. (1998) 'The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis', *Nature*, 395(6704), pp. 801-804.

Michels, K.B., Giovannucci, E., Joshipura, K.J., Rosner, B.A., Stampfer, M.J., Fuchs, C.S., Colditz, G.A., Speizer, F.E. and Willett, W.C. (2000) 'Prospective study of fruit and vegetable consumption and incidence of colon and rectal cancers', *Journal of the National Cancer Institute*, 92(21), pp. 1740-1752.

Miyaki, M., Konishi, M., Tanaka, K., Kikuchi-Yanoshita, R., Muraoka, M., Yasuno, M., Igari, T., Koike, M., Chiba, M. and Mori, T. (1997) 'Germline mutation of MSH6 as the cause of hereditary nonpolyposis colorectal cancer', *Nat Genet*, 17(3), pp. 271-2.

Mohammed, A., Janakiram, N.B., Li, Q., Choi, C.-I., Zhang, Y., Steele, V.E. and Rao, C.V. (2011) 'Chemoprevention of colon and small intestinal tumorigenesis in APCMin/+ mice by licofelone, a novel dual 5-LOX/COX inhibitor: potential implications for human colon cancer prevention', *Cancer prevention research*, 4(12), pp. 2015-2026.

Mortensen, E.L., Wang, T., Malte, H., Raubenheimer, D. and Mayntz, D. (2010) 'Maternal preconceptional nutrition leads to variable fat deposition and gut dimensions of adult offspring mice (C57BL/6JBom)', *Int J Obes (Lond)*, 34(11), pp. 1618-24.

Moser, A.R., Pitot, H.C. and Dove, W.F. (1990) 'A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse', *Science*, 247(4940), pp. 322-4.

Murphy, E.A., Davis, J.M., McClellan, J.L. and Carmichael, M.D. (2011) 'Quercetin's effects on intestinal polyp multiplicity and macrophage number in the Apc min/+ mouse', *Nutrition and cancer*, 63(3), pp. 421-426.

Murphy, N., Norat, T., Ferrari, P., Jenab, M., Bueno-de-Mesquita, B., Skeie, G., Dahm, C.C., Overvad, K., Olsen, A. and Tjønneland, A. (2012) 'Dietary fibre intake and risks of cancers of the colon and rectum in the European prospective investigation into cancer and nutrition (EPIC)', *PloS one*, 7(6), p. e39361.

Myzak, M.C., Dashwood, W.M., Orner, G.A., Ho, E. and Dashwood, R.H. (2006) 'Sulforaphane inhibits histone deacetylase in vivo and suppresses tumorigenesis in Apc-minus mice', *Faseb j*, 20(3), pp. 506-8.

Nagaoka, T., Banskota, A.H., Tezuka, Y., Harimaya, Y., Koizumi, K., Saiki, I. and Kadota, S. (2003) 'Inhibitory effects of caffeic acid phenethyl ester analogues on experimental lung metastasis of murine colon 26-L5 carcinoma cells', *Biological and Pharmaceutical Bulletin*, 26(5), pp. 638-641.

Nautiyal, J., Banerjee, S., Kanwar, S.S., Yu, Y., Patel, B.B., Sarkar, F.H. and Majumdar, A.P.N. (2011) 'Curcumin enhances dasatinib-induced inhibition of growth and transformation of colon cancer cells', *International Journal of Cancer*, 128(4), pp. 951-961.

Neel, J.V. (1962) 'Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"?', *American journal of human genetics*, 14(4), p. 353.

Negrini, S., Gorgoulis, V.G. and Halazonetis, T.D. (2010a) 'Genomic instability--an evolving hallmark of cancer', *Nat Rev Mol Cell Biol*, 11(3), pp. 220-8.

Negrini, S., Gorgoulis, V.G. and Halazonetis, T.D. (2010b) 'Genomic instability—an evolving hallmark of cancer', *Nature reviews Molecular cell biology*, 11(3), pp. 220-228.

Neufeld, K.L., Zhang, F., Cullen, B.R. and White, R.L. (2000) 'APC-mediated downregulation of β-catenin activity involves nuclear sequestration and nuclear export', *EMBO reports*, 1(6), pp. 519-523.

