PREVENTIVE EFFECTS OF FATTY ACIDS AND PHYTOCHEMICALS IN ApcMin/+ MOUSE, A MODEL OF COLORECTAL CANCER

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ABSTRACT

Colorectal cancer is the third most common cancer worldwide, and nutrition has been reported to play an important role on its incidence, development and prevention. Moreover, a high fat diet has been related to the risk of obesity, inflammation and cancer. This thesis tests the possible preventive effects of fatty acids, and phytochemicals on the prevention of colorectal cancer in two separate experiments. The animal model used in this work was the ApcMin/+ mouse, which has been described as an excellent model for chemopreventive studies of colorectal cancer, because it mimics the rapid development of adenomatous polyposis in humans. In both studies a high fat diet was used and the endpoints of interest were body and organs weight, mitosis and apoptosis in the small intestine and colon tissue, local and systemic cytokines levels, and tumour number and size. In the fatty acids experiment mice were treated for ten weeks with diets rich in corn, palm or fish oil. While in the phytochemical experiment mice were treated with a mix of coffee, cocoa, walnuts, turmeric, thyme and berries. The results of the present study is not consistent with those showing a beneficial effect of fish oil or phytochemical on cancer prevention. Although fish oil reduced tumour size, it was unable to reduce tumour number, cell proliferation and levels of pro-inflammatory cytokines. The phytochemical diet also failed to show any protective effect against colorectal cancer development, but instead increased tumour size and number, as well as the levels of pro-inflammatory cytokines. In the present study fish oil and phytochemicals did not have a positive effect on prevention of colorectal cancer in ApcMin/+ mouse.

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ABREVIATIONS

AA	Arachidonic acid
ACF	Aberrant crypt foci
AIF	Apoptosis-inducing factor
ALA	Alfa Linolenic acid
AOM	Azoxymethane
APC	Adenomatous Polyposis Coli
BAX	Bcl-2 associated protein X
BHT	Butylated hydroxyltoluene
BMI	Body mass index
CHCL3	Chloroform
CIMP	CpG island methylation phenotype
CIN	Chromosomal instability
CO	Corn oil
COX	Cyclooxigenase
CRC	Colorectal cancer
DHA	Docosahexaenoic acid
DMH	Dimethylhydrazine
DMU	Disease Modeling Unit
DNA	Deoxyribonucleic acid
EGCG	Epigallocatechin gallate
ENU	N-ethyl-N-nitrosourea
EPA	Eicosapentaenoic acid
EPIC	European prospective investigation into cancer
FAMES	Fatty acids methyl esters
FAP	Adenomatous Polyposis Coli
FO	Fish oil
GC	Gas chromatography
GMC-SF	Granulocyte-macrophage colony stimulating factor
HCL	Hydrochloric acid
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IFNγ	Interferon-γ
IGFRIIR	Insulin-like growth factor II receptor
IL	Interleukin
IP-10	Interferon-inducible protein-10
K2CO3	Potassium carbonate
KC	Keratinocyte chemoattractant
KC	Keratinocyte derived chemokine
KHCO3	Potassium bicarbonate
LA	Linolenic acid

LOX	Lipooxygenase
LT	Leukotrienes
MCP-1	Macrophage inflammatory protein 1
MCP-1	Monocyte chemoattractant protein-1
MeOH	Methanol
MIG	Monokine induced by gamma-interferon
MIP-1α	Macrophage inflammatory protein-1 alpha
MIS-H	Microsatellite instability high
MMR	Mismatch repair
MSI	Microsatellite instability
NF-kB	Nuclear Factor kB
Nrf2	Nuclear factor erythroid-derived 2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE	Prostaglandin
PHY	Phytochemical
PLA2	Phospholipase
PO	Palm oil
PPARγ	Peroxisome proliferator-activated receptor γ
PUFA	Polyunsaturated fatty acid
RIZ	Retinoblastoma protein interacting zinc
RNI	Reactive nitrogen intermediates
ROS	Reactive oxygen species
SDA	Stearidonic acid
SPSS	Statistical package for the social sciences
TF4	T-cell factor 4
TGF-β1RII	Transforming growth factor β1 receptor II
TNF-α	Tumour necrosis factor-alpha
TX	Thromboxanes
UEA	University of East Anglia
VEGF	Vascular endothelial growth factor
WCRF	Wold Cancer Research Fund

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1. GENERAL INTRODUCTION

1.1. COLORECTAL CANCER

1.1.1. Basic concepts and epidemiology

Cancer is a group of more than 100 diseases characterized by uncontrolled cellular growth as a result of changes in the genetic information of cells. Cells and tissues are complex systems with critical stages and checkpoints to ensure normal growth, development, and function. Normally the division, differentiation, and death of cells are carefully regulated. All cancers start as a single cell that has lost control of its normal growth and replication process.

Cancer of prostate, female breast, colon and lung are the most common cancers and comprise almost half of the total cancer burden. In men, prostate cancer continues to be the most frequent cancer, followed by lung and colorectal cancer, while breast cancer remains the most frequent neoplasm in women, followed by colorectal cancer. In Norway, in 2008, 26,121 people were diagnosed with cancer, for which 14,000 occurred among men and 12,121 among women. The most common cancers in Norway are the same as the ones observed in the United Kingdom (Norway 2009; UK 2010).

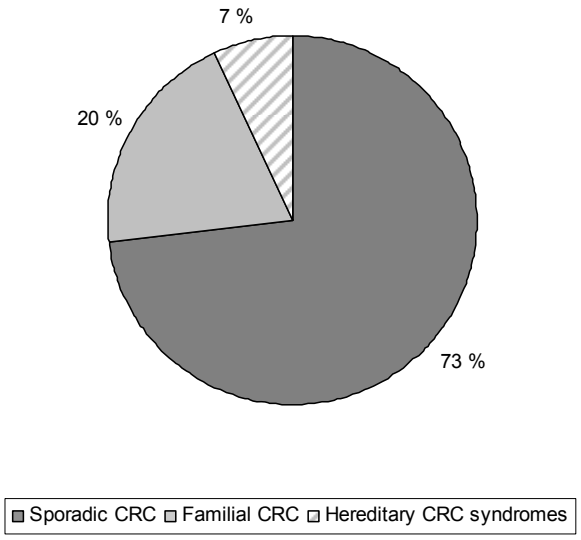

Figure 1.1 Classification of colorectal cancer (Rodriguez-Bigas, Cutait et al. 2010).

Colorectal cancer (CRC) is a cancer that forms either in the tissues of the colon or of the rectum, and the main types are: i) adenocarcinomas; ii) squamous cell carcinomas; iii) carcinoid tumours; iv) sarcomas; and v) lymphomas. More than 95% of colorectal cancers are adenocarcinomas with the cancer starting in the gland cells in the lining of the intestinal wall. Colorectal cancer can be classified into three forms according to the way that it has developed, which are hereditary, familial and sporadic (Figure 1.1). The proportion of each form may be different in different populations, but generally the majority of colorectal cancer cases are considered sporadic. The most common hereditary syndrome is Hereditary Non-Polyposis Colorectal Cancer (HNPCC), and the second most common is the Familial Adenomatous Polyposis Coli (FAP), in which disease occurs due to germline mutation of the Adenomatous Polyposis Coli (APC) gene (Jankowski 2008).

Colorectal cancer is more prevalent in North America, Argentina, Australia, New Zealand and parts of Europe, Japan, and Israel, and for this reason, is commonly regarded as a Western lifestyle disease. Colorectal cancer is one of the most frequent cancers worldwide.

In 2007, 298,000 new cases of cancer were recorded in the United Kingdom according to the report from Cancer Research UK (UK 2011).

1.1.2. Types of colorectal cancer

1.1.2.1. Hereditary non-polyposis colorectal cancer (HNPCC)

Hereditary non-polyposis colorectal cancer is also known as Lynch syndrome and cancer family syndrome. In hereditary non-polyposis colorectal cancer, colon malignancies usually occur from a single or a few adenomas. HNPCC is an autosomal dominant condition caused by mutation of one of the mismatch repair (MMR) genes. These genes are responsible for maintaining the fidelity of DNA by correcting base-pair mismatches that occur during cell replication. MutL homolog 1 (MLH1), MutS homolog 2 (MSH2), MutS homolog 3 (MSH3), MutS homolog 6 (MSH6) and postmeiotic segregation increased 2 (PMS2) are the main proteins involved in this system. When a mismatch is detected, a number of steps take place: MSH2 associates with either MSH6 or MSH3, and MLH1 couples with PMS2, PMS1, or MLH3. The recognition of mismatches and insertion-deletion loops is carried out by the complex formed by a MutS and a MutL, which interacts with replication factor C. Excision of the mismatch is performed by proteins such as exonuclease 1 and proliferating cell-nuclear antigen. Finally, resynthesis and re-ligation of the DNA strand is carried out by DNA polymerase σ and DNA ligase (Figure 1.2) (Vilar and Gruber 2010).

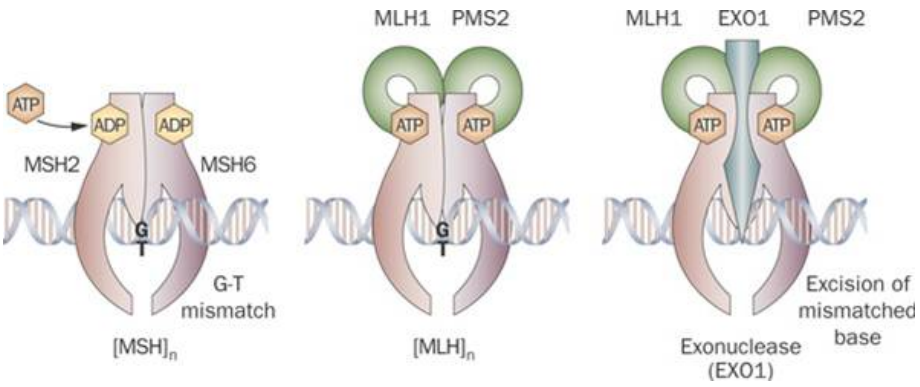

Figure 1.2 Model of the proposed mechanism of mismatch repair proteins (Vilar and Gruber 2010).

If the repair process is damaged the microsatellite sequences can lose or gain nucleotides and this phenomenon is termed microsatellite instability (MSI). Ultimately, loss of function of DNA repair proteins results in genomic instability that promotes mutations in other genes and facilitates malignant transformations. HNPCC accounts for about 1% to 3% of all colorectal cancer. The lifetime risk of colorectal cancer in persons with a MMR gene mutation approaches 80%, and the mean age at diagnosis of colorectal cancer in this syndrome is 44 years old (Gearhart and Ahuja 2010).

HNPCC is caused by mutation in one of four DNA MMR genes: MSH2, MLH1, PMS2, and MSH6. The germline mutations of MSH2 and MLH1 account for more than 90% of the mutations found in HNPCC families. It seems that the genetic pathway leading to tumorigenesis in HNPCC considerably overlaps with the sporadic and FAP cancer, but there are differences in the involvement of the different genes. Tumours of the HNPCC pathway also show mutations of APC, p53 and K-ras genes, but the incidence of p53 and K-ras mutation is less frequent than in chromosomal instability (Gearhart and Ahuja 2010).

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In addition, there are certain genes involved with important roles in the regulation of cell growth that have coding microsatellite sequences, such as transforming growth factor β1 receptor II (TGF-β1RII), retinoblastoma-protein-interacting zinc fingers (RIZ), T-cell factor 4 (TF4), bcl-2-associated X protein (BAX), and insulin-like growth factor II receptor (IGFRIIR). Among these, the tumorigenic role of TGF-β1RII and BAX mutations is the best characterized. Mutation of TGF-β1RII is the most common among the above-mentioned genes, occurring in about 90% of MSI-associated cancers. Change of function of this receptor leads to dysregulation of the SMAD protein pathway that has an important role in transcription regulation. The BAX gene is affected in about 33% of MSI-associated cancers, and the mutation of this gene leads to disruption of the function of B-cell CLL/lymphoma 2 (bcl-2) mediated apoptosis pathway (Jankowski 2008).

Histologically, HNPCC is more likely to be poorly differentiated, abundant in extra-cellular mucin, and distinguished by a lymphoid host response to the tumour. HNPCC patients and family members can develop a wide range of extracolonic malignancies. The highest risk for other tumours is that for endometrial cancer (40-70% lifetime risk). Also, these patients can have adenocarcinomas of the stomach, small bowel, biliary tract or ovary and transitional cell carcinoma of the ureter. The lifetime risk for these cancers range between 2% and 13% (Rozen 2001).

1.1.2.2. Familial Adenomatous polyposis (FAP)

Familial adenomatous polyposis is a rare autosomal dominant disease with almost 100% penetrance. The disease is maintained in the population at a frequency of about 1 in 8000, and is characterized by a large number of colorectal adenomas, more than 100, and accounts for approximately 1% to 2% of all colorectal cancer. FAP is inherited as an

autosomal dominant disease caused by a germline mutation of the tumour suppressor gene APC. In this condition, one mutated APC allele is inherited as a germline mutation from the affected parent, and adenomas develop when the second allelle (from the unaffected parent) becomes mutated or lost. The identification of an interstitial deletion on chromosome 5q21 in a patient with Garder's syndrome, followed by linkage analysis, led to the positional cloning of the adenomatous polyposis coli gene in 1991. APC is a large gene, encompassing 15 exons with an open reading frame of 8538 base pair. It encodes a protein of 2843 amino acids with a molecular weight of 310kDa. More than 800 different germline mutations in the APC gene have been published and the most (>90%) are nonsense mutations and frameshift mutations that lead to premature stop codons. The resulting protein is truncated and presumably non-functional (Spigelman, Phillips et al. 1994).

The APC protein (Figure 1.3) is a multifaceted regulator of colonic epithelial cell homeostasis and participates in the process of cell proliferation, migration, differentiation, apoptosis, and chromosomal segregation (Sieber, Tomlinson et al. 2000) . The proximal portion of the APC protein contains regions that enable oligomerization as well as binding to proteins that regulate the actin cytoskeleton, thereby affecting cell morphology, polarity, and migration. In its central portion, the APC protein binds to β-catenin, a protein that normally maintains cell-cell junctions by anchoring a cell surface adhesion molecule, E-cadherin. The carboxy-terminal portion of APC contains a domain that contributes to proper chromosomal segregation and cytoskeleton regulation. Because the vast majority of APC mutations truncate the protein, this has the dual effect of disrupting the constitutive breakdown of β-catenin, leading to activation of cancer-associated genes, and creating abnormal chromosomal segregation, leading to chromosomal instability (Fodde, Smits et al. 2001)

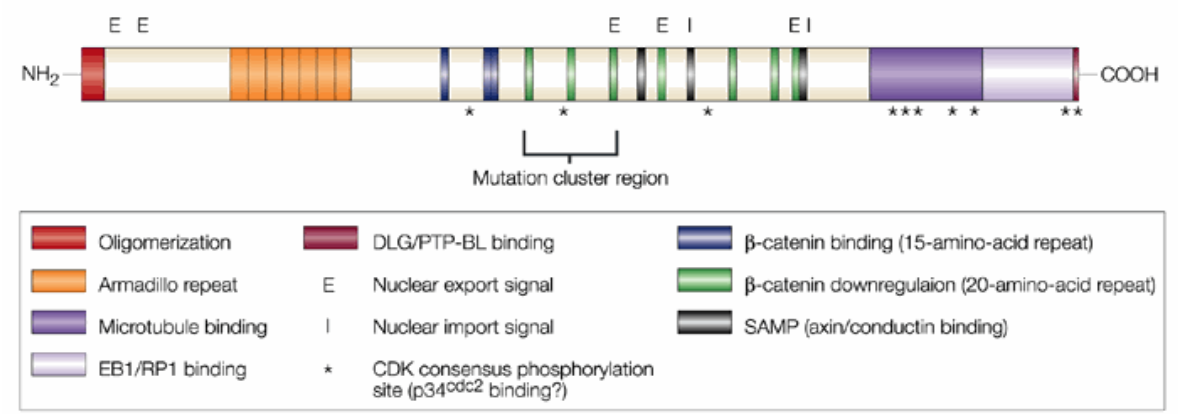

Figure 1.3 The adenomatous polyposis coli (APC) protein. (Fodde, Smits et al. 2001).

It is not the mutation itself that is of relevance, but its localization within the gene, since it defines the length of the mutant APC protein. The FAP phenotype (age of onset, type and number of intestinal polyps and extracolonic tumours) correlates somewhat with the type and location of APC mutations. Generally, mutations in the central region of the gene give a profuse polyposis phenotype with thousands of intestinal polyps. A deletion of 5 base pairs at codon 1309 is the most frequent mutation (18% of all FAP patients). Another frequent mutation is a deletion of 5 base pairs at codon 1061 (12% of all FAP patients). Despite the above genotype-phenotype correlations, various reports suggest that the location of the mutation along the APC gene is not the sole determinant of phenotype, and that other genetic and environmental factors may play a role (Jankowski 2008).

The majority of patients with FAP develop hundreds to thousands of colorectal polyps. Polyp development starts in the distal colorectum at an average age of 15 years, and most patients become symptomatic by the age of 25-30 years. The number and size of these polyps increases with time, ultimately reaching 100 to 5000 in number. Usually there is a positive family history. However, up to 25% of FAP patients do not have a family history, suggesting spontaneous germline mutations in these patients (Spigelman, Phillips et al. 1994).

APC mutations occur in up to 80% of adenomas and adenocarcinomas and 4.3% of the preneoplastic lesion called an aberrant crypt foci or ACF (Powell, Zilz et al. 1992). An important function of the APC gene is the prevention of accumulation of β-catenins. APC protein translated from the APC gene is a major factor in the WNT signalling pathway (Figure 1.4) and APC regulates cell proliferation by binding and degrading β-catenin protein that promotes cell proliferation (Aoki and Taketo 2007). In the absence of a WNT signal, the level of free intracellular β-catenin is minimized by sending it for degradation in the proteasome. Free cytoplasmic β-catenin, which is in equilibrium with β-catenin at adherens junctions, is recruited to a 'destruction complex' containing APC, axin/conductin and glycogen synthase kinase 3β (GSK3β). GSK3β phosphorylates β-catenin, allowing it to be recognized by an SCF (Skp1-Cul1-Fbox) complex containing the F-box protein β-TrCP. Other proteins in the SCF complex catalyse the addition of a polyubiquitin chain to β-catenin, allowing β-catenin to be recognized and degraded by the proteasome. Consequently, β-catenin cannot reach the nucleus, and cannot co-activate TCF-responsive genes. Groucho, a corepressor, also prevents the activation of TCF-responsive genes in the absence of β-catenin (Fodde, Smits et al. 2001).

However, In the presence of WNT, its receptor, Frizzled, in complex with LRP6, is activated. This leads to a poorly understood signalling cascade in which Dishevelled activates GBP — an inhibitor of GSK3β. Consequently, β-catenin cannot be targeted for destruction and is free to diffuse into the nucleus, where it acts as a co-activator for TCF-responsive genes, which targets c-myc, cyclin D1 and c-jun genes and promotes cell proliferation (Fodde, Smits et al. 2001; Watson 2006; Tanaka 2009).

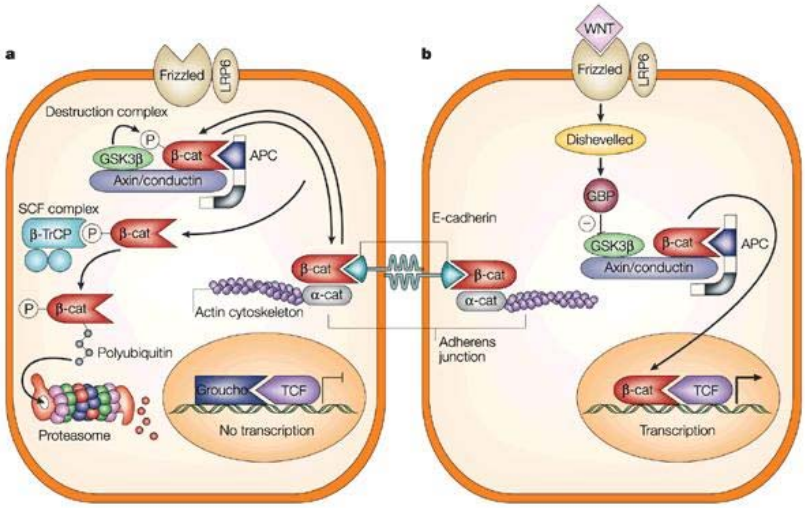

Figure 1.4 WNT signalling pathway. (a) In the absence of a WNT signal. (b) In the presence of WNT. (Fodde, Smits et al. 2001).

1.1.2.3. Sporadic Colorectal Cancer

Colorectal cancer not associated with hereditary cancer syndromes are defined as sporadic. The lifetime risk of developing sporadic colorectal cancer after age 50 is approximately 5%. In addition to genetic predisposition, environmental factors including a diet low in fibre, vegetables and folate but high in fat, red meat and alcohol, sedentary occupation, and cigarette smoking are believed to be associated with an increased colorectal cancer risk (Jankowski 2008).

Genomic destabilization is an early step in sporadic tumour development. Inactivation of the APC gene is the first molecular event in sporadic colorectal cancer. Somatic APC mutation is identified in approximately 60-80% of sporadic colorectal cancers and adenomas. About 50% of sporadic tumours with intact APC are reported to show mutations of β-catenin. P53 mutations have been identified in 40-50% of sporadic colorectal cancers. P53 mutation occurs at the time of transition from adenoma to

carcinoma. Patients with cancers involving a p53 mutation have a worse outcome and shorter survival time than patients without p53 mutation. K-ras mutation has been found in 15-68% of sporadic colorectal adenomas and in 40-65% of cancers. Mutated k-ras protein activates a variety of effectors pathways that leads to constitutive growth promotion. Point mutations of the DCC (Deleted in Colorectal Carcinoma) gene have been identified in approximately 6% of sporadic colorectal cancers. Mutations on SMAD, a tumour suppressor gene has been associated with progression of cancers (Gearhart and Ahuja 2010).

1.1.2.4. Sporadic MSI-Colorectal Cancer

Most sporadic colorectal cancer follows the chromosomal instability (CIN) pathway, but about 15% of them show a different pathway and these are associated with deficient mismatch repair. In contrast to HNPCC, these sporadic microsatellite instability high (MIS-H) associated colorectal cancer carry no germline mutation of DNA repair genes, the underlying mechanism is usually epigenetic silencing of the genes. Epigenetic changes affect the gene function by methylation of cytosine residues of cytosine- and guanine-rich 0.5-2-kb regions, so called CpG islands in promoter sequences. As a consequence, there is no transcription of the gene, hence the gene is silenced. Methylation in MSI-H-associated colorectal cancer most commonly occurs at the promoter regions of hMLH1 (Jankowski 2008).

Methylation of the genome on one hand is considered as a normal aging process, in as much it shows an increased frequency with progressive age. It is also more commonly found in females. As a consequence, MSI-H-associated sporadic colorectal cancer is more frequent in elderly females. On the other hand it is possible that in some instances there is a

predisposition to increased genomic methylation. Colorectal tumour development against a background of increased genomic methylation is designated CpG island methylation phenotype (CIMP). CIMP is characterized by promoter methylation of multiple genes that are methylated in cancer but not in normal tissue. This type of methylation is termed Type C in contrast to age-related Type A methylation (Malkhosyan, Yamamoto et al. 2000).

Sporadic MSI-H-associated colorectal cancer differs from HNPCC not just in their genetic background but also in clinical and pathologic features. Sporadic MSI-H-associated colorectal cancer, as mentioned, tends to be more common in females and is seen in older individuals, usually 65 years of age. They also have a greater tendency to develop in the proximal colon than it is seen in HNPCC. There is also increasing evidence that while HNPCC develops from traditional colorectal adenomas, most if not all sporadic MSI-H colorectal cancers arise from serrated polyps. The sporadic MSI-H tumours show high frequency of b-raf mutation and microsatellite instability, while k-ras mutation rate was found to be very low. B-raf is part of the mitogen activating protein kinase activating cascade, which regulates various cellular activities, such as gene expression, mitosis, differentiation, and cell survival/apoptosis (Moran, Ortega et al. 2010).

1.1.3. Adenocarcinoma Sequence

Accumulation of alterations during carcinogenesis leads to impairment of normal growth inhibition by increased cell growth and by inhibition of apoptosis, resulting in clonal expansion of tumour cells. Normally the balance between cell proliferation and apoptosis in the colonic mucosa is tightly regulated in order to maintain a constant cell number. The disturbance of the balance results in an escape from the normal homeostasis of cell number and favours the survival of mutated and undifferentiated cells. Inhibition of proliferation

and increase in apoptosis of these aberrant cells are important mechanisms of prevention of colon cancer (Leslie, Carey et al. 2002).

Figure 1.5 shows a schematic representation of the adenocarcinoma sequence in colorectal cancer first described by Vogelstein (Vogelstein, Fearon et al. 1988). The process of cancer development can be divided into different steps. It starts with DNA damage and mutations in the initiation phase, followed by growth of transformed cells in the promotion stage, leading to malignant growth and invasion in the progression stage (Li and Lai 2009). During development of colon cancer normal colonic epithelium transforms into hyperproliferative epithelium and then further on into adenoma, carcinoma and eventually metastasis, through accumulation of genetic alterations. Crucial genes involved in this process include APC, K-ras, SMAD, DCC, p53, c-myc, and COX-2 (Li and Lai 2009). The animal model of colorectal cancer that was used in this thesis was the ApcMin/+ mouse, which has a mutation on the APC gene. Moreover, genetic studies using mutant mice have demonstrated that mutations in the APC gene are responsible for intestinal tumorigenesis (Nandan and Yang 2010). This is discussed in detail in section 1.3.2.

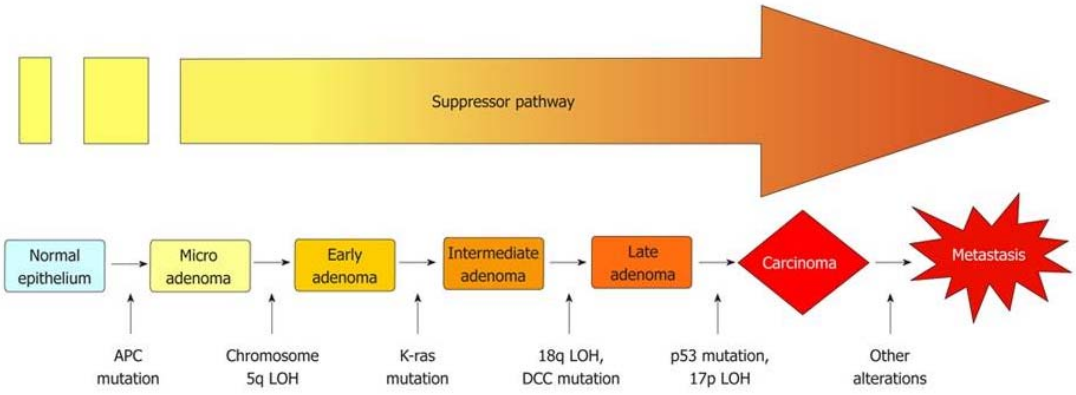

Figure 1.5 A schematic representation of the adenocarcinoma sequence in colorectal cancer (Moran, Ortega et al. 2010).

Cell proliferation and cell death can also be affected by some mediators of inflammation that have a pro-tumorigenic or anti-tumorigenic inflammatory response. Inflammation can also be a result of activation of oncogenes. For example, the activation of the oncogene RAS (rat sarcoma oncogene) turns it into a dominant oncogene capable of inducing cell proliferation. RAS activation results in a pro-tumorigenic microenvironment surrounding tumour cells and induces the expression of various inflammatory cytokines and chemokines (Borrello, Degl'Innocenti et al. 2008). Moreover, NF-kB is a transcription factor and key mediator of inflammation-induced carcinogenesis which also affects cell proliferation (Shen and Tergaonkar 2009). NF-kB, under normal cellular conditions, binds to and is negatively regulated by inhibitor of kappa B in the cytoplasm. Following an inflammatory stimulus, inhibitor of kappa B is phosphorylated and undergoes proteosomal degradation. This allows activated NF-kB to translocate to the nucleus where it activates the transcription of target genes, including inflammation-related genes (cytokines, chemokines, NOS, COX-2, and TNF-α). By increasing the expression of several cell cycle genes, activated NF-kB leads to increased cell proliferation (Pikarsky, Porat et al. 2004).

Another mechanism that can also affect cell proliferation is the prostaglandin pathway s. Cyclooxygenases (COX) are enzymes required for the production of prostaglandins from fatty acids. Prostaglandins, especially PGE2, are produced by COX and are key mediators in inflammation and inflammation-associated cancers (Schetter, Heegaard et al. 2010). There are two cycloxygenase isoforms, COX-1 and COX-2. COX-1 is constitutively expressed at relatively low levels, whereas COX-2 is the inducible from of the enzyme and is primarily responsible for the increased COX activity due to chronic inflammation. Initially found to be highly expressed in colon adenocarcinomas, COX-2 has now been found to be highly expressed in nearly every tumour type examined. Expression of COX-2 activity is necessary and sufficient to cause a malignant transformation in multiple in vitro

and animal models. COX-2 activity and the subsequent increase of PGE2 can affect cell proliferation, DNA mutation, angiogenesis, apoptosis and metastasis (Harris 2009).

Apoptosis, may be considered one of the important targets in a preventive approach against cancer and this programmed-cell death is a complex process that involves the active participation of affected cells in a self-destruction cascade (Ramos 2008). Apoptosis seems to be an essential defense against the appearance of somatic mutations that constitute the earliest steps in tumorigenesis. At later stages in the adenoma-carcinoma sequence, mutations or gene-silencing events that cause an inhibition of apoptosis are thought to be critical for the survival and growth of tumours (Johnson 2002).

1.1.4. Risk Factors for Colorectal Cancer

1.1.4.1. Obesity

The World Health Organization defines obesity as an abnormal or excess fat accumulation in adipose tissue, to the extent that health is impaired (Donohoe, Pidgeon et al. 2010). Obesity is a result of positive energy balance and prevails in conditions of energy excess. As a consequence of major economic, social and technological changes, many populations find themselves in environments characterized by abundant calorie-rich food and low physical activity requirements. As a result, obesity is rapidly approaching epidemic proportions in many parts of the world and has become a major public health concern (Gunter and Leitzmann 2006).

Epidemiological studies have provided evidence for the association of obesity with cancer (Calle, Rodriguez et al. 2003; Calle and Kaaks 2004). In parallel to the geographic variation seen in obesity rates worldwide, colorectal cancer incidence is highest in affluent industrialized countries such as the United States, Australia, and Western Europe (Gunter and Leitzmann 2006). The World Cancer Research Fund (WCRF) used a standardized approach to analysis of the evidence and concluded that there is convincing evidence of an association between obesity and oesophageal adenocarcinomas, pancreas, colorectal, breast, endometrial and kidney cancer (WCRF 2007).

The largest meta-analysis to date includes 282,000 patients from prospective observational studies with over 133 million person-years of follow up (Renehan, Tyson et al. 2008). This analysis showed that high BMI is associated with an increased incidence of many types of cancer, which is in agreement with others results (Lin, Zhang et al. 2004; Moore, Bradlee et al. 2004; Tamakoshi, Wakai et al. 2004). There is a modest but consistently reported relationship between in the incidence of colonic cancer and obesity, with a relative risk of 1.24-1.59 in obese men and 1.09-1.22 in obese women, as previously reported in published meta-analyses (Donohoe, Pidgeon et al. 2010). The risk is greater for cancer of the colon than of the rectum. Colonic adenomas, precursor lesions for colorectal cancer, have also been associated with increased BMI (Bird, Frankl et al. 1998; Kono, Handa et al. 1999; Wang, Lin et al. 2005). Most studies demonstrate a stronger association between obesity and colorectal cancer in men than in women.

In accordance to the epidemiological results, data from animal studies have also shown that a high fat diet augments colorectal carcinogenesis, whereas caloric restriction reduces colorectal tumour incidence (Steinbach, Kumar et al. 1993; Premoselli, Sesca et al. 1998; Baltgalvis, Berger et al. 2009; Erdelyi, Levenkova et al. 2009). Based on these

observations, it was decided to use a high fat diet in the present experiment in order to increment the carcinogenesis process in the animal model used as well as assess whether the type of fat was important.

The biological mechanisms by which obesity has been linked to colorectal cancer may be by the fact that excess adipose tissue has been associated with a chronic state of systemic low-grade inflammation (Mohanty, Hamouda et al. 2000; Wajchenberg 2000; Esposito, Nappo et al. 2002). The relation between obesity and inflammation was demonstrated by the finding that adipocytes constitutively express the pro-inflammatory cytokine tumour necrosis factor (TNF-α), and that TNF-α expression in adipocytes of obese rodents is markedly increased (Hotamisligil, Shargill et al. 1993). Although the immune system plays a fundamental role in antitumour immunity, under certain circumstances it can also aid tumour development and progression (Donohoe, Pidgeon et al. 2010).

1.1.4.2. Dietary Fibre

A systematic review of 13 case-control studies supports the hypothesis that intake of dietary fibre may be protective against colorectal cancer (Howe, Ghadirian et al. 1992) by a consistent reduction in colorectal cancer risk in the highest fibre intake quintile compared with the lowest (odds ratio [OR] 0.53; 95% CI 0.47-0.61). However, large cohort studies have been less consistent. The European Prospective Investigation in Cancer and Nutrition study suggested that dietary fibre was protective (Bingham, Day et al. 2003), whilst the Pooling Project of Prospective Studies of Diet and Cancer found no association between fibre intake and colorectal cancer risk (Park, Hunter et al. 2005).

1.1.4.3. Meat Consumption

Ecologic data from 27 countries showed a strong correlation between colorectal cancer incidence and average per capita meat consumption (Ognjanovic, Yamamoto et al. 2006). This is supported by case-control and cohort studies, and the World Health Organization concluded that the consumption of red meat is likely to be associated with CRC (Scheppach, Bingham et al. 1999). This decision is consistent with systematic reviews that report an increased risk of CRC in those in the highest red and processed meat categories compared with the lowest (Norat and Riboli 2001; Sandhu, White et al. 2001). There have been several biologic explanations for this apparent association, and the most common ones are the high-fat intake; the formation of heterocyclic amines and polycyclic aromatic hydrocarbons during high-temperature cooking; formation of N-nitroso compounds; and heme iron as a promoter of carcinogenesis (Ferguson 2010)

1.1.4.4. Fruits and Vegetable Intake

Fruits and vegetables are an important source of antioxidants and fibre and have been reported to reduce the risk of a variety of cancers. The impact on colorectal cancer is less clear with both positive and negative studies (Voorrips, Goldbohm et al. 2000; Terry, Giovannucci et al. 2001). A systematic review identified 28 case-control and 12 cohort studies evaluating vegetable and/or fruit intake and colorectal cancer risk (Riboli and Norat 2003). Overall the case-control studies reported a reduction in colorectal cancer risk for both fruit and vegetables but there was no statistically significant association in cohort studies.

1.1.4.5. Alcohol

Alcohol is associated with a variety of cancers including the oral cavity, pharynx, larynx, and oesophagus (Boffetta and Hashibe 2006). Alcohol may have a general effect on increasing the risk of neoplasia due to direct genotoxic effects, production of reactive oxygen species, and interference with folate metabolism (Boffetta and Hashibe 2006). A review of 16 cohort studies found that there was an increased risk of colorectal cancer in those in the highest compared to the lowest alcohol intake category (Moskal, Norat et al. 2007).

1.1.4.6. Physical Activity

A sedentary lifestyle increases the risk of a number of chronic diseases including cancer and is associated with reduced survival (Warburton and Bredin 2006). The reasons for this are unclear but exercise has effects on the immune function, hormones and prostaglandin synthesis (Quadrilatero and Hoffman-Goetz 2003). Physical activity may influence these molecules to protect against colorectal cancer as may the effects of exercise on gut transit time. A review of 19 cohort studies found that increased physical-activity consistently reduced the risk of colorectal cancer (Samad, Taylor et al. 2005).

1.2. INFLAMMATION AND COLORECTAL CANCER

1.2.1. Overview of Inflammation

In response to injury, a multifactorial network of chemicals signals initiate and maintain a host response designed to heal the afflicted tissue. This involves activation and directed migration of leukocytes (neutrophils, monocytes and eosinophils) from the venous system to sites of damage, and tissue mast cells also have a significant role (Grivennikov, Greten et al. 2010). For neutrophils, a four-step mechanism is believed to coordinate recruitment of these inflammatory cells to sites of tissue injury and to the provisional extracellular matrix that forms a scaffolding upon which fibroblast and endothelial cells proliferate and migrate, thus providing a nidus for reconstruction of the normal microenvironment (Coussens and Werb 2002).

A family of chemotactic cytokines, named chemokines, which possess a relatively high degree of specificity for chemoattraction of specific leulocyte populations, recruits downstream effectors cells and dictates the natural evolution of the inflammatory response (Grivennikov and Karin 2010). The profile of cytokine/chemokines persisting at an inflammatory site is important in the development of chronic disease. The pro-inflammatory cytokine TNF-α controls inflammatory cell populations. As well as mediating many of the other aspects of the inflammatory process. The key concept is that normal inflammation, for example, inflammation associated with wound healing, is usually self-limiting. However, dysregulation of any of the converging factors can lead to abnormalities and ultimately, pathogenesis (Coussens and Werb 2002).

The presence of leukocytes within tumours, observed in the 19th century by Rudolf Vichow, provided the first indication of a possible link between inflammation and cancer. It is only during the last decade that clear evidence has been obtained that inflammation plays a critical role in tumorigenesis, and some of the underlying mechanisms have been elucidated (Karin 2006). A role for inflammation in tumorigenesis is now generally accepted, and it has become evident that an inflammatory microenvironment is an essential component of all tumours, including some in which a direct causal relationship with inflammation is not yet proven (Mantovani, Allavena et al. 2008).

Several types of inflammation differing by cause, mechanism, outcome, and intensity can promote cancer development and progression. The inflammatory response triggered by infection precedes tumour development and is a part of normal host defence, whose goal is pathogen elimination. However, in tumorigenesis pathogens subvert host immunity and establish persistent infections associated with low-grade but chronic inflammation (Grivennikov and Karin 2010).

1.2.2. Chronic Inflammation Associated with Cancer

Epidemiological data demonstrate a strong connection between chronic inflammation and developing cancer. Both endogenous (inherited diseases and obesity) and exogenous (acquired infections) inducers of inflammation contribute to chronic inflammation-associated cancer (Mantovani, Allavena et al. 2008; Medzhitov 2008). Several chronic inflammatory diseases lead to increased risk of cancer. Inflammatory bowel diseases (Crohn's disease and ulcerative colitis) are associated with increased rates of colon adenocarcinomas (Ekbom, Helmick et al. 1990; Gillen, Andrews et al. 1994; Gillen, Walmsley et al. 1994). However, not all chronic inflammatory diseases increase cancer

risk equally. Ulcerative colitis imposes much greater risk for colitis-associated cancer then Crohn's disease (Grivennikov, Greten et al. 2010). Even cancers that evolve without underlying chronic inflammation exhibit tumour-associated inflammation and contain inflammatory infiltrates. Oncogene activation (Ras and Myc), or cell senescence induced by DNA damage or oncogene activation, can enhance the transcription of pro-inflammatory genes, coding for cytokines and chemokines (Ancrile, Lim et al. 2007; Mantovani, Allavena et al. 2008).

However, it remains to be determined whether chronic inflammation can cause tumour-initiating genetic alterations or can only act in conjunction with carcinogen exposure. In the case of colitis-associated cancer, it was suggested that chronic inflammation and colonic injury can directly cause DNA alterations (Meira, Bugni et al. 2008). However, chronic inflammation and loss of protective mucus can also increase intestinal permeability for environmental toxins and mutagens, which induce mutations in stem cells that give rise to cancer (Sakaguchi, Brand et al. 2001). Furthermore, inflammation can stimulate the proliferation of cells that harbour oncogenic mutations induced by carcinogens rather than induce mutations themselves. Nonetheless, inflammation can result in the production of reactive oxygen and nitrogen species (ROS and RNI) by immune cells as well as immune mediated stimulation of ROS production in pre-malignant cells, induction of mutagenic enzymes, and inactivation of DNA damage gatekeeper pathways, such as the mismatch repair response or p53 (Niu, Wright et al. 2005; Colotta, Allavena et al. 2009; Takai, Toyoshima et al. 2009). Inflammation may also lead to epigenetic modifications including DNA and histone methylation that eventually lead to silencing of tumour suppressor loci (Cooper and Foster 2009). In summary, it remains to be fully established whether inflammation alone can result in tumour initiation, but there is ample evidence that inflammation is tumour promoting.

1.2.3. Inflammation and Tumour Growth

Tumour growth or tumour promotion is the sum total of malignant cell proliferation vs. malignant cell death. Both processes are strongly impacted by inflammation and inflammatory cytokines produced by tumour infiltrating immune cells, such as IL-6 and TNF-α, can serve as mitogens and survival factors for pre-malignant and fully established cancer cells (Grivennikov, Greten et al. 2010). Inflammation also contributes to the induction of angiogenesis, which is crucial for supplying the growing tumour with necessary nutrients and oxygen (Zumsteg and Christofori 2009).

Much of the growth stimulating cross-talk between immune and malignant cells is mediated by cytokines that activate the oncogenic transcription factors NF-kB. Activation of NF-kB is found in over 50% of all cancers (Karin 2006) and is a pre-requisite for the expression of a variety of target genes important for tumorigenesis, including anti-apoptotic genes (c-IAP, Bcl-xl, Bcl-2, c-FLIP), proliferative genes (Cyclins, c-Myc), stress-response genes, chemokines, and pro-angiogenic molecules (VEGF, bFGF, CXCL12) (Karin 2008; Naugler and Karin 2008).On the contrary , in immune cells, NF-kB promotes the production of pro-inflammatory cytokines, which mediate NF-kB activation in cancer cells, including IL-1, TNF, IL-6, and IL-2 (Karin 2008; Naugler and Karin 2008; Grivennikov and Karin 2010).

Cytokines are signalling molecules that are key mediators of inflammation or an immune response. These signalling molecules have a wide variety of cellular functions and are stimulated when tissue homeostasis is altered. Cytokines can be generally classified as pro-inflammatory (IL-1, IL-6, IL-15, IL-17, IL-23, and TNF-α) or anti-inflammatory (IL-4, IL-10, IL-13, TGF-β, IFN-α) (Schetter, Heegaard et al. 2010). Depending on the balance of

cytokines, their collective effect can be either pro- or anti-tumorigenic. Upon binding their membrane receptor, cytokines activate signal transduction pathways that lead to apoptosis, cell proliferation, angiogenesis, and cellular senescence. In general, constitutive exposure to high levels of pro-inflammatory cytokines is thought to be pro-tumorigenic. For example, TNF-α is a pro-inflammatory cytokine for which there is convincing evidence of a role in tumours promotion (Balkwill 2009). TNF-α knockout mice are more resistant to certain tumours (Pasparakis, Alexopoulou et al. 1996). IL-6 is another pro-inflammatory cytokine that has been implicated in pro-tumorigenic activity for many cancers, and has been found to be required for colitis-associated colon cancer in a mouse model (Grivennikov, Karin et al. 2009).

As a result, it is evident that inflammatory cells can have a powerful effect on tumour development. Early in the neoplastic process, these cells are powerful tumour promoters, producing an attractive environment for tumour growth, facilitating genomic instability and promoting angiogenesis. The inflammatory cells, and the chemokines and cytokines that they produce, influence the whole tumour organ, regulating the growth, migration, and differentiation of all cell types in the tumour microenvironment.

1.2.4. Diet and Inflammation

It is known and accepted that obesity is associated with low-grade chronic inflammation and that inflammation contributes to risk of other diseases linked to obesity. Circulating and adipose derived cytokines such as tumour necrosis factor-α or IL-6 have been shown to be elevated in obese humans (Kern, Ranganathan et al. 2001). In animal models, several studies have demonstrated that diet-induced obesity is associated with increased expression of a number of pro-inflammatory cytokines or biomarkers of inflammation in adipose

tissue (Xu, Barnes et al. 2003; Brake, Smith et al. 2006). The mechanisms underlying obesity-associated inflammation are not fully defined, however it is known that a high-fat diet induces expression of adhesion molecules in adipose tissue, which are associated with leukocyte migration and adherence (Brake, Smith et al. 2006). Din et al. (Ding, Chi et al. 2010) have shown that high fat diets and bacteria interact to promote pro-inflammatory changes in the small intestine, which preceded weight gain and obesity. The gastrointestinal tract is another potential source of inflammation associated with diet or obesity that has not been extensively explored.

Consumption of the n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (C20:5 n-3) and docosahexaenoic acid (C22:6 n-3), as mainly present in fish, may be associated with anti-inflammatory effects (Calder 1997; Calder 2006).The key link between PUFAs and inflammation is that eicosanoids, which are among the mediators and regulators of inflammation, are generated from 20-carbon PUFAs. In section 1.4.2 the metabolism of PUFAs are discussed into more detail as well as the formation of eicosanoids. Because inflammatory cells typically contain a high proportion of the n-6 PUFA arachidonic acid (C20:4 n-6) and low proportions of other 20-carbon PUFAs, arachidonic acid is usually the major substrate for eicosanoid synthesis. Eicosanoids, which include prostaglandins (PGs), thromboxanes, leukotrienes, and other oxidized derivatives, are generated from arachidonic acid are involved in modulating the intensity and duration of inflammatory responses (Figure 1.6) (Lewis, Austen et al. 1990; Tilley, Coffman et al. 2001).

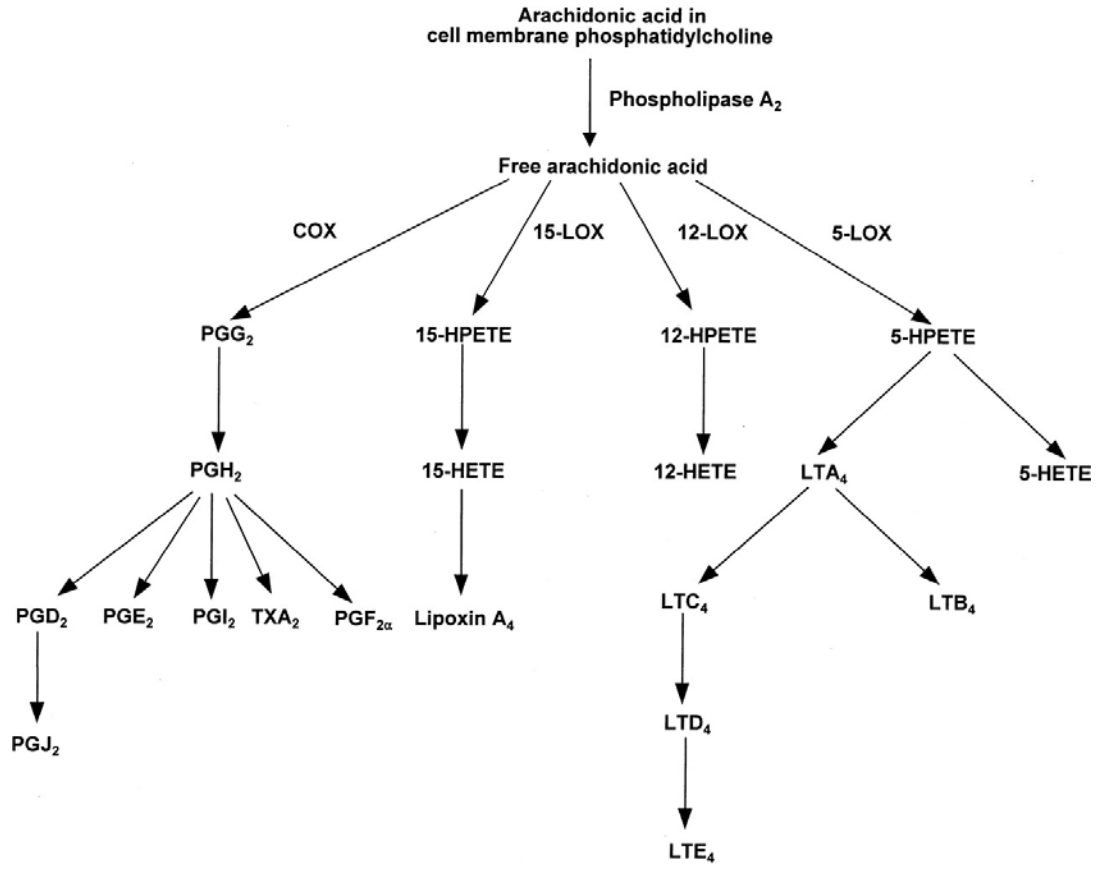

Figure 1.6 Arachidonic acid (AA) pathways. AA can be converted to prostaglandins (PG) or leukotrieness (LT), depending on the enzymes that are present. Each metabolite affects its biological action through receptors. COX: Cyclooxygenase; LOX: Lipoxigenase; TX: Thromboxane; HETE: Hydroxyeicosatetraenoic acid; HPETE: Hydroperoxyeicosatetraenoic acid. (Yoon and Baek 2005).

