Modulation of colon carcinogenesis by dietary $\omega$-6/$\omega$-3 fatty acid ratios: A chemopreventive strategy?

Celeste H. Abrahams

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophiae

at the

Department of Medical Bioscience
Faculty of Natural Sciences
University of the Western Cape

Supervisor: Dr Stefan Abel,
Co-supervisors: Prof Maryna de Kock
Prof Wentzel C.A. Gelderblom

November 2015
DECLARATION

I declare that Modulation of colon carcinogenesis by dietary $\omega$-6/$\omega$-3 fatty acid ratios: A chemopreventive strategy? is my own work, that it has not been submitted before for any degree or assessment in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

______________________     ________________  
Celeste H. Abrahams     Date
ABSTRACT

The aim of this study was to determine whether dietary fats constituting specific ω-6/ω-3 fatty acids (FA) ratio has chemopreventive modulating effects on the development of colon cancer. Western diets intake of saturated FA (SATS) and ω-6 polyunsaturated FA (PUFA) are very high relative to low ω-3 PUFA consumption. This high ω-6 and low ω-3 FA intake, resulting in a high ω-6/ω-3 FA ratio, appears to have a promoting effect on disease outcome, whilst increased ω-3 FA intake exhibiting anti-cancer effects. An animal cancer model was employed to evaluate the effects of dietary fat ratios on chemically induced carcinogenesis during cancer promotion. This was to determine whether the FA diets have a promoting or inhibitory effect on early neoplastic lesions by quantifying aberrant crypt foci (ACF) development and monitoring the crypt cells proliferative and apoptotic indices. The expressions of genes associated with changes in cells redox balance were also assessed. Common dietary fats were combined to produce the dietary fat ratios: sunflower oil (S), borage oil (B) and fish oil (F). Combinations of these oils generated the different ω-6/ω-3 FA ratios: SB (ω-6/ω-3: 38:1), SF (ω-6/ω-3: 13:1) and SBF (10:1). To represent the Western diet’s high ω-6/ω-3 FA ratio profile, S (ω-6/ω-3: 501: 1) was used as a control, and canola oil and olive oil as additional reference. The dietary fats had no toxic effects on the liver and kidney based on serum clinical biochemical measurements. Diets containing borage oil (SB and SBF diets), canola and olive oil decreased (p<0.05) the crypt multiplicity of large (≥7 crypts/focus) ACF, exhibiting anti-cancer effects by decreasing (p<0.05) the proliferative activity of the rat colon crypts. Borage oil’s protective effect resulted from the enhanced supply of C18:3ω-6 that has anti-inflammatory and anti-proliferative properties. The observed decrease (p<0.05) in apoptosis in the ACF was also facilitated by the up- and down-regulation of DNA repair and DNA replication associated genes, Xpa and Ercc2 by borage oil, respectively. Canola oil and olive had the largest inhibitory effect on suppressing crypt multiplicity by reducing (p<0.05) proliferation in the colon. Both oils effected the up-regulation (p<0.05) of the expression of several oxidative stress and anti-oxidant defence genes mediating the regulation of cell proliferation. The increased supply of C18:1ω-9 (canola and olive) and total polyphenolic content (olive) protected cells against oxidative stress induced apoptosis, which provided interesting interactive effects between FA and polyphenolic oil constituents that
should be further elucidated. In contrast, the fish oil containing (SF diet) and the control sunflower (S diet) increased (p<0.05) the total ACF and colon crypt multiplicity (≥7 crypts/focus) when compared to the SB, SBF, olive oil and canola oil diets. An increased resistance to oxidative stress induced apoptosis appears to facilitate fish oil’s enhancing effect on crypt multiplicity despite the increased supply of LC ω-3 FA, which are prone to oxidation and leads to increased oxidative stress. This protective effect on crypt multiplicity and ACF development was mainly due to enhanced cellular antioxidant and DNA repair responses through the up-regulation (p<0.05) of \(Gpx4\) and \(Nudt1\), which favoured the increase (p<0.05) of crypt cells proliferation.

The \textit{in vitro} study demonstrated that oil ratio emulsions (S: \(ω-6/ω-3 = 249:1\); SB: \(ω-6/ω-3 = 28:1\); SF: \(ω-6/ω-3 = 12:1\) and SBF: \(ω-6/ω-3 = 12:1\)) had differential effects on the survival indices of HT-29 and Caco-2 colon cancer cells. Contrary to the \textit{in vivo} model, fish oil (SF and SBF emulsions) significantly (p<0.05) reduced the viability and proliferation of both cell lines, with the HT-29 cells showing greater sensitivity to the oil’s anti-proliferative effect. The HT-29 cells exposure to increased levels of \(C20:5ω-3\) and \(C22:6ω-3\) predisposes it to lipid peroxidation that increases the potential for cell removal via apoptosis. However, apoptotic effects were absent due to the HT-29 cells removal via necrosis as the cells energy status (ATP production) was significantly (p<0.05) depleted. Similar to the animal cancer model, borage oil (SB and SBF emulsions) had a reducing (p<0.05) effect on cell proliferation in both cell lines. However, as ATP was decreased (p<0.05), the S, SF and SBF emulsions resulted in an increased (p<0.05) apoptotic response in the Caco-2 cells in a dose dependent manner. This response resulted from the altered FA and lipid composition effected by the oil emulsions. Increased (p<0.05) incorporation of \(C20:5ω-3\) and \(C22:6ω-3\) in membrane phospholipid, phosphatidylethanolamine (PE), resulted in a significant decrease (p<0.05) in total SATS and MUFA content. A decrease (p<0.05) in membranes \(ω-6/ω-3\) FA ratio was noted as well. This effect seems to selectively favour the induction of apoptosis by borage oil (SB and SBF). Similarly, an increase (p<0.05) in the PC/PE ratio by all oil emulsions, and a decrease (S and SB) and increase (SF and SBF) (p<0.05) in the chol/PL ratio appears to facilitate apoptosis too. A different threshold of the FA and
lipid composition parameters elicits the inhibition of cell proliferation utilising lower oil emulsion concentrations. Therefore, the dietary supply of fats characterised by a defined low ω-6/ω-3 FA ratio can selectively modulate the growth indices of colon cancer. Specific oil ratio combinations by incorporating borage oil and fish oil hereby provide a selective strategy for chemoprevention in the colon, although underlying interactions and threshold effects of specific FA seems to prevail that should be further unravelled.
DEDICATION

This thesis is dedicated to my Mom, who was my greatest supporter and source of encouragement throughout this PhD journey!
ACKNOWLEDGEMENTS

I wish to extend my appreciation and gratitude to the following individuals and institutions:

- Dr Stefan Abel (supervisor and mentor): Stefan, thank you for giving me the chance to grow as a scientist. You have taught me a lot, and for that I thank you.

- Prof Wentzel Gelderblom (co-supervisor): Blom, your wisdom and input has always been appreciated. Thanks for being a great example of an individual who strives for excellence in science, something we all aim for as the "juniors" in the field.

- Prof Maryna de Kock (co-supervisor): Prof, thank you for your continuous moral support and encouragement throughout my years as a student at UWC, and for acting as a liaison whilst I was based at the Medical Research Council (MRC). You input has been greatly appreciated!

- The MRC Research and Capacity development programme for the financial support throughout 4 years of my PhD studies.

- The Cancer Association of South Africa (CANSA) for the funding provided during this study.

- Lorraine Moses: Thanks so much for all your input regarding the molecular work. All your assistance and moral support was greatly appreciated!

- Samira Ghoor (MRC Diabetes Development Platform): For her assistance with the RNA bioanalysis.

- Nathan McGregor (PhD student – Stellenbosch Medical Biochemistry): For his assistance with the PCR microarrays.

- Prof Dirk van Schalkwyk: Much gratitude to you Prof, for doing some of the statistical analyses.

- To my friends and lab mates Lana Keet and Sedicka Davids: Thanks ladies for your moral support, especially the last few chaotic months of my PhD journey! You filled the endless days in the lab with much fun and laughter, and were always there to bounce off ideas on. To Sedicka, in particular, I cannot express enough gratitude to you for your support and encouragement. You
understood everything I was going through, since we walked this PhD journey together! Choma, thank you for your friendship always. It is my blessing.

- To my family and friends: Your support and prayers throughout my studies has always been needed and welcomed. Thank you.

- To my mother: Moeks, it has been a tough few years, but your love and support has been unwavering, even at times when I was a complete boar to be around! Thank you for always being there, praying for me and encouraging me always. Words cannot express how much I appreciated all that you are and what you’ve done for me. Thank you and I love you lots.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACF</td>
<td>aberrant crypt foci</td>
</tr>
<tr>
<td>AIN</td>
<td>American Institute of Nutrition</td>
</tr>
<tr>
<td>ALP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate aminotransferase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BBOT</td>
<td>2,5-bis(5'-tert-butylbenz-oxazolyl-[2'])thiophene</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromodeoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BW</td>
<td>body weight</td>
</tr>
<tr>
<td>C18:1ω-9</td>
<td>oleic acid</td>
</tr>
<tr>
<td>C18:2ω-6</td>
<td>linoleic acid</td>
</tr>
<tr>
<td>C18:3ω-6</td>
<td>γ-linolenic acid</td>
</tr>
<tr>
<td>C18:3ω-3</td>
<td>α-linolenic acid</td>
</tr>
<tr>
<td>C20:3ω-6</td>
<td>dihomo-γ-linolenic acid</td>
</tr>
<tr>
<td>C20:4ω-6</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>C20:5ω-3</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>C22:6ω-3</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>CANSA</td>
<td>Cancer Association of South Africa</td>
</tr>
<tr>
<td>CDKs</td>
<td>cyclin dependent kinases</td>
</tr>
<tr>
<td>CM</td>
<td>chloroform:methanol</td>
</tr>
<tr>
<td>CMS</td>
<td>chloroform:methanol:saline</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DMH</td>
<td>1,2-dimethylhydrazine</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>EFA</td>
<td>essential fatty acids</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagles’s minimal essential medium</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid(s)</td>
</tr>
<tr>
<td>FAME</td>
<td>fatty acid methyl esters</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FO</td>
<td>fish oil</td>
</tr>
</tbody>
</table>
g  grams
GC  gas chromatography
GSH  glutathione
\( \text{H}_2\text{O}_2 \)  hydrogen peroxide
HDL  high density lipoprotein cholesterol
IARC  International Agency of Cancer Research
IC50  inhibitory concentration at 50% activity
LC  long-chain
LC PUFA  long chain polyunsaturated fatty acids
LDH  lactose dehydrogenase
LDL  low density lipoprotein cholesterol
LOX  lipoxygenases
LPO  lipid peroxidation
mg  milligrams
MRC  Medical Research Council
MUFA  monounsaturated fatty acids
PC  phosphatidylcholine
PE  phosphatidylethanolamine
PG  prostaglandin(s)
\( \text{PGE}_1 \)  prostaglandin \( E_1 \)
\( \text{PGE}_2 \)  prostaglandin \( E_2 \)
\( \text{PGE}_3 \)  prostaglandin \( E_3 \)
PKC  protein kinase C
PLA\(_2\)  phospholipase \( A_2 \)
PPAR  peroxisome proliferator activated receptors
PUFA  polyunsaturated fatty acids
ROS  reactive oxygen species
RT  room temperature
S  sunflower oil
SATS  saturated fatty acids
SB  sunflower oil + borage oils
SBF  sunflower oil + borage oil + fish oil
SF  sunflower oil + fish oil
SOD  superoxide dismutase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TPP</td>
<td>total polyphenols</td>
</tr>
<tr>
<td>μg</td>
<td>micrograms</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
<tr>
<td>ω-3</td>
<td>omega-3</td>
</tr>
<tr>
<td>ω-6</td>
<td>omega-6</td>
</tr>
</tbody>
</table>
# List of Tables

## Chapter 3

| Table 3.1: | Composition of the AIN-76A purified rat diet per kilogram. | 54 |
| Table 3.2: | The fatty acid profile of dietary oils expressed as a percentage of total content. | 59 |
| Table 3.3: | The individual fatty acid intake of the rats with the dietary oil feeds. | 62 |
| Table 3.4: | Total ACF and crypt multiplicity in the colon of rats as a function of the dietary oil treatments. | 64 |
| Table 3.5: | Proliferative Ki-67 activity of the colon crypts in response to diets. | 65 |
| Table 3.6: | Apoptotic caspase-3 activity of the colon crypts in response to diets. | 66 |
| Table 3.7: | Effect of the dietary oil feeds on the animals' blood clinical chemistry. | 67 |

## Chapter 4

| Table 4.3: | Average body weight gain, feed and fatty acid intake parameters. | 92 |
| Table 4.4: | Select genes expression significantly affected in the colon of carcinogen treated Fisher 344 rats by the experimental dietary oils relative to the control, sunflower oil only. | 97 |

## Chapter 5

| Table 5.1: | Fatty acid profile of oil-in-water emulsions expressed as a percentage of total content. | 133 |
| Table 5.2: | Oil emulsion concentrations in mg/ml required to produce 50% cell activity as indicated by ATP release and BrdU incorporation in the colon cancer cells after 48 hour exposure. | 137 |
| Table 5.3: | Modulation of cytotoxicity, induction of apoptosis and cell viability by oil emulsions in different colon cancer cells. | 139 |

## Chapter 6

| Table 6.1: | Effects of the dietary oil ratios on ACF development and cell survival indices. | 175 |
| Table 6.2: | Dietary oil ratios protective effects against increased oxidative stress during the promotion phase. | 177 |
Table 6.3: The anti-cancer effects of canola and olive oil relating to its modification of oxidative stress and antioxidant defence genes expression in ACF. ..... 179

ADDENDUM

Addendum 2

Table 4.1 Total polyphenol content of the dietary oils. .............................................. 196

Table 4.2: Complete list of genes associated with oxidative stress and antioxidant defences in the RT2 Profile™ PCR array (PARN-065). ......................... 198

Addendum 3

Table 5.4: Cholesterol and phospholipids content in Caco-2 cell membranes after 48 hours exposure to oil emulsions. ................................................................. 200

Table 5.5: Saturated fatty acids content of Caco-2 cell membranes after 48 hours exposure to oil emulsions ................................................................. 201

Table 5.6: Monounsaturated fatty acids content of Caco-2 cell membranes after 48 hours exposure to oil emulsions ................................................................. 202

Table 5.7: Polyunsaturated fatty acid (ω-3) content of Caco-2 cell membranes after 48 hours exposure to oil emulsions ................................................................. 203

Table 5.8: Polyunsaturated fatty acid (ω-6) content of Caco-2 cell membranes after 48 hours exposure to oil emulsions ................................................................. 204

Table 5.9: Total polyunsaturated fatty acid (ω-3 and ω-6) and ratios content of Caco-2 cell membranes after 48 hours exposure to oil emulsions......... 205
LIST OF FIGURES

CHAPTER 2

Figure 2.1: Estimates of the commonly diagnosed cancers worldwide in 2012............. 8

Figure 2.2: The cell cycle involving sequential phase transitions from quiescence (G0 phase) to proliferation (G1, S, G2, and M phases) of the cell. G0 phase accounts for a non-growing, non-proliferating stage in the cycle (i.e. cell cycle arrest); DNA synthesis occurs during the synthesis (S) phase, which is preceded by a gap phase called G1, where the cell prepares for DNA synthesis, and is followed by G2 during which the cell prepares for mitosis (M phase). Checkpoint G1S and G2M induces a temporary halt in cell cycle progression to check for chromosomal errors and ensures DNA repair before transitioning to the following phase. ................................................................. 11

Figure 2.3: The human colon: Organ facilitating the final process of digestion. (a) Structure and location in the lower abdominal cavity, (b) Cross section of the intestine illustrating the multiple tissue layers. .............................. 13

Figure 2.4: The multistep model of colon carcinogenesis illustrating some of the genetic and physiological changes that occur during the process. ...................... 15

Figure 2.5: Microscopic identification of aberrant crypt foci (ACF) with methylene blue staining............................................................ 15

Figure 2.6: The nomenclature and chemical structure of essential fatty acids (a) α-linolenic acid (C18:3ω 3), and (b) linoleic acid (C18:2ω-6). .................. 17

Figure 2.7: The biosynthesis of longer chain PUFA from EFA α-linolenic acid (ω-3) and linoleic acid (ω-6) involving sequential desaturation and elongation steps. .................................................................................. 20

Figure 2.8: Metabolic pathway depicting the biosynthesis of eicosanoids derived from C20:4ω-6 and C20:5ω-3. The 20-carbon chains are released from membrane phospholipids by action are phospholipase A2. Subsequent reactions involve cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450-controlled monooxygenases to generate the multitude of eicosanoids that elicit various cellular responses. .............. 23

Figure 2.9: The 3 step pathway of free radical mediated lipid peroxidation. Initiation: 1) Unsaturated FA reacts with free radical (X•) to generate a carbon centred radical through hydrogen abstraction. Propagation: 2) Addition of oxygen to the pentadienyl radical to give rise to lipid peroxyl radicals. Termination: 3) Release of oxygen from peroxyl radical to give pentadienyl radicals, and 4) intramolecular addition of the peroxyl radical to yield bicyclic prostaglandin-type products (i.e. aldehydes and alkenals). .................................................................................................................. 26
CHAPTER 3

Figure 3.1: Average rat total body weight gain (A), and feed intake in grams/100 gram body weight (B) over the 4 month experimental period. Diets contained the following oil mixtures: S = sunflower oil only, SB = sunflower oil + borage oil, SF = sunflower oil + fish oil, SBF = sunflower oil + borage oil + fish oil, Canola = canola oil and Olive = olive oil.  

