# CHAPTER 8
# Conclusions and future directions

## 8.1. Introduction

The incidence of colorectal cancer is the highest among westernised countries and it is now one of the most common type of cancer affecting Australians in particular [4]. It has long been recognised that preventative measures including changing one's lifestyle and diet can reduce the risk of developing this disease. Therefore, the identification of dietary agents that help in promoting bowel health is highly relevant and provides individuals with the knowledge to help improve their colorectal cancer risk.

In addition to the identification of dietary agents, it is also important to understand the molecular mechanisms by which dietary agents interact with the colonic luminal environment. Identifying how they may affect biological responses involved with the instigation of the carcinogenesis process, such as the formation and removal of acute DNA damage is of great value. Such information can greatly contribute to the general field of knowledge regarding the process of colorectal oncogenesis. Furthermore, such information may lead to the use of these acute host responses as biomarkers of CRC risk. These then have the potential to assist in establishing the preventative effects of dietary modulation in future human intervention studies.

Fish oil was originally associated with having chemopreventative properties due to epidemiological associations made with high fish eating populations and low cancer risk. As a result, fish oil has been tested for its chemopreventative properties with varied results, as detailed in sections 1.4.4-6. While *in vitro* studies provide strong evidence for the protection of fish oil against CRC, this correlation weakens as data from *in vivo* studies and randomised human trials are compiled.

*In vivo* studies that specifically explore the effects of fish oil on the acute homeostatic response in colonic cells are minimal. These acute responses to a carcinogen have been associated with CRC incidence and therefore, they are

considered to play a crucial role in determining the eventual consequences for the onset and development of oncogenesis.

## 8.2. Summary of aims and hypothesis of this thesis

The general objective of this thesis was to explore the potential of fish oil as a regulator of the host responses in the colon after an insult of a DNA damaging agent and then associate this to the possible outcomes of CRC.

The first area of study dealt with the concept of the host responses to carcinogen. Prior to any studies using fish oil as a dietary intervention it was important that the acute host responses to AOM were established under controlled conditions. In short, the following aims were completed to allow us to establish a better understanding of the response patterns over time;

- Establish a functional and reproducible immunostaining assay and image analysis system that allows the detection and quantification of $O^6$medG in rat colonic epithelial cells.
- Measure the level of $O^6$medG formation, apoptosis and cell proliferation in response to an insult of an alkylating carcinogen in rat colonic epithelial cells over a set time course of 48h.
- Analyse the pattern of $O^6$medG formation, apoptosis and cell proliferation over the 48h time period and identify points of significant change for each response.
- To investigate whether the onset of apoptosis in the acute AOM rat model is influenced by the BER pathway

The completion of these aims not only allowed us to better understand the response of the colonic cellular environment to DNA damaging agents, but an insight into their possible interplay was also gained.

The next areas of study were central to this thesis and involved investigating whether dietary fish oil regulated these acute responses to AOM. The host responses that were established in the time course study were measured in the

colons of rats fed a wide range of diets that incorporated both different forms and doses of fish oil. In conjunction to this, lipid uptake in animal tissues was also analysed in order to be able to relate any promising regulatory effects with the physiological effects of the fish oil diets. The following aims were completed to determine if modulation occurred;

- Establish the physiological effects of free or microencapsulated dietary tuna oil on the lipid composition the phospholipid membrane.
- Measure $O^6$medG formation, apoptosis and cell proliferation in the colon to an insult of alkylating carcinogen in animals fed different doses of both free and encapsulated tuna oil.
- Determine if a diet of either free or encapsulated tuna oil can regulate any of these host responses and identify the optimal tuna oil dose and form for a positive effect.

Finally, the consequences of regulation by fish oil were explored by conducting a longer term animal study that looked at the effect of fish oil on the formation of preneoplastic lesions in the colon. Essentially, this final component of research allowed us to determine whether free fish oil was protective against ACF formation and whether fish oil, when encapsulated, protected against ACF formation. This study also allowed us to determine whether the regulation of an early host response ($O^6$medG) as observed in the acute AOM model translated to suppression of oncogenesis as reflected in ACF incidence. The following aims were completed to help answer these questions;

- Carry out a longer term study that measures the effect of fish oil on the development on preneoplastic ACF lesions in rat colon
- To determine whether the regulation the acute host responses by encapsulated fish oil can be translated into an overall effect on the suppression of oncogenesis as reflected in ACF incidence.