Nichenametla, S.N., Taruscio, T.G., Barney, D.L. and Exon, J.H. (2006) 'A review of the effects and mechanisms of polyphenolics in cancer', *Critical reviews in food science and nutrition*, 46(2), pp. 161-183.

Niculescu, M.D. and Zeisel, S.H. (2002) 'Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline', *The Journal of nutrition*, 132(8), pp. 2333S-2335S.

Nomura, A.M.Y., Wilkens, L.R., Murphy, S.P., Hankin, J.H., Henderson, B.E., Pike, M.C. and Kolonel, L.N. (2008) 'Association of vegetable, fruit, and grain intakes with colorectal cancer: the Multiethnic Cohort Study', *The American journal of clinical nutrition*, 88(3), pp. 730-737.

Nugent, K.P., Phillips, R.K., Hodgson, S.V., Cottrell, S., Smith-Ravin, J., Pack, K. and Bodmer, W.F. (1994) 'Phenotypic expression in familial adenomatous polyposis: partial prediction by mutation analysis', *Gut*, 35(11), pp. 1622-1623.

Olschwang, S., Tiret, A., Laurent-Puig, P., Muleris, M., Parc, R. and Thomas, G. (1993) 'Restriction of ocular fundus lesions to a specific subgroup of< i> APC</i> mutations in adenomatous polyposis coli patients', *Cell*, 75(5), pp. 959-968.

Olson, J.A. and Hayaishi, O. (1965) 'The enzymatic cleavage of beta-carotene into vitamin A by soluble enzymes of rat liver and intestine', *Proceedings of the National Academy of Sciences of the United States of America*, 54(5), p. 1364.

Omenn, G.S., Goodman, G.E., Thornquist, M.D., Balmes, J., Cullen, M.R., Glass, A., Keogh, J.P., Meyskens Jr, F.L., Valanis, B. and Williams Jr, J.H. (1996) 'Effects of a combination of beta carotene and vitamin A on lung cancer and cardiovascular disease', *New England journal of medicine*, 334(18), pp. 1150-1155.

Osbourne, J.W. (2002) 'Notes on the Use of Data Transformation', *Practical Assessment, Research & Evaluation*, 8(6), p. n6.

Osburn, D.L., Shao, G., Seidel, H.M. and Schulman, I.G. (2001) 'Ligand-dependent degradation of retinoid X receptors does not require transcriptional activity or coactivator interactions', *Mol Cell Biol*, 21(15), pp. 4909-18.

Oshima, M., Dinchuk, J.E., Kargman, S.L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J.M., Evans, J.F. and Taketo, M.M. (1996) 'Suppression of intestinal polyposis in Apc Δ716 knockout mice by inhibition of cyclooxygenase 2 (COX-2)', *Cell*, 87(5), pp. 803-809.

Osmond, C. and Barker, D.J. (2000) 'Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women', *Environmental Health Perspectives*, 108(Suppl 3), p. 545.

Palozza, P., Serini, S., Maggiano, N., Tringali, G., Navarra, P., Ranelletti, F.O. and Calviello, G. (2005) 'β-Carotene Downregulates the Steady-State and Heregulin-α–Induced COX-2 Pathways in Colon Cancer Cells', *The Journal of nutrition*, 135(1), pp. 129-136.

Papas, M.A., Giovannucci, E. and Platz, E.A. (2004) 'Fiber from fruit and colorectal neoplasia', *Cancer Epidemiology Biomarkers & Prevention*, 13(8), pp. 1267-1270.

Park, Y., Subar, A.F., Kipnis, V., Thompson, F.E., Mouw, T., Hollenbeck, A., Leitzmann, M.F. and Schatzkin, A. (2007) 'Fruit and vegetable intakes and risk of colorectal cancer in the NIH–AARP diet and health study', *American journal of epidemiology*, 166(2), pp. 170-180.

Parvin, S.G. and Sivakumar, B. (2000) 'Nutritional status affects intestinal carotene cleavage activity and carotene conversion to vitamin A in rats', *The Journal of nutrition*, 130(3), pp. 573-577.