CHAPTER 4

Figure 4.2: Rats total polyphenol intake expressed as mg gallic acid per 100 gram body weight. Animal feed diets contained the following oil mixtures: S = sunflower oil only, SB = sunflower oil + borage oil, SF = sunflower oil + fish oil, SBF = sunflower oil + borage oil + fish oil, Canola = canola oil and Olive = olive oil. Significant differences compared to the sunflower oil only (control) diet set at p<0.05 (*), p<0.001 (**) and p<0.0001 (***)....  

Figure 4.3: Functional classification of genes significantly altered in the colon mucosa of carcinogen treated male Fischer 344 rats exposed to different diets.  

Figure 4.4: Summary of possible anticancer properties of canola and olive oils resulting from the up-regulation (+) of redox sensitive genes expression. Canola oil induced effects on gene expressions are underlined; olive oil in bold.  

CHAPTER 5

Figure 5.1: Caco-2 cells dose response relative to borage oil.  

Figure 5.2: Cholesterol and phospholipids content in the Caco-2 cells membranes after 48 hours exposure to different oil emulsion concentrations (ATP IC50 and BrdU IC50). (A) Cholesterol, (B) Phosphatidylcholine (PC) content, (C) Phosphatidylethanolamine (PE) content, (D) PC/PE ratio and (E) cholesterol/phospholipids (chol/PL) ratio. Significant differences between groups set at p<0.05 are indicated by different letters: lowercase – differences between oil emulsions within assay (ATP IC50 or BrdU IC50); uppercase - corresponding lipid variable compared at different concentrations (i.e. ATP IC50 vs. BrdU IC50) within the same oil emulsion treatment.  

Figure 5.3: Summary of the modulation of Caco-2 cells SATS composition by the oil emulsions when compared to the control. The dose responses indicated are the apoptosis inducing concentrations effects on the FA relative to the BrdU IC50 treatments.  

Figure 5.4: Summary of the modulation of Caco-2 cells MUFA composition by the oil emulsions when compared to the control. The dose responses
indicated are the apoptosis inducing concentrations effects on the FA relative to the BrdU IC50 treatments. ................................................................. 147

Figure 5.5: Summary of the modulation of Caco-2 cells ω-3 FA composition by the oil emulsions when compared to the control. The dose responses indicated are the apoptosis inducing concentrations effects on the FA relative to the BrdU IC50 treatments. ................................................................. 150

Figure 5.6: Summary of the modulation of Caco-2 cells ω-6 FA composition by the oil emulsions when compared to the control. The dose responses indicated are the apoptosis inducing concentrations effects on the FA relative to the BrdU IC50 treatments. ................................................................. 152

Figure 5.7: Summary of the modulation of Caco-2 cells total PUFA and FA ratios composition by the oil emulsions when compared to the control. The dose responses indicated are the apoptosis inducing concentrations effects on the FA relative to the BrdU IC50 treatments. ................................................................. 155

CHAPTER 6

Figure 6.1: Summary of the Caco-2 cell membranes altered FA content required to elicit apoptosis or inhibit cell proliferation in response to oil emulsion exposure. .................................................................................................. 182

Figure 6.2: Summary of the Caco-2 cell membranes altered lipid content required to elicit apoptosis or inhibit cell proliferation in response to oil emulsion exposure. .................................................................................................. 183

ADDENDUM

Addendum 2

Figure 4.1  PCR microarray plate layout. ................................................................................. 197
CONTENTS

Declaration i
Abstract ii
Dedication v
Acknowledgements vi
Abbreviations viii
List of tables xi
List of figures xiii

CHAPTER 1
GENERAL INTRODUCTION 2

CHAPTER 2
LITERATURE REVIEW

2.1 Cancer ........................................................................................................ 8
2.1.1 Biology of cancer ...................................................................................... 9
   (a) Nomenclature of cancers ........................................................................... 9
   (b) Gene mutations and carcinogenesis .......................................................... 
   (c) Critical cellular processes disrupted during cancer development ...........

2.2 Colon Cancer ............................................................................................. 12
   2.2.1 The Colon: Structure, function and morphology .................................. 12
   2.2.2 Colon carcinogenesis: The process of transformation from normal tissue to a tumour ................................................................. 13
   2.2.3 The role of diet in colon carcinogenesis .............................................. 16

2.3 Polyunsaturated fatty acids ..................................................................... 16
   2.3.1 Nomenclature and chemical structure ............................................... 17
   2.3.2 Dietary sources and intake recommendations ..................................... 18
   2.3.3 Metabolism of PUFA .......................................................................... 19
   2.3.4 Structural and biological functions of PUFA ................................... 20
CHAPTER 3
PROMOTION MODULATING EFFECTS OF DIFFERENT DIETARY FAT MIXTURES ON THE DEVELOPMENT OF ABERRANT CRYPT FOCI IN 1, 2-DIMETHYLHYDRAZINE-INDUCED COLON CARCINOGENESIS IN RATS.

ABSTRACT ......................................................................................................................... 50
3.1 INTRODUCTION ........................................................................................................ 51
3.2 METHODS AND MATERIALS .................................................................................. 53
3.3 RESULTS .................................................................................................................... 58
3.4 DISCUSSION .............................................................................................................. 68
3.5 CONCLUSIONS ......................................................................................................... 74
3.6 REFERENCES ............................................................................................................ 75

CHAPTER 4
MODULATION OF THE EXPRESSION OF OXIDATIVE STRESS AND ANTIOXIDANT DEFENCE GENES BY DIETARY FAT DURING 1, 2-DIMETHYLHYDRAZINE INDUCED COLON CARCINOGENESIS IN RATS.

ABSTRACT ......................................................................................................................... 83
4.1 INTRODUCTION ........................................................................................................ 84
4.2 MATERIALS AND METHODS .................................................................................. 86
4.3 RESULTS .................................................................................................................... 90
4.4 DISCUSSION .............................................................................................................. 100
4.5 SUMMARY AND CONCLUSIONS ............................................................................ 111
4.6 REFERENCES ............................................................................................................ 113
CHAPTER 5
ALTERATIONS IN MEMBRANE FATTY ACID PROFILE BY DIETARY FAT EMULSIONS AND THE EFFECT ON SURVIVAL INDICES OF HUMAN COLON CANCER CELLS IN VITRO.

ABSTRACT .................................................................................................................. 126
5.1 INTRODUCTION .............................................................................................. 127
5.2 MATERIALS AND METHODS ....................................................................... 129
5.3 RESULTS ......................................................................................................... 137
5.4 DISCUSSION .................................................................................................... 156
5.5 CONCLUSIONS .............................................................................................. 164
5.6 REFERENCES ................................................................................................. 164

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSIONS

6.1 GENERAL DISCUSSION ............................................................................... 173
6.2 CONCLUSIONS ............................................................................................. 186
6.3 REFERENCES ................................................................................................. 186

ADDENDUM .............................. 194
1.1 GENERAL INTRODUCTION

Dietary intake is an essential part of our existence as humans, providing sources of nutrients and energy that are required to sustain our physiology. However, coupled to sedentary living, inadequate or excessive dietary practices have impacted negatively on the maintenance of good health. This fact is demonstrated by the continuous and increasing pandemic of diet related chronic ailments worldwide (Nishida et al., 2004). Excessive caloric intake of fats above the recommended level is a characteristic feature of the modern Western diet (Simopoulos, 1999). The diet comprises a high proportion of saturated fats (>10%), is rich in ω-6 polyunsaturated fatty acids (PUFA) and low in ω-3 PUFA, resulting in dietary ω-6/ω-3 ratios in the range 20-30:1 (Gómez Candela et al., 2011; Simopoulos, 1999). This dietary pattern appears to impact significantly on the development of cancers in the colon, breast and prostate (Bartsch et al., 1999; Leitzmann et al., 2004).

The cancer promoting effects of certain dietary fatty acids (FA) are linked to their functional role in maintaining cellular homeostasis under optimal cellular conditions. FA are important macromolecules influencing cell membranes structure, as well as affecting cellular functions (Ibarguren et al., 2014). The interaction of PUFA from both ω-6 and ω-3 classes greatly influence mechanisms regulating cell proliferation and apoptosis that are critical processes determining cell survival (Bartsch et al., 1999; Calviello et al., 1999). Some of the responses elicited by these FAs include altering signal transductions, generating eicosanoids, as well as modifying gene expressions and cells oxidative status (Abel et al., 2004; Cathcart et al., 2011; Jump and Clarke, 1999).

The metabolism of diet supplied essential FA linoleic acid (C18:2ω-6) increases arachidonic acid (C20:4ω-6), which is a key regulator of normal cellular function (Brash, 2001). With regards to colon cancer development though, colonic cells C20:4ω-6 levels are high (Nicholson et al., 1991). This has been shown to enhance the synthesis of eicosanoids such prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) that has a mitogenic effect, associated with the increased proliferative characteristic of cancers (Chan, 2006; James et al., 2000; Murff et al., 2012). Conversely, increased intake of long chain ω-3 PUFA such as eicosapentaenoic acid (C20:5ω-3) and docosahexaenoic
acid (C22:6ω-3) have shown anticancer effects by eliciting anti-inflammatory responses by increasing PGE$_3$ production, reducing C20:4ω-6 accumulation and cell proliferation (Barham et al., 2000; Chapkin et al., 2007; Huang et al., 1996) and increasing apoptosis (Giros et al., 2009).

The aforementioned opposing effects rely greatly on the proportion of ω-6 and ω-3 FA (i.e. ω-6/ω-3 and C20:4ω-6:C20:5ω-3 ratios) available (Barham et al., 2000), and thus could be regulated by dietary FA intake. Dietary PUFA can also increase the level of oxidative stress, which acts as a critical modulator of cell survival by inhibiting cell proliferation and enhancing apoptosis (Alexander-North et al., 1994; Martindale and Holbrook, 2002). The ω-3 FA PUFA composition of cell membranes increases the susceptibility of cells to lipid peroxidation (LPO), an indicator of oxidative stress (Song and Miyazawa, 2001). LPO is generally very low in cancer cells as a result of reduced PUFA content in cell membranes (Horrobin, 1990) and an adaptive increase in antioxidant activity also provides cancer cells with protection against any deleterious events initiated by oxidative stress (Landriscina et al., 2009). Therefore, increasing cancer cells supply of PUFA via dietary intakes could potentially prevent cancer development by modulating the intensity of oxidative stress to undermine any tumour protective responses (e.g. increased antioxidant activity). Dietary sources of these biologically important FAs are abundant in nature. Thus the potential for eliciting selective responses to inhibit cancer growth is possible by the dietary manipulation of the supply of these macromolecules.

Studies suggest that in addition to the quantity of dietary FA consumed, adjusting the type of FA constituting the ω-6/ω-3 ratio may have a chemopreventive effect against cancer development (Simopoulos, 2008). Therefore, the PUFA ratio (ω-6/ω-3) composition requires critical assessment in the development of such a dietary intervention strategy. This current study investigates the chemopreventive potential that specific dietary fat sources may have on the process of colon carcinogenesis. With each fat source featuring varied ω-6/ω-3 FA content, the theory is that the dietary FA supply would elicit different responses with respect to cancer promoting and/or protective effects. Chapter 2 will briefly outline current knowledge pertaining to colon cancer development, the role of FA regarding their potential harmful role in promoting and/or preventing carcinogenesis. The influence of experimental dietary
fat ratios on early biomarkers of colon cancer development such as aberrant crypts foci (ACF), histological indicators of proliferative and apoptotic indices in colonic crypts, as well as blood biochemistries will be critically assessed in Chapter 3 utilizing a rat colon carcinogenesis model. Chapter 4 highlights the experimental fat ratios modulatory effects on the expression of genes responding to oxidative stress and antioxidant defences in the same animal model. In Chapter 5, utilizing an in vitro model, the survival of human colon cancer cell lines will be monitored in terms of the modulation of cell viability, proliferative responses and the induction of apoptosis utilising the different experimental dietary fat emulsions with varying ω-6/ω-3 FA ratios in relation to the degree of FA incorporation into cellular membranes. The main findings and conclusions of the investigations will be critically discussed in Chapter 6.

1.2 REFERENCES


2.1 Cancer

Cancer is a disease that affects individuals worldwide, regardless of the boundaries of class, race, gender or age. Although cardiovascular disease, strokes and lower respiratory infections accounts for the top 3 causes of global deaths (Mathers et al., 2009), as a non-communicable disease the mortality rate resulting from cancer incidences remains high. In a recent report by the International Agency for Research on Cancer (IARC), according to GLOBOCAN 2012, an estimated 14.1 million new cases and 8.2 million cancer related deaths occurred in 2012 (Ferlay et al., 2015).

According to this report, the most commonly diagnosed cancers were those of the lungs, breast and colorectum (Fig. 2.1), with the incidence rate for cancer development being 25% higher in males than females. Furthermore, worldwide trends illustrate the greater increasing burden of cancer in the more affluent countries, compared to poor developing nations. However, there appears to be discrepancies in the incidence numbers reported due to the lack of early detection and access to treatment facilities in the poorer countries.

![Figure 2.1: Estimates of the commonly diagnosed cancers worldwide in 2012.](adapted from Ferlay et al., 2015)
2.1.1 Biology of cancer

(a) Nomenclature of cancers

The uncontrolled proliferative state of cells, a feature of all tumours, can occur in various types of tissue. Hence the types of cancer can be subdivided into 4 groups:

1) Carcinomas: malignancies of epithelial cell origin (e.g. colon, breast)
2) Sarcomas: solid tumours of the connective tissue (e.g. muscle, bone, cartilage and fibrous tissue)
3) Lymphomas: cancers arising in lymph tissue
4) Leukemia: cancer of blood cells

Tumours that arise from these cells can be defined as either being benign or malignant. Slow growing benign tumours are non-invasive growths that remain confined to its original location of development. Conversely, malignant tumours proliferate rapidly, and are capable of both invading surrounding normal tissue and spreading throughout the body via the circulatory or lymphatic systems (metastasis) (Mareel and Leroy, 2003).