The following hypotheses were made and addressed throughout various stages of this thesis;

- The initiation of $O^6$medG triggered apoptosis is mediated through a mechanism in the BER repair pathway.

- The microencapsulation of tuna oil results in greater omega-3 PUFA incorporation into the phospholipid of the distal colon.

- A high dose tuna oil diet will increase the apoptotic response and decrease the colonic $O^6$medG load in response to an insult of AOM.

- Omega-3 PUFAs do not interfere with the metabolism of AOM in the rat.

- A reduction in the total $O^6$medG load translates into a reduction of pre-neoplastic ACF lesions.

- Fish oil, high in EPA, has a greater protective effect against ACF formation than fish oil, high in DHA.

## 8.3. Acute host responses in rat colonic epithelium in response to an insult of alkylating agent

Functional acute host responses to DNA damaging agents ensure that the cellular environment retains its normal phenotype by avoiding potential mutational events. This has implications for the development of colorectal cancer.

This research has explored these acute host responses in colonic epithelial cells after an insult of a colon specific carcinogen. Data from this thesis shows that time taken for colonic cells to respond to a DNA damaging agent is quite rapid. $O^6$medG DNA adducts are first formed in colonic epithelial cells only 2h following an insult of AOM. The $O^6$medG load continues to build in the nuclei of epithelial cells until the 6h mark after the insult of genotoxin, at which point the $O^6$medG load then steadily declines.

The decline in $O^6$medG damage might be the result of a combination of removal of the adduct via repair by MGMT, removal of the affected cell via apoptosis and

the general maturation and discarding of older cells into the colonic lumen. The latter is unlikely though because cell lifespan is of the order of 60-72h [173] and too slow to explain a reduction of adducts, given they first occur primarily in the proliferative zone, as early as 2-4h.

The concept that older cells still contain $O^6$medG when they are sloughed off into the colonic lumen is supported by data in this thesis that shows a clear shift in distribution of $O^6$medG throughout the crypt over time. The development of the successful $O^6$medG immunochemical assay has not only allowed the measurement of the amount of $O^6$medG present in cells, but the high-quality staining results have also allowed the pattern of $O^6$medG in the cells of a colonic crypt to be established.

Initial $O^6$medG damage at 2h is concentrated in the lower proliferative compartment of the colonic crypt. As the load of $O^6$medG increases to its maximum level, so does its distribution throughout the crypt. This distribution becomes more uniform along the crypt over time, while the later stages of 36-48h post AOM sees the distribution of $O^6$medG skewed in favour of the more mature cells situated towards the surface. The fact that these cells still have $O^6$medG lesions within their DNA implies that not all damaged cells containing this adduct are repaired or removed before they are shed from the colonic crypt.

The pattern of apoptosis follows the pattern of $O^6$medG formation quite closely. These two important host responses are only separated by a delay of 2h. Hence, the onset of apoptosis is observed 4h post AOM and peaks at 6-8h post AOM with rates declining from there on.

Unlike *in vitro* studies that observe a late onset of apoptosis after exposure to an alkylating agent [97] and *in vivo* studies that have reported a late second wave of apoptosis after irradiation in small intestine [172], a late second wave of apoptosis was not observed. This was supported by additional TUNEL staining that also failed to show a second wave. These data collected from the time course experiment support the results of other studies [61, 77] that have observed a similar pattern of AARGC in the distal colon.

Interestingly, the 6h point following an insult of AOM seems to be a significant time point in which all endpoints measured recorded their maximal response. This was also the case with the rate of cell proliferation. The cell cycle was effectively stalled at the 6h mark also, with a significant reduction in the number of actively proliferating cells being observed. This stalling effect was retained for a further 10h at which point cell proliferation rates increased above their normal base line levels.

It appears that there is an interplay between these measured host responses (and also other repair processes) that may be suited to best manage the effects of damaging agents when exposed to DNA. In a summary of these responses, it appears that following the formation of $O^6$medG, apoptosis is initiated to remove heavily damaged, unrepairable cells. At the same time, the cell cycle is slowed down. This may assist in stalling the replication of damaged cells, allowing DNA repair to proceed and hence, preventing the fixation of mutations in damaged DNA.

