CHAPTER 1.

INTRODUCTION AND LITERATURE REVIEW

1.1. Rationale

1.1.1. The Burden of Colorectal Cancer

Cancer of the colon and rectum is the third most common type of cancer worldwide. It is estimated that just under one million people develop this cancer each year, and though recent improvements in early detection and treatment have been made, mortality figures are approximately one half that of incidence, with a 5 year survival rate of 40-50% [1].

The geographical spread of colon cancer cases is perhaps one of the more striking aspects of this disease. It is very much a cancer that is widespread in the more affluent, developed countries. Though 5-10% of colorectal cancer (CRC) cases can be attributed to strong hereditary dispositions and a further 20% of cases occur in people who have a family history of the disease, high CRC rates in the developed world can be heavily attributed to a westernized lifestyle. Risk factors include living a sedentary lifestyle, obesity, smoking, high alcohol intake and a diet high in fat and low in polysaccharides and vegetables [2].

The influence of environmental factors on CRC risk become even more significant when taking migrant studies into consideration [3]. When populations move from low-risk to high-risk areas, the incidence of CRC increases significantly and within the first generation individuals adopt the country's higher risk rates for this disease.

Within Australia, CRC has become a major health problem and we now lead the world in CRC incidence rates. More specifically, South Australian figures now see CRC representing approximately 16% of total cancer cases, coming in at the second most common for women and men, behind breast (28.8%) and prostate (24.6%) respectively [4]. As a result of its extensiveness, CRC directly or

indirectly affects many people, placing a large emotional and financial burden on the community.

The multi staged progression of colon cancer combined with a relatively good prognosis when diagnosed early makes this particular form of carcinogenesis optimal for screening programs. Fortunately, this process is quite effective and has the potential to significantly reduce the mortality load from CRC. Hence, recent efforts in the battle against CRC have concentrated on screening programs and early detection. There are a variety of screening procedures and the risks, costs and benefits have to be taken into account when weighing up the different options.

In the 2005-06 Australian budget, $43.4 million dollars was allocated over 3 years for the implementation of a national bowel screening program [5]. Commencing from mid 2006, faecal occult blood tests (FOBTs) were offered to Australians turning 55 and 65, with general practitioner referrals and follow up colonoscopies for those returning positive tests. Participation rates are of course fundamental to the success of a screening program and recent developments in test kit technology such as the introduction of Faecal Immunohistochemical tests (FITs) has seen participation rates substantially improve [6]. It has been estimated that with perfect compliance using current screening methods, mortality from colorectal cancer could be reduced by 50% [7]. Though this would be an ideal outcome, this type of success requires continuous commitment from all participants and an ongoing effectiveness of all tests. Therefore, though ideal, screening programs are not fail proof.

For that reason and because of the potential to avoid the onset of colorectal oncogenesis completely, primary prevention by the individual through lifestyle and dietary changes would compliment screening programs and have great merit in further assisting with the reduction of CRC and its associated costs. Changing behavioural patterns, such as increasing physical activity, being of a healthy weight and reducing alcohol intake can contribute to lowering one's risk. However, perhaps one of the most relevant and well studied environmental influences in relation to lowering CRC risk is one's dietary habits.

1.1.2. The Effect of Diet on Colorectal Cancer

Foods that are encouraged to be eaten and that correlate well with lower CRC risk include dietary polysaccharides, fruits and vegetables. Though results for fruit consumption are less persuasive, most published studies demonstrate a strong inverse association for these foods [8].

Dietary polysaccharides including resistant starch and fibre act to lessen one's risk through a number of possible ways. These dietary agents may reduce risk by decreasing faecal pH, increasing stool bulk or reducing the transition time through the colon as reviewed by D. Topping and P. Clifton [9]. As a result, it has been estimated that the risk of developing CRC may be reduced by up to 10% for every 10g of dietary fibre consumed per day [8]. As a fermentable substrate for colonic microflora, resistant starch can also cause an increase in colonic fermentation thereby increasing the production of short chain fatty acids (SCFA) in the large bowel [10]. SCFA, specifically butyrate is a primary energy source for colonocytes and has been reported to induce apoptosis and inhibit growth both in animal studies and in tumour cell lines [11-13].

Vegetables and fruits also contain fibre and therefore support favourable bacterial fermentation. Other mechanisms suggested to be responsible for the chemopreventative effects of vegetables include their antioxidant capabilities, dilution and binding of carcinogens, alteration of hormone metabolism and the induction of detoxification enzymes by various cruciferous vegetables [14]. Other compounds obtained through dietary means that have warranted investigation and have shown some promising results include folate, calcium, vitamin D and selenium [15].

At the other end of the spectrum, foods that may be considered as high risk foods include substantial amounts of red meat (usually >140g/d) [8]. Rates of CRC correlate with the national per capita consumption of animal fat and meat when compared between countries [16]. While many studies demonstrate an association between a high intake of animal fat or red meat and a raised CRC risk [17], debate surrounding this topic still continues, with some studies

showing no such association [18]. The mechanism responsible for this possible increased risk remains unclear and popular theory in the past has focused on the formation of carcinogenic agents when cooking red meat.

When cooked at high temperatures, a reaction between creatinine and amino acids occurs in meat resulting in the formation of polycyclic aromatic hydrocarbons and heterocyclic amines [19]. These products are thought to have carcinogenic properties and therefore, they may provide some reason behind the association between red meat and higher CRC risk. Other possible explanations behind this link include the presence of the haem component, formation of N-nitrosocompounds, the high protein content or the high amount of saturated animal fat consumed in a high red meat diet [20-22].

Dietary fat and associated nutrients play an essential role in maintaining the functions and overall health of the body. However, an association between excessive amounts of dietary fat and saturated fat in particular has been long associated with the development of a variety of diseases. Originally it was the strong link found between a high fat diet and cardiovascular disease that lead to the introduction of public heath recommendations advising a reduction in dietary fat. This recommendation has been strengthened by the further numbers of ailments arising as a result of obesity including diabetes mellitus, dyslipidemia and hypertension [23].

Excessive intake of fat has also been associated with certain types of cancer. This association has since been supported by a number of experimental studies. However the importance of not only the quantity of fat intake, but rather the quality and type of fat has been a primary observation to come out of these studies with regard to carcinogenesis. Issues relating to dietary fat and colorectal cancer in particular are influenced by the type of fat as much or more than the quantity of dietary fat consumed [24]. At present, it is understood that diets rich in saturated fatty acids or omega-6 fatty acids have tumour promoting properties while diets rich in omega-3 fatty acids lack such effects and may even help to reduce risk [25].

1.1.3. Omega-3 polyunsaturated fatty acids and chemoprevention

Epidemiological studies in high fish eating populations produced some of the earliest data highlighting a correlation between low CRC risk and polyunsaturated omega-3 fats in particular [26, 27]. The long chain polyunsaturated fatty acids (PUFAs), docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) are important membrane components and precursors to a number of eicosanoids which affect numerous cellular physiological processes. Primarily found in oils derived from marine species, DHA and EPA have also been strongly associated with a number of other health benefits surrounding cardiovascular disease, arthritis and asthma [25].

Though the data are not yet over whelming, the potential of fish oil containing DHA and EPA as a chemopreventative agent for colorectal cancer is significant. While the mechanisms behind this protective role remain unclear, studies have demonstrated a protective role from diets high in these essential fatty acids by reducing both tumour incidence and multiplicity.

This project will complement these previous studies and aims to add to the knowledge in this area in an attempt to clarify the importance of omega-3 in CRC. Furthermore, rather than looking at tumour formation as an endpoint in the later stages of the carcinogenesis pathway, the core focus of this project is instead on the formation, removal and repair of DNA damage – damage that might initiate oncogenesis in the colon.

A better understanding of the early colonic host responses to a genotoxic agent will be of great benefit as these mechanisms are thought to play a vital role in regulating the initiation and early development of CRC. Furthermore, by using a dietary intervention such as omega-3 PUFAs in this acute model we can establish if their reported chemopreventative effects also take place during the initiation phase of CRC. The specific aims and hypotheses of this project are further discussed in section 4.1.

1.2. The Development of Colorectal Cancer

The progression of colorectal cancer follows a multi-staged adenoma-carcinoma pathway that can evolve over a time period of approximately 5 to10 years in sporadic CRC, although it may develop more rapidly in certain inherited syndromes. The change from normal epithelial cells to adenocarcinoma has been well described and is characterized by a progressively disordered genome. The accumulation of genetic changes in colonic cells results in a progressively altered phenotype that eventually gives rise to invasive cancer.

The following section outlines the biology of neoplastic transformation in the colon. It briefly outlines the histological changes observed in the colonic luminal epithelium at the various stages of tumour growth and ends with a summary describing some of the known genetic changes experienced during carcinogenesis development.

1.2.1. The biology of tumour formation

Approximately 99% of colorectal cancers are classed as adenocarcinomas. These arise from epithelial cells that line the crypts present in the mucosal layer of the colon. These epithelial cells act as a barrier between the contents in the lumen and the muscular layers. Colonic epithelial cells have a rapid turnover rate with each crypt producing up to 11 new cells per hour [28]. Generated at the bottom third of the crypt, cells mature and travel up the crypt length to the surface where they are shed into the lumen and excreted with other waste products. The proliferation of these cells is normally tightly regulated by various control mechanisms. The loss of proliferative control and/or defective apoptosis (programmed cell death) is central to neoplasia; the capacity to invade is central to the cancer (i.e. adenocarcinoma) phenotype. All these changes however, are generally considered to result in an unstable genome.

When proliferation increases and the colonic epithelium accumulates, elongated crypts lined by tall columnar cells are formed. This increased proliferation of normal cells together with increase in total cell mass is termed hyperplasia. This

process however, can occur in the normal human colon due to a number of different ailments and physiological stimuli and is not necessarily considered a premalignant condition as the genome is not unstable [29].

The increased proliferative activity or hyperplasia that can occur within single colonic crypts can give rise to aberrant morphological changes. These lesions are known as aberrant crypt foci (ACF) and are the first sign of a pre-neoplastic state [30]. The increased proliferative activity attributed to ACF colonic lesions give them specific characteristics including irregular shaped and dilated luminal openings, increased crypt height and width when compared to normal neighbouring crypts and a thickened epithelial lining around the crypt . From these actively proliferating ACF lesions, adenomas can form, and these are a strong risk factor for the development of CRC.

Adenomas, which sometimes take on the shape of a polyp and then are termed adenomatous polyps, are not homogeneous and are classified by several different schemes including their size, histological characteristics, number and the patient's familial background. Cells in an adenoma are neoplastic and show features of genomic instability. Certain adenomas are considered to have more malignant potential than others, for example, villous adenomas have long been recognised as being more likely to be advanced and at a higher stage of progression than tubular adenomas [31].

The development from adenomas to frank cancers and the possible further progression on to metastasis occurs at a time in which the genome is becoming increasing altered and unstable. The cancer phenotype is reached when the cell becomes invasive. At the stage of metastasis, the tumour has attracted a blood supply, invaded surrounding basal membrane and possibly invaded the venous or lymphatic circulation and then shed cells to distant sites. In the worst case scenarios, cancerous cells will migrate to many distant sites in the body and implant themselves giving rise to secondary cancers else where in the body [32]. At this point, the clinical prognosis for patients is quite poor and long-term survival is unlikely.