Perkins, S., Clarke, A.R., Steward, W. and Gescher, A. (2003a) 'Age-related difference in susceptibility of Apc(Min/+) mice towards the chemopreventive efficacy of dietary aspirin and curcumin', *Br J Cancer*, 88(9), pp. 1480-3.

Perkins, S., Clarke, A.R., Steward, W. and Gescher, A. (2003b) 'Age-related difference in susceptibility of ApcMin/+ mice towards the chemopreventive efficacy of dietary aspirin and curcumin', *British journal of cancer*, 88(9), pp. 1480-1483.

Pham, D.N., Leclerc, D., Levesque, N., Deng, L. and Rozen, R. (2013) 'beta,beta-carotene 15,15'-monooxygenase and its substrate beta-carotene modulate migration and invasion in colorectal carcinoma cells', *Am J Clin Nutr*, 98(2), pp. 413-22.

Pietinen, P., Malila, N., Virtanen, M., Hartman, T.J., Tangrea, J.A., Albanes, D. and Virtamo, J. (1999) 'Diet and risk of colorectal cancer in a cohort of Finnish men', *Cancer Causes & Control*, 10(5), pp. 387-396.

Pretlow, T.P., Brasitus, T.A., Fulton, N.C., Cheyer, C. and Kaplan, E.L. (1993) 'K-ras mutations in putative preneoplastic lesions in human colon', *Journal of the National Cancer Institute*, 85(24), pp. 2004-2007.

Prior, R.L. and Cao, G. (2000) 'Antioxidant phytochemicals in fruits and vegetables: diet and health implications', *HortScience*, 35(4), pp. 588-592.

Purup, S., Larsen, E. and Christensen, L.P. (2009) 'Differential effects of falcarinol and related aliphatic C17-polyacetylenes on intestinal cell proliferation', *Journal of agricultural and food chemistry*, 57(18), pp. 8290-8296.

Pyper, S.R., Viswakarma, N., Yu, S. and Reddy, J.K. (2010) 'PPARalpha: energy combustion, hypolipidemia, inflammation and cancer', *Nucl Recept Signal*, 8, p. e002.

Rajamanickam, S. and Agarwal, R. (2008) 'Natural products and colon cancer: current status and future prospects', *Drug development research*, 69(7), pp. 460-471.

Rajamanickam, S., Kaur, M., Velmurugan, B., Singh, R.P. and Agarwal, R. (2009) 'Silibinin suppresses spontaneous tumorigenesis in APC min/+ mouse model by modulating beta-catenin pathway', *Pharm Res*, 26(12), pp. 2558-67.

Rajamanickam, S., Velmurugan, B., Kaur, M., Singh, R.P. and Agarwal, R. (2010) 'Chemoprevention of intestinal tumorigenesis in APCmin/+ mice by silibinin', *Cancer research*, 70(6), pp. 2368-2378.

Rao, C.V., Cooma, I., Rodriguez, J.G.R., Simi, B., El-Bayoumy, K. and Reddy, B.S. (2000) 'Chemoprevention of familial adenomatous polyposis development in the APCmin mouse model by 1, 4-phenylene bis (methylene) selenocyanate', *Carcinogenesis*, 21(4), pp. 617-621.

Rapta, P., Mišík, V., Staško, A. and Vrábel, I. (1995) 'Redox intermediates of flavonoids and caffeic acid esters from propolis: an EPR spectroscopy and cyclic voltammetry study', *Free Radical Biology and Medicine*, 18(5), pp. 901-908.

Razin, A. (1998) 'CpG methylation, chromatin structure and gene silencing—a three-way connection', *The EMBO journal*, 17(17), pp. 4905-4908.

Reddy, B.S. (2000) 'Novel approaches to the prevention of colon cancer by nutritional manipulation and chemoprevention', *Cancer Epidemiology Biomarkers & Prevention*, 9(3), pp. 239-247.