To complete this host response story and complement the observations made for AARGC, the measurement of MGMT repair protein levels in colonic epithelial cells would have provided a valuable insight into the role this type of enzymatic repair has in the removal of $O^6$medG lesions from DNA. Published data referring to the levels of this protein after an insult of genotoxin are conflicting. Particular immunoassay data seem to support the concept that MGMT levels increase during this time [61]. However, the majority of research suggests that MGMT levels are completely depleted and are only detectable again 24-48h after the insult of the alkylating agent [60, 64, 85].

Further research that provides an insight into MGMT repair levels in colonic epithelial cells would help in clarifying the pattern of repair during this time and is recommended. By comparing this response to the other measured host responses in the colon, a better understanding of all mechanisms that act to retain the normal phenotype of cells could be established.

In addition to understanding the pattern of the host responses in greater detail, an important observation was taken from the data from this time course study concerning $O^6$medG mediated apoptosis.

As discussed in section 4.2.2, the prevailing theory pertaining to the mechanism of $O^6$medG mediated apoptosis, has been that following adduct formation, a round of S-phase is needed to create the mispair, then a second round is needed to trigger mismatch repair and if this fails only then is apoptosis triggered. This concept has been derived from *in vitro* work and is heavily supported by experimental *in vitro* data present in the current literature as reviewed by Kaina [213]. Previous findings lead to this pathway being questioned due to the discrepancy with regard to the time in which AARGC first began and its peak as early as 6-8h in the *in vivo* setting.

The analysis of these acute host responses in the colon of rats in this body of work confirmed this reservation. This thesis raises questions about the ability to relate this *in vitro* concept to the *in vivo* setting. Our research shows only a 2 hour delay between $O^6$medG and apoptosis. The mere fact that apoptosis is seen at 6-8h in the rat-AOM model and at 24h in a cell culture model indicates the innate differences that are present between these two models. It is suggested that the application of information between these two different settings should be made cautiously.

The short time period between the formation of $O^6$medG and the initiation of apoptosis suggested that an alternative and more direct trigger of an $O^6$medG mediated form of apoptosis may occur *in vivo*. Somehow, the cell can sense the presence of adducts and in some cases, clearly not all as cells with adducts persist as they migrate up the crypt, this triggers apoptosis directly.

To try and understand how this might occur, the hypothesis that apoptosis is mediated via BER repair was tested in this thesis, but this was not supported. AARGC was measured in BER proficient rats and also in rats with a deficient BER pathway, BER having been disabled by methoxyamine. While the BER deficient rats did tend to have slightly lower apoptotic rates, the apoptotic rate in

these groups were not significantly different from each other. Therefore it was concluded that BER has no significant effect on the initiation of apoptosis via either the $O^6$medG adduct directly or through other signalling processes.

The fact that the inhibition of BER did not significantly alter the apoptotic response also implies that apoptosis is not mediated through other DNA adducts that are primary repaired by this pathway. Therefore, it can also be implied that the slowly repaired N7meG adduct and the 3MeA DNA adduct are unlikely to play a role in the initiation of apoptosis *in vivo*.

Though unlikely, one must consider the possibility that the formation of $O^6$medG and the initiation of AARGC are not directly related at all in this model. Rats fed a high dose tuna oil diet in the dietary intervention study had significantly lower levels of $O^6$medG damage, but no affect on the level of the apoptotic response was observed. This summary of data seems to work against the concept that $O^6$medG initiates apoptosis directly. One could assume that if the AARGC were to be mediated directly through $O^6$medG lesions, then the level of $O^6$medG would correlate with the apoptotic response. This was not observed in the data gathered from the dietary intervention study. Therefore, although acute responses followed a similar pattern over time, they may be parallel events and not necessarily causally linked.

It is apparent that this research has perhaps created more questions than answers surrounding the relationship between $O^6$medG and apoptosis. Further research into the pathway of $O^6$medG mediated apoptosis in the rat-AOM model would definitely assist in clarifying this relationship and help to better understand how the host responses interact to eliminate DNA damage.