Recent studies have shown that prostaglandin E2 (PGE2) induces cycloxygenase 2 (COX-2) in fibroblasts cells and so up-regulates its own production, and induces the production of IL-6 by macrophages, inhibits 5-lipoxygenase and so decreases production of the 4-series LTs, and induces l5-LOX and so promotes the formation of lipoxins, which have been found to have an anti-inflammatory or inflammation resolving effect (Calder 2006).

However, increased consumption of n-3 PUFAs, such as EPA and DHA, results in increased proportions of those fatty acids in inflammatory cell phospholipids (Caughey,

Mantzioris et al. 1996; Healy, Wallace et al. 2000; Yaqoob, Pala et al. 2000). Because less substrate is available for synthesis of eicosanoids from arachidonic acid, fish oil supplementation of the human diet has been shown to result in decreased production of PGE2 (Endres, Ghorbani et al. 1989; Caughey, Mantzioris et al. 1996; Trebble, Wootton et al. 2003).

In addition to long-chain n-3 PUFAs modulating the generation of eicosanoids from arachidonic acid and to EPA acting as a substrate for the generation of alternative eicosanoids, recent studies have identified a novel group of mediators, termed E-series resolvins, formed from EPA by COX-2 that appear to exert anti-inflammatory or inflammation resolving actions (Serhan, Clish et al. 2000; Serhan, Hong et al. 2002). DHA-derived mediators termed D-series resolvins, docosatrienes and neuroprotectins, also produced by COX-2 have been identified and also appear to be anti-inflammatory (Mukherjee, Marcheselli et al. 2004). Although the action in antagonizing arachidonic acid is the main key anti-inflammatory effect of n-3 PUFAs, these fatty acids have several other anti-inflammatory effects, such as decreased leukocyte chemotaxis, decreased production of reactive oxygen species and pro-inflammatory cytokines, and decreased adhesion molecule expression, which might result from altered eicosanoid production or might be independent of this (Mukherjee, Marcheselli et al. 2004).

Diets containing a variety of fresh fruits and vegetables, whole grains, nuts, legumes and plant-based foods are rich in phytochemicals, and they have also shown to be protective against diseases and in lowering inflammation (Sanchez-Moreno, Cano et al. 2004). In fact, dietary vegetables and fruits have been regarded as rich sources of chemopreventive agents and potent anti-inflammatory properties. Food phytochemicals such as curcumin, EGCG, resveratol, genistein and isothiocyanates, with strong anti-inflammatory activity,

have been shown to inhibit carcinogenesis in preclinical animal models. Epidemiological studies have been clearly documented that the frequent consumption of diet high in fruits and vegetables has been linked to a lower risk of many types of cancers including prostate, colon, oral cavity, stomach, lung and esophagus (Chen, Yu et al. 2000; Chan and Giovannucci 2001).

The nuclear factor-erythroid 2-related factor 2 (Nrf2) is a transcription factor mediating the antioxidant response and acting as the major signalling pathway for many food phytochemicals to exert their anti-inflammatory effect (Khor, Yu et al. 2008). There is a growing body of evidence that the Nrf2 signalling pathway is closely involved with the regulation of inflammation. Khor et al. (Khor, Yu et al. 2008) have shown that Nrf2-deficient mice have increased susceptibility to DSS-induced colitis, and have also loss of colonic crypts, massive infiltration of inflammatory cells and anal breeding. In addition, immunocytochemical staining of nitrotyrosine, a biomarker of inflammation, was more intense in Nrf2-deficient mice. Concomitantly, more intense induction of pro-inflammatory biomarkers, such as IL-1β, IL-6, and TNF-α, as well as pro-inflammatory mediators like inducible nitric oxide synthetase (iNOS) and COX-2, was observed in Nrf2-deficient mice (Khor, Huang et al. 2006).

It is postulated that attenuation of inflammation through induction of anti-oxidative enzymes and suppression of pro-inflammatory mediators in an Nrf2-dependent manner in acute inflammation animal models, results in decreased sensitivity of wild-type mice towards inflammatory oxidative damage (Khor, Yu et al. 2008). In vivo data have indicated that ablation of Nrf2 resulted in increased expression of pro-inflammatory mediators like IL-1β, IL-6, COX-2, which are modulated by NF-kB (Khor, Yu et al. 2008) . Despite these findings, the possible mechanism linking Nrf2 and inflammation is still

unclear. Phytochemicals can also affect inflammation by other pathways such as the arachidonic acid (AA)-dependent pathways (cycloxygenase, COX; lipooxygenase, LOX; and phospholipase A2, PLA2) (Yoon and Baek 2005).

Several phytochemicals have been reported to inhibit PLA2. Quercitin, flavonoids and retinoids have been shown to inhibit the activity of different isoforms of PLA2 in several cell types and animal models via direct interaction with the enzyme (Lindahl and Tagesson 1993; Kim, Pham et al. 2001). Hong et al. (Hong, Bose et al. 2004) have shown that curcumin inhibited COX and LOX enzymes, supporting the hypothesis that phytochemicals might simultaneously affect multiple targets in one or more pathways. Several phytochemicals exert multiple cellular effects involving one or more signalling pathways. These effects may be synergistic, additive, or even antagonistic depending on the cell type, the concentration of the phytochemical and its surrounding environment. A better understanding of these cellular effects is vital to properly utilize the phytochemicals as promising agents that promote health and prevent disease.

1.3. APCMIN/+ MOUSE MODEL OF COLORECTAL CANCER

1.3.1. Basic concepts

The study of experimental colon carcinogenesis in rodents has had a remarkably long history, dating back almost 80 years, and the study of human disease has been greatly facilitated by the use of animal model systems (Rosenberg, Giardina et al. 2009). Mutant strains of mice have largely come from three sources: naturally occurring variation; phenotypic screening following germline mutagenesis; and targeted mutation of cloned genes (Shoemaker, Gould et al. 1997). N-ethyl-N-nitrosourea (ENU), the most potent known germline mutagen in the mouse, has been used to induce germline mutations resulting in mouse models for a variety of human disorders. It was through phenotypic screening following ENU mutagenesis that the Min mutant mouse was discovered (Moser, Dove et al. 1992). The ApcMin/+ mouse is the pioneer genetic model for colorectal cancer, although admittedly it primarily serves as a model for small intestinal cancers. This genetically engineered model is widely used, and patterns the human condition familial adenomatous polyposis (Tammariello and Milner 2010).

1.3.2. Genetic and Phenotype characteristics in ApcMin/+ Mouse Model

ApcMin (Min= multiple intestinal neoplasia) is an autosomal dominant mutation that predisposes mice to develop adenomas throughout the intestinal tract. On the C57BL/6J genetic background, Min/+ mice develop, on average, more than 50 tumours throughout the entire length of the intestinal tract and rarely live past 150 days of age (Su, Kinzler et al. 1992). Since all intestinal tumours in C57BL/6J Min/+ mice are benign adenomas, the

premature death of these animals is associated with secondary effects of tumour growth, including severe chronic anaemia and intestinal blockage.

The phenotypic similarities between FAP patients and Min mice led scientists to examine whether the Min phenotype is due to germline mutation of the mouse APC gene. Genetic mapping localized Min to the region of mouse chromosome 18 that also carries APC. Sequence analysis of the entire 8535 bp APC cDNA identified a nonsense mutation in Min/+ mice that results from a T/A: A/T transversion at nucleotide 2549 (codon 850) of APC (Corpet and Pierre 2003; Yamada and Mori 2007).

A number of genes that modify intestinal tumorigenesis have been identified using APC mutant mice. Deletion in the genes related to arachidonic acid metabolism has been shown to suppress intestinal tumorigenesis, whereas deletions in genes related to genomic stability increases tumorigenesis in APC mutant mice (Edelmann, Yang et al. 1999; Kuraguchi, Yang et al. 2001). The phenotype and genetic similarities between Min mice and humans with FAP make Min an excellent animal model for chemopreventive studies of colorectal cancer because it mimics the rapid development of adenomatous polyposis in humans. However, adenocarcinomas are seldom observed in this model, and no typical aberrant crypt foci (ACF) arise above the intestinal mucosa. Consequently, the ACF to carcionoma progression is not established in this model. Moreover, the K-ras mutations observed in many human tumours were not detected in Min mouse polyps (Shoemaker, Gould et al. 1997), and p53 inactivation, considered to play an important role in the conversion of adenomas to adenocarcinomas in humans, does not raise tumour number in Min mice nor indicate evidence of malignant transformation (Fazeli, Steen et al. 1997; Yamada and Mori 2007).

The human and mouse APC genes are 86 and 90% identical at the nucleotide and amino acid levels, respectively. Min/+ mice and FAP patients share several phenotypic characteristics, most notably the presence of multiple intestinal tumours. In Min/+ mice, most of these tumours occur in the small intestine, whereas in humans, tumours of the colon tend to be much more prevalent (Mai, Colbert et al. 2003). Regarding the intestinal tumour formation in Min mice, work from a number of laboratories has led to an understanding of tissue development in the mouse intestine (Shoemaker, Gould et al. 1997). Intestinal crypts in adult mice are clonal units, whereas early in postnatal development intestinal crypts are polyclonal. Another important change that occurs in the mouse intestine during the first several weeks of life include a significant increase in crypt number, suggesting the tumours are initiated early in life (Shoemaker, Gould et al. 1997). Dietary treatments are typically begun in the mice by the age of 4-5 weeks, when tumours may already be present. Only a few studies have exposed the animals in utero, when neoplastic foci may not be already present. The timing also mimics some clinical intervention trials, when dietary interventions are given to adults likely to have some Min polyps remaining after the visible ones have been removed. In most mouse studies it is the number of tumours in the small intestine that is the primary end point, although some authors have split the small intestine between proximal, mid and distal parts, and showed differences on the protective effect according to the region of the intestine (Corpet and Pierre 2003).

In ApcMin/+ mice, the number of colonic microadenomas per area was reported to be higher than that of adenomatous lesions in the small intestine, suggesting that the loss of APC occurs frequently in colonic crypts as well as in the epithelium of the small intestine (Yamada, Hata et al. 2002). Despite the frequent development of microadenomas, the number of macroscopic colonic tumours is much less than that of small intestinal tumours.

Interestingly, the size of microadenomas in the colon of ApcMin/+ mice does not increase with time. Based on the multistep carcinogenesis theory in the colon, the findings indicate that there are at least two distinct stages for intestinal tumorigenesis in ApcMin/+ mice (Yamada and Mori 2007). In contrast to colonic lesions, the adenomatous lesions in the small intestine show various sizes, and the mean size of the lesions is significantly larger than in the colon (Yamada, Hata et al. 2002). As the loss of APC is also involved in the earliest lesions in the small intestine, it is possible that in the small intestine, the loss of function of APC results in the formation of microadenomas that could develop directly into intestinal tumours by aging. This findings suggests that the mechanism of tumorigenesis involved in the small intestine may differ from those in the colon (Yamada and Mori 2007).

1.3.3. Other types of ApcMin/+ Mouse Model of Colorectal Cancer

After the discovery that the Min mouse phenotype was caused by truncated APC in position 850, other mice have been genetically modified so that one or more oncogenes hold a germline mutation (truncated APC in positions 716, 1309, or 1638, and mutated MSH2 or MLH1). As in humans, different mutations lead to different phenotypes, for instance more adenomas are found in the gut of Apc716 mutant mice (250 polyps ± 95) than in classical Min mice (40 ± 20) (Corpet and Pierre 2003). Apc1638 mice tend to develop the least amount of tumours all together, and Apc1309 develop a slightly higher number of polyps than the ApcMin/+ mouse (Smits, Kielman et al. 1999). While there are no perfect models, varieties of the APC model incorporate conditions that reflect phenotypic responses in humans.

1.4. FATTY ACIDS AND COLORECTAL CANCER

1.4.1. Definition

Triacylglycerols make up the largest proportion of dietary lipids consumed by humans. A triacylglycerol is composed of three fatty acids esterified to a glycerol molecule in one of three stereochemically distinct bonding positions. These fatty acids are generally nonbranched hydrocarbon chains with an even number of carbons ranging from 4 to 26 carbons atoms (Shils, Shike et al. 2006). In addition to differences in chain length, fatty acids vary in the number and arrangement of double bonds along the hydrocarbon chain. Double bonds identified relative to the methyl end use the terms "n" or "ω" to indicate distance of the first bond along the carbon chain. Addition of further double bonds produces a polyunsaturated fatty acid (PUFA) (Wall, Ross et al. 2010). Fatty acids of 18 carbon atoms or greater that possess more than one double bond contain the first bond of their series only at the n-9, n-6 or n-3 position(Shils, Shike et al. 2006). The human body can produce all but two of the fatty acids it requires. Thus, linoleic acid (LA, C18:2n-6), precursor of the n-6 series of fatty acids, and α-linolenic acid (ALA, C18:3n-3), precursor of the n-3 series of fatty acids, are essential in the human diet (Wall, Ross et al. 2010). A dietary (n-6):(n-3) ratio of 4:1 is recommended as optimal. However, actual dietary intakes of these fatty acids are in excess of 15-16:1, particularly in Western countries, due to increased consumption of LA-rich vegetable oils (sunflower, safflower, and corn oils) and animal fat (Simopoulos 2003). Figure 1.7 shows the chemical structures of EPA, DHA, LA and ALA acid. In parallel with increased LA intakes, increased rates of many diseases that involve inflammatory process have occurred, including cancer. This imbalance can be corrected by increasing the consumption of foods rich in n-3 fatty acids such as eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3).

Marine fish are the principal sources of EPA and DHA. However, the content of marine n-3 fatty acids varies greatly according to the species of fish (Paulsen, Elvsaas et al. 1997). An alternative approach might be to replace n-6 PUFAs with mono-unsaturated fatty acids such as oleic acid (C18:1). It is interesting to note that high intakes of n-6 PUFAs has recently been shown to be associated with increased risk of ulcerative colitis, supporting the concept that type of PUFA may affect inflammation in the colon (de Silva, Olsen et al. 2010).

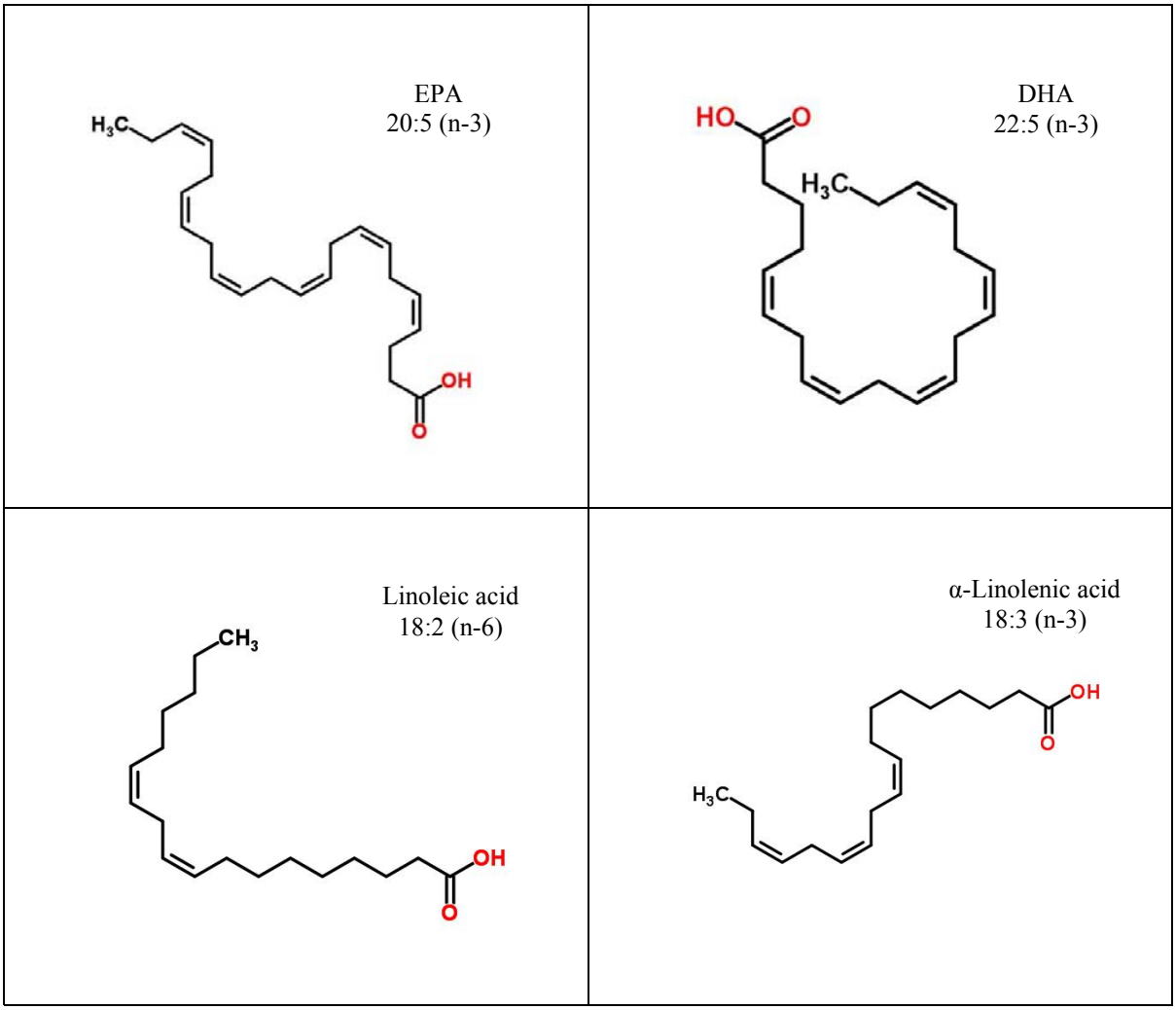

Figure 1.7 Chemical structures of EPA, DHA, Linoleic acid and α-Linolenic acid.

1.4.2. Metabolism of Fatty Acids

Although mammalian cells cannot synthesize LA and ALA, they can metabolize them into more physiologically active compounds by the introduction of further double bonds (desaturation) via desaturases and by lengthening the acyl chain (elongation) via elongases. The Figure 1.8 shows the metabolism of n-6 and n-3 fatty acids. LA is converted into γ-linolenic acid (C18:3n-6) via the action of desaturase, and γ-linolenic acid is elongated via elongase to dihome-γ-linolenic acid (C20:3n-6), which is further converted to arachidonic acid (AA, C20:4n-6). Arachidonic acid is either metabolized to docosatetraenoic acid (C22:4n-6) or to eicosanoids via cycloxygenase (COX) and lipoxygenase (LOX) (Wall, Ross et al. 2010).

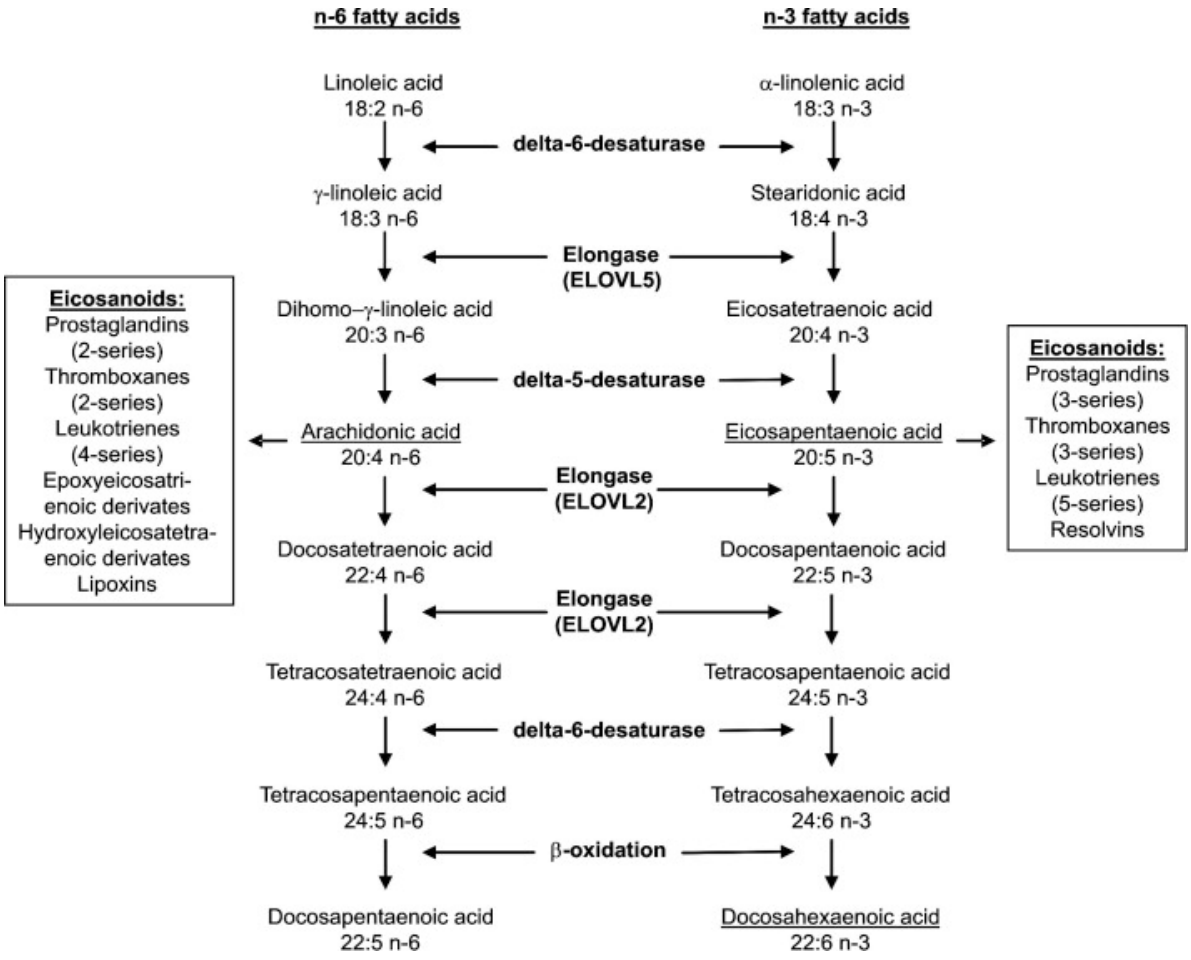

Figure 1.8 Metabolism of n-6 fatty acids and n-3 fatty acids (Wall, Ross et al. 2010).

Using the same series of enzymes as those used to metabolize n-6 PUFAs, ALA is converted to stearidonic acid (C18:4n-3), and then elongated to eicosatetraenoic acid (C20:4n-3), which is converted to EPA via desaturase. EPA is either metabolized to DHA or to eicosanoids via COX and LOX. In mammals, the pathway of desaturation and elongation of n-6 and n-3 fatty acids occurs mainly in the liver. Since LA and ALA are metabolized by the same set of enzymes, competition exists between these two fatty acids, with an excess of one causing a decrease in the metabolism of the other. Normally, desaturases and elongases exhibit affinity to metabolize n-3 over n-6 PUFAs, provided that both exist in a physiological ratio of 1:1-4. However, the higher concentration of LA typically found in the Western diet results in a greater conversion of LA to arachidonic acid (Simopoulos 2003). Nonetheless, increasing ALA intake appears to result in significant enhancements in the EPA content of plasma phospholipids (Mantzioris, James et al. 1994). Furthermore, decreasing the LA content of the diet has been shown to result in increased metabolism of ALA to its longer chain derivatives. For example, a recent study showed that decreasing LA in the diet to an optimal LA:ALA ratio of 4:1 resulted in higher plasma phospholipid EPA and a 40% lower arachidonic acid: EPA ratio than a diet containing a LA:ALA ratio of 10:1 (Liou, King et al. 2007). Approximately 8-20% of ALA is converted to EPA in humans, while conversion of ALA to DHA is less and estimated to be around 0.5-9% (Burdge 2006). Consequently, this route is unlikely to provide sufficient levels of EPA and DHA for optimal human health, which emphasizes the importance of dietary intake of EPA and particularly DHA.

1.4.3. Animal and Epidemiological evidence of the effects of Fatty Acids

In several animal models, such as carcinogenic induced models using DMH (1,2-dymethylhydrazine) and AOM (azoxymethane), the ApcMin/+, and implantation of tumour cells, fatty acids has shown to be protective against colorectal cancer (Chang, Chapkin et al. 1997; Calder, Davis et al. 1998; Paulsen, Ø.Elvsaas et al. 2007). Such studies also provide evidence for the relative protective effects of different fatty acids, such that EPA and DHA provide most protection compared with a sunflower or safflower oil, or even an olive oil diet (Lund 2006).

Several studies done in chemically induced mice/rats have also demonstrated a positive effect of fish oil on preventing tumour development in the colon (Reddy and Sugie 1988; Latham, Lund et al. 1999; Latham, Lund et al. 2001; Rao, Hirose et al. 2001). One recent example is the study performed by van Beelen et al. (van Beelen, Spenkelink et al. 2009) in which AOM-induced rats were treated with 20% by weight of fish oil diet for 8 weeks. They found that animals given fish oil had only half the total number of aberrant crypt foci compared to the animals given the corn oil diet, but also had a significantly lower number of large aberrant crypt foci. In the rat DMH model, fish oil suppressed cell proliferation and increased apoptosis relative to corn oil-fed animals when fed after exposure to the carcinogen (Latham, Lund et al. 1999). Experiments in ApcMin/+ mouse have also demonstrated that n-3 PUFA have an anti-carcinogenic effect (Paulsen, Namork et al. 2000; Bommareddy, Zhang et al. 2009).

One of the dietary habits that may reduce colorectal cancer risk is the consumption of fish. Several observational studies have shown that fish consumption could be related to a decreased risk of colorectal cancer. Recently, a meta-analysis of 19 prospective cohort

studies showed a borderline significant 12% decrease in the relative risk of colorectal cancer 0.88 (95% CI 0.78-1.00) in a comparison of high fish consumption with low fish consumption (Geelen, Schouten et al. 2007). The largest contribution study to this meta-analysis was the European Prospective Investigation into Cancer (EPIC) study, which supported the evidence that increased intake of fish reduce risk of colorectal cancer (Norat, Bingham et al. 2005). However, the evidence of an inverse association between colon cancer and fish intake is less consistent than the evidence of a positive association with red meat (WCRF 1997). A 22-year prospective study of n-3 fatty acid intake and colorectal cancer risk in men was recently published by Hall et al. (Hall, Chavarro et al. 2008). They have found that intake of fish and long-chain n-3 fatty acids from fish may decrease the risk for colorectal cancer.

Despite the possible positive effect of fish consumption on prevention of colorectal cancer, contradictory results were also observed. Pot et al. (Pot, Majsak-Newman et al. 2009) performed a multicentre randomized controlled trial to investigate the effects of a six months intervention with oil-rich or lean fish on markers of colorectal cancer. However, no preventive effect was observed.

The World Cancer Research Report of 2007 (WCRF 2007) have assessed 19 cohort studies and 55 case-control studies on fish and colorectal cancer. They found that 9 cohort studies showed decreased risk for the highest intake group when compared to the lowest intake group, however only two were statistically significant. They concluded that a substantial amount of data is available but the results are inconsistent, therefore, the evidence suggesting that eating fish protects against colorectal cancer was limited. Many of these studies were however limited by a generally low intake of fish in the populations studied.

1.4.4. Chemopreventive Mechanism of Fatty Acids

Several studies indicate that diets high in saturated fatty acids (beef tallow and lard) and n-6 PUFAs (corn oil or safflower) increase the concentration of colonic luminal secondary bile acids (which act as promoters in colon carcinogenesis), whereas dietary fish oil high in n-3 PUFAs had no such enhancing effect (Reddy 1995). The mechanisms by which n-3 PUFA may inhibit the promotion and progression stages of carcinogenesis include 1) suppression of AA-derived eicosanoid biosynthesis, which results in altered immune response to cancer cells and modulation of inflammation, cell proliferation, apoptosis, metastasis, and angiogenesis; 2) influences on transcription factor activity, gene expression, and signal transduction, which leads to changes in metabolism, cell growth, and differentiation; 3) alteration of estrogen metabolism, which leads to reduce estrogen-stimulated cell growth; 4) increased or decreased production of free radicals and reactive oxygen species; and 5) mechanisms involving insulin sensitivity and membrane fluidity (Larsson, Kumlin et al. 2004). In the present review I will focus on the suppression of AA-derived eicosanoid production mechanism, as it is directly related to the scope of my experiment, and it is directly related to inflammation, and consequently carcinogenesis.

As previous mentioned in section 1.2.4, one of the more important functions of PUFAs is related to their enzymatic conversion into eicosanoids, which are short-lived, hormone-like lipids with chain lengths of 20 carbon atoms. Eicosanoids are biologically potent and have a wide array of activities: they modulate inflammatory and immune responses and play a critical role in platelet aggregations, cellular growth, and cell differentiation. The precursor fatty acids for the formation of eicosanoids are dihomo-γ-linolenic acid, AA and EPA. LA and ALA are the predominant plant-derived dietary PUFAs and are the precursors of dihomo-γ-linolenic acid, AA and EPA. The production of eicosanoids begins with the

liberation of PUFAs from membrane phospholipids by the action of various phospholipases. Since inflammatory cells typically contain a high proportion of AA and low proportions of EPA, AA is usually the major substrate for eicosanoids synthesis, and for COX and LOX (Calder 2001). Metabolism of AA by COX gives rise to the 2-series protaglandins (PGs), and the 2-series thromboxanes (TXs). Metabolism of AA by 5-LOX pathway gives rise to hydroxyl and hudroperoxy derivates and to 4-series leukotrienes. The eicosanoids derived from AA are generally ascribed to be pro-inflammatory (Bagga, Wang et al. 2003).

For example, PGE2 induces production of the proinflammatory cytokine IL-6 in macrophages and causes pain and vasodilation (Bagga, Wang et al. 2003). Leukotriene B4 is a potent chemotactic agent for leukocytes and an activator of neutrophils. It also leads to the production of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 by macrophages (Nataraj, Thomas et al. 2001). Indeed, a common characteristic of chronic inflammatory diseases is excessive production of AA-derived eicosanoids (Calder 2001).

EPA also acts as a substrate for COX and LOX enzymes and gives rise to a different family of eicosanoids, the 3-series PGs and TXs, the 5-series leukotrienes (LTs), and the hydroxyl-EPAs. The eicosanoids derived from EPA are considered to be less inflammatory or even anti-inflammatory, compared to eicosanoids derived from AA (Robinson and Stone 2006). The 5-series LTs from EPA are 10 to 100-fold less potent as a neutrophil chemotactic agent than the 4-series LTs from AA, and thus a much weaker inducer of inflammation (Lee, Menica-Huerta et al. 1984). Moreover, Bagga et al. (Bagga, Wang et al. 2003) demonstrated that PGE3 was a less potent inducer of IL-6 production by macrophages than PGE2.

DHA can be metabolized to resolvins via LOX-initiated mechanisms. Resolvins are endogenous, local acting mediators possessing potent anti-inflammatory and immunoregulatory properties. At the cellular level, these include reducing neutrophil infiltration and regulating the cytokine-chemokine axis and reactive oxygen species, as well as lowering the magnitude of he inflammatory response (Serhan, Lu et al. 2007).

Overexpression of COX-2 plays an important role in colon carcinogenesis and it has been implicated in the regulation of apoptosis of rat intestinal epithelial cells and at high concentrations it may suppress apoptosis (Tsujii and DuBois 1995). Elevated levels of COX-2 have been observed in human colon tumours and chemically induced colon tumours in rodents (Kargman, O'Neill et al. 1995; DuBois, Radhika et al. 1996). Studies have shown that a high fat enhanced AOM-induced expression of COX-2 and eicosanoids formation from AA in colon tumours of rats, whereas the high fat fish oil inhibited the levels of COX-2 (Rao, Hirose et al. 2001). The inhibition of eicosanoids production through the modulation of COX-2 activity may be important for the ability of n-3 PUFAs to inhibit colon tumorigenesis. In colon tumours, lowering the levels of PGs may be enough to slow growth by inhibiting proliferation and induction of apoptosis and thus tumour inhibition. Moreover, the reduction in generation of AA-derived mediators that accompanies n-3 PUFAs consumption has led to the idea that fish oil is anti-inflammatory (Calder 2006).

1.5. PHYTOCHEMICAL AND COLORECTAL CANCER

1.5.1. Definition

The “phyto” of the word phytochemicals is derived from the Greek word phyto, which means plant. Therefore, phytochemicals are plant chemicals. Phytochemicals are defined as bioactive non-nutrient plant compounds in fruits, vegetables, grains, and other plant foods that have been linked to reducing the risk of major chronic diseases. It is estimated that >5000 individual phytochemicals have been identified in fruits, vegetables, and grains, but a large percentage still remain unknown and need to be identified before we can fully understand the health benefits of phytochemicals in whole foods (Surh 2003). However, more and more convincing evidence suggests that the benefits of phytochemicals in fruits and vegetables may be even greater than is currently understood.

The phytochemicals can be divided into subgroups of carotenoids, alkaloids, nitrogen-containing compounds, organosulphur containing compounds and phenolics (Figure 1.9). The most studied of the phytochemicals are the phenolics. A benzene ring with at least one hydroxyl substitute is the common denominator of all phenolics, and based on additional structural component the phenolics can be futher divided into phenolic acids, flavonoids, stilbenes, coumarins and tannins (Liu 2004).

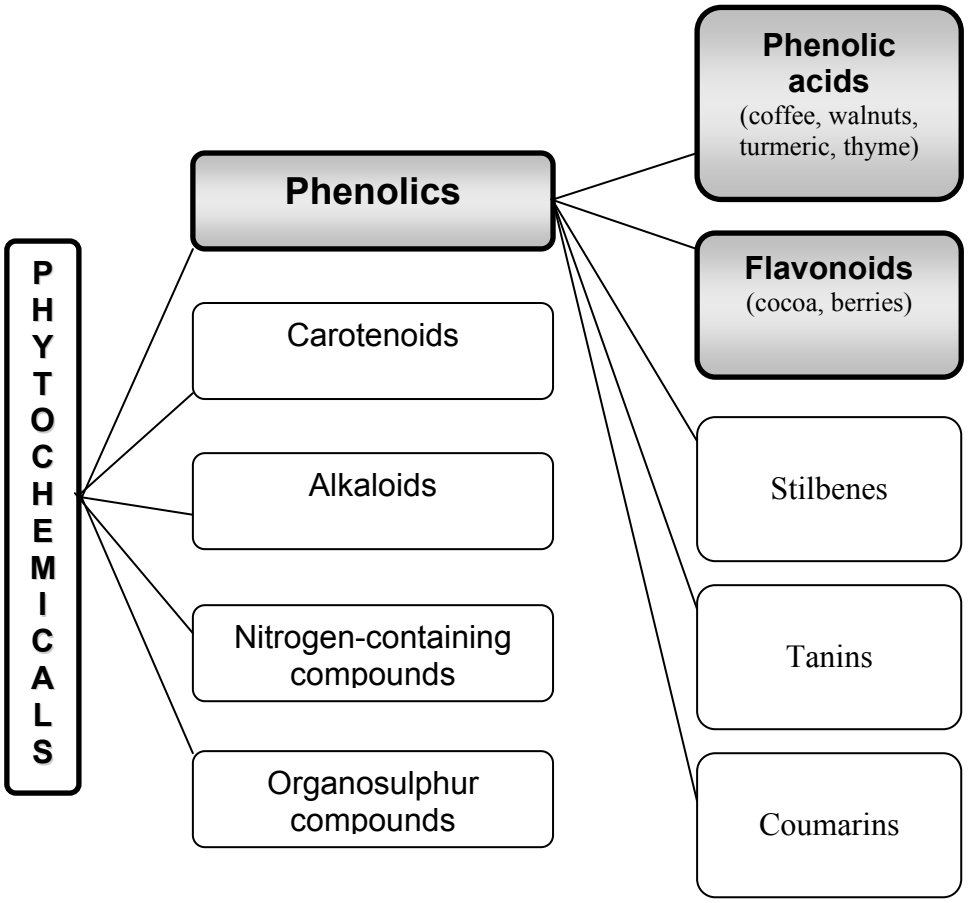

Figure 1.9 Classification of phytochemicals and dietary foods used in the present study.

It is estimated that the total intake of phenolics in our diet is composed of one third phenolic acids and two thirds of flavonoids (Liu 2004), and the main sources are fruits, beverages such as tea, coffee, wine and fruit juices, chocolate and, to a lesser extent, vegetables, cereals and legumes seeds (Scalbert, Morand et al. 2002). The phytochemicals used in this study are mostly classified as phenolic acids or flavonoids. Flavonoids have been identified in fruits, vegetables, and other plant foods and have been linked to reducing the risk of major chronic diseases. Differences in the generic structure classify them as flavonols (quercetin, kaempferol, myricetin), flavones (luteolin, apigenin), flavonols (catechin, epicatechin, epigallocatechin, epicatechin gallate, epigallocatechin gallate),

flavanones (naringenin), anthocyanidins, and isoflavonoids (genistein) are common flavonoids in the diet. Phenolic acids can be subdivided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids.

1.5.2. Metabolism of Phenolics

Indirect evidence of the benefits of phenolic absorption through the gut barrier is the increase in the antioxidant capacity of the plasma following the consumption of phenolic-rich foods. Once absorbed in the small intestine, phytochemicals are transported to the liver or direct to the circulation depending on the solubility (Yeum and Russell 2002). The liver is the main site of metabolism of xenobiotics, however small amounts can be metabolized by the intestinal mucosa. Many phytochemicals share metabolic pathways with xenobiotics, and are thus handled by the same enzymes (the Phase I, II and III enzymes) (Scalbert and Williamson 2000). These phytochemicals are reconjugated in the enterocytes or liver. Phytochemicals are seldom consumed and absorbed in sufficient quantity to saturate their metabolic pathways, and hence they are, by and large metabolized in the intestine and found conjugated in tissue other than intestine and liver (Scalbert and Williamson 2000). Recovery in urine after ingestion of given amounts of a particular polyphenol allows the comparison of the bioavailability of the different molecules present in diets (Scalbert, Morand et al. 2002). The few existing studies show that the quantities of polyphenols found intact in urine vary from one phenolic compound to another. Most dietary phenolics are quickly eliminated in both urine and bile after ingestion. In man, a post-prandial peak is observed 1-2h after ingestion of various flavonols and flavanols but is longer for isoflavones while other polyphenols are only absorbed after partial degradation by the colon microflora (Scalbert and Williamson 2000).

Phenolics are extensively metabolised either in the tissues, once they are absorbed through the gut barrier, or, for the non-absorbed fraction and the fraction re-excreted in the bile, by the colonic microflora (Scalbert and Williamson 2000). All phenolics are conjugated to form O-glucuronides, sulphate esters and O-methylether, however very little is known about the biological properties of these metabolites. Phenolics, once they reach the colon, are extensively metabolised by the microflora. The flavonoid glycosides such as rutin, that are not absorbed in the upper part of the intestinal tract, can be hydrolysed and the aglycone absorbed (Bokkenheuser, Shackleton et al. 1987).

1.5.3. Epidemiological Evidence

Vegetables and fruits, nuts and seeds, and herbs and spices, wherever they grow or can be cultivated, have always been part of human diets. In the past, vegetables were the main source of many vitamins, and fruits the main source of energy for hunters and pastoral peoples. However, recently, the consumption of vegetables and fruits has been decreasing because of the higher consumption of other foods and food products commonly used in a Western diet. Evidence that vegetables and fruits might be protective against some cancers emerged in a greater depth in the 1990's (WCRF 1997). However, the evidence for a large preventive effect of vegetable and fruits came primarily from case-control studies, which can be readily biased by differences in recall of past diet by patients with cancer and healthy control subjects (Willett 2010). Prospective studies largely avoid bias because of recall and selective participation, but results of large prospective cohort studies of diet and cancer began to come together, and these did not confirm the strong inverse associations found in most case-control studies (Willett 2010).

Riboli and Norat (Riboli and Norat 2003) have published in 2003 a meta-analysis of epidemiologic studies of the protective effect of fruit and vegetable on colorectal cancer risk. They found 28 case-control and 12 cohort studies on colorectal cancer, and only three case-control studies reported a significant protective effect of high vegetable intake and the remaining reported no association. In the case of fruits, only two of the case-control studies reported significant protective effects of citrus fruits. Their conclusion was that the pooled relative risk indicates that there is a moderate but significantly decreased risk of colorectal cancer with high intake of vegetables and fruit for all studies combined. That protective effect was significantly stronger in case-control studies, while cohort studies did not support the hypothesis of a protective effect of vegetable and fruit consumption on colorectal cancer.

Miller et al. (Miller, Lesko et al. 2010) have published a very recent review of the epidemiological evidence of the protective effects from dietary patterns on colorectal cancer mostly form cohort studies. A total of 16 articles published up to January 2009 were included in the review, 10 were cohort, and 6 were case-control studies. They found 1 cohort and 2 case-control studies reporting the beneficial effects of high intake of fruits and vegetables against colorectal cancer. However, they concluded that the association of fruits and vegetables with colorectal cancer risk were modest and not consistently statistically significant, which is in agreement with much of the previous literature.

Furthermore, a European cohort study performed by Boffetta et al. (Boffetta, Couto et al. 2010) of nearly 400 000 men and woman who developed approximately 30 000 cancers at all sites combined over nearly 9 years of follow-up showed a 4% lower incidence of all cancers combined for an increment of 200g of total fruits and vegetables per day.

The results from the European Prospective Investigation into Cancer and Nutrition (EPIC) related to colorectal cancer was published by van Duijnhoven et al. (van Duijnhoven, Bueno-De-Mesquita et al. 2009). They found that higher consumption of fruit and vegetables may protect against the development of colorectal cancer, especially colon cancer. They also concluded that the association was stronger in never and former smokers, whereas current smokers showed a positive association.

1.5.4. Coffee

Coffee (Coffea robusta) is one of the most widely consumed beverages in the world and is the major contributor of redox active phytochemicals in the diet (Tosetti, Noonan et al. 2009). The main constituents of coffee include caffeine, diterpenes, phenolic acids, and melanoids and acrylamide produced during roasting of coffee beans (Bekedam, Loots et al. 2008). Coffee can potentially impact on the aetiology of cancer of various sites along multiple pathways, ranging from carcinogenesis to cellular apoptosis (Arab 2010). However, the World Cancer Research Report (WCRF), addressed only the relationships between coffee and risk of pancreatic and kidney cancer (WCRF 2007). The effect of coffee on colorectal cancer prevention has been contradictory. Case-control studies showed a potential effect of coffee against colorectal cancer (Tavani and Vecchia 2004). While some cohort studies showed no association (Tavani and Vecchia 2004), others showed a positive effect (Oba, Shimizu et al. 2006; Lee, Inoue et al. 2007). Coffee has been shown to have anti-inflammatory effect (Paur, Balstad et al. 2010), however a lack of effect has been reported in relation to carcinogenesis in animal models of colorectal cancer (Oikarinen, Erlund et al. 2007; Park, Davis et al. 2010).

1.5.5. Walnuts

Walnuts (Juglans regia) are the subject of increasing interest as a source of phytochemicals mainly due to the fact that their regular consumption has been reported to reduce the risk of coronary heart disease (Blomhoff, Carlsen et al. 2006) and some types of cancers (Mathew, Peters et al. 2004). Walnuts are good sources of essential fatty acids (linoleic acid is its major fatty acid), tocopherols and tocotrienols, proteins, fibre, melatonin, sterols, folate, tannins and other phenolics (Pereira, Oliveira et al. 2008). Among several nut types, walnuts have the highest content of phenolics and most of the compounds are located in the pellicles (Blomhoff, Carlsen et al. 2006). Despite some studies showing a positive association between regular consumption of nut and reduced incidence of several chronic diseases, the World Cancer Research Report (WCRF) considered the evidence was too limited in amount, consistency, or quality to draw any conclusions (WCRF 2007). Nevertheless, an anti-cancer effect of walnuts has been demonstrated in MDA-MB 231 human breast cancers implanted into nude mice (Hardman and Ion 2008).

1.5.6. Cocoa

Cocoa (Theobroma cacao L.) is rich in soluble flavonoids (catechin and epicatechin), which are reducing agents, and one of their effects is protection against oxidative stress, an imbalance between the concentrations of reactive oxygen species (ROS) and the defence mechanisms of the body (Maskarinec 2009). Cocoa also has high concentration of procyanidins (polymeric condensation products of catechins) formed during fermentation. These bioactive compounds constitute 60% of the total phenolics content in cocoa products (Hammerstone, Lazarus et al. 2000). The majority of the human studies on cocoa products and disease risk have reported on cardiovascular disease (Cooper, Donovan et al. 2008).

However, in experimental settings, catechins and procyanidins have been shown to influence the immune response by modulating the activation of NF-kB, and consequently might be preventing cancer by damping inflammation (Maskarinec 2009).

1.5.7. Turmeric and Thyme

Turmeric is prepared from the root of the Curcuma longa plant, a member of the ginger family, and curcumin is its principal constituent, and is one of the principal ingredients in curry powder. Curcumin is in modern use worldwide as a cooking spice, flavouring agent and colorant. The active ingredient of curcumin is diferuloylmethane, a hydrophobic phenolic with a characteristic yellow colour (Epstein, Sanderson et al. 2010). Curcumin has chemo-preventive and therapeutic properties against many tumours in *in vitro* and *in vivo* models and has been tested in a number of clinical trials (Khor, Keum et al. 2006). The safety, tolerability and non-toxicity of curcumin at high doses have been established, and in human trials, only minor side effects have been reported (Anand, Kunnumakkara et al. 2007; Johnson and Mukhtar 2007). In colon cancer cell lines, curcumin has shown anti-inflammatory (Chen and Xu 2005), pro-apoptotic (Song, Mao et al. 2005), and anti-cancer effects (Chen and Xu 2005; Song, Mao et al. 2005). In animal models curcumin has also been shown to have chemopreventive efficacy in several studies (Rao, Rivenson et al. 1995; Pereira, Grubbs et al. 1996; Collett, Robson et al. 2001; Kim and Hellerstein 2007).

Thyme is among the commercially available spices with the highest total antioxidant capacity, and rosmarinic acid is among the phytochemical compounds present in thyme. Thyme has been reported to reduce inflammation in a model of colitis (Bukovska, Cikos et al. 2007).

1.5.8. Berries

Berries such as blackberries, cranberries, strawberries, raspberries and blueberries contain significant amounts of phenolics, especially flavonoids. Anthocyanins are particularly prevalent in berries, reaching concentrations in excess of 10g/kg in some cultivars (Coates, Popa et al. 2007). Moreover, anthocyanins can modify cancer biomarkers *in vitro* (Coates, Popa et al. 2007), and also inhibit cancer cell growth in culture (Kamei, Kojima et al. 1996). In the present study, the berries used in the experimental diet were bilberries (Vaccinium myrtillus L) and blackcurrant (Ribes nigrum). Bilberries and blackcurrants are rich in redox active compounds especially anthocyanins, which give rise to the colour, but also contain flavonols, phenolic acids, stilbenes, catechins and lignans (Hakkinen, Karenlampi et al. 1999; Sandell, Laaksonen et al. 2009). Several *in vivo* studies have demonstrated the anti-carcinogenic effect of berries in different models of colorectal cancer (Hagiwara, Miyashita et al. 2001; Gee, Hara et al. 2002; Bobe, Wang et al. 2006). Regarding the substantial amount of inconsistent evidence from several cohort studies on the protective effect of fruits, the World Cancer Research Report (WCRF) concluded there is limited evidence suggesting that fruits protect against colorectal cancer (WCRF 2007).

1.5.9. Chemopreventive Mechanism of Phytochemicals

The need for biologically active compounds with low profiles of adverse reactions compared to pharmacological drugs has triggered an extensive investigation of plants and phytochemicals and their mechanisms of action. The biological activities of these phytochemicals are often determined by their physical and chemical properties, which in turn are determined by the structures of the compounds, the type of functional groups present and the utilized concentration. Several phytochemicals exert multiple cellular

effects involving one or more signalling pathways. These effects can be synergistic, additive or even antagonistic depending on the cell type, the concentration of the phytochemical and its surrounding environment (Issa, Volate et al. 2006).