Reddy, B.S. and Maeura, Y. (1984) 'Dose-response studies of the effect of dietary butylated hydroxyanisole on colon carcinogenesis induced by methylazoxymethanol acetate in female CF1 mice', *Journal of the National Cancer Institute*, 72(5), pp. 1181-1187.

Replication, D.N.A. (2003) 'The hereditary nonpolyposis colorectal cancer syndrome: genetics and clinical implications', *Ann Intern Med*, 138, pp. 560-570.

Ribot, J., Felipe, F., Bonet, M.L. and Palou, A. (2004) 'Retinoic acid administration and vitamin A status modulate retinoid X receptor α and retinoic acid receptor α levels in mouse brown adipose tissue', *Molecular and cellular biochemistry*, 266(1-2), pp. 25-30.

Rice-Evans, C.A., Miller, N.J. and Paganga, G. (1996) 'Structure-antioxidant activity relationships of flavonoids and phenolic acids', *Free Radical Biology and Medicine*, 20(7), pp. 933-956.

Richter, M., Weiss, M., Weinberger, I., Fürstenberger, G. and Marian, B. (2001) 'Growth inhibition and induction of apoptosis in colorectal tumor cells by cyclooxygenase inhibitors', *Carcinogenesis*, 22(1), pp. 17-25.

Rijnkels, J.M. and Alink, G.M. (1998) 'Effects of a vegetables–fruit mixture on liver and colonic 1, 2-dimethylhydrazine-metabolizing enzyme activities in rats fed low-or high-fat diets', *Cancer letters*, 128(2), pp. 171-175.

Roseboom, T.J., Van Der Meulen, J.H.P., Ravelli, A.C.J., Osmond, C., Barker, D.J.P. and Bleker, O.P. (2001) 'Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview', *Molecular and cellular endocrinology*, 185(1), pp. 93-98.

Rosenberg, C.L., Motokura, T., Kronenberg, H.M. and Arnold, A. (1993) 'Coding sequence of the overexpressed transcript of the putative oncogene PRAD1/cyclin D1 in two primary human tumors', *Oncogene*, 8(2), pp. 519-521.

Rosin-Arbesfeld, R., Townsley, F. and Bienz, M. (2000) 'The APC tumour suppressor has a nuclear export function', *Nature*, 406(6799), pp. 1009-1012.

Rosin-Arbesfeld, R., Cliffe, A., Brabletz, T. and Bienz, M. (2003) 'Nuclear export of the APC tumour suppressor controls β-catenin function in transcription', *The EMBO journal*, 22(5), pp. 1101-1113.

Rustgi, A.K. (2007) 'The genetics of hereditary colon cancer', *Genes & development*, 21(20), pp. 2525-2538.

Saleh, H., Garti, H., Carroll, M. and Brandt, K. (2013) 'Effect of carrot feeding to APC Min mouse on intestinal tumours', *Proceedings of the Nutrition Society*, 72(OCE4), p. E183.

Schoen, R.E. (2002) 'The case for population-based screening for colorectal cancer', *Nature Reviews Cancer*, 2(1), pp. 65-70.

Seckl, J.R. (2004) 'Prenatal glucocorticoids and long-term programming', *European Journal of Endocrinology*, 151(Suppl 3), pp. U49-U62.

Segditsas, S. and Tomlinson, I. (2006) 'Colorectal cancer and genetic alterations in the Wnt pathway', *Oncogene*, 25(57), pp. 7531-7537.

Sharoni, Y., Stahl, W., Danilenko, M. and Levy, J. (2004) 'Anticancer activity of carotenoids: from human studies to cellular processes and gene regulation', *OXIDATIVE STRESS AND DISEASE*, 13, pp. 165-196.

Shen, G., Khor, T.O., Hu, R., Yu, S., Nair, S., Ho, C.T., Reddy, B.S., Huang, M.T., Newmark, H.L. and Kong, A.N.T. (2007) 'Chemoprevention of familial adenomatous polyposis by natural dietary compounds sulforaphane and dibenzoylmethane alone and in combination in ApcMin/+ mouse', *Cancer research*, 67(20), pp. 9937-9944.