1.2.2. Genomic instability in the adenoma-carcinoma sequence

At each stage, the biological progression of colon cancer is accompanied with a variety of diverse genetic alterations. As the tumourigenic process becomes more advanced and involved, genetic damage progresses. During the very early stages however, a normal cell can eradicate the majority of potentially harmful DNA damage through either cell deletion or DNA repair mechanisms. It is when DNA damage escapes these processes, or when the cell is not able to repair such damage or delete itself through apoptosis, that mutations may persist. This in turn, can give rise to progressively altered clones that have the capacity to progress on to cancer.

Genetic alterations that escape repair or removal mechanisms result in a variety of modifications including genetic mutations, deletions and chromosomal rearrangements. Most of the time, such alterations in DNA result in no significant change. However, if located in significant regions of a genome, this damage can alter the function of genes that carry out important cell regulatory functions. Important genes, that if damaged have the potential to influence the carcinogenic process include both proto-oncogenes and tumour suppressor genes.

Proto-oncogenes encode normal cellular proteins, however, when spontaneously affected by a genetic rearrangement or a point mutation, usually caused by damaging environmental exposures, proto-oncogenes are activated and turn into oncogenes. Oncogenes can be involved in the production of growth factors and their receptors and with no form of regulation their uncontrolled activity is oncogenic. One of the most commonly studied oncogenes involved with membrane associated proteins and involved with cellular signal transduction are the RAS oncogenes [33]. The family of RAS oncogenes are activated by point mutations and maintain the configuration of a protein in such a way that constant activation of signal transduction events occur with any ligand or receptor, regardless of their suitability [34].

In contrast to oncogenes which require activation, tumour suppressor genes can contribute to the carcinogenesis process due to their inactivation or deletion by DNA mutations. The normal role of the tumour suppressor gene p53, is to stop cells from beginning the process of DNA replication and activate either cell death or repair if DNA damage has occurred. Its regulatory role in preventing inappropriate cell proliferation is key as it causes cell cycle arrest at the G1 phase in response to DNA damage, suppressing potential tumour formation [35]. When inactivated this regulatory role is lost and cells are free to replicate without any form of control. Unlike oncogenes, the inactivation of tumour suppressor genes, requires the mutation of both alleles, and therefore, this defect can be influenced by one's hereditary disposition as well as from exogenous influences [36].

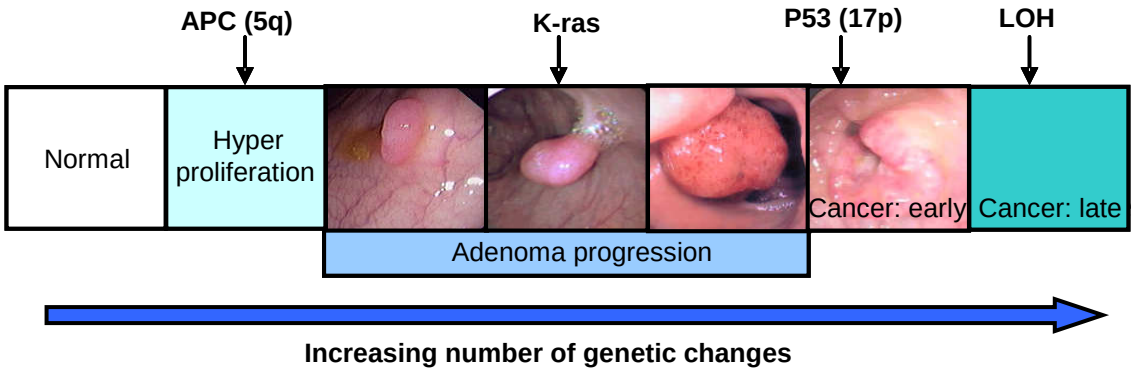
Particular genetic events involving both oncogenes and tumour suppressor genes are known to be associated with either the early, middle or late stages of CRC development. No single gene has been identified as being solely responsible for tumour development, rather, it is more likely that malignancy develops due to a complicated network of molecular pathways involving a variety of these genes and more.

A set sequence of events has been described that relate to genetic changes and the development of tumourigenesis in the human colon as summarised in figure 1. One of the earliest events in this process seems to be global hypomethylation of DNA [37, 38]. The methylation patterns of certain genes are known to be significantly different between normal tissue and adenomas, suggesting a potential role in the initiation of carcinogenesis. The mutation of the APC gene, which is associated with dysfunctional proliferation, is also found in small adenomas and is considered to be an early event in the pathway [39, 40].

Mutations involving the K-ras and p53 genes that support the process of neoplastic growth occur at later stages of the carcinogenesis pathway and are found in larger adenomas (K-ras) and invasive cancer cells (p53) [41, 42]. The later stages are also accompanied with a rise in the overall instability of the genome. A key characteristic of tumourigenesis, genomic instability steadily

increases with neoplastic progression, contributing to the loss of numerous chromosomal segments in the later stages, otherwise known as a loss of heterozygosity (LOH) as observed in the chromosomal instability pathway.

Figure 1: The multi-staged development of colorectal cancer and accompanying affected genes.



1.2.3. Types and causes of genomic instability

It is known that as cancer progresses, the genome becomes more unstable. But what causes normal cellular genes to mutate in the first place?

It is well understood that up to 5% of colorectal cancers are due to a hereditary disposition from either the mutation of the adenomatous poliposis coli (APC) gene as found in Familial Adenomatous Polyposis (FAP), or the mutation of the mismatch repair gene (MMR) in Hereditary Non-Polyposis Colorectal Cancer (HNPCC) [43]. Hence, in these cases, the genome is already primed for initiation and progression to cancer. For healthy individuals however, the spontaneous change from a healthy cell to one containing oncogenic mutations can arise from either a number of spontaneous endogenous reactions or environmental exposures.

As the primary function of the digestive system is to process and absorb the food we consume, the colonic luminal environment is constantly exposed to adverse compounds. When coming into contact with the cellular environment in the colon particular chemical groups have the potential to attach to and alter the structure of DNA.

Though not considered to be mutations at this point, these structural changes, known as DNA adducts, have the potential to give rise to fixed mutations and as a result may play a significant role in the initiation of carcinogenesis at a molecular level. The following section reviews the knowledge surrounding DNA adducts and the potential role they play in the initiation of carcinogenesis.

1.3. DNA Adducts

DNA adducts have become a subject of significant interest in cancer research over the last thirty years. The formation, repair and biology of individual adducts were initially the primary focus of such research, with more recent experimental work looking at the use of adducts as biomarkers in response to various carcinogenic substances [44]. Such data gathered from the past combined with continuing research provides an insight into DNA adducts and their role in the initiation of carcinogenesis.

The following section outlines the biological relevance of DNA adducts, their formation, their role in the initiation of carcinogenesis and their removal and repair. It looks at a specific adduct, O^6methyldeoxyGuanosine (O^6medG) in this relationship and its effect on homeostatic responses. To finish with, the impact of dietary agents on the levels of O^6medG is summarised.

1.3.1. What is an adduct?

A DNA adduct is formed when a chemical intermediate covalently reacts with a DNA base. The chemical intermediate can arise from a variety of sources, both exogenous and endogenous, and may include alkylating agents, oxidative reactive species or bifunctional aldehydes [45]. When one such chemical intermediate becomes bound to a nucleic acid in DNA, the resulting structurally altered DNA is known as a DNA adduct.

Low levels of DNA adducts are expected in all tissues at all times, partially due to the ongoing formation of endogenous adducts. This background DNA damage is the result of a balance between the rates of formation, removal, repair,

cell death and 'dilution' by DNA replication. It is estimated that the level of adduct formation in normal human DNA is approximately 1 modification per 10^7 nucleotides and according to Farmer's calculations [46], this means that there can be anywhere from 600 adducts upwards in one human, adult cell at a time.

Endogenous influences can give rise to oxidative DNA adducts. These are formed when reactive oxygen intermediates (ROI), that arise from oxidative processes in the cell, interact with DNA to form adducts such as 8-oxo-7,8-dihydro2-deoxyguanosine (8-oxodG) [47]. This well-studied oxidative adduct is the main product formed from the reaction between ROIs and DNA and if not repaired, is capable of causing miscodes by DNA polymerase and transversion mutations in human DNA. ROIs also react with proteins and lipids in the cell, however, unlike DNA adducts, formation of these types of adducts are thought to have little impact on the operations of the cell [48].

DNA adducts caused by alkylating agents rather than oxidative processes can also affect proteins and lipids, however again, it is the damage caused to DNA and the genome which has serious implications. The level and nature of an alkylating adduct is dependent on the type of agent the DNA has come into contact with and naturally, the quantity of adduct formation can vary greatly depending on one's level of exposure to the various exogenous alkylating compounds [45]. Exposure to these agents can come from a variety of sources including certain environmental processes, tobacco smoke, occupational exposures or through one's diet.

1.3.2. The *O*^{6}methyldeoxyGuanosine DNA adduct

DNA damage by toxic exogenous alkylating compounds is known to produce over a dozen different types of alkylating adducts which either affect an oxygen or a nitrogen group belonging to a DNA base [49]. Attention has largely focused on *O*^{6}alkylguanine and to a lesser extent, *O*^{4}alkylthymine, with the methyl version of these adducts being the most commonly studied. The N7-methylguanine DNA adduct has also been commonly studied as it is an adduct that facilitates depurination and which is formed most readily in the highest

amounts. However, the most significant biological effects with regards to carcinogenesis is mediated by the adduct, O^6methyldeoxyGuanosine (O^6medG). This pro-carcinogenic adduct has a tendency to pair with thymine during DNA replication, facilitating GC to AT transition, i.e. a change from a paired G and C to a paired A and T [50].

1.3.2.1. Formation of O^6medG

The ability that O^6medG residues have to form miscodes in human DNA makes this an extremely significant adduct when investigating the relationship between adduct formation and the initiation of carcinogenesis.

The formation of O^6meG by alkylating species is not indiscriminate. Several factors influence the creation of this adduct, namely the attraction of methylating ions to the oxygen atom at position 6 of the DNA base guanine. Methylating ions, such as the methyldiazonium ions metabolised from the carcinogenic agent azoxymethane (AOM), have an intermediate reactivity level. This reactivity level is very similar to the nitrogen and oxygen atoms present in DNA. Furthermore, oxygen atoms are slightly stronger bases, as are these methylating species, and therefore, as species of similar properties are attracted to one another, oxygen atoms in guanine bases are more likely to become methylated [51]. A particular DNA sequence itself may also influence the binding of methylating ions [52]. Data demonstrates that several alkylating carcinogens fail to show preferential binding in single stranded oligonucleotides, suggesting that secondary structure of guanine is a major factor in causing sequence dependant binding [53].

Methyl adducts on the oxygen atoms of guanine are formed relatively more extensively in DNA than RNA. O^6medG comprises approximately 25% and 15% of the total amount of alkylated products formed in DNA and RNA respectively when a cell is subjected to alkylating agents during experimental work [54]. Total O^6medG formation does depend however, on the particular alkylating agent used in each study. For example, data shows that O^6medG is formed in greater amounts when alkylating carcinogens such as N-ethyl-N-

nitrosourea (ENU) and N-Nitroso-N-methylurea (MNU) are used when compared to agents such as Methyl methane-sulfonate (MMS).