There is growing evidence that a number of different classes of food constituents such as flavonoids, can regulate the process of cell proliferation and death *in vitro*, and in colorectal crypts *in vivo* (Johnson 2002). The flavonoids consumed in the diet are metabolized and the metabolites appear in the circulation soon after consumption, but the epithelial tissues of the gastrointestinal tract are probably exposed to much higher local concentrations (Halliwell, Zhao et al. 2000).

As discussed in section 1.1.2.2 an important pathway involved in the regulation of cell proliferation in colorectal epithelia is that involving the intracellular protein β-catenin (Byun, Karimi et al. 2005). In normal epithelial cells there is a relatively large and stable pool of β-catenin immobilized by interactions with cytoskeletal proteins, and a small labile pool in the cytoplasm. However, in cancer cells the balance is altered in favour of the cytoplasmic pool because the regulatory mechanism for β-catenin is disrupted by various mutations or epigenetic events (Gregorieff and Clevers 2005).

The survival of most cancer cells depends critically upon their ability to divide continuously, and to evade apoptosis (Pierini, Gee et al. 2008). A variety of different flavonoids have been shown to inhibit proliferation and enhance apoptosis *in vitro*. Despite the great variety of naturally occurring compounds shown to be active *in vitro*, it has proven much more difficult to establish that food-borne flavonoids increase apoptosis in animals or humans (Johnson 2002).

Many dietary chemopreventive polyphenols, including quercetin, EGCG, curcumin, resveratrol and others, are believed to evoke their inhibitory effect on carcinogenesis though the induction of apoptosis (Surh 2003). Ramos (Ramos 2008) has described in her review how phytochemicals can affect apoptosis by describing the effect of EGCG which induces ROS generation and may mediate apoptosis by inducing DNA fragmentation, activation of caspases-3 and -9, release of the apoptogenic cytochrome c, Smac and apoptosis-inducing factor (AIF), in concert with diminished levels of anti-apoptotic proteins such as Bcl-2 and myeloid cell leukaemia-1 (Mcl-1). Polyphenol treatment of several cancer cell lines induced apoptosis through activation of proteins related to programmed-cell death pathways such as caspases-3, -9 and -8, as well as through the inhibition of other proteins such as the inhibitor of apoptosis protein-2 (c-IAP2), X-linked IAP, Bcl-2, Bcl-xl and Bid (Hayakawa, Saeki et al. 2001; Nishikawa, Nakajima et al. 2006). Moreover, promotion of apoptotic cell death by EGCG in the sarcoma 180 cell line was induced through G2/M cell cycle arrest, down-regulation of Bcl-2 and c-myc, up regulation of p53 and Bax (Manna, Banerjee et al. 2006). Hastak et al. (Hastak, Agarwal et al. 2005) has demonstrated that inactivation of p53 by using small interfering RNA generated resistance against EGCG-induced apoptosis primarily via a p53-dependent pathway which involved the function of both p21 and Bax. Curcumin activated caspases-3, -7, -8 and -9 in several colon cancer cell lines, but a reduced activation of caspases related to the mitochondrial pathway together with a partial blocking of AIF (Rashmi, Santhosh Kumar et al. 2003).

There are several other chemopreventive mechanisms of phytochemicals against carcinogenesis. Possibly different mechanisms will be associated with particular phytochemicals and may affect tumorigenesis at different stages of cancer development (initiation, promotion, and progression). Another possible mechanism by which

phytochemicals may suppress tumour development may be involved in their antioxidants properties.

During all the stages of cancer development many key proteins related to cellular antioxidant defences, cellular proliferation and survival transduction pathways are up-regulated or down-regulated (Ramos 2008). A better understanding of these mechanisms is vital to properly utilize the phytochemicals as promising agents that promote health and prevent diseases (Ramos 2008; Randi, Edefonti et al. 2010).

2. AIMS OF THE STUDY

It is known that colorectal cancer is enhanced by a high fat diet, which plays an important role on tumour development. The present thesis was proposed to test the hypothesis that the effect of a high fat diet on the development of colorectal cancer would vary according to the type of fat consumed by ApcMin/+ mouse. Moreover, this thesis was also proposed to test the hypothesis that phytochemicals would have positive effect by preventing colorectal cancer development. To test these hypothesis, two separate animal experiments were performed, one to evaluate the effects of a high fat diet (25%) rich in dietary n-3 fatty acids (fish oil diet, FO) or n-6 fatty acids (corn oil diet, CO) on colorectal cancer development in ApcMin/+ mouse model. The aim of the second experiment was to test the hypothesis that a high anti-oxidant phytochemical mix would protect against intestinal cancer development in ApcMin/+ mouse. Both experiments had the same control (palm oil diet, PO) and the same endpoints. The following effects were analysed:

- ✓ Effect of sex, genotype and diet on body and organs weight.
- ✓ Effect of genotype and diet on mitosis, apoptosis and crypt length in the small and large intestine as early markers of cancer risk.
- ✓ Effect of genotype and diet on cytokine levels, as a marker of inflammatory status, in the small and large intestine, and in the plasma.
- ✓ Effect of diet on tumour size and tumour number in the small and large intestine.

3. MATERIAL AND METHODS

In this chapter the details regarding the diet composition used in both experiments is presented. The mouse breeding programme is also described, as well as the experimental design. Moreover, it describes the methods of the analysis done in the present study, such as body weight, tissue and blood collection; DNA extraction; polymerase chain reaction; fatty acids measurement; cell proliferation and apoptosis; protein assay; scoring tumour size and number; and cytokines measurements are described in detail. The statistical method used to analyse the data in the study is also presented in the chapter.

3.1. DIET

The treatment diets were supplied by Special Diets Services (Essex, UK). The composition of the diet is summarized in Table 3.1 and the fatty acids content in Table 3.2. All the treatment diets had 20 % energy as protein, 55 % energy as carbohydrate, and 25% energy as fat. The palm oil group was used as control diet, which contained the following fatty acids: 500 mg/g saturated, 400 mg/g monounsaturated, and 100 mg/g polyunsaturated. The corn oil diet contained 130 mg/g saturated, 270 mg/g monounsaturated, and 600 mg/g polyunsaturated. The fish oil used for the preparation of the fish oil diet contained 202 mg/g of EPA and 260 mg/g of DHA and the weight of fish oil was 11.25g/kg diet. Consequently, for each kilo of the treatment fish oil diet the content of EPA and DHA was 112.5 x 0.202g = 22.7g and 112.5 x 0.260g = 29.3g, respectively, a total of 52g/kg diet.

Palm oil was chosen as a control diet because it hasn't previously been shown to have a specific carcinogenic effect in animal models of colorectal cancer. However, corn oil has been shown to be carcinogenic (Whelan and McEntee 2004), while fish oil had

demonstrated protective effects on colorectal cancer in several experiments (Paulsen, Elvsaas et al. 1997; West, Clark et al. 2010) . The average intake of diet was 2.8g per mouse/day, and the average intake of EPA+DHA for animals fed the fish oil diet was 145.6 mg/mouse/day (63.6 mg/mouse/day of EPA and 82 mg/mouse/day of DHA).

The amount of EPA + DHA in this diet was high compared to the dietary recommendations for humans whether calculated based on per kg body weight or per kg diet.

Firstly the amount of EPA+DHA given to the mice per kg of body weight is converted to the same dose for a human with an average weight of 70kg. Each mouse weighed on average 20g (0.02kg) and ingested 0.146g of EPA+DHA per day or 7.3g/kg body weight. Converting the same dose of fatty acids to a human with an average weight of 70kg, the amount would be equivalent to 509.6 g of EPA+DHA per day. Considering the present recommendation of EPA and DHA for a healthy adult being around 500mg/day, or a maximum of 2-3g per day in specific cases, it demonstrates that the dose used per kg of body weight in the experiment was extremely high.

Alternatively it can be argued on the basis of dietary content and that the amount of EPA and DHA in the mouse diet was 52 g/Kg diet. Humans eat approx. 500g/d dry weight of food (Denomme, Stark et al. 2005). A high EPA+DHA supplement may provide as much as 3g of EPA+DHA per day (Engeset, Alsaker et al. 2005) which would be equivalent to 6g/Kg dry weight. Alternatively a Norwegian traditional diet contains about 200g/d fish of which about half is oily fish plus fish roe and cod liver oil. Oil-rich fish may contain about 2g/100g EPA+DHA so such people would be eating perhaps over 2g EPA+DHA per day. On this basis the diet had about ten times the concentration in an ideal human diet.

The phytochemical diet had palm oil as a source of fat, but also contained 100g/kg of phytochemical mix containing coffee (*Coffea robusta*), cocoa (*Theobroma cacao L.*), turmeric (*Curcuma domestica*), thyme (*Thymus pulegioides*), walnuts (*Juglans regia*), and mix of bilberries and blackcurrant (*Vaccinium myrtillus* and *Ribes nigrum*). The content of phenols in the phytochemical diet was calculated by the Phenol-Explorer, which is a comprehensive electronic database on polyphenol content in foods Table 3.3 (Neveu, Perez-Jiménez et al. 2010).

The phytochemical mix was added in place of cellulose (100g/kg) as previous studies have shown that 10% non-fermentable polysaccharides have no effect on adenoma number in the small intestine or colon (Moser, Dove et al. 1992), leaving all other nutrients unchanged except those added as part of the phytochemical mix.

The phytochemical mix was constituted by six different food ingredients with the following quantities: coffee (31% of 100g/kg of diet), cocoa (31% of 100g/kg of diet), and walnuts (31% of 100g/kg of diet). Only a small part of the mix was constituted by turmeric and thyme (3.1% of 100g/kg of diet) and berries (0.16% of 100g/kg of diet). According to the phytochemical classification (Liu 2004), the mix used in the present diet was mainly rich in phenolic acids and flavonoids, which are a subgroup of phenolics.

The content of phytochemicals in the diet was 442 mg of flavonoids, phenolic acids and other polyphenols/ kg of diet. As the average food consumption was 2.8g per mouse, the animals consumed a total of 442 x 2.8/1000 mg =1.2 mg of polyphenols per day, which equals to 62 mg of polyphenols/ kg of mouse body weight. Converting the same amount of phytochemicals ingested by the mice to a human weighing 70kg, it would be equivalent to 4.3 g of polyphenols per day. A study performed by Saura-Calixto et al. (Saura-Calixto,

Serrano et al. 2007) found that the content of total polyphenols in a whole Spanish diet was between 2.6 and 3. 02 g/person/day, Therefore in this part of the study the intake was not unduly high compared to human consumption. The concentration in the human diet is approx. 3 g/500g dry weight or 6 g/kg while the mouse diet contained only 0.4g so on this basis the intake was low compared to the Spanish diet.

The content of Vitamin E (33IU/kg), zinc (10mg/kg) and selenium (150µg/kg) were reduced to a minimum level in all diets because of its possible protective effect against colorectal cancer (Kucharzewski, Braziewicz et al. 2003; Peters and Takata 2008).

The phytochemical mix used in the present study was chosen based on the study performed by Paur et al (Paur, Austenaa et al. 2008). They have screened 35 dietary plant extracts looking for the best candidates that would modulate basal or induced NF-kB in vitro and in vivo. The reason they have chosen NF-kB as the molecular target is because this protein has been identified as a promising therapeutic target both in cancer and chronic inflammation. At the end of their study, they found five efficient inhibitors of NF-kB, and ten extracts with the dual effect of increasing basal and inhibiting LPS/TNF-α induced NF-kB activity. Based on that, the phytochemical mix used for the present experiment was formulated with the expectation that it would be a good mixture of chemopreventive phytochemicals likely to oppose the promotion of tumorigenesis in the ApcMin/+ mouse model. Paur et al. (Paur, Balstad et al. 2010) have also used a mix of phytochemicals from dietary plants in an animal study. However, they have used a single dose of the dietary extracts via oral gavage corresponding to an amount of 1.38g of the dietary plant.

Dietary plants contain a vast number of phytochemicals that are divided into several subgroups. However, in the present study the phytochemicals present in the phytochemical

mix belong mainly to the phenolic group, which is also subdivided into phenolic acids, flavonoids, stilbenes, lignans, and coumarins (Liu 2004). The foods used in the formulation of the phytochemical mix are mainly a source of phenolic acids and flavonoids.

Diet composition	**Palm oil**		**Corn oil**		**Fish oil**		**Phytochemical**	
	gm%	*kcal%*	*gm%*	*kcal%*	*gm%*	*kcal%*	*gm%*	*kcal%*
Protein	20	20	20	20	20	20	20	20
Carbohydrate	55	55	55	55	55	55	55	55
Fat	11.25	25	11.25	25	11.25	25	11.25	25
Other	11	1	11	1	11	1	11	1
Total	100	100	100	100	100	100	100	100
kcal/gm	4.1		4.1		4.1		4.1	
	gm	*kcal*	*gm*	*kcal*	*gm*	*kcal*	*gm*	*kcal*
Casein, Iatic	200	800	200	800	200	800	200	800
L-cystine	3	12	3	12	3	12	3	12
Corn starch	272.61	1194.6	272.61	1194.6	272.61	1194.6	272.61	1194.6
Maltodextrin	91.66	366.64	91.66	366.64	91.66	366.64	91.66	366.64
Sucrose	162.98	651.92	162.98	651.92	162.98	651.92	162.98	651.92
Cellulose	100	0	100	0	100	0	0	0
Soybean oil	31.25	281.25	31.25	281.25	31.25	281.25	31.25	281.25
Palm oil	81.5	733.5	0	0	0	0	81.5	733.5
Corn oil	0	0	81.5	733.5	0	0	0	0
Fish oil	0	0	0	0	81.5	733.5	0	0
Phytochemical mix	0	0	0	0	0	0	100	0
Mineral mix	10	0	10	0	10	0	10	0
DiCalcium phosphate	13	0	13	0	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0	5.5	0	5.5	0
Potassion citrate	16.5	0	16.5	0	16.5	0	16.5	0
Vitamin mix	10	40	10	40	10	40	10	40
Choline bitartrate	2	0	2	0	2	0	2	0
Total	1000	4079.91	1000	4079.91	1000	4079.91	1000	4079.91

Table 3.1 Dietary composition of the treatment diets given to ApcMin/+ and wild-type mice for 10 weeks.

C:D	**Fatty Acids**	**Palm oil Diet (%)**	**Corn oil Diet (%)**	**Fish oil Diet (%)**	**Phytochemical Diet (%)**
C14:0	Myristic acid	0.87	0.27	0.21	0.28

C14:1	Myristoleic acid	0.52	0.21	0.23	0.17
C16:0	Palmitic acid	33.10	11.42	27.71	27.04
C18:0	Stearic acid	5.97	2.35	6.75	4.10
C16:1	Palmitoleic acid	0.01	0.04	7.16	0.02
C18:1	Oleic acid	33.15	30.11	18.02	29.67
C18:2	Linoleic acid	22.13	62.21	15.62	30.09
C18:3	Gama-Linolenic acid	1.82	2.65	2.25	4.15
C20:0	Arachidic acid	0.44	0.43	0.39	0.34
C20:1	Gadoleic acid	0.11	0.08	0.55	0.15
C20:2	Eicosedienoic acid	0.06	0.04	0.11	0.03
C20:4	Arachidonic acid	0.01	0.02	0.58	0.02
C20:3	Eicosatrienoic acid	0.02	0.00	0.02	0.00
C22:0	Behenic acid	0.21	0.20	0.17	0.12
C22:1	Erucic acid	0.02	0.00	0.39	0.00
C20:5	Eicosapentaenoic acid - EPA	0.03	0.16	9.71	0.19
C24:0	Lignoceric acid	0.01	0.09	0.10	0.02
C24:1	Nervonic acid	0.01	0.00	0.20	0.00
C22:6	Docosahexaenoic acid - DHA	0.01	0.07	4.77	0.13

Table 3.2 Fatty acids content of the treatment diets.

Ingredients	Flavonoids	Phenolic acids	Other polyphenols	Stilbenes
Cocoa (*Theobroma cacao L.*)	159.62	11.56	0.14	0
Coffee (*Coffea robusta*)	0	63.83	0	0
Turmeric (*Curcuma longa*)	0	0	169.52	0
Thyme (*Thymus spp*)	0	27.41	0	0
Walnuts (*Juglans regia*)	0	8.89	0	0
Bilberry (*Vaccinium myrtillus*)	0.002	0.004	0	0.001
Blackcurrant (*Ribes nigrum*)	0.96	0.02	0	0
Total	**160.58**	**111.71**	**169.66**	**0.001**

Table 3.3 Total content of flavonoids, phenolic acids, other polyphenols and stilbenes in the phytochemical dietary group. Values are presented as mg/kg of diet. Values were calculated based on the Database on Polyphenol content in Foods.

3.2. MOUSE BREEDING

The ApcMin/+ mouse is a transgenic model of human intestinal tumorigenesis that bears a germline mutation at codon 850 of the mouse homologue of the human Adenomatous Polyposis Coli gene, which is frequently mutated in human colon cancer. By age 4-5 months, these mice bear numerous visible tumours in the intestinal tract and usually die due to intestinal blockage, bleeding, and severe anemia (Moser, Dove et al. 1992). Fifteen

males *Min* heterozygote mice (C57BL/6L ApcMin/+) and thirty females inbred C57BL/6L were obtained from Jackson Laboratory in USA. Animals arrived at the Disease Modeling Unit (DMU) at the University of East Anglia (UEA) at eight weeks of age. Fifteen breeding 'pairs' (one C57BL/6L ApcMin/+ male and two inbred C57BL/6L females per cage) were set up two weeks after the animal's arrival. The *Min* mutation cannot be propagated through the female, because anemia and intestinal adenomas interfere with pregnancy (Paulsen, Ø.Elvsaas et al. 2007). As reproductive performance generally decreases with age, breeders who did not produce litters within six to eight week had the males exchanged between the female's breeders. Also, if the male breeder was visually sick, it was also replaced by a younger breeder. All mice used in the experiment were related within the number of generations necessary for securing their status as inbred. The animals were housed in plastic cages with temperature (23 ± 2C) and humidity (40-60%) controlled with a 12h light-dark light cycle. All mice had continual access to food and water and were observed daily for health status. The total number of mice for the experiment was obtained from 69 breeding cages during a period of twelve months. Offspring were genotyped by polymerase chain reaction, and then used as breeder or put on the treatment diet.

3.3. EXPERIMENTAL DESIGN

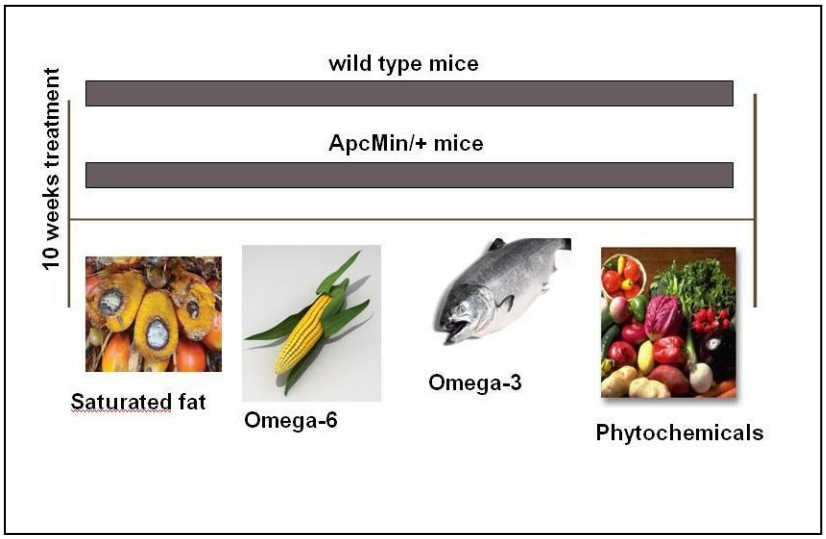

Figure 3.1 Study design

The animal experiment was carried out according to a protocol approved by the DMU ethical committee at the UEA and covered by Prof Ian Johnson's Home Ofice Project Licence. A total of 128 mice were randomly assigned to four different diet groups (n=32/group): palm oil as control diet; corn oil; fish oil; or phytochemical (Figure 3.1). The composition of the diet is described in section 2.1. Each dietary group had 16 ApcMin/+ mice and 16 wild-type mice, half males and half females. The animals were allowed to consume the diets *ad libitum* for 10 weeks. At the end of the 10 weeks, animals were anaesthetized by inhalation of Isoflurane (IsoFlo – Abbott Animal-Health, Abbott Laboratories Ltd. Queensborough, Kent, UK), opened ventrally and blood collected by cardiac puncture before cervical dislocation to ensure death prior to organ removal for further analysis. The animal experiment was designed to have the data analyzed as two separate studies. One study focusing on the effects of fatty acids on gastrointestinal cancer, and the other study on the effects of phytochemical. Due to ethical reasons and the need to reduce the total number of animals used in the experiment, as requested by the Home Office, it was decided to have only one control group (PO diet), which was shared between

both studies. Tail snips were taken at weaning for the genotype analysis by polymerase chain reaction (PCR).

3.4. BODY WEIGHT, TISSUE AND BLOOD COLLECTION

During the experiment the animals were weighed at the beginning of the study, at week 5 and at the end, week 10 before being killed. Blood was centrifuged at 14,000 rpm for 5 min., the plasma collected and transferred into another labeled microfuge tube and stored at -80°C for future analysis. Spleen, liver, heart, kidney, stomach, and caecum were removed, weighed, immediately put on dry ice, and stored at -80°C until further analysis. The intestines were also removed for scoring the tumours as described in section 2.10.

3.5. DNA EXTRACTION

The DNA was extracted from the tail snips by using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). The tail snip after having being thawed slightly was placed in a 1.5mL microcentrifuge tube, and 180µL of lysis solution was added, followed by 20µL of the Proteinase K solution to the rodent tail. After vortexing, the sample was incubated at 55°C until the tail tissue was completely digested. The sample was vortexed again after the digestion was completed. 200µL of lysis solution was added to the sample and vortexed. 500µL of the Column Preparation Solution was added to each pre-assembled GenElute Miniprep Binding Column and centrifuged at 12,000 x g for 1 min. 200µL of ethanol was added to the lysate. The entire contents of lysate was transferred into the treated binding column and centrifuged at 6500 x g for 1 min. The collection tube was

discarded and the binding column was placed in a new 2 mL collection tube. 500µL of Wash Solution was added to the binding column and centrifuged for 1 min at 6500 x g. The collection tube was discarded and the binding column was placed in a new 2 mL collection tube. Another 500µL of Wash Solution was added to the binding column and centrifuged for 3 min at maximum speed 16,000 x g to dry the binding column. The column was centrifuged for one additional minute at maximum speed. The flow-through liquid was discarded and the binding column was placed in a new 2 mL collection tube. 200µL of the Elution Solution was added directly to the center of the binding column and centrifuged for 1 min at 6,500 x g to elute the DNA. The pure genomic DNA was frozen in -20°C until further analysis.

3.6. POLYMERASE CHAIN REACTION (PCR)

PCR is an in vitro DNA amplification that involves a repeated cycling process of defined stages, termed denaturation, annealing, and extension. The reagents required for the PCR include DNA polymerase, each of four nucleotides (dNTPs), a primer, magnesium source, buffer solution and DNA template (Maurer 2006). The DNA to be amplified was isolated from tail snips of mice as described in section 3.5.

The PCR method used in the present study has been previously described by Leu at al. (Hu, Le Leu et al. 2007). 5 µL of genomic DNA was amplified in a 57.7 µL reaction volume per sample containing final concentrations of 100 µM oIMR0033, 100 µL oIMR0034, 100 µL oIMR0758 (Table 3.4, primers purchased from Sigma, UK), 2.5 µM each of dATP, dCTP, dGTP, dTTP (Promega Corporation, USA), 24µM $MgCl_2$, 5x Green GoTaq Flexi Reaction Buffer (Promega Corporation, USA), autoclaved water, 5 U/µL Taq

polymerase (Promega Corporation, USA). The amplification conditions were 36 cycles of 3 min at 94°C, 30 seconds at 94°C, 1 min at 55°C, 1 min at 72°C, followed by a final extension at 72°C for 2 min.

The primers oIMR0033 and oIMR0034 were used to amplify the wild-type allele and primers oIMR0034 and oIMR0758 was used to amplify the disrupted allele. The ApcMin/+ mice could be identified by the wild-type allele generated a 600bp fragment and the disrupted allele generated a 340bp fragment.

When DNA is heated in excess of 94-95°C for at least 60 seconds, the double strands come apart to produce two single strands. This process is called denaturation, which allows the primers to bind to the DNA template (annealing), whose sequences are complementary to the primers. In the next stage the temperature is reduced to 35-60°C for 30-120 seconds. Subsequently, the polymerase makes a complementary copy of the template DNA started from each primer, thereby creating a double strand of the target region. Known as extension, this step usually takes places at 72°C for 60-180 seconds. In the next cycle, the DNA produced from the previous cycle becomes new template to produce a double new DNA (Maurer 2006)

The PCR products were visualized by eletrophoresis. The prepared 1.5% agarose gel (Promega Corporation, USA) on a supporting plate is submerged into a chamber containing electrophoresis buffer (TAE 1x). The wells were created in the agarose gel with the aid of a comb inserted into the cooling agarose. Into the wells, samples with have been mixed with a loading dye were loaded. Eletrophoresis was carried out for 45min at room temperature followed by staining with ethidium bromide (Sigma-Aldrich, UK). A

successful PCR amplification results a strong band of targeted amplified product with faint bands of residual nucleotides and primers (Figure 3.2).

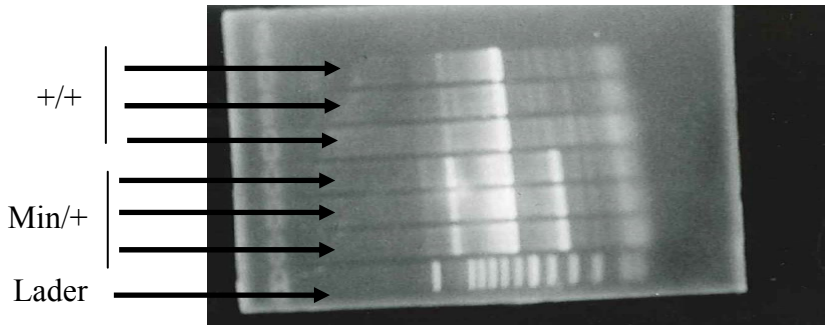

Figure 3.2 PCR products from DNA from mice tails runned on 1,5% agarose gel.

Primer	Sequence	Primer type
oIMR0033	5'-GCC ATC CCT TCA CGT TAG	Wild-type
oIMR0034	5'-TTC CAC TTT GGC ATA AGG C	Common
oIMR0758	5'-TTC TGA GAA AGA CAG AAG TTA	Mutant

Table 3 4 Primers used in amplification and sequencing of the samples.

3.7. FATTY ACIDS MEASUREMENT

Lipids were extracted using the Bligh and Dyer method (Bligh and Dyer 1959). Diets were homogenised in water. In a pre-labelled and pre-weighed vial 250 µL of sample was added to 550 µL water, 800 µL Butylated hydroxyltoluene/Chloroform (10 mg BHT/ 100 ml $CHCL_3$) and 1.6 mL Methanol (MeOH). Samples were vortexed and centrifuged at 2,000 rpm at 4°C for 7 min. The bottom layer was removed and put into a pre-weighed vial and dried under oxygen-free nitrogen. Then, 1 mL of BHT/$CHCL_3$ was added to the dried extracts and re-drying the remainder for total lipid analysis. The vacuum tank was assembled with 2 dram vials in place for each sample and a syringe was placed above the cartridge column of dram 'vial 1'. The syringe was rinsed with chloroform without allowing it to run dry. Then 1 mL chloroform was added to the syringe followed by the 0.5

mL total lipid extract sub-sample. 1 mL chloroform was added, then 2 mL chloroform/methanol (49:1). The syringe was moved to a second position on the vacuum and the phospholipid fraction collected by washing with 2.5 mL methanol and added to the syringe until dry. Samples were then dried under oxygen free nitrogen and stored at -20°C.

The extracted phospholipid and total lipid samples were then converted to fatty acid methyl esters (FAMES) by acid methylation prior to gas chromatography (GC) analysis. Firstly 0.5 ml 'dry' toluene was added to dried lipid extractions and vortexed to dissolve the lipids. Then 1 ml of 2%(v/v) sulphuric acid in methanol was added and the solution was heated at 50 °C for 2h. After cooling 1ml neutralising solution (0.025M $KHCO_3$, 0.5M K_2CO_3) and 1ml dry hexane were added and the solution was vortexed. The sample was centrifuged at 1000rpm for 2 min at room temperate with low brake and the upper phase collected in a dram vial. The methylated sample was dried under oxygen-free nitrogen at 40 °C and resuspended in 150 µl hexane. The sample was then transferred to a gas chromatography vial and stored at -20 °C. Lipids were quantified by gas chromatography (GC) using Trace MS plus with GC ultra and triplus autosampler (Thermo Electron Corporation). GC column specifications were 30m x 0.22 mm x 0.25mm (SG BPX70) and samples were injected split flow 22 ml/min. Program temperature was 140 °C to 200 °C at 5 °C/min for 11 min, then to 220 °C at 10 °C/min for 5 min with a helium flow rate at constant pressure (1 ml/min). Samples were calibrated using a standard FAME mix (Supelco PA-USA, F.A.M.E Mix GLC-10) and each peak using mass spectrometer data to predict the compound using NIST mass spectral library with search program (version 2.0). Areas beneath each peak were converted to percentages of the total area of all fatty acid methyl ester peaks; those found not to be a fatty acid methyl ester were omitted from the total area calculation.

3.8. CELL PROLIFERATION AND APOPTOSIS

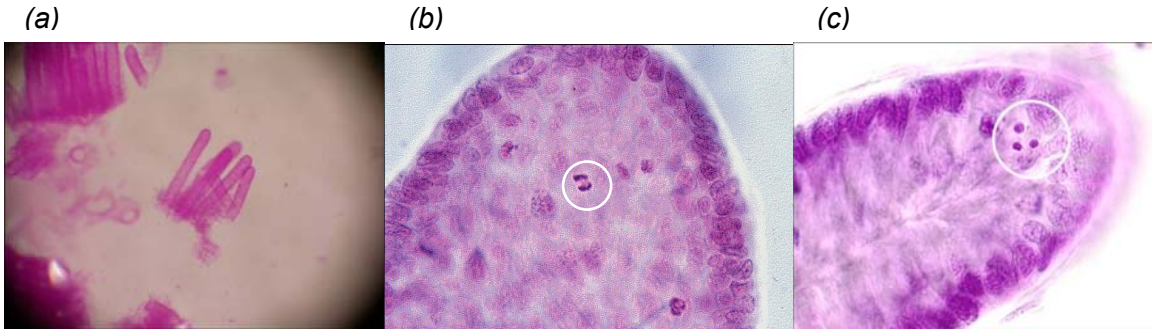

Figure 3.3 Picture of crypt dissected, mitosis and apoptosis analysis. (a) Crypt dissected. (b) Mitosis at x40 objective. (c) Apoptosis at x40 objective.

The number of mitotic and apoptotic cells in the gut tissue were determined using the morphological criteria method (Smith, Lund et al. 1998; Latham, Lund et al. 1999). After the animals have been sacrificed, the gut was removed and washed with PBS. The intestine was divided into proximal, middle, and distal small intestine, and colon. Samples from the distal small intestine and colon was removed and fixed in ethanol:acetic acid (75:25) prior staining. A small section (2-4mm2) of tissue was transferred to a pre-labelled vial containing about 2mls of 50% (aq) ethanol for 15min at room temperature. Afterwards, 50% ethanol was removed and added 2 mls of distilled water for 10 minutes at room temperature. Water was removed and 2mls of 1N HCL was added and vial was in the water bath at 60°C for 7 min. The tissue was rinsed with water, then added 2 mls of feulgens reagent for 20-40 min at room temperature. Tissue was rinsed again with water, and 2 mls of 45% (aq) acetic acid was added for storage prior to micro-dissection.

Under a standard dissection microscope, the muscle layers were teased from the gut mucosa and thin strips of crypts micro-dissected from the tissue using a fine gauge needle. The strips were then lightly compressed under a cover slip to separate and flatten the crypts, and 10 randomly chosen intact crypts per animal (320 crypts/group) were viewed

under a light microscope. Apoptotic cells were identified by the presence of condensed chromatin and spherical apoptotic bodies containing nuclear material. Nuclei, which were visibly in prophase, metaphase, anaphase, and telophase, were classified as mitosis. The length of each crypt was determined by comparison with a calibrated linear eyepiece graticule and the positions of mitotic and apoptotic nuclei were recorded and allocated to one of the 10 equally spaced longitudinal compartments. All crypts were analyzed blinded to the groups from which the samples were obtained. (Figure 3.3).

3.9. PROTEIN ASSAY

The protein assay was done using the standard procedure for microtiter plates by the Bradford method (Bradford 1976). Firstly, the Dye Reagent Concentrate was prepared by diluting 1 part of dye and 4 parts of water, and then filtered through a Whatman #1 filter to remove any un-dissolved particles. 5 dilutions of a protein standard were prepared and the linear range of the microtiter plate assay was 0.05 mg/ml to 0.5 mg/ml. The samples were assayed in duplicates. 10 μL of each standard and sample solution was added into separate microtiter plate wells. For plasma samples it was used at a dilution factor of 1:1000. Tissue samples were previously homogenized by using 'Protease Inhibitor cocktail' (Sigma-UK), and a dilution factor of 1:500 was used for the protein assay. 200 μL of diluted dye reagent was added to each well. The samples and reagent were mixed using a microplate mixer. The microtiter plate was incubated at room temperature for at least 5 min. Absorbance was measured at 595nm.

3.10. SCORING OF TUMOURS

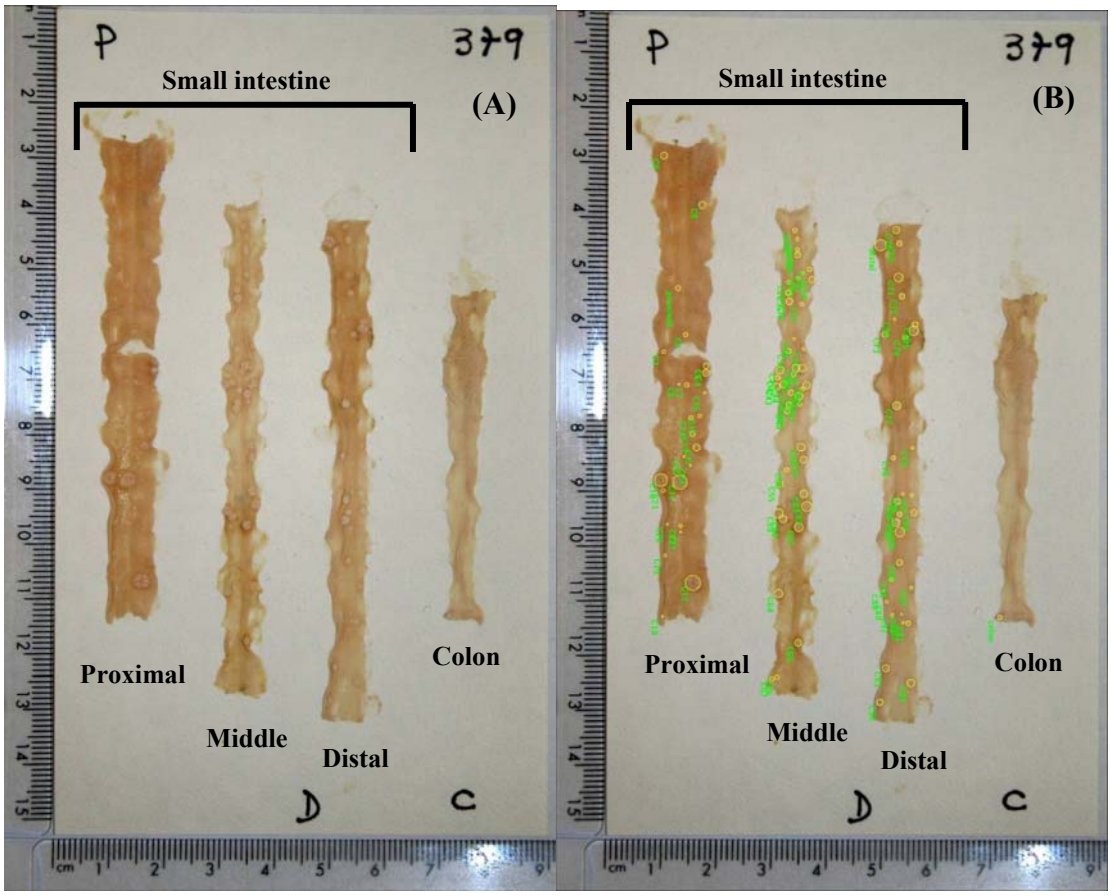

Figure 3.4 Picture of the intestinal tract from ApcMin/+ mouse. (A) Without scoring the tumours. (B) Scoring tumour size and number with Image Pro.

After mice were killed, the small intestine and colon were isolated and rinsed with cold phosphate buffered saline (PBS). The small intestine was divided into three equal sections (proximal, middle, distal) and the colon was left uncut. A gut cutting device was used to cut the gut longitudinally (Rudling, Hassan et al. 2006). The stainless steel rods were threaded through the segments and then placed over a piece of filter paper in the device. The lid of the device was then fitted and its angled bars used to guide a scalpel blade to longitudinally dissect the tissues.

The tissue was gently held to ensure that it did not move with the knife. The lid was then removed. The tissue was dampened with a gloved fingertip, dipped into PBS and rubbed along the segments. Rods were then rolled side to side to open up the gut and spread it flat on the filter paper, which was placed on dry ice briefly to freeze. On freezing the tumours displayed a distinct white color denser than normal tissue. Afterwards a picture was taken using a camera (Canon 350D) and the tumours were scored by size and location (proximal, middle, and distal small intestine and colon) using image analysis software Image Pro (Figure 3.4) (Media Cybernetics, Inc., USA). Gut area and gut length was also recorded.

3.11. CYTOKINE MEASUREMENTS

Cytokines were detected using a sandwich immunoassay based protein array system (Mouse Cytokine/Chemokine Multiplex Immunoassay Kit, MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Cat# MPXHCYTO-70K, Milllipore, Billerica, USA) with 21 distinctly coloured bead sets for Interleukin (IL) 1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-15, IL17, α-immunoprotein 10 (IP-10), granolyte/macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1 (MCP-1), keratinocyte derived chemokine (KC), tumour necrosis factor-alpha (TNFα), monokine induced by interferon gamma (MIG), vascular endothelial growth factor (VEGF), macrophage inflammatory protein-1 alpha (MIP-1α). Cytokine detection was performed according to the manufacturer's instruction. All reagents were allowed to warm to room temperature and were prepared before starting the assay. The microtiter filter plate was prewet by adding 200 µL of wash buffer into each well, which was sealed and shaken on a plate shaker for 10 min at room temperature. The wash buffer was removed by vacuum. And 25 µL of each standard and controls were added into the wells. Then, 25 µL of

appropriate matrix solution was added to the background, standards, and control wells. When plasma was assayed the diluted 'Serum Matrix' provided in the kit was used. When tissue was assayed the Tissue Extraction Reagent I (Sigma) was used as matrix solution. 25 µL of sample was added into the appropriate wells. The bottle with the premixed beads was vortexed and 25 µL was added into each well. The plate was sealed and incubated with agitation on a plate shaker overnight incubation at 4°C. Next day, the fluid was gently removed by vacuum and the plate washed 2 times by adding 200 µL of wash buffer and removing by vacuum. Then 25 µL of detection antibodies was added into each well, and the plate was sealed and incubated with agitation on a plate shaker for 1 hour at room temperature. After the incubation, 25 µL of streptavidin-phycoerythrin was added into each well containing the 25 µL of detection antibodies. The plate was sealed and incubated with agitation on a plate shaker for 30 minutes at room temperature. The fluid was then gently removed by vacuum and washed 2 times by adding 200 µL of wash buffer into each well. After having wiped any excess of fluid on the bottom of the plate with a tissue, 150 µL of sheath fluid was added into all wells. To resuspend the beads the plate was shaken for 5 min before running the plate on the Luminex machine. The Bio-Plex 100 System with the BIORAD manager acquisition program (BIORAD manager 4.1) was used to run the samples and process the data. Controls, standards and the samples were run in duplicates.

3.12. STATISTICAL ANALYSIS

Results are reported as means ± SD, means ± SE, or median and 95% CI. The models applied to analyse the data were the analysis of variance (ANOVA), non-parametric tests, and the standard diagnostics (outlier removal, checks for leverage, influence and normality of residuals). If the assumption of normality failed, a Kruskal-Wallis and Mann-Whitney

ANOVA on ranks was done. Post hoc analyses were done with the Tukey's method. Significance was set at $p < 0.05$. The statistical packages used were the statistical package for the social sciences (SPSS) software version 18 (SPSS, Chicago, IL, USA).

Analysis of variance is a procedure for assigning sample variance to different sources and deciding whether the variation arises within or among different population groups. In the present study one-way and two-way ANOVA was used when appropriate. A one-way ANOVA means that there is only one independent variable, and that the independent variable has two or more level, while the two-way ANOVA test indicates two independent variables. The assumptions for ANOVA is that the dependent variable should be a continuous variable that is normally distributed, the groups should be mutually exclusive (independent of each other), and the groups should have equal variances (homogeneity of variance requirement) (McKillup 2005). ANOVA was used to analyze the following data: body and organs weight; apoptosis and cell proliferation; as well as the tumour development.

Non-parametric test was also used in the present experiment. Non-parametric tests do not use the predictable distribution of sample means, which is the basis of most parametric tests, to infer whether samples are from the same population. Consequently non-parametric tests are generally not as powerful as their parametric equivalents. When data are significantly skewed, thus failing the assumption of a normal distribution, non-parametric techniques might be used (Munro 2005). In the present experiment, the non-parametric test was used to analyze data from the cytokines profile, which were not normally distributed. Two commonly used techniques are the Mann-Whitney U, which was used to compare two groups, and the Kruskal-Wallis H, which was used to compared two or more groups (which is analogous to the parametric technique analysis of variance).

4. FATTY ACIDS STUDY

In this chapter the results and the discussion of the animal intervention using ApcMin/+ mouse model to study the effects of fatty acids on the development of colorectal cancer are presented. A high-fat diet (25% kcal) rich in corn oil (n-6 fatty acids) and fish oil (n-3 fatty acids) were the treatment diets tested, while a high-fat diet rich in palm oil was used as the control group. Wild-type animals (n=16) and ApcMin/+ animals (n=16) were exposed to the treatment diet for 10 weeks. The endpoints evaluated were body and organs weight, mitosis and apoptosis, local and systemic inflammation, as well as tumour development.

4.1. RESULTS

4.1.1. Effects on body and organs weight

Animals were fed ad lib and the average consumption of diet was 19.41g/animal/week for PO group, 17.40g/animal/week for CO group, and 19.60g/animal/week for the FO group. Female mice were smaller and gained less weight than males. The initial, second and final weight did not differ from each other by genotype of the animals. However, a two-way ANOVA showed significant interaction for initial weight between genotype and diet, $p=0.02$. There was also a statistically significant main effect for diet, $p=0.02$. Animals from the fish oil group were significantly heavier at the start of the experiment (16.91 ± 2.89g), compared to animals fed the palm oil (12.47 ± 4.46g). In addition, animals fed the fish oil were significantly older (37.58 ± 4.12 days) than animals fed the palm oil (30.96 ± 6.77 days) and the corn oil diet (31.78 ± 6.30 days). Also, mice from the fish oil group had a significant smaller body weight change during the treatment (7.41 ± 2.76g) than the other

groups (palm oil = 10.95 ± 3.81g; corn oil = 9.99 ± 5.51g). Table 4.1 summarizes the age, body and organs weight according to sex and genotype of the animals.

There was no significant difference between the dietary groups for all the organs (heart, liver, spleen, kidney, stomach, and caecum) (Table 4.2). However, there was a significant interaction between genotype and diet for kidney, but no main effect was observed. Moreover, Figure 4.1 shows that ApcMin/+ mice had significantly larger spleens compared to wild-type animals.

Variables	Sex		Genotype	
	Male	Female	ApcMin/+	Wild-type
Age (days)	34.32 ± 5.97 [a]	33.46 ± 7.33 [a]	33.58 ± 6.45 [a]	34.21 ± 6.94 [a]
Initial body weight (g)	**16.08 ± 4.39 [a]**	**13.38 ± 3.94 [b]**	14.42 ± 4.59 [a]	15.06 ± 4.14 [a]
Final body weight (g)	**26.88 ± 2.18 [a]**	**20.60 ± 1.98 [b]**	23.14 ± 3.41 [a]	24.38 ± 4.05 [a]
Change in body weight (g)	**10.79 ± 4.50 [a]**	**7.22 ± 3.85 [b]**	8.71 ± 4.38 [a]	9.32 ± 4.72 [a]
Stomach (g)	**4.87 ± 1.11 x10^{-3} [a]**	**5.98 ± 1.30 x10^{-3} [b]**	5.57 ± 1.28 x10^{-3} [a]	5.27 ± 1.36 x10^{-3} [a]
Caecum (g)	**2.80 ± 1.01 x10^{-3} [a]**	**3.24 ± 1.00 x10^{-3} [b]**	3.00 ± 0.91 x10^{-3} [a]	3.03 ± 1.14 x10^{-3} [a]
Liver (g)	4.45 ± 0.69 x10^{-2} [a]	4.54 ± 0.51 x10^{-2} [a]	4.61 ± 0.71 x10^{-2} [a]	4.47 ± 0.46 x10^{-2} [a]
Heart (g)	5.04 ± 0.93 x10^{-3} [a]	5.30 ± 0.81 x10^{-3} [a]	5.28 ± 0.81 x10^{-3} [a]	5.06 ± 0.93 x10^{-3} [a]
Spleen (g)	**5.83 ± 3.43 x10^{-3} [a]**	**7.93 ± 5.47 x10^{-3} [b]**	**8.97 ± 4.34 x10^{-3} [a]**	**4.66 ± 3.94 x10^{-3} [b]**
Kidney (g)	**1.19 ± 0.10 x10^{-2} [a]**	**1.27 ± 0.08 x10^{-2} [b]**	1.22 ± 0.09 x10^{-2} [a]	1.24 ± 0.10 x10^{-2} [a]

Table 4.1 Age, body and organ weight by sex and genotype. Values are mean ± SD. Values within a row were analysed by sex and strain. Values having a different superscript are significantly different by one-way analysis of variance, $p<0.05$. Sex n= 63, Genotype n= 61-65. All organs weight was corrected for body weight.

Variables	Diet			Significant effects		
	Palm oil	Corn oil	Fish oil	Geno	Diet	Geno*Diet
Age (days)	**30.96 ± 6.77 [a]**	**31.78 ± 6.30 [a]**	**37.58 ± 4.12 [b]**	ns	0.03	ns
Initial body weight (g)	**12.47 ± 4.46 [a]**	**13.34 ± 4.35 [a]**	**16.91 ± 2.89 [b]**	ns	0.02	0.02
Final body weight (g)	23.42 ± 3.94	23.33 ± 3.77	24.32 ± 3.67	ns	ns	ns
Change in body weight (g)	10.95 ± 3.81	9.99 ± 5.51	7.41 ± 2.76	ns	ns	ns
Heart (g)	5.45 ± 0.98 x10^{-3}	4.28 ± 0.76 x 10^{-2}	4.91 ± 0.69 x 10^{-3}	ns	ns	ns
Liver (g)	4.37 ± 0.47 x10^{-2}	1.24 ± 0.09 x 10^{-2}	4.28 ± 0.76 x 10^{-2}	ns	ns	ns
Spleen (g)	7.36 ± 5.92 x10^{-3}	6.50 ± 3.71 x 10^{-3}	6.50 ± 3.02 x 10^{-3}	0.000	ns	ns
Kidney (g)	1.24 ± 0.09 x10^{-2}	1.21 ± 1.26 x 10^{-3}	1.24 ± 0.09 x 10^{-2}	ns	ns	0.023
Stomach (g)	5.34 ± 1.29 x10^{-3}	4.93 ± 0.95 x 10^{-3}	5.33 ± 1.26 x 10^{-3}	ns	ns	ns
Caecum (g)	2.50 ± 0.58 x10^{-3}	2.58 ± 0.59 x 10^{-3}	2.95 ± 0.95 x 10^{-3}	ns	ns	ns

Table 4.2 Effect of dietary palm oil, corn oil and fish oil on organ weight of experimental animals. Values are mean ± SD, n=31-32. ApcMin/+ and wild-type mice combined. Significant effects of genotype (Geno), ApcMin/+ and wild-type, and diet were identified by two-way analysis of variance $p<0.05$. NS, not significant. All organs weight was corrected for body weight.