Sherr, C.J. (2000) 'The Pezcoller lecture: cancer cell cycles revisited', *Cancer research*, 60(14), pp. 3689-3695.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben-Ze'ev, A. (1999) 'The cyclin D1 gene is a target of the beta-catenin/LEF-1 pathway', *Proc Natl Acad Sci U S A*, 96(10), pp. 5522-7.

Shureiqi, I. and Lippman, S.M. (2001) 'Lipoxygenase modulation to reverse carcinogenesis', *Cancer Research*, 61(17), pp. 6307-6312.

Sie, K.K.Y., Medline, A., Van Weel, J., Sohn, K.-J., Choi, S.-W., Croxford, R. and Kim, Y.-I. (2011) 'Effect of maternal and postweaning folic acid supplementation on colorectal cancer risk in the offspring', *Gut*, 60(12), pp. 1687-1694.

Sierra, J., Yoshida, T., Joazeiro, C.A. and Jones, K.A. (2006) 'The APC tumor suppressor counteracts beta-catenin activation and H3K4 methylation at Wnt target genes', *Genes Dev*, 20(5), pp. 586-600.

Singh, A. (2013) 'Effect of dietary pattern on coronary heart disease (CHD) patients', *Biomedical Research (0970-938X)*, 24(3).

Sinicrope, F.A. and Gill, S. (2004) 'Role of cyclooxygenase-2 in colorectal cancer', *Cancer and Metastasis Reviews*, 23(1-2), pp. 63-75.

Sinicrope, F.A. and Sargent, D.J. (2012) 'Molecular pathways: microsatellite instability in colorectal cancer: prognostic, predictive, and therapeutic implications', *Clinical Cancer Research*, 18(6), pp. 1506-1512.

Slavin, J.L. and Lloyd, B. (2012) 'Health benefits of fruits and vegetables', *Advances in Nutrition: An International Review Journal*, 3(4), pp. 506-516.

Smith, A.J., Stern, H.S., Penner, M., Hay, K., Mitri, A., Bapat, B.V. and Gallinger, S. (1994) 'Somatic APC and K-ras codon 12 mutations in aberrant crypt foci from human colons', *Cancer research*, 54(21), pp. 5527-5530.

Smith, G., Carey, F.A., Beattie, J., Wilkie, M.J.V., Lightfoot, T.J., Coxhead, J., Garner, R.C., Steele, R.J.C. and Wolf, C.R. (2002) 'Mutations in APC, Kirsten-ras, and p53—alternative genetic pathways to colorectal cancer', *Proceedings of the National Academy of Sciences*, 99(14), pp. 9433-9438.

Smith, K.J., Johnson, K.A., Bryan, T.M., Hill, D.E., Markowitz, S., Willson, J.K., Paraskeva, C., Petersen, G.M., Hamilton, S.R. and Vogelstein, B. (1993) 'The APC gene product in normal and tumor cells', *Proceedings of the National Academy of Sciences*, 90(7), pp. 2846-2850.

Society, A.C. (2013) 'Cancer facts and figures 2013'. American Cancer Society Atlanta.

Söreide, K., Janssen, E.A.M., Söiland, H., Körner, H. and Baak, J.P.A. (2006) 'Microsatellite instability in colorectal cancer', *British journal of surgery*, 93(4), pp. 395-406.

Soslow, R.A., Dannenberg, A.J., Rush, D., Woerner, B.M., Khan, K.N., Masferrer, J. and Koki, A.T. (2000) 'COX-2 is expressed in human pulmonary, colonic, and mammary tumors', *Cancer*, 89(12), pp. 2637-45.

Steele, R.J.C., Thompson, A.M., Hall, P.A. and Lane, D.P. (1998) 'The p53 tumour suppressor gene', *British journal of surgery*, 85(11), pp. 1460-1467.

Su, L.-K., Kinzler, K.W., Vogelstein, B., Preisinger, A.C., Moser, A.R., Luongo, C., Gould, K.A. and Dove, W.F. (1992) 'Multiple intestinal neoplasia caused by a mutation in the murine homolog of the APC gene', *Science*, 256(5057), pp. 668-670.