1.3.2.2. Mispair and mutation properties of O^6medG

The mere attachment of a methyl group to the O^6 position of guanine does not influence or affect the initiation of carcinogenesis. Rather, it is the mispairing properties that ensue as a result of this adduct that can lead to fixation of a mutation, which might then be oncogenic.

If the methylation of guanine has occurred by a particular alkylating agent and the adduct is not removed by repair mechanisms before DNA synthesis proceeds, a base mispair can ensue. The actual base mispair involving the O^6medG results in a thymine base becoming paired to the modified guanine instead of its normal cytosine partner. Hence, instead of retaining the correct and normal G to C base pair in DNA, the mispaired G to T base is now in place [55, 56].

In saying this however, it is also important to note that though mispairs can occur as a result of the changes caused by O^6medG, the base insertion does remain a competitive process with the proper cytosine base-pair still having a high affinity for pairing to the modified (i.e. adducted) guanine. *In vitro* studies have shown that one third of O^6medG lesions pair with the incorrect thymine base while two-thirds pair with cytosine. Therefore, the base pairing process is actually precursor concentration dependant.

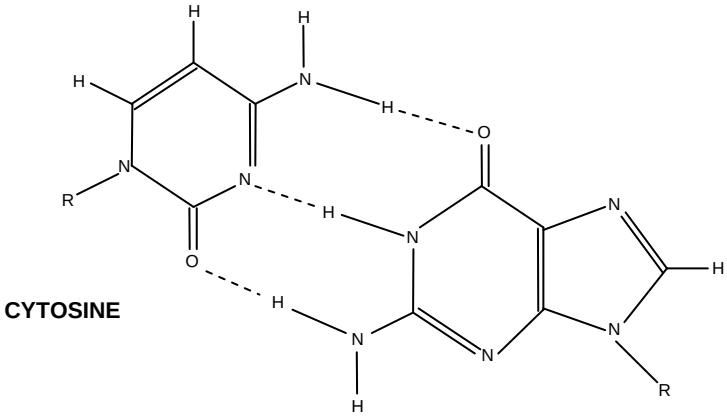
DNA adducts have the ability to form base mispairs for one of three main reasons as reviewed by Margison and Connor [54]. The first involves steric interference. Certain adducts form on DNA in such a way that the modified structure affects surrounding atoms and creates interference with the corresponding base pair. The impact of these adducts often involve the inhibition of DNA polymerase during replication and give rise to errors in RNA more than DNA. The second reason covers mispairs caused by adducts which do not involve hydrogen bond positions and the third reason, and that which

O^6medG is pertinent to, involves the changing positions of the hydrogen bonds between base pairs.

It is very likely that the hydrogen bonding positions of each nucleotide base play a significant role during DNA synthesis. These hydrogen bonds between bases assist the DNA polymerase in identifying which particular base needs to be inserted for its corresponding partner. When the hydrogen bond positions between bases change, as is the case with the introduction of O^6medG, the polymerase reads and amplifies the tautomeric form of the base in the template instead. As a result, allosteric changes are caused in the polymerase and the affinity for the incoming nucleotide is changed (see figure 2). It is likely that the exonuclease activity (proof reading) of polymerase I is also changed, as in order to become a mispair a modified base has to escape the normal selection and corrective processes involved in DNA synthesis. If no repair mechanism is put into place and the mispair remains, with a second round of DNA synthesis the mispair can then be converted into a "fixed" A to T mutation.

Figure 2: From O^6medG adduct to mutation – the mispairing properties of O^6medG

1.



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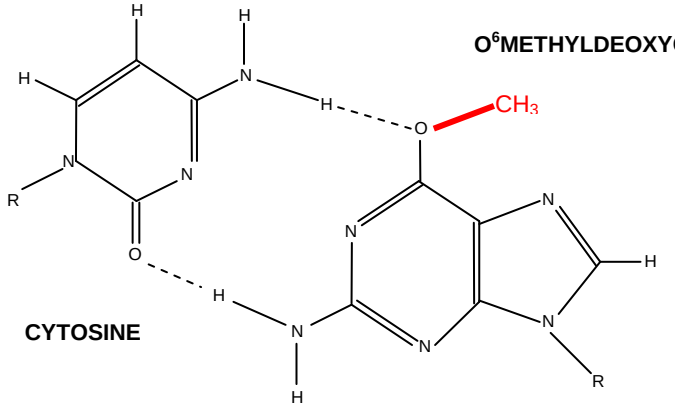
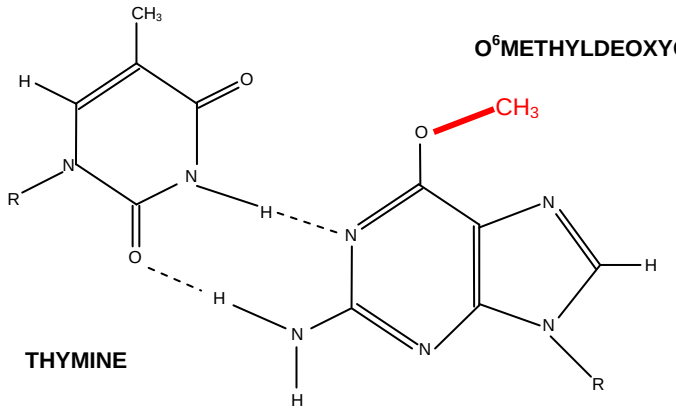
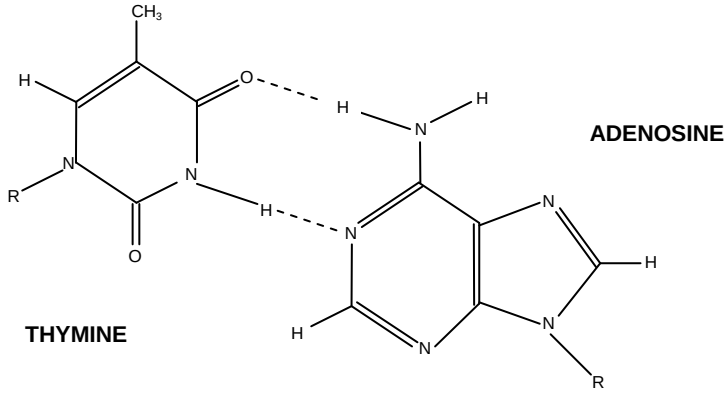


3. **O^{6}METHYLDEOXYGUANOSINE**



THYMINE

4. **ADENOSINE**



THYMINE

These 4 structures demonstrate the bonding changes and creation of a mispair which take place following the formation of the *O*^{6}MedG adduct. 1) A normal guanosine paired with a cytosine 2) The attachment of a methyl group on the *O* position of the guanosine affects hydrogen bonding and creates an *O*^{6}methyldeoxyguanosine and cytosine base pair 3) With changed bonding positions, the *O*^{6}methyldeoxyguanosine is mispaired to a thymine 4) Finally, the mispair is complete with the corresponding adenine base pairing up with the incorrect thymine base. *R denotes sugar and phosphate backbone of DNA. (From Margison *et al.*, 1979, [54])

Following the introduction of a mispair after one round of DNA synthesis the MGMT repair protein can still operate to remove the methylated lesion from the damaged guanine and return the base to its normal structure. However, if this mechanism is not put into place and a second round of DNA synthesis goes ahead, the mispaired base can be converted into a “fixed” A to T mutation.

The frequency in which this GC to AT “point” mutation occurs does depend on the extent, location and nature of a reaction as well as the frequency of DNA synthesis in cells. More specifically, studies have found that particular guanines that have been methylated are more likely to mutate than others. Burns *et al.*

[57] demonstrated through the use of oligonucleotides that a mutation in the first guanine in a run of two or more guanine residues is not often observed. In addition to this, guanine residues preceded by another guanine or an adenine were more likely to mutate than those preceded by pyrimidine residues. Nevertheless, all that is needed to make this point mutation biologically significant to carcinogenesis initiation is its presence in a region of a gene which plays a role in this process.

For example, the transformation of the significant K-ras proto-oncogene to an active oncogene is very likely to be related to the methylation of the normal gene and its eventual point mutation. A normal section of the gene sequence found in K-ras includes a GGA-CCT codon that encodes the protein glycine. If the second guanine in this particular sequence is methylated and progresses on to become a mutation, the codon will change to one that reads GAA-CTT and a glutamine acid will now take the place of the original glycine protein. This change in protein configuration, initially brought on by an O^6medG residue, can cause the activation of a K-ras proto-oncogene and by doing so expose the cellular environment to various other oncogenic effects.

1.3.2.3. O^6medG levels in human and animal studies

Human studies have demonstrated a considerable amount of variation of O^6medG levels in the DNA of tested human populations. This variation not only differs from person to person, but also within different regions of the body. O^6medG adducts are readily detectable in the human colon and levels tend to be higher in the more cancer prone distal colon as opposed to the proximal colon [58]. This variation may be a consequence of personal exposure to specific alkylating agents, derived from either exogenous sources, or alternatively, endogenous agents arising from the chemical or bacterial colonic nitrosation of amines.

Rao completed a study in which Fischer rats were injected with two doses of 15mg/kg of the alkylating carcinogen azoxymethane (AOM). The entire colon

of each rat was resected and adducts level were quantified at 232 ± 24μmol O^6MedG /mol of guanine in DNA [59].

Jackson *et al*. looked at the presence of O^6medG adduct in mouse colon after the administration of DMH and found that O^6medG levels varied with regard to location, being higher in the distal region of the colon, and also time after the carcinogen injection. Different dosages of carcinogen however, did not intensify or change O^6medG levels in any way. When carried out to a 20 week tumour study, tumour yield correlated well with the cumulative amount of O^6medG measured in DNA over the treatment period [60].

O^6medG data obtained by immunohistochemical work done by Hong [61] indicate that levels of this particular adduct are barely detectable at 0h following the administration of the carcinogen AOM and then gradually increase over a 12 hour time course. Adducts levels were slightly, but significantly higher in the bottom third of the crypt which can be attributed to either a greater amount of damage or lower levels of repair.

1.3.2.4. O^6medG as an early biomarker for CRC

Experimental data shows that O^6medG levels in the colon correlate well with eventual tumour formation. Consequently, the presence of this adduct in colonic cells can be considered as an early biomarker of DNA damage and ultimate colon cancer risk. The measurement of O^6medG levels in tissue is therefore a valuable tool in analysing the early effects of various dietary agents on carcinogenesis initiation at a molecular level.

A few studies have looked at the effect that amounts of various foods or food components have on adduct levels in the hope that particular dietary agents either have a positive or negative effect on DNA damage which can then be interpreted into possible CRC risk. O^6medG in particular has been measured in a few animal studies using different dietary components. These studies are briefly described below.

Dithiolthione, a compound that naturally occurs in cruciferous vegetables was tested for its potential anti-carcinogenic properties by Rao *et al*. [59]. The agent oltipraz was given to a group of rats at a level of 300p.p.m and compared to a control group fed the standard AIN-76A diet. Adduct levels were measured 15h after AOM administration and analysis of both O^6medG and N7meG showed that the addition of dietary oltipraz did significantly inhibit these induced adduct species.