(b) Gene mutations and carcinogenesis

Malfunctions within cells regulatory processes arises from dynamic changes occurring in the genome that provides cancer cells with the ability to proliferate independent of a growth stimulus. This unregulated growth is caused by damage to DNA, resulting in mutations in genes that encode for proteins controlling cell division (Dixon and Kopras, 2004). These mutations can be caused by chemical or physical agents called carcinogens, or by exposure to UV radiation or by certain viruses that can insert their DNA into the human genome (Chang et al., 2014; Mancebo and Wang, 2014; Shubik, 1995). Also, mutations can occur spontaneously (sporadic), or are passed down generations as a result of germline mutations (Kim et al., 2007; Zhang et al., 2014).

At least 3 important classes of genes play key roles in tumour initiation: proto-oncogenes, tumour suppressor genes, and genes involved in DNA repair mechanisms (Tysnes and Bjerkvig, 2007). Mutations, amplifications or deletions in these genes may lead to the disruption of biological mechanisms involved in the regulation of normal cell growth.
Alteration in the genetic material is only the first, irreversible step in the multi-staged process of carcinogenesis. Following this initiation step is a promoting phase in which initiated or transformed cells are stimulated to undergo further proliferation and is desensitized to cell regulatory processes such as apoptosis (Evan and Vousden, 2001). Numerous environmental factors, including diet, have been identified as acting as tumour promoters by activating signal transductions that gives the initiated condition a proliferative advantage over normal neighbouring cells (Bode and Dong, 2014; Van Engeland et al., 2003). Thus, at this stage cancer development can be reversed by the selective modulation of cancer stimulatory mechanisms (Lee and Herceg, 2014; Shukla et al., 2014). Progression is the final stage, and is characterized by the accumulation of additional mutations in tumour suppressor genes and oncogenes that enables tumour cell invasion and metastasis (Yokota, 2000). This stage is also irreversible, and can ultimately lead to death.

(c) Critical cellular processes disrupted during cancer development

Abnormalities in the regulation of critical signaling pathways mediating cell proliferation and apoptosis are typical behaviours in the establishment of all cancers or tumours (Evan and Vousden, 2001). Under normal conditions these processes are tightly regulated in the cell cycle, which is subdivided into 5 sequential phases that go from quiescence (G₀ phase) to proliferation (G₁, S, G₂, and M phases) (Fig. 2.2). Regulation of these phases involves the controlled interaction of key regulatory cyclin-dependent kinases (CDKs), cyclins and checkpoints that ensures the transition of one phase to the next. In cancer these interactions and checkpoints are deregulated (Foster, 2008; Hanahan and Weinberg, 2011). Therefore, cancer is essentially the result of defects occurring in the cell cycle, which usually maintains a balance between proliferation, growth arrest and apoptosis to ensure cellular homeostasis.
Figure 2.2: The cell cycle involving sequential phase transitions from quiescence (G\(_0\) phase) to proliferation (G\(_1\), S, G\(_2\), and M phases) of the cell. G\(_0\) phase accounts for a non-growing, non-proliferating stage in the cycle (i.e. cell cycle arrest); DNA synthesis occurs during the synthesis (S) phase, which is preceded by a gap phase called G\(_1\), where the cell prepares for DNA synthesis, and is followed by G\(_2\) during which the cell prepares for mitosis (M phase). Checkpoint G\(_1\)S and G\(_2\)M induces a temporary halt in cell cycle progression to check for chromosomal errors and ensures DNA repair before transitioning to the following phase (adapted from [http://medicinembbs.blogspot.com/2011/02/tissue-repair.html](http://medicinembbs.blogspot.com/2011/02/tissue-repair.html), accessed 11 September 2013).

(i) Cell proliferation
In the cell cycle, during the M (mitosis) phase, cells divide and replicate their genome in order to generate new cells to replace old and damaged cells. This process is regulated by the interaction of cyclins and CDKs that assist in controlling cell growth and proliferation and inhibits apoptosis (Kishimoto, 1994). In normal cells proto-oncogenes contain the codes for proteins that transmit signals to the cell nucleus to stimulate cell division (Oshima and Campisi, 1991). Mutations in these genes (i.e. oncogenes) results in the continuous and increased activation of growth stimulatory effects that is typical in all cancers (Yokota et al., 1986)

(ii) Apoptosis
Apoptosis, or programmed cell death, is an essential component of cell number regulation that prevents damaged or mutated cells from surviving and dividing (Elmore, 2007). It is distinctly different from necrotic cell death in which cells lose their membrane integrity and lyse when subjected to toxic stimuli (Zong and Thompson, 2006). Meanwhile apoptosis occurs in a well ordered sequence of events that involves the activation of proteolytic enzymes that result in cell degradation (Elmore, 2007). In pathological conditions such as cancer, the deregulation of
apoptosis allows damaged cells to live beyond their lifespan (Reed, 1999). Deregulation of this process normally results from mutations in tumour suppressor genes such as p53, which usually causes cell cycle arrest in the event of DNA damage and prevent its replication into progeny cells under normal conditions (Duffy et al., 2014). Consequently, modulation of the apoptotic pathway has been identified as a key target for chemopreventive strategies.

2.2 Colon Cancer
Malignant neoplasms arising in the colon or bowel is one of the leading causes of death in both men and women in the developed world (Jemal et al., 2011). Although advances in radiotherapy, chemotherapy and surgery have improved survival rates, approximately 1 in 17 people over the age of 50 years will develop colon cancer in their lifetime, with more than 50% of these individuals eventually dying from the disease (Harewood and Lawlor, 2005).

There are enormous country-to-country differences in the incidence of the disease throughout the world. International correlation studies have shown that the highest incidences of colon cancer occur in North America, Great Britain and parts of Europe, and that the lowest incidences occur in Asia, Latin America and Africa (Center et al., 2009). According to the National Cancer Registry, in South Africa the risk of developing colorectal cancer for individuals aged between 0-74 years are 1 in 115 men and 1 in 199 women. However, as traditional diets are replaced by so-called “western” diets, a rise in the incidence of colon cancer have been experienced in these low risk population groups (Center et al., 2009; Kanavos, 2006).

2.2.1 The Colon: Structure, function and morphology
The colon, also referred to as the large intestine or bowel, is a muscular U-shaped tube located within the lower abdominal cavity (Fig. 2.3a). The major function of the organ is to extract water, salts and nutrients from partially digested food and to propel the residue to the rectum and anus for expulsion. Also, the colon serves as a critical barrier for the organism, protecting it from toxic agents found within the luminal environment (Ashida et al., 2012). Therefore, in addition to being exposed to luminal nutrients, the intestinal mucosa is constantly challenged by diet-derived oxidants, mutagens and carcinogens, as well as endogenously generated reactive
oxygen species (ROS) (Sanders et al., 2004; Wakabayashi et al., 1992). As a result, the colon has developed a highly regulated system to ensure for the maintenance of cellular integrity and tissue homeostasis.

**Figure 2.3:** The human colon: Organ facilitating the final process of digestion. (a) Structure and location in the lower abdominal cavity ([www.newshealthguide.org(Rectum-Function.html](www.newshealthguide.org/Rectum-Function.html), accessed 22 October 2014), (b) Cross section of the intestine illustrating the multiple tissue layers (adapted from [www.purdue.edu/uns/html4ever/0002.Badylak.SIS.html](www.purdue.edu/uns/html4ever/0002.Badylak.SIS.html), accessed 22 October 2014).

The organ is composed of multiple tissue layers: the mucosa, the submucosa, the muscle layer/muscularis propria (i.e. containing circular and smooth muscle) and the serosa (Fig. 2.3b). Within the inner colon mucosal wall a complex organization of epithelial cells is situated. Here crypts, folds of the colonic epithelium lining the inner surface, are continuously renewed by stem cell division (Lipkin, 1974). These epithelial cells have a much higher turnover rate compared to tissues in the rest of the body (Lipkin, 1974). Under optimal growth conditions, undifferentiated precursor epithelial cells at the base of crypts proliferate and rapidly undergo morphological and functional differentiation as they migrate to the surface (Lamprecht and Lipkin, 2002). Here at the surface the epithelial cells undergo apoptosis and extrude into the lumen of the gastrointestinal tract. These functional compartments along the crypt axis are maintained within precise boundaries by multiple homeostatic signals. Disruptions of these processes can lead to colon carcinogenesis (Boman et al., 2008).

### 2.2.2 Colon carcinogenesis: The process of transformation from normal tissue to a tumour

The development of tumours in the colon is dependent on the progressive perturbation of the normal signalling pathways involved in cell proliferation, thus
conferring a growth advantage to the cells (Ferrand *et al*., 2005). To commence cancer initiation and progression, first several genetic changes occur in the epithelial cells lining the intestinal tract (Michor *et al*., 2005). Besides familial genetic predispositions, these genomic changes can be caused by multiple environmental factors (Ponz de Leon and Roncucci, 2000). In the multi-step carcinogenesis model proposed by Fearon and Volgestein (1990), genetic alterations occur in a stepwise fashion such that a clone that has growth advantage proliferates, acquires more genetic alterations and undergoes another selection for survival and growth, eventually resulting in cancer (Fig. 2.4). In this model, the accumulation of mutations in oncogenes and several tumour suppressor genes are the critical catalysts facilitating normal colonic mucosal cells conversion to malignancy. The most common changes noted are K-ras point mutations, mutations in growth suppressor gene p53 on chromosome p17 and allelic loss in chromosome 5 (apc gene), and the growth suppressor gene DCC on chromosome 18q (Armaghany *et al*., 2012; Wilmink, 1997). The clonal expansion of these genetically altered cells is further propagated by reducing inhibitory signals produced by normal cells (Hanahan and Weinberg, 2011). Furthermore, these responses may be enhanced by dietary influences that result in an increase in DNA damaging oxidative stress and subsequent modulation of cell regulatory signalling pathways favouring an increase in proliferation (Bartsch and Nair, 2004; Caderni *et al*., 1999). Clinical and histopathological manifestations of the disease’s stepwise development are demonstrated through a series of stages ranging from single crypt lesions through small benign tumours (adenomatous polyps) to malignant cancers (carcinomas). This adenoma-to-carcinoma sequence of colon carcinogenesis has provided the baseline for studies into the mechanisms pertaining to the disease’s progress.
Small areas of focal damage termed aberrant crypt foci (ACF) develop as an early physical evidence of this mutation process as seen in rodents and humans (Di Gregorio et al., 1997; Roncucci et al., 2000), and can be identified microscopically as a localised cluster of enlarged crypts (Fig. 2.5). These early neoplastic lesions display an enhanced proliferative state compared to normal colon crypts (Corpet et al., 1997). The total number of ACF may be considered to be a valid biomarker in rats at a very early stage of carcinogenesis, while in subsequent weeks (later stages of development) ACF with higher crypt multiplicity (i.e. >4 crypts per focus) are considered a more specific biomarker than the total number of ACF (Bird, 1995). A small percentage of these will develop into tumours over a period of 10-20 years (Johnson, 2004) with environmental influences such as diet having a significant effect on the process (Rao et al., 2001).

Figure 2.4: The multistep model of colon carcinogenesis illustrating some of the genetic and physiological changes that occur during the process (adapted from Aspinall and Taylor-Robinson, 2000).

Figure 2.5: Microscopic identification of aberrant crypt foci (ACF) with methylene blue staining (adapted from Takayama et al., 1998).
2.2.3 The role of diet in colon carcinogenesis

Migrant studies show an elevation in the risk of developing colon cancer in populations that have moved from low incidence (Japan, China) to high incidence (United States) areas (Haggar and Boushey, 2009). These findings suggest that the variation in colon cancer incidence is strongly influenced by environmental factors. Numerous reports have shown associations between diet and colon cancer (Kasdagly et al., 2014; Key et al., 2002; Lipkin et al., 1999). A relation commonly found in epidemiological studies is an increase in risk associated with a high fat and low fibre dietary pattern (Pericleous et al., 2013; Woutersen et al., 1999).

Numerous mechanisms whereby dietary fat intake influences the carcinogenesis process have been proposed. A common hypothesis is that it induces the excretion of bile salts (Breuer and Goebbell, 1985). Increased secretions of bile salts have a non-specific effect on the colon lumen, damaging the colon mucosa and resulting in an increased risk for endogenous mutations (Bernstein et al., 2005). In many cases, however, colon cancer is considered as a disease resulting from chronic inflammation (Itzkowitz and Yio, 2004). Certain dietary fatty acids can irritate colon tissue by increasing pro-inflammatory responses (Chan, 2006). Prostaglandin E₂, a derivative of the ω-6 fatty acid arachidonic acid sourced either directly by diet or de novo synthesis, is widely accepted as playing a significant role in promoting cancer cell growth (Pai et al., 2002).

Dietary fat can also influence colon cancer cells oxidative status by generating lipid radicals that lead to DNA damage that initiates the transformation of normal cells to a neoplastic phenotype (Bartsch and Nair, 2006), as well as prompting mechanisms that maintain its survival (Erdelyi et al., 2009). Furthermore, dietary fats may also influence colon cancer risk through its involvement with insulin resistance, altered immunological responses and changes in the fatty acid composition of membranes (Bruce et al., 2000; Szachowicz-Petelska et al., 2007).

2.3 Polyunsaturated fatty acids

Fatty acids (FA) both free and as part of complex lipids, are important macromolecules that play a major role in cellular function (Tvrzicka et al., 2011). Most mammals, including humans, are able to synthesize saturated FA and
monounsaturated FA (Mashima et al., 2009). However, they are unable to manufacture unsaturated FA with more than a single carbon double bond due to the lack of Δ12- and Δ15-desaturases (Huang et al., 2004). As a result, polyunsaturated fatty acids (PUFA) linoleic acid (C18:2ω-6) and α-linolenic acid (C18:3ω-3) can only be obtained through dietary intake (Simopoulos, 1999). Both these FA serve as precursors for the synthesis of longer chain unsaturated FA. Therefore, the term essential fatty acid (EFA) is applied only to these two PUFA that are necessary for good health and cannot be synthesized by the body.

2.3.1 Nomenclature and chemical structure
FA are long-chain (LC) hydrocarbon molecules containing a carboxylic acid moiety at one end and a methyl group at the other, with either no double bond or at least one double bond in the molecule. There are 3 classes of FA: i) saturated fatty acids (SATS) with no double bond, ii) monounsaturated fatty acids (MUFA) with a single double bond, and iii) PUFA with 2 or more double bonds. Unsaturated FA are classified according to the location of the last double bond relative to the terminal methyl group (–CH₃) at one end of the molecule. MUFA and PUFA are subdivided into additional families (i.e. ω-3, ω-6, ω-7 and ω-9). For example, EFA C18:3ω-3 has 18 carbon (C)-atoms with three double bonds, with the first bond at the 3rd carbon atom counted from the methyl group at the end (Fig. 2.6a). Similarly, C18:2ω-6 has 18 C-atoms and two double bonds, with the first double bond located at the 6th carbon atom counted from the methyl end (Fig. 2.6b).

![Figure 2.6: The nomenclature and chemical structure of essential fatty acids (a) α-linolenic acid (C18:3ω-3), and (b) linoleic acid (C18:2ω-6) (adapted from http://lipidlibrary.aocs.org/Lipids/whatlip/index.htm, accessed 27 October 2014).](http://lipidlibrary.aocs.org/Lipids/whatlip/index.htm)
2.3.2 Dietary sources and intake recommendations

FA represent 30-35% of total energy intake in many industrial countries and the most important dietary sources of FA are vegetable oils, dairy products, meat products, grain and fatty fish or fish oils (Rustan and Drevon, 2001). The FA C18:2ω-6 is abundant in all vegetable oils, including corn, sunflower, safflower and olive oil (Kaur et al., 2012). The recommendation for intake of this FA is set at 2.5-9% of total fat intake (Elfmadfa and Kornsteiner, 2009). But in the United States alone, for example, the average intake of C18:2ω-6 is 84-89% of the total PUFA energy (Kris-Etherton et al., 2000), illustrating the excessive intake pattern.