Sun, D.F., Gao, Z.H., Liu, H.P., Yuan, Y. and Qu, X.J. (2012) 'Sphingosine 1-phosphate antagonizes the effect of all-trans retinoic acid (ATRA) in a human colon cancer cell line by modulation of RARbeta expression', *Cancer Lett*, 319(2), pp. 182-9.

Sutherland, R.L. and Musgrove, E.A. (2004) 'Cyclins and breast cancer', *J Mammary Gland Biol Neoplasia*, 9(1), pp. 95-104.

Swamy, M.V., Patlolla, J.M.R., Steele, V.E., Kopelovich, L., Reddy, B.S. and Rao, C.V. (2006) 'Chemoprevention of familial adenomatous polyposis by low doses of atorvastatin and celecoxib given individually and in combination to APCMin mice', *Cancer research*, 66(14), pp. 7370-7377.

Takitani, K., Miyazaki, H., Fukunishi, S., Takaya, R., Yoden, A., Higuchi, K. and Tamai, H. (2011) 'Altered expression of both beta-carotene 15,15' monooxygenase and lecithin:retinol acyltransferase in obese Zucker rats', *J Nutr Sci Vitaminol (Tokyo)*, 57(1), pp. 108-13.

Tanaka, T., Kojima, T., Kawamori, T., Wang, A., Suzui, M., Okamoto, K. and Mori, H. (1993) 'Inhibition of 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis by the naturally occurring plant phenolics caffeic, ellagic, chlorogenic and ferulic acids', *Carcinogenesis*, 14(7), pp. 1321-1325.

Tanaka, T., Shnimizu, M. and Moriwaki, H. (2012) 'Cancer chemoprevention by carotenoids', *Molecules*, 17(3), pp. 3202-3242.

Temple, N.J. and Basu, T.K. (1987) 'Protective effect of β-carotene against colon tumors in mice', *Journal of the National Cancer Institute*, 78(6), pp. 1211-1214.

Terry, P., Giovannucci, E., Michels, K.B., Bergkvist, L., Hansen, H., Holmberg, L. and Wolk, A. (2001) 'Fruit, vegetables, dietary fiber, and risk of colorectal cancer', *Journal of the National Cancer Institute*, 93(7), pp. 525-533.

Thibodeau, S.N., Bren, G. and Schaid, D. (1993) 'Microsatellite instability in cancer of the proximal colon', *Science (New York, NY)*, 260(5109), p. 816.

Thibodeau, S.N., French, A.J., Cunningham, J.M., Tester, D., Burgart, L.J., Roche, P.C., McDonnell, S.K., Schaid, D.J., Vockley, C.W. and Michels, V.V. (1998) 'Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1', *Cancer Research*, 58(8), pp. 1713-1718.

Toyota, M., Ohe-Toyota, M., Ahuja, N. and Issa, J.-P.J. (2000) 'Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype', *Proceedings of the National Academy of Sciences*, 97(2), pp. 710-715.

Tsubono, Y., Otani, T., Kobayashi, M., Yamamoto, S., Sobue, T. and Tsugane, S. (2005) 'No association between fruit or vegetable consumption and the risk of colorectal cancer in Japan', *British journal of cancer*, 92(9), pp. 1782-1784.

Tsujii, M., Kawano, S. and DuBois, R.N. (1997) 'Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential', *Proceedings of the National Academy of Sciences*, 94(7), pp. 3336-3340.

Umar, A., Boland, C.R., Terdiman, J.P., Syngal, S., de la Chapelle, A., Rüschoff, J., Fishel, R., Lindor, N.M., Burgart, L.J. and Hamelin, R. (2004) 'Revised Bethesda Guidelines for hereditary nonpolyposis colorectal cancer (Lynch syndrome) and microsatellite instability', *Journal of the National Cancer Institute*, 96(4), pp. 261-268.

van Breda, S.G., van Agen, E., van Sanden, S., Burzykowski, T., Kleinjans, J.C. and van Delft, J.H. (2005a) 'Vegetables affect the expression of genes involved in carcinogenic and anticarcinogenic processes in the lungs of female C57BL/6 mice', *The Journal of nutrition*, 135(11), pp. 2546-2552.