Barch and Fox looked further into the association between oesophageal cancer and the combination of a zinc deficient diet and the chemical methylbenzylnitrosamine (MBN). This combination is known to increase the incidence of human oesophageal cancer and this increased incidence was supported when reproduced in a rat model. When oesophageal O^6medG was measured, rats sustained on a zinc deficient diet had significantly higher amounts than the control group, correlating well with eventual carcinoma formation [62].

A supplement of 50p.p.m. of beta-naphthoflavone (BNF) was trialed in the diet of Wistar rats for 1 week by Tacchi-Bedford *et al.* [63]. O^6medG were measured in the colon at 1, 12 and 24h following the subcutaneous administration of DMH and O^6medG levels were found to increase in the colon of BNF treated rats when compared to control levels. One explanation for this unexpected rise as discussed by authors was the potential of this supplement to impact the rate of metabolic activation of DMH, possibly raising its binding potential to DNA.

Jacoby *et al*. tested a supplement of calcium at dosages of 0.87 and 1.8% by weight on a Sprague-Dawley-DMH animal model. Calcium supplementations at both dosages were found to have no significant effect on O^6medG levels, repair protein or proliferation rates in the colon either before or after the administration of DMH [64].

Shimpo *et al*. fed male Fischer rats a basal diet with the added supplement of either 5% Aloe arborescence (ALOE) or 0.25% of a commercially available crude aloin [65]. Animals were sacrificed 6h after the injection of AOM and O^6medG levels were measured in the colon. While aloin supplementation did

slightly decrease O^6medG levels when compared with controls, the ALOE addition significantly inhibited the O^6medG levels measuring a 50% reduction of positively stained cells. These results coincide with experimental data observing an inhibitory effect of aloin and ALOE on the AOM induced ACF formation in rat colon.

Lemon grass extract has also been tested in a rat-AOM model, with doses of 0.5 or 5g/kg body wt administered by gavage, significantly inhibiting DNA adduct formation in the colonic mucosa of rats. In addition, this inhibitory effect was also carried through and observed with regard to ACF and tumour formation [66].

Maintaining Fischer rats on a diet supplemented with 5p.p.m. selenium and subjected to the administration of the methylating tobacco specific agent N-nitosamine 4-(methylnitrosamino)-1-3-(3-pyridyl)-1-butanone (NNK) recorded lower levels of O^6medG by 16.5% when measured at 4h post carcinogen injection and compared to control groups. When fed an increased level of selenium at 15p.p.m O^6medG inhibition in liver tissue rose significantly from 16.5% to 69.5%. O^6medG levels in lung tissue were also significantly reduced by 73.1% in the selenium fed groups [67].

Using this same tobacco carcinogen, el-Bayoumy *et al*. found that a high fat diet significantly enhanced the formation of O^6medG in the rat lung 4h after the final carcinogen administration [68].

A diet high in total fat however seemed to promote O^6medG formation in previous studies, Hong (2000) found that a diet using fish oil as the primary fat source had an inhibitory effect on adduct levels when compared to a control diet using corn oil as a fat source. O^6medG levels were lower at 6,9 and 12h after AOM administration [69].

1.3.3. The removal and repair of DNA adducts

Generally, all DNA adducts including O^6medG are repaired or removed by mechanisms that are well established in the cell before a mutation can be established. The effectiveness of these mechanisms is vital in maintaining a relatively low steady state of DNA adducts. Active removal or repair of DNA adducts can occur by several different processes. One of the most vital and innate mechanisms of removal includes apoptosis, while repair of adducts or their effects are carried out by either a direct repair protein (such as MGMT as is the case with O^6medG), Base Excision Repair (BER) or the Mismatch Repair system (MMR). The mechanisms of these systems are explained in more detail below.

1.3.3.1. Apoptosis

Apoptosis is a form of programmed cell death or suicide that is central to maintaining tissue homeostasis in all animals. Spontaneous apoptosis can occur in healthy tissue as a means of removing an unwanted or old cell in order to maintain a healthy tissue structure. Alternatively, apoptosis can also occur in cells that have suffered DNA damage such as a DNA adduct [70].

The process of removing these damaged cells through apoptosis is an intrinsic defence mechanism against tumourigenesis. During CRC development it has been shown that the pathways which regulate apoptosis become increasingly disordered [71]. As a result, genetically damaged cells escape deletion and this can lead to an increase in further clones with similar defects. Ultimately, the break down of the apoptotic pathway adds to the overall instability of the genome during the initiation and progression of CRC.

There are numerous genes which play an important role in the onset of apoptosis. The p53 gene, deemed the “guardian of the genome” provides a form of surveillance and initiates a rapid response to DNA damage [72]. It can either cause cell cycle arrest at G1, allowing for time to assess any damage or it can act directly to initiate apoptosis by affecting Bcl-2 proteins[73, 74]. The Bcl-2

family of proteins including the pro-apoptotic BAX and anti-apoptotic Bcl-2 proteins are then able to control mitochondrial permeability. Cytochrome C is then released from the mitochondria and this in turn initiates a downstream activation of caspase enzymes which then carry out then degradation of the chosen cell [75].

The degradation of the cell occurs in a characteristic way that is unique to apoptosis. These distinctive morphological changes include cell shrinking, nuclear fragmentation and chromatin condensation [76]. All of which are identifiable under the light microscope and which set apoptosis clearly apart from other forms of cell death such as necrosis.

When subjected to an insult of genotoxic carcinogen such as AOM, colonic epithelial cells undergo an acute apoptotic response to a genotoxic carcinogen (AARGC) [77]. This early host response is important in deleting any damaged cells containing mutagenic DNA adducts that have the potential to initiate neoplastic transformation. By increasing this AARGC response, the likelihood of any damaged cells escaping removal is lowered. Therefore, any agent that induces apoptosis can be considered beneficial to reducing the risk of tumourigenesis initiation.

1.3.3.2. Methylguanine methyltransferase (MGMT)

MGMT (also referred to as ATase, AGT and AGAT) is a repair protein that not only repairs unwanted methyl groups from DNA, but is also capable of removing other alkyl, 2-chloroethyl, benzyl and pyridyloxobutyl adducts. However, the removal rate of the adduct does depend on the size of the alkyl group. Thus, MGMT repairs methyl adducts much faster than other larger alkyl groups.

MGMT has little or no sequence specificity and its sole function is to remove the adduct lesion from the affected guanine. It does not have the capabilities to interfere with or replace nucleotide bases with their correct partners and it is able to repair O^6medG independent of whether it is paired with cytosine or thymine.

As a result, if repair does occur post-replicatively, a guanine-thymine mismatch may be left behind.

The action of this repair protein involves the direct transfer of the unwanted methyl group from a guanine residue to a cysteine acceptor group in the protein itself. The cysteine residue is located internally within the repair protein and the O^6medG residues are located within the internal structure of the DNA helix. As a result, a specific amino acid is needed to bring the methyl residue and the acceptor group closer together in order to see the reaction eventuate. Once this amino acid is inserted into the targeted base it causes the base to rotate and the methyl group gets expelled out of the DNA helix and into a binding pocket containing the cysteine acceptor site. This in turn, removes the adduct and in doing so inactivates the protein. The inactivated MGMT protein is then subsequently degraded through ubiquitination pathways [78].

One MGMT molecule can only repair one alkyl adduct before being inactivated, therefore, the cells capacity for removing O^6medG depends on the number of MGMT molecules present in the cell and the rate at which the cell can re-synthesize MGMT [79].

MGMT is not an essential protein as knockout mice are viable and phenotypically normal, but they do show increased gene toxicity and a high frequency of tumours following treatment with a genotoxin [80-82]. While in the AOM rat model, MGMT inhibition was shown to increase the formation of colonic tumour incidence and multiplicity [83].

Immunostaining of rat colonic tissue has shown the presence of MGMT primarily located in the upper non-proliferative compartment of crypts. Following the insult of an alkylating agent, these levels peak at 9h and level off through to 12h [61]. Studies by Zaidi reported similar findings with regard to distribution in which MGMT levels were greatest towards the top of the colonic crypt in normal tissues [84].

However, other studies using a tritium based assay suggest that MGMT levels are depleted in the colon to non detectable levels following the administration of an alkylating agent. Jacoby *et al*. put base line MGMT levels in the colon at approximately 0.9fmol/ug DNA. Following DMH administration MGMT levels were not again detectable until 72h post injection [64]. Jackson *et al*. also measured the base line MGMT activity in colonic epithelial cells at 1fmole/ug DNA and at 56h post DHM administration MGMT levels were still only 50% that of the control tissue [60]. While Margison *et al.* [85] reported a peak induction of MGMT only 24h after exposure to the inducing agent.

It has also been suggested that epigenetic silencing of the MGMT gene may occur in normal tissue and that this may have an effect on tumourigenesis. Polymorphisms in the MGMT have been described however their significance currently remains unclear.

1.3.3.3. Base excision repair (BER)

The base excision repair pathway focuses on the repair of a mispaired base rather than the removal of an adduct itself. While it remains unclear as to whether or not O^6medG is repaired via this pathway, it is known that other less oncogenic alkylation adducts such as N7meG, N3meA and N3MeG are [86].

Initially, a specific DNA glycosylase recognises the distortion of the affected base and a further glycosylase is enlisted to remove the modified base by hydrolysing the N-glycosidic bond, thereby leaving an apurinic site. DNA polymerase β then inserts a single nucleotide in the repair patch while also releasing a lyase which releases the 5` dRP residue from the incised AP site. Longer patches of up to 10 nucleotides can also be repaired with the additional input from the Polε, Polδ and PCNA proteins [87].

While MGMT appears to play a more vital role in removing the O^6medG lesion specifically and preventing its mutagenic effects, the BER pathway appears to be more important in the defence against adducts in a non-replicating cell.

1.3.3.4. The mismatch repair pathway (MMR)

Like BER, MMR does not deal directly with the repair or removal of an adduct itself, but rather repairs the consequence of an adduct such as a base mispair. The extensively studied MMR pathway is critical to maintaining the stability of the genome and deals with base mismatches that arise from a number of sources including base oxidation, demination, oxidation or methylation caused by the formation of adducts or otherwise [88].

The MMR pathway has been extensively reviewed [89-92]. In brief, there are 9 mammalian MMR genes including MLH1, MLH3, MSH2-6 and PMS1-2. These proteins interact with one another to create a variety of different complexes that each have there own specific function. While the exact mechanism of identifying each incorrect mispair remains unclear, it is thought that the MutSα homolog (a MSH2 and MSH6 heterodimer) in the MSH family of proteins directly scans the length of double stranded DNA in a 'sliding clamp' action in search for any base mispairs [93]. When a mispair is recognised the assembly of other complexes including a MutL homolog of MLH1 and PMS2 occurs [94]. The excision of the inaccurate DNA strand is then performed by endonuclease 1 followed by the re-synthesis of the correct sequence by Polδ.

This MutSα homolog is able to repair single base substitutions and 1 bp mutations, while the MSH2 and MSH3 heterodimer can repair insertion and deletion mutations of up to 4bp. It is these mechanisms that are thought to be the most important in repairing mutations and preventing cancer formation in the MMR pathway.