Sources of EFA C18:3ω-3 includes soybeans, walnuts, and dark green leafy vegetables such as kale, spinach, broccoli and Brussels sprouts. Also seeds or their oils such as flaxseed, mustard seed and rapeseed (canola) contain C18:3ω-3 (Abedi and Sahari, 2014). Most of these oils, however, are also rich in C18:2ω-6 (Larsson et al., 2004). The marine food chain is based on ω-3 FA that are present in plankton and algae on which fish feed on (Bartsch et al., 1999). Several species of cold water fish are rich in ω-3 LC PUFA eicosapentaenoic acid (C20:5ω-3) and docosahexaenoic acid (C22:6ω-3). These include fatty fish such as salmon, tuna, herring, mackerel and anchovy. Recommendations regarding the daily intake of ω-3 FA vary between 400-1000 mg C20:5ω-3 + C22:6ω-3 in the form of food or supplements (Kris-Etherton et al., 2009; Meyer et al., 2003; Vannice and Rasmussen, 2014).

However, with the Western diet intake of fish is minimal, thus the predominant intake of ω-3 FA is C18:3ω-3. With this dietary pattern the dominant intake of ω-6 FA (C18:2ω-6) coupled to minimal ω-3 FA (C20:5ω-3 and C22:6ω-3) provides a high dietary ratio of ω-6/ω-3 FA that has been linked to the onset of numerous diseases including colon cancer (Simopoulos, 2008). Thus, to circumvent any negative effects caused by excessive ω-6 PUFA intake, an increase in fish or fish oils intake is recommended to elevate tissue levels in C20:5ω-3 and C22:6ω-3 that have numerous protective effects (Bartram et al., 1993).
2.3.3 Metabolism of PUFA

PUFA, largely consumed in the form of triglycerides from various food sources, are digested in the small intestine (Iqbal and Hussain, 2009). These macromolecules are considered important nutrients required by the body in order to function. Thus the digestion process allows for the absorption of these FA by enterocytes and their transport in the blood to various organs for assimilation (Niot et al., 2009). Some of these FA are degraded through the multistep process of β-oxidation in mitochondria to generate energy or are esterified to lipid molecules (Sprecher, 2000). Furthermore, while mammals cannot synthesize ω-3 and ω-6 EFA de novo, mammalian cells can manufacture longer carbon chain PUFA by elongation and desaturation (Nakamura and Nara, 2003).

The liver and adipose tissue are the major sites for FA biosynthesis, however it does occur in other tissue as well (Bonet et al., 2012). The ω-3 and ω-6 FA cannot be metabolically interconverted but the metabolism of one can moderately impede the metabolism of the other (Simopoulos, 1999). The first part of the synthesis pathway leading to eicosapentaenoic acid (C20:5ω-3) and arachidonic acid (C20:4ω-6) takes place in the endoplasmic reticulum (ER) and consists of sequential alternating elongation and desaturation steps catalysed by FA elongase, Δ6- and Δ5-desaturases (Nakamura and Nara, 2003). C18:3ω-3 is desaturated and elongated to eicosapentaenoic acid (C20:5ω-3), while C18:2ω-6 is metabolized into arachidonic acid (C20:4ω-6) using these same enzymes (Fig. 2.7). The enzyme Δ6-desaturase seems to be the rate limiting step in the pathway due to its greater affinity for ω-3 FA (Larsson et al., 2004). Therefore the FA synthesis outcome is dependent on competition between C18:3ω-3, C18:2ω-6 and some of their longer chain products for Δ6-desaturation, and negative feedback regulation of LC PUFA on both Δ5- and Δ6-desaturases (Horrobin, 1993).
Figure 2.7: The biosynthesis of longer chain PUFA from EFA α-linolenic acid (ω-3) and linoleic acid (ω-6) involving sequential desaturation and elongation steps (adapted from Kaur et al., 2012).

2.3.4 Structural and biological functions of PUFA

FA have diverse roles in cells. They are important components of lipid molecules that are the building components for plasma membranes (Abbott et al., 2012). Consequently, any PUFA produced in the ER may be removed from the biosynthetic cascade and used for membrane lipid synthesis by enzymes localized in the ER. Therefore, the bioavailability of these FA in membranes has great influence on both their structural integrity and critical membrane associated functions (Ibarguren et al., 2014).

2.3.4.1 Structural components

The plasma membrane represents the barrier of life, the structure that separates living cells from their surroundings. Its bilayer structure is composed of several phospholipid, cholesterol and protein molecules (Spector and Yorek, 1985). The lipid types and their distribution within membranes may be unique to different cell types, but are altered by the availability of dietary FA, particularly PUFA (Alexander, 1998). Phospholipids are the dominant lipid in all membranes, only varying in their FA content (Abbott et al., 2012). These lipid molecules include a hydrophilic “head” (i.e. choline, ethanolamine, serine or inositol) connected through phosphoric acid to a hydrophobic part. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the most abundant phospholipids in membranes containing a SATS or MUFA at the
sn-1 position on the glyceride backbone and a PUFA at the sn-2 position (Escribá et al., 2008). The FA content in individual lipid class affects membrane fluidity substantially, thus impacting on cellular function (Kullenberg et al., 2012). Besides affecting membrane fluidity, the lipid composition greatly influences the formation of receptors, binding of ligands to their receptors, and the activation of intracellular pathways (Alexander, 1998).

PUFA esterified to membrane phospholipids occupy greater space in the hydrophobic head due the cis configuration of the molecules, hereby increasing membrane fluidity, whereas SATS and MUFA have a trans configuration (i.e. “flat”) and cause more rigid membrane structures (Kremmyda et al., 2011). PUFA are also involved in cholesterol transport and metabolism. Cholesterol is transported in the body largely in the form of fatty esters. Found in large amounts in plasma membranes, cholesterol also has a rigidifying effect on the membrane by inhibiting the overall flexing motion of the acyl hydrocarbon chains (Petrache et al., 2005). Dietary intakes of ω-6 PUFA has been shown to lower plasma cholesterol (Horrobin and Manku, 1983), whilst an increase in its content in rat platelet membranes has also been noted with ω-6 and ω-3 FA rich diets (Heemskerk et al., 1995). Therefore, the type of dietary FA consumed impacts significantly on the lipid composition (structure), as well as membrane fluidity and associated functions. Aberrations in these features have been shown to participate in disease development (Hulbert et al., 2005).

2.3.4.2 Biological functions
(a) Precursor for eicosanoid biosynthesis
Eicosanoids are important hormone-like molecules that stimulate critical cellular responses in all mammalian cells (Tapiero et al., 2002). These lipid derived signalling molecules include prostaglandins (PG), leukotrienes, thromboxanes, prostacyclins, lipoxins and hydroperoxy FA. The effects elicited by these molecules include the modulation of inflammation, cytokine release, immune responses, platelet aggregation, vascular reactivity, thrombosis and allergic reactions (Tapiero et al., 2002). Biosynthesis of these critical stimulants is dependent on the bioavailability of Δ5-desaturation products LC PUFA C20:4ω-6 (Leslie, 2004). In unstimulated cells, this 20-carbon FA is found sequestered in phospholipids from which it is
released upon stimulation. It is cleaved from membrane phospholipids by phospholipase A2 (PLA$_2$) and is released as a free FA to react with enzymes that cause their oxygenation and is further modified to yield the cell stimulatory eicosanoids (Fig 2.8). For example, C20:4ω-6 is the precursor of 2-series PG that promote inflammation, which is generated by the metabolizing action of cyclooxygenases on this FA (James et al., 2000). Eicosanoids are produced only on demand. Therefore, their synthesis is tightly regulated by the cellular concentrations and activities of the enzymes involved in their biosynthesis (Funk, 2001). These enzymes are subdivided into 4 major groups: phospholipases for the signal induced release of C20:4ω-6 from membrane phospholipids; prostaglandin endoperoxidase synthases or cyclooxygenases (COX) for the metabolism of C20:4ω-6 to prostanoids; lipoxygenase (LOX) for the formation of hydroxylated eicosatetraenoic acids (HETEs), leukotrienes and cytochrome P450-controlled monooxygenases for epoxidation and hydrolation of C20:4ω-6 (Marks et al., 2000).

Eicosanoids derived from ω-3 FA have less biological potency for inducing cellular responses than those derived from C20:4ω-6. Consequently, the metabolism of ω-3 FA is associated with anti-inflammatory responses. For example, prostanoids derived from C20:5ω-3 (PG of the 3-series) (Fig. 2.8) have been shown to provide anti-inflammatory, protective effects in cancer tissue (Chapkin et al., 2007). Also, C20:3ω-6, an elongation product in the ω-6 metabolism pathway, provides a less prominent cascade and yields PG of the 1-series (Wang et al., 2012). Although the production of eicosanoids from C20:5ω-3 is formed at a slower rate, it can suppress the production C20:4ω-6 derived eicosanoids by competing for a common enzyme (e.g. COX) in the eicosanoid biosynthesis pathway (Schmitz and Ecker, 2008). The balance of these FA precursors in membranes is regulated by dietary supply; therefore diet can influence the eicosanoid response (von Schacky et al., 1985).
Figure 2.8: Metabolic pathway depicting the biosynthesis of eicosanoids derived from C20:4ω-6 and C20:5ω-3. The 20-carbon chains are released from membrane phospholipids by action are phospholipase A$_2$. Subsequent reactions involve cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450-controlled monooxygenases to generate the multitude of eicosanoids that elicit various cellular responses (adapted from Larsson et al., 2004).

(b) PUFA regulation of cell signalling pathways and gene expression

FA are important secondary messengers or mediators of cell signal transductions, particularly of signals triggered at the level of cell membranes (Sumida et al., 1993). As briefly highlighted in the previous section, modulation of inflammatory responses is a key example of PUFAs role in metabolic cascade pathways (Awada et al., 2013). The stimulatory action of PLA$_2$, for example, releases unsaturated FA from the sn-2 position on the glycerol backbone of membrane phospholipids. These free unsaturated FA act directly as secondary messengers (Dennis et al., 1991). For example, C20:4ω-6 plays an important role as a secondary messenger by activating pathways regulating cell proliferation and apoptosis (Cao et al., 2000; Khan et al., 1995; Wolf and Laster, 1999).

PUFA also have a role in regulating gene expressions by directly binding to transcription factors and controlling its activity (Duplus et al., 2000). The peroxisome proliferator activated receptors (PPAR), which function as transcription factors regulating genes expression, are the best recognized sensor systems for FA
These ligand-activated nuclear transcription factors play important roles in cellular differentiation, insulin sensitization and cancer (Krishnakumar and Kraus, 2010). Various PUFA, especially those in the class of ω-3 PUFA, are natural ligands for PPAR (Jump and Clarke, 1999). C20:5ω-3 and C22:6ω-3 are potent activators of the alpha isoform of the molecule compared to ω-6 FA (Heuvel et al., 2006). Subtypes PPARα and PPARγ appear to play an important role in FA catabolism and storage of FA, respectively (Kliewer et al., 1997).

Alternatively, PUFA act indirectly on gene expression through their effects on a) specific enzyme-mediated pathways, such as COX, LOX, protein kinase C (PKC), or sphingomyelinase signal transduction pathways; or b) pathways that involve changes in membrane lipid composition that affect G-protein receptor or tyrosine kinase-linked receptor signalling (Jump, 2004). For example, the activation of PKC is dependent on the release and interaction of PUFA from lipid membranes. PKC belongs to a family of phospholipid depend serine/threonine kinases that play important roles in signal transduction by phosphorylating target protein associated with a variety of cellular responses, including cell growth and differentiation, gene expression, hormone secretion, apoptosis and membrane function (Newton, 1995). Also, it participates in immune responses and inflammatory processes, and is essential for the activation of numerous specialised cells such as neutrophils, lymphocytes and macrophages (Giroux and Descoteaux, 2000; Tan and Parker, 2003). There are numerous isoforms of PKC, which may explain the different effects of different LC PUFA (Newton, 1995).

(c) Substrates for lipid peroxidation
Reactive oxygen species (ROS) and other free radicals are constitutively generated in cells as a by-product of numerous metabolic processes (Valko et al., 2007). These reactive species include free radicals such as superoxide anion (O$_2^-$), perhydroxyl radical (HO$_2^-$), hydroxyl radical (‘OH), nitric oxide (NO) and other reactive species. In the absence of adequate antioxidant defences, the accumulation of ROS generates oxidative stress in cells, and can lead to the damage of DNA, proteins and lipids (Jacob and Burri, 1996).
Plasma membranes PUFA content, particularly in ω-3 FA, are prone to oxidation reactions due to the double bond nature of these macromolecules (Song and Miyazawa, 2001). Lipid peroxidation (LPO) is a free radical mediated chain reaction (Fig. 2.9) that starts with the abstraction of a hydrogen atom from the methyl group (−CH₂−) on PUFA molecules by •OH (Catalá, 2009). Lipid molecules can be oxidized by both enzymatic and non-enzymatic free radical mediated LPO (Niki et al., 2005). Although the resultant lipid hydroperoxides have a relatively short lifespan, their breakdown results in the formation of secondary products of LPO (aldehydes such as malondialdehyde (MDA) and the 4-hydroxyalkenals) that last longer and can attack a variety of cellular targets (Barrera et al., 2008). This process of oxidative lipid damage produces a gradual loss of cell membrane fluidity, reduces membrane potential and increases permeability to ions like Ca²⁺ (Guéraud et al., 2010).

The responses elicited by these LPO products are dependent on their concentration levels in tissue: low level PUFA-induced oxidative stress is cytostatic, higher levels result in apoptosis, and extreme levels cause cellular necrosis (Niki, 2009). Due to the potentially toxic nature of lipid radicals, organisms have developed free radical scavenging mechanisms that protect macromolecules from oxidative damages. These include enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (Gpx) and catalase, as well as non-enzymatic glutathione (GSH) and other antioxidant (Valko et al., 2007). Dietary antioxidants also supply a measure of protection against LPO (Ashokkumar and Sudhandiran, 2008; Bonanome et al., 1992; Kasdallah-Grissa et al., 2008).
2.3.5 Role of PUFA in cancer development

The potential role of PUFA in cancer development was first observed in the Greenland Eskimo population in the 1970s. The dietary patterns of this population were rich in marine species, that contain high levels of LC PUFA C20:5ω-3 and C22:6ω-3 (Bang et al., 1976). The incidence of cancer was much lower in these populations when compared to Eskimos who had migrated to Denmark, where individuals consumed a high fat, animal and plant based diet (Boysen et al., 2008; Dyerberg et al., 1975).

However, since then further studies into the role dietary PUFA have in human cancer development has yielded inconsistent reports (Arem et al., 2013; Bartsch et al., 1999; O’Doherty et al., 2012; Wynder et al., 1997). This may be related to the complexity of the human genome and its interaction with a diverse number of environmental exposures (Lee and Herceg, 2014; Liu et al., 2008; Willett, 2002). Consequently, to elucidate the potential role of PUFA in cancer development,
numerous *in vitro* and animal cancer models have provided great insight into their role in the process.

(a) *Altered lipid profile and FA metabolism*

A study by Meng *et al.* (2004) demonstrated that the membranes of malignant cell lines have a significantly altered FA composition compared to normal cells. Similar effects have been observed in both animal and human cancer studies (Abel *et al.*, 2001; Oraldi *et al.*, 2009; Shim *et al.*, 2005). Cancer cells appear to have a higher proportion of SATS and MUFA esterified to membrane phospholipids than PUFA (Horrobin, 1990). This reduction in PUFA content is due to a loss or reduction in Δ6-desaturase activity (Horrobin, 1990). Impairment of this enzyme reduces the synthesis of biologically functional LC PUFA. Consequently, the loss in availability of these FA may play an important role in abnormal cellular growth that is found in malignant transformations.