In particular, it is recommended that an animal study using a MMR deficient model would be useful in clarifying the relationship between these two responses. The importance of the MMR complex in the direct initiation of apoptosis via $O^6$medG is well established *in vitro,* with apoptosis being disabled in MMR deficient lines [214, 215]. Hence, measuring these acute responses to

AOM in a MMR deficient animal would help in either support or dismiss the $O^6$medG mediated apoptosis pathway as supported *in vitro.*

Another way to pursue this possibility would be with the use of a conditional or dose-dependent inhibitor of MGMT, or some other means of precisely regulating adduct load, followed by careful quantification of the associated apoptotic response. A very simple way to do this would be to give different doses of AOM and measure the related adduct formation and apoptotic response. Such has never been reported in the literature.

## 8.4. Benefits of the microencapsulation of fish oil

Tuna oil was used as the primary dietary agent in this thesis. However the amount of tuna oil and the form in which it was added to the diet was varied in the acte study. This allowed us to trial a variety of diets and therefore, increased the chances of finding a specific tuna oil intervention that would achieve optimal regulation while potentially lowering CRC risk.

As an aside to the primary aim of investigating fish oil, the testing of a novel microencapsulated product containing tuna oil was also carried out at a variety of doses. It was hypothesised that this microencapsulated product could deliver tuna oil directly to the colon, thereby resulting in increased omega-3 PUFA levels in the phospholipid membrane of the distal colon. It was thought that this action may potentiate the possible chemopreventative properties of regular tuna oil.

LCFA data however did not support the potential for this product to achieve direct delivery of oil to the colon. This was reflected in the products' inability to alter the lipid content of the phospholipid membrane of colonic tissue.

Though the phospholipid membrane of the colon and also all other tissues measured had equivalent levels of omega-3 PUFA incorporation in the comparative free and encapsulated oil diets, this does not necessarily dismiss the initial hypothesis of direct delivery completely.

It is possible that the claims of direct delivery to the colon may still be supported and that the failure of the data to show this lies in the use of the LCFA assay to demonstrate this. It is likely that some of the oil was absorbed in the small intestine and hence systemically delivered plus a portion of the encapsulated oil may have still been directly delivered to the colonic cells. This process of absorption over the 4 week feeding period may have lead to an eventual equilibration of omega-3 incorporation in all tissues, masking any effects of direct delivery. If this is the case, the potential of this product to deliver any health claims as a result of direct delivery may be questioned if it simply achieves incorporation levels comparable to what regular fish oil achieves over time.

In saying this however, it is still possible that any potential benefit coming from the encapsulation of tuna oil may be achieved via mechanisms other than the uptake and systemic action of additional omega-3 PUFAs in the colon. Though this research does not support the concept of using microencapsulation technology to increase colonic omega-3 PUFA incorporation into tissues, this product still has the potential for the regulation of cellular responses and possibly disease via other means. The potential of this is discussed further in section 8.4.

## 8.5. The regulation of acute homeostatic responses by fish oil

Having established the pattern of acute host responses in colonic cells after exposure to a DNA damaging agent, this information was used and a dietary intervention study was conducted that sacrificed animals at the 6h time point after an insult of AOM. Previous dietary studies have showed the promising chemopreventative effects of fish oil, and for this reason in combination with the fact that further clarification in this field was needed, fish oil was chosen as the dietary agent to be tested. More specifically high DHA tuna oil was tested.

This research conflicts with the previously supported concept [69, 132] and also our hypothesis that dietary fish oil regulates the apoptotic response to an insult of AOM. No regulation of apoptosis was observed with any dose or form of tuna

oil. Furthermore, there was no clear trend or impact with regard to the dose of tuna oil used and the apoptotic response. These data support the concept that tuna oil in the diet has no regulatory effect at all on the acute apoptotic response to an insult of alkylating carcinogen.

In addition to this, a diet containing tuna oil had no affect on the proliferative activity of cells in the distal colon. This implies that the balance of marine oils (EPA and DHA) in tuna oil does not modulate the cell cycle after an insult of AOM.

However, regulation of the acute $O^6$medG DNA adduct load in the distal colon was observed in animals fed a high dose tuna oil diet. More specifically, the 15% free tuna oil and the 7% encapsulated tuna oil diets resulted in the down regulation of $O^6$medG 6h post AOM. While the low dose tuna oil diets did not have any significant affect on the $O^6$medG load, a trend was noted in that $O^6$medG levels were lower in tuna oil fed animals when compared to animals fed control sunflower oil diets.