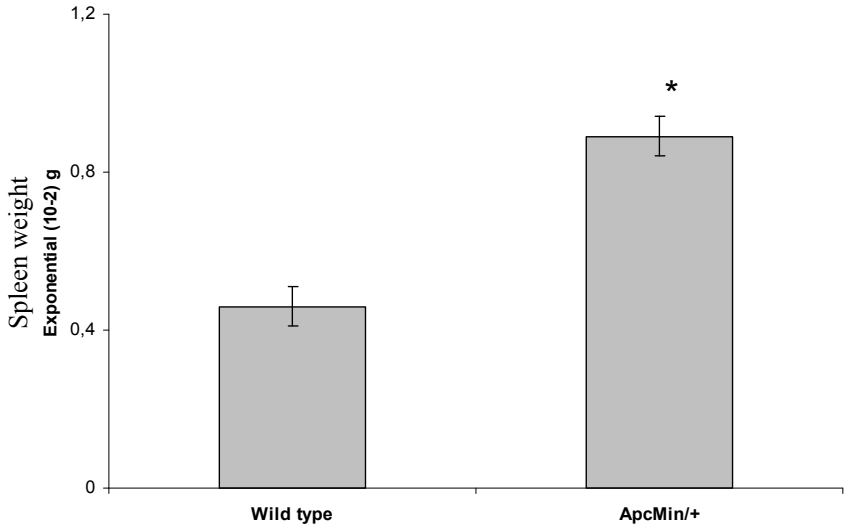

Figure 4.1 The effect of genotype on spleen weight after the 10 weeks of treatment, male and female mice combined (mean ± SEM, n=61-65). * one-way analysis of variance showed significant difference $p<0.01$.

4.1.2. Effects on mitosis, apoptosis and crypt length

ApcMin/+ mice fed the palm oil diet showed a significantly greater cell proliferation in both distal small intestine (2.99 ± 0.28 mitotic cell/ crypt) and colon (1.58 ± 0.15 mitotic cell/ crypt) compared with the wild-type mice (distal SI =2.06 ± 0.17 mitotic cell/ crypt and colon =0.98 ± 0.18 mitotic cell/ crypt). However, this effect of genotype was only seen in the palm oil group. Cell proliferation was found significantly lower in the colon tissue of ApcMin/+ mice fed the corn oil and fish oil diet, compared to animals fed the palm oil (Figure 4.2). No difference was observed in cell proliferation in the colon tissue in any of the groups for the wild-type mice. However, in the distal tissue, wild-type animals fed the corn oil diet had a significant increase in cell proliferation compared to the fish oil and palm oil group (Figure 4.2).

The number of apoptotic cells/crypt in the distal colon was higher in the ApcMin/+ mice fed the corn oil, and to a lesser extent fish oil, when compared to the wild-type within the same diet (Figure 4.3). No differences were observed between genotype for the animals fed the palm oil diet. Also, no significant effect was found within either the ApcMin/+ and wild-type mice by the different treatment diet. In the colon tissue, no significant effect was found regarding the level of apoptosis.

The different dietary treatments had no significant effect on crypt length between strains (Figure 4.4). Although there was a significant increase in crypt length for all ApcMin/+ mice compared to wild-type mice, both in the distal tissue ($p=0.01$) and in the colon ($p=0.03$).

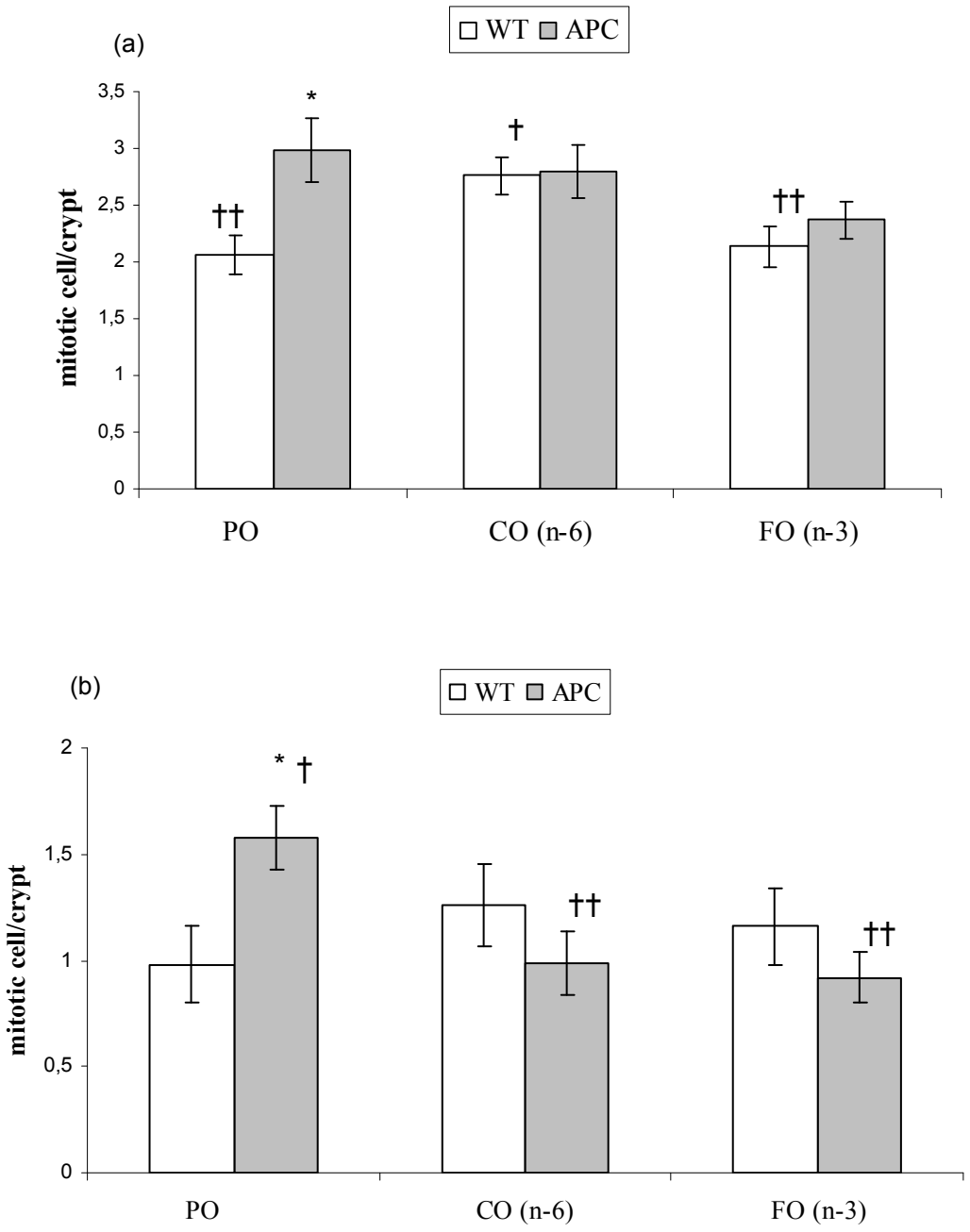

Figure 4.2 Effects of palm oil (PO), corn oil (CO) and fish oil (FO) on cell proliferation in the distal (a), and in the colon (b) of wild-type and ApcMin/+ mice. The values represent the mean (n=32) and standard error of the mean. The values not sharing the same symbol are significantly different ($p<0.05$) by two-way ANOVA general linear model. Data analysed within the same diet by different genotype and is significantly different is indicated by *. Data that is significantly different when analysed within the same genotype by different diet is indicated by †. Ten crypts were analyzed per animal.

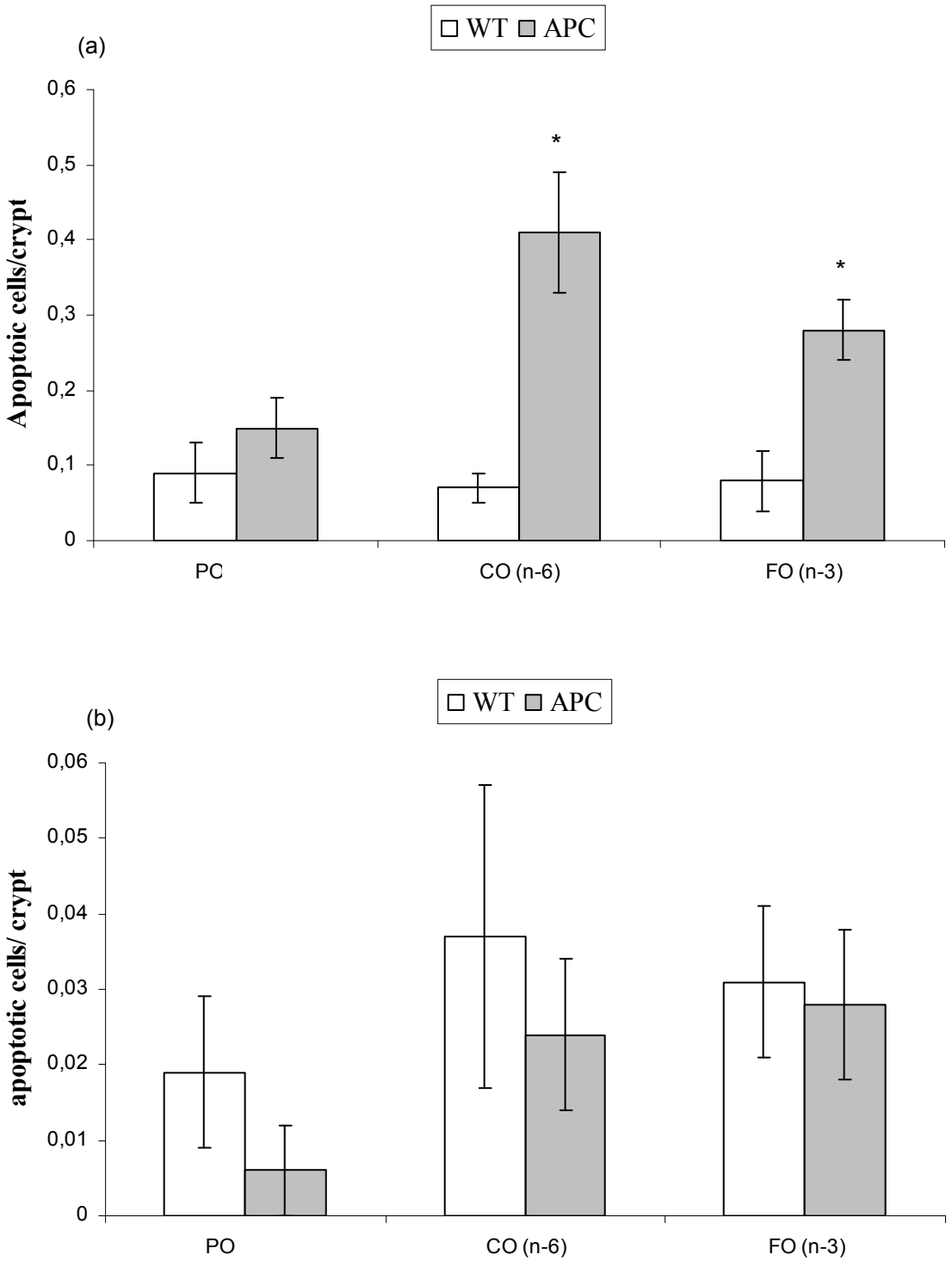

Figure 4.3 Effects of palm oil (PO), corn oil (CO) and fish oil (FO) on cell apoptosis in the distal (a), and in the colon (b) of wild-type and ApcMin/+ mice. The values represent the mean (n=32) and standard error of the mean. The values not sharing the same symbol are significantly different ($p<0.05$) by two-way ANOVA general linear model. Data analyzed within the same diet by different genotype (*). Data analyzed within the same genotype by different diet (†). Ten crypts were analyzed per animal.

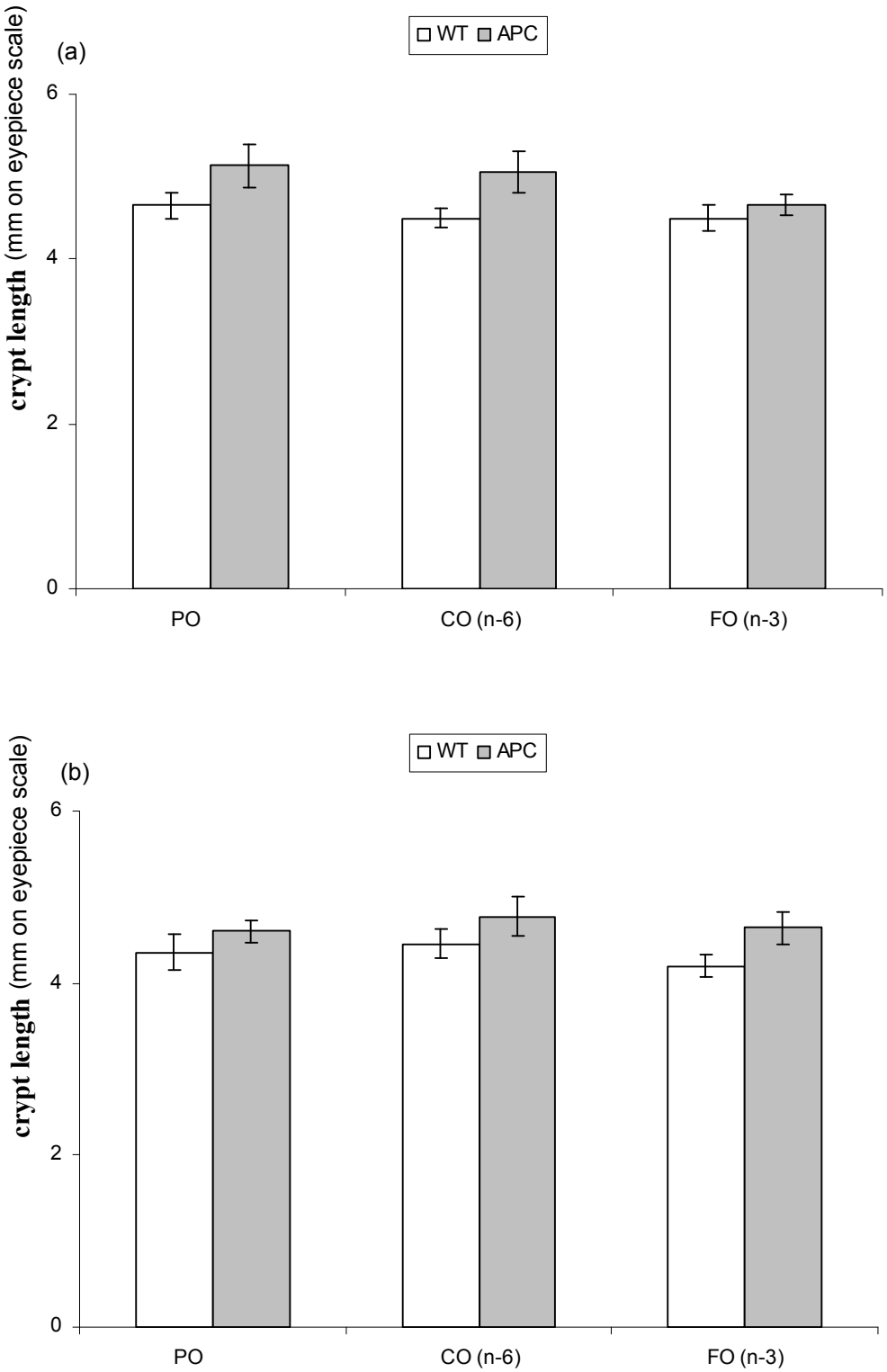

Figure 4.4 Effects of palm oil (PO), corn oil (CO) and fish oil (FO) on crypt length in the distal (a), and in the colon (b) of wild-type and ApcMin/+ mice. The values represent the mean in mm on eyepiece scale (n=32) and standard error of the mean. Each unit equals 40μm. No significant effect was observed by two-way ANOVA general linear model. Ten crypts were analyzed per animal.

4.1.3. Effects on local and systemic inflammation

4.1.3.1. Distal - small intestine tissue

The effect of dietary treatment on cytokines levels from the distal tissue was significant for most of the cytokines measured when wild-type and ApcMin/+ were combined (INFγ, IL-1α, IL-2, IL-5, IL-10, IL-12p40,IL-12p70, IL-15, IP-10, KC, GMC-SF, TNFα, MIP-1α and VEGF. While the cytokines IL-4, IL-6, IL-17, IL-1β, MCP-1, and MIG were not significant (Table 4.3).

Analyzing the data within each diet by the genotype (Table 4.4) showed that ApcMin/+ mice treated with palm oil had significant higher levels of KC (p=0.03) and IL-1β (p=0.01) compared with the wild-type. However the wild-type had higher IL-5 (p=0.00). In the corn oil diet, ApcMin/+ mice also had higher KC (p=0.00) and MIG (p=0.04) when compared to the wild-type. While, the wild-type animals had higher levels of IL-12p40 (p=0.00), IL-12p70 (p=0.02) and IP-10 (p=0.00) than the ApcMin/+ mice fed the corn oil diet. Moreover, the fish oil treatment seemed to increase the levels of IL-15 (p=0.02), KC (p=0.00), IL-1β (p=0.00), and MIG (p=0.00) in the ApcMin/+ mice, while IL-12p40 (p=0.04) and IP-10 (p=0.02) were decreased. In Table 4.4 is summarized the effects of the diets on cytokines by each genotype.

In Table 4.5 the effects of different treatment diet by each genotype is summarized. The effects of dietary treatments within the wild-type animals was significantly different for IL-5, IL-10, IL-12p40,IL-12p70, IL-15, IP-10, KC, TNFα, MIP-1α and VEGF, at a p-value equal or lower then 0.02. Wild-type animals fed the corn oil and fish oil treatment diet had significantly decreased levels of IL-5, IL-10, IL-15, KC, and TNFα, compared to mice fed

the palm oil diet. However, corn oil and fish oil diet increased the levels of IL-12p40 and IP-10 in wild-type mice. Moreover, the fish oil diet significantly increased the levels of IL-12p70, and MIP-1α in wild-type animals compared to those fed the palm oil. Animals fed corn oil diet has lower levels of VEGF compared to animals fed the palm oil and fish oil.

On the other hand, within the ApcMin/+ mice the majority of the cytokines also appeared to be significantly affected by diet. Animals fed the fish oil diet had increased levels of IFNγ, IL-12p40, IP-10, MIP-1α, but lower levels of IL-5 and GMC-SF compared to ApcMin/+ mice fed the palm oil diet. Animal fed the corn oil diet significantly reduced the levels of IL-2, IL-12p40, IL-12p70, IL-1β and VEGF compared to mice fed the palm oil diet. When mice fed the corn oil and the fish oil diets were compared, the main significant differences observed were that fish oil increased the levels of IL-1α, IL-2, IL-5, IL-12p40, IL-12p70, IL-15, IP-10, IL-1β, MIP-1α, and VEGF. The only cytokine reduced with fish oil compared to corn oil was GMC-SF. ApcMin/+ mice had no significant effect on the levels of IL-4,IL-6, IL-10,IL-17, KC, TNF-α, MCP-1 and MIG in any of the treatment diets.

Cytokines	Palm oil *Median (95% CI)* *pg/mg protein*	Corn oil *Median (95% CI)* *pg/mg protein*	Fish oil *Median (95% CI)* *pg/mg protein*	*p-value*
(A) IFN-γ	**0.08(0.03-0.10)** [a]	**0.06(0.00-0.21)** [ab]	**0.14(0.11-0.18)** [b]	0.01
(P) IL-1α	**0.46(0.37-0.62)** [a]	**0.37(0.30-0.78)** [a]	**0.87(0.50-1.25)** [b]	0.02
(P) IL-2	**0.16(0.11-0.19)** [ac]	**0.08(0.01-0.10)** [b]	**0.28(0.10-0.38)** [c]	0.00
(A) IL-4	0.03(0.02-0.05) [a]	0.02(0.004-0.04) [a]	0.03(0.02-0.04) [a]	0.18
(P) IL-5	**0.01(0.004-0.13)** [a]	**0.003(0.002-0.004)** [b]	**0.002(0.002-0.003)** [c]	0.001
(P) IL-6	0.26(0.19-0.32) [a]	0.24(0.14-0.29) [a]	0.21(0.14-0.33) [a]	0.75
(A) IL-10	**1.58(0.60-2.17)** [a]	**1.01(0.82-1.21)** [a]	**0.71(0.56-0.83)** [b]	0.001
(P) IL-12p40	**0.77(0.004-1.34)** [a]	**0.34(0.002-1.70)** [a]	**2.29(1.62-4.16)** [b]	0.001
(P) IL-12p70	**1.03(0.78-1.16)** [a]	**0.64(0.35-1.17)** [a]	**1.50(1.16-3.08)** [b]	0.001
(P) IL-15	**0.48(0.01-0.92)** [ac]	**0.004(0.003-0.20)** [b]	**0.004(0.003-0.95)** [bc]	0.01
(P) IL-17	0.23(0.16-0.38) [a]	0.19(0.11-0.24) [a]	0.21(0.13-0.26) [a]	0.20
(A) IP-10	**1.59(0.91-2.57)** [a]	**1.54(1.09-2.91)** [a]	**3.15(2.66-3.93)** [b]	0.001
(P) KC	**0.57(0.44-0.85)** [ac]	**0.29(0.22-0.54)** [b]	**0.37(0.28-0.74)** [bc]	0.02
(P) GMC-SF	**0.003(0.002-0.003)** [a]	**0.003(0.002-0.004)** [a]	**0.002(0.002-0.003)** [b]	0.02
(P) TNF-α	**0.13(0.12-0.20)** [ac]	**0.07(0.02-0.13)** [b]	**0.13(0.08-0.16)** [c]	0.001
(P) IL-1β	0.63(0.44-0.99) [a]	0.46(0.22-0.74) [a]	0.62(0.42-0.98) [a]	0.13
(P) MCP1	0.63(0.44-0.90) [a]	0.50(0.41-0.71) [a]	0.77(0.69-0.86) [a]	0.20
(A) MIG	26.38(16.49-38.46) [a]	24.01(11.03 -36.44) [a]	23.53(12.40-37.27) [a]	0.76
(P) MIP1α	**0.22(0.12-0.26)** [a]	**0.12(0.01-0.27)** [a]	**0.57(0.45-0.64)** [b]	0.001
(P) VEGF	**2.59(2.29-3.67)** [ac]	**1.18(1.01-1.71)** [b]	**2.89(2.01-4.88)** [c]	0.001

Table 4.3 Effect of dietary palm oil (n=28), corn oil (n=30) and fish oil (n=27) on cytokines levels (pg/mg protein) in the distal tissue of the small intestine in mice after 10-week treatment. (A) Anti-inflammatory cytokines; (P) pro-inflammatory cytokines. Values are presented as median and 95% confidence interval. ApcMin/+ and wild-type mice combined. p-value of Kruskal-Wallis test. Values having different superscript are significantly different by post-hoc Mann-Whitney test $p<0.05$.

4.1.3.2. Colon - large intestine tissue

Animals fed the palm oil had a higher level of IL-6 (p=0.04) compared to the corn oil and fish oil diet. However, TNFα (p=0.04) and MCP-1 (p=0.05) were higher in animals treated with the fish oil diet comparing to animals fed the palm oil diet (Table 4.6). There was no significant difference for palm oil, corn oil, and fish oil when data was analyzed by strain (Table 4.7). Analyzing all wild-type mice by the different dietary treatment, no statistical difference where shown. However, within the ApcMin mice, fish oil diet showed higher levels of IL-1β (p=0.05) and TNFα (p=0.26), and a lower level of IL-6 (p=0.02) compared to the animals fed the palm oil diet (Table 4.8).

Cytokines	Palm oil: **Wild-type** *(n=14)* *Median (95% CI)* *pg/mg protein*	Palm oil: **ApcMin** *(n=14)* *Median (95% CI)* *pg/mg protein*	*p-value*	Corn oil: **Wild-type***(n=15)* *Median (95% CI)* *pg/mg protein*	Corn oil: **ApcMin** *(n=15)* *Median (95% CI)* *pg/mg protein*	*p-value*	Fish oil: **Wild-type** *(n=13)* *Median (95% CI)* *pg/mg protein*	Fish oil: **ApcMin** *(n=14)* *Median (95% CI)* *pg/mg protein*	*p-value*
(A) IFNγ	0.06 (0.004-0.35)	0.08 (0.003-0.10)	0.33	0.16 (0.003-0.63)	0.004 (0.002-0.16)	0.14	0.17 (0.11-0.35)	0.13 (0.07- 0.18)	0.06
(P) IL-1α	0.41 (0.25-0.59)	0.60 (0.31-1.13)	0.09	0.51 (0.004-1.88)	0.32 (0.004-0.54)	0.19	0.79 (0.24-1.99)	0.88 (0.46- 1.35)	0.59
(P) IL-2	0.15 (0.004-0.19)	0.16 (0.11-0.50)	0.09	0.03 (0.004-0.14)	0.09 (0.01-0.10)	0.46	0.19 (0.003-0.41)	0.33 (0.13- 0.38)	0.22
(A) IL-4	0.04 (0.01-0.07)	0.03 (0.01-0.06)	0.46	0.01 (0.003-0.08)	0.02 (0.002-0.04)	0.29	0.03 (0.02-0.05)	0.03 (0.02- 0.06)	0.62
(P) IL-5	**0.09 (0.004-0.25)**	**0.003 (0.003-0.21)**	0.00	0.0033 (0.003-0.004)	0.0027 (0.002-0.02)	0.57	**0.0030 (0.0025-0.0033)**	**0.0024 (0.0021- 0.0029)**	0.03
(P) IL-6	0.26 (0.08-0.42)	0.26 (0.16-0.47)	0.89	0.23 (0.06- 0.29)	0.26 (0.11-0.34)	0.54	0.21 (0.11-0.45)	0.20 (0.10- 0.36)	0.80
(A) IL-10	1.97 (0.60-2.66)	0.89 (0.35-2.17)	0.60	1.00 (0.74- 1.12)	1.01 (0.82-1.94)	0.25	0.74 (0.45-0.92)	0.68 (0.50- 0.88)	0.93
(P) IL-12p40	0.31 (0.003-4.44)	1.10 (0.003-1.43)	0.52	**2.07 (0.31-8.03)**	**0.002 (0.002-0.37)**	0.001	**3.88 (1.91-7.05)**	**1.66 (1.44- 3.46)**	0.04
(P) IL-12p70	1.01 (0.54-1.34)	1.11 (0.63-1.56)	0.52	**1.09 (0.64-2.50)**	**0.38 (0.25-0.65)**	0.02	2.80 (1.17-3.58)	1.23 (0.90- 3.18)	0.08
(P) IL-15	0.43 (0.004-1.28)	0.59 (0.00-1.06)	0.89	0.003 (0.003- 0.01)	0.20 (0.002-0.55)	0.25	**0.003 (0.002-0.61)**	**0.96 (0.002- 1.45)**	0.02
(P) IL-17	0.21 (0.14-0.48)	0.25 (0.12-0.52)	0.67	0.13 (0.00-0.22)	0.23 (0.16-0.29)	0.08	0.18 (0.08-0.31)	0.22 (0.13- 0.30)	0.35
(A) IP-10	0.91 (0.57-2.89)	1.96 (1.02-2.81)	0.12	**3.22 (1.53-4.94)**	**0.98 (0.78-1.55)**	0.001	**3.85 (3.02-4.66)**	**2.76 (2.26- 3.80)**	0.02
(P) KC	**0.47 (0.25-0.87)**	**0.71 (0.49-1.24)**	0.03	**0.22 (0.003- 0.30)**	**0.68 (0.29-0.87)**	0.001	**0.32 (0.14-0.37)**	**0.76 (0.34- 1.28)**	0.001
(P) GMC-SF	0.003 (0.002-0.004)	0.003 (0.002-0.003)	0.29	0.003 (0.002-0.004)	0.002 (0.002-0.15)	0.91	**0.003 (0.002-0.003)**	**0.002 (0.002- 0.002)**	0.001
(P) TNFα	0.17 (0.11-0.26)	0.13 (0.09-0.24)	0.29	0.05 (0.004- 0.15)	0.08 (0.02-0.21)	0.22	0.10 (0.08-0.16)	0.13 (0.04- 0.18)	0.66
(P) IL-1β	**0.48 (0.25-0.80)**	**1.03 (0.44-1.27)**	0.01	0.56 (0.15- 0.81)	0.45 (0.35-0.76)	0.78	**0.44 (0.30-0.62)**	**1.32 (0.55- 2.16)**	0.001
(P) MCP1	0.50 (0.32-0.79)	0.79 (0.44-1.01)	0.15	0.59 (0.26- 0.93)	0.45 (0.41-0.83)	0.66	0.72 (0.57-0.88)	0.81 (0.72- 1.08)	0.13
(A) MIG	20.17 (1.67-48.14)	33.03 (18.67-53.58)	0.21	**11.03 (1.18-32.44)**	**35.66 (16.51-42.13)**	0.04	**16.76 (6.23-23.53)**	**35.25 (22.26- 59.32)**	0.001
(P) MIP1α	0.21 (0.004-0.27)	0.22 (0.08-0.40)	0.43	0.01 (0.003- 0.80)	0.13 (0.07-0.27)	0.49	0.51 (0.35-0.64)	0.61 (0.43- 0.86)	0.10
(P) VEGF	2.50 (1.31-3.99)	2.95 (2.29-6.16)	0.08	1.09 (0.08-1.72)	1.48 (1.07-2.03)	0.15	2.64 (1.69-7.97)	3.08 (1.86- 4.88)	0.80

Table 4.4 Effect of palm oil (PO), corn oil (CO), and fish oil (FO) on cytokine level (pg/mg protein) in the distal tissue of the small intestine of wild-type (WT) and ApcMin (APC) mice. (A) Anti-inflammatory cytokines; (P) pro-inflammatory cytokines. Values are presented as median and 95% confidence interval (CI). Significant p-value ($p<0.05$) was identified by Mann-Whitney test.

	Wild-type				ApcMin/+			
Cytokines	**Palm oil** *(n=14)* Median (95% CI) *pg/mg protein*	**Corn oil** *(n=15)* Median (95% CI) *pg/mg protein*	**Fish oil** *(n=13)* Median (95% CI) *pg/mg protein*	*p-value*	**Palm oil** *(n=14)* Median (95% CI) *pg/mg protein*	**Corn oil** *(n=15)* Median (95% CI) *pg/mg protein*	**Fish oil** *(n=14)* Median (95% CI) *pg/mg protein*	*p-value*
(A) IFNγ	0.06 (0.004-0.35)	0.16 (0.003-0.63)	0.17 (0.11-0.35)	ns	**0.08 (0.00-0.10)** [a]	**0.004 (0.002-0.16)** [ab]	**0.13 (0.07- 0.18)** [b]	0.03
(P) IL-1α	0.41 (0.25-0.59)	0.51 (0.004-1.88)	0.79 (0.24-1.99)	ns	**0.60 (0.31-1.13)** [ac]	**0.32 (0.004-0.54)** [a b]	**0.88 (0.46- 1.35)c**	0.001
(P) IL-2	0.15 (0.004-0.19)	0.03 (0.004-0.14)	0.19 (0.003-0.41)	ns	**0.16 (0.11-0.50)** [ac]	**0.09 (0.01-0.10)** [b]	**0.33 (0.13- 0.38)** [c]	0.001
(A) IL-4	0.04 (0.01-0.07)	0.01 (0.003-0.08)	0.03 (0.02-0.05)	ns	0.03 (0.01-0.06)	0.02 (0.002-0.04)	0.03 (0.02- 0.06)	Ns
(P) IL-5	**0.09 (0.004-0.25)** [a]	**0.003 (0.003-0.004)** [b]	**0.003 (0.002-0.003)** [c]	0.001	**0.003 (0.003-0.21)** [a]	**0.002 (0.002-0.02)** [a]	**0.002 (0.0021- 0.0029)** [b]	0.001
(P) IL-6	0.26 (0.08-0.42)	0.23 (0.06- 0.29)	0.21 (0.11-0.45)	ns	0.26 (0.16-0.47)	0.26 (0.11-0.34)	0.20 (0.10- 0.36)	Ns
(A) IL-10	**1.97 (0.60-2.66)** [a]	**1.00 (0.74- 1.12)** [b]	**0.74 (0.45-0.92)** [b]	0.001	0.89 (0.35-2.17)	1.01 (0.82-1.94)	0.68 (0.50- 0.88)	Ns
(P) IL-12p40	**0.31 (0.003-4.44)** [a]	**2.07 (0.31-8.03)** [b]	**3.88 (1.91-7.05)** [b]	0.01	**1.10 (0.003-1.43)** [a]	**0.002 (0.002-0.37)** [b]	**1.66 (1.44- 3.46)** [c]	0.001
(P) IL-12p70	**1.01 (0.54-1.34)** [a]	**1.09 (0.64-2.50)** [a]	**2.80 (1.17-3.58)** [b]	0.001	**1.11 (0.63-1.56)** [ac]	**0.38 (0.25-0.65)** [b]	**1.23 (0.90- 3.18)** [c]	0.001
(P) IL-15	**0.43 (0.004-1.28)** [a]	**0.003 (0.003- 0.01)** [b]	**0.003 (0.002-0.61)** [b]	0.001	**0.59 (0.00-1.06)** [ac]	**0.20 (0.002-0.55)** [ab]	**0.96 (0.002- 1.45)** [c]	0.04
(P) IL-17	0.21 (0.14-0.48)	0.13 (0.00-0.22)	0.18 (0.08-0.31)	ns	0.25 (0.12-0.52)	0.23 (0.16-0.29)	0.22 (0.13- 0.30)	ns
(A) IP-10	**0.91 (0.57-2.89)** [a]	**3.22 (1.53-4.94)** [b]	**3.85 (3.02-4.66)** [b]	0.001	**1.96 (1.02-2.81)** [a]	**0.98 (0.78-1.55)** [a]	**2.76 (2.26- 3.80)** [b]	0.001
(P) KC	**0.47 (0.25-0.87)** [a]	**0.22 (0.00- 0.30)** [b]	**0.32 (0.14-0.37)** [b]	0.001	0.71 (0.49-1.24)	0.68 (0.29-0.87)	0.76 (0.34- 1.28)	ns
(P) GMC-SF	0.003 (0.002-0.004)	0.003 (0.002-0.004)	0.003 (0.002-0.003)	ns	**0.003 (0.002-0.003)** [a]	**0.002 (0.002-0.15)** [a]	**0.002 (0.002- 0.002)** [b]	0.001
(P) TNFα	**0.17 (0.11-0.26)** [ac]	**0.05 (0.004- 0.15)** [b]	**0.10 (0.08-0.16)** [c]	0.001	0.13 (0.09-0.24)	0.08 (0.02-0.21)	0.13 (0.04- 0.18)	ns
(P) IL-1β	0.48 (0.25-0.80)	0.56 (0.15- 0.81)	0.44 (0.30-0.62)	ns	**1.03 (0.44-1.27)** [ac]	**0.45 (0.35-0.76)** [b]	**1.32 (0.55- 2.16)** [c]	0.001
(P) MCP1	0.50 (0.32-0.79)	0.59 (0.26- 0.93)	0.72 (0.57-0.88)	ns	0.79 (0.44-1.01)	0.45 (0.41-0.83)	0.81 (0.72- 1.08)	ns
(A) MIG	20.17 (1.67-48.14)	11.03 (1.18-32.44)	16.76 (6.23-23.53)	ns	33.03 (18.67-53.58)	35.66 (16.51-42.13)	35.25 (22.26- 59.32)	ns
(P) MIP1α	**0.21 (0.00-0.27)** [a]	**0.01 (0.003- 0.80)** [ab]	**0.51 (0.35-0.64)** [b]	0.02	**0.22 (0.08-0.40)** [a]	**0.13 (0.07-0.27)** [a]	**0.61 (0.43- 0.86)** [b]	0.001
(P) VEGF	**2.50 (1.31-3.99)** [ac]	**1.09 (0.08-1.72)** [b]	**2.64 (1.69-7.97)** [c]	0.001	**2.95 (2.29-6.16)** [ac]	**1.48 (1.07-2.03)** [b]	**3.08 (1.86- 4.88)** [c]	0.001

Table 4.5 Effect of strain on cytokine level (pg/mg protein) in the distal tissue of the small intestine according to the diet treatment. (A) Anti-inflammatory cytokines; (P) pro-inflammatory cytokines. Values are presented as median and 95% confidence interval. *Significant p-value ($p<0.05$) was identified by Kruskal-Wallis test. Values having different superscript are significantly different by post-hoc Mann-Whitney test $p<0,0$

Cytokines	**Palm oil** *Median (95% CI)* *pg/mg protein*	**Corn oil** *Median (95% CI)* *pg/mg protein*	**Fish oil** *Median (95% CI)* *pg/mg protein*	*p-value*
(P) GMC-SF	0.02 (0.01-0.09)	0.01 (0.01-0.10)	0.01 (0.01-0.06)	0.60
(P) IL-1β	0.02 (0.01-0.10)	0.07 (0.01-0.21)	0.12 (0.01-0.18)	0.10
(P) IL-6	**1.00 (0.17-6.13)** [a]	**0.52 (0.04-1.03)** [b]	**0.36 (0.05-1.16)** [b]	0.04
(P) TNFα	**0.01 (0.00-0.98)** [a]	**0.30 (0.01-4.31)** [ab]	**0.28 (0.01-3.40)** [b]	0.04
(P) MCP-1	**0.55 (0.43-0.62)** [a]	**0.66 (0.45-0.83)** [ab]	**0.81 (0.48-1.46)** [b]	0.05

Table 4.6 Effect of dietary palm oil (n=16), corn oil (n=16) and fish oil (n=16) on cytokines levels (pg/mg protein) in the colon tissue of the large intestine in mice after 10-week treatment. (P) pro-inflammatory cytokines. Values are presented as median and 95% confidence interval. Male ApcMin/+ and wild-type mice combined. *P-value of Kruskal-Wallis test. Values having different superscript are significantly different by post-hoc Mann-Whitney test p<0,05.

	Palm oil			**Corn oil**			**Fish oil**		
Cytokines	**Wild-type** *(n=8)* Median (95% CI) *pg/mg protein*	**ApcMin/+** *(n=8)* Median (95% CI) *pg/mg protein*	p-value	**Wild-type** *(n=8)* Median (95% CI) *pg/mg protein*	**ApcMin/+** *(n=8)* Median (95% CI) *pg/mg protein*	p-value	**Wild-type** *(n=6)* Median (95% CI) *pg/mg protein*	**ApcMin/+** *(n=10)* Median (95% CI) *pg/mg protein*	p-value
(P) GMC-SF	0.02 (0.01-0.10)	0.02 (0.01-0.18)	0.75	0.01 (0.01-0.13)	0.04 (0.01-0.13)	0.25	0.01 (0.01-0.02)	0.03 (0.01-0.19)	0.66
(P) IL-1β	0.02 (0.01-0.27)	0.02 (0.01-0.20)	0.75	0.02 (0.01-0.29)	0.09 (0.02-0.26)	0.46	0.08 (0.01-0.18)	0.12 (0.01-0.22)	0.23
(P) IL-6	1.00 (0.03-8.63)	1.00 (0.09-11.46)	0.24	1.00 (0.04-12.31)	0.34 (0.04-5.75)	0.53	0.22 (0.05-1.00)	0.69 (0.04-1.29)	0.38
(P) TNFα	0.01 (0.00-7.95)	0.01 (0.00-1.18)	0.60	0.01 (0.00-7.74)	0.37 (0.00-6.31)	0.60	0.88 (0.01-3.40)	0.28 (0.01-5.39)	0.59
(P) MCP-1	0.55 (0.29-1.17)	0.55 (0.39-0.60)	0.60	0.53 (0.21-1.03)	0.74 (0.30-0.87)	0.34	0.79 (0.48-1.11)	0.81 (0.41-1.74)	0.83

Table 4.7 Effect of diet on cytokine level (pg/mg protein) in the colon tissue of the large intestine by diet. (P) pro-inflammatory cytokines. Values are presented as median and 95% confidence interval (CI). Significant p-value (p<0.05) was identified by Kruskal-Wallis test.

	Wild-type				ApcMin/+			
Cytokines	**Palm oil** *(n=8)* *Median (95% CI)* *pg/mg protein*	**Corn oil** *(n=8)* *Median (95% CI)* *pg/mg protein*	**Fish oil** *(n=6)* *Median (95% CI)* *pg/mg protein*	*p-value*	**Palm oil** *(n=8)* *Median (95% CI)* *pg/mg protein*	**Corn oil** *(n=8)* *Median (95% CI)* *pg/mg protein*	**Fish oil** *(n=10)* *Median (95% CI)* *pg/mg protein*	*p-value*
(P) GMC-SF	0.02 (0.01-0.10)	0.01 (0.01-0.13)	0.01 (0.01-0.02)	0.51	0.02 (0.01-0.18)	0.04 (0.01-0.13)	0.03 (0.01-0.19)	0.97
(P) IL-1β	0.02 (0.01-0.27)	0.02 (0.01-0.29)	0.08 (0.01-0.18)	0.86	0.02 (0.01-0.20)	0.09 (0.02-0.26)	0.12 (0.01-0.22)	0.07
(P) IL-6	1.00 (0.03-8.63)	1.00 (0.04-12.31)	0.22 (0.05-1.00)	0.58	**1.00 (0.09-11.46)** [a]	**0.34 (0.04-5.75)** [b]	**0.69 (0.04-1.29)** [b]	0.04
(P) TNFα	0.01 (0.00-7.95)	0.01 (0.00-7.74)	0.88 (0.01-3.40)	0.63	0.01 (0.00-1.18)	0.37 (0.00-6.31)	0.28 (0.01-5.39)	0.08
(P) MCP-1	0.55 (0.29-1.17)	0.53 (0.21-1.03)	0.79 (0.48-1.11)	0.23	0.55 (0.39-0.60)	0.74 (0.30-0.87)	0.81 (0.41-1.74)	0.14

Table 4.8 Effect of strain on cytokine level (pg/mg protein) in the colon tissue of the large intestine according to the diet treatment. (P) pro-inflammatory cytokines. Values are presented as median and 95% confidence interval. Significant p-Value (p<0,05) was identified by Kruskal-Wallis test.

4.1.3.3. Plasma

The concentration of IL-1α, IL-5, IL-6, and MCP-1 were significantly different ($p<0.05$) when all animals were combined (ApcMin/+ and wild-type mice) and compared between the dietary treatments (Table 4.9). Animals treated with fish oil had higher levels of IL-5 ($p=0.01$) and MCP-1 ($p=0.00$) when compared to the corn oil and palm oil group. IL-6 ($p=0.03$) and GM-CSF ($p=0.03$) were also higher in the fish oil group, but only compared with animals fed the corn oil diet. IL-1α was significant lower ($p=0.02$) in the fish oil group compared to corn oil and palm oil.

Cytokines	**Palm oil** *Median (95% CI)* pg/ml	**Corn oil** *Median (95% CI)* pg/ml	**Fish oil** *Median (95% CI)* pg/ml	*p-value*
(A) IFNγ	3.20 (3.07-3.20)	3.20 (2.46-3.20)	3.20 (3.20-3.20)	0.98
(P) IL-1α	**45.96 (3.20-97.19)** [ac]	**83.67 (3.20-175.78)** [a]	**38.21 (3.20-56.23)** [bc]	0.05
(P) IL-2	3.20 (3.20-15.85)	3.20 (3.20-3.20)	3.20 (3.20-13.38)	0.07
(A) IL-4	3.20 (1.79-3.20)	3.20 (2.46-3.20)	3.20 (1.23-3.20)	0.28
(P) IL-5	**8.99 (4.58-13.24)** [a]	**6.02 (4.36-11.01)** [a]	**11.20 (6.54-18.31)** [b]	0.01
(P) IL-6	**9.89 (3.20-30.00)** [ac]	**3.20 (3.20-7.67)** [b]	**10.50 (3.20-17.73)** [c]	0.03
(A) IL-10	3.20 (3.20-10.73)	3.20 (3.20-3.20)	3.20 (3.20-17.05)	0.12
(P) IL-12p40	58.74 (3.75-116.16)	51.33 (13.99-64.81)	39.88 (16.87-86.17)	0.36
(P) IL-12p70	3.20 (3.20-478.92)	3.20 (3.20-24.66)	3.20 (3.20-496.29)	0.91
(P) IL-15	57.81 (3.20-211.73)	3.20 (3.20-101.02)	3.20 (3.20-187.11)	0.43
(P) IL-17	3.20 (3.20-53.09)	3.20 (3.20-3.20)	3.20 (3.20-53.59)	0.13
(A) IP-10	200.30 (169.59-268.32)	215.61 (183.45-282.66)	257.30 (216.71-286.36)	0.23
(P) KC	77.99 (3.20-127.49)	74.64 (5.35-78.29)	43.91 (3.20-118.13)	0.17
(P) GMC-SF	3.20 (3.20-41.24) [ab]	3.20 (3.20-3.20) [a]	3.20 (3.20-63.63) [b]	0.07
(P) TNFα	3.20 (1.37-3.20)	3.20 (1.93-3.20)	3.20 (0.91-3.20)	1.00
(P) IL-1β	3.20 (3.20-17.68)	5.04 (3.38-32.54)	8.54 (2.97-32.54)	0.33
(P) MCP1	**14.27 (5.22-41.78)** [a]	**19.93 (9.31-30.26)** [a]	**42.98 (30.34-59.06)** [b]	0.00
(A) MIG	144.09 (120.62-311.93)	157.04 (111.82-185.84)	157.15 (124.67-205.98)	0.13
(P) MIP1α	3.20 (3.20-24.42)	3.20 (3.20-13.73)	5.37 (3.20-10.16)	0.91
(P) VEGF	3.20 (0.65-3.20)	3.20 (0.68-3.20)	3.20 (0.83-3.20)	0.69

Table 4.9 Effect of dietary palm oil (n=29), corn oil (n=29) and fish oil (n=29) on cytokines levels (pg/ml) in the plasma of mice after 10-week treatment. (A) Anti-inflammatory cytokines; (P) pro-inflammatory cytokines. Values are presented as median and 95% confidence interval. Male ApcMin/+ and wild-type mice combined. *P-value of Kruskal-Wallis test. Values having different superscript are significantly different by post-hoc Mann-Whitney test p<0,05.

When the cytokines were analysed for each dietary treatment by strain, the following results were observed (Table 4.10). In the palm oil group, IL-2 (p=0.02) was significantly higher in the wild-type mice compared to the ApcMin/+ mice. Moreover, wild-type mice also showed a trend to have higher concentration of MCP-1 (p=0.09) compared to the ApcMin/+ mice also fed the palm oil diet. ApcMin/+ mice from the corn oil group had significantly (p=0.03) higher levels of IL-6 and KC compared to the wild-type animals. However, the wild-type animals fed the corn oil diet, showed a trend towards high concentrations of MCP-1 (p=0.09) and MIG (p=0.08) compared to ApcMin mice fed the same diet. Animals treated with fish oil were only different for IL-17, which was higher in the wild-type (p=0.02) compared to the ApcMin/+ mice.

When the cytokines were analysed by strain the following results were observed (Table 4.11). Within the wild-type animals, the fish oil treatment increased the levels of IL-4 (p=0.04) and IL-17 (p=0.00) compared to animals fed the palm oil. Wild-type animals fed the corn oil diet decreased the levels of IL-6 (p=0.03) and KC (p=0.01) compared to animal fed the palm oil diet. When mice fed the corn oil diet were compared to mice fed the fish oil diet, the levels of IL-4 (p=0.04), IL-6 (p=0.01), IL-17 (p=0.05) were significantly higher for the fish oil group.

Within the ApcMin/+ mice, animals fed the fish oil diet had levels of IL-5 (p=0.02), IL-1β (p=0.03), and MCP-1 (p=0.007) higher than the animals fed the corn oil diet. When comparing the levels of cytokines in animals fed the fish oil and corn oil diet, only MCP-1 was significant (p=0.01) higher for the fish oil group. No significant effect was observed for ApcMin/+ animals fed the corn oil diet compared to palm oil.