van Breda, S.G.J., van Agen, E., van Sanden, S., Burzykowski, T., Kienhuis, A.S., Kleinjans, J.C.S. and van Delft, J.H.M. (2005b) 'Vegetables affect the expression of genes involved in

anticarcinogenic processes in the colonic mucosa of C57BL/6 female mice', *The Journal of nutrition*, 135(8), pp. 1879-1888.

van Kranen, H.J., Van Iersel, P.W., Rijnkels, J.M., Beems, D.B., Alink, G.M. and van Kreijl, C.F. (1998) 'Effects of dietary fat and a vegetable-fruit mixture on the development of intestinal neoplasia in the ApcMin mouse', *Carcinogenesis*, 19(9), pp. 1597-1601.

Verma, A.K., Shoemaker, A., Simsiman, R., Denning, M. and Zachman, R.D. (1992) 'Expression of retinoic acid nuclear receptors and tissue transglutaminase is altered in various tissues of rats fed a vitamin A-deficient diet', *J Nutr*, 122(11), pp. 2144-52.

Voorrips, L.E., Goldbohm, R.A., van Poppel, G., Sturmans, F., Hermus, R.J.J. and Van Den Brandt, P.A. (2000) 'Vegetable and fruit consumption and risks of colon and rectal cancer in a prospective cohort study The Netherlands Cohort Study on Diet and Cancer', *American Journal of Epidemiology*, 152(11), pp. 1081-1092.

Vulcan, A., Brandstedt, J., Manjer, J., Jirstrom, K., Ohlsson, B. and Ericson, U. (2015) 'Fibre intake and incident colorectal cancer depending on fibre source, sex, tumour location and Tumour, Node, Metastasis stage', *Br J Nutr*, pp. 1-11.

Wang, P., Zhang, K., Zhang, Q., Mei, J., Chen, C.J., Feng, Z.Z. and Yu, D.H. (2011) 'Effects of quercetin on the apoptosis of the human gastric carcinoma cells', *Toxicology In Vitro*, 26(2), pp. 221-228.

Wang, X., Ouyang, Y., Liu, J., Zhu, M., Zhao, G., Bao, W. and Hu, F.B. (2014) 'Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis of prospective cohort studies'.

Waterland, R.A. and Jirtle, R.L. (2003) 'Transposable elements: targets for early nutritional effects on epigenetic gene regulation', *Mol Cell Biol*, 23(15), pp. 5293-300.

Weber, J.L. (1990) 'Informativeness of human (dC-dA) n.(dG-dT) n polymorphisms', *Genomics*, 7(4), p. 524.

Weinberg, R.A. (1991) 'Tumor suppressor genes', *Science (New York, NY)*, 254(5035), p. 1138.

Weisenberger, D.J., Siegmund, K.D., Campan, M., Young, J., Long, T.I., Faasse, M.A., Kang, G.H., Widschwendter, M., Weener, D. and Buchanan, D. (2006) 'CpG island methylator

phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer', *Nature genetics*, 38(7), pp. 787-793.

Weyant, M.J., Carothers, A.M., Mahmoud, N.N., Bradlow, H.L., Remotti, H., Bilinski, R.T. and Bertagnolli, M.M. (2001) 'Reciprocal expression of ERα and ERβ is associated with estrogen-mediated modulation of intestinal tumorigenesis', *Cancer Research*, 61(6), pp. 2547-2551.

Wheeler, J.M.D., Bodmer, W.F. and Mortensen, N.J.M.C. (2000) 'DNA mismatch repair genes and colorectal cancer', *Gut*, 47(1), pp. 148-153.

Williams, C.S., Mann, M. and DuBois, R.N. (1999) 'The role of cyclooxygenases in inflammation, cancer, and development', *Oncogene*, 18(55), pp. 7908-7916.

Wilson, C.L., Heppner, K.J., Labosky, P.A., Hogan, B.L. and Matrisian, L.M. (1997) 'Intestinal tumorigenesis is suppressed in mice lacking the metalloproteinase matrilysin', *Proc Natl Acad Sci U S A*, 94(4), pp. 1402-7.