It is currently thought that MMR proteins also play a role in preventing carcinogenesis by initiating an apoptotic response to damaged DNA as reviewed by B. Kaina [95]. Knockout mouse models have been used to demonstrate a significant reduction of apoptosis in a mouse deficient in MMR. The mechanism behind the influence MMR has on apoptosis remains unclear, however, initiation from the formation of double strand breaks via failed MMR repair of O^6medG-

created base mispairs has been proposed [96-98]. This O^6medG-mediated apoptotic pathway requires the completion of 2 cell cycles to occur.

Germline mutation in one or the other of the MMR genes, hMLH1 or hMSH2, is a known cause of hereditary non-polyposis colorectal cancer (HNPCC), with up to 85% of HNPCC families having identifiable mutations in these two genes. Additional data highlights the importance of MMR in colorectal cancer cases with somatically acquired defects in MMR genes attributed for up to17,000 new CRC cases each year in the United States [99, 100].

1.4. Diet and disease interactions

The primary prevention of colorectal cancer through lifestyle, nutrition or exercise has been the core focus for many researchers over the past two decades. The large international variation in CRC incidence and many population comparisons imply that one's lifestyle, diet or amount of physical activity can significantly impact the risk of developing CRC.

General lifestyle behaviours including exposure to tobacco products, excess alcohol intake, physical inactivity and excess body weight are consistent risk factors for colon cancer [2]. Diet and nutritional factors are also clearly important, particularly with CRC, as any type of food consumed has a direct relationship with the luminal environment in the bowel.

A large range of different types of foods and micronutrients have been tested to try and determine their beneficial or detrimental effects in relation to CRC development. While many tested components can give conflicting or inconclusive results, others, by means of repeated studies, demonstrate correlations to either an increased risk, such as a high fat or red meat diet or a decreased risk, such as vegetables and a high fibre diet.

A diet high in fat has long been linked to various health related problems including cardiovascular disease, diabetes and obesity. As a result the amount and type of fat consumed has warranted high levels of investigation into its link

with various types of carcinogenesis. Numerous studies have looked into the connection between fat and colon cancer in particular and this relationship is discussed in more depth in the following section.

1.4.1. Dietary fats and CRC

Accumulating evidence concerning the implication of dietary fat in cancer aetiology suggests an association between the two. Moreover, inconsistencies in both epidemiological and experimental studies highlight the fact that not only the amount of fat is important but the origin and type also play a significant role. Fats obtained through the diet can be separated into two categories; saturated and unsaturated. If we then continue to further divide these two categories depending on fatty acid chain length, we can then more specifically analyse the relationship between type of fat and CRC risk.

An increased CRC risk has been associated with diets high in saturated fat and cholesterol. Medium chain fatty acids (MCFAs) are saturated fatty acids including lauric and myristic acids (C14:0). The proportion of these MCFA are determined by the relative contributions of fats and carbohydrates in one's diet and like total saturated fat, have been associated with a possible elevated risk of CRC. However, unlike the MCFAs, experimental data on the longer chain saturated fatty acids (LCFAs), that are predominantly found in vegetable oils and that fall in the length category of C16:0 to C22:0, have displayed no consistent pattern associated with risk of CRC [16].

A similar type of pattern between the unsaturated fatty acids has also been observed. Monounsaturated fatty acids (MUFAs) found in a variety of nuts, red meat and dairy products such as palmitoleic, oleic and erucic have also given unconvincing data when studying their relationship to carcinogenesis. However, the polyunsaturated fatty acids (PUFAs), have given promising experimental and epidemiological data showing an association between this type of unsaturated fatty acid and a decreased CRC risk.

1.4.2. Omega-3 polyunsaturated fatty acids

Long chain PUFAs are classified by their chain length (18-26 carbon atoms) and the 2 or more double bonds they possess (degree of unsaturation). From a nutritional point of view, the most important PUFAs are linoleic acid (LA 18:2n-6), linolenic acid (LNA 18:3n-3), arachnidonic acid (AA 20:4n-6), eicosapentaenoic acid (EPA 20:5n-3) and docosahexaenoic acid (DHA 22:6n-3). Both LA and LNA are considered to be essential fatty acids and must be obtained through dietary means as we do not possess the desaturase enzymes required for their synthesis.

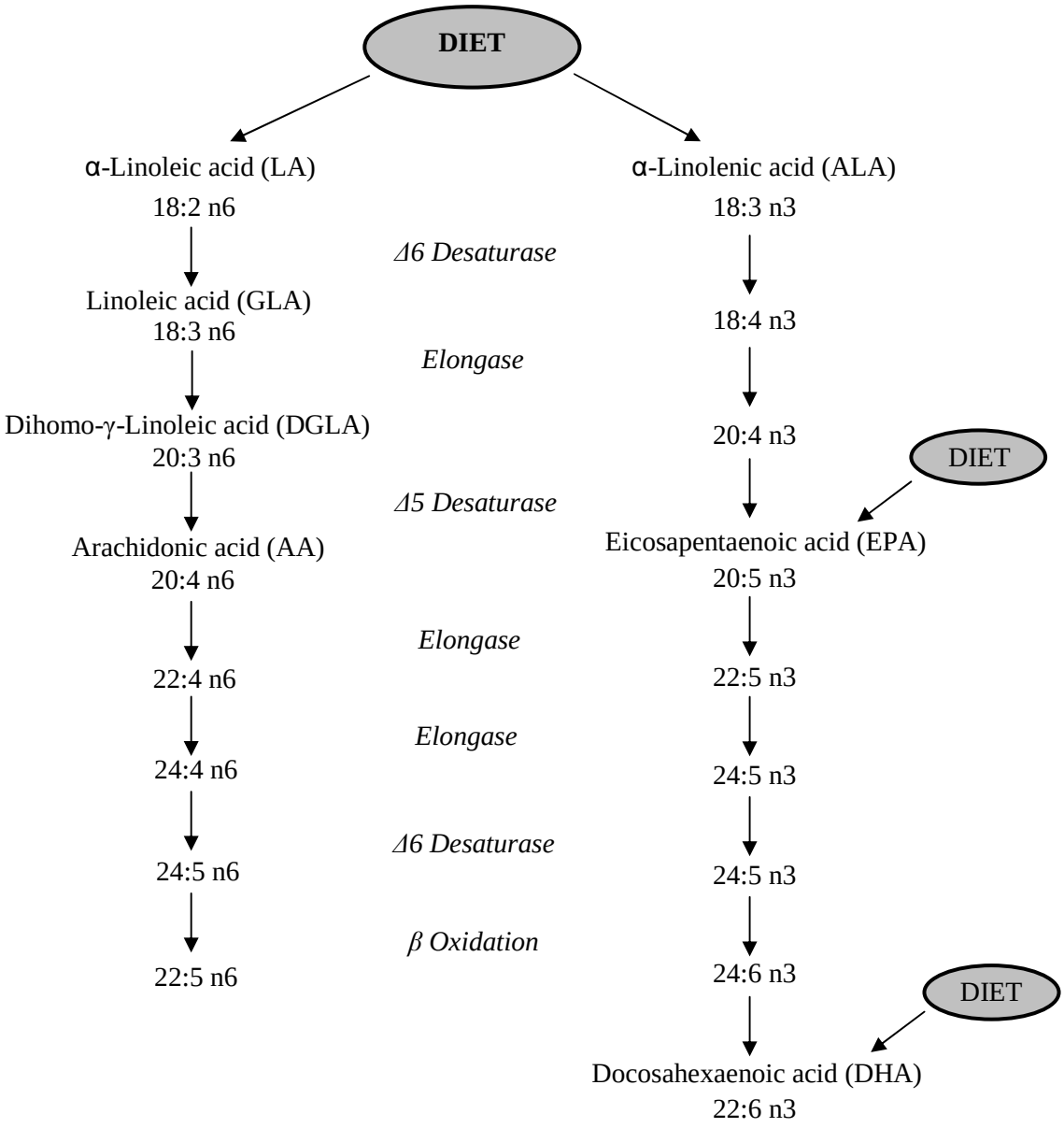
The down stream derivatives of both omega-6 LA and omega-3 LNA (see figure 3) are critical components of cell membranes and precursors of eicosanoids. However, it has been shown that the conversion of LNA to EPA and DHA is quite limited in humans and therefore, due to the numerous beneficial effects on health these two omega-3 PUFAs have, a diet high in both EPA and DHA is highly recommended [101].

The best source of both DHA and EPA is from marine species. These omega-3 PUFAs are synthesised in the chloroplast of marine phytoplankton, zooplankton, microalgae and autotrophic bacteria [102]. Fish species then incorporate omega-3 PUFAs into their visceral organs, their adipose tissue and into their muscle fat by feeding on these micro-organisms. Therefore, fish with higher fat ratios such as the sardine, menhaden, mackerel and salmon have higher levels of both DHA and EPA.

However, this ratio can still vary within species depending on the place and season of capture. Water temperature does seem to have an influence on omega-3 oil content of fish. The theory behind this suggests that the degree of unsaturation of fatty acids in the tissues of fish is increased in colder waters to compensate for the reduction in the fluidity of membranes. Though the lipid content in edible parts of marine fish is known to vary greatly, they can still be classified into four groups depending on their common lipid content. These groups include lean fish (<2% fat), low fat fish (2-4% fat), medium fat fish (4-

8%) and the high fat fish (>8%). In general, it is accepted that fish with high lipid content and that breed in colder waters are the best source of essential omega-3 fatty acids [103].

Figure 3: The pathway of omega-6 and omega-3 PUFA synthesis



The various PUFA derivatives from omega-6 LA and omega-3 ALA fatty acids are listed above. EPA (20:5n-3) and DHA (22:6n-3) can be synthesised from ALA, however this is not an efficient process and it is recommended that these essential omega-3 PUFAs be obtained through the diet.

1.4.3. Potential mechanisms of chemoprevention via omega-3 PUFAs

Growing evidence supports the chemopreventative action of omega-3 fatty acids. Though their inhibitory effect against the promotion and progression of colon cancer has been shown, conjecture surrounding the mechanisms behind this action still remains. Several molecular mechanisms have been proposed, with explanations involving influences on eicosanoid biosynthesis, gene regulation, transcription factor activity, membrane properties and the creation of lipid peroxidation products and their affect on apoptosis. Some of these mechanisms are revised and discussed in more detail in the following section.