Cytokines	Palm oil		Corn oil		Fish oil	
	Wild-type *Median (95% CI)* pg/ml	**ApcMin/+** *Median (95% CI)* pg/ml	**Wild-type** *Median (95% CI)* pg/ml	**ApcMin/+** *Median (95% CI)* pg/ml	**Wild-type** *Median (95% CI)* pg/ml	**ApcMin/+** *Median (95% CI)* pg/ml
(A) IFNγ	3.20 (2.25-3.20)	3.20 (1.58-3.42)	3.20 (1.12-3.20)	3.20 (2.28-3.44)	3.20 (3.20-3.20)	3.20 (2.65-3.20)
(P) IL-1α	50.64 (3.20-408.51)	35.49 (3.20-85.90)	111.37 (3.20-265.83)	41.67 (3.20-249.97)	40.11 (3.20-90.16)	27.11 (3.20-56.23)
(P) IL-2	**3.20 (3.20-21.21)** [a]	**3.20 (1.79-15.85)** [b]	3.20 (2.51-3.20)	3.20 (3.20-22.70)	3.20 (3.20-22.06)	3.20 (3.20-25.69)
(A) IL-4	3.20 (1.09-3.20)	3.20 (1.16-3.20)	3.20 (0.81-3.20)	3.20 (1.09-3.20)	3.20 (0.54-131.73)	3.20 (0.89-3.20)
(P) IL-5	9.87 (5.48-15.64)	5.25 (3.42-15.26)	6.02 (3.72-12.05)	5.77 (3.71-12.60)	9.02 (5.27-20.82)	13.15 (5.93-18.31)
(P) IL-6	28.64 (3.20-62.01)	7.10 (3.20-18.79)	**3.20 (1.68-3.20)** [a]	**7.22 (3.20-20.77)** [b]	11.21 (3.20-42.66)	8.49 (3.19-54.47)
(A) IL-10	3.20 (3.20-12.99)	3.20 (3.20-12.20)	3.20 (3.20-3.20)	3.20 (3.20-8.67)	3.20 (3.20-34.92)	3.20 (3.20-17.05)
(P) IL-12p40	58.74 (3.20-215.81)	66.74 (3.20-116.75)	49.70 (3.20-103.82)	51.84 (5.48-81.60)	24.40 (3.20-120.30)	48.90 (16.87-108.41)
(P) IL-12p70	18.13 (3.20-534.56)	3.20 (3.20-494.83)	3.20 (3.20-523.79)	3.20 (3.20-520.46)	3.20 (3.20—555.38)	3.20 (3.20-544.99)
(P) IL-15	64.75 (3.20-318.12)	41.31 (3.20-316.07)	3.20 (3.20-226.81)	3.20 (3.20-223.22)	29.54 (3.20-351.51)	3.20 (3.20-187.11)
(P) IL-17	3.20 (1.08-53.37)	3.20 (3.20-53.48)	3.20 (3.20-53.09)	3.20 (0.54-53.34)	**3.20 (3.20-55.16)** [a]	**3.20 (3.20-53.59)** [b]
(A) IP-10	200.83 (167.65-321.32)	192.41 (163.47-268.32)	215.61 (199.18-298.39)	227.91 (160.30-348.67)	257.30 (165.13-297.86)	250.53 (200.85-328.67)
(P) KC	77.99 (3.20-143.42)	72.44 (3.20-155.49)	**46.43 (3.20-76.25)** [a]	**78.13 (3.20-127.51)** [b]	54.86 (3.20-148.61)	33.58 (3.20-125.19)
(P) GMC-SF	3.20 (3.20-49.51)	3.20 (3.20-67.85)	3.20 (3.20-3.20)	3.20 (3.20-3.20)	3.20 (3.20-63.63)	3.20 (3.20-103.47)
(P) TNFα	3.20 (1.37-3.20)	3.20 (0.75-3.20)	3.20 (3.20-3.20)	2.30 (0.85-3.20)	3.20 (1.36-3.20)	1.51 (0.51-3.20)
(P) IL-1β	4.27 (3.20-35.22)	3.20 (2.94-19.53)	5.04 (3.38-27.66)	7.15 (3.20-39.46)	5.84 (2.34-36.11)	16.13 (2.97-45.27)
(P) MCP1	**31.05 (3.20-49.19)** [a]	**12.77 (3.20-41.78)** [b]	**23.64 (6.73-56.02)** [a]	**16.67 (3.20-35.39)** [b]	52.62 (3.20-82.50)	41.17 (9.07-59.06)
(A) MIG	127.20 (105.75-688.79)	149.97 (110.56-360.71)	**184.00 (151.26-301.02)** [a]	**116.07 (73.65-185.84)** [b]	155.22 (101.52-263.50)	160.78 (102.58-202.07)
(P) MIP1α	3.20 (3.20-28.11)	6.17 (3.20-35.67)	3.20 (3.20-13.73)	3.20 (3.20-49.06)	5.68 (3.20-24.42)	4.29 (3.20-9.00)
(P) VEGF	3.20 (0.26-3.20)	3.20 (0.44-3.20)	3.20 (0.44-3.20)	3.20 (0.54-3.20)	3.20 (0.32-3.20)	3.20 (0.66-3.20)

Table 4.10 Effect of palm oil (WT n=13; APC n=16), corn oil (WT n=15; APC n=14), or fish oil (WT n=13; APC n=16) on cytokine level in the plasma of wild-type and ApcMin/+ mice. (A) Anti-inflammatory cytokines; (P) pro-inflammatory cytokines. Values are presented as median (pg/ml) and 95% confidence interval (CI). Values having different superscript are significantly different ($p<0.05$) by Mann-Whitney test within the diet.

Cytokines	Wild-type			ApcMin/+			WT	APC
	Palm oil *Median (95% CI)* pg/ml	**Corn oil** *Median (95% CI)* pg/ml	**Fish oil** *Median (95% CI)* pg/ml	**Palm oil** *Median (95% CI)* pg/ml	**Corn oil** *Median (95% CI)* pg/ml	**Fish oil** *Median (95% CI)* pg/ml	**p**	**p**
(A) IFNγ	3.20 (2.25-3.20)	3.20 (1.12-3.20)	3.20 (3.20-3.20)	3.20 (1.58-3.42)	3.20 (2.28-3.44)	3.20 (2.65-3.20)	0.94	0.92
(P) IL-1α	50.64 (3.20-408.51)	111.37 (3.20-265.83)	40.11 (3.20-90.16)	35.49 (3.20-85.90)	41.67 (3.20-249.97)	27.11 (3.20-56.23)	0.21	0.11
(P) IL-2	3.20 (3.20-21.21)	3.20 (2.51-3.20)	3.20 (3.20-22.06)	3.20 (1.79-15.85)	3.20 (3.20-22.70)	3.20 (3.20-25.69)	0.14	0.14
(A) IL-4	**3.20 (1.09-3.20)** [a]	**3.20 (0.81-3.20)** [a]	**3.20 (0.54-131.73)** [b]	3.20 (1.16-3.20)	3.20 (1.09-3.20)	3.20 (0.89-3.20)	0.05	1.00
(P) IL-5	9.87 (5.48-15.64)	6.02 (3.72-12.05)	9.02 (5.27-20.82)	**5.25 (3.42-15.26)** [a]	**5.77 (3.71-12.60)** [ab]	**13.15 (5.93-18.31)** [b]	0.11	0.05
(P) IL-6	**28.64 (3.20-62.01)** [a]	**3.20 (1.68-3.20)** [b]	**11.21 (3.20-42.66)** [ac]	7.10 (3.20-18.79)	7.22 (3.20-20.77)	8.49 (3.19-54.47)	0.01	0.83
(A) IL-10	3.20 (3.20-12.99)	3.20 (3.20-3.20)	3.20 (3.20-34.92)	3.20 (3.20-12.20)	3.20 (3.20-8.67)	3.20 (3.20-17.05)	0.16	0.58
(P) IL-12p40	58.74 (3.20-215.81)	49.70 (3.20-103.82)	24.40 (3.20-120.30)	66.74 (3.20-116.75)	51.84 (5.48-81.60)	48.90 (16.87-108.41)	0.45	0.46
(P) IL-12p70	18.13 (3.20-534.56)	3.20 (3.20-523.79)	3.20 (3.20—555.38)	3.20 (3.20-494.83)	3.20 (3.20-520.46)	3.20 (3.20-544.99)	0.87	0.72
(P) IL-15	64.75 (3.20-318.12)	3.20 (3.20-226.81)	29.54 (3.20-351.51)	41.31 (3.20-316.07)	3.20 (3.20-223.22)	3.20 (3.20-187.11)	0.85	0.30
(P) IL-17	**3.20 (1.08-53.37)** [ab]	**3.20 (3.20-53.09)** [b]	**3.20 (3.20-55.16)** [c]	3.20 (3.20-53.48)	3.20 (0.54-53.34)	3.20 (3.20-53.59)	0.01	0.86
(A) IP-10	200.83 (167.65-321.32)	215.61 (199.18-298.39)	257.30 (165.13-297.86)	192.41 (163.47-268.32)	227.91 (160.30-348.67)	250.53 (200.85-328.67)	0.99	0.11
(P) KC	**77.99 (3.20-143.42)** [ac]	**46.43 (3.20-76.25)** [b]	**54.86 (3.20-148.61)** [ba]	72.44 (3.20-155.49)	78.13 (3.20-127.51)	33.58 (3.20-125.19)	0.04	0.81
(P) GMC-SF	3.20 (3.20-49.51)	3.20 (3.20-3.20)	3.20 (3.20-63.63)	3.20 (3.20-67.85)	3.20 (3.20-3.20)	3.20 (3.20-103.47)	0.31	0.22
(P) TNFα	3.20 (1.37-3.20)	3.20 (3.20-3.20)	3.20 (1.36-3.20)	3.20 (0.75-3.20)	2.30 (0.85-3.20)	1.51 (0.51-3.20)	0.84	0.87
(P) IL-1β	4.27 (3.20-35.22)	5.04 (3.38-27.66)	5.84 (2.34-36.11)	**3.20 (2.94-19.53)** [a]	**7.15 (3.20-39.46)** [ab]	**16.13 (2.97-45.27)** [b]	0.93	0.05
(P) MCP1	31.05 (3.20-49.19)	23.64 (6.73-56.02)	52.62 (3.20-82.50)	**12.77 (3.20-41.78)** [a]	**16.67 (3.20-35.39)** [a]	**41.17 (9.07-59.06)** [b]	0.15	0.01
(A) MIG	127.20 (105.75-688.79)	184.00 (151.26-301.02)	155.22 (101.52-263.50)	149.97 (110.56-360.71)	116.07 (73.65-185.84)	160.78 (102.58-202.07)	0.37	0.20
(P) MIP1α	3.20 (3.20-28.11)	3.20 (3.20-13.73)	5.68 (3.20-24.42)	6.17 (3.20-35.67)	3.20 (3.20-49.06)	4.29 (3.20-9.00)	0.90	0.65
(P) VEGF	3.20 (0.26-3.20)	3.20 (0.44-3.20)	3.20 (0.32-3.20)	3.20 (0.44-3.20)	3.20 (0.54-3.20)	3.20 (0.66-3.20)	0.81	0.80

Table 4.11 Effect of genotype on cytokine level in the plasma of wild-type and ApcMin/+ mice. Values are presented as median (pg/ml) and 95% confidence interval (CI). (A) Anti-inflammatory cytokines; (P) pro-inflammatory cytokines. Values having different superscript are significantly different ($p<0.05$) by Kruskal-Wallis test. (WT: wild-type; APC: ApcMin/+; P: p-value).

4.1.4. Effects on tumour development

Tumour development was measured only in the ApcMin/+ mice because wild-type animals did not develop tumours either in the small intestine nor colon.

4.1.4.1. Effect of diet on tumour size

Tumour size varied according to the treatment diet (Figure 4.5). Animals fed fish oil (1.00 ± 0.40 mm^2) had smaller tumour size compared to the control palm oil (1.51 ± 0.81 mm^2) and to the animals fed corn oil (1.42 ± 0.71 mm^2), p=0.0001 and p=0.005 respectively.

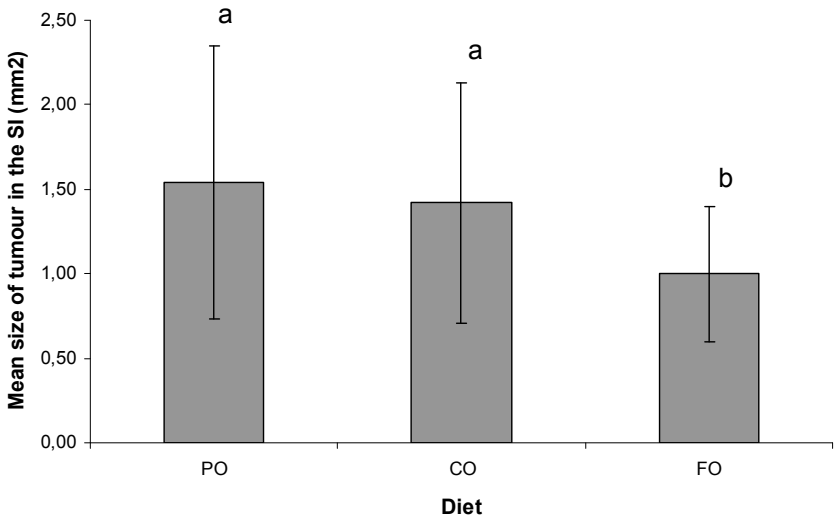

Figure 4.5 Effect of treatment diets on mean size of tumour in the small intestine (SI) of the ApcMin/+ mice fed for 10 weeks. (n=16 mice/diet). Values (mean ± SD) having different superscript indicate statistically significant difference between the treatment groups by ANOVA at $p< 0.05$.

4.1.4.2. Effect of intestinal position on tumour size

In Figure 4.6 is shown that tumour size varies with position in the small intestine. Tumours at the distal part of the small intestine (2.1 ± 0.45 mm^2) was significantly bigger than tumours at the middle part (1.2 ± 0.45 mm^2), p=0.0004. However, the tumour size at the middle part of the intestine was significantly smaller than the proximal (1.72 ± 0.92 mm^2), p=0.0002. There was no difference in tumour size between proximal and distal. This figure showed that tumour size did vary with position in the small intestine.

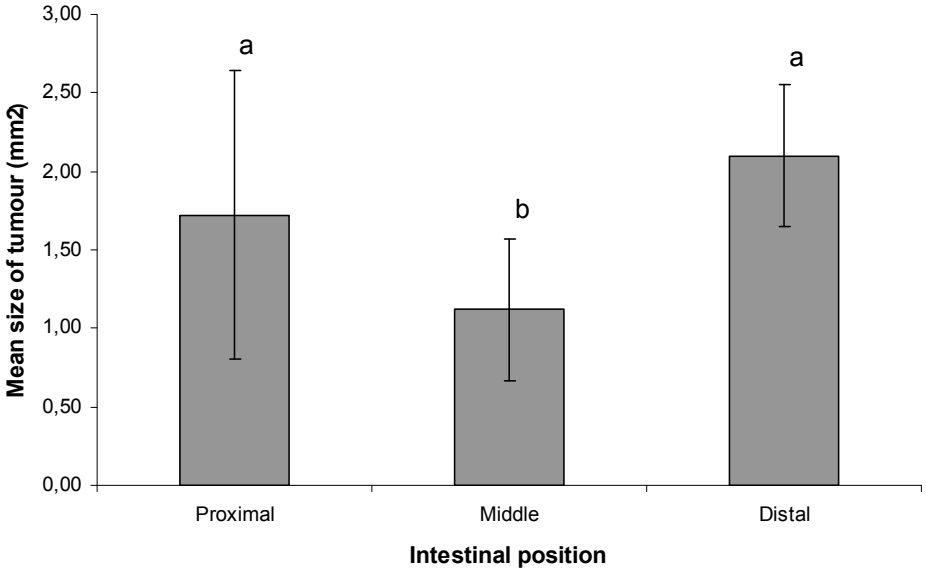

Figure 4.6 Mean tumour size (mm2) according to the intestinal position in the small intestine of ApcMin/+ mice (n=48). Values show means and SD and those having different superscript indicate statistically significant difference between the treatment groups by ANOVA at $p< 0.05$.

In the proximal part of the small intestine (Figure 4.7), tumour size was smaller for animals fed fish oil (1.1 ± 0.4 mm^2) compared not only to the control palm oil (2.1 ± 0.9 mm^2), but also to corn oil (1.9 ± 1.0 mm^2), p<0.0001.

Figure 4.8 shows the results of tumour size in the middle part of the small intestine. Animals fed the fish oil diet (0.9 ± 0.4 mm^2) had smaller tumours compared to the control palm oil diet (1.2 ± 0.5 mm^2) and corn oil (1.2 ± 0.4 mm^2) in both cases, p=0.0001.

Figure 4.9 shows the results of tumour size at the distal part of the small intestine. Mice fed the fish oil diet (0.9 ± 0.4 mm^2) had smaller tumour size compared to control palm oil (1.2 ± 0.6 mm^2) and to corn oil (1.2 ± 0.4 mm^2), p=0.0001.

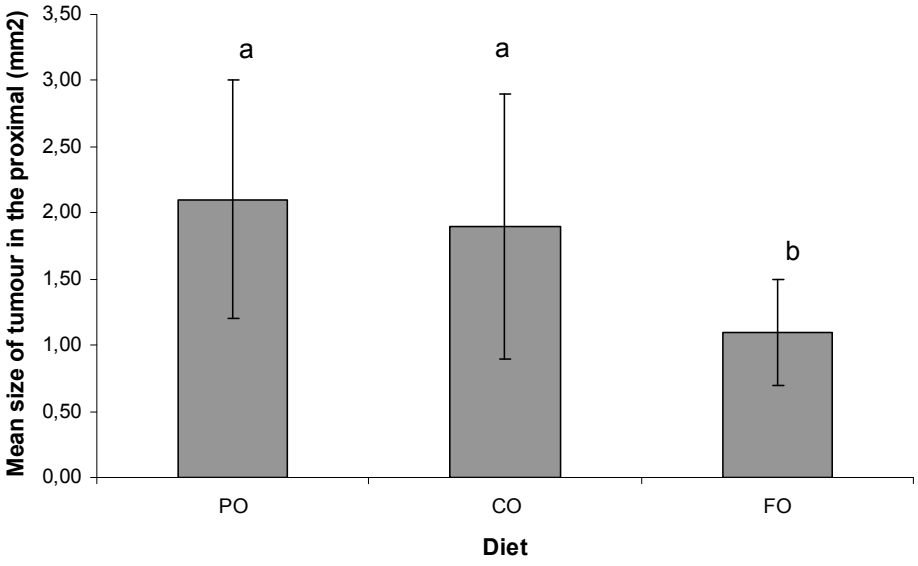

Figure 4.7 Effect of treatment diets mean size of tumour (mm2) in the proximal part the small intestine of the ApcMin/+ mice fed for 10 weeks (n=16 mice/diet). Values (mean ± SD) having different superscript indicate statistically significant difference between the treatment groups by ANOVA at $p< 0.05$.

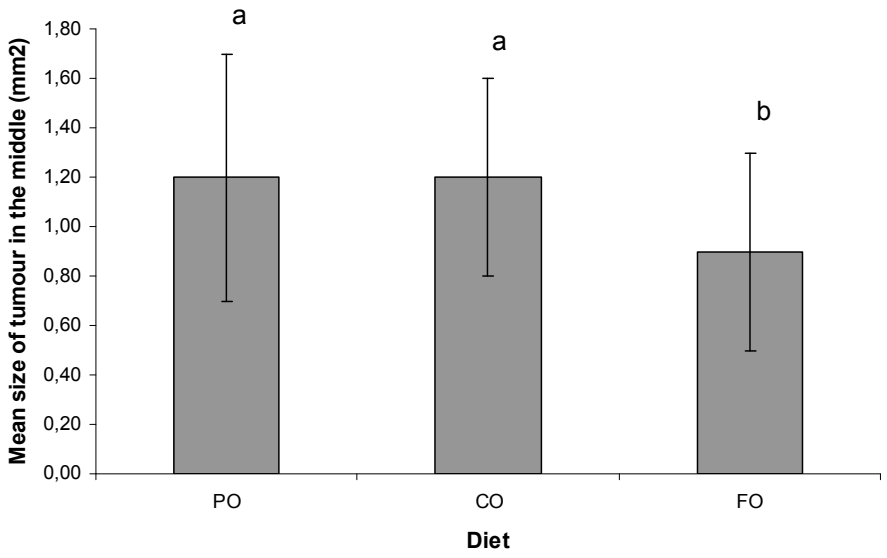

Figure 4.8 Effect of treatment diets mean size of tumour (mm2) in the middle part the small intestine of the ApcMin/+ mice fed for 10 weeks (n=16 mice/diet). Values (mean ± SD) having different superscript indicate statistically significant difference between the treatment groups by ANOVA at $p< 0.05$.

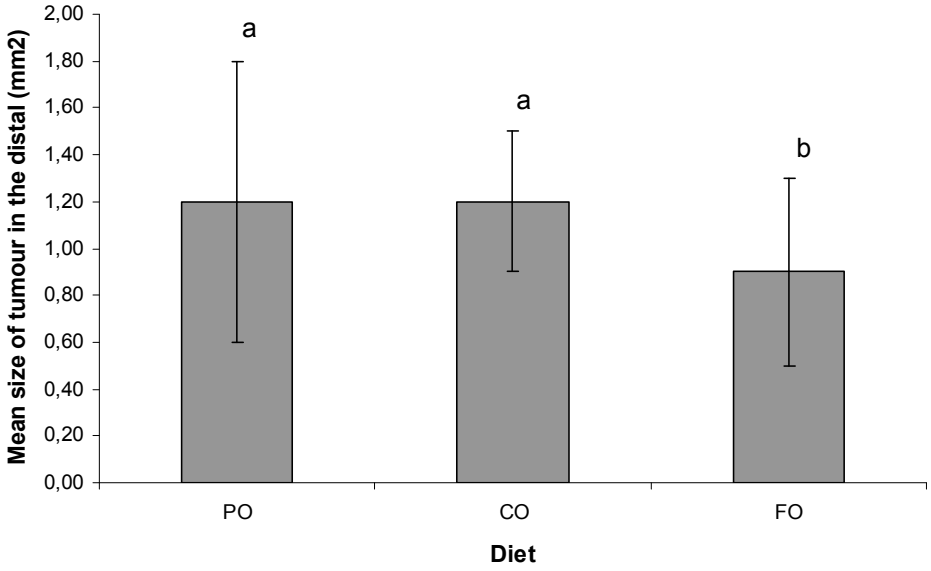

Figure 4.9 Effect of treatment diets mean size of tumour (mm2) in the distal part the small intestine of the ApcMin/+ mice fed for 10 weeks (n=16 mice/diet). Values (mean ± SD) having different superscript indicate statistically significant difference between the treatment groups by ANOVA at $p< 0.05$.

4.1.4.3. Effect of diet on total tumour area

In figure 4.10 is shown the effects of treatment diet on total tumour area across all the small intestine. Animals fed the corn oil (1621 mm^2) and fish oil diet (1246 mm^2) were not significant different from the palm oil group (1355 mm^2) regarding total tumour area.

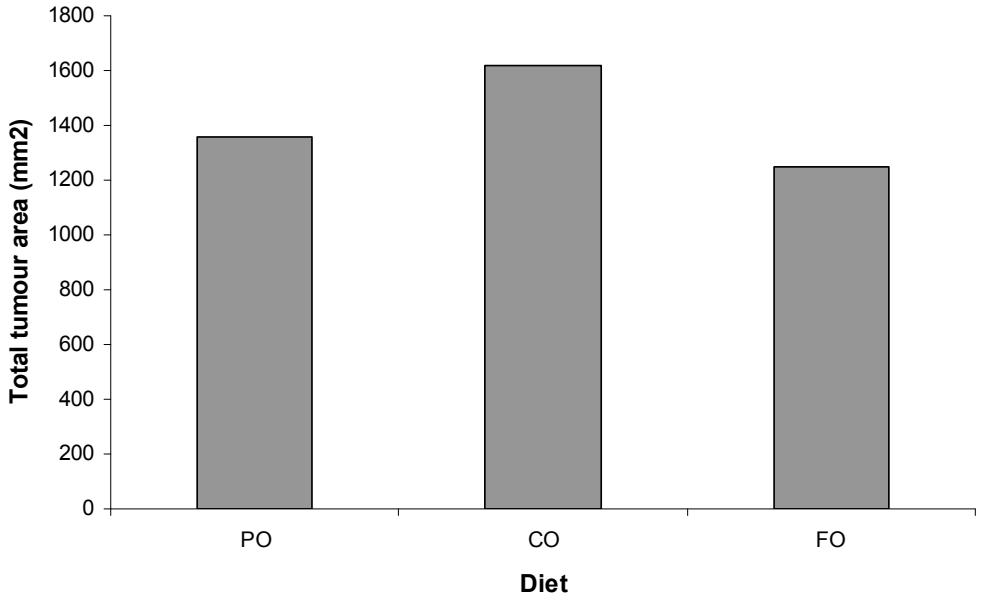

Figure 4.10 Effect of treatment diets on total tumour area (mm2) across all the small intestine of the ApcMin/+ mice fed for 10 weeks (n=16 mice/diet). No differences statistically significant were found between the treatment groups by ANOVA at $p< 0.05$.

However, when the total tumour area was categorized by the position in the small intestine, corn oil significantly increased the total tumour area in the proximal (471 mm^2) compared to palm oil (287 mm^2). Moreover, fish oil diet significantly reduced the total tumour area in the distal part of the small intestine (427 mm^2) compared to palm oil (597 mm^2) and corn oil (630 mm^2) (Figure 4.11).

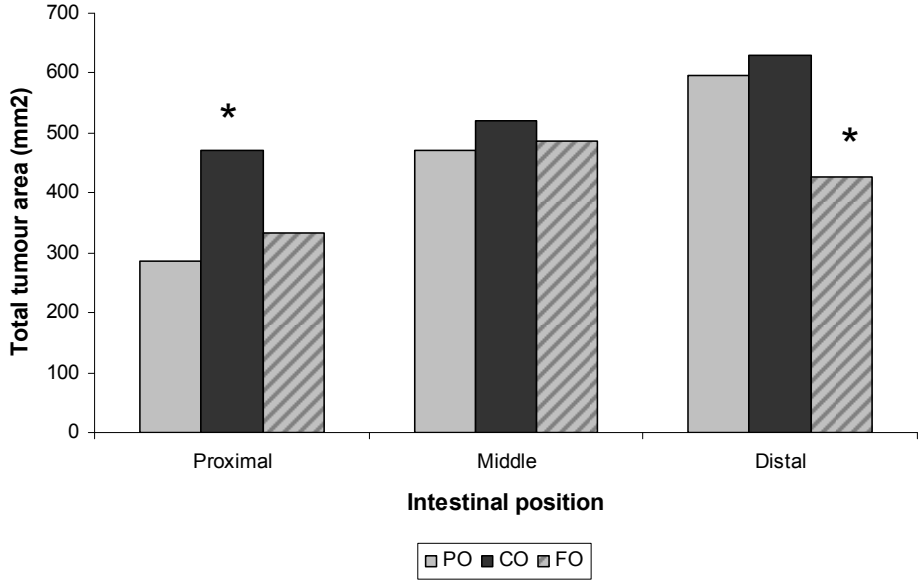

Figure 4.11 Effect of treatment diets on total tumour area (mm2) in the proximal, middle and distal parts of the intestinal position of the ApcMin/+ mice fed for 10 weeks (n=16 mice/diet). Values having different superscript indicate statistically significant difference between the treatment groups within the same intestinal position by ANOVA at $p< 0.05$.

4.1.4.4. Effect of diet on total tumour number

The diet treatments had no significant difference on total tumour number across all the small intestine (PO=1044; CO=1168; FO=1284). However, when the tumours were separated by different positions of the small intestine (Figure 4.12), and categorized by different sizes: small (<1mm^2), medium (1-5mm^2), and big (>5mm^2) (Table 4.13), significant effects were observed. The incidence of total tumours in ApcMin/+ given fish

oil was significantly greater in the middle region compared with the proximal (middle 531 vs. proximal 308, p=0.008). Animals fed with corn oil and palm oil had significantly more tumours in the distal compared to proximal (palm oil: distal 427 vs. proximal 239, p=0.008 and corn oil: distal 543 vs. proximal 170, p=0.0001).

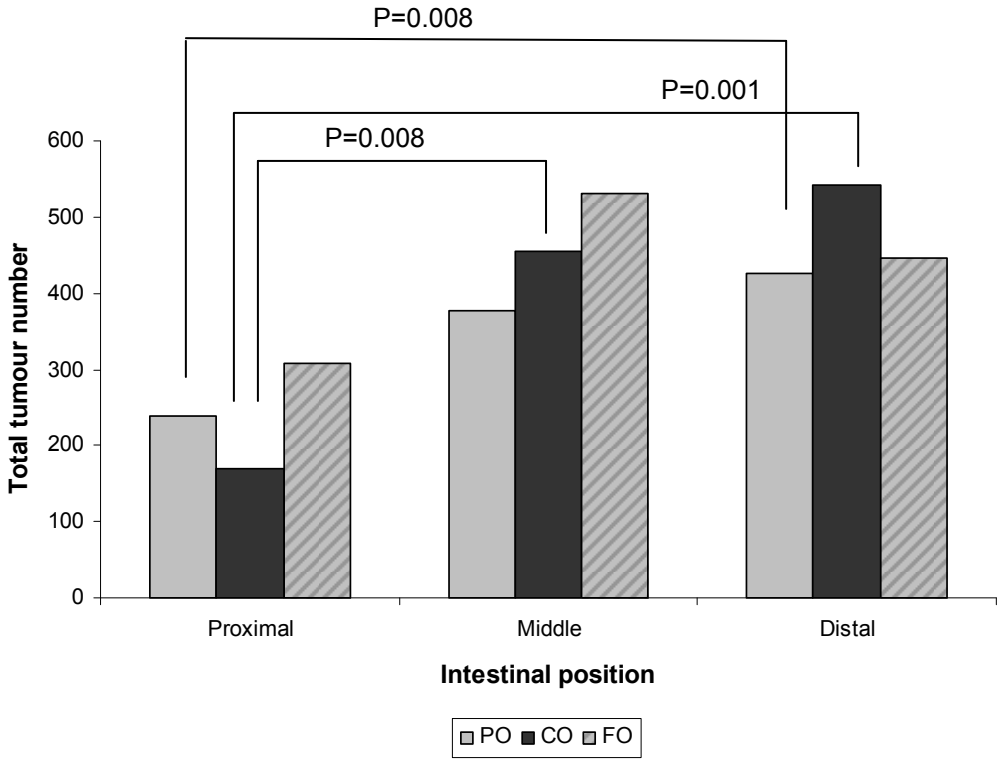

Table 4.12 Effect of treatment diets on total tumour area (mm2) in the proximal, middle and distal parts of the intestinal position of the ApcMin/+ mice fed for 10 weeks (n=16 mice/diet). Values having different superscript indicate statistically significant by ANOVA at $p< 0.05$.

Considering the size of tumours, ApcMin/+ fed fish oil had significantly greater numbers of small tumours (<1mm^2) in all regions (proximal, middle, distal) in the small intestine compared to control palm oil (proximal: fish oil 204 vs. palm oil 108, p=0.0001; middle: fish oil 360 vs. palm oil 204, p=0.0001; distal: fish oil 301 vs. palm oil 207, p=0.0002).

Tumour region and size	Palm oil	Corn oil	Fish oil
Proximal			
< 1 mm2	108[a]	85[a]	204[b]
1-5 mm2	104[a]	75[b]	98[a]
> 5 mm2	27[a]	10[b]	6[b]
Middle			
< 1 mm2	204[a]	269[a]	360[b]
1-5 mm2	170	184	164
> 5 mm2	4	2	7
Distal			
< 1 mm2	207[a]	290[b]	301[b]
1-5 mm2	212[a]	248[a]	141[b]
> 5 mm2	8	5	5

Table 4.13 Effect of palm oil, corn oil and fish oil on tumour number of ApcMin/+ mice. Values are presented as numbers of tumours and its percentage by each position of the small intestine. Male and female ApcMin/+ mice combined (n=16 animals/diet). Values having different superscript are significantly different by ANOVA, $p<0.05$.

4.1.4.5. Effect of diet on tumour development in the colon

There was no significant difference in mean tumour size in the colon (PO=0.3 ± 0.5 mm^2; CO=2.6 ± 4.9 mm^2; FO=0.8 ± 1.2mm^2). The number of tumours in the colon was too small to perform statistical analysis. It is shown in Table 4.14 that animals fed the fish oil diet had greater numbers of small tumours compared to the other treatment diets, however it was not significant.

Tumour size	Palm oil	Corn oil	Fish oil
Colon			
Total	11	13	29
< 1 mm2	9	6	19
1-5 mm2	2	2	9
> 5 mm2	0	5	1

Table 4.14 Effect of palm oil, corn oil and fish oil on tumour number and size in the colon of ApcMin/+ mice. Values are presented as numbers of tumours and its percentage categorized by size. Male and female ApcMin/+ mice combined (n=16 animals/diet). Values were too small to perform any statistical analysis.

4.2. DISCUSSION

4.2.1. Mitosis, apoptosis and crypt length

The mammalian intestinal epithelium is constantly renewed by cells derived from a rapidly dividing population in the crypts. Chronic hyperproliferation may be an early step in the progression from normal mucosa to malignant transformation. Normal colonic stem cells, which reside at the base of the crypt, generate the colonic mucosa that has an incredible rate of cell production and turnover. Cancer may therefore develop as a result of alteration of this process though the accumulation of mutations and damage within the controlling stem cells. The cellular division in cancer is driven by internal cellular events regardless of external stimuli (Anti, Marra et al. 1992; Salama and Platell 2009).

Epidemiological studies indicate that there is an inverse association between intake of n-3 polyunsaturated fatty acids (PUFAs) and incidence of colon cancer, although these studies are not uniformly conclusive (Nkondjock, Shatenstein et al. 2003). Also, cancer xenografts in immunosuppressed mice, as well as cell culture studies demonstrate that PUFAs may slow down cancer cell growth (Schonberg, Rudra et al. 1997; Clarke, Lund et al. 1999; Latham, Lund et al. 1999; Chen and Istfan 2000; Chamras, Ardashian et al. 2002; Diggle 2002; Kato, Hancock et al. 2002).

In the present study, the fish oil diet had no significant effect on cell proliferation in the distal small intestine tissue of ApcMin/+, compared to wild-type mice fed the same diet. The lack of positive effect of fish oil on reducing cell proliferation seems to contradict various studies where fish oil had positive effect on promoting an inhibition of cell proliferation in cell lines (Llor, Pons et al. 2003; Fukunaga, Hossain et al. 2008; Habbel,

Weylandt et al. 2009) and in animal studies (Chang, Chapkin et al. 1998; Calviello, Palozza et al. 1999; Fukunaga, Hossain et al. 2008; Kramer, Johnson et al. 2009). Even in normal rat colonic mucosa fish oil was shown to decrease cell proliferation when rats were fed low doses of purified EPA or DHA ethyl esters (1g/Kg body weight) (Calviello, Palozza et al. 1999). In this experiment, the cell proliferation analysis was carried out in healthy wild-type intestinal tissue, and in ApcMin/+ tissue, in macroscopically normal intestinal tissue. Cell proliferation was not measured in the tumour tissue. Perhaps, if it was measured it in the actual tumour tissue a significant effect of fish oil on cell proliferation in the carcinogenic animal model might have been found. However, this would have been difficult to analyse using the methods employed in this study due to the disrupted tissue architecture found in tumours and thus lack of clearly defined crypts to be dissected out.

The effect of fish oil in human studies also seems to be contradictory. Cheng et al. found no effect on crypt proliferation as measured by Ki-67 labelling with low dose of fish oil supplementation (100mg of EPA and 400mg of DHA per day) in patients with colorectal adenomas after 12 or 24 months of treatment (Cheng, Ogawa et al. 2003). Similarly, Gee et al. (Gee, Watson et al. 1999) giving 1.4g of EPA and 1.0g of DHA per day for 2 weeks found no evidence of any effect of supplementation with fish oil in patients requiring surgery for carcinoma of the colon, despite the clear evidence of incorporation of EPA into epithelial cells. However, Courtney at al. (Courtney, Matthews et al. 2007) found a significantly decreased cell proliferation in patients with colorectal adenomas treated with highly purified EPA (2g/day) in the free fatty acid form for 3 months. Moreover, Anti et al. (Anti, Marra et al. 1992) reported that relatively high doses of fish oil (EPA 4.1g/day, DHA 3.6g/day) reduced the rate of proliferation as assessed by autoradiographic analysis of [H]thymidine incorporation in the flat rectal mucosa of patients with previously resected sporadic adenoma. In a subsequent study (Anti, Armelao et al. 1994) they reported that in

15 adenoma patients receiving 2.5g of fish oil per day both the frequency and spatial distribution of labelled cells were normalized compared to control patients given a placebo. These discrepancies between different studies suggest that not only the duration of treatment could be responsible for the different results, but also the type of EPA used in the experiment. Differences in efficiency of absorption and the biological activity of different forms of EPA and DHA may be interfering significantly in the results of several studies (Patten, Augustin et al. 2009).

While many studies have reported the protective effect of fish oil, others report that corn oil, which is high in n-6 polyunsaturated fatty acids, especially linoleic acid, have strong tumour enhancing effects (Reddy, Burill et al. 1991). Reddy and Sugie (Reddy and Sugie 1988) found that a 20% energy corn oil diet showed a tumour promoting effect in AOM-treated mice in comparison with the low fat diet, whereas a 20% fish oil had no such effect. A recent study performed by Solanas et al. (Solanas, Grau et al. 2009) confirms the procarcinogenic effect of the corn oil by showing an increased mitotic index in cancer cells. The present results are in contrast with these previous studies. In the present experiment, corn oil had no significant effect on increasing cell proliferation in ApcMin/+ mice compared to wild-type fed the same diet. However, wild-type animals fed the corn oil diet showed an increase of cell proliferation compared to wild-type fed the palm oil or fish oil. This result is line with the results found by Pell et al. where they fed healthy rats for only 14 days and corn oil also increased the cell proliferation compared to the fish oil treatment but not compared to saturated fat (Pell, Brown et al. 1994). Thus, corn oil seems to increase the carcinogenic effect in healthy animals, instead of enhancing it in the animal model of colorectal cancer. A high fat diet rich in corn oil has been shown to promote colon carcinogenesis, particularly in the post-initiation or promotional phases (Reddy, Burill et al. 1991; Rao, Hirose et al. 2001; Dai, Liu et al. 2002). It is known that in

ApcMin/+ mice that tumour development occurs very early in life with the development of intestinal polyclonal crypts, which during the second or third weeks of life become clonal units (Shoemaker, Gould et al. 1997). There are several explanations that suggest the initiation of tumour in ApcMin/+ mice, but it may be that the polycolonal nature of intestinal crypts in young mice may mean that there is a larger population of target cells per crypt that can acquire a tumour-initiating mutation prior to 14 days of age. This means that when the treatment diets were given at weaning (21d) to ApcMin/+ mice the tissue will already be in a pre-neoplastic state such that any post-initiation carcinogenic effect of corn oil reported in the literature should be easily observable in the experiment. Wu et al.(Wu, Iwakiri et al. 2004) have found that corn oil did have a carcinogenic effect in an animal model of colorectal cancer. They found in their study that a 10% corn oil diet induced an increase in cell proliferation in AOM-treated rats, both in the colonic mucosa and in the tumours. Nevertheless, no similar effect of corn oil occurred in the experiment with the ApcMin/+ mice.

One of the mechanisms by which corn oil may promote carcinogenesis has been demonstrated by Singh et *al.* (Singh, Hamid et al. 1997) using animal models. They have shown that a high fat corn oil diet enhances activities of diverse enzymes, including protein kinases that have been implicated directly or indirectly in colon promotion and progression. These kinases have been also shown to participate in *ras*-mediated growth-promoting signal transduction pathways. In their study corn oil enhanced the expression of *ras-p21*, a guanine nucleotide-binding protein, in AOM-induced mice. This protein is a product of *ras* genes that function in the regulation of cell proliferation. This mechanism was observed in chemically induced colon cancer of mice, but perhaps corn oil might not have the same mechanistic effect in the ApcMin/+ mouse model, as this model has a different genetic modification regarding its phenotype and tumour formation.

Furthermore, in the current study, ApcMin/+ mice fed the palm oil diet had a 45% higher level of cell proliferation in intestinal distal tissues and 60% higher in the colon, compared to wild-type mice from the same dietary group, which might be expected to lead to increased tumour number. On the contrary, Boateng et al. (Boateng, Verghese et al. 2006) showed that palm oil decreased the incidence of AOM induced aberrant crypt foci (ACF) compared to soybean oil and may therefore have a beneficial effect in reducing the incidence of colon cancer. While, in the present experiment, animals fed the corn oil and fish oil diets showed no difference between strains in their mitotic index either in the distal small intestine or in the colon. However, cell proliferation rates in the colon of ApcMin/+ mice fed the corn oil and the fish oil diet, was significant inhibited by -1.6-fold and -1.7-fold respectively, compared to ApcMin/+ fed the palm oil. It should be remembered that the ApcMin/+ animal model develops its tumours mainly in the small intestine, and not in the colon. As a result, a decrease in the level of cell proliferation in a tissue where there is almost no tumour development in progress is unlikely to be relevant.

Another mechanism that is important to mention is the Wnt/β-catenin pathway, already described in section 1.1.2.2, which also plays a critical role in the development of the gastrointestinal tract, especially in relation to murine models of cancers with Apc germline mutations. Central to this pathway is a multiprotein scaffold consisting of adenomatous polyposis coli (APC), which when malfunctioning allows for the accumulation of β-catenin in the cytoplasm. When β-catenin enters the nucleus it triggers the cell cycle. To further enhance the oncogenic potential, nuclear β-catenin increases the expression of protein that promote cellular proliferation and resistance to apoptosis (Salama and Platell 2009). Nonetheless, dietary fat seems to influence Wnt/β-catenin pathways (Chang, Chapkin et al. 1998; Fujise, Iwakiri et al. 2007). Vanamala et al. (Vanamala, Glagolenko et al. 2008)

found that diets containing both fish oil and pectin suppressed levels of peroxisome proliferator-activated receptor γ (PPARγ), and this was associated with a downregulation of Wtn/β-catenin in azoxymethane (AOM)-induced CRC in mice. While Jansson et al. (Jansson, Are et al. 2005) showed that ApcMin/+ mice had increased levels of PPARγ in the polyps, showing that Wnt/β-catenin signalling may play a role in the regulation of PPARγ function. Although fish oil didn't have an extraordinary effect on cell proliferation inhibition in the present study, it did have a positive effect on the overall tumorigenesis in ApcMin/+ mice, which will be discussed further on.

Apoptosis plays an important role in the elimination of damaged cells. There is increasing evidence to support the hypothesis that failure of apoptosis may be an important factor in the evolution of colorectal cancer. Apoptosis provides a route for elimination of stem cells carrying potentially procarcinogenic mutations (Latham, Lund et al. 2001). Different potential mechanisms have been shown to modulate the dysregulation of apoptosis, and considerable controversy exists as to whether the frequency of apoptosis increases or decreases during the adenoma-carcinoma sequence. Although the apoptosis mechanisms are still unclear, many studies have demonstrated that dietary components also affect the modulation of colonic cytokinetics, in which fat and fiber seem to be the most significant dietary components affecting the intestinal mucosa (Bartsch, Nair et al. 1999). Specially, n-3 polyunsaturated fatty acids (PUFAs) enhance colonic apoptosis, and are considered to be protective against colon cancer in various animal and cell experimental studies (Latham, Lund et al. 1999; Chen and Istfan 2000; Latham, Lund et al. 2001; Cheng, Ogawa et al. 2003; Hofmanova, Vaculova et al. 2005; Engelbrecht, Toit-Kohn et al. 2008; Toit-Kohn, Louw et al. 2009).

In the present study, ApcMin/+ animals fed both the corn oil and fish oil diets had increased numbers of apoptotic cells in the distal small intestine, however the differences observed were not significant when compared to ApcMin/+ mice fed the palm oil diet. Even though the increase in apoptotic cells was insignificant, it shows an interesting pattern. Animals fed the fish oil diet had a 1.8-fold increase in apoptotic cells, but this effect was less prominent when compared to the effect of corn oil diet, which also increased the number of apoptotic cells in the distal small intestine tissue, but by 2.7-fold. This contrasts to previous studies showing a greater pro-apoptotic effect of fish oil than corn oil (Chang, Chapkin et al. 1997; Latham, Lund et al. 1999; Vanamala, Glagolenko et al. 2008; Bommareddy, Zhang et al. 2009).

Latham et al. (Latham, Lund et al. 1999) found a positive effect of fish oil compared to corn oil on increasing the apoptosis not only in DMH-induced rats, but also in rats given a sham injection. Chang et al. (Chang, Chapkin et al. 1997) also found that AOM-induced rats fed with fish oil diet had a greater increase in apoptosis than corn oil, and this was observed at each level of the crypt and also in overall number of apoptotic cells/crypt in the proximal and distal colon. A most recent study, also done in AOM-induced rats, showed that animals fed with fish oil diets for 37 weeks had enhanced apoptosis levels when compared to animals fed the corn oil diet (Vanamala, Glagolenko et al. 2008). It has also been shown in the literature that fish oil is more effective in promoting apoptosis in colon tissue of healthy rats than corn oil (Latham, Lund et al. 2001). In contrast, Bommareddy et al. studied the effect of corn meal, corn oil and flaxseed oil, which is a source of the n-3 PUFA alpha linolenic acid (ALA), on apoptosis cell in the small intestine and colon of ApcMin/+ mice, but no significant difference was found among the all groups (Bommareddy, Zhang et al. 2009). However, ALA has not shown potential benefit in many in vitro assessments. In the present experiment fish oil was used as a source of n-3 PUFA,

but even though no effect was observed on enhancing apoptosis within the ApcMin/+ mice fed different diets. ApcMin/+ fed the fish oil diet had significantly enhanced levels of apoptosis by 3.5-fold when compared to wild-type mice fed the same treatment diet. This enhanced apoptosis was not observed to the same extent in mice fed palm oil, but was also seen in those fed corn oil.

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Some recent epidemiological and clear experimental data have linked a high dietary intake of n-6 polyunsaturated fatty acids (PUFAs), such as linoleic acid, to an increased risks of colon cancer (Bartsch, Nair et al. 1999). Despite this other case-control and prospective cohort studies have failed to show a consistent association between intake of n-6 polyunsaturated fatty acids and colon cancer. Llor et *al*. (Llor, Pons et al. 2003) found that linoleic acid had no effect on inducing apoptosis in HT-29 and Caco-2 cells. Similar results were found by Habermann et *al*. (Habermann, Schon et al. 2010) in LT97 human colon adenoma and HT29 human colon adenocarcinoma, where linoleic acid failed to induce apoptosis in both cell types. Latham et *al*. (Latham, Lund et al. 1999) has also shown in DMH-induced colon cancer in rats that corn oil, rich in n-6 polyunsaturated fatty acids, did not increase apoptosis, and in fact decreased it, in either the distal and mid colon tissue compared to DMH-induced rats fed the fish oil. This is in contrast to the evidence presented in the present study where ApcMin/+ fed the corn oil diet increased the number of apoptotic cells in the distal small intestine per crypts by 32% compared to ApcMin/+ fed the fish oil diet, even though this was a not significant increase. However, apoptosis was significantly increased by 5.8-fold when compared to wild-type mice also fed the corn oil diet.

Wild-type animals fed the palm oil, corn oil or fish oil diet had no difference in the number of apoptotic cells in the distal small intestine in this present study. Which again contradicts

results found in the literature, especially regarding the effect of fish oil in healthy intestinal tissues which I have already mentioned (Latham, Lund et al. 1999; Latham, Lund et al. 2001).

No significant difference was observed in the numbers of apoptotic cells/crypt in the colon tissue of either wild-type and ApcMin/+ animals. Nevertheless, there was an apparent increase in apoptosis in both wild-type and ApcMin/+ mice fed the corn oil and fish oil diet compared to those fed the palm oil. Again these results are in contrast to those described previously in the colon of rats and human experiments treated with fish oil diet. It is has been reported in the literature that n-3 PUFAs have been found not only to increase apoptosis in normal rat mucosa (Calviello, Palozza et al. 1999), but also to have its protective effect against colorectal cancer associated with increased mucosal apoptosis in carcinogen-induced cancer in rats (Chang, Chapkin et al. 1998; Latham, Lund et al. 1999). Regarding epidemiological studies, Anti et al. (Anti, Armuzzi et al. 2001) and Martin et al. (Martin, Connelly et al. 2002) have described a generalized decreased level of mucosal apoptosis throughout the colon in subjects with colorectal adenomas, which may predispose these subjects to the development of polyps. Additionally, n-3 PUFAs have been shown to be protective in humans against colorectal cancer by modulating cell death. Courtney et al. (Courtney, Matthews et al. 2007) found an increase of apoptotic cells in the normal appearing mucosa from patients with colorectal adenomas who received EPA supplementation for 3 months. Furthermore, Cheng et al. (Cheng, Ogawa et al. 2003) also found that low dose of omega-3 fatty acid supplementation significantly increased rates of apoptosis in the normal colonic mucosa of patients with colorectal cancer after 2 years of supplementation.