(b) *Increases pro-inflammatory eicosanoid synthesis*

Disturbances in eicosanoid production have been highlighted as a major factor in the development of cancers (Marks *et al.*, 2000). LC PUFA C20:4ω-6 is a critical bioactive that plays a significant role in numerous biological functions (Brash, 2001). Under normal conditions cells utilize unesterified C20:4ω-6 as an important signal for inducing apoptosis (Cao *et al.*, 2000). However, cancer cells favour an increase in cell production, thus utilizes C20:4ω-6 and other FA to construct membrane lipids. As a result, the level of unesterified or free C20:4ω-6 becomes depleted. Hereby, a potential pro-apoptotic signal is removed, and thus may promote carcinogenesis. Different products of C20:4ω-6 metabolism are also implicated in carcinogenesis. As much as 80-90% of colon carcinomas show an enhanced cyclooxygenase-2 (COX-2) expression compared with normal mucosa (Cathcart *et al.*, 2011). COX-2 is the enzyme that catalyses the rate limiting step in eicosanoid synthesis, converting C20:4ω-6 to PG. Consequently, the level of PG, particularly PGE$_2$, is high in colon cancers (Castellone *et al.*, 2005). Conversely, ω-3 FA have been reported to inhibit the production of the 2-series of eicosanoids, including PGE$_2$ (Chapkin *et al.*, 2007; James *et al.*, 2000).
(c) Lipid peroxidation in cancer

Oxidative stress has been identified as having a critical role in the development of cancers by both promoting tumour cell initiation and its progression towards a malignancy (Bakalova et al., 2013; Costa et al., 2014; Jrah-Harzallah et al., 2013; Perše, 2013). LPO, a consequence of oxidative stress, has been implicated as a significant causative factor. In patients with cancer prone diseases, the formation of ethno (ε)-modified DNA bases were a consequence of increased trans-4-hydroxy-2-nonenal, a major product of LPO (Bartsch and Nair, 2004). However, during the early phases of development or the promotion stage, the level of LPO is found to be low in colon adenomas (Kondo et al., 1999). This is likely due to cancer cells reduction in esterified ω-3 and ω-6 FA (Cheeseman et al., 1988), which reduces their susceptibility to LPO and provides a stable environment for cancer cell growth and survival. Furthermore, the activation of exogenous antioxidant systems may also play a role (Valko et al., 2007). In Morris hepatoma cells, changes in membranes PUFA content and the loss of protective enzymes against oxygen radicals, such as SOD, increased membranes resistance to LPO (Galeotti et al., 1986). Enhancing membranes PUFA content can impact on cell regulatory processes that control cell numbers by inhibiting cell growth and/or inducing apoptosis (Bartsch et al., 1999). Thus increasing cancer cell membranes resistance to LPO may be a critical factor for promoting tumour growth, depending on the stage of development.

(d) PUFA induced changes in gene expression in cancers

In human prostate cancer cell line PC3 it has been demonstrated that certain FA can enhance cell growth by activating specific gene expressions (Hughes-Fulford et al., 2001). The addition of ω-6 PUFA C18:2ω-6 and C20:4ω-6 to prostate cancer cell significantly increased their growth by inducing the expressions of c-fos and cox-2 genes, whilst MUFA C18:1ω-9 and PUFA C20:5ω-3 had a reducing effect on cell growth. Other studies also show that LC PUFA ω-3 FA C22:6ω-3 has a protective effect against cancer development by down-regulating the family of prostaglandin genes, as well as COX-2 expression and several cell cycle related genes (Narayanan et al., 2001; Singh et al., 1997). Thus evidently, the type of FA supplied has a significant influence on which signal transductions (stimulatory or inhibiting)
are activated. The activation of PARP, for example, which is up-regulated in cancers, can modulate tissue specific responses dependent on the FA supply (Heuvel, 1999).

**Biomarkers of oxidative stress**

As noted earlier, the interaction of ROS with lipid membrane components (e.g. PUFA) elevates oxidative stress levels, which may lead to the induction of apoptosis or necrosis in normal/healthy cells (Chandra *et al.*, 2000). Low or transient ROS exposure can affect several cellular responses, including its influence on transcriptional factors and the expression of genes encoding growth factors and proto-oncogenes that regulates cell proliferation (Allen and Tresini, 2000). The activation of the redox sensitive nuclear transcriptional factor κB (NF-κB) by lipid peroxides, for example, plays an important role in the molecular responses of enterocytes that initiates signals which stimulates cells to proliferate or die during increased oxidative stress (Aw, 1999). Cancer cells, however, have an adaptive nature that allows it to thrive under conditions of high oxidative stress, which support and sustains a persistent proliferative profile (Klaunig *et al.*, 2010). Identifying target biomarkers such as genes that are responsive to changes in cancer cells pro-oxidant/antioxidant (redox) balance, is therefore critical in understanding the disease’s pathology. Microarray technologies have provided an invaluable tool in determining the modification in cancer gene expressions associated with these effects (Cortes *et al.*, 2011; Kachroo *et al.*, 2011).

The up-regulation of several stress-responsive genes is a common feature in cancer cells, many of which include genes encoding antioxidant enzymes that assist their survival under extensive ROS onslaught (Mates *et al.*, 1999). The expression of manganese-SOD was shown to be increased in both gastric adenocarcinomas and oesophageal carcinomas when comparing normal and cancerous cells (Janssen *et al.*, 2000). In colorectal cancer, an increase in lipoperoxide levels coincided with increased activities in of Gpx, SOD and γ-glutamylcysteine synthase (Ozdemirler *et al.*, 1998). Such responses illustrate cancer cells nature to ensure its protection against oxidative damage by eliciting selective gene expressions. Peroxiredoxins (Prdx 1, Prdx2, Prdx3, Prdx4, Prdx5), a family of abundant and ubiquitously expressed thiol-dependent peroxidases, acts as a redox sensor of hydrogen
peroxide (H$_2$O$_2$), and regulates its associated effects on cell signalling (Poynton and Hampton, 2014; Riquier et al., 2014). Prdx1 is commonly overexpressed in many human tumour types, in which it may suppress apoptosis via interactions with the enzyme Jun N-terminal kinase (JNK). Prdx1 suppresses the pro-apoptotic activity of JNK by suppressing its release from a glutathione S-transferase Pi/JNK complex. Furthermore, activation of oxidative stress responsive genes such as the NADPH oxidases (Nox1, Nox2, Nox3, Nox4, Nox5, Duox1 and Duox2) also plays a role in the negative regulation of signalling pathways such as (phosphatidylinositol kinase) PI3-kinase/Akt that affects apoptotic responses (Smolková et al., 2011). Genes encoding for ROS metabolizing proteins like the redox sensitive transcriptional factor NF-E2-related factor 2 (Nrf2) promotes cell survival through the regulation of several other oxidative stress inducible genes: Prdx1, catalase, Gpx, SOD and Trx (Ishii et al., 2000). An increase in Nrf2 expression has also been noted in numerous tumour cells (Ohta et al., 2008; Wang et al., 2008). With changes in cancer cells redox balance having a significant role in promoting mechanisms sustaining its survival, modulating the level of oxidative stress to infer the expression of specific stress responsive genes provides an ideal target for cancer prevention strategies.

2.6 Chemopreventive effects of dietary FA

The multi-staged nature of cancer development provides numerous targets for intervention strategies due to each stage profiling distinct biochemical responses (Pitot, 1993). Initiation and progression, the first and last stages in cancer development, involves irreversible modifications to cells genome. However, promotion, the reversible intermediate stage, is characterized by alterations in gene expressions that provide transformed cells with a proliferative advantage. This makes promotion an ideal target for intervention since its control mechanisms can be manipulated (Russo, 2007).

Therefore, the aim of chemoprevention is to utilize natural or chemical agents that either inhibits the process entirely or retards the growth of malignant cells to reduce the risk of invasive cancers. Key characteristics of a chemopreventive agent is that i) it should target malignant cells with minimal damage to the surrounding, healthy cells, ii) impede or block the mechanisms initiating cells neoplastic transformation or
sustaining cancer cells survival and iii) the chemopreventive agent should be pharmacologically safe, inexpensive and freely available.

As highlighted in previous sections, both ω-3 and ω-6 PUFA have a significant role in both healthy and pathological conditions by participating in the regulation of cell membranes structure and their associated functions. It was also noted that FA of both classes appear to induce opposing responses in cells, demonstrating the importance of tissues ω-6/ω-3 FA ratio that likely determines a disease’s outcome.

The supply of these PUFA in cells is greatly influenced by dietary intake. As mentioned before, most ω-3 and ω-6 FA are provided in the form of triglycerides from various food sources including vegetable, marine and seed oils. Fish oil (FO), high in the LC PUFA C20:5ω-3 and C22:6ω-3 has been extensively investigated for its numerous health benefits. In animal cancer models, FO have been shown to effectively reduce the initiation of tumour development by minimizing the formation of DNA adducts, as well as inducing apoptosis as a chemoprotective mechanism (Hong et al., 2005). Enhancing membrane ω-3 PUFA content also appears to sensitize cancer tissue to the toxic effects of pro-oxidant agents generated by LPO (Das, 1999; Stoll, 2002). Furthermore, the protective effects of FO’s dominant FA are also related to their ability to diminish inflammatory responses in tissue due to their effects on eicosanoid production (Wall et al., 2010).

The potential cancer protective effects of ω-6 FA C18:3ω-6 (γ-linolenic acid) has also been noted in other pathological conditions. In rheumatic conditions that are characterized by an increase in inflammatory mediators (e.g. 4-series leukotriene (LTB₄) and 2-series prostaglandins), treatments with primrose oil and borage oil induced a protective, anti-inflammatory effect (Belch and Hill, 2000). The anti-inflammatory effects provided by these oils are largely due to the supply of C20:3ω-6, a metabolite derived from C18:3ω-6 in the ω-6 FA synthesis pathway, which blocks the transformation of C20:4ω-6 into pro-inflammatory eicosanoids by increasing the supply in 1-series PG (Kapoor and Huang, 2006). In cancer cells the incorporation of C18:3ω-6 into cell membranes effectively blocks cell proliferation in a dose dependent manner (Hrelia et al., 1996). This FA has also been shown to selectively kill cancer cells due to its ability to initiate oxidative stress induced LPO in these cells (Bégin, 1987; Horrobin, 1992).
Canola oil and olive oil are other dietary oils that have also been recognized for their protective effects. Olive oil is best known for its high content in phenolic compounds and MUFA, C18:1ω-9 that displays antioxidant properties (Cicerale et al., 2010; Lee et al., 1998). A major component of the Mediterranean diet, olive oil has been shown to effectively modulate colon carcinogenesis during both early and late stages in the process by reducing aberrant lesions, as well as minimizing pro-inflammatory metabolites derived from C20:4ω-6 (Bartolí et al., 2000). In addition, the antioxidant content of the oil minimizes oxidative damage (de Kok et al., 1994). The oil’s bioactive components also appears to induce apoptotic events in colon cancer cell lines by decreasing the expression of Bcl-2 whose translated protein is a potent inhibitor of programmed cell (Llor et al., 2003). Meanwhile, canola oil contains a high proportion of EFA C18:3ω-3, and has displayed anticancer effects by suppressing cancer growth and reducing pro-inflammatory responses (Bhatia et al., 2011; Hardman, 2007)

2.7 Summary and aims of the study
The Western diet is characterised by high fat intake, particularly in ω-6 FA that is supplied by vegetable oils such as sunflower oil, which is commonly used in South Africa (MacKenzie, 2007). Coupled to low ω-3 FA intake, this dietary pattern generates a high ω-6/ω-3 FA ratio that has been associated with the onset of numerous diseases, including colon cancer. It has been suggested that by incorporating more ω-3 FA into the diet in the form of C20:5ω-3 and C22:6ω-3, this ratio will decrease and minimize the likelihood of pathological events associated with high ω-6 FA intake. The ensuing investigations utilizes combinations of the some of the oils/dietary fats mentioned above (i.e. sunflower + borage oil, sunflower + fish oil, sunflower + borage + fish oils) to generate varying dietary ω-6/ω-3 FA ratio. The aim of the current investigation is to evaluate whether these dietary fats with varying FA ratios can impede colon carcinogenesis by modulating selective biomarkers of colon carcinogenesis.
2.8 References


fatty acid composition differs between normal and malignant cell lines. P. R. Health Sci. J. 23, 103–106.


Website references


CHAPTER 3
PROMOTION MODULATING EFFECTS OF DIFFERENT DIETARY FAT MIXTURES ON THE DEVELOPMENT OF ABERRANT CRYPT FOCI IN 1, 2-DIMETHYLHYDRAZINE-INDUCED COLON CARCINOGENESIS IN RATS.
ABSTRACT

The modulating effect of dietary fat mixtures composed of different ω-6/ω-3 fatty acid (FA) ratios on colon carcinogenesis was investigated by monitoring the development of aberrant crypt foci (ACF), colonic crypt cell proliferation and apoptosis. Male Fischer rat weanlings (50g) were maintained on a modified AIN-76A diet containing sunflower oil as a fat source (5%) until a body weight (BW) of 150 g was reached. Following cancer initiation utilising the colon carcinogen 1, 2-dimethylhydrazine (20 mg/100 g BW subcutaneously once a week for 5 consecutive weeks), rats were fed the modified AIN-76A diet composed of different dietary fat sources: (i) sunflower oil only (S), (ii) S + borage oils (SB), (iii) S + fish oils (SF), (iv) S + B + F oils (SBF) and the reference dietary oils, (v) canola oil and (vi) olive oil for 4 months. Blood chemistry markers of liver function, total bilirubin, direct bilirubin, alanine aminotransferase and creatinine were significantly (p<0.05) reduced suggesting that the dietary oils did not have any adverse effects on liver and kidney function. The total number of early neoplastic lesions or ACF developed was significantly (p<0.05) increased by the SF oil diet. The fish oil diet also tended to increase (p<0.05) the crypts multiplicity (≥7 crypts/focus) of the larger ACF, which was also noticed with the sunflower (s) only control diet. In contrast, the crypt multiplicity (≥7 crypts/focus) was significantly (p<0.05) reduced by the SB, SBF, olive and canola oil based diets when compared to the S and SF containing diets. Aberrant crypt cells proliferative and apoptotic activity was significantly reduced (p<0.05) by the borage oil containing (SB, SBF) diets as well as canola and olive oil based diets when compared to the S and SF diets. The aforementioned oils exhibited a chemoprotective effect when considering crypt multiplicity, presumably by reducing cell proliferation. The SF oil diet, however, appears to enhance colon carcinogenesis as indicated by the increase in the total number of ACF, mainly due to the increased multiplicity of larger ACF lesions. The underlying mechanism involved is likely related to an increased resistance of the ACF towards increased oxidative stress, which is triggered by the SF diet due to the susceptibility of ω-3 long-chain PUFA to lipid peroxidation. Threshold effects regarding the mucosal membrane levels of C20:4ω-6, C18:3ω-6 and C20:5ω-3 and the effect of their prostanoid metabolites on cell growth parameters are likely to be main regulators of the development of persistent ACF when considering the different oil mixtures. This became evident when considering the resultant stimulating effect of the SF diet on ACF development during cancer promotion, whilst the SBF oil combination significantly reduced ACF multiplicity. Differential fat diet-induced effects of the borage/fish oil mixtures on cell proliferation and apoptosis therefore play a determining role in the modulation of cell survival indices of ACF in the colon. Specific FA and polyphenolic interactions when utilising the reference canola and olive oils provide interesting futuristic avenues of research on the chemopreventive potential against colon carcinogenesis.

Key words: colon carcinogenesis, dietary fats, ω-6 and ω-3 fatty acids, ω-6/ω-3 ratio, aberrant crypt foci, proliferation, apoptosis, oxidative stress
3.1 INTRODUCTION

Early detection of colon cancer remains a critical step in preventing further development of the disease, which results in the 3rd largest proportion of cancer related deaths in the Western world (Jemal et al., 2011). With colon carcinogenesis being a multi-step process (Fearon and Vogelstein, 1990), identifying significant biomarkers are key points of interest for researchers in planning preventive measures at specific stages of development. In animal studies numerous biomarkers for colon cancer has been identified (Anti et al., 1994; Bird et al., 1996; Ferrand et al., 2005; Srivastava et al., 2001).