Wong, Y.-N., Chang, W.-C., Clapper, M. and Engstrom, P.F. (2007) 'Chemoprevention of colorectal cancer', in *Colorectal Cancer*. Springer, pp. 33-49.

Woods, L.L., Weeks, D.A. and Rasch, R. (2004) 'Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis', *Kidney international*, 65(4), pp. 1339-1348.

World Health Organization (2005) *Fruit and vegetables for health: Report of a Joint FAO/WHO Workshop, 1-3 September, 2004, Kobe, Japan*. WHO.

Wu, J., Omene, C., Karkoszka, J., Bosland, M., Eckard, J., Klein, C.B. and Frenkel, K. (2011) 'Caffeic acid phenethyl ester (CAPE), derived from a honeybee product propolis, exhibits a diversity of anti-tumor effects in pre-clinical models of human breast cancer', *Cancer letters*, 308(1), pp. 43-53.

Xiao, J.H., Ghosn, C., Hinchman, C., Forbes, C., Wang, J., Snider, N., Cordrey, A., Zhao, Y. and Chandraratna, R.A. (2003) 'Adenomatous polyposis coli (APC)-independent regulation of beta-catenin degradation via a retinoid X receptor-mediated pathway', *J Biol Chem*, 278(32), pp. 29954-62.

Xu, X.M., Qian, J.C., Cai, Z., Tang, T., Wang, P., Zhang, K.H., Deng, Z.L. and Cai, J.P. (2012) 'DNA alterations of microsatellite DNA, p53, APC and K-ras in Chinese colorectal cancer patients', *European journal of clinical investigation*.

Yamashita, N., Minamoto, T., Ochiai, A., Onda, M. and Esumi, H. (1995) 'Frequent and characteristic K-< i> ras</i> activation and absence of p53 protein accumulation in aberrant crypt foci of the colon', *Gastroenterology*, 108(2), pp. 434-440.

Young, G.P., Hu, Y., Le Leu, R.K. and Nyskohus, L. (2005) 'Dietary fibre and colorectal cancer: a model for environment–gene interactions', *Molecular nutrition & food research*, 49(6), pp. 571-584.

Young, L.E., Rees, W.D., Sinclair, K.D. and Langley-Evans, S.C. (2004) 'Programming in the pre-implantation embryo', *Fetal nutrition and adult disease: programming of chronic disease through fetal exposure to undernutrition*, pp. 333-351.

Young, M., Ordonez, L. and Clarke, A.R. (2013) 'What are the best routes to effectively model human colorectal cancer?', *Molecular oncology*, 7(2), pp. 178-189.

Zeng, Z.-S., Shu, W.-P., Cohen, A.M. and Guillem, J.G. (2002) 'Matrix Metalloproteinase-7 Expression in Colorectal Cancer Liver Metastases Evidence for Involvement of MMP-7 Activation in Human Cancer Metastases', *Clinical Cancer Research*, 8(1), pp. 144-148.

Zhang, T., Nanney, L.B., Luongo, C., Lamps, L., Heppner, K.J., DuBois, R.N. and Beauchamp, R.D. (1997) 'Concurrent overexpression of cyclin D1 and cyclin-dependent kinase 4 (Cdk4) in intestinal adenomas from multiple intestinal neoplasia (Min) mice and human familial adenomatous polyposis patients', *Cancer research*, 57(1), pp. 169-175.

Zidorn, C., Jöhrer, K., Ganzera, M., Schubert, B., Sigmund, E.M., Mader, J., Greil, R., Ellmerer, E.P. and Stuppner, H. (2005) 'Polyacetylenes from the Apiaceae vegetables carrot, celery, fennel, parsley, and parsnip and their cytotoxic activities', *Journal of agricultural and food chemistry*, 53(7), pp. 2518-2523.

Zucker, S. and Vacirca, J. (2004) 'Role of matrix metalloproteinases (MMPs) in colorectal cancer', *Cancer and Metastasis Reviews*, 23(1-2), pp. 101-117.