In contrast, there are also a few studies which did not find positive effects of fish oil on enhancing the number of apoptotic cells in human colon tissue. For example, Pot et al. (Pot, Majsak-Newman et al. 2009) found no effect on apoptosis in patients with colorectal polyps receiving oil-rich fish or lean fish for 6 months. Moreover, Gee at al. (Gee, Watson et al. 1999) found no effect on colorectal cytokinetics in patients with colorectal neoplasms being supplemented with fish oil.

The precise mechanism by which n-3 PUFA triggers apoptosis in intestinal epithelial cells is not yet established. Under *in vitro* conditions, the increased cell death brought about by treatment with EPA is considerably amplified by depletion of cellular glutathione and blocked by antioxidants (Clarke, Lund et al. 1999;Latham, Lund et al. 2001). This suggests that the effect of incorporating n-3 PUFA into the cellular lipid pool is to increase the production of reactive oxygen species, which are known to induce apoptosis in a number of different cell lines (Antunes and Cadenas 2001). A similar mechanism has been proposed to account for the induction of apoptosis in a human adenocarcinoma line by β-carotene (Palozza, Calviello et al. 2001). A role for reactive oxygen species in the induction of colonic epithelial cell apoptosis *in vivo* is suggested by the fact that induction of apoptosis by dietary fish oil in the intact rat colon is also enhanced by depletion of glutathione (Latham, Lund et al. 2001).

An alternative possible mechanism may be via the activation of PPARγ, at least in HT-29 human colon cancer cells. Yang and Frucht (Yang and Frucht 2001) demonstrated that activation of the PPAPγ pathway by a specific ligand (ciglitazone), induced colon cancer cells (HT-29) to undergo apoptosis. However, the receptor activation of PPARγ in the ApcMin/+ mouse enhanced tumorigenesis and increased the number of polyps (Yang and Frucht 2001). Fatty acids are known to bind and activate PPARs (Forman, Tontonoz et al.

1995; Xu, Lambert et al. 1999; Thoennes, Tate et al. 2000), and fish oil is known to be an agonist for PPARγ, and thus may influence apoptosis by the cyclooxygenase (COX) and Wnt/β-catenin pathways. Prostaglandin E2 (PGE2) formed from the n-6 PUFA arachidonic acid via COX, transactivates PPARγ. In addition, β-catenin, a downstream effector of the Wtn/β-catenin pathway, transcriptionally upregulates PPARγ. Interestingly, PGE2 can also upregulate β-catenin levels, which again upregulates PPARγ (He, Chan et al. 1999; Wang, Wang et al. 2004; Castellone, Teramoto et al. 2005).

Reddy (Reddy 2004) has shown evidence that a high fat fish oil diet inhibited the levels of COX and the production of eicosanoids in the colon tumours, suggesting that inhibition of eicosanoid production through the modulation of COX activity may be important for the ability of n-3 PUFAs to inhibit colon tumorigenesis by inducing apoptosis. Moreover, Vanamala et al.(Vanamala, Glagolenko et al. 2008) showed for the first time that suppression of the COX (PGE2) and Wnt/β-catenin pathways by the fish oil diet are associated with a concurrent suppression of the PPARγ. It should be noted that that EPA is also a substrate for COX but PGE3 rather than PGE2 is produced.

No significant differences in crypt length were observed in the present experiment, either in the distal small intestine or colon of either wild-type or ApcMin/+ mice fed different diets, although it was observed that crypt length was higher in ApcMin/+ mice.

4.2.2. Inflammation

4.2.2.1. Small Intestine

In the small intestine twenty cytokines (IFNγ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-15, IL-17, IP-10, KC, GCM.SF, TNFα, IL-1β, MCP-1, MIG, MIP-1α, and VEGF) were measured in the tissue from the distal position after ten weeks of the dietary treatment. Animals (wild-type and ApcMin/+ combined) fed the corn oil diet had only six cytokines (30%) significantly changed, IL-2, IL-5, IL-15, KC, TNFα and VEGF, which were reduced compared to animals fed the palm oil diet. Analysing the levels of cytokines by strain from animals fed the corn oil diet, it was observed that out of 20 only 5 cytokines were significantly different, 3 cytokines were lower (IL-12p40, IL-12p70, IP-10) and 2 cytokines (KC and MIG) were higher, both changes being in the ApcMin/+ mice compared to wild-type. Corn oil is rich in linoleic acid, which is converted to arachidonic acid and can be metabolized by cyclooxygenases and lipoxygenases to eiconsanoids, such as the proinflammatory and procarcinogenic PGE2 (Kawamori, Uchiya et al. 2003). Erdelyi et al. (Erdelyi, Levenkova et al. 2009) fed C57Bl/6J mice (model for sporadic colon cancer) a 20% corn oil diet for 3 and 6 months and analysed pro and anti-inflammatory protein expression profiling in the mid colon. They found that after animals had been fed the diet for 3 months there was increased expression of IgA, C-reactive protein, fibrinogen, MCP-5 and MIP-1γ, when compared to the control diet (5% corn oil). However, those changes at 3 months were no longer apparent at 6 months. Mehl et al. (Mehl, Davis et al. 2005) have demonstrated that ApcMin/+ mice have increased levels of IL-6 which could be reduced by exercise training. However, in the present study ApcMin/+ mice fed corn oil did not have increased levels of IL-6, but KC and MIG when compared to wild-type mice. Song et al. (Song, Ito et al. 1999) have studied intestinal inflammation in IL-10 knock-out mice and found that KC production may provide a reliable marker to objectively evaluate the development of inflammation. In humans with inflammatory bowel disease, one of the characteristic lesions is the presence of crypt abscesses, accumulations of neutrophils within the luminal space of the intestinal epithelial crypt.

While in humans IL-8 is responsible for the accumulation of neutrophils, KC is a chemokine in mice that is similarly capable of recruiting and activating murine neutrophils (Lira, Zalamea et al. 1994). Besides, MIG (monokine induced by IFN-γ) is a potent chemoattractant for tumour-infiltrating lymphocytes and it functions as an angiostatic agent in the tumour vasculature, and it has been used in chemokine gene therapy in order to prolong life span of mice with colon carcinoma by avoiding tumour development (Ruehlmann, Xiang et al. 2001). Thus, the change of the pro-inflammatory KC and the anti-tumour development chemokine MIG in ApcMin/+ mice fed the corn oil diet were proportional (200% increased compared to the wild-type mice). Not only MIG, which is considered to be beneficial on cancer prevention were significantly changed in these animals, but also IP-10, which is an anti-inflammatory cytokine. However IP-10 was decreased in ApcMin/+ mice compared to wild-type. IP-10 functions as a major chemoattractant for activated T cells and natural killer cells, and it has also been involved with anti-tumour activities into tumour sites and antagonizes the vascularizing effects of powerful angiogenic factors (Zipin-Roitman, Meshel et al. 2007). On the other hand, the pro-inflammatory IL-12 which activates and induces proliferation, cytotoxicity and cytokine production of natural killers (Borish and Steinke 2003) was reduced in ApcMin/+ mice fed corn oil.

Animals (wild-type and ApcMin/+ combined) fed the fish oil diet were compared to those fed the palm oil, had 50% of the cytokines significantly different. IFNγ, IL-1α, IL-12p40, IL-12p70, IL-13, IP-10 and MIP-1α were significantly higher, whereas IL-15, IL-10, IL-15 and GM-CSF were found lowered compared to the palm oil group. Fish oil affected changes in the cytokines levels by 83% more than the changes observed by the corn oil treatment diet. Analyzing the cytokines levels in the fish oil group by strain, it showed that ApcMin/+ mice had significantly lower levels of IL-12p40, IP-10, and GM-CSF, but

higher levels of IL-15, KC, IL-1β and MIG. Similar to the changes observed in the corn oil group, fish oil also decreased the levels of IL-12p40 and IP-10, and increased levels of KC and MIG in ApcMin/+ mice compared to wild-type. However, ApcMin/+ mice fed the fish oil also had decreased levels of GM-CSF and increased IL-15, IL-1β.

The cytokine IL-1β which is cleaved by interleukin-1β-converting enzyme has been implicated as a factor in tumour progression via the expression of metastatic and angiogenic genes and growth factors, as well as being associated with a more virulent tumour phenotype (Lewis, Varghese et al. 2006). IL-1 induces expression of metastatic genes such as matrix metalloproteinases and stimulates nearby cells to produce angiogenic proteins and growth factors such as VEGF, IL-6 and TNFα. However, in the present study animals fed the fish oil did not change the levels of VEGF, IL-6 and TNFα, despite the significantly increased level of Il-1β. Reimund et al. (Reimund, Wittersheim et al. 1996) also described an increase of IL-1β in the serum and intestinal mucosa of subjects with chronic inflammatory bowel disease. Meydani et al. (Meydani, Lichtenstein et al. 1993) also studied the cytokine production capacity but in healthy subjects before and after 24 weeks of a low or high n-3 PUFAs diet. They found that ex-vivo production of IL-1β by isolated peripheral blood mononuclear cells fell significantly in the high n-3 PUFA diet, whereas IL-1β was significantly increased in the low n-3 PUFA diet. Based on the findings of these studies it is possible to observe that the levels of IL-1β might be related to disease, in this case colorectal cancer. Lewis et al. (Lewis, Varghese et al. 2006) have reported that solid tumours in which IL-1β has been shown to be up regulated includes colon cancers and melanomas, and patients with IL-1β producing tumors have generally bad prognoses.

IL-15 is a multifunctional cytokine that stimulates T cell proliferation and activates cytokine production. IL-15 is also a potent inhibitor of apoptosis of both lymphocytes and

epithelial cells, as well as possessing a proangiogenic effect (Kuniyasu, Oue et al. 2001). In the present study IL-15 was found high in ApcMin/+ fedh the fish oil compared to the wild-type fed the same diet. Kuniyasu et al. (Kuniyasu, Oue et al. 2001) have investigated the role of IL-15 in the development of colorectal cancer and found that this cytokine was expressed in all colorectal carcinoma cell lines (TCO, Colo320, WiDr, DLD1). Moreover, they also found an increase between 40-80% on cell growth in those colorectal carcinoma cell lines. Overall this study indicates that IL-15 exhibits multiple functions in support of cancer cells: cell growth, cell survival under stress and anticancer drugs, cell invasion, and a proangiogenic effect by inducing VEGF production. Angiolillo et al. (Angiolillo, Kanegane et al. 1997) have administrated IL-15 subcutaneously into nude mice and they found that IL-15 induced a vigorous local angiogenic response. However, in the present study VEGF and TNFα, which are also angiogenic, were not found elevated in the disease animal model used.

Regarding the level of GMC-SF, Mroczko et al. (Mroczko, Lawicki et al. 2003) have investigated the level of this cytokine in colorectal cancer patients and found it to be significantly increased in the serum compared to healthy subjects. Moreover, the higher level of GMC-SF was also found in more advanced tumours. GMC-SF stimulates the survival of monocytes and their maturation into macrophages in vitro. Monocytes have several roles in the immune system including responding to inflammation signals. Terada et al. (Terada, Takizawa et al. 2002) studied the effect of EPA on GMC-SF-induced monocyte survival and macrophages and they found that EPA induced cell death of the monocyte via apoptosis and could be delaying the inflammatory and immune reactions, which are involved in the development of disease, including colorectal cancer. In the present study, fish oil reduced the level of GMC-SF in ApcMin/+ mice compared to wild-type animals. This cytokine drives hematopoietic precursor cells to become mature

granulocytes, macrophages, or dentritic cells. It is also used clinically to accelerate bone marrow recovery and increase the production of white blood cells to facilitate host defense. Moreover, GMC-SF's immunostimulatory properties may be beneficial in cancer therapy because the presence of neoplasia is associated with macrophage dysfunction (Eubank, Roberts et al. 2004). Hill and Redmond (Hill and Redmond 1996) studied the antineoplastic effect of GM-CSF on tumour growth in a murine carcinoma model and they found a significant reduction on tumour growth in tumour bearing mice, and this antineoplastic effect also occurred during the postoperative period. In the present study the level of GM-CSF in tumour bearing mice was reduced for the fish oil group, and this fact might be affecting the macrophages defense against tumours and explain increased tumour number.

In the animal group fed the palm oil diet, which is high in palmitic acid a saturated fat, few changes in the cytokine levels were found between strains. The only two significant changes observed were the reduction of IL-5 in ApcMin/+ mice, and the increase of KC in the ApcMin/+mice compared to the wild-type fed the same diet. IL-5 is a pro-inflammatory cytokine that has been associated with colitis and which is associated with increased risk of colorectal cancer. Makins and Ballinger (Makins and Ballinger 2005) have performed an in vitro study using Caco-2 cells and they found that when the cells were cultured with IL-5 it potentiated IGF-II induced cellular growth by 40%. In general, palm oil did not seem to increase inflammation in the ApcMin/+ mice, apart from the high level of KC, which was consistently observed in the other treatment groups. KC is a chemokine member of the CXC family which is known to play a major role in inflammation, angiogenesis, tumorigenesis and wound healing (Dhawan and Richmond 2002) but also in maintenance of tissue integrity in the presence of normal gut microflora (Rakoff-Nahoum- cell 2004).

Slattery et al. (Slattery, Potter et al. 1997) have demonstrated in their epidemiological study that palmitic acid was the second most common type of fat consumed in the diet, however it was not associated with the incidence of colorectal cancer. Some other studies have also found similar results (Neoptolemos, Husband et al. 1991; Hietanen, Bartsch et al. 1994; Baro, Hermoso et al. 1998). Although an association may have been missed, because the small number of subjects did not allow statistical power to be achieved, the evidence seems consistent with no relationship between palmitic acid and colorectal cancer. On the other hand, there are some studies showing contradictory results, like the one performed by Sundram et al. (Sundram, Khor et al. 1989) where they showed a positive effect of palm oil on chemically induced mammary carcinogenesis by preventing the development of tumours. Clinical and epidemiologic studies have suggested a strong association between chronic infection, inflammation and cancer. However in the present study ApcMin/+ mice fed palm oil did not have increased the inflammatory markers compared to wild-type animals fed the same diet.

When the cytokine levels in the distal small intestine of all wild-type mice were compared by each treatment diet it was found that animals fed the corn oil mostly decreased the significant cytokines (IL-5, IL-10, IL-15, KC, TNFα, VEGF). Only IL-12p40 and IP-10 was increased in the wild-type animals fed corn oil. On the other hand, animals fed fish oil also reduced levels of IL-5, IL-10, IL-15, KC, and TNFα, but significantly increased levels of IL-12p40 and IL-12p70, IP-10, MIP-1α. It seems that fish oil had a greater pro-inflammatory effect on wild-type animals than corn oil. Pischon et al. (Pischon, Hankinson et al. 2003) performed a prospective cohort investigation in healthy volunteers to observe the habitual n-3 and n-6 fatty acids intake and its effect on inflammatory markers. They found a significant association between n-3 fatty acid intake and levels of TNF receptors.

Blok et al. (Blok, Katan et al. 1996) have also investigated the effects of n-3 fatty acids on cytokines in animal studies and they reported 7 studies with an increase and 3 with a decrease in TNF-α. Therefore, results regarding the effects of n-3 fatty acids on cytokines secretion are quite conflicting.

When ApcMin/+ mice were analyzed by diets it was found that the animals fed corn oil had all the significant cytokines decreased (IL-2, IL-12p40, IL-12p60, IL-1β and VEGF) compared to the ApcMin/+ mice fed palm oil. However, animals fed fish oil had increased levels of many pro-inflammatory cytokines IFNγ, IL-12p40, IP-10, MIP-1α. Analysing the cytokines in the ApcMin/+ mice by the different treatment diets, corn oil and fish oil had a different effect on inflammation compared to the ApcMin/+ mice fed the palm oil. While corn oil significantly reduced the levels of cytokines considered to be pro-inflammatory (IL-2, IL-12p40, IL-12p60, IL-1β and VEGF), fish oil significantly increased the levels of IFNγ, IL-12p40, IP-10, MIP-1α and only had a reduction of IL-5 and GMC-SF.

Read et al. (Read, Beale et al. 2007) have performed a study in patients with advanced colorectal cancer to observe the effect of n-3 fatty acids (EPA) and energy dense oral nutrition supplement on cytokine profile in the plasma at baseline, and after 3 and 9 weeks. They found little change in cytokines between the three time points, and only 3 of the 16 recorded cytokines changed significantly (Eotaxin, GM-CSF, RANTES). However they concluded that those cytokines changed are unlikely to be clinically relevant. A more recent study by Pot et al. (Pot, Geelen et al. 2009) in patients with colorectal adenomas and nonactive ulcerative colitis receiving additional fish in their diets for 6 months reported no effect on local markers of inflammation in colon biopsies or faeces of a dose equivalent to 1.2g/day of EPA + DHA.

4.2.2.2. Colon

The levels of pro-inflammatory cytokines in colon tissue of both strains (ApcMin/+ and wild-type combined) were analyzed by the treatment diets, and an increased level of IL-6, TNFα and MCP-1 was observed in animals fed the fish oil compared to animals fed palm oil. However, no significant differences were observed when the cytokines levels were analyzed by strain by each treatment diet. All wild-type and ApcMin/+ mice were also analyzed by diet, and the only significant difference was observed in the ApcMin/+ group in which fish oil has reduced the level of IL-6 compared to the ApcMin/+ fed palm oil. As previously mentioned, tumour development in the ApcMin/+ mice occurs predominantly in the small intestine. The observation that the cytokine prolife in the colon tissue did not differ much between wild-type and ApcMin/+ mice suggests that the changes may have been in response to the tumours rather than related to the underlying genetic defect. This idea is supported by the fact that both normal and tumour tissue was included in the analysis of the small intestine as it was not practicable to separate the two but in the colon the few large tumours were removed before analysis. Analysis of this tumour tissue however did reveal much higher cytokine levels but there was insufficient data to include in the final analysis (data not included).

4.2.2.3. Plasma

The systemic cytokine profile was also analysed in the wild-type and ApcMin/+ mice in present study. When the strains were combined and analysed by treatment diet it was found that out of 20 only 4 cytokines appeared to be significantly changed, but many were at levels below detection limits. Corn oil decreased the level of IL-6, while fish oil increased the level of IL-5 and MCP-1, both compared to the palm oil group. None of the diets

dramatically changed the cytokine profile in the plasma. When data were analysed within each treatment diet by strain the following were observed. ApcMin/+ mice fed palm oil had decreased levels of IL-2 and MCP-1 compared to wild-type fed the same diet. While in the fish oil fed group IL-17 was lower in the ApcMin/+ mice compared to wild-type. Only ApcMin/+ mice fed corn oil had a pro-inflammation effect associated with the ApcMin/+phenotype in which IL-6 and KC were raised, even though MCP-1 and MIG were decreased. Within the wild-type animals fish oil increased level of IL-4 and IL-17, whereas corn oil decreased level of IL-6 and KC. Fish oil also seemed to increase the cytokines level within the ApcMin/+ mice, as IL-5, IL-1β and MCP-1 were significantly changed compared to ApcMin+ mice fed palm oil.

4.2.2.4. General conclusion

In the present study, local and systemic cytokine profile seems to be differently affected by the different treatment diets. The small intestine had the greatest changes in the cytokines profile, which is possibly explained by the tumour development process happening in this region. According to Coussens and Werb (Coussens and Werb 2002) tumour cells produce various cytokines and chemokines that attract leukocytes, and the inflammation component of a developing neoplasm may include a diverse leukocyte population (neutrophils, dendritic cells, macrophages, eosinophils, mast cells and lymphocytes). All these cells are capable of producing an assorted array of cytokines, cytotoxic mediators, interleukins, interferons, etc., which creates a tumour microenvironment that is indispensable participant in the neoplastic process. The different fatty acids tested in the present experiment had different effects on cytokines levels in the small intestine. Corn oil diet changed 28% of the cytokine levels, while fish oil diet changed 50%, and almost no effect of palm oil diet was observed on the level of cytokines between strains. Moreover, the fish oil diet seemed to

increase the pro-inflammatory cytokines both in the healthy animals, as in the disease model. Whereas, the corn oil diet had the opposite effect and reduced the levels of pro-inflammatory cytokines, both in the healthy and disease model.

In the colon tissue, not much effect was observed, apart from fish oil diet increasing the levels of TNF-α and MCP-1, when strain was combined. However, in the ApcMin/+ mice both corn oil and fish oil diet reduced the level the pro-inflammatory cytokine IL-6. In the plasma there was almost no effect on the levels of cytokines either. However among the significant changes, fish oil diet increased levels of pro-inflammatory cytokines both in the ApcMin/+ and wild-type animals.

4.2.3. Tumour Development

The present study demonstrates that a 10 weeks dietary treatment with fish oil, rich in EPA (average intake of 64 mg/mouse/day) and DHA (average intake of 82 mg/animal/day), suppressed the growth of tumours in the intestine of ApcMin/+ mice. Tumour size and tumour number was analysed in the small and large intestine of the animals. In the small intestine, tumour size analysis was done by measuring: the mean size of tumours for each treatment diet, mean size of tumours for each position of the small intestine (proximal, middle, distal), and the mean tumour size in each position of the small intestine by each treatment diet. Tumour size was also summarized across all the small intestinal positions and analysed by the treatment diet. Moreover, total tumour number was analysed by measuring the mean tumour number across all the small intestine, but it was also analysed in respect to each position of the small intestine for each treatment diet. The large intestine was not divided into different positions, so tumour number and tumour size was analysed in the colon as a whole tissue.

Tumour size in the small intestine varied according to the treatment diet. The mean of tumour size across all the small intestinal positions was significantly smaller for animals fed the fish oil diet compared to animals fed the corn oil or palm oil diet. However, when tumour size was summarized across all the small intestinal position and analysed by the treatment diets no significant effect of fish oil or corn oil was observed compared to the palm oil. Although Paulsen et al. (Paulsen, Elvsaas et al. 1997) did not mentioned the effect of fish oil on total tumour burden in ApcMin/+ mice, they have mentioned that animals fed the K85 diet rich in fish oil had a shift in the cumulated frequency distribution curves to the left compared to the control curve, which illustrates inhibited tumour growth. Data supporting the positive effect of fish oil on tumour burden in humans is reported in the West et al. (West, Clark et al. 2010) study. They found that individuals with familial adenomatous polyposis (FAP) receiving EPA (2g/day) for 6 months had a modest significant improvement compared to the placebo group, which had an increased rectal polyp burden.

It was very important in the present study to have analysed the tumour data by each position of the small intestine and the effect of the treatment diet, so significant effects could be observed. When the mean of tumour size was analysed by each position of the small intestine, it was found that the distal position had significantly larger tumours compared to the middle position. Taking the treatment diets into consideration for each position of the small intestine, the following results were found. In the proximal position animals fed the fish oil diet had smaller mean tumour size compared to animals fed the corn oil and the palm oil diet. In the middle position, animals fed the fish oil diet had smaller mean tumour size compared to animals fed the corn oil and palm oil diet. In the distal position, animals fed the fish oil diet also had significantly smaller tumours

compared to animals fed the corn oil or palm oil diet. Some significant results were observed when the tumour size was summarized by each position of the small intestine and analysed by the treatment diets. Animals fed the fish oil diet had a smaller summarized tumour size in the proximal, middle and distal position compared to the corn oil and palm oil group.

Thus, it is clearly consistent in this experiment that animals fed the fish oil diet had reduced mean tumour size in all the positions of the small intestine. This result is in line with the study performed by Paulsen et *al*. (Paulsen, Elvsaas et al. 1997), where they also showed a decreased tumour size in ApcMin/+ mice treated with n-3 PUFA ethyl ester enriched fish oil K85 (54.4% of EPA and 30.3% of DHA). They reported that the suppressing effect of enriched fish oil K85 was even more pronounced when only medium size tumours (>1mm) were scored. Tumour size was also reduced in a study performed by Oshima et *al*. (Oshima, Takahashi et al. 1995), in which they fed Apc^{716} knockout mice, a different murine FAP model, with a DHA dietary treatment (3% DHA with an average intake of 78mg/mouse/day). The effect of DHA in that study was more significant in females than in males, which the proportion of larger polyps decreased.

There is also a very recent study using ApcMin/+ mice, which has been performed by Bommreddy et al. (Bommareddy, Zhang et al. 2009). In their study they have used a diet rich in n-3 PUFA, but using flaxseed as the source of n-3 fatty acids. They found that dietary flaxseed (15% flaxseed meal or 15% flaxseed oil) suppressed intestinal polyp formation by about 45% compared with AIN and corn oil diet after 12 weeks treatment. Moreover, they also found that tumour area was significantly smaller (53%) compared to animals fed the AIN and corn oil diet.

Studies performed to analyse the effect of fish oil on the growth of human colon carcinoma xenograft in nude mice have also demonstrated a suppressive effect on tumour growth. Kato et al. (Kato, Hancock et al. 2002) injected nude mice with WiDr colon adenocarcinoma and treated them with a high fat fish oil diet (16% fish oil) for 53 days (7,5 weeks). They found the growth of the tumour significantly suppressed by the fish oil treatment compared with that of tumours in mice fed the corn oil diet. A most recent study from the same group (Kato, Kolenic et al. 2007) was performed using a different human colon carcinoma xenograft (COLO 205). They also found similar results, in which a high fat fish oil diet inhibited the tumour growth by 80%, while a high fat diet rich in DHA resulted in 93% growth inhibition. Bordreau et al. (Boudreau, Sohn et al. 2001) also performed a study treating nude mice with fish oil (18% w/w) for 2 weeks before the human colon cancer cell line (HCT-116) was injected into the mice. After injection the animals continued on the diet for three more weeks. They found that the growth of tumour cell xenografts was significant suppressed by the fish oil treatment compared to animals fed the safflower diet.

Several studies done in chemically induced mice/rats have also demonstrated a positive effect of fish oil on preventing tumour development in the colon (Reddy and Sugie 1988; Latham, Lund et al. 1999; Latham, Lund et al. 2001; Rao, Hirose et al. 2001). One recent example is the study performed by van Beelen et al. (van Beelen, Spenkelink et al. 2009) in which AOM-induced rats were treated with 20% by weight of fish oil diet for 8 weeks. They found that animals given fish oil had only half the total number of aberrant crypt foci (ACF) compared to the animals given the corn oil diet, but also had a significantly lower number of large ACF.

ACF like those described after carcinogen treatment dot not occur spontaneously in the colon of ApcMin/+ mice, and therefore are not analyzed in ApcMin/+ mice (Paulsen, Namork et al. 2000). However Paulsen et al. (Paulsen, Elvsaas et al. 1997; Paulsen, Namork et al. 2000) discovered small flat dysplastic lesions, which they denoted ACFM in contrast to classical elevated ACF, These lesions were not elevated above the surrounding mucosa and their detection by surface examination in whole mount colon preparation was totally dependent on both methylene blue staining and transillumination. The ACFMin exhibited dysplastic crypts were similar to those found in adenomas, and like the adenomas, they responded to chemoprevention with dietary fish oil. In their experiment performed in 1997 (Paulsen, Elvsaas et al. 1997) showed in females that the mean number of ACFMin per animal was reduced by 73%, and the growth of ACFM in was reduced by 60% with 2.5% of n-3 PUFA ethyl ester enriched fish oil K85.

Overall, the results reported in the literature and those from my study with the ApcMin/+ mice, that fish oil reduces the size of the tumour in the small intestine are very consistent. However, regarding the number of tumours, in the present study there was no significant difference when number of tumours was analyzed across all the small intestine.

Nevertheless, when tumours were categorized by size and position in the small intestine, it was possible to observe the differences in tumour number caused by the treatment diets. Tumours were categorized into 3 classes of size (small: $<1mm^2$; medium: 1-5 mm^2; large: $>5mm^2$) for each position of the small intestine (proximal, middle, and distal). Animals fed the fish oil had a greater number of tumours in all the positions of the small intestine compared to animals fed the palm oil diet, which is in contrast with some studies where there was a reduction of the tumour number for animals fed a fish oil diet (Petrik, McEntee et al. 2000; Whelan and McEntee 2004). However, the majority of tumours found in the

animals fed the fish oil diet were small (< 1mm^2). This result together with the fact that mean tumour size was consistently smaller in all the positions of the small intestine for the fish oil group, suggests that fish oil may be having an important effect on delaying or avoiding the promotion of the tumours. A similar pattern was observed by Paulsen et al. (Paulsen, Elvsaas et al. 1997) when they found a growth retarding effect of the tumours from animals treated with K85 rich fish oil diet. Oshima et al. (Oshima, Takahashi et al. 1995) also found that Apc716 mice fed the diet rich in DHA also had the polyp size shifted toward the smaller side. So, it is convincing that fish oil does have an effect on suppressing the growth of intestinal polyps in ApcMin/+ mice.

Paulsen et al. (Paulsen, Elvsaas et al. 1997) have performed a study giving fish oil to neonatal ApcMin/+ mice during the initiation of tumour development. They have demonstrated an effect of fish oil on suppressing the total number of monocryptal adenomas (<0.1mm in diameter) in the small intestine. In the present experiment, animals received the treatment diet after weaning (3-4 weeks after birth), by which time tumours may already be present. However, the timing mimics that of clinical intervention trials, when dietary interventions are often given to adults that already bear Min polyps. Nevertheless, treating animals at initiation or at post-initiation stage, fish oil in both cases seems to be beneficial against colorectal cancer.

On the other hand, animals fed the corn oil diet did not differ from the palm oil group regarding the number of small tumours in the proximal and middle position of the small intestine. In the distal position the corn oil group had a significantly higher number of small tumours compared to the palm oil group, and this result was similar to the effect of the fish oil group. However no difference was found between corn oil and fish oil. Whelan et al. (Whelan and McEntee 2004) did not classify different size of tumours in their

experiment but they did find that the mean size of tumour was significantly bigger for ApcMin/+ mice fed the high omega-6 diet compared to fish oil diet.

When the medium size of tumours from the small intestine were analysed, it was found that in the proximal position only the corn oil group had a significant effect on reducing the number of medium size tumours by 27% compared to the palm oil group. In the middle position there was no difference, but in the distal position only animals fed the fish oil diet had fewer tumours (33% less) compared to palm oil group. The number of large tumours in the small intestine was the least frequently observed. However there was a significant effect in the proximal position, where the corn oil group had the number of large tumours reduced by 63% compared to the palm oil group, while fish oil led to a 77% reduction.

Looking at the tumour number in the different positions of the small intestine, it was found that the distal position was the part of the tissue with the highest number of total tumours in the palm oil and corn oil group, and this fact was also observed by others authors (Paulsen, Elvsaas et al. 1997; Sang, Ju et al. 2006; van Beelen, Spenkelink et al. 2009).

Furthermore, there was no significant difference in the average total number of tumours in the small intestine between diets. However, the results show an interesting pattern of effect. Fish oil seemed to increase the number of small tumours in the first 2/3 of the intestine by having increased the total number of tumours by 28% in the proximal, 40% in the middle, while only 4% increase was observed in the distal position of the small intestine. This observation may be related to its metabolism and its bioavailability, which could be an explanation for the consistent effects observed. In the present study, fish oil consistently increased the total number of tumours in all the position of the small intestine. There was also a consistent effect of fish oil on being associated with smaller mean size of

tumours. Also, fish oil led to a consistently greater number of small tumours (<1mm2) in each of the position in the small intestine.

Patten et al. (Patten, Augustin et al. 2009) performed a very extensive experiment on fish oil digestion and bioavailability in Sprague-Dawley rats, and they found significant levels of plasma EPA and DHA at 1.5h in rats gavaged with fish oil that was evident for up to 6h. The total n-3 PUFA plasma level was significantly higher at 2h and was maintained until 6h. Moreover, significant amounts of EPA and DHA were recovered from fish oil fed animals up to 6h in the small intestine, while small amounts were recovered in the cecum. Their experiment was done in fasted rats, but they speculated that if rats were not in the fasted state during gavage, it was likely that fish oil would have been absorbed completely in the small intestine before reaching the cecum.

Based on the findings described above, it could be speculated that fish oil diet given to ApcMin/+ mice has a quite fast metabolism and is well absorbed and bioavailable across all the small intestine, especially at the beginning of the small intestine. This fact could explain the higher activity of the fish oil in the first 2/3 of the intestine. And this availability of the fish oil in these animals could be having an effect on preventing promotion and/or progression of the tumours by a very specific mechanism.

The ApcMin/+ mouse as a model of colorectal cancer is limited as although it has many tumours in the small intestine it only has a few tumours in the colon. By contrast, human tumours are rarely found in the small intestine but frequently in the colon. The reason for this discrepancy is unclear, but it may be related to differences in key enzymes between the small and large bowel of ApcMin/+ mice. Phospholipases A-2 and cyclooxygenase-2 are up regulated in colonic tumours of humans and rats, and in small intestinal tumour of

ApcMin/+ mice. The resulting increase in PGE2 level would promote cancer growth, and this up-regulation is not seen in the colon of ApcMin/+. These considerations would then suggest that the small intestine of ApcMin/+ mice is a reasonable model of human colon and better than the ApcMin/+ mouse colon. (Corpet and Pierre 2003). However, these authors ignore the role of intestinal bacteria which may play a key role in carcinogenesis and are several fold more numerous in the colon than in the small intestine.

In the present study, the incidence of tumours in the colon was 98% less than the incidence of tumours observed in the small intestine. Because of the very low number of tumours present in the colon, statistical analysis on number of tumour by tumour size was not possible. However, it was possible to observe that fish oil increased the absolute number of all sizes of tumours compared to palm oil. In contrast, the total tumour size in the colon was significantly smaller in animals fed the fish oil compared to corn oil diet, but not to the palm oil group. The same pattern was observed in the small intestine, where fish oil also increased the number of tumours, but reduced the tumour size. The effect of fish oil in the colon was not significant however it shows the effect on preventing promotion of the tumours in the large intestine. This is a similar effect of fish oil to that observed in the small intestine.

Paulsen et *al*. (Paulsen, Elvsaas et al. 1997) were also unable to statistically prove the observed tendency in reduced incidence, formation and growth of tumours in the colon of ApcMin/+ mice fed dietary fish oil enriched in EPA and DHA. Despite the low number of tumours in the colon, Bommareddy et *al*, (Bommareddy, Zhang et al. 2009) were able to show a significantly reduced number and size of tumour in colon of ApcMin/+ mice fed flaxseed oil (rich in the n-3 PUFA ALA) compared to corn oil. However, Petrik et *al*. (Petrik, McEntee et al. 2000) tested different n-3 PUFA (ALA, SDA, EPA, DHA) in

ApcMin/+ mice, and they found that stearidonic acid (SDA) was the only n-3 PUFA associated with significantly fewer tumour in the colon, although EPA was effective in the small intestine.

Animal models of colorectal cancer have provided convincing evidence that a western-style diet high in saturated and animal fats and n-6 PUFAs promotes colorectal carcinogenesis, whereas diets rich in n-3 PUFAs would be preventive. Moreover, the present experiment also supports the benefits of the consumption of a diet rich in n-3 PUFAs. Several studies performed in humans have also found positive effect of n-3 PUFAs on preventing colorectal cancer (Anti, Marra et al. 1992; Tiemersma, Kampman et al. 2002; Busstra, Siezen et al. 2003; Norat, Bingham et al. 2005; Courtney, Matthews et al. 2007; Geelen, Schouten et al. 2007; Hall, Chavarro et al. 2008). However, studies with contradictory results also can be found (Kobayashi, Tsubono et al. 2004; Lin, Zhang et al. 2004; Pot, Majsak-Newman et al. 2009). According to the present scenario, more research has to be carried out on the effect of fish oil and colorectal cancer and at what doses, as well as finding out the precise mechanism of its effect.

4.2.4. General conclusion of the effects of fatty acids

Increasing the consumption of fish or fish oil in the diet has been suggested to prevent colorectal cancer development. However, the evidences of its beneficial effects are still limited. The results of the present study did not show promising effects of fatty acids on colorectal cancer prevention either, especially regarding the n-3 polyunsaturated fatty acids from fish or fish oil. Increased cell proliferation has been considered an early step in colorectal cancer development and it was one of the end points analysed in this study. However, the fish oil diet did not show any beneficial effect in reducing cell proliferation

in the gut mucosa, either in the colorectal cancer disease animal model or in the wild-type animals, as was expected. Nevertheless, corn oil diet seemed to increase cell proliferation in the wild-type animals. The number of apoptotic cells was also measured in the present study because of its important role in the elimination of damaged cells. However, no effect of any fatty acids was found in the gut mucosa of the animals.

Local and systemic cytokine profile was also performed in the experiment, as inflammation is regarded as an important factor in the development of cancer. Most changes in the cytokine profile were found in the small intestinal tissue, and the reason for that is believed to be in response to the presence of the intense tumour development in the tissue. In general, not only in the tissue of the small intestine from the disease animals, but also from the wild-type animals, the fish oil diet significantly increased mainly pro-inflammatory cytokines. Whereas, animals fed the corn oil diet showed an opposite effect by decreasing the levels of pro-inflammatory cytokines, both in the ApcMin/+ mice and wild-type animals. In the colon tissue, there was no effect of fatty acids on the cytokine profile, apart from the unique reduction of IL-6 caused by the fish oil diet in the ApcMin/+ mice. In the plasma, the number of cytokines significantly changed was 45% less compared to the cytokine changes observed in the small intestine of the disease model. These changes were related to an increase in pro-inflammatory cytokines in the ApcMin/+ mice fed the fish oil diet. Overall, it seems that the fish oil diet in the present study promoted or allowed the increase of pro-inflammatory cytokines in both animal strains.

The result regarding tumour development was the only endpoint analysed that fish oil diet seemed to have a slightly positive effect. In general, animals fed the fish oil diet had the mean size of tumour smaller in all the positions of the small intestine compared to the control diet. However, when the total tumour area was summarized across the small

intestine and analysed by the different treatment diets, no significant results were observed. Similar results were also previously found (Paulsen, Elvsaas et al. 1997). Regarding tumour number, none of the treatment diets reduced the total tumour number in the small intestine. On the contrary, fish oil diet consistently increased the number of small tumours in all the intestinal positions.

All in all, the effects of fish oil in the present experiment were not promising in respect to any protective effects against the development of colorectal cancer in the ApcMin/+ mouse model.

5. PHYTOCHEMICAL STUDY

The results and discussion of the animal intervention using ApcMin/+ mouse model to study the effects of phytochemicals on the prevention of colorectal cancer development are presented in this chapter. A high-fat diet (25% energy as fat) enriched with 10% of a phytochemical mix containing coffee, cocoa, turmeric, thyme, walnuts, and red berries was compared to high-fat palm oil diet (25% energy as fat) control diet. Wild-type animals (n=16) and ApcMin/+ animals (n=16) were exposed to the treatment diet for 10 weeks. The endpoints evaluated were body and organ weights, mitosis and apoptosis, local and systemic inflammation, as well as tumour development.

5.1. RESULTS

5.1.1. Effects on body and organs weight

The effect of sex, genotype and diet on age, body weight and organs are summarized in Table 5.1 and Table 5.2. Females had a smaller body weight when compared to the males (12.83 g ± 4.32 g vs. 15.95 ± 4.48 g, p=<0.05). However, they had a significantly greater stomach weight (6.42 ± 1.55 x10-3 g vs. 4.98 ± 0.95 x10-3 g, p=<0.05) and spleen (8.95 ± 6.90 x10-3 g vs. 5.51 ± 3.37 x10-3 g). Genotype also had a significant effect on the animals, such that wild-type mice were bigger than the ApcMin/+ mice at the end of the 10 weeks dietary treatment. However, ApcMin/+ had a significantly heavier liver, heart, and spleen. Data analysis revealed an apparent interaction between genotype and diet on age (p=0.01), that might also have contributed to effects on final body weight (p<0.01) and kidney weight (p=0.01) despite initial weights being similar. The dietary treatment was

also significant among genotypes. Mice fed the phytochemical diet were younger and larger than the animals fed the palm oil. Moreover, the phytochemical treatment apparently led to an increase in stomach, caecum, and liver size, while the heart was larger in the mice fed the control diet.

Variables	Sex		Genotype	
	Male	**Female**	**ApcMin/+**	**Wild-type**
Age (days)	34.16 ± 6.24 [a]	32.15 ± 8,03 [a]	32.23 ± 5.84 [a]	33.97 ± 8.28 [a]
Initial body weight (g)	**15.95 ± 4.48** [a]	**12.83 ± 4.32** [b]	13.84 ± 4.38 [a]	14.84 ± 4.88 [a]
Final body weight (g)	**26.72 ± 2.29** [a]	**20.70 ± 2.45** [b]	**22.56 ±3.50**[a]	**24.66 ± 3.92**[b]
Change in body weight (g)	**10.76 ± 4.50** [a]	**7.86 ± 4.23** [b]	8.72 ± 4.43 [a]	9.81 ± 4.70 [a]
Stomach (g)	**4.98 ± 0.95 x10^{-3}** [a]	**6.42 ± 1.55 x10^{-3}** [b]	5.84 ± 1.44 x10^{-3} [a]	5.60 ± 1.51 x10^{-3} [a]
Caecum (g)	3.09 ± 1.10 x10^{-3} [a]	3.45 ± 1.19 x10^{-3} [a]	3.19 ± 0.91 x10^{-3} [a]	3.34 ± 1.35 x10^{-3} [a]
Liver(g)	4.65 ± 0.61 x10^{-2} [a]	4.67 ± 0.58 x10^{-2} [a]	**4.89 ± 0.54 x10^{-2}** [a]	**4.45 ± 0.57 x10^{-2}** [b]
Heart(g)	5.01± 0.68 x10^{-3} [a]	5.34 ± 1.04 x10^{-3} [a]	**5,47 ± 0.99 x10^{-3}** [a]	**4.91 ± 0.71 x10^{-3}** [b]
Spleen(g)	**5.51 ± 3.37 x10^{-3}** [a]	**8.95 ± 6.90 x10^{-3}** [b]	**9.54 ± 5.32 x10^{-3}** [a]	**5.18 ± 5.26 x10^{-3}** [b]
Kidney(g)	0.012 ± 0.001[a]	0.012 ± 0.0001[a]	0.012 ± 0.001 [a]	0.012 ± 0.0001[a]

Table 5.1 Age, body and organ weight by sex and genotype. Values are mean ± SD. Values within a row were analysed separately by sex and genotype. Values having a different superscript are significantly different by one-way analysis of variance and are indicated using bold letters and different superscripts, $p<0.05$. Male n= 31, Female n=32, ApcMin/+ n= 30, Wild-type n=33.

Variables	Palm oil	Phytochemical	Genotype*Diet
Age (days)	**35.38 ± 7.08** [a]	**30.96 ± 6.77** [b]	0.01
Initial body weight (g)	**12.47 ± 4.46** [a]	**16.32 ± 4.02** [b]	ns
Final body weight (g)	23.42 ± 3.94 [a]	23.91 ± 3.78 [a]	0.001
Change in body weight (g)	**10.95 ± 3.81** [a]	**7.58 ± 4.71** [b]	ns
Stomach (g)	**5.34 ± 1.29 x10^{-3}** [a]	**6.10 ± 1.56 x10^{-3}** [b]	ns
Caecum (g)	**2.50 ± 0.58 x10^{-3}** [a]	**4.07 ± 1.05 x10^{-3}** [b]	ns
Liver (g)	**4.37 ± 0,47 x10^{-2}** [a]	**4.96 ± 0.56 x10^{-2}** [b]	ns
Heart (g)	**5.45 ± 0.96 x10^{-3}** [a]	**4.89 ± 0.73 x10^{-3}** [b]	ns
Spleen (g)	7.36 ± 5.92 x10^{-3} [a]	7.15 ± 5.52 x10^{-3} [a]	ns
Kidney (g)	0.012 ± 0.000 [a]	0.012 ± 0.001 [a]	0.01

Table 5.2 Differences in age, body and organ weight of mice on the phytochemical-rich and control diet. Values are mean ± SD, Control n= 32; Phytochemical mix n=31. ApcMin/+ and wild-type mice combined. Significant effects of Genotype*Diet were identified by two-way analysis of variance $p<0.05$. ns=not significant

5.1.2. Effects on mitosis, apoptosis and crypt length

ApcMin/+ mice had higher cell proliferation in the distal small intestine compared to the wild-type in both treatment diets, (control diet: ApcMin/+ 2.99 ± 0.28 mitotic cell/crypt vs. wild-type 2.06 ± 0.17 mitotic cell/crypt, p=0.00; phytochemical diet: ApcMin/+ 2.72 ± 0.24 mitotic cell/crypt vs. wild-type 2.10 ± 0.19 mitotic cell/crypt, p=0.05), Figure 5.1(a). ApcMin/+ also had a greater cell proliferation rate in the colon, as compared to the wild-type, but this was only significant for the palm oil control diet (ApcMin/+ 1.58 ± 0.15 mitotic cell/crypt vs. wild-type 0.98 ± 0.19 mitotic cell/crypt, p=0.01), Figure 5.1 (b). There was no significant difference in the colon tissue for the phytochemical group (ApcMin/+ 1.31 ± 0.20 mitotic cell/crypt vs. wild-type 1.00 ± 0.14 mitotic cell/crypt).

The effect of apoptosis on wild-type and ApcMin/+ are summarized in Figure 5.2. There was no significant difference in apoptosis in the distal tissue of animals fed with palm oil (ApcMin/+ 0.12 ± 0.03 apoptotic cell/crypt vs. wild-type 0.03 ± 0.01 apoptotic cell/crypt). However, ApcMin/+ mice fed with the phytochemical-rich diet had a significantly higher rate of apoptosis compared to wild-type (2.72 ± 0.24 apoptotic cell/crypt vs. 2.10 ± 0.19 apoptotic cell/crypt). No significant difference was observed in the colon between the treatment diets or genotypes. There was no significant effect on crypt length at the distal or colon tissue among diets or genotypes (Figure 5.3).

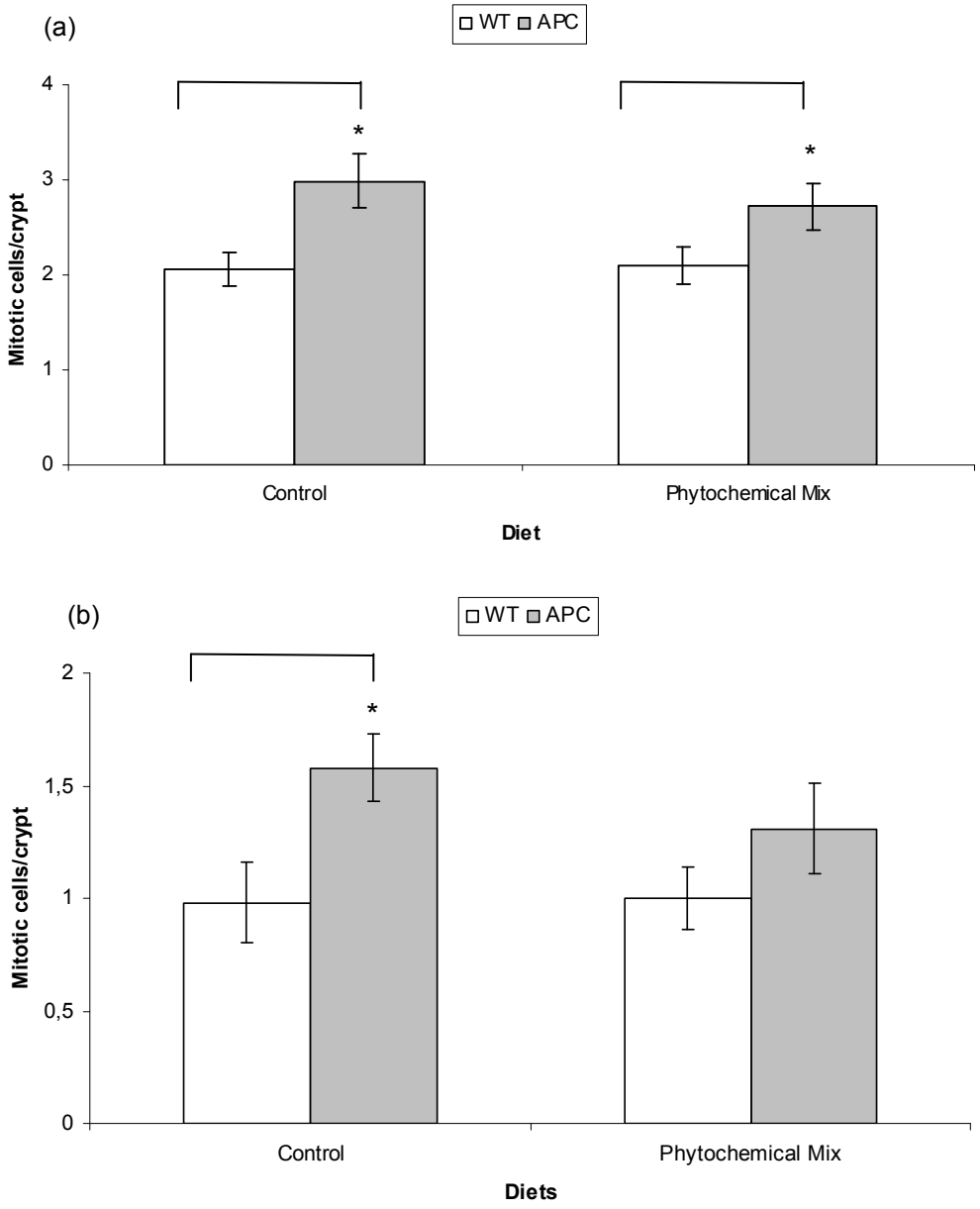

Figure 5.1 Effects of phytochemicals on cell proliferation in the distal (a), and in the colon (b) of wild-type and ApcMin/+ mice. The values represent the mean (n=32) and standard error of the mean. The values not sharing the same symbol are significantly different ($p<0.05$) by two-way ANOVA general linear model. Data analysed within the same diet by different genotype and is significantly different is indicated by *. No significant effect on same genotype by different diet. Ten crypts were analysed per animal.