One of these biomarkers includes the aberrant crypt foci (ACF) that were first identified by Bird in mice via colorimetric microscopy (Bird, 1987). ACF have been regarded as precancerous lesions and are widely used as a biomarker of colon cancer in a number of studies (Cademi et al., 1995; Higurashi et al., 2012; Raju, 2008; Wu et al., 2004; Zheng et al., 1999). These precancerous lesions provide a quantitative approach to assess the disease progress in response to either preventive or cancer promoting agents treatments (Pereira et al., 1994; Takayama et al., 1998). Under experimental conditions, these surface abnormalities on the colon appear within weeks after the treatment with carcinogens such as 1, 2 dimethylhydrazine (DMH) or azoxymethane (McLellan and Bird, 1988).

Although the disease evolves from genetic alterations to specific genes (Rustgi and Podolsky, 1992), the influence of numerous environmental factors including dietary patterns, has been associated with the development of colon cancer (Bruce et al., 2000; Slattery et al., 1998). High fat intake, in particular, is considered a major contributor towards the development of the disease (Giovannucci and Goldin, 1997). More so, the type of fat, rather than just the quantity consumed, is a critical factor for consideration (Reddy and Maeura, 1984; Reddy et al., 1996). Diets high in omega-6 (ω-6) polyunsaturated fatty acids (PUFA) appear to exhibit colon tumour promotion activity (Whelan and McEntee, 2004). These effects are demonstrated usually in conjunction with low ω-3 PUFA consumption, of which studies have shown to suppress the development of tumours (Fan et al., 2011; Reddy and Maruyama, 1986; Roynette et al., 2004).
Omega-6 PUFA such as C18:2ω-6 (linoleic acid) are mainly sourced from vegetable oils and seeds such as those from sunflowers and corn (Meyer et al., 2003). Deep cold-water fish, e.g. salmon, mackerel, are a rich source of the long-chain (LC) ω-3 PUFA C20:5ω-3 (eicosapentaenoic acid) and C22:6ω-3 (docosahexaenoic acid), whilst green leafy plants and nuts are a good source of C18:3ω-3 (α-linolenic acid) (Meyer et al., 2003). Apart from being structural components in cellular membranes, the fatty acids (FA) from both classes give rise to a variety of functional metabolites that have a significant role in normal physiological and pathological conditions (Lunn and Theobald, 2006; Murff et al., 2012; Simopoulos, 1999). Modulation of inflammatory effects and oxidative status are the commonly demonstrated responses influenced by the availability of these FA (Barham et al., 2000; Chapkin et al., 2007; Udilova et al., 2003). Metabolism of the 20-carbon ω-6 PUFA C20:4ω-6 (arachidonic acid) via the cyclooxygenase-2 (COX-2) pathway generates pro-inflammatory compounds (Goodman et al., 2004; Williams et al., 1999). The excessive supply of C20:4ω-6 and uncontrolled generation of pro-inflammatory bioactive compounds are positively linked to colon carcinogenesis (Tapiero et al., 2002). The opposite effect is observed with ω-3 metabolites derived from C20:5ω-3, which generates anti-inflammatory compounds that have a suppressive effect on the survival of cancer cells (Chapkin et al., 2007). The generation of metabolites derived from ω-6 and ω-3 PUFA is dependent on the same enzymatic action (James et al., 2000). Consequently, the interactive balance between the available FA from both polyunsaturated classes (i.e. ω-6/ω-3 ratio) plays a significant role in determining the disease’s progress. With diet being a major source of these FA, the supply of specific, biologically functional PUFA can be tightly regulated, and thus influence disease outcome.

A study by Sarotra et al. (2010) further illustrates the interactive effects of specific dietary FA ratios at the initiation and post-initiation phase of chemically induced carcinogenesis. In the study, 1, 2 DMH-induced animals displayed differential responses to fish and corn oil ratio (1:1 and 2.5:1) diets. During the initiation phase, the 1:1 ratio diet increased antioxidant activity to effectively reduce ROS generation and apoptosis, whilst post-initiation, the opposite effects was observed. These responses significantly decreased the number of ACF. A similar result was seen in
the liver, where diets with increased ω-3 FA content modulated the tissue’s oxidative status and reduced the formation of nodules (Abel et al., 2004). Both studies highlight the chemopreventive effect diets constituting low ω-6/ω-3 FA ratios, with a specified FA composition, have on disease progression.

The assessment of cellular responses to specific dietary manipulations serves as a practical tool and/or early biomarkers to monitor the efficacy of diet in disease prevention. Such an approach is particularly important when assessing the potential delay in disease onset. Therefore, the objective for the current study was to evaluate the modulating effect of different dietary fat mixtures and natural oils with specific FA ω-6/ω-3 ratios on the presentation of certain tissue biomarkers post-initiation. ACF development and cell growth parameters related to cell proliferation and apoptosis were determined as prognostic indicators of cancer development in rat colon.

3.2 MATERIALS AND METHODS

3.2.1 Materials
Fish oil rich in ω-3 FA was obtained from Equazen UK Ltd. (St Petersburg Place, London, UK). Borage oil was purchased from Créde Natural Oils (Strand, South Africa). Sunflower oil was supplied by RFS Catering Supplies (Milnerton, South Africa). The Cancer Association of South Africa (CANSA) approved brands of canola and olive oils were sourced locally. The carcinogen, 1, 2-dimethyl hydrazine (DMH), as well as the tissue staining agent methylene blue, was purchased from Sigma-Aldrich (St Louis, USA).

3.2.2 Preparation of diets
A rat diet (AIN-76A) (Table 3.1) with a total fat content of 5% (w/w) was used throughout the experiment with commercially bought sunflower oil being used as a common fat source in 4 of the diets, and canola and olive oil as 2 other individual fat sources. For the experimental fat mixture diets, the AIN-76A diet fat content was modified with fish oil and borage oil to obtain fats with varying ω-6/ω-3 FA ratios for the duration of the experiment. The experimental fat sources consisted of: (i) sunflower oil only (S) – the control diet with respect to the ensuing oil mixtures made, (ii) sunflower oil supplemented with fish oil (SF), (iii) sunflower oil supplemented with
borage oil (SB) and (iv) sunflower oil supplemented with fish oil and borage oil (SBF). Two additional dietary fats, canola oil and olive oil, comprising a total 5% (w/w) fat content, were also used separately as reference oils. The diets were kept at -20 °C under nitrogen until use. The FA profile of the dietary oil mixtures were analysed using gas chromatography (Varian 3300) based on the method described by Opperman et al. (2011). Briefly, 10 mg oil (mixtures/reference) was dissolved in 10 ml chloroform/methanol (2:1) containing butylated hydroxytoluene as an antioxidant. A volume of 100 μl was removed and dried under nitrogen at 37 °C, and 2 ml sulphuric acid in methanol was added for transmethylation and incubated at 70 °C for 2 hours. After cooling, 1 ml distilled water and 2 ml hexane were added and the solution vortexed for 1 minute. After phase separation, the hexane layer containing the FA methyl esters was transferred to a glass tube for solvent evaporation under nitrogen at 37 °C. The dried residue was redissolved in 100 μl hexane and 1 μl was injected into a gas chromatograph. A Varian model 3300 chromatograph was fitted with a BPX-70 fused silica capillary column (30 m x 0.32 mm i.d., 0.25 mm film thickness, SGE). The injector and flame ionisation detector was at 240 °C and 280 °C, while column temperature was programmed from 160 °C to 220 °C at 3 °C per minute. The hydrogen column flow rate was 30 cm/s. Heptadecanoic acid was used as an internal standard and added to all samples. The FA content of the samples were quantified and expressed as a percentage of total FA.

Table 3.1: Composition of the AIN-76A purified rat diet per kilogram.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Grams/kg batch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>200</td>
</tr>
<tr>
<td>D-L-methionine</td>
<td>3</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>150</td>
</tr>
<tr>
<td>Sucrose</td>
<td>500</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
</tr>
<tr>
<td>Dietary oil</td>
<td>total fat content of 5% (w/w) (see Table 3.2)</td>
</tr>
<tr>
<td>Mineral mix</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
</tr>
</tbody>
</table>
3.2.3 Animals and treatment
The Ethics Committee of the Medical Research Council (MRC) of South Africa approved the use of laboratory animals for this study (Addendum 1). At weaning (body weight 50 g), male Fischer rats, strain 344 (n = 120) were divided and housed individually in wire-bottomed cages under controlled lighting (12 hr. cycles) and temperature (23-25°C) with free access to water. The animals were initially fed ad libitum the AIN-76A diet containing sunflower oil as a fat source (5% w/w) until reaching a body weight of approximately 150g.

3.2.4 Experimental design
After 4 weeks at a body weight (BW) of approximately 150 g, the animals were injected subcutaneously with 20 mg/100 g BW 1, 2 dimethylhydrazine (DMH), once weekly for 5 consecutive weeks to induce colon carcinogenesis. The animals remained on the sunflower oil diet throughout the initiation process.

After a week of acclimatization post-induction, the animals were divided into their respective experimental dietary fat mixture groups with 20 animals per treatment: SB; SF and SBF. Another group of animals (N=20) was maintained on the sunflower oil only diet (S) as a control group. Canola oil and olive oil based diets were also used as comparison reference groups. Throughout the experiment, changes in the BW and feed intake were recorded. After 4 months (16 weeks post-initiation), animals were terminated by exsanguination after an anaesthetising injection (i.p.) with sodium pentobarbital (0.15 ml /100 g body weight, 6% solution). Colon samples from all dietary groups were excised, sliced open and washed in a phosphate buffer solution. Samples were collected and fixed between filter lined microscope glass slides in formalin for both the analysis of pre-neoplastic lesion formation (i.e. ACF scoring; N = 10/group) and immunohistochemistry analyses (i.e. Ki-67 and caspase-3 activity; N = 10/group). Blood samples were collected from the abdominal aorta in heparin-coated blood vials, centrifuged and the serum sequestered for clinical chemical analysis. Samples were stored at - 80 °C until analyses.

3.2.5 Methylene blue staining and ACF scoring
Colon samples preserved in formalin were stained with 0.2% methylene blue according to the method of Bird (1995). Briefly, the methylene blue dye was
prepared in Kreb's Ringer buffer and filtered. Samples were blot dried and transferred to the dye solution for 2 minutes. This step was repeated once more to ensure effective staining. The stained colon was divided into 3 segments for scoring under a light microscope (x20 magnification), and the total number of ACF and the corresponding number of crypts within the foci (multiplicity) were quantified.

3.2.6 Colon immunohistochemistry analyses

For the identification of Ki-67 (cell proliferation) and caspase-3 (apoptosis) activity, colon samples were cut into sections, and fixed with 4% paraformaldehyde for 5 hours at room temperature (RT). Samples were then dehydrated and embedded in paraffin according to standard histological protocols. Slides were kindly prepared by Mrs Charna Chapman at the MRC Diabetes Discovery Platform. Tissue paraffin blocks were sliced into 4 µm sections, dewaxed with xylene, and dehydrated by gradient alcohol treatment. Endogenous peroxidase activity in the colon samples were blocked with 3% hydrogen peroxide (H₂O₂) for 5 minutes, and then washed with distilled water for 5 minutes. Sample slides were treated under high pressure (Pascal chamber: 125 °C for 3 minutes; 90 ° for 30 seconds) with 0.01 M citrate buffer (pH 6.0), and rinsed with 0.05 M Tris Buffered Solution (TBS), pH 7.2 for 5 minutes. Normal horse serum and normal goat serum was added to the Ki-67 and caspase-3 slide samples, respectively at a 1:20 ratio in a moisture chamber for 20 minutes at RT. Excess serum was blotted off, and samples were incubated with 100 µl of the required antibody (anti-Ki-67 or anti-caspase-3) at a ratio of 1:50 per section in the moisture chamber, overnight at 4 °C. Slides were then rinsed with 0.05 M TBS, pH 7.2 for 5 minutes. For anti-Ki-67 activity, slides were stained with biotinylated anti-Mouse immunoglobulin G (IgG), and for caspase-3 activity with biotinylated anti-Rabbit IgG for 20 minutes in a moisture chamber at a ratio of 1:200 for 1 hour and 30 minutes, respectively. Samples were rinsed with 0.05 M TBS (pH 7.2) for 5 minutes, and then stained with the Vectastain for 2 hours (anti-Ki-67 activity) or 1 hour (anti-caspase-3 activity). Samples were rinsed with 0.05 M TBS pH 7.2 for 5 minutes, dried and developed with a 3, 3'-diaminobenzidine tetrahydrochloride substrate solution (1 mg/ml). Developing time was controlled under microscope (approximately 10 minutes). Samples were then washed with distilled water for 5 minutes and counterstained with haemotoxylin for 2 minutes. Slides were washed and dried
completely before mounting with DPX. Viewed via microscopy (x20 magnification), positive Ki-67 and caspase-3 activity was identified by the presence of dark brown particles in the nucleus of the colonic crypts. For each section, Ki-67 or caspase-3 positive cells were scored in 5-10 longitudinal crypts, and expressed as a percentage of the total number of colonic crypt cells.

3.2.7 Blood serum clinical biochemistry
Post termination process, blood samples were immediately centrifuged at 1000g for 10 minutes at 4°C. Serum was collected and stored at 4°C for clinical pathology. Serum parameters were measured using kits and a Technicon RA automated analyser at the Nutritional Intervention Research Unit (NIRU) (MRC, Tygerberg, South Africa). The following serum parameters were analysed: total and direct bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine, glucose, triglycerides, total cholesterol, high density lipoprotein cholesterol (HDL) and low density lipoprotein cholesterol (LDL).

3.2.8 Statistical analyses
The results presented are expressed as the mean±standard deviation (i.e. graphs or tables). Normal distribution amongst all the dietary groups means were determined by the Kolmogorov-Smirnov test, with variances homogeneity being tested as well (Bartlett’s test). One way analysis of variance (1-ANOVA) was used to investigate any differences between all group means. A post hoc Tukey-Kramer test determined the significant differences between comparison groups. A 2x2 factor design (2-ANOVA) was used to determine interaction and main effects with the dietary oil mixtures. Interactive effects within these 4 dietary groups (i.e. different combinations of oil factors with each other → S, SB, SF and SBF) were determined to assess an intervention fish and/or borage oil effect. Analyses were determined with GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Significant values were set at p<0.05 or p<0.0001 where indicated.
3.3 RESULTS

3.3.1 Dietary oils fatty acid compositions (Table 3.2)

In control diet (S), the FA content included C18:1ω-9 (26.90%) and saturated FA C16:0 and C18:0 at 6%, respectively. The total PUFA (59.42%) content was comprised of mainly C18:2ω-6 (59.01%), with a ω-6/ω-3 FA ratio of approximately 502:1.

The SB oil mixture consisted of C18:1ω-9 (23.79%), C18:2ω-6 (51.42%), and C18:3ω-6 (6.53%) with a ω-6/ω-3 FA ratio of approximately 38:1. The total PUFA content in the oil was 59.85%, while C16:0 and C18:0 content was 7.34% and 5.33%, respectively. The SF dietary oil contained mainly C18:1ω-9 (25.38%), C18:2ω-6 (36.54%), C16:0 (5.73%), C18:0 (5.62%), C20:5ω-3 (3.53%) and C22:6ω-3 (0.55%) with a ω-6/ω-3 FA ratio of 13:1 and a total PUFA content of 61.50%.

The combination SBF dietary oil provided a FA profile consisting of C18:1ω9 (22.82%), C18:2ω-6 (48.31%), C18:3ω-6 (6.33%), C18:3ω-3 (1.49), C20:5ω-3 (3.31%) and C22:6ω-3 (0.54%). The SBF oil mixture had a ω-6/ω-3 ratio of approximately 10:1 with a total PUFA of 60.80% and a saturated FA content of C16:0 at 6.90% and C18:0 at 5.12%.