1.4.3.1. Inhibition of unfavourable eicosanoid biosynthesis

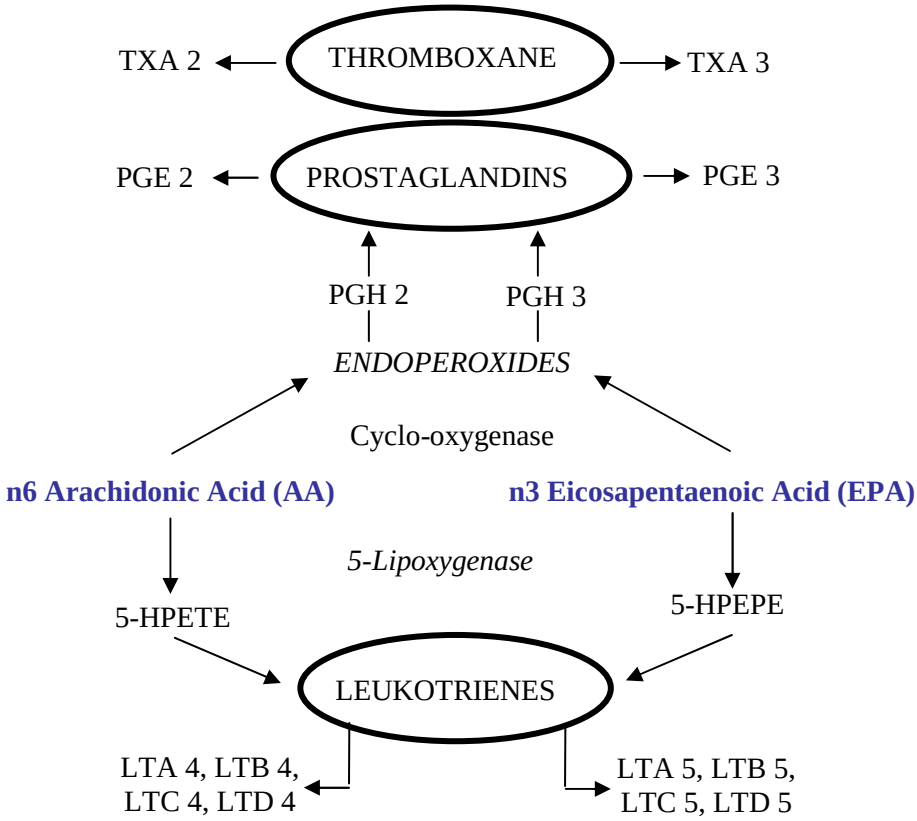
An important function of PUFAs involves their enzymatic conversion into eicosanoids. Once freed from membrane phospholipids, PUFAs serve as substrates for cyclooxygenases, lipoxygenases or cytochrome P450 monooxygenases. The cyclooxygenases give rise to prostaglandins and thromboxanes and the lipoxygenases produce leukotrienes, hydroxyl fatty acids and lipoxins. All of these are short lived hormone like lipids that are biologically potent and have an effect on a wide range of activities including the modulation of inflammatory and immune responses, cellular growth and cellular differentiation.

Linoleic acid (LA, 18:2n-6) and α-Linolenic acid (α-LNA, 18:3n-3) are the prodominent plant derived dietary PUFAs. They are also the precursors of dihomo-γ-linolenic acid (DGLA, 20:3n-6), Arachnidonic acid (AA) and eicosapentaenoic acid (EPA), which are the three fatty acids from which these eicosanoids are formed. The most important factor here is that eicosanoids derived from the omega-6 fatty acids are quite different and have shown to have inflammatory and tumour promoting activity when compared to those derived from the omega-3 fatty acids [104].

Ordinarily, as the majority of cell membrane is usually composed of omega-6 PUFAs, the various eicosanoids generated would be from omega-6 PUFAs

including the 2-series prostanoids and the 4-series leukotrienes. These particular eicosanoids in general have proinflammatory properties, are proliferation stimulants and inhibit apoptosis, with some being positively linked to carcinogenesis. Whereas, eicosanoids derived from omega-3 substrates are of the 3-series prostanoids and 5-series leukotrienes and have the opposite effect, being anti-inflammatory, suppressing proliferation and inducing apoptosis (see figure 4) [105, 106].

Figure 4: Metabolism of AA and EPA via cyclooxygenase and 5-Lipoxygenase



Omega-6 PUFAs (AA) and omega-3 PUFAs (EPA) give rise to different classes of prostaglandins and leukotrienes. The prostaglandins and leukotrienes derived from omega-3 PUFAs are considered to be more beneficial to cellular health having pro-apoptotic and anti-inflammatory properties.

As a high dietary intake of omega-3 PUFAs is likely to result in a higher incorporation of omega-3s into membrane phospholipids, they can partially replace omega-6 PUFAs and as a result decrease their availability as eicosanoid precursors. Therefore, the biosynthesis of these tumourigenesis promoting eicosanoids is suppressed by means of substitution in favour for the more favourable omega-3 derived eicosanoids [107].

In addition to reducing these unfavourable eicosanoids by means of substitution, omega-3 PUFAs also actively compete with omega-6 PUFAs for desaturases and elongases, with which they also have a higher affinity for. This in turn means that dietary the conversion from dietary LA into AA is also reduced as is the production of omega-6 eicosanoids.

1.4.3.2. Influence on COX-2 Expression

Another mechanism thought to be behind the chemopreventative actions of omega-3 PUFAs is their ability to suppress levels of the prostaglandin endoperoxide synthase, known as cyclooxygenase or COX. This enzyme resides in the cell membrane and is involved in the conversion of PUFAs to prostaglandins. It has 2 iso-forms, COX-1 and COX-2. While COX-1 is expressed in all tissue types, COX-2 is a pro-inflammatory enzyme found in the gut that is generally only expressed following some form of pro-inflammatory or mitogenic stimulation.

The COX-2 enzyme has been shown to be important in colorectal cancer. It is upregulated in 80-90% of colorectal carcinomas and 40-50% of adenomas in humans [108] and is also up-regulated in rat tumours produced following the administration of carcinogen [109, 110]. Furthermore, a variety of COX inhibitors have been shown to protect against cancer development [111, 112].

The mechanism behind COX-2 promoting colorectal cancer is uncertain but it is thought that either its ability to down regulate the apoptotic pathway or its role in converting AA PUFAs to pro-inflammatory prostaglandins may be involved [113].

1.4.3.3. Influence on gene expression and transcription factors

Long chain PUFAs through either their eicosanoid derivatives, as free fatty acids or as a result of there conformational changes to membrane structure have the ability to affect the transcription of significant genes. Originally, these PUFAs were only thought to act on a single subfamily of nuclear receptors known as

peroxisome proliferators activated receptors (PPAR's) [114, 115]. However, it is now known that they have the ability to either directly or indirectly affect many genes through additional transcriptional factors such as nuclear factor-κβ (NF-κβ), retinoid-X-receptor-α (RXR-α), sterol regulatory element binding protein-1c (SREBP-1c) and liver X receptors. The influence that omega-3 PUFAs have on some of these transcriptional factors in favouring a less malignant cellular environment is discussed in more detail below.

PPAR is a family of transcription factors that have been identified as possible fatty acid receptors. The three isoforms of PPAR are expressed in adipose and muscular tissues and in high amounts in the colonic mucosa. They form interactions with an array of nuclear proteins, known as co activators, which mediate contact between PPAR and the chromatin and basal transcriptional machinery which promote and repress gene activation. The activation of PPAR is said to inhibit NF-κβ function, cytokine and COX2 expression and as omega-3 PUFAs readily bind to PPARs and activate this transcription factor, they thereby indirectly create a setting less likely to promote tumourigenesis [116].

The nuclear transcription factor-κβ is involved in cytokine gene expression, cellular adhesion and cell cycle activation and apoptosis. The activation of this NF-κβ has been shown to be involved in tumour growth and the incorporation of omega-3 PUFAs has been reported to decrease NF-κβ activation thereby, affecting tumour formation and growth [117].

1.4.3.4. Formation of lipid peroxidation products and apoptosis

Recent evidence suggests that oxidative stress caused by omega-3 PUFAs may favourably modulate the apoptotic response in cells. More specifically, when DHA is incorporated into the inner mitochondrial membrane phospholipid, membrane lipid oxidation occurs and this event is correlated well with the induction of oxidative stress and apoptotic signalling [118]. Furthermore, the addition of antioxidants partially reverses this effect, giving more weight to this particular case.

The opening of the mitochondrial permeability transition pore is mechanistically linked to cytochrome c release in certain models of mitochondrial apoptosis. Little is known about the effect that fatty acids have on the dynamics of this pore, but it has been shown in recent studies that DHA pre treated cells have a higher resting membrane potential when compared to LA pre-treated cells [119, 120]. This effect on membrane potential via mitochondrial membrane lipid oxidation may then favour the opening of the pore and encourage apoptosis. The rise in apoptosis is favourable as cells with DNA damage that have the potential to mutate are more likely to be eradicated [121].

1.4.4. Population, cohort and case control studies and omega-3 PUFAs

Epidemiological studies provide much of the available information about diet and cancer risk. Early assumptions on the health benefits of fish oils and omega-3 fats originated from population studies highlighting the good health of the Eskimos, a community with a high intake of fatty marine fish [27].

Like the Eskimos, the Japanese have, historically, had a relatively low incidence of colon cancer. A diet abundant in fish is a normal occurrence in these cultures, with daily consumption levels being 10 to 30 times higher than that consumed in the average western diet. The case for omega 3 PUFAs gains considerably more momentum when looking at migration studies of the Japanese. These studies show that Japanese migration to a western country and adaptation to a western diet high in omega-6 PUFAs is accompanied by an increase in colon cancer incidence [3].

Trends such as these support the potential role of omega 3 as chemopreventative agents. In contrast however, other studies show little or no association between omega-3 consumption and colon cancer risk. Instead, numerous population studies show an increased risk with the consumption of a diet high in total fat as discussed below.

Drasar and Irving showed a positive correlation between total fat consumption and colon cancer risk in a study that looked at the eating habits of people

throughout 47 countries [122]. Jain also reported similar results with a diet high in cholesterol and saturated fat [123]. A study carried out by Haenszel that looked at Hawaiian Japanese subjects also noted an increase in colorectal cancer risk with a change of diet that saw fish being replaced with intake of higher amounts of red meat. Either the reduction in omega-3 fatty acids or the inclusion of extra animal fat or possibly the red meat itself may play a role in the reason behind these results [124].

Perhaps, the most convincing data set suggesting a protective effect of fish oil was gathered by Caygill [103]. Not only did this study demonstrate the association between a diet lower in fat and a decreased colon cancer risk, but the type of fat was also identified as an important determinant of cancer risk. Fatty acids derived from fish oil were inversely correlated with CRC and it was suggested that a 3 fold increase in the consumption of fish oil could reduce the mortality rate of colon cancer by approximately 30%.

This however, is perhaps one of the few epidemiological studies that reports on such a significant inverse association, with many other cohort and case control studies reporting weaker correlation or no association at all between omega-3 fatty acids and colorectal cancer risk.

Hursting [125] carried out correlation analysis on international data, looking at the relationship between total fat and more specific PUFAs with the incidence rates of a variety of cancers including breast, prostate and colon. While omega-3 PUFAs did have a negative association with colon cancer incidence, it was not significant. Furthermore, correlations between total fat and an increased colon cancer risk were not found in this particular study, unlike those mentioned previously. A Dutch cohort study published by Busstra also found that the intake of fish was inversely associated with colorectal adenomas, though again, these results did not reach significance [126].

A cohort study by Terry looking at the dietary habits of Swedish women showed no association between cancer risk and a variety of components including total fat, omega-3 fatty acids and the omega-3/omega-6 fatty acid ratio [127]. A

Japanese cohort study by Kobayashi returned similar results with no trends found between dietary fat and cancer risk. This study did not support the role of fish oils and omega-3 PUFAs in the aetiology of cancer [128]. A more recent cohort study by Oh involving 34 451 US women came to the same conclusions with regard to any possible protective effects by omega-3 fats for colorectal cancer. This study did however, suggest that an increased omega-3 intake may reduce the progression of small to large adenomas [129].