The mechanism by which $O^6$medG is regulated by these high dose fish oil diets remains unclear and requires further detailed investigation. As the higher tuna oil dose achieved the highest levels of omega-3 incorporation into tissues it is possible that the effect on the lipid profile in tissues may play a role in the reduction of $O^6$medG.

However, our findings suggest that this effect would not be the sole reason as the LCFA lipid profile in the 7% encapsulated tuna oil diet was equivalent to the 7% free tuna oil diet and the regulation of $O^6$medG was not achieved in the later diet. Nevertheless, the incorporation of omega-3 PUFAs at the expense of omega-6 PUFAs may have still primed tissues in the pre-feeding period. This may have promoted the production of more favourable compounds that may have provided an advantage in dealing with the insult of DNA damaging agents.

An alternative theory that may directly explain the reduction of the total colonic load of $O^6$medG in the distal colon may involve an increase in the actual physical

removal of $O^6$medG lesions in colonic epithelial cells. This may be accomplished through either their removal via cell deletion or their direct repair. As no significant effect of these high dose tuna oil diets were observed on the apoptosis rate, the theory pertaining to the reduction of $O^6$medG via increased cell death is not supported by our findings.

Unfortunately, the possibility that an increased level of repair may contribute to the reduction of $O^6$medG can not be tested in the available tissues collected from this rat-AOM study. An insult of AOM to an animal effectively reduces all traces of MGMT in the colon immediately after its administration. This is supported by the various published works on this area [60, 64, 85]. This was also supported by our investigations. Using a radiolabelled microtitre assay, no detectable trace of colonic MGMT protein could be found at the 6h time point post AOM. As a result, the effect of diet on this specific type of repair can not be measured when using the rat-AOM model.

This is not to say however, that fish oil cannot modulate the level of MGMT or other repair systems. It is still possible that a diet containing high dose tuna oil may assist in the up regulation of MGMT in the colon. This could be measured in rats not administered AOM or alternatively measured at a much later time point. If tuna oil did increase the amount of MGMT present in cells, this would give the cellular environment a greater capacity to repair damage and hence reduce their total $O^6$medG load. It is suggested that levels of MGMT in rats fed a high dose tuna oil diet should be measured prior to an insult of AOM to determine whether this dietary agent does in fact up regulate the repair protein MGMT and hence reduces the $O^6$medG load.

This body of work has also investigated the concept that the reduction of $O^6$medG as seen in these high dose fish oil diets may be an effect of the interference in the metabolism of AOM by fish oil. The level of the N7meG adduct, which was effectively used as a biomarker of the dose of the carcinogen insult given to tissues, was found to be significantly lower in the high fish oil group. Therefore, these data do support the theory that omega-3 PUFAs may inhibit enzymes important to the metabolism of this carcinogen. It is proposed

that the conflicting results from several studies that use the rat-AOM *in vivo* model to test fish oil may be explained through the potential of this agent to cause the interference of AOM metabolism.

This theory however, still needs more clarification in the rat-AOM model and further detailed work identifying and analysing more accurate markers of AOM metabolism are required. Of particular interest is the effect of a fish oil diet on the drug metabolising enzyme, CYP450 2E1. It is recommended that such investigations are undertaken and the soundness of the rat-AOM model is ensured before embarking on any further dietary intervention studies using fish oil in this model.

The down regulation of $O^6$medG was observed in animals fed both a high dose free fish oil diet and a slightly lower dose of the encapsulated form of fish oil. This instigated some further thought into the effectiveness of the encapsulated tuna oil product as a regulator of host responses to AOM.

$O^6$medG regulation was achieved with a dose of 7% tuna oil when encapsulated but at the higher dose of 15% when tuna oil was given in its regular free oil form. This suggests that the microencapsulation of tuna oil does in fact have an advantage over the free tuna oil in modifying the acute $O^6$medG response to AOM. Even though the encapsulated tuna oil did not increase the omega-3 content in the lipid membrane of tissues when compared to its free oil equivalent, it is possible that this regulation might have been achieved by other means not dependant on the composition of the phospholipid membrane of tissues. Whether this involves the product's ability to potentially deliver the tuna oil directly to the colon or by entirely other unrelated means is unclear.