Canola oil had a low PUFA content (29.9%) due to a low C18:2ω6 (19.18%), with a ω-6/ω-3 FA ratio of approximately 2:1. The SATS, C16:0 (4.49%) and C18:0 (2.23%) were also the lowest of all the oils used. The oil contained mainly C18:1ω-9 (59.19%) and C18:3ω-3 (10.28%). Olive oil had the highest percentage of C16:0 (9.9%), C18:1ω-9 (77.41%) and the lowest C18:2ω-6 with a total PUFA content of only 6.23% and a ω-6/ω-3 FA ratio of 9:1.
Table 3.2: The fatty acid profile of dietary oils expressed as a percentage of total content.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Dietary oils</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S (N=2)</td>
<td>SB (N=2)</td>
<td>SF (N=2)</td>
<td>SBF (N=2)</td>
<td>Canola (N=2)</td>
<td>Olive (N=2)</td>
</tr>
<tr>
<td>C16:0</td>
<td>6.00±0.01</td>
<td>7.34±0.07</td>
<td>5.73±0.01</td>
<td>6.90±0.21</td>
<td>4.49±0.01</td>
<td>9.91±0.01</td>
</tr>
<tr>
<td>C18:0</td>
<td>5.94±0.01</td>
<td>5.33±0.08</td>
<td>5.62±0.18</td>
<td>5.12±0.14</td>
<td>2.23±0.01</td>
<td>3.70±0.01</td>
</tr>
<tr>
<td>C18:1ω-9</td>
<td>26.90±0.01</td>
<td>23.79±0.53</td>
<td>25.38±0.20</td>
<td>22.82±0.43</td>
<td>59.19±0.01</td>
<td>77.41±0.01</td>
</tr>
<tr>
<td>C18:3ω-3</td>
<td>0.12±0.01</td>
<td>1.52±0.03</td>
<td>0.10±0.01</td>
<td>1.49±0.08</td>
<td>10.28±0.01</td>
<td>0.61±0.01</td>
</tr>
<tr>
<td>C20:5ω-3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>3.53±0.17</td>
<td>3.31±0.15</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C22:6ω-3</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.10±0.07</td>
<td>0.54±0.04</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ω-3 total</td>
<td>0.12±0.01</td>
<td>1.52±0.03</td>
<td>4.37±0.29</td>
<td>5.45±0.22</td>
<td>10.28±0.01</td>
<td>0.61±0.01</td>
</tr>
<tr>
<td>C18:2ω-6</td>
<td>59.01±0.01</td>
<td>51.42±0.49</td>
<td>36.54±0.68</td>
<td>48.31±0.65</td>
<td>19.18±0.01</td>
<td>5.62±0.01</td>
</tr>
<tr>
<td>C18:3ω-6</td>
<td>&lt;0.01</td>
<td>6.53±0.08</td>
<td>&lt;0.01</td>
<td>6.33±0.26</td>
<td>0.08±0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C20:4ω-6</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.21±0.01</td>
<td>0.15±0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ω-6 total</td>
<td>59.31±0.01</td>
<td>58.33±0.41</td>
<td>57.13±0.54</td>
<td>55.35±0.53</td>
<td>19.50±0.01</td>
<td>5.62±0.01</td>
</tr>
<tr>
<td>PUFA total</td>
<td>59.42±0.01</td>
<td>59.85±0.42</td>
<td>61.50±0.51</td>
<td>60.80±0.64</td>
<td>29.78±0.01</td>
<td>6.23±0.01</td>
</tr>
<tr>
<td>ω-6/ω-3</td>
<td>501.82±0.01</td>
<td>38.36±0.72</td>
<td>13.11±0.96</td>
<td>10.16±0.37</td>
<td>1.90±0.01</td>
<td>9.24±0.01</td>
</tr>
</tbody>
</table>

Values represent the mean ± standard deviation of the dietary oils fatty acids profile expressed as a percentage of total content. Dietary oils: S = sunflower oil only, SB = sunflower oil + borage oil; SF = sunflower oil + fish oil; SBF = sunflower oil + borage oil + fish oil.

3.3.2 Body weight gain and feed intake

No significant differences in body weight gain (Fig. 3.1A), nor total feed intake (Fig. 3.1B) were observed with the diets during the 4 month experimental period.

Figure 3.10: Average rat total body weight gain (A), and feed intake in grams/100 gram body weight (B) over the 4 month experimental period. Diets contained the following oil mixtures: S = sunflower oil only, SB = sunflower oil + borage oil, SF = sunflower oil + fish oil, SBF = sunflower oil + borage oil + fish oil, Canola = canola oil and Olive = olive oil.
3.3.3 Intake of individual fatty acids

Based on the feed intake and FA analyses of the oil and oil mixtures, the intake of individual FA was calculated. Due to the different oil combinations provided by the experimental dietary FA ratio groups, sunflower + borage oils (SB), sunflower + fish oils (SF), sunflower + borage + fish oils (SBF), as well as the sunflower oil only diet (S) and the reference oils canola and olive, altered levels of FA intake were observed (Table 3.3).

3.3.3.1 C16:0 (palmitic acid)

No significant fish/borage oil interaction effects were observed in modulating C16:0 intakes. Animals’ intake of C16:0 increased significantly (p<0.05) with the SB oil and SBF oil diets when compared to the S oil diet (Table 3.3). Borage oil demonstrated an increasing (p<0.0001) main effect on C16:0 intake, whilst fish oil resulted in a decrease (p<0.05). C16:0 intakes with the diets containing the reference oils canola and olive, decreased (p<0.05) and increased (p<0.05), respectively relative to the S oil and oil mixture diets.

3.3.3.2 C18:0 (stearic acid)

No significant fish/borage oil interaction effects influenced the animals’ intake of C18:0. Intake of the FA decreased significantly (p<0.05) with diets containing the SB and SBF oil mixtures (Table 3.3). Borage oil had a significant decreasing (p<0.0001) main effect on C18:0 intake, while fish oil increased (p<0.05) the FA. Diets containing canola oil or olive oil reduced (p<0.05) animals’ intake of C18:0 further.

3.3.3.3 C18:1ω-9 (oleic acid)

No significant fish/borage oil interaction effects were noted. The SBF oil diet resulted in a reduced (p<0.05) supply in C18:1ω-9 relative to the S oil diet (Table 3.3). Both fish oil and borage oil had a significant decreasing main effect (p<0.05 and p<0.0001, respectively) on C18:1ω-9 intake, with the former reducing the FA more. Canola and olive oils provided a significantly greater supply of this monounsaturated FA, with the latter dietary group demonstrating the highest (p<0.05) intake of C18:1ω-9.
3.3.3.4 C18:3ω-3 (α-linolenic acid)
No significant fish/borage oil interaction effects were observed. C18:3ω-3 intake was significantly higher (p<0.05) with the SB oil and SBF oil containing diets when compared to the S oil and SF oil diets (Table 3.2). The elevated intake of the FA was due to borage oil’s increasing (p<0.0001) main effect. Animals exposed to the canola oil diet ingested the highest level (p<0.05) of C18:3ω-3.

3.3.3.5 C20:5ω-3 (eicosapentaenoic acid)
Significant fish/borage oil interaction effects were observed with C20:5ω-3 intake. The fish oil containing diets, SF and SBF, provided a significantly high (p<0.05) level of FA intake, whilst only trace levels were consumed with the S oil and SB oil diets (Table 3.2). A significantly higher (p<0.05) intake of C20:5n-3 was noticed with the SF oil diet as compared to the SBF oil diet. Overall, individually both fish oil and borage oil had a significant main effect (Addendum 1D), with fish oil resulting in an increase (p<0.0001) in C20:5ω-3 intake, whilst borage oil reduced (p<0.05) the FA. The reference oils canola and olive only provided trace levels of C20:5ω-3.

3.3.3.6 C22:6ω-3 (docosahexaenoic acid)
No significant fish/borage oil interaction effects were observed. Only trace levels of C22:6ω-3 was consumed with the S oil and SB oil diets (Table 3.3). The fish oil containing diets, SF and SBF, provided significantly higher (p<0.05) levels of the FA, with the highest of C22:6ω-3 being observed with the SF oil diet. This was due to fish oil’s increasing (p<0.0001) main effect on C22:6ω-3 intake. Only trace levels of the FA was consumed with the canola and olive oil diets.

3.3.3.7 C18:2ω-6 (linoleic acid)
No significant fish/borage oil interaction effects were noted. Animals exposed to dietary treatments containing either SB oil or SBF oil consumed significantly less (p<0.05) C18:2ω-6 when compared to the SF oil and S oil containing diets (Table 3.3). The reduced intake levels in C18:2ω-6 was due to borage oil’s lowering (p<0.0001) main effect. C18:2ω-6 intake from the other reference oils diet, canola and olive, was significantly reduced (p<0.05) compared to that of S oil diet.
Table 3.3: The individual fatty acid intake of the rats with the dietary oil feeds.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Diet</th>
<th>F/B main effects</th>
<th>Canola (N=20)</th>
<th>Olive (N=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S (N=17)</td>
<td>SB (N=20)</td>
<td>SF (N=20)</td>
<td>SBF (N=20)</td>
</tr>
<tr>
<td>C16:0</td>
<td>13.72±1.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.19±1.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.07±1.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.46±1.45&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:0</td>
<td>13.59±1.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.74±0.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.82±1.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.45±1.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:1ω-9</td>
<td>61.53±6.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.39±3.27&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>57.89±6.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.12±4.81&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:3ω-3</td>
<td>0.27±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.35±0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.23±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.36±0.30&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:5ω-3</td>
<td>&lt;0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.04±0.87&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.35±0.62&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:6ω-3</td>
<td>&lt;0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.25±0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.18±0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values represent the mean ± standard deviation of the animals’ fatty acid intake expressed in mg FA/100 grams BW. Diets contained the following oil mixtures: S = sunflower oil only (control), SB = sunflower oil + borage oil, SF = sunflower oil + fish oil, SBF = sunflower oil + borage oil + fish oil, Canola = canola oil and Olive = olive oil. Values in a row followed by different letters differed significantly (p<0.05). Main effects caused by either fish oil (F) or borage oil (B) are indicated as follows: increase = ↑, decrease = ↓. Significant main effects set at p<0.0001 (#) and p<0.05 (*)
Lower levels of C18:2ω-6 intake was observed with the olive oil based diet compared with canola oil (p<0.05).

**3.3.3.8 C18:3ω-6 (γ-linolenic acid)**
No significant fish/borage oil interaction effects were observed. Only trace levels of C18:3ω-6 (i.e. <0.01 mg) was provided with the S oil diet, while animals maintained on diets containing oil mixtures SB and SBF demonstrated significantly higher (p<0.05) intake levels of the FA (Table 3.3). The borage oil enriched diet had a significant elevating (p<0.0001) main effect on C18:3ω-6 intake. Trace intake levels of the FA were observed with the SF, canola and olive oil diets.

**3.3.3.9 C20:4ω-6 (arachidonic acid)**
The fish/borage oil interaction effects significantly (p<0.0001) influenced C20:4ω-6 intake. Only trace levels of the FA were consumed with the S oil and SB oil diets, whilst the SF and SBF oil mixture diets presented significantly higher (p<0.05) intake levels (Table 3.3). Both fish oil and borage oil exerted significant main effects, with fish oil having the greater effect in increasing (p<0.0001) the FA intake, whilst borage oil had a reducing (p<0.0001) effect. Canola and olive oil only provided trace levels of C20:4ω-6.

**3.3.3 Total ACF and multiplicity**
The total number of early pre-neoplastic lesions (total ACF) increased (p<0.05) significantly with the SF diet when compared the S, SB, SBF, canola and olive oil diets (Table 3.4). With regards to ACF multiplicity, a great number of small (1-3 crypts/ focus) to medium (4-6 crypts/focus) sized lesions were observed amongst all the dietary groups. However, their tally was not significantly different in all the dietary treatment groups’ comparisons. The multiplicity of lesions (≥7 crypts/focus) were significantly (p<0.05) decreased by the SBF oil diet, illustrating an interaction between the fish and borage oils, despite the fact that the SF oil diet tended to increase the multiplicity (not significantly). Overall borage oil showed a decreasing (p<0.05) main effect. Diets containing canola and olive oils exhibited a similar decreasing effect.
Table 3.4: Total ACF and crypt multiplicity in the colon of rats as a function of the dietary oil treatments.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Total ACF</th>
<th>Multiplicity (crypts/focus)</th>
<th>Main effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Small (1-3)</td>
<td>Medium (4-6)</td>
</tr>
<tr>
<td>S (N=8)</td>
<td>57.50±8.89a</td>
<td>25.67±7.17a</td>
<td>27.80±6.53a</td>
</tr>
<tr>
<td>SB (N=10)</td>
<td>63.20±13.88a</td>
<td>23.71±7.78a</td>
<td>26.50±6.66a</td>
</tr>
<tr>
<td>SF (N=9)</td>
<td>98.67±4.51b</td>
<td>23.33±9.31a</td>
<td>27.29±6.87a</td>
</tr>
<tr>
<td>SBF (N=9)</td>
<td>57.75±9.00a</td>
<td>24.00±7.93a</td>
<td>23.17±5.42a</td>
</tr>
<tr>
<td>Canola (N=9)</td>
<td>67.75±5.85a</td>
<td>35.83±8.42a</td>
<td>27.60±7.60a</td>
</tr>
<tr>
<td>Olive (N=10)</td>
<td>49.00±7.78a</td>
<td>26.00±4.30a</td>
<td>19.20±4.60a</td>
</tr>
</tbody>
</table>

Values represent the mean±standard deviation of the total aberrant crypt foci (ACF) and crypt multiplicity. Diets contained the following oil mixtures: S = sunflower oil only (control), SB = sunflower oil + borage oil, SF = sunflower oil + fish oil, SBF = sunflower oil + borage oil + fish oil, Canola = canola oil and Olive = olive oil. Total ACF = total aberrant crypt foci. Significant differences between diet groups in the column Large (≥7 crypts/focus) indicated by different superscript letters (p <0.05). Main effects caused by either fish oil (F) or borage oil (B) are indicated as follows: increase = ↑, decrease = ↓, no interactive main F oil effects determined = n/a. Significant main effects set at p<0.05 (*).

3.3.4 Colon immunohistochemistry

3.3.4.1 Effect on cell proliferation (Ki-67 activity)

Tissue samples displayed a significant reduction (p<0.05) in Ki-67 positive cells with the oil mixture diets SB and SBF when compared to the diet containing the S oil (Table 3.5). Borage oil had a significant reducing (p<0.0001) main effect on the extent of proliferative activity within the colonic crypts. A similar reducing effect was noted with the canola oil and olive oils based diets.
Table 3.5: Proliferative Ki-67 activity of the colon crypts in response to diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Proliferation</th>
<th>F/B main effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Ki-67 positive cells per total crypt cells</td>
<td></td>
</tr>
<tr>
<td>S (N=7)</td>
<td>100.00±31.31(^a)</td>
<td>n/a</td>
</tr>
<tr>
<td>SB (N=8)</td>
<td>66.77±25.19(^b)</td>
<td>(\downarrow) B#</td>
</tr>
<tr>
<td>SF (N=9)</td>
<td>89.50±34.12(^a)</td>
<td>n/a</td>
</tr>
<tr>
<td>SBF (N=8)</td>
<td>65.85±22.32(^b)</td>
<td>(\downarrow) B#</td>
</tr>
<tr>
<td>Canola (N=8)</td>
<td>63.43±26.80(^b)</td>
<td>n/a</td>
</tr>
<tr>
<td>Olive (N=7)</td>
<td>62.31±19.14(^b)</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Values represent the mean±standard deviation of the percentage of Ki-67 positive cells relative to the total number of colon crypt cells. Diets contained the following oil mixtures: S = sunflower oil only (control), SB = sunflower oil + borage oil, SF = sunflower oil + fish oil, SBF = sunflower oil + borage oil + fish oil, Canola = canola oil and Olive = olive oil. Values in a column followed by different letters (superscript) differed significantly (p<0.05). Main effects caused by either fish oil (F) or borage oil (B) are indicated as follows: increase = \(\uparrow\), decrease = \(\downarrow\), n/a = no interactive main F/B oil effects determined. Significant effects set at p<0.0001 (#) and p<0.05 (*).