At the other end of the spectrum, only one cohort study in the Netherlands by Brink suggested that total, saturated and monounsaturated fat was not significantly associated with colon or rectal cancer, whereas, a high intake of polyunsaturated fat is associated with an increased risk of mutated K-ras-containing tumours in the colon [130]. In saying this however, it must be highlighted that this association involved polyunsaturated fats as a whole, with focus on omega-6, not omega-3 fatty acids.

1.4.5. *In vivo* studies and omega-3 PUFAs

The evidence for the protective effect of fish oil is more persuasive when it comes to *in vivo* studies. With the majority of studies overall, largely supporting the suggestion that omega-3 fatty acids have chemopreventative qualities. The following section will review the literature dealing with omega-3 and *in vivo* studies. The large majority of these studies were carried out using either the Sprague-Dawley or Fischer rat model, but the measured end points are varied. To begin with, the effect of omega-3 on initiation data (ie. adduct formation, AARGC) will be revised followed by ACF and tumour data.

1.4.5.1. Acute animal studies

Much of the published data in the area of omega-3 and acute *in vivo* models has been completed by a research group lead by Hong, MY. More specifically, a key paper by this group in 2000 [69] concentrates on the effect of omega-3 on acute apoptosis and adduct load. Using a Sprague-Dawley – AOM model, rats were fed either corn oil or fish oil at 15% of the total diet. Following AOM

administration, groups of rats were killed at 3,6,9 and 12h and apoptosis, MGMT repair protein and O^6medG levels were measured in colonic epithelial cells. While the apoptotic change throughout the whole crypt was not significant, a significant apoptotic increase was observed in the top 1/3 of the crypt in the fish oil group. A further key finding in this group included significantly lower levels of O^6medG. Diet had no effect in the colon with regard to the MGMT repair protein.

Their data was strengthened by a similar second experiment [131], which again demonstrated an increase in apoptosis levels, and a reduction of O^6medG load in the colon of rats fed 15% fish oil. This particular group has also looked into the mechanisms associated with the increase in apoptosis due to fish oil [132]. When measuring Bcl-2 in fish oil fed rats, they found that significantly lower levels of Bcl-2 expression correlated with the increase in apoptosis. Furthermore, fish oil primed crypts that had also been exposed to butyrate experienced a significant drop in mitochondrial membrane potential and an increase in caspase 3. When analysing the fish oil in more detail, it was found that both EPA and DHA levels correlated with the decrease in MMP and increase in caspase 3.

The latest experimental data from this group has focused on the formation of oxidative DNA damage, rather then alkylating damage. However, the pattern is consistent, in that a fish oil diet given to rats administered DSS, has resulted in a significantly lower level of 8OHdG adducts, significantly increased apoptosis counts and in addition, decreased cell proliferation counts when compared to a high corn oil diet [133]. Bancroft's work on the oxidative adduct 8OHdG correlates well with this data also suggesting that fish oil can significantly lower the amount of oxidative adduct load in the colonic cells of rats fed a diet of 15% fish oil [134].

These acute experiments all included measurements of the AARGC between 3 and 12h. The summarised data demonstrate a significant difference between the AARGC responses between fish oil and high corn oil diets during this time period. However, it is important to note that of those reporting a difference, the

majority only observed the increase of apoptosis in the top third of the colonic crypts, rather than an overall increase.

Furthermore, the AARGC response was measured using the TUNEL assay in these studies. This immunohistochemical assay identifies apoptotic cells by labelling the open ends of DNA which are exposed during the process of apoptosis. Apoptotic cells counted this way may be deceptive as cells that are breaking down by other forms of cell death may be falsely identified as being truly apoptotic. This is emphasised even more so in the top third of the crypt where many mature cells may be going through the process of anoikis.

1.4.5.2. Long-term animal studies

Studies looking at apoptosis at longer time points after carcinogen administration display various results. Apoptotic counts completed in fish oil fed groups at 24h, 48h and 18 weeks were significantly higher than those from corn oil fed rats [135]. While, the only study found not to show a difference in the apoptotic response between dietary groups including a 17% mixed lipid diet, a 17% fish oil diet and a 5% low fat corn oil diet was Rao *et al.* [136]. In this case, apoptosis was measured at 8, 23 and 38 weeks and at no time did diet have an affect on the apoptotic response. In saying this however, tumour incidence from this study did vary within the different dietary groups. All animals fed the mixed lipid diet had ACF's and tumours, while in the fish oil and corn oil diets, tumours were found in 69% and 63% of rats respectively. Therefore, the fish oil diet did result in a lower number of tumours when compared to the mixed lipid diet, but not when compared to the low fat corn oil diet.

The influence of fish oil on ACF formation has been described by Coleman (2002) and Dommels (2003). Dommels compared a high fat corn oil and fish oil diet in Fischer rats and found that the incidence of AOM induced ACF's were significantly lower in rats fed fish oil, however, this decrease was only reported in the proximal colon [137]. Coleman compared a 10% fish oil diet with a 10% sunflower oil diet and at 13 weeks the fish oil diet had 19% fewer ACF's. This

protective effect was amplified when alpha-cellulose was added to the fish oil diet [138].

Numerous tumour studies comparing the effects of fish oil and other types of oils have been reported with mixed results. While the majority has seen a protective effect associated with a fish oil diet, it is important to note the type of control diet that the fish oil is being compared to, as these are quite variable with regards to both the dosage and type of oil used.

Reddy *et al*. have carried out a number of studies comparing fish oil to a variety of diets with different fat sources. One of their earliest studies [139] compared a high fat diet at 22.5% and a low fat diet at 5% with both corn oil and menhaden oil. At 34 weeks tumour incidence and multiplicity in the high and low menhaden groups was significantly decreased only to the high fat corn oil diet. This data was supported by an additional study five years later, which looked at tumour incidence between 5% low fat corn oil, 23% high fat corn oil and an 18.5% menhaden oil diet. Again no difference was found between the menhaden oil and low corn oil groups, however tumour incidence and multiplicity was significantly lower when compared to the 23% high fat corn oil diet.

Again in 2005, Reddy compared high fat corn and fish oil diets only and demonstrated the same trend as was reported in his previous data. With the addition of celecoxib at 250ppm, it was shown that the inhibition of tumours in the fish oil group become more pronounced, suggesting a synergistic effect between the fish oil diet and drug administration [111].

Numerous other studies also found a significant protective effect of fish oil on tumour load when compared to a high saturated or high mixed lipid diet. Kim reported that a diet of 12% fish oil reduced the incidence of colonic tumours in DMH treated rats compared to a diet of 12% beef tallow, a highly saturated fat [140]. Zhou also reported data in which tumour incidence and multiplicity was significantly lower in the MNN affected rats fed fish oil when compared to those fed similar amounts of beef tallow, soy oil or alkana oil [141].

These studies have often found significance in the results when comparing high saturated fat diet to a fish oil diet of various dosages. However, studies have also revealed the protective affects of fish oil when comparing it a standard corn oil control diet. A paper by Singh measured tumour incidence at 36 weeks in AOM treated F344 rats. A significant difference in tumour incidence and multiplicity was found between the fish oil diet and both the high fat and low fat corn oil diet, with incidence levels being detailed at 40%, 57% and 76% respectively [142].

When comparing a corn and fish oil diet of similar dosages in AOM treated F344 rats, Dwivedi [143] found a tumour load of 90% and 75% respectively, suggesting a possible protective effect of fish oil. Chang compared a 15% fish oil and corn oil diet and at 36 weeks rats fed a fish oil diet had developed significantly less tumours than the corn oil fed group with an incidence of 56.1% compared to 70.3% [144].

This study also had additional dietary groups that tested the synergistic effect of a fibre source and a fish oil diet. Unlike Coleman (2002), Chang found that a combination of pectin and fish oil, rather than cellulose, gave the lowest incidence of tumours in the colon at 51.5%. Joosun *et al*. also tested this synergistic theory and while it was found that the normal fish oil group did give significantly lower tumour numbers than the normal corn oil group, it was reported that the addition of dietary fibre, either in the form of cellulose or pectin, had no effect at all on tumour numbers [145].

Linder *et al*. (1991) studied the effect of different dietary lipids on DMH induced tumourigenesis in rats. By looking at a variety of different fats, including saturated, monounsaturated and polyunsaturated, a negative association between omega-3 polyunsaturated fats and tumour incidence was found [146].

Concentrating on polyunsaturated fatty aids alone, Minoura looked into the effects of 5% linoleic acid with or without the inclusion of 5% EPA. Rats fed EPA had a significantly lower tumour incidence at 33% compared to 69% [147]. On the other hand, Takahasi gavaged 0.7ml of DHA or 0.7ml of water to F344 rats injected with AOM and though tumour incidence was lower in the DHA

group, the results were not significant after 36 weeks [148]. These results were followed by a similar study by the same group in which 1ml of DHA was gavaged instead of the lower amount of 0.7ml. Again, though tumour multiplicity in rats given DHA was lower, there was no significance between the DHA and control group, with figures of 92% and 96% respectively [149].

In summary, the effect of fish oil on long term apoptosis, ACFs and tumours seems to be mixed with studies both supporting and disagreeing with the proposed health benefits of fish oil. While fish oil has in certain studies shown to be protective against tumourigenesis when compared to control diets with a similar fat content, the most convincing data is seen when a diet of fish oil is compared to high total fat diets or diets high in saturated fat.

1.4.6. *In vitro* studies and omega-3 PUFAs

Research using polyunsaturated fatty acids in a cell culture setting has been much more convincing and unified in presenting results that suggest a protective effect of fish oil and its components. Of course, results in cells removed from their environment might, however, be misleading in terms of effect on cancers even though they might provide great insight into mechanisms of action.

A number of studies using the colon cancer cell line HT-29 have demonstrated the induction of apoptosis as a result of either fish oil, EPA or DHA treatment. The administration of these fatty acids resulted in the induction of apoptosis and cell differentiation and also diminished cell proliferation. Furthermore, Chen showed this induction occurring in a time-dose dependent manner in response to DHA [118]. Hofmanova also used HT-29 cells to demonstrate a synergistic effect between DHA and sodium butyrate. It was found that this combination had the highest incidence of apoptosis and it was suggested that the DHA treatment sensitised the cells, making them more susceptible to sodium butyrate induced apoptosis [150].

Another colon cancer cell line CaCo-2, has also been used to show the effects of fish oil *in vitro*. Results concur with those observed with the HT-29 cell line.

Jordan [151] observed the inhibition of cell growth and increase of apoptosis in fish oil treated cells, as did Narayann [152] who used 5mM of DHA to obtain the same results. Nano also noted similar responses in omega-3 treated cells and also reported an increase in the membrane fluidity and lipid peroxidation of these cells [153].

Swamy used the HCA-7 cell line, which is a type of culture that has been modified to express COX 2. When using a range of different DHA quantities on these cells, apoptosis was induced and cell proliferation was inhibited at all dosages ranging from 150 to 225mM [112].