One such possible unrelated explanation is that the combination of the capsule ingredients and the tuna oil interacted to achieve regulation. An increase in both caecal and faecal butyrate levels were observed with respect to the 7% encapsulated oil groups. Butyrate is derived from fermentation in the colon of the carbohydrate. Therefore, this increase was attributed to the formulation of the protein and carbohydrate matrix that forms the outer capsule product. Butyrate is

a known regulator of the apoptotic response and promotes colonic health [77, 195]. Therefore, it is possible that the combination of the high dose tuna oil and the butyrate-generating carbohydrate outer capsule may have regulated this effect.

While the outer capsule product and the oil were separated and tested individually at a dose of 3.5% in this thesis, regulation was not observed at this level and hence this information was not of use. In hindsight, this same dietary group at a dose of 7% would have been of great value in investigation this concept. To eliminate the possibility of this interplay between capsule and oil it is recommended that a study testing the individual components of the encapsulated oil product at dose of 7% is carried out.

## 8.6. The consequences of regulation of acute homeostatic responses by fish oil for oncogenesis

It is important in the scheme of this research to translate any early regulatory effect observed in the cellular environment to an actual effect on oncogenesis. Both the $O^6$medG load and apoptotic acute host responses have been associated with eventual cancer incidence in past studies as summarised in section 1.3.2.4. ACF incidence is commonly accepted as a biomarker of tumourigenesis as outlined above in section 7.1.2. Therefore, it was hypothesised that any regulation of these acute responses in the colon would be translated into regulation of the number of colonic ACF lesions.

Following on from the acute host response data which showed that a reduction of $O^6$medG was achieved in the 7% encapsulated tuna oil group, an ACF study was carried out to determine what consequences this may have for colorectal oncogenesis.

ACF incidence however, was not reduced by 7% encapsulated tuna oil and therefore the findings did not support the concept that a lowered $O^6$medG load is associated with suppression of oncogenesis. Several explanations might account

for the lack of association between these two biomarkers as shown by this research and these are discussed below.

First, it is possible that the lack of association means that there is no association of adduct load with consequent oncogenesis. It seems premature to come to such a conclusion, however, not all ACF are destined to proceed to cancer and it has been shown by Young *et al.* that some dietary interventions are stage-specific [216]. Furthermore, the biological consequences of oncogenic mutations induced by $O^6$medG adducts might be for progression (or lack of) after the ACF stage. Thus, studies that measure cancer as a final endpoint should be undertaken to fully explore this possibility.

Second, there is a possibility that dietary omega-3 PUFAs may have impeded the amount of carcinogen delivered to rat tissues and this, in turn may have unknowingly affected the results of the ACF study. To further explore this possibility, levels of AOM metabolising enzymes would have to be measured as previously discussed.

Third, it is also feasible that a clear association was not established due to limitations in the experimental design. $O^6$medG was measured in the acute model 6h post AOM. This time point was decided on for the dietary intervention as it was the peak time of $O^6$medG formation to the colonic epithelial cells. However, the measurement of this DNA adduct at a single time point after AOM may not be appropriate in predicting the consequence of this regulation for oncogenesis. A more appropriate measure of $O^6$medG may include a cumulative measure taken over time as shown by Jackson *et al.* [60] or alternatively a measure of $O^6$medG some time after 6h when unrepaired or unremoved adducts are likely to be of greater biological consequence. Such a later measurement may better reflect an association as the additional time in which the cells will have been subjected to repair and removal mechanisms may actually give rise to more biologically significant levels of $O^6$medG.

Fourth, the association may have been lost in the transfer of data from the $O^6$medG stain to the image analysis ratio. While the image analysis system was

suitable in picking up different intensities of $O^6$medG positive staining in the nuclei of cells, the appropriateness of then relating this intensity into a level that is biologically significant is unknown. It is possible that while different staining intensities were expressed as different ratios, the power of this immunochemical assay is not sufficient to differentiate between levels of damage that give rise to different consequences. To achieve this, a highly specific, quantitative but more cost intensive assay such as a mass spectrometry - gas chromatography method may have to be considered. Such methods are currently not sufficiently sensitive to enable this.

Apart from the inability to demonstrate a clear relationship between $O^6$medG and ACF lesions, the long term ACF study did support fish oil as being protective against ACF formation. This result is promising for the role of fish oil as a possible chemopreventative agent in the fight against colorectal cancer.