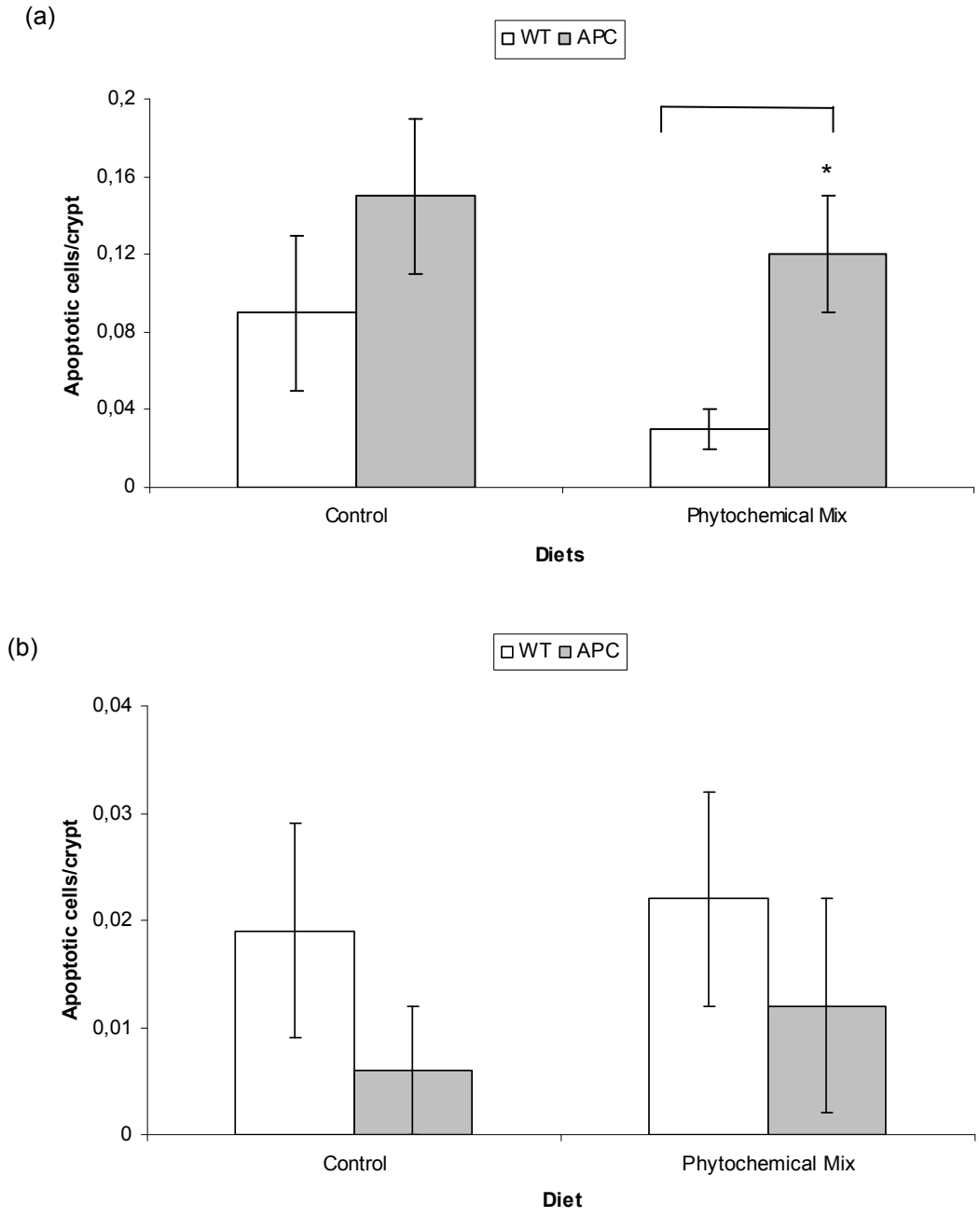

Figure 5.2 Effects of phytochemicals on apoptosis in the distal small intestine (a), and in the colon (b) of wild-type and ApcMin/+ mice. The values represent the mean (n=32) and standard error of the mean. The values not sharing the same symbol are significantly different ($p<0.05$) by two-way ANOVA general linear model. Data analyzed within the same diet by different genotype (*).No significant effect on same genotype by different diet. Ten crypts were analysed per animal.

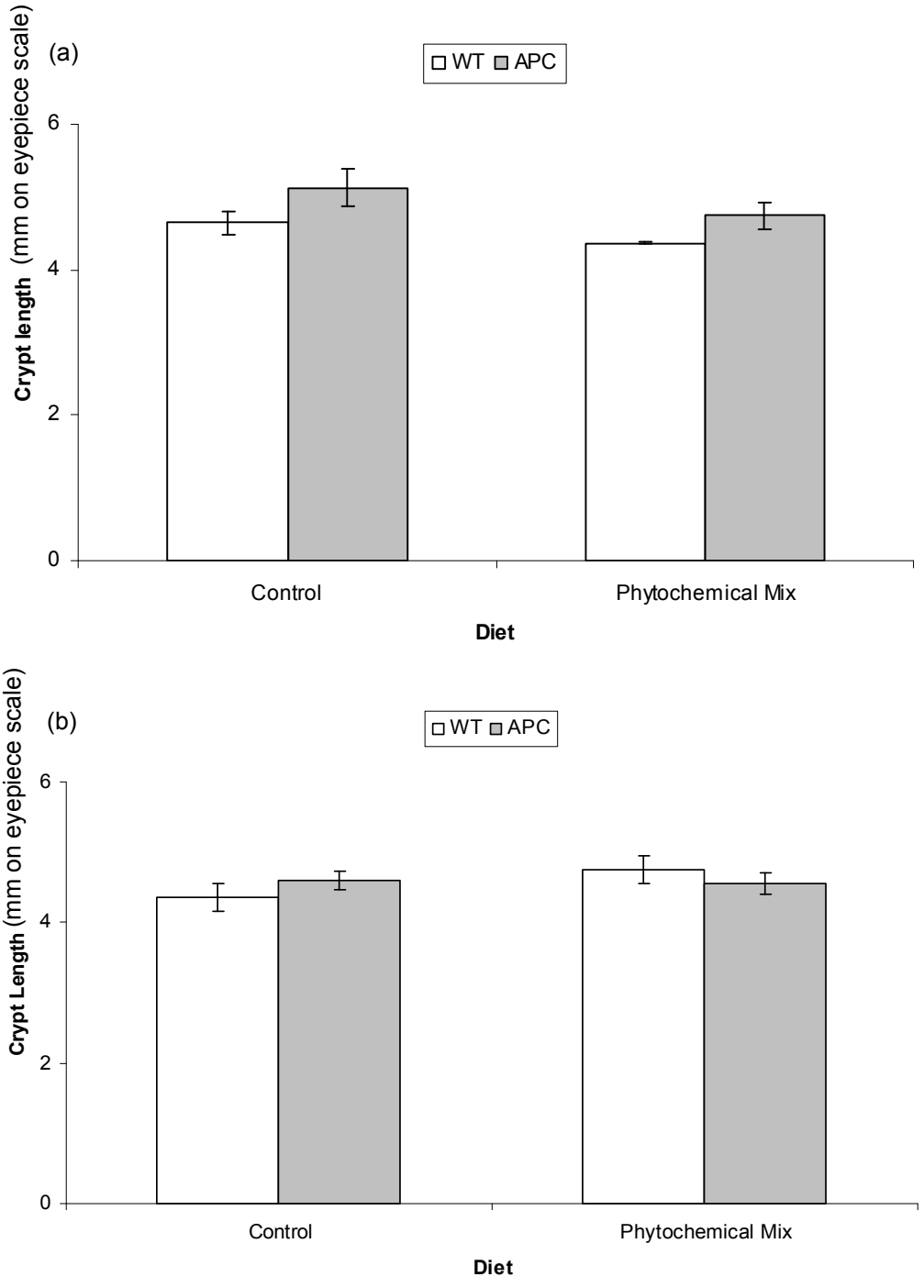

Figure 5.3 Effects of phytochemicals on crypt length in the distal small intestine (a), and in the colon (b) of wild-type and ApcMin/+ mice. The values represent the mean in mm on eyepiece scale (n=32) and standard error of the mean. Each unit equals 40μm. No significant effect was observed by two-way ANOVA general linear model. Ten crypts were analysed per animal.

5.1.3. Effects on local and systemic inflammation

5.1.3.1. Distal small intestine tissue

Of the total number of cytokines, 52% were significantly affected by diet, and the results are summarized in Table 5.3. The phytochemical diet increased the levels of seven cytokines (IFN-γ, IL-1α, IL-2, IL-12p40, IP-10, IL-1β, and MIP-1α) compared to the control palm oil ($p \leq 0.05$). Whereas, the control group had higher levels of IL-5, IL-10 and IL-1β compared to the phytochemical group.

Table 5.4 summarizes the effects of palm oil and phytochemicals on the distal cytokine levels separated by strain. ApcMin/+ mice fed with the palm oil had higher levels of KC and IL-1α compared with palm oil wild-type animals. ApcMin/+ mice fed phytochemicals had not only higher levels of KC and IL-1α, but also MCP1, IL-6 and IL-1α compared to wild-type mice on the same diet. For the palm oil group, wild-type animals had higher levels of IL-5, while for the phytochemicals group the wild-type animals had higher levels of INF-γ and IL-2.

Wild-type animals fed with the phytochemicals had significantly higher levels of IL-1α, IL-2, and MIP-1α, and lower levels of IL-5, IL-10, KC, and TNF-α compared to the control group ($p \leq 0.05$). As with the wild-type mice fed phytochemicals the ApcMin/+ mice also had significantly higher levels of IL-1α, and MIP-1α.. Moreover, the levels of cytokines were also higher for IP-10 and MCP-1 within the ApcMin/+ fed phytochemicals. The anti-inflammatory cytokine IL-10 was significantly lower in mice fed with the phytochemicals (Table 5.5).

Cytokines	**Palm oil** *Median (95% CI)* *pg/mg protein*	**Phytochemicals** *Median (95% CI)* *pg/mg protein*	p-value
(A) IFN-γ	**0.08 (0.03-0.10)**	**0.16 (0.08-0.19)**	0.03
(P) IL-1α	**0.46 (0.37-0.62)**	**0.95 (0.81-1.24)**	0.001
(P) IL-2	**0.16 (0.11-0.19)**	**0.33 (0.19-0.44)**	0.04
(A) IL-4	0.03 (0.02-0.05)	0.02 (0.01-0.04)	0.29
(P) IL-5	**0.01 (0.004-0.13)**	**0.003 (0.003-0.004)**	0.001
(P) IL-6	0.26 (0.19-0.32)	0.18 (0.09-0.30)	0.54
(A) IL-10	**1.58 (0.60-2.17)**	**0.23 (0.14-0.35)**	0.001
(P) IL-12p40	**0.77 (0.004-1.34)**	**1.23 (0.91-1.63)**	0.03
(P) IL-12p70	1.03 (0.78-1.16)	0.83 (0.64-1.14)	0.32
(P) IL-15	0.48 (0.01-0.92)	0.26 (0.00-1.00)	0.20
(P) IL-17	0.23 (0.16-0.38)	0.18 (0.10-0.28)	0.24
(A) IP-10	**1.59 (0.91-2.57)**	**2.69 (2.31-2.88)**	0.001
(P) KC	0.57 (0.44-0.85)	0.33 (0.23-1.29)	0.39
(P) GMC-SF	0.0039 (0.0029-0.0038)	0.0029 (0.0026-0.032)	0.07
(P) TNF-α	**0.13 (0.12-0.20)**	**0.08 (0.04-0.13)**	0.01
(P) IL-1β	**0.63 (0.44-0.99)**	**0.72 (0.31-0.81)**	0.04
(P) MCP1	0.63 (0.44-0.90)	0.62 (0.47-1.91)	0.28
(A) MIG	26.38 (16.49-38.46)	28.74 (17.37-35.86)	0.77
(P) MIP1α	**0.22 (0.12-0.26)**	**0.40 (0.31-0.57)**	0.001
(P) VEGF	2.59 (2.29-3.67)	2.85 (2.47-3.30)	0.89

Table 5.3 Comparison of the levels of 20 different cytokines (pg/mg protein) n mice fed the control diet (n=28) vs. phytochemicals (n=29) in the distal small intestine tissue of mice after 10-week treatment. (A) Anti-inflammatory cytokine; (P) pro-inflammatory cytokine Values are presented as median and 95% confidence interval. ApcMin/+ and wild-type mice combined. Significant p-value was identified by Kruskal-Wallis non-parametric at $p<0.05$.

	Palm oil			Phytochemical		
	Wild-type *(n=14)* Median (95% CI)	**ApcMin/+** *(n=14)* Median (95% CI)		**Wild-type** *(n=15)* Median (95% CI)	**ApcMin/+** *(n=15)* Median (95% CI)	
Cytokines	*pg/mg protein*	*pg/mg protein*	p-value	*pg/mg protein*	*pg/mg protein*	p-value
(A) IFN-γ	0.06 (0.004-0.35)	0.08 (0.003-0.10)	0.33	**0.19 (0.13-0.22)**	**0.08 (0.003-0.18)**	0.01
(P) IL-1α	0.41 (0.25-0.59)	0.60 (0.31-1.13)	0.09	**0.82 (0.56-1.24)**	**1.11 (0.82-2.05)**	0.04
(P) IL-2	0.15 (0.004-0.19)	0.16 (0.11-0.50)	0.09	**0.39 (0.26-0.68)**	**0.25 (0.003-0.43)**	0.04
(A) IL-4	0.04 (0.01-0.07)	0.03 (0.01-0.06)	0.46	0.03 (0.01-0.05)	0.02 (0.01-0.04)	1.00
(P) IL-5	**0.09 (0.004-0.25)**	**0.003 (0.003-0.21)**	0.001	0.0034 (0.0031-0.0045)	0.0036 (0.0031-0.0043)	0.97
(P) IL-6	0.26 (0.08-0.42)	0.26 (0.16-0.47)	0.89	**0.11 (0.08-0.28)**	**0.27 (0.10-0.79)**	0.05
(A) IL-10	1.97 (0.60-2.66)	0.89 (0.35-2.17)	0.60	0.17 (0.08-0.30)	0.23 (0.15-0.53)	0.14
(P) IL-12p40	0.31 (0.003-4.44)	1.10 (0.003-1.43)	0.52	1.23 (0.73-1.66)	1.32 (0.77-1.79)	0.48
(P) IL-12p70	1.01 (0.54-1.34)	1.11 (0.63-1.56)	0.52	0.78 (0.43-1.14)	0.90 (0.61-1.97)	0.29
(P) IL-15	0.43 (0.004-1.28)	0.59 (0.003-1.06)	0.89	0.34 (0.002-1.11)	0.003 (0.002-1.40)	0.83
(P) IL-17	0.21 (0.14-0.48)	0.25 (0.12-0.52)	0.67	0.18 (0.06-0.27)	0.21 (0.09-0.72)	0.32
(A) IP-10	0.91 (0.57-2.89)	1.96 (1.02-2.81)	0.12	2.57 (1.88-2.86)	2.77 (2.29-3.25)	0.15
(P) KC	**0.47 (0.25-0.87)**	**0.71 (0.49-1.24)**	0.03	**0.23 (0.11-0.33)**	**1.46 (0.30-2.96)**	0.001
(P) GMC-SF	0.0035 (0.0029-0.0045)	0.0030 (0.0027-0.0038)	0.29	0.0030 (0.0026-0.0035)	0.0028 (0.0025-0.0034)	0.65
(P) TNF-α	0.17 (0.11-0.26)	0.13 (0.09-0.24)	0.29	0.06 (0.01-0.10)	0.09 (0.04-0.29)	0.32
(P) IL-1β	**0.48 (0.25-0.80)**	**1.03 (0.44-1.27)**	0.01	**0.20 (0.02-0.54)**	**0.79 (0.72-1.39)**	0.001
(P) MCP1	0.50 (0.32-0.79)	0.79 (0.44-1.01)	0.15	**0.49 (0.38-0.55)**	**2.02 (1.03-3.08)**	0.001
(A) MIG	20.17 (1.67-48.14)	33.03 (18.67-53.58)	0.21	28.74 (13.08-35.86)	28.47 (14.26-60.00)	0.60
(P) MIP1α	0.21 (0.004-0.27)	0.22 (0.08-0.40)	0.43	0.33 (0.22-0.81)	0.54 (0.26-0.79)	0.28
(P) VEGF	2.50 (1.31-3.99)	2.95 (2.29-6.16)	0.08	3.08 (2.46-3.46)	2.71 (1.57-3.37)	0.46

Table 5.4 Effect of phytochemicals on cytokine level (pg/mg protein) in the distal small intestine tissue of wild-type and ApcMin/+ mice. (A) Anti-inflammatory cytokine; (P) pro-inflammatory cytokine Values are presented as median and 95% confidence interval. Significant p-Value ($p<0.05$) was identified by Kruskal-Wallis test.

	Wild-type			ApcMin/+		
	Palm oil *(n=14)*	**Phytochemical** *(n=15)*		**Palm oil** *(n=14)*	**Phytochemical** *(n=14)*	
	Median (95% CI)	*Median (95% CI)*		*Median (95% CI)*	*Median (95% CI)*	
Cytokines	*pg/mg protein*	*pg/mg protein*	p-value	*pg/mg protein*	*pg/mg protein*	p-value
(A) IFN-γ	0.06 (0.00-0.35)	0.19 (0.13-0.22)	0.12	0.08 (0.003-0.10)	0.08 (0.00-0.18)	0.41
(P) IL-1α	**0.41 (0.25-0.59)**	**0.82 (0.56-1.24)**	0.01	**0.60 (0.31-1.13)**	**1.11 (0.82-2.05)**	0.001
(P) IL-2	**0.15 (0.00-0.19)**	**0.39 (0.26-0.68)**	0.001	0.16 (0.11-0.50)	0.25 (0.00-0.43)	0.82
(A) IL-4	0.04 (0.01-0.07)	0.03 (0.01-0.05)	0.48	0.03 (0.01-0.06)	0.02 (0.01-0.04)	0.25
(P) IL-5	**0.09 (0.00-0.25)**	**0.003 (0.003-0.004)**	0.001	0.003 (0.003-0.21)	0.003 (0.003-0.004)	0.54
(P) IL-6	0.26 (0.08-0.42)	0.11 (0.08-0.28)	0.22	0.26 (0.16-0.47)	0.27 (0.10-0.79)	0.52
(A) IL-10	**1.97 (0.60-2.66)**	**0.17 (0.08-0.30)**	0.001	**0.89 (0.35-2.17)**	**0.23 (0.15-0.53)**	0.001
(P) IL-12p40	0.31 (0.00-4.44)	1.23 (0.73-1.66)	0.11	1.10 (0.00-1.43)	1.32 (0.77-1.79)	0.15
(P) IL-12p70	1.01 (0.54-1.34)	0.78 (0.43-1.14)	0.26	1.11 (0.63-1.56)	0.90 (0.61-1.97)	0.89
(P) IL-15	0.43 (0.00-1.28)	0.34 (0.00-1.11)	0.51	0.59 (0.00-1.06)	0.003 (0.002-1.40)	0.33
(P) IL-17	0.21 (0.14-0.48)	0.18 (0.06-0.27)	0.19	0.25 (0.12-0.52)	0.21 (0.09-0.72)	0.82
(A) IP-10	0.91 (0.57-2.89)	2.57 (1.88-2.86)	0.07	**1.96 (1.02-2.81)**	**2.77 (2.29-3.25)**	0.02
(P) KC	**0.47 (0.25-0.87)**	**0.23 (0.11-0.33)**	0.01	0.71 (0.49-1.24)	1.46 (0.30-2.96)	0.07
(P) GMC-SF	0.003 (0.002-0.004)	0.003 (0.002-0.003)	0.18	0.003 (0.002-0.003)	0.002 (0.002-0.003)	0.26
(P) TNF-α	**0.17 (0.11-0.26)**	**0.06 (0.01-0.10)**	0.01	0.13 (0.09-0.24)	0.09 (0.04-0.29)	0.46
(P) IL-1β	0.48 (0.25-0.80)	0.20 (0.02-0.54)	0.11	1.03 (0.44-1.27)	0.79 (0.72-1.39)	0.96
(P) MCP1	0.50 (0.32-0.79)	0.49 (0.38-0.55)	0.54	**0.79 (0.44-1.01)**	**2.02 (1.03-3.08)**	0.01
(A) MIG	20.17 (1.67-48.14)	28.74 (13.08-35.86)	0.54	33.03 (18.67-53.58)	28.47 (14.26-60.00)	0.85
(P) MIP1α	**0.21 (0.00-0.27)**	**0.33 (0.22-0.81)**	0.001	**0.22 (0.08-0.40)**	**0.54 (0.26-0.79)**	0.001
(P) VEGF	2.50 (1.31-3.99)	3.08 (2.46-3.46)	0.29	2.95 (2.29-6.16)	2.71 (1.57-3.37)	0.20

Table 5.5 Effect of strain on cytokine level (pg/mg protein) in the distal small intestine tissue of according to the diet treatment. (A) Anti-inflammatory cytokine; (P) pro-inflammatory cytokine. Values are presented as median and 95% confidence interval. *Significant p-Value ($p<0.05$) was identified by Kruskal-Wallis test. Values having different superscript are significantly different by post-hoc Mann-Whitney test $p<0.05$.

5.1.3.2. Colon – large intestine tissue

There were no effects of diet on the cytokine levels in the large intestinal tissue (Table 5.6). In addition, there were also no significant effects of diet when compared within strain (Table 5.7). The only significant effect observed in the colon is shown in the Table 5.8, in which the level of MCP-1 was 30% higher in ApcMin/+ fed phytochemicals when compared to the ApcMin/+ animals from the control diet (p=0.04). A trend of having a higher level of GMC-SF was also observed for the ApcMin/+ mice fed with phytochemicals (p=0.07).

Cytokines	**Palm oil** *(n=16)* *Median (95% CI)* *pg/mg protein*	**Phytochemical** *(n=18)* *Median (95% CI)* *pg/mg protein*	p-value
(P) GMC-SF	0.02 (0.01-0.09)	0.07 (0.01-0.12)	0.17
(P) IL-1β	0.02 (0.01-0.10)	0.03 (0.01-0.14)	0.21
(P) IL-6	1.00 (0.17-6.13)	1.00 (0.61-5.77)	0.94
(P) TNF-α	0.01 (0.00-0.98)	0.01 (0.00-0.13)	0.39
(P) MCP-1	0.55 (0.43-0.62)	0.62 (0.50-0.90)	0.10

Table 5.6 Comparison of the control palm oil diet (n=16) with a phytochemical enriched diet (n=18) on cytokines levels (pg/mg protein) in the colon in mice after a 10-week treatment. (P) Pro-inflammatory cytokine. Values are presented as median and 95% confidence interval. Male ApcMin/+ and wild-type mice combined. *P-value of Kruskal-Wallis test.

	Palm oil			Phytochemicals		
Cytokines	**Wild-type** *(n=8)* *Median (95% CI)* *pg/mg protein*	**ApcMin/+** *(n=8)* *Median (95% CI)* *pg/mg protein*	p-value	**Wild-type***(n=9)* *Median (95% CI)* *pg/mg protein*	**ApcMin/+** *(n=9)* *Median (95% CI)* *pg/mg protein*	p-value
(P) GMC-SF	0.02 (0.01-0.10)	0.02 (0.01-0.18)	0.75	0.05 (0.01-0.12)	0.08 (0.06-0.14)	0.12
(P) IL-1β	0.02 (0.01-0.27)	0.02 (0.01-0.20)	0.75	0.01 (0.01-0.08)	0.07 (0.01-0.29)	0.15
(P) IL-6	1.00 (0.03-8.63)	1.00 (0.09-11.46)	0.24	1.00 (0.79-5.77)	3.23 (0.21-11.36)	0.96
(P) TNF-α	0.01 (0.00-7.95)	0.01 (0.00-1.18)	0.60	0.01 (0.00-0.10)	0.02 (0.00-1.53)	0.69
(P) MCP-1	0.55 (0.29-1.17)	0.55 (0.39-0.60)	0.60	0.60 (0.35-0.78)	0.72 (0.50-1.18)	0.23

Table 5.7 Effect of diet on cytokine level (pg/mg protein) in the colon tissue of the large intestine according to mouse strain. (P) Pro-inflammatory cytokine. Values are presented as median and 95% confidence interval (CI). Significant p-value ($p<0.05$) was identified by Kruskal-Wallis test.

	Wild-type			ApcMin/+		
Cytokines	**Palm oil** *(n=8)* Median (95% CI) *pg/mg protein*	**Phytochemical** *(n=9)* Median (95% CI) *pg/mg protein*	p-value	**Palm oil** *(n=8)* Median (95% CI) *pg/mg protein*	**Phytochemical** *(n=9)* Median (95% CI) *pg/mg protein*	p-value
(P) GMC-SF	0.02 (0.01-0.10)	0.05 (0.01-0.12)	0.77	0.02 (0.01-0.18)	0.08 (0.06-0.14)	0.07
(P) IL-1β	0.02 (0.01-0.27)	0.01 (0.01-0.08)	0.92	0.02 (0.01-0.20)	0.07 (0.01-0.29)	0.10
(P) IL-6	1.00 (0.03-8.63)	1.00 (0.79-5.77)	0.63	1.00 (0.09-11.46)	3.23 (0.21-11.36)	0.85
(P) TNF-α	0.01 (0.00-7.95)	0.01 (0.00-0.10)	0.92	0.01 (0.00-1.18)	0.02 (0.00-1.53)	0.21
(P) MCP-1	0.55 (0.29-1.17)	0.60 (0.35-0.78)	0.85	**0.55 (0.39-0.60)**	**0.72 (0.50-1.18)**	0.04

Table 5.8 Effect of strain on cytokine level (pg/mg protein) in the colon tissue of the large intestine according to the diet treatment. (P) Pro-inflammatory cytokine. Values are presented as median and 95% confidence interval. Significant p-Value ($p<0.05$) was identified by Kruskal-Wallis test.

5.1.3.3. Plasma

Animals fed with phytochemicals had significantly lower levels of IL-6 and IL-2 compared to the control (Table 5.9). The ApcMin/+ fed palm oil had significantly lower (30%) level of IL-1α compared to the wild-type mice fed the same diet (p=0.02). However, the ApcMin/+ mice fed phytochemicals had a 3 times higher level of IL-6 than the wild-type (p=0.02), Table 5.10. Wild-type animals fed the phytochemical diet had significantly lower levels of IL-1α and IL-6 when compared to ApcMin/+ fed the palm oil diet, whereas ApcMin/+ mice fed the phytochemical diet has significantly higher level of the growth factor VEGF compared to the palm oil group.

Cytokines	**Palm oil** *(n=29)* *Median (95% CI)* *pg/ml*	**Phytochemicals** *(n=8)* *Median (95% CI)* *pg/ml*	p-value
(A) IFN-γ	3.20 (3.07-3.20)	3.20 (3.20-3.20)	0.97
(P) IL-1α	45.96 (3.20-97.19)	3.20 (3.20-33.03)	0.60
(P) IL-2	**3.20 (3.20-15.85)**	**3.20 (3.20-20.77)**	0.02
(A) IL-4	3.20 (1.79-3.20)	3.20 (1.19-3.20)	1.00
(P) IL-5	8.99 (4.58-13.24)	8.85 (5.72-16.23)	0.19
(P) IL-6	**9.89 (3.20-30.00)**	**3.20 (3.20-7.68)**	0.04
(A) IL-10	3.20 (3.20-10.73)	3.36 (3.20-15.00)	0.09
(P) IL-12p40	58.74 (3.75-116.16)	65.96 (16.33-97.57)	0.47
(P) IL-12p70	3.20 (3.20-478.92)	3.20 (3.20-529.82)	0.88
(P) IL-15	57.81 (3.20-211.73)	25.42 (3.20-260.45)	0.13
(P) IL-17	3.20 (3.20-53.09)	3.20 (3.20-54.16)	0.17
(A) IP-10	200.30 (169.59-268.32)	243.27 (178.34-288.01)	0.45
(P) KC	77.99 (3.20-127.49)	42.13 (3.20-77.18)	0.29
(P) GMC-SF	3.20 (3.20-41.24)	3.20 (3.20-45.85)	0.29
(P) TNF-α	3.20 (1.37-3.20)	3.20 (0.91-3.20)	0.71
(P) IL-1β	3.20 (3.20-17.68)	10.61 (3.27-25.40)	0.09
(P) MCP1	14.27 (5.22-41.78)	31.96 (27.92-42.90)	0.30
(A) MIG	144.09 (120.62-311.93)	201.56 (167.16-274.27)	0.48
(P) MIP1α	3.20 (3.20-24.42)	3.20 (3.20-16.29)	0.75
(P) VEGF	3.20 (0.65-3.20)	3.20 (2.82-3.20)	0.06

Table 5.9 Effect of phytochemicals on cytokines levels (pg/ml) in the plasma of mice after 10-week treatment. (A) Anti-inflammatory cytokine; (P) pro-inflammatory cytokine. Values are presented as median and 95% confidence interval. Male ApcMin/+ and wild-type mice combined. Palm oil n=29 and phytochemicals n=28. *P-value of Kruskal-Wallis test significant at $p<0.05$.

Cytokines	Palm oil			Phytochemical		
	Wild-type (*n=13*) *Median (95% CI) pg/ml*	**ApcMin/+** (*n=16*) *Median (95% CI) pg/ml*	p-value	**Wild-type** *(n=16) Median (95% CI) pg/ml*	**ApcMin/+** *(n=12) Median (95% CI) pg/ml*	p-value
(A) IFN-γ	3.20 (2.25-3.20)	3.20 (1.58-3.42)	0.82	3.20 (3.20-3.20)	3.20 (3.20-3.20)	0.92
(P) IL-1α	**50.64 (3.20-408.51)**	**35.49 (3.20-85.90)**	0.02	3.20 (3.20-28.98)	18.11 (3.20-100.71)	0.36
(P) IL-2	3.20 (3.20-21.21)	3.20 (1.79-15.85)	0.33	3.20 (3.20-22.25)	3.20 (3.20-24.06)	0.89
(A) IL-4	3.20 (1.09-3.20)	3.20 (1.16-3.20)	0.27	3.20 (1.19-3.20)	3.20 (0.55-3.20)	0.39
(P) IL-5	9.87 (5.48-15.64)	5.25 (3.42-15.26)	0.51	12.51 (3.20-17.26)	6.71 (4.42-22.45)	0.61
(P) IL-6	28.64 (3.20-62.01)	7.10 (3.20-18.79)	0.29	**3.20 (3.20-3.23)**	**8.42 (3.20-42.36)**	0.02
(A) IL-10	3.20 (3.20-12.99)	3.20 (3.20-12.20)	0.69	3.20 (3.20-30.17)	8.21 (3.20-21.64)	0.57
(P) IL-12p40	58.74 (3.20-215.81)	66.74 (3.20-116.75)	0.44	70.02 (12.07-132.12)	65.22 (16.33-92.52)	0.44
(P) IL-12p70	18.13 (3.20-534.56)	3.20 (3.20-494.83)	0.82	3.20 (3.20-555.38)	9.02 (3.20-530.53)	0.78
(P) IL-15	64.75 (3.20-318.12)	41.31 (3.20-316.07)	0.56	11.65 (3.20-343.56)	119.78 (3.20-264.02)	0.74
(P) IL-17	3.20 (1.08-53.37)	3.20 (3.20-53.48)	0.65	3.20 (3.20-54.48)	3.20 (0.78-54.51)	0.85
(A) IP-10	200.83 (167.65-321.32)	192.41 (163.47-268.32)	0.33	262.39 (185.14-360.58)	195.15 (47.49-275.42)	0.09
(P) KC	77.99 (3.20-143.42)	72.44 (3.20-155.49)	0.98	45.54 (3.20-113.79)	36.80 (3.20-100.30)	0.92
(P) GMC-SF	3.20 (3.20-49.51)	3.20 (3.20-67.85)	0.89	3.20 (3.20-62.42)	3.20 (3.20-68.92)	0.91
(P) TNF-α	3.20 (1.37-3.20)	3.20 (0.75-3.20)	0.69	3.20 (0.79-3.20)	3.20 (0.79-3.20)	0.94
(P) IL-1β	4.27 (3.20-35.22)	3.20 (2.94-19.53)	0.22	15.76 (3.11-30.07)	4.94 (3.20-25.31)	0.11
(P) MCP1	31.05 (3.20-49.19)	12.77 (3.20-41.78)	0.09	29.83 (9.67-51.98)	34.17 (6.99-42.90)	0.26
(A) MIG	127.20 (105.75-688.79)	149.97 (110.56-360.71)	0.16	209.38 (185.04-319.62)	153.98 (100.00-267.75)	0.21
(P) MIP1α	3.20 (3.20-28.11)	6.17 (3.20-35.67)	0.82	3.20 (3.20-22.02)	8.35 (3.20-23.18)	1.00
(P) VEGF	3.20 (0.26-3.20)	3.20 (0.44-3.20)	0.91	3.20 (0.57-3.20)	3.20 (0.39-4.96)	0.11

Table 5.10 Effect of dietary palm oil, or phytochemicals on cytokine level in the plasma of wild-type and ApcMin/+ mice. (A) Anti-inflammatory cytokine; (P) pro-inflammatory cytokine. Values are presented as median (pg/ml) and 95% confidence interval (CI). Significant p-value ($p<0.05$) was identified by Mann-Whitney test.

Cytokines	Wild-type			ApcMin/+		
	Palm oil *(n=13)* *Median (95% CI)* *pg/ml*	**Phytochemical** *(n=16)* *Median (95% CI)* *pg/ml*	p-value	**Palm oil** *(n=16)* *Median (95% CI)* *pg/ml*	**Phytochemical** *(n=12)* *Median (95% CI)* *pg/ml*	p-value
(A) IFN-γ	3.20 (2.25-3.20)	3.20 (3.20-3.20)	0.87	3.20 (1.58-3.42)	3.20 (3.20-3.20)	0.90
(P) IL-1α	**50.64 (3.20-408.51)**	**3.20 (3.20-28.98)**	0.02	35.49 (3.20-85.90)	18.11 (3.20-100.71)	0.24
(P) IL-2	3.20 (3.20-21.21)	3.20 (3.20-22.25)	0.24	3.20 (1.79-15.85)	3.20 (3.20-24.06)	0.07
(A) IL-4	3.20 (1.09-3.20)	3.20 (1.19-3.20)	0.85	3.20 (1.16-3.20)	3.20 (0.55-3.20)	0.83
(P) IL-5	9.87 (5.48-15.64)	12.51 (3.20-17.26)	0.79	5.25 (3.42-15.26)	6.71 (4.42-22.45)	0.13
(P) IL-6	**28.64 (3.20-62.01)**	**3.20 (3.20-3.23)**	0.001	7.10 (3.20-18.79)	8.42 (3.20-42.36)	0.64
(A) IL-10	3.20 (3.20-12.99)	3.20 (3.20-30.17)	0.45	3.20 (3.20-12.20)	8.21 (3.20-21.64)	0.09
(P) IL-12p40	58.74 (3.20-215.81)	70.02 (12.07-132.12)	0.58	66.74 (3.20-116.75)	65.22 (16.33-92.52)	0.56
(P) IL-12p70	18.13 (3.20-534.56)	3.20 (3.20-555.38)	0.91	3.20 (3.20-494.83)	9.02 (3.20-530.53)	0.96
(P) IL-15	64.75 (3.20-318.12)	11.65 (3.20-343.56)	0.39	41.31 (3.20-316.07)	119.78 (3.20-264.02)	0.22
(P) IL-17	3.20 (1.08-53.37)	3.20 (3.20-54.48)	0.21	3.20 (3.20-53.48)	3.20 (0.78-54.51)	0.42
(A) IP-10	200.83 (167.65-321.32)	262.39 (185.14-360.58)	0.33	192.41 (163.47-268.32)	195.15 (47.49-275.42)	0.58
(P) KC	77.99 (3.20-143.42)	45.54 (3.20-113.79)	0.37	72.44 (3.20-155.49)	36.80 (3.20-100.30)	0.62
(P) GMC-SF	3.20 (3.20-49.51)	3.20 (3.20-62.42)	0.37	3.20 (3.20-67.85)	3.20 (3.20-68.92)	0.51
(P) TNF-α	3.20 (1.37-3.20)	3.20 (0.79-3.20)	0.91	3.20 (0.75-3.20)	3.20 (0.79-3.20)	0.60
(P) IL-1β	4.27 (3.20-35.22)	15.76 (3.11-30.07)	0.25	3.20 (2.94-19.53)	4.94 (3.20-25.31)	0.32
(P) MCP1	31.05 (3.20-49.19)	29.83 (9.67-51.98)	0.77	12.77 (3.20-41.78)	34.17 (6.99-42.90)	0.39
(A) MIG	127.20 (105.75-688.79)	209.38 (185.04-319.62)	0.66	149.97 (110.56-360.71)	153.98 (100.00-267.75)	0.58
(P) MIP1α	3.20 (3.20-28.11)	3.20 (3.20-22.02)	0.89	6.17 (3.20-35.67)	8.35 (3.20-23.18)	0.78
(P) VEGF	3.20 (0.26-3.20)	3.20 (0.57-3.20)	0.49	**3.20 (0.44-3.20)**	**3.20 (0.39-4.96)**	0.03

Table 5.11 Effect of genotype on cytokine level in the plasma of wild-type and ApcMin/+ mice. (A) Anti-inflammatory cytokine; (P) pro-inflammatory cytokine.Values are presented as median (pg/ml) and 95% confidence interval (CI). Significant p-value ($p<0.05$) was identified by Mann-Whitney test

5.1.4. Effects on tumour development

5.1.4.1. Effect of diet on tumour size

In figure 5.4 it shows that mice fed with phytochemical diet (1.93 ± 0.90 mm^2) had significantly larger tumours across the whole small intestine compared to the palm oil diet (1.54 ± 0.81 mm^2), $p<0.0001$.

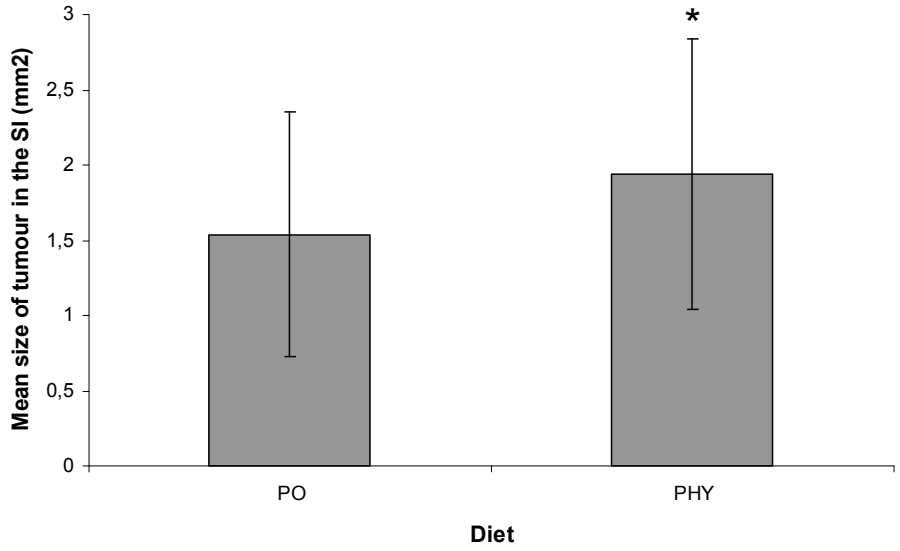

Figure 5.4 Effect of treatment diet on mean size of tumour (mm2) in the small intestine (SI) of the ApcMin/+ mice fed for 10 weeks. (n=16 mice/diet). Values having different superscript indicate statistically significant difference between groups by ANOVA at $p<0.05$.

5.1.4.2. Effect of intestinal position on tumour size

Figure 5.5 summarizes the effect of intestinal position on tumour size, which varied significantly. Larger tumours developed in the proximal (2.43 ± 1.10 mm^2) small intestine compared to tumours sized in the middle(1.37 ± 0.61 mm^2) or distal (1.61 ± 0.71 mm^2), ($p<0.0001$, $p<0.002$ respectively).

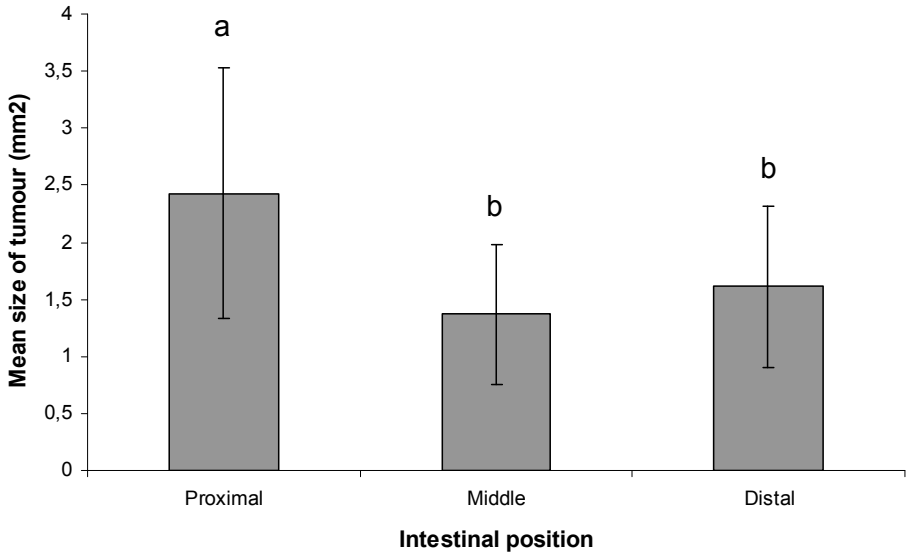

Figure 5.5 Mean tumour size (mm2 ± stdev) according to the intestinal position in the small intestine of ApcMin/+ mice (n=32). Values having different superscript indicate statistically significant difference between the intestinal positions by ANOVA at $p<0.05$.

There was no significant effect of phytochemicals on tumour size in the proximal (PHY= 2.76 ± 1.20 mm^2 *vs*. PO= 2.10 ± 0.90 mm^2) and in the middle intestine (PHY= 1.58 ± 0.69 mm^2 *vs*. PO= 1.20 ± 0.40 mm^2) (Figure 5.6 and Figure 5.7). In the distal position of the small intestine (Figure 5.8) the phytochemical group (2.03 ± 0.54 mm^2) had significant larger tumours compared to control palm oil (1.20 ± 0.60 mm^2).

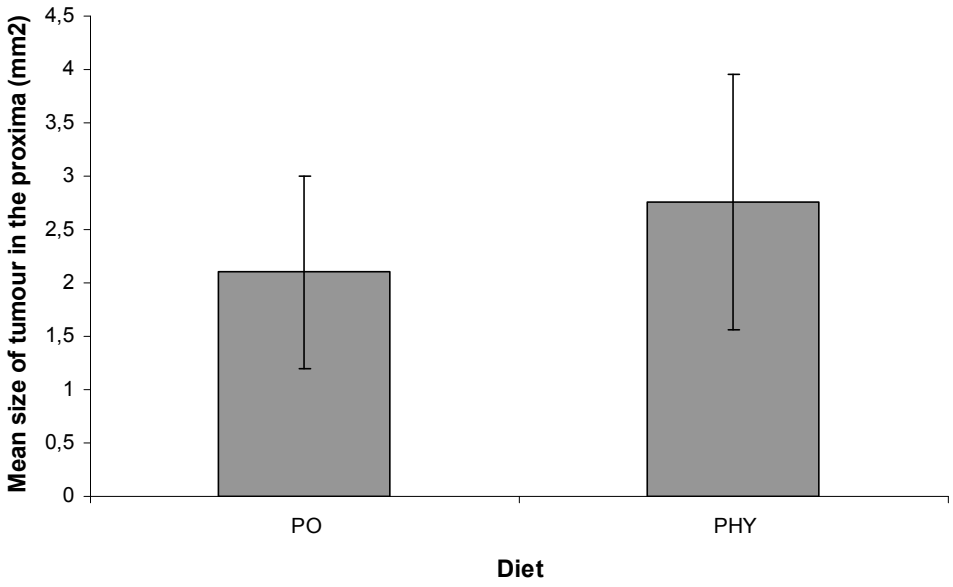

Figure 5.6 Effect of treatment diet on mean size of tumour (mm2 ± stdev) in the proximal part the small intestine of the ApcMin/+ mice (n=16 mice/diet). PO=palm oil; PHY=phytochemical. No statistically significant difference were found between the treatment groups by ANOVA at $p<0.05$.

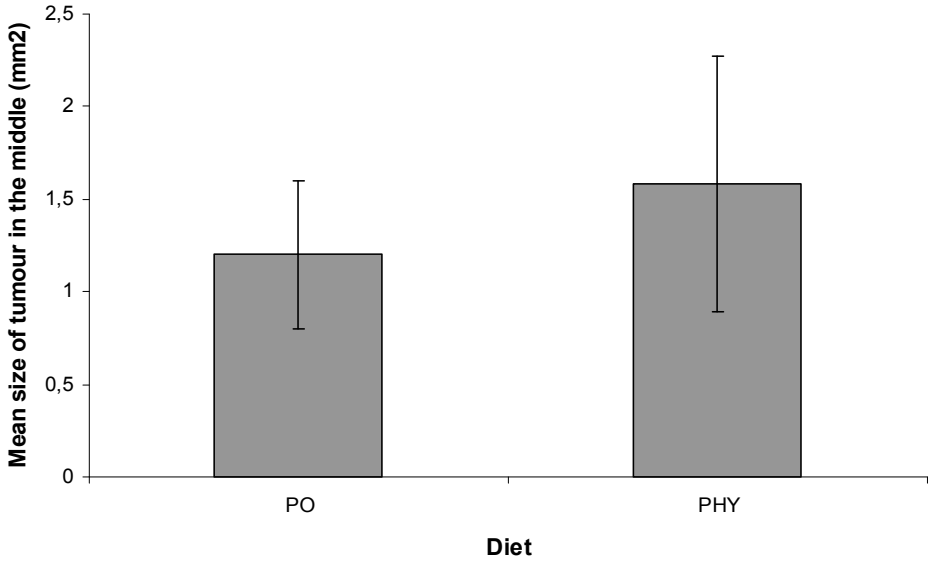

Figure 5.7 Effect of treatment diet on mean size of tumour (mm2 ± stdev) in the middle part the small intestine of the ApcMin/+ mice (n=16 mice/diet). PO=palm oil; PHY=phytochemical. No statistically significant difference were found between the treatment groups by ANOVA at $p<0.05$.