1.5. Novel food technologies involving omega-3 PUFAs

Over the last decade the food technology industry has supplied the consumer with a large choice of new products which offer greater health benefits and with a seemingly high demand for these products, the current market is aware of the role diet plays in maintaining one's health.

Traditional foods have been altered to include additional healthy components and examples of these can be seen in the various juices, breads and cereals that have been fortified with iron, folate and also omega-3 PUFAs. While simply adding compounds to food products is one way of ensuring products have a greater health benefits, new and novel technology is also being used to produce different forms of healthy food components that contain health benefits. One such technology includes the encapsulation of fish oil.

1.5.1. Microencapsulation of fish oil

Designed by Food Sciences Australia (FSA) microencapsulation is the process of transforming a free compound such as a lipid into an encapsulated powered product. Though the completion of various emulsion and drying processes, micro droplets of lipid end up becoming coated in a specifically designed protein-carbohydrate complex. Essentially, by surrounding each droplet of lipid

by this complex, a dry, stable emulsion is created with the end product resembling a fine, soft powder-like substance.

The applications of this technology in the food science industry are diverse. This product protects the chosen lipid from oxidation and therefore, increases its shelf life. Furthermore, due to the specific formulation of the outer layer of capsule this product has the potential to remain intact after ingestion when exposed to the various gastric fluids in the digestive system, only releasing its contents when reaching the colon (unpublished data). This feature has promising potential for the delivery of chemopreventative agents such as omega-3 PUFAs directly to the lumen of the colon in the hope that direct delivery may enhance protection against tumourigenesis.

1.6. Research questions and aims

This thesis reflects the need to better understand how, 1) the colonic epithelial cell acutely responds to DNA damaging agents, 2) to what extent the capacity of dietary agents, fish oil in particular, regulate this and 3) what, if any, are the consequences of this regulation for colorectal oncogenesis. Given the information available in the current literature combined with our previous experimental observations, it was decided that this thesis will explore the following core areas of work. The rationale behind the undertaking of each section along with some general aims is briefly discussed.

1.6.1. The pattern of acute homeostatic responses in rat colonic epithelium following an insult of alkylating carcinogen

To study whether a dietary agent may possibly regulate the acute host responses to AOM, one must first understand the general pattern of these responses under controlled conditions. Though some information can be found for particular responses, it was important to this body of work that a detailed time course of the host responses in the rat-AOM model were measured and understood.

The essential responses that take place in colonic epithelial cells in response to an insult of the AOM alkylating agent include the formation of O^6medG DNA

adducts, the apoptotic response and the rate of cell proliferation. The O^6medG adduct in particular was considered to be a key measurement in representing the level of early DNA damage in the rat-AOM model. With information lacking in regard to this response in the rat-AOM model, it was vital that a functional and reliable assay be established in order to understand when and where O^6medG formation occurred in the colon.

Therefore, the first essential aim of this project related to establishing a way of measuring the O^6medG adduct in colonic cells and is as follows. This is addressed in chapter 3;

- To establish a functional, consistent and reproducible immunostaining assay and image analysis system that allows the detection and quantification of O^6medG in rat colonic epithelial cells.

With a functional assay set up, the pattern of O^6medG formation and persistence could be measured over an extended time course of 48h. In addition to this, measurements of apoptosis and cell proliferation were also collected. The collection and analysis of this data fulfilled the next 2 general aims as were addressed in chapter 4.1;

- To measure the host responses in the colon, including O^6medG formation, apoptosis and cell proliferation, to an insult of an alkylating carcinogen in rat colonic epithelial cells over a set time course of 48h.
- To analyse the pattern of O^6medG formation, apoptosis and cell proliferation over the 48h time period and identify points of significant change for each response.

Once the relationship between these homeostatic responses was established and the onset of formation, maximum response and then decline were noted for each response a suitable time point could be selected for a dietary intervention study. Furthermore, a better understanding of the possible interplay between the responses could be developed.

Results from this time course experiment justified previous reservations that were held with regard to a current theory that links the O^6medG adduct with the onset of apoptosis.

Previous studies carried out using *in vitro* models point towards a late onset of apoptosis at least 24h after the exposure to an insult of an alkylating agent and the induction of O^6medG adducts. *In vitro* studies support the idea that two rounds of cell replication and DNA synthesis are needed during this time period to trigger apoptosis.

Data gathered from the time course study however, suggests that this process is not feasible in an *in vivo* model. This is due to an insufficient period of time between the O^6medG formation and the commencement of apoptosis. As a result an additional study was designed out of interest to determine whether the initiation of acute apoptosis may be through an alternate pathway. This study is detailed in section 4.2 and addresses the following aim;

- To determine whether the onset of apoptosis in the acute AOM rat model is influenced by the BER pathway by comparing apoptotic rates in AOM injected BER efficient and BER deficient rats.

1.6.2. The effect of both free and encapsulated tuna oil on the lipid profile in animal tissue

Following on from the time course study, a dietary intervention study in the rat-AOM model was carried out. Before determining whether any of these experimental diets successfully modulated the acute homeostatic responses to AOM, it was important to explore what changes each of the diets had caused to the physiology of the animals.

One possible theory as to how fish oil may protect against oncogenesis suggests that a fish oil diet changes the composition of the phospholipid of tissues by increasing the omega-3 PUFA content. This altered omega-3 to omega-6 ratio then contributes to a more beneficial class of anti-inflammatory and pro-apoptotic eicosanoids being made in the body. Given this information, the lipid

profiles of tissues were measured. Given a wide range of fish oil diets would be tested, this information would also allow us to explore the potential correlation between the amount of fish oil in the diet and the level of omega-3 uptake in different tissues.

Measuring the lipid profile of tissues also allowed us to investigate the properties of the new microencapsulated form of fish oil being tested. This novel microencapsulated product was selected to be tested in an *in vivo* model because of preliminary data that showed a preferential delivery mechanism of oil directly to the colon. It was hypothesised that by passing absorption in the small intestine and delivering fish oil directly to the colon, the level of omega-3 incorporation into the colonic phospholipid may be higher than that achieved in a free oil diet.

The general aims for this section are addressed in chapter 5 and are as follows:

- To establish any differential effects of a tuna oil or microencapsulated tuna oil diet on the weight and the colonic fermentation properties in the colon.

- To establish the effects of free or microencapsulated dietary tuna oil on the level of omega-3 PUFA incorporation into the phospholipid membrane by establishing tissue long chain fatty acid profiles.

In order to fulfil these aims, a dietary intervention animal study first had to be carried out. This was done by feeding groups of rats specifically composed diets containing either free tuna oil or encapsulated tuna oil for a period of 4 weeks. Following the euthanasia of all rats 6h after an AOM insult, a range of tissue samples were collected including the distal colon and these were analysed for their long chain fatty acid profiles.

Additionally, a number of other physiological endpoints were also measured (weight, faecal pH and SCFA profiles) to determine any other physiological effects caused by either the free or microencapsulated fish oil diets.

1.6.3. Regulation of acute homeostatic responses in rat colonic epithelium following an insult of alkylating carcinogen using dietary fish oil

The third core area of this thesis drew the first two elements together. The host responses to AOM that were established in the time course study would now be analysed in the colonic tissue of animals from the dietary intervention study. Effectively, the level of O^6medG, apoptosis and cell proliferation would be analysed in these animals to determine whether any of the fish oil diets had a regulatory effect on these responses following AOM.

Dietary fish oil has shown promising results in previous studies that suggest that this agent can cause an inhibitory effect on colorectal carcinogenesis. However, few studies have looked at the effect of fish oil on the very early host responses in the colon to an insult of alkylating carcinogen. These responses can be indicative of eventual carcinogenesis development and therefore, they can provide an insight into the mechanism by which fish oil may protect against colorectal oncogenesis.

It was hypothesised that a fish oil diet high in omega-3 fatty acids would increase apoptosis and reduce the level of O^6medG DNA adduct load in the distal colon of rats. And that these early effects on reducing the acute levels of DNA damage in the colon, may in part explain the potential chemopreventative qualities of fish oil that have been observed in longer term tumourigenesis studies. This hypothesis was then extended to suggest that the encapsulation of fish oil may potentiate any regulatory effects on O^6medG due a possible increase in omega-3 levels when compared to a free fish oil diet.

Additionally, the wide range of fish oil diets that were trialled incorporated diets of both different forms and doses. As well as gaining a possible insight into the mode of acute regulation that fish oil promotes, it was hoped that an optimal fish oil diet could be identified that gave the most promising regulatory results.

The general aims pertaining to this section were addressed in chapter 6 of this thesis and are summarised below;

- To measure the host responses in the colon, including O^6medG formation, apoptosis and cell proliferation, to an insult of alkylating carcinogen in animals fed both free and microencapsulated tuna oil.

- To determine if a diet of either free or microencapsulated tuna oil can regulate any of these host responses, and if so, to identify the optimal tuna oil dose and form for this effect.

In light of data gathered from this study, a small additional experiment was conducted that explored the effect of fish oil on the rat-AOM model itself. This study aimed to confirm that any regulation of the host responses was in fact the direct result of the fish oil and not the result of any interference with the metabolism of the AOM carcinogen in the rat.

The following aim was address in section 6.2;

- To establish whether a diet high in omega-3 PUFAs can influence the metabolism of AOM in the *in vivo* rat–AOM model of colorectal carcinogenesis by measuring the N7meG DNA adduct load in the colon.

1.6.4. The regulation of acute homeostatic responses in rat colonic epithelium and possible consequences for colorectal carcinogenesis

Having established whether or not dietary fish oil can regulate the acute host responses to AOM, it was important to extend these findings further and relate them to the consequences for colorectal oncogenesis.

A longer term study was designed over a 12 week period in which rats were fed an assigned diet and given an AOM dose sufficient to produce colonic aberrant crypt foci in the colon of these rats. Aberrant crypt foci are widely accepted as a type of preneoplastic lesion that can give rise to more advance cancerous tumours. ACF incidence and size have been correlated to the eventual development of colorectal cancer. Therefore, this ACF study allows us to explore the concept that any regulatory effects by fish oil on the acute host

responses to AOM can be translated into an overall protective effect against colorectal carcinogenesis.

The total level of O^6medG adduct formation has been correlated with ACF incidence and tumour incidence previously as has the apoptotic response. Therefore, we hypothesise that the regulation of any of these responses in animals fed fish oil will be translated to a reduction in ACF incidence in animals fed the corresponding diet.

In general, it is hoped that as well as gaining an insight into the relationship between the acute responses to AOM and the formation of ACF, this study will also allow us to identify a specific fish oil diet that can be recommended as protective against colorectal oncogenesis.

The following aims were addressed in the final chapter that dealt with the ACF study;

- To conduct a longer term study that measures preneoplastic ACF lesions in rat colon following a double insult of an alkylating carcinogen.

- To determine whether the regulation of any of the measured host responses by fish oil is translated into an overall effect on the early developmental stages of colorectal cancer.

- To identify an optimal fish oil diet that is protective against the formation of ACF.

In addition to these central aims, this study was also extended in that two types of fish oil were trialled in the ACF study. Tuna oil, high in DHA was this time tested along side menhaden oil, which is high in EPA, to allow us to explore the concept that one type of fish oil may deliver more promising results with regard to the prevention of ACF formation.