## 8.7. Implications of this research and future steps

This body of work has resulted in the creation of novel technologies and the initiation of new concepts. The implications for these outcomes are listed below.

1. The design and implementation of the successful immunohistochemical assay allowed the detection of $O^6$medG in rat colonic epithelial cells. The quantifiable data that this specific and sensitive assay produces, regarding both the amount and distribution of $O^6$medG, is invaluable in the field of acute DNA damage. It is a tool that can be adapted and applied to measure $O^6$medG in other tissues as well as other time points. Furthermore, with an alternative stable and specific primary antibody, the protocol can be used to detect other DNA adducts.

2. The fact that the findings do not support the prevailing theory pertaining to the mechanism of $O^6$medG mediated apoptosis, namely that following adduct formation, a round of S-phase is needed to create the mispair, then a second round is needed to trigger mismatch repair and if this fails only then is apoptosis triggered is not reason to dismiss this *in vitro* concept. Rather, in this case, the

validity of translating *in vitro* data into an *in vivo* setting has been questioned. As extensive work has been carried out on this area *in vitro,* the concept of $O^6$medG mediated apoptosis should be extensively studied in rat colonic epithelial cells so a more comprehensive assessment can be made. Somehow, the cell can sense the presence of adducts and in some cases this triggers apoptosis directly. Data from this thesis has initiated such research and has concluded that apoptosis is not triggered via the BER pathway directly or indirectly through other adducts such as N7meG, 3MeA or potentially $O^6$medG.

3. This research has clearly demonstrated the effect of a fish oil diet upon the physiological effects of tissues. This thesis supports the concept that a high tuna oil diet is not toxic and that the level of omega-3 PUFA equilibrated throughout the body and incorporated into tissues is determined on a dose-response relationship.

4. This research did not support the concept that fish oil, when encapsulated, selectively increases the omega-3 content of tissue phospholipid membranes in the colon. Though this does not completely dismiss this product's concept of direct colonic delivery, it is suggested that further *in vitro* and *in vivo* studies are undertaken to more clearly understand the properties of the capsules and their release mechanism.

5. It has been established that doses below 15% of free dietary tuna oil do not regulate any acute host response in the rat-AOM model. A diet of 15% and 7% encapsulated tuna oil does however significantly reduce the colonic $O^6$medG load following an insult of AOM. This demonstrates that a dietary agent such as fish oil can in fact modulate these early responses to a DNA damaging agent. This supports the concept that dietary regulation can influence the cellular mechanisms and can therefore potentially impact disease outcomes.

6. The down regulation of the colonic $O^6$medG adduct load was not obviously associated with a protective effect against the formation of pre neoplastic colonic ACF. This implies that regulation of the total load of $O^6$medG induced by AOM is therefore, not protective against oncogenesis. This

casts doubt on the concept that the total $O^6$medG DNA adduct load is a biomarker for consequent oncogenesis. It may be, however, that measurement of the adduct load at a subsequent time point, when "repair" mechanisms have had time to regulate the adduct load, might be a better time to assess the consequences of adduct load for oncogenesis. Studies that further investigate a wide range of adduct loads measured at various times, against consequent cancer formation, are essential to fully investigate the regulatory effects of host responses and their consequences for colorectal oncogenesis.

7. While dietary agents can regulate measured cellular responses, this thesis highlights the potential for dietary agents to also influence other unidentified compounds in the body, such as enzymes that regulate carcinogen metabolism. This is of importance when testing dietary agents and must be considered especially when using the rat-AOM model. The potential of fish oil to interfere with the metabolism of AOM can influence the endpoints. The need for a thorough investigation into the exact enzymes involved in the metabolism of AOM is crucial as is further research into the direct effects of omega-3 PUFA on drug metabolising enzymes.

8. Although an association between a reduction in total $O^6$medG damage 6h after carcinogen and a reduction in ACF could not be established, this research has supported the concept that a diet of 7% regular tuna oil and menhaden oil is protective against the formation of pre-neoplastic ACF lesions. This implies protection against oncogenesis by including fish oil in the diet. While the mechanism of this protective effect remains unclear, the use of fish oil as a chemopreventative dietary agent is supported by this research.

L.S. Nyskohus, 2009.