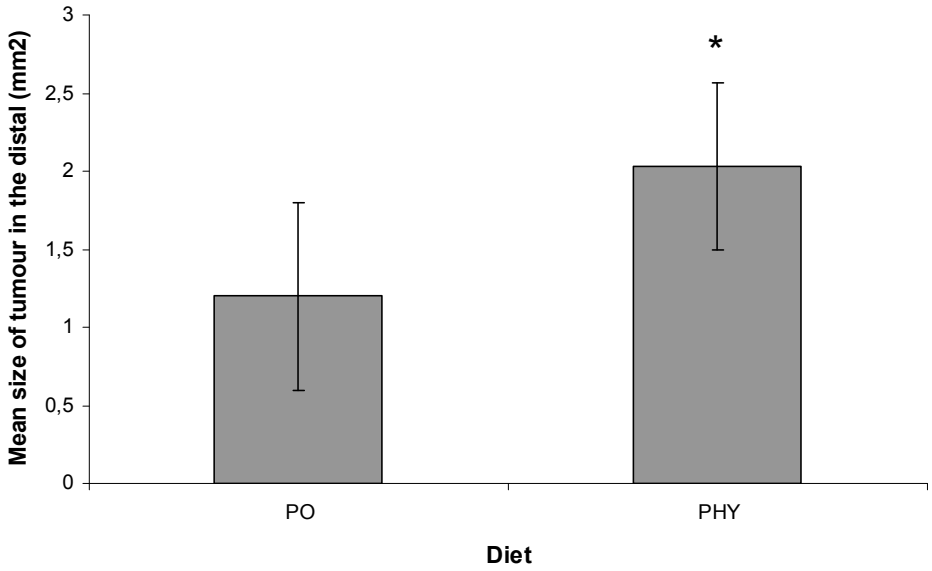

Figure 5.8 Effect of treatment diet on mean size of tumour (mm2 ± stdev) in the distal part the small intestine of the ApcMin/+ mice (n=16 mice/diet). PO=palm oil; PHY=phytochemical. Values having different superscript indicate statistically significant difference between the treatment groups by ANOVA at $p<0.05$.

5.1.4.3. Effect of diet on total tumour area

Figure 5.9 summarises the total tumour area (mm^2) across all the small intestine and the effect of the treatment diet. Total tumour size was significantly increased in animals fed with the phytochemical diet (2559.7 mm^2) compared to the control diet (1355 mm^2), $p=0.003$.

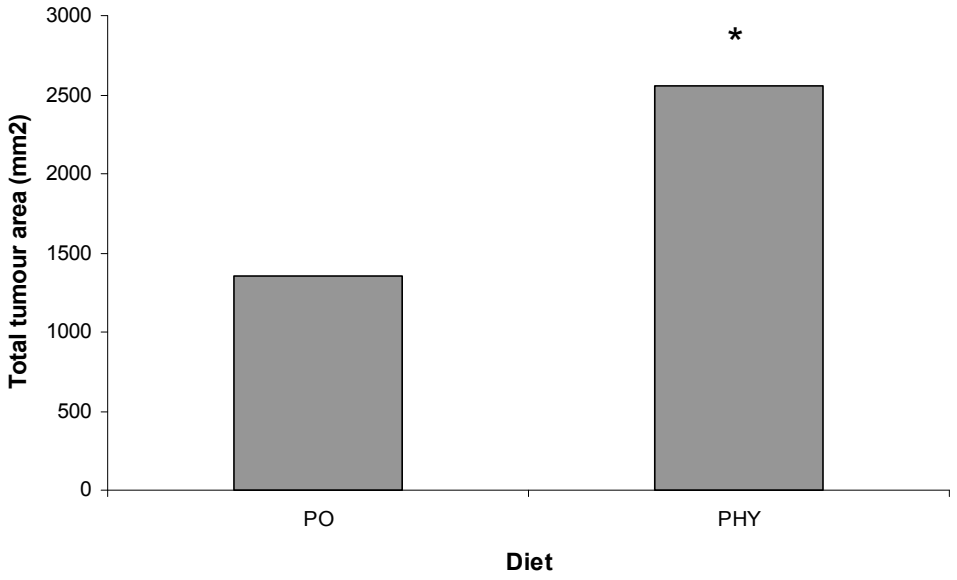

Figure 5.9 Effect of treatment diet on total tumour area (mm2) in the across all the small intestine of the ApcMin/+ mice (n=16 mice/diet). PO=palm oil; PHY=phytochemical. Values having different superscript indicate statistically significant difference between the treatment groups by ANOVA at $p<0.05$.

5.1.4.4. Effect of diet on total tumour number

There was no significant difference on total tumour number in the small intestine of mice fed the phytochemical diet (1321 tumours) compared to the palm oil control (1044 tumours). However, when the number of tumours was compared separately in each region of the small intestine (proximal, middle, distal) by different sizes (<1mm2; 1-5mm2; >5mm2), many significant differences were observed (Table 5.12). In the proximal position animals fed the phytochemical diet had significantly fewer medium size tumours compared to palm oil (53 *vs.* 104 tumours, p=0.0001). However, in the middle position animals fed the phytochemical diet increased the number of large tumours by 300% compared to control (16 *vs.* 4 tumours, p=0.007). Moreover, in the distal position, animals fed the phytochemical diet had significantly increased numbers of tumours of all sizes

compared to control, (small: 275 *vs.* 207 tumours, p=0.002; medium: 404 *vs.* 212 tumours, p=0.0001; distal: 57 *vs.* 8 tumours, p=0.0001).

Tumour region and size	Palm oil	Phytochemicals	p-value
Proximal			
< 1 mm2	108	89	0.176
1-5 mm2	**104**	**53**	0.0001
> 5 mm2	27	30	0.691
Middle			
< 1 mm2	204	214	0.625
1-5 mm2	170	184	0.457
> 5 mm2	**4**	**16**	0.007
Distal			
< 1 mm2	**207**	**275**	0.002
1-5 mm2	**212**	**404**	0.0001
> 5 mm2	**8**	**57**	0.0001

Table 5.12 Incidence of tumours in the small intestine of ApcMin/+ mice fed with palm oil and phytochemicals for 10 weeks. Values are presented as numbers of tumours. Male and female ApcMin/+ mice combined (n=16 animals/diet). Values are significantly different by General Linear Model $p<0.05$.

5.1.4.5. Effect of diet on tumour area and tumour number in the colon

No significant difference was seen in the colon for tumour area, although the phytochemical group had a larger mean tumour area compared to the animals fed palm oil, (3.68 ± 2.98 mm^2 *vs.* 0.84 ± 0.52 mm^2). However, when tumour number was categorized by size (<1mm^2; 1-5mm^2; >5mm^2), significant differences were seen (Table 5.13). Animals fed the phytochemicals had increased numbers of tumours of all sizes compared to control animals (small: 28 *vs.* 9 tumours, p=0.002; medium: 9 *vs.* 2 tumours, p=0.035; large: 9 *vs.* 0 tumours, p=0.012).

Colon Tumour region and size	Palm oil	Phytochemicals	p-value
< 1 mm2	**9**	**28**	0.002
1-5 mm2	**2**	**9**	0.035
> 5 mm2	**0**	**9**	0.012

Table 5.13 Incidence of tumours in the colon of ApcMin/+ mice fed with palm oil and phytochemicals for 10 weeks. Values are presented as numbers of tumours. Male and female ApcMin/+ mice combined (n=16 animals/diet). Values are significantly different by General Linear Model $p<0.05$.

5.2. DISCUSSION

5.2.1. General effects of the phytochemical mix

5.2.1.1. Body and organs weight

As expected, female mice were smaller compared to males. In relation to the organs weight, liver, heart, and spleen showed no difference between sex, however when compared between strains, ApcMin/+ mice had larger organs, and lower final body weight. Animals fed the phytochemical diet did not show difference in the final body weight. However they had larger organs (stomach, caecum, liver, and heart).

5.2.1.2. Mitosis, apoptosis, and crypt length

ApcMin/+ mice fed the phytochemical diet had increased the number of mitoses in the small intestine in ApcMin/+ mice compared to the wild-type fed the same diet, but not compared to ApcMin/+ mice fed the control diet. The same was observed in the control group, ApcMin/+ mice fed palm oil had higher number of mitosis compared to wild-type animals fed the same diet. In the colon tissue, animals fed the phytochemical had no effect regarding the number of mitoses. However, within the control group, the ApcMin/+ mice had higher numbers of mitoses compared to wild-type animals.

On the other hand, ApcMin/+ mice fed the phytochemical diet had increased number of apoptosis in the small intestine by 4-fold increased compared to the wild-type animals fed the same diet. However, no difference was observed between strains in the control group. Animals fed the phytochemical did not change in the number of apoptosis in the colon. In

resume, the ApcMin/+ mice fed the phytochemical diet did not show a positive effect on reducing the proliferation of intestinal cells or increasing the cell death in the intestine tissues in the present experiment. All the significant changes were observed only when the strains were being fed the same diet.

5.2.1.3. Inflammation

Small intestine

Distal tissue from animals (wild-type and ApcMin/+ combined) fed the palm oil and the phytochemical treatment diet were analyzed for 20 cytokines. Significant changes were observed in ten of the cytokines, which were mostly increased in the phytochemical group. IFN-γ, IL-1α, IL-2, IL-12p40, IP-10, IL-1β, and MIP-1α were all increased by the phytochemical diet while IL-5, IL-10, and TNF-α were reduced. When the cytokine profile was analyzed by strain the following was observed. ApcMin/+ mice fed the palm oil diet had KC, the mouse equivalent to IL8, and IL-1β increased, and IL-5 decreased compared to the wild-type. ApcMin/+ mice fed the phytochemical diet had KC, IL-1β, MCP-1, IL-6 and IL-1α increased, whereas IFN-γ and IL-2 were decreased. These results clearly show that the phytochemical diet has increased the inflammation in ApcMin/+ mice, which contradicts the hypothesis that phytochemicals can act as anti-inflammatory agents.

Colon

In the colon tissue the only significant change in the cytokine level was in the ApcMin/+ mice fed the phytochemical diet, which had the level of MCP-1 increased compared to the ApcMin/+ mice fed the control diet. In the ApcMin/+ mice the tumour development occurs mainly in the small intestine, as a result changes in the cytokine profile in the colon tissue might be less affected, when compared to the small intestine.

Plasma

Minimal changes were observed in the plasma in response to the phytochemical diet. When both strains were combined, animals fed the phytochemical diet increased levels of IL-2 and reduced levels of IL-6. When the strains were observed separately, the phytochemical diet only increased level of VEGF in the ApcMin/+ animals, while in the wild-type animals, the phytochemical diet decreased IL-1α and IL-6.

5.2.1.4. Tumour development

Phytochemicals have been associated with a decrease in colorectal cancer in several cell culture and animal cancer models by inhibiting carcinogenesis at different stages. However, in the present study the results seem to be contradictory when compared to several other studies. The phytochemical diet containing coffee, cocoa, turmeric, thyme, walnuts and berries, fed for 10 weeks did not inhibit carcinogenesis in ApcMin/+ mice. Furthermore, phytochemicals have increased the size of tumour throughout the small intestine. It was also observed that the phytochemical diet had a different effect on tumour size according to the position of tumours in the small intestine. For example, in the distal part of the small intestine the tumour size was larger in ApcMin/+ mice fed the phytochemical diet when compared to control. Moreover, when all the tumour size was summarized, animals fed the phytochemical diet also showed increased value.

In respect to the total number of tumours in the small intestine, a 26% increase was observed in the group fed the phytochemical diet compared to the control diet, but it was not significant. However, when the region of the intestine was taken into consideration a tremendous increase was observed in the number of tumours in the distal part of animals fed the phytochemical diet by 72% compared to the control. The ApcMin/+ mouse is

regarded as a model of colorectal cancer. However it does not develop a significant carcinogenic process in the colon. Besides, phytochemical also increased the number of tumours in the colon tissue of animals by 4.1-fold compared to the control diet.

5.2.2. Discussion of the effects by each phytochemical

The phytochemical diet was enriched with a mix of dietary foods such as coffee, cocoa, turmeric, thyme, walnuts, bilberries, and blackcurrant, rich in different phytochemicals. Because of that, it is difficult to attribute a specific effect to a particular food ingredient. However, when the animals consumed a mix of dietary foods within the diet, it mimics the manner of a real human consumption of phytochemicals.

5.2.2.1. Coffee

Coffee (Coffea robusta) is one of the major commodities worldwide, and also one of the most commonly consumed beverages. The main constituents of coffee include caffeine, diterpenes, phenolic acids, and melaniods and acrylamide produced during roasting of coffee beans.

A meta-analysis of colorectal cancer and coffee consumption was completed and published in 2004 (Tavani and La Vecchia 2004). That work compiled 3 cohort and 15 case-control studies, together were experiences from over 8,713 cases among 147,227 subjects. The 3 cohort studies showed no association, however in the case-control studies there was a potentially protective effect of coffee and colorectal cancer. Two other recent cohort studies from Japan have found 60% reductions in risk of colorectal cancer with coffee consumption among women only (Oba, Shimizu et al. 2006; Lee, Inoue et al. 2007). Due

to the heterogeneity of effect and the lack of dose-response effect, there is very weak evidence of a preventive effect (Arab 2010).

Effect of coffee on mitosis and apoptosis

Grubben et al. (Grubben, Van Den et al. 2000) have performed a randomized trial in healthy subjects to study the effect of drinking coffee on cell proliferation as a biomarker of colorectal cancer. They found that the consumption of 1 L of unfiltered coffee per day did not affect the colorectal cell proliferation. On the other hand, Wang et al. (Wang, Dashwood et al. 2008) performed a study using rats treated with 3 cycles of 2-amino-1-methyl-6-phenylimidazol pyridine carcinogen to evaluate the effect of caffeine on tumorigenesis. They found that caffeine altered colonic crypt homeostasis by not only enhancing cell proliferation, but by inhibiting apoptosis. Both studies contradict the epidemiological evidence that coffee may be protective against colorectal cancer by inhibiting cell proliferation. However, they are consistent with the current study.

There is limited literature available regarding the effect of coffee on apoptosis in cell lines or animal models of colorectal cancer. However, Oh et al. (Um, Oh et al. 2010) performed a study to evaluate the effect of kahweol (coffee-specific diterpene) on apoptosis in human renal carcinoma Caki cell line. They found that cell treating cells with kahweol had no effect on enhancing apoptosis, however when kahweol was combined with tumour necrosis factor-related apoptosis-inducing ligand, which is a potent anti-cancer drug, apoptosis was significantly increased.

Effect of coffee on inflammation

Kempf et al. (Kempf, Herder et al. 2010) studied the effect of drinking coffee on human serum inflammatory markers. They found that the consumption of 8 cups of coffee/day decreased the levels of IL-18, but had no effect on IL-6 and C-reactive protein. However, there are a few studies that have shown contradictory results (Zampelas, Panagiotakos et al. 2004). The effect of kahweol, which is present in coffee beans, was studied by Kim et al. (Kim, Kim et al. 2006) to confirm the anti-inflammatory effects of kahweol by examining its effect on the inflammatory response induced in a rat using an acute air pouch inflammation model. They found that kahweol significantly reduced the levels of the inflammatory process as well as the levels of TNF-α and prostaglandin E2 (Kim, Kim et al. 2006).

Only a few papers can be found on coffee and its effect on cytokines prolife in animal models of colorectal cancer, however, it had been shown that coffee does affect key molecules such as NF-kB, which is directly related to the production of cytokines. NF-kB is a transcription factor that plays a critical role in inflammatory responses, and its aberrant regulation had been noted in several human cancers and inflammatory bowel disease (Karin 2006; Karin 2008). Paur et al. (Paur, Balstad et al. 2010) have studied the effect of coffee on the activity of NF-kB in vitro (U937-3xkB-LUC cells) stimulated with LPS, treated with 30mg/mL of coffee) and in vivo (mice injected D-luciferin with PBS, treated with a single dose extract of 600mg coffee). They found coffee inhibited NF-kB activity by more than 80% in vitro, and by 63% in mice.

Effects of coffee on tumour development

Park et al. (Park, Davis et al. 2010) have studied the effect of coffee compounds in the tumour development in the colon of AOM-induced mice. They fed the animals for 20 weeks a 0.01% of chlorogenic acid, which is equivalent to ~0.5 servings of coffee consumed/day in humans, or 0.1% of chlorogenic acid, which is equivalent to ~5 servings. They found no effect of coffee on tumour number, but found an increase in total number of ACF in animals fed the 0.01% chlorogenic acid compared to control.

The effects of coffee have also been studied in the ApcMin/+ mouse model. Oikarinen et al. (Oikarinen, Erlund et al. 2007) fed the ApcMin/+ mice with filtered (low levels of kahweol/cafestol) and unfiltered coffee (high levels of kahweol/cafestol) for 9 weeks. That study was similar to the present study in that they also used a high-fat diet (40%), and because the coffee was served as a dietary ingredient and not as a drink. However, comparing the amount of coffee ingested per mice/day was very different between their and the present experiment. Their animal consumption was 2.4g/day, in which 0.24g was coffee, whereas in the present experiment animals were fed the equivalent of 0.09g of coffee per day. This means that in their experiment, the ApcMin/+ mice received 2.5 times more coffee than the animals in the present study. However, they found no effects on total tumour number or size in the small intestine and in the colon of ApcMin/+ mice compared to control. Hong et al. (Ju, Hong et al. 2005) have also treated ApcMin/+ mice with an administration of caffeine at a dose of 0.044% in drinking fluid and they also found no effect on tumour formation.

Mutations in the APC gene lead to the accumulation of hypo-phosphorylated β-catenin protein in the cytosol and later in the nucleus, which contributes to the activation of cyclin

D1 gene and is related to the progression of cancer (Korinek, Barker et al. 1997). Oikarinen et al. (Oikarinen, Erlund et al. 2007) tested if coffee could interfere in the levels of β-catenin and cyclin D1 in ApcMin/+ mice. Therefore, they concluded that coffee consumption does not affect cancer progression in the adenoma tissue in animals, as the level of β-ccatenin and cyclin D1 did not change with coffee, as well as the number of tumours which were not different from control diet.

5.2.2.2. Walnuts

Walnuts (Juglans regia) are rich source of both n-3 and n-6 polyunsaturated fatty acids with known anti-inflammatory properties, but also rich in phenolic acids. Epidemiological studies have shown a positive association between regular consumption of nuts and the prevention of some types of cancer.

Effects of walnuts on mitosis and apoptosis

Carvalho et al. (Carvalho, Ferreira et al. 2010) have studied the anti-proliferative activities from walnuts (seed, green husk, and leaf) in colon cancer Caco-2 cell line. They found an inhibition of cell proliferation between 23-78% by the walnuts extracts. The level of cell growth inhibition observed with leaf extracts ranged from 13% to 78%, followed by 15-41% with green husk extracts, and 0-23% with seed extracts. In the present study, the phytochemical mix used in the experiment contained 31g of walnuts/ kg of diet, and so animals may have ingested on average 0.09g of walnuts/ day.

Yang et al. (Yang, Liu et al. 2009) have also studied the effect of different nuts on cell proliferation of Caco-2 cell line. They found that walnuts had the highest phenolics content

and was among the nuts that had the highest anti-proliferative effect on the Caco-2 colorectal cancer cell line, and that effect was seen to be dose-dependent.

Hardman and Ion. (Hardman and Ion 2008) performed a study by implanting MDA-MB 231 human breast cancers into nude mice, which were fed an 18% (calorie) walnut diet for 35 days. The quantity of the walnut in the animal diet was used to mimic two servings of walnuts per day in humans. In contrast to the present study they found significantly decreased cell proliferation in mice that consumed walnuts, however, no effect was found in number of apoptotic cells/crypt.

Effects of walnuts on inflammation

Not much is found in the literature about the effects of walnuts on inflammation in animal model of colorectal cancer. However, it has been reported in the literature that walnuts can reduce inflammation related to other chronic diseases such as cardiovascular (Cortés, Núñez et al. 2006). However, recent studies have demonstrated that walnuts inhibit NF-kB in vitro and in vivo (Paur, Austenaa et al. 2008; Paur, Balstad et al. 2010). NF-kB has recently been demonstrated as a pivotal factor connecting inflammation and cancer (Greten, Eckmann et al. 2004).

Effects of walnuts on tumour development

Hardman and Ion. (Hardman and Ion 2008) found a positive effect of walnuts in their *in vivo* study. Nude mice implanted with MDA-MB 231 human breast cancers had a decreased tumour growth rate after 10 days of intervention with 18% of the dietary calories from walnuts (11.3g of walnuts). Considering a mouse average intake of 2.8 g/day, that

would be equal to a consumption of 0.03g of walnuts/day. In the present experiment, the ApcMin/+ mice ingested the equivalent to 0.09g of walnuts/day. However, tumour number and tumour area were not reduced in the group treated with phytochemicals. That positive effect on preventing tumour development attributed in Hardman and Ion's study, may be a result the alpha linolenic acid (ALA, C18:3), an n-3 fatty acids, present in walnuts. However, many of the mechanisms proposed for suppression of cancer growth by omega 3 fatty acids required the presence of long chain (20 or 22C) omega 3 fatty acids (Hardman 2002). In the study performed by Hardamn and Ion, EPA and DHA had no effect despite significantly higher levels of EPA and DHA being found in the liver of mice fed the walnuts diet compared to control. Because the only source of the omega 3 bond was the ALA of the walnuts, the increased EPA and DHA indicates that the ALA of the walnut was probably being effectively elongated and desaturated to EPA and DHA in the liver. As a consequence, it is likely that the arachidonic acid was significantly decreased, reducing the amount of substrate available for synthesis of inflammatory prostaglandin E2 (PGE2) in the tumour. PGE2 has been found to be promotional to tumour cell proliferation (Amano, Hayashi et al. 2003).

The beneficial effects of ALA from walnuts on tumour suppression has also been shown in ApcMin/+ mice, however using a different dietary source of ALA. Bommareddy et al. (Bommareddy, Zhang et al. 2009) fed ApcMin/+ mice for 12 weeks with a diet with 15% of the dietary calorie from flaxseed meal or flaxseed oil, which is also rich in ALA. They found that flaxseed meal and oil not only significantly suppressed intestinal polyp formation by about 45%, but also lowered tumour multiplicity and tumour size compared to control diet. They also found lower levels of AA, COX-1 and COX-2 expression in the animal group fed the flaxseed oil and meal compared to control. As a result, they

speculated that the conversion of AA to prostaglandins (tumour promoters) mediated by COX might have also been decreased.

5.2.2.3. Cocoa

Although numerous polyphenolic compounds are present in the (Theobroma cacao L.), cocoa powder is particularly rich in a subclass of polyphenolics known as flavonoids (catechin, epicatechin, procyanidins), which can be also found in tea. There is limited epidemiological data that shows interesting effects of cocoa in relation to cancer (Maskarinec 2009). On the other hand, several experiments have demonstrated a variety of anti-cancer effects of phytochemicals present in the cocoa (Ramljak, Romanczyk et al. 2005; Gu, House et al. 2006; Aron and Kennedy 2008).

Effects of cocoa on mitosis and apoptosis

Flavonols and procyanidins from cocoa have shown to have positive effects against colonic cancer in cell line. Carnesecchi et al. (Carnésecchi, Schneider et al. 2002) have treated Caco-2 cells with 50ug/ml of cocoa powder (CC), crude procyanidin (CP) and procyanidin-enriched extracts (PE). They found 25% inhibition of cell proliferation by CP, whereas PE extracts inhibited 75% of cell growth. CC which had the lowest content had no effect on mitosis. No effect on apoptosis, measured by DNA fragmentation was observed in the cells treated with PE extracts. However, cocoa flavonols and procyanidins caused Caco-2 cell death by a non-apoptotic process, which as measured by the lactate dehydrogenase release into the culture medium. Procyanidin from cocoa have also demonstrated its anti-proliferative effect in human breast cancer cells (Ramljak, Romanczyk et al. 2005).

An animal study with ApcMin/+ mouse has also shown a positive effect of a catechin on cell proliferation inhibition. Bose et al. (Bose, Hao et al. 2007) have fed the ApcMin/+ mice with a high fat (20% (w/w) diet added 0.16% of (-)-epigallocatechin-3-gallate (EGCG) or high fat fish oil diet in combination of EGCG for 9 weeks. They found that EGCG decreased the proliferation index by 53%, and the combination of EGCG with fish oil reduced by 25% compared to its control. The apoptotic index was also significantly higher in the tumours of all treatment groups.

Effects of cocoa on inflammation

In that same study performed by Bose et al. (Bose, Hao et al. 2007), they have tested the effect of EGCG on PGE2 level on the small intestine, and a significant decrease was observed. They found that the dietary EGCG combined with fish oil reduced PGE2 levels by 81%. However, in the present study PGE2 levels were not measured. PGE2 mediates the inflammation process, and is also involved in the production of cytokines (Dooper, Wassink et al. 2002).

ECGC has also been shown to decrease NF-kB levels and activity in a dose dependent manner in cancer and normal human epidermal keratinocytes (Ahmad and Mukhtar 1999). In accordance with that result, other studies have reported the inhibitory effect of ECGC on NF-kB activation, which is also directely connected with cytokines production (Ahmad and Mukhtar 1999; Wenzel, Kuntz et al. 2000; Nishikawa, Nakajima et al. 2006).

While NF-kB can be modulated by phytochemicals, and consequently may be affecting cytokines production (Paur, Austenaa et al. 2008; Paur, Balstad et al. 2010; Paur, Balstad

et al. 2010), Crouvezier et al. (Crouvezier, Powell et al. 2001) has shown that phenolic components of tea may modulate pro- and anti-inflammatory cytokine production. They studied the effect of 4 different catechins (epicatechin EC; epicatechin gallate ECG; epigallocatechin EGC; and EGCG) and tea extract (TE) on cytokines production by human leukocytes in vitro. Their results in whole blood cultures showed that EC or ECG had no effect on the production of IL-1β, IL-6 or TNF-α. However EGC and EGCG inhibited IL-1β. The TE thus produced a small, but inconsistent, inhibition of IL-1β. Moreover, the combination of all 4 catechins had no effect on any of the pro-inflammatory cytokines. They speculated that tea-derived catechin may not affect inflammation through mechanism involving the NF-kB, as the most of the pro-inflammatory cytokines were not affected in their study.

Effects of cocoa on tumour development

The effect of EGCG on tumorigenesis in ApcMin/+ mice has been reported in the study performed by Bose et al. (Bose, Hao et al. 2007). They fed the mice an EGCG plus fish oil diet for 9 weeks and found that 0.16% of EGCG combined with fish oil reduced multiplicity of tumours by 53%, while EGCG alone reduced by 19%, and fish oil alone had no effect. They found the distal part of the small intestine was most affected by the EGCG + fish oil diet, and tumours were reduced by 58% compared to control. In the present study, the distal part was also the most affected by the phytochemical diet; however, the result went in the opposite direction and the phytochemical diet increased the multiplicity by 72% compared to control.

There is another study in which the effect of white and green tea on tumorigenesis in ApcMin/+ was also studied. Orner et al. (Orner, Dashwood et al. 2003) have given a

standard diet plus 1.5% of white and green tea as drinking water for 12 weeks. They found that both teas reduced the number of tumours. In the distal part of the small intestine, they found that white tea was more effective than green tea in reducing tumour number. Interestingly, they reported that when both teas were combined with the chemopreventive pharmaceutical sulindac (a non-steirodal anti-inflammatory drug, NSAIDs) the chemopreventive effect of the diet was 70% more effective in reducing the number of tumours in the ApcMin/+ mice.

In contrast with the results found by Oikarinen et al. (Oikarinen, Erlund et al. 2007), in which coffee had no effect on levels of β-catenin in tumours of ApcMin/+ mice, Bose et al. showed that catechins from tea significantly reduced the level of β-catenin translocation from the cell membrane to the nucleus in the group fed not only the EGCG diet, but also in the group fed the EGCG plus fish oil. In accordance to that result, Orner et al (Orner, Dashwood et al. 2003) have also found reduced level of β-catenin when tea was combined with sulindac.

5.2.2.4. Turmeric and Thyme

Turmeric (Curcuma longa) is a dietary plant rich in phenolic acids, especially curcumin, which is a yellow pigment, also found in mustard, and is widely used for flavouring and colouring in foods. Thyme (Thymus spp.) is an herbal plant used for flavouring and it is also rich in phenolic acid, such as rosmarinic acid.

Effects of turmeric and thyme on mitosis and apoptosis

Mahmoud et al. (Mahmoud, Carothers et al. 2000) performed a study with ApcMin/+ mice for 15 weeks to in order to observe the effect of plant derived-phenolics on cell proliferation and cell death. They have analysed the enterocyte proliferation and found that treatment with curcumin increased enterocyte proliferation in the intestinal tissue of ApcMin/+ mice, resulting in a normalisation of enterocyte proliferation. It has been previously demonstrated in the literature that a normal migration of enterocytes from the crypts to the villus tips is inhibited in the ApcMin+ mice, and consequently, the mucosal enterocytes of these animals have a reduced turnover rate (Mahmoud, Bilinski et al. 1999). So in that study, curcumin restored the enterocyte migration rate to the level of wild-type animals.

The same study also showed that 1.5% of curcumin in the diet led to a 10-fold increase in the number of apoptotic cells in ApcMin/+ mice when compared to the untreated ApcMin/+ mice. However no difference was found when compared to wild-type animals. Their result is in contradiction to the result found in the present experiment, in which the phytochemical mix did not show any difference between treated and untreated ApcMin/+ mice.

Effects of turmeric and thyme on inflammation

Lim and Kwon (Lim and Kwon 2010) showed that curcumin induced a down regulation of MCP-1 expression in human monocytic cell lines from lymphoma (U937). In animal model that develop colitis, Nones et al. (Nones, Knoch et al. 2009) have shown that adding curcumin to the diet significantly reduced intestinal inflammation. Moreover, curcumin has

been demonstrated to inhibit NF-kB (Gong, Li et al. 2003; Shishodia, Amin et al. 2005; Paur, Austenaa et al. 2008). Thyme has also been reported to inhibit NF-kB (Paur, Balstad et al. 2010).

Effects of turmeric and thyme on tumour development

A very recent study performed by Mudduluru et al. (Mudduluru, George-William et al. 2010) has shown the anti-carcinogenic effects of curcumin in colonic epithelial cell line (HCT116). The cancer cells treated with curcumin reduced the expression of miR-21, which is normally over expressed in tumours, inhibited tumour growth and invasion. Nautiyal et al. (Nautiyal, Banerjee et al. 2010) have also found similar results using the same cell HCT116 cell line.

There are only two studies performed in the ApcMin/+ mouse to study the chemopreventive effect of curcumin (Mahmoud, Carothers et al. 2000)(Perkins, Verschoyle et al. 2002). In the first study 0.15% curcumin in the diet reduced intestinal tumour formation by 64% after 10 weeks. In the second study various concentrations of curcumin in the diet were tested (0.1, 0.2, or 0.5%) for a period of 15 weeks and they found that although the concentration of 0.1% curcumin had no effect on tumour burden diets containing 0.2 and 0.5% curcumin significantly reduced tumour burden by 40% and tumour number by 30% but not significantly. They also found differences in the effect of curcumin on tumour size according to the dose and region of the intestine.

Similarly, Perkins et al. (Perkins, Verschoyle et al. 2002) have found that curcumin have to be administered in a certain amount in the diet to be able to affect tumorigenesis, at least in ApcMin/+ mouse. They found no effect of curcumin in the diet with 150 mg/kg body

weight/day, whereas at ~300 mg/kg/day curcumin prevented, or retarded, adenoma formation. A further increase to ~750 mg/kg/day failed to yield any additional gain in efficacy. In the present study the dietary turmeric was present at 3.1 g/kg (0.3%) of diet, and the daily consumption was ~9mg per day/mouse (450 mg/kg body weight/ day). However they have used pure curcumin. It is suggested that in 100g of turmeric there is 3-5g of curcumin. Based on that, the amount of curcumin in the present diet was at least 0.09g/kg. The amount of curcumin ingested by the animals in the present study was well below the lowest dose used by Perkins et al. (Perkins, Verschoyle et al. 2002) (~0.42mg/animal/day), in which they found no effect. The positive effect of curcumin in ApcMin/+ mouse was found when animals ingested at least 0.84mg of curcumin in the diet (Perkins, Verschoyle et al. 2002). Based on that, the amount of curcumin present in the present study would have no effect on adenoma formation.

5.2.2.5. Berries

Berries present in the phytochemical mix used in this study were bilberries (Vaccinium myrtillus L) and blackcurrant (Ribes Nigrum). Both berries are among the fruits with the highest content of phytochemicals, such as anthocyanins, but also contain flavonols (quercetin, kaempferol), and isoflavonoids.

Effects of berries on mitosis and apoptosis

Musk et al. (Musk, Stephenson et al. 1995) have studied the effect of quercetin on cell proliferation of human colorectal adenocarcinomas cell line HT29, and found a significant inhibition by the treatment with quercetin. Quercitin not only inhibited mitosis but also induced apoptosis.

Gee et al. (Gee, Hara et al. 2002) tested the effect of quercetin in tissue of rats treated with quercetin in the diet for 7 days. They found that quercetin significantly reduced the frequency of crypt cell mitosis in the proximal, mid, and distal small intestine, and the distal colon. However, a trend for quercetin to induce mitosis in the caecum was also observed. Regarding the apoptosis levels, that study showed no effect of quercetin either in the small or large intestine. In the present study cell proliferation was not measured in the proximal and mid small intestine, but only in the distal small intestine and in the colon. The reason for choosing only the distal part for the cytokinetics measurements was because the tumour load in the distal part was the highest compared to the other regions of the small intestine. In that same experiment, Gee et al. injected rats with DMH and rats fed 1g/kg quercetin had reduced cell proliferation after 30h of exposure. At 6 weeks after a second exposure of DMH, lower levels of cell proliferation were still evident. However, the frequencies of apoptosis in DMH-induced rats were not affected by quercetin.

Blueberry extract has also been shown to reduce cell proliferation in endothelial cell neoplasms by oral treatment of mice with hemaangioendothelioma (Gordillo, Fang et al. 2009). In that same study, Gordillo et al. reported that blueberry extract inhibited the NF-kB signalling pathway. Berries have also been reported to inhibit cell proliferation in cell lines of colorectal cancer. Blackberry juice and blackberry extract inhibited HT29 colon cancer cell proliferation (Kang, Seeram et al. 2003). Proliferation of breast cancer line MCF-7 and the colon cancer cell line HT29 was reduced by up to 74% following exposure to extracts of several fruits and berries including blueberries, blackcurrant, black chokeberries and raspberries at concentrations ranging from 0.025 to 0.5% (Olsson, Gustavsson et al. 2004).

Effects of berries on inflammation

Camuesco et al. (Camuesco, Comalada et al. 2006) performed a study in rats with intestinal inflammatory disorder (DSS-induced colitis) to evaluate the effect of the bioflavonoid quercetin combined with fish oil on the concentration of colonic pro-inflammatory mediators. They found a significant reduction in TNF-α and IL-1β, and that effect was greater when compared with rats receiving a fish oil diet without the flavonoid. That study brings attention to the fact that synergism may occur between phytochemicals and the type of fatty acids consumed in the diet.

Ziegler et al. (Ziegler, Rainwater et al. 2004) have performed a study in ApcMin/+ mice in which they analysed the levels of COX-2 and PGE2 in tumours from mice treated with dietary resveratrol, which is a phytochemical present in grapes, berries and red wines. They found that animals treated with resveratrol for 7 weeks had decreased levels of PGE2 in tumour tissue by 42%, but no effect was observed in COX-2 protein expression. Prostaglandins and COX-2 are key mediators in inflammation and inflammation-associated tumours (Schetter, Heegaard et al. 2010).

Effects of berries on tumour development

The chemopreventive effect of quercetin on the number of ACF in the colon was analysed by Gee et al. (Gee, Hara et al. 2002). They fed DMH-induced rat with quercetin in the diet and found that after 42 days the numbers of single aberrant crypts in the distal part of the colon, the total area of aberrant crypt foci, and the numbers of larger ACF were significantly lower in animals fed quercetin than in control animals. A study performed by Bobe et al. (Bobe, Wang et al. 2006) was done with a slightly different approach from the

previous study. They combined a dietary phytochemical therapy with an anticarcinogenic pharmaceutical at a suboptimal dosage to minimize any potential adverse side effects, to study the chemopreventive effect on tumorigenesis. ApcMin/+ mice were fed various dosages of anthocyanin-rich tart cherry extract with 0.01% sulindac for 19 weeks. The small intestine of ApcMin/+ mice fed the anthocyanin-rich tart cherry with sulindac had a 20% reduced total tumour area compared to animals fed sulindac alone. Tumour number was also reduced by 22% in the intestine of animals treated with the phytochemical and sulindac. Similarly to my own observation, they also found decreased tumorigenesis in the proximal and in the mid small intestine, while increased tumour number and tumour area was found in the distal SI. There was no effect between different dosages (0, 375, 750, 1500, 3000mg of the anthocyanin-rich tart cherry extract on tumour number or area in any of the three intestinal sections.

In the present experiment the phytochemical mix had a huge effect on increasing tumorigenesis in the colon of ApcMin/+ mice, as mentioned before. The present result is in accordance with that found by Bobe et al. when no sulindac was added to the diet (Bobe, Wang et al. 2006). They found that the prevalence of papillary tumours in the large intestine were similar between the treated and untreated group. However, when different doses of anthocyanin-rich tart cherry extract was considered, they found animals receiving the highest dose, 1500mg of anthocyanin-rich extract/kg of diet had or tended to have a greater prevalence and number of papillary tumours when compared to the group receiving the lowest dose of anthocyanin-rich extract. These results show that the amount of phytochemicals used in the diet might have a great influence on tumorigenesis in the colon of ApcMin/+ mouse.

Another study performed by Cooke et al. (Cooke, Steward et al. 2005) has also used the ApcMin/+ mouse model but showed a positive effect of an anthocyanin-rich diet on preventing tumorigenesis. Furthermore, anthocyanin has also been shown to decrease colonic ACF formation, which is a biological markers for tumorigenesis in carcinogen-induced rat models (Hagiwara, Miyashita et al. 2001; Harris, Gupta et al. 2001; Hagiwara, Yoshino et al. 2002)

Misikangas et al. (Misikangas, Pajari et al. 2007) have also shown a positive chemopreventive effect of 3 different berries on colorectal cancer in ApcMin/+ mice. They have fed ApcMin/+ mice for 10 weeks with a high fat diet with added bilberry (10%), lingonberry or cloudberry. They found that all the berries significantly inhibited the formation of intestinal adenomas by 15-30%. However, only lingonberry and cloudberry inhibited adenoma growth by over 60% in the distal small intestine. In the present study, the phytochemical mix had 0.16% of bilberries and blackcurrant in the phytochemical diet, and maybe this amount was not enough to have an effect however synergisms can occur between the phytochemicals and produce an unpredictable effect. Although no tests were performed in the present experiment to study the synergism between the phytochemical compounds used in the experimental diet.

5.2.3. General conclusion on the effects of phytochemicals

Despite the positive effects of several phytochemicals shown in the literature by different studies including epidemiological, in cell lines and in animals, the real effects of phytochemicals are still unclear, especially in human studies. Another point that should be considered is that in many studies only 1 or 2 phytochemicals are tested. But, in real life people eat daily a combination of phytochemicals of which little is known of their effects in combination or on bioavailability and dose.

In the present experiment, the mix of phytochemicals used in the phytochemical treatment diet significantly increased inflammation, not only in the small intestine, where all the tumour development process was taking place in the animal model used, but also in the colon, and in the plasma. However, in the wild-type mice the phytochemical diet actually had a positive anti-inflammatory effect. Based on these results, it could be speculated that these phytochemicals might be acting in a more specific way. For example their antioxidant effect may adversely modify the carcinogenesis process happening in the ApcMin/+ mice, which might also have a direct effect on the inflammatory markers. The mechanisms of action of phytochemicals in inflammation and carcinogenesis are still unclear, but it has been shown that these chemical compounds present in several foods and plants are considered to have an effect on inhibition of signalling through NF-kB, which its activation plays a central role in inflammation, which consequently affects carcinogenesis. The expression of NF-kB proteins can provide site and event-specificity in response to a particular stimulus, such as activation of pro-inflammatory genes, production of TNF-α, IL-1β, IL-6 and IL-8, and also expression of COX-2 (Tak and Firestein 2001). NF-kB has also been implicated in the induction of pro-inflammatory cytokines in animal models of inflammation, however it is not certain whether its activation in colonic epithelium and

increases in pro-inflammatory cytokine production are the cause or result of NF-kB binding (Tak and Firestein 2001). Overall, more studies on the preventive effects of phytochemicals against the development of colorectal cancer are necessary to be performed in order to clarify by which mechanisms phytochemicals could affect carcinogenesis.

6. CONCLUSION

The present thesis tested in vivo the chemopreventive effects of dietary foods such as fatty acids and phytochemicals on colorectal cancer. The animal model chosen to perform the experiment was the ApcMin/+ mouse because of its similarities to the development of adenomatous polyposis in humans. Thus, the present work contributes to the understanding of how fatty acids and phytochemicals can affect the tumour development of colorectal cancer.

The fatty acids experiment has demonstrated contradictory results. It has been widely published and accepted in several previous studies that n-3 PUFAs may module cytokinetic activities by reducing cell proliferation and increasing apoptosis in models of colorectal cancer. However, in the present study n-3 PUFAs did not have a positive effect in ApcMin/+ mice regarding these endpoints. Besides, contradicting the several studies in the literature, n-6 PUFAs did not look to be pro-carcinogenic by up regulating the cell proliferation activities and inhibiting apoptosis in the animal model used in the present work. The results on inflammation was also contradictory, because n-3 PUFAs increased local inflammation, acting as a pro-inflammatory agent while n-6 PUFAs reduced local inflammation in ApcMin/+ mice. Despite these unexpected results relating to cytokinetics and inflammation, the n-3 PUFAs did inhibit tumour promotion in ApcMin/+ mice by reducing the size of tumours, but not the numbers of tumours. In order to really elucidate the effects of fatty acids on colorectal cancer prevention, more studies are needed as outlined below.

As regards the effects the phytochemicals, the results were not promising either. While there were no effects of the phytochemical mix used in the diet on the cytokinetic activities

in the small or large intestine, phytochemicals did increase the local and systemic inflammation in the disease model. Moreover, phytochemicals also promoted tumour development in ApcMin/+ mice by increasing the number and size of tumours, both in the small and large intestine. Despite all the anti-proliferative, anti-inflammatory and anti-carcinogenic effects that have been attributed to coffee, walnuts, cocoa, turmeric, thyme, and berries in the literature, the present study showed that this specific dietary phytochemical mix did not prevent the promotion of colorectal cancer in ApcMin/+ mice, but instead promoted tumour development. Using dietary foods as source of phytochemicals, the information on individual components and their potential effect are lost, however the relevance to nutrition is increased, as it mimics the human food consumption.

In conclusion, more studies are needed to really understand the effects and the mechanisms by which fatty acids and phytochemicals may or may not be preventive against colorectal cancer prevention.

6.1. LIMITATION OF THE STUDY

The present study has certain limitations that need to be taken into account when considering the study and its contributions. The first limitation to be considered is in regards the animal breeding. Breeding ApcMin/+ mice can be quite challenging, which in the present study this process took a very long time until the total amount of animals were obtained for each treatment group. Moreover, mice at weaning age (3 weeks) were not always ready to go on a treatment diet, as they were too small. So a longer time had to be waited until they could have gone on the treatment diet. Regarding the experimental

design, the dietary treatment diets had to be limited to only a high fat composition diet at 25%kcal fat. However it would have been interesting to also have a normal fat diet (7-9% kcal fat) to be used in the experiment. However it would have doubled the size and the cost of the experiment. Regarding the cytokinetics analysis, cell proliferation and apoptosis were measured at the normal looking gut tissue. However, if the measurements had also been done in the adenoma tissue maybe different effect would have being observed. Moreover, those measurements were done only in the distal part of the small intestine, but having the measurements done in all the parts of the intestine could have shown a better picture of what was happening regarding cell proliferation and apoptosis. The measurement of the cytokines present was also a limitation in the present study. The profile of cytokines was only measured after the dietary treatment at week 10, but not at baseline. Besides, an accurate effect of the dietary treatments could not be verified.

6.2. FUTURE WORK

Following the investigations described in the thesis, a number of further analyses could be carried out. Regarding the fatty acids experiment, a gene expression study could be done to find out how different fatty acids could affect the expression of genes related to cell proliferation, apoptosis, inflammation, and tumorigenesis. Regarding the phytochemical study, separate animal experiments could be done to test the effect of individual dietary foods on tumour development in ApcMin/+ mouse, as well as different mixes of those dietary compounds.

7. CANDIDATE'S INFORMATION

7.1. CURRICULUM VITAE

Jucineide Matos Lima was born in Brazil in 25 January 1978. At the end of the year 2000 she obtained the Bachelor Degree in Nutrition in Brazil, at the Filadelfia University. In 2001 enrolled a post-graduate specialization course in Management Food Quality Control at the State University of Londrina. In 2002 entered a master program in Food Technology at the Federal University of Paraná, where have defended her thesis in 2003 on "Effects of storage temperature and modified atmosphere on physico-chemical and microbiological parameters of yacon tubers". In 2001 started working as a lecturer at the Department of Nutrition at the University of Central East, where she has worked until moving to England to start her PhD studies at the University of East Anglia / Institute of Food Research, in October 2006. During the studies at the University of East Anglia, the candidate had established a collaboration work with Prof. Rune Blomhoff from the University of Oslo, where she has been carrying out her work since 2009.

7.2. GRANT APPLICATIONS

Programme Alban. PhD Scholarship (€ 54 000)	2006-2009
NuGO – Nutragenomics Organisation. Exchange grant (€ 6 600)	2009
Norwegian Research Council. Exchange grant (NOK 130 000)	2010

7.3. PHD TRAINING

Overview of conferences and courses attended during PhD

Introduction to postgraduate research	2006
In vitro and in vivo course on NF-kB-luciferase transgenic mice (Oslo, NO)	2006
Seminar: The Metabolic Syndrome (London-UK)	2006
Developing professional writing skills	2006
Microsoft excel I	2006
Radiation safety induction course	2006
Workshop on Gilson pipette	2006
Computer orientation course	2006
EndNote. Bibliographies management course	2006
Workshop on Nutrition and Inflammation (Florence-IT)	2007
European Nutrition Conference (Paris, FR)	2007
Showcase of Postgraduate Research (UEA)	2007
Animal Licence Home Office. Module I, II, III.	2008
Microsoft excel II	2008
European Nutrition Leadership Programme (Luxembourg, LU)	2009
Sixth European Nutrigenomics Conference (Montecatine, IT)	2009
19th International Congress of Nutrition (Bangkok, TH)	2009
Seminar on Conflict management in different cultural setting (Bangkok, TH)	2009
Challenges from the World Summit on Food Security (Oslo, NO)	2010

7.4. PUBLICATIONS

Lima, J.M. Lecture for workshop. Project Presentation: Impact of obesity on intestinal health and potential protective effects of fatty acids. Workshop in Nutrition and Inflammation. Florence, IT. 2007

Lima, J.M., Johnson I.T., Dainty, J.R., Lund, E.K. Effects of high fat diet on intestinal carcinogenesis. Showcase of Postgraduate Research. Institute of Food Research. Norwich, UK. March, 2009

Lima, J.M., Johnson I.T., Dainty, J.R., Lund, E.K. Effects of different fatty acids on intestinal carginogenesis in ApcMin/+ mice. Sixth European Nutrigenomics Conference. Monticatine, IT. Sept 2009.

Lima, J.M., Johnson I.T., Dainty, J.R., Lund, E.K. Abstract for oral presentation. Effects of high fat diet on intestinal carcinogenesis. 19th International Congress of Nutrition. Bangkok, TH. Jul 2009.

Lima, J.M., Johnson I.T., Dainty, J.R., Lund, E.K. Abstract for distinguished poster presentation. Effects of fish oil on cell proliferation and apoptosis in ApcMin/+ mice. 19th International Congress of Nutrition. Bangkok, TH. Jul 2009.

Lima, J.M., Bøhn, S.K., Johnson, I.T., Blomhoff, R., Lund, E.K. Abstract for poster. Cytokine profiles of plasma and intestinal mucosa can differentiate between wild-type and ApcMin/+ mice and between dietary fatty acids. Nutrition Society Summer Meeting. Edinburgh, GB. Jun 2010

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