3.3.4.2 Induction of apoptosis (Caspase-3 activity)

The inclusion of fish oil (diet SF) did not alter the percentage of cell undergoing cell death when compared to the S oil diet (Table 3.6). However, diets containing oil mixtures SB and SBF significantly reduced (p<0.05) the level of caspase-3 positive cells, illustrating borage oil’s reducing (p<0.0001) main effect. The canola oil and olive oils based diets also decreased (p<0.05) apoptosis with the former having the greater reducing effect. Canola oil exposure generated the lowest number of caspase-3 positive cells relative to the SBF diet as well.
Table 3.6: Apoptotic caspase-3 activity of the colon crypts in response to diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Apoptosis % caspase-3 positive per total cells per crypt cells</th>
<th>F/B main effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>S (N=7)</td>
<td>100.00±32.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n/a</td>
</tr>
<tr>
<td>SB (N=8)</td>
<td>46.67±21.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>↓ B#</td>
</tr>
<tr>
<td>SF (N=9)</td>
<td>91.18±31.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>n/a</td>
</tr>
<tr>
<td>SBF (N=7)</td>
<td>49.11±19.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>↓ B#</td>
</tr>
<tr>
<td>Canola (N=7)</td>
<td>23.57±14.86&lt;sup&gt;c&lt;/sup&gt;</td>
<td>n/a</td>
</tr>
<tr>
<td>Olive (N=7)</td>
<td>32.15±8.34&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Values represent the mean±standard deviation of the percentage of Ki-67 positive cells relative to the total number of colon crypt cells. Diets contained the following oil mixtures: S = sunflower oil only (control), SB = sunflower oil + borage oil, SF = sunflower oil + fish oil, SBF = sunflower oil + borage oil + fish oil, Canola = canola oil and Olive = olive oil. Values in a column (super script) followed by different letters differed significantly (p<0.05). Main effects caused by either fish oil (F) or borage oil (B) are indicated as follows: increase = ↑, decrease = ↓, n/a = no interactive main F/B oil effects determined. Significant main effects set at p<0.0001 (#).

3.3.5 Blood clinical biochemistry parameters

Only total and direct bilirubin, alanine aminotransferase (ALT) and creatinine levels were significantly altered in response to the dietary oil treatments (Table 3.7). Total bilirubin content reduced significantly (p<0.05) with the SBF oil diet when compared to that of SB. Hereby fish oil demonstrated a significant decreasing (p<0.05) main effect. Canola oil had a similar reducing (p<0.05) effect. Both canola and olive oil based diets lowered (p<0.05) the level of direct bilirubin relative to the SB diet.
Table 3.7: Effect of the dietary oil feeds on the animals’ blood clinical biochemistry.

<table>
<thead>
<tr>
<th>Clinical Biochemical parameter</th>
<th>S</th>
<th>SB</th>
<th>SF</th>
<th>SBF</th>
<th>F/B main effect</th>
<th>Canola oil</th>
<th>Olive oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>total bilirubin</td>
<td>0.89±0.42</td>
<td>1.15±0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.81±0.18</td>
<td>0.60±0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>↓ F*</td>
<td>0.62±0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.78±0.35</td>
</tr>
<tr>
<td>Direct bilirubin</td>
<td>4.01±0.32</td>
<td>5.16±1.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.38±0.79</td>
<td>4.13±0.76</td>
<td>–</td>
<td>3.74±0.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.13±0.88&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALP</td>
<td>61.50±9.33</td>
<td>49.50±8.79</td>
<td>64.50±13.30</td>
<td>52.80±9.80</td>
<td>–</td>
<td>66.90±12.33</td>
<td>65.87±22.39</td>
</tr>
<tr>
<td>ALT</td>
<td>64.83±9.13</td>
<td>96.63±32.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78.38±20.64</td>
<td>60.00±14.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>–</td>
<td>61.00±15.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.50±17.33</td>
</tr>
<tr>
<td>AST</td>
<td>99.26±21.33</td>
<td>166.53±50.47</td>
<td>103.71±8.00</td>
<td>119.25±35.11</td>
<td>–</td>
<td>93.30±19.39</td>
<td>110.64±29.71</td>
</tr>
<tr>
<td>creatinine</td>
<td>53.65±4.61&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>52.39±8.72&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>47.29±9.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.78±7.18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>↓F#, ↓B*</td>
<td>40.87±8.51</td>
<td>43.17±16.85</td>
</tr>
<tr>
<td>glucose</td>
<td>9.20±0.68</td>
<td>9.26±1.09</td>
<td>9.28±0.55</td>
<td>9.37±0.60</td>
<td>–</td>
<td>9.43±0.78</td>
<td>8.93±0.78</td>
</tr>
<tr>
<td>triglycerides</td>
<td>0.50±0.17</td>
<td>0.64±0.29</td>
<td>0.42±0.14</td>
<td>0.45±0.15</td>
<td>–</td>
<td>0.68±0.28</td>
<td>0.68±0.38</td>
</tr>
<tr>
<td>cholesterol</td>
<td>1.75±0.16</td>
<td>1.67±0.29</td>
<td>1.62±0.22</td>
<td>1.58±0.29</td>
<td>–</td>
<td>1.64±0.23</td>
<td>1.61±0.53</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.10±0.21</td>
<td>1.18±0.14</td>
<td>1.17±0.14</td>
<td>1.26±0.25</td>
<td>–</td>
<td>1.23±0.17</td>
<td>1.17±0.40</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.59±0.19</td>
<td>0.44±0.13</td>
<td>0.47±0.23</td>
<td>0.42±0.09</td>
<td>–</td>
<td>0.37±0.07</td>
<td>0.34±0.17</td>
</tr>
</tbody>
</table>

Values are the mean ± standard deviation of rat blood chemistry components. Diets contained the following oil mixtures: S = sunflower oil only, SB = sunflower oil + borage oil, SF = sunflower oil + fish oil, SBF = sunflower oil + borage oil + fish oil, Canola = canola oil and Olive = olive oil. Blood serum components: ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; HDL = high density lipoprotein; LDL = low density lipoprotein. Values in a row followed by different letters (superscript) differed significantly (p<0.05). Main effects caused by either fish oil (F) or borage oil (B) are indicated as follows: increase = ↑; decrease = ↓. Significant main effects set at p<0.001 (#) and p<0.05 (*).
Blood serum levels in ALT was significantly reduced (p<0.05) with the diet containing oil mixture SBF when compared to the SB oil diet. The canola oil diet had a similar decreasing (p<0.05) effect on ALT content. Creatinine levels were reduced (p<0.05) with the SF oil and SBF oil diets when compared to the S oil. Borage oil and fish oil had a significant reducing (p<0.05 and p<0.0001, respectively) main effect on creatinine, with fish oil enhancing the decrease.

3.4 DISCUSSION

Aberrant crypt foci (ACF) are one of the widely accepted biomarkers used to monitor the progress of early precancerous cells in the colon. These neoplastic lesions, appearing on the colon mucosal surface, result from the deregulation of mechanisms controlling cellular homeostasis that arise from genetic alterations (Bird, 1995; Chen et al., 2005; Michor et al., 2005). Chemopreventive studies demonstrate that numerous dietary components, which include dietary fats and its FA constituents, modulate the development of ACF (Rao et al., 2001; Schmelz et al., 2000; Volate et al., 2005; Zheng et al., 1999). Of interest are the cancer modulating effects of ω-6 PUFA found in common seed oils and long-chain (LC) ω-3 PUFA, found in fish oils. Both classes of PUFA and their metabolites have a variety of physiological roles (Zamara, 2004). However, individual PUFA have been identified for their enhancing or suppressive role in cancer development (Dommels et al., 2002). Thus, the interaction of specific ω-6/ω-3 PUFA dietary FA ratios is an important factor to consider in determining disease outcome.

The current study demonstrated that the FA profile of the different dietary oils consumed by the animals elicited differential effects on the selected biomarkers identified including ACF formation. Certain oil diets (SF, SBF and canola) reduced the level of select serum enzymes (total and direct bilirubin, ALP, creatinine), which serves as markers of hepato- and nephrotoxicity in the DMH-induced animals (Swaroop and Gowda, 2012). Thus, a decrease demonstrated the non-toxic effects of the dietary oils with an increase in liver and kidney function during the dietary treatment period. Regarding ACF development, a great number of small lesions (1-3 crypts/focus) tended to develop in response to the effect of canola oil. Medium sized lesions (4-6 crypts/focus) appear to decrease with the SBF and olive oil based diets.
Interestingly, with the inclusion of fish oil (SF diet) and to some extent the sunflower (S) oil only diet, animals displayed a distinct dietary fat input response favouring an increase in the multiplicity of the larger ACF ($\geq 7$ crypts/focus), while the SF significantly increased the induction in the total number of ACF. Studies suggest that ACF with higher crypt multiplicity are more likely to persist and progress into a more aggressive cancer phenotype, (Raju, 2008; Srivastava et al., 2001). Thus, it is suggested to be a more favourable predictor of tumour incidence, whereas the smaller category foci and ACF tend to regress (Perše and Cerar, 2011; Roncucci et al., 2000).

ACF arise as a consequence of an imbalance between cell proliferation and apoptosis with these lesions favouring increased proliferative activity (Boman et al., 2008; Yamashita et al., 1994). In other animal studies, exposure to fish oil has demonstrated anticancer effects by reducing total ACF and cell proliferation (Latham et al., 1999; Rao et al., 2001; Reddy et al., 1991). In the current study, an opposite effect was noticed with the SF oil based diet, as indicated by the increased level of higher crypt multiplicity. However, the experimental models from the aforementioned anticancer fish oil effect differed from the current study with regards to the stage of dietary intervention. Most of the studies dietary interventions occurred at a pre-carcinogen treatment period as opposed to the effects of the dietary oil mixtures during the cancer promotion stage in the current investigation. A possible reason for the discrepancy in effect is likely associated with the resistance towards oxidative stress of persistent ACF in the colon. An increased level of oxidative stress is associated with the induction of the initiated cells, which makes them more susceptible to the induction of apoptosis and reduces carcinogen-induced initiation as reported previously (Latham et al., 1999). A similar effect is noticed in the liver where initiated cell populations are very susceptible to undergo apoptosis following cancer initiation (Schulte-Herman et al., 1997). However, once established these carcinogen initiated cell populations displayed a highly resistant phenotype during cancer promotion in the liver, suggesting that they survive in stressful microenvironments (Farber et al., 1991). In the colon, the carcinogen DMH is a potent inducer of DNA damage (Klaunig et al., 2011; Sasaki et al., 1998), which imparts selective growth advantages to the initiated cells during cancer promotion.
Therefore, to sustain the altered phenotype, protective mechanisms are activated to adapt the cancer cells metabolisms which included resistance towards oxidative stress, allowing their growth and survival (Cairns et al., 2011; Sarotra et al., 2010).

3.4.1 Modulation of cell proliferation

The modulating effect of fish oil is related to its FA content in LC ω-3 FA such as C20:5ω-3 and C22:6ω-3. These FA are potent inducers of apoptosis due to their susceptibility to free radical attack and consequent initiation of oxidative stress (Das, 1999; Hong et al., 2005). A recent study indicated that the fish oil containing diet significantly increases oxidative stress in the colon mucosa of rats when fed for 4 months, which was related to an increase in oxidative stress that resulted from an enhanced incorporation of C20:5ω-3 and C22:6ω-3 (Abrahams, 2009 - Addendum 4). Despite the increase in oxidative stress, cell proliferation was increased in the current study, suggesting towards higher proliferative activity in these ACF (Bird, 1995). This response may be a consequence of an altered oxidative status, e.g. increased antioxidant capacity in the pre-cancerous lesions that perpetuates the transformed condition's resistance against oxidative stress. Hereby, an increased cell proliferative index is supported as suggested elsewhere (Klaunig and Kamedulis, 2004).

Diets containing the oil mixtures SB and SBF, and also the reference oils canola and olive, exerted a protective effect by reducing cell proliferation, highlighting different underlying mechanistic effects in response to the dietary fat supply. This became apparent as the SF and control sunflower (S) only oils diets proportionally showed a higher proliferative response in the colon under the current experimental conditions. The S only diet is known to promote colon carcinogenesis due to the high levels of C18:2ω-6 FA that is likely to modulate the levels of the C20:4ω-6-derived PGE₂ prostanoids metabolites known to stimulate cell proliferation (Kawarimori et al., 2003). Both the S and the SF oil diets provide similar C20:4ω-6 levels in the colon mucosa phosphatidylcholine (PC) and phosphatidylethanolamine (PE) phospholipids to sustain such an increased proliferative response (Abrahams, 2009 – Addendum 4). Borage oil contains the PUFA C18:3ω-6, whose metabolism to C20:3ω-6
(dihomo-γ-linolenic acid) generates metabolites, such as PGE$_1$, that have both anti-inflammatory and anti-proliferative properties (Kapoor and Huang, 2006; Wang et al., 2012). Of interest is that borage oil significantly (main effect) reduced the intake of C20:4ω-6 and C20:5ω-3, while selectively increasing the intake of C18:3ω-6. However, detailed FA analyses of the colon mucosa of rats fed the same dietary oil mixtures used in the current study for 4 months, indicated that borage oil increased both C18:3ω-6 and C20:4ω-6 in the colon mucosa in PC and PE phospholipids (Abrahams, 2009 – Addendum 4). Borage oil also significantly increased C20:5ω-3, which provides interesting interactive responses when considering the reduction of ACF in the current study. This became evident as the combination of C18:3ω-6 with C20:5ω-3 FA in the SB and SBF oil diets serves as the precursor FA for the anti-inflammatory PGE$_1$ and PGE$_3$ prostaglandins, respectively, which reduces the synthesis of pro-inflammatory prostanoid PGE$_2$, derived from C20:4ω-6 (Barham et al., 2000; Pham et al., 2006). Therefore, the combined effect of fish and borage oil seems to be a more efficient approach for inhibiting of ACF development. This suggests that a threshold of introducing ω-3 FA exists, above which adverse responses can be recorded as demonstrated with the SF oil only diet. Olive and canola oils, although not dominant in PUFA content, appears to have similar mechanistic effects by reducing the synthesis of PGE$_2$ (Bartoli et al., 2000; Bhatia et al., 2011).

3.4.2 Modulation of apoptosis

Apoptosis plays a critical role in the maintenance of the cellular environment in colon tissue by providing a controlled removal of damaged cells to balance cell renewal (Hall et al., 1994). Inhibition of this process is considered a critical feature in the development of colorectal cancer (Bedi et al., 1995; Thompson et al., 1992). However, coinciding with the decrease in proliferative activity in the current study, apoptosis was also reduced by the diets containing the oils SB, SBF canola and olive oils, with canola oil having the largest effect. However, the relative balance between the two growth regulatory parameters is not known, but it would appear that the reduction in cell proliferation is also associated with a decrease in apoptosis in order to maintain cellular homeostasis. With respect to the SB and SBF oils, though, subtle changes in the FA profiles could stimulate the induction of apoptosis relatively
to the cell proliferative index, and hence the reduction in ACF multiplicity. In this regard, the increase in C20:4ω-6 in the colon mucosa by SB (Abrahams, 2009 – Addendum 4) is of interest as it is associated with the modulation of ceramide levels, a key determinant of apoptosis (Jayadey et al., 1994).

The level of apoptosis in the colon crypts was significantly (p<0.05) higher in the rats fed the SF and the control S diet when compared to the other oil mixture diets. This coincided with an enhanced cell proliferative index and the increased apoptosis is likely to maintain cellular homeostasis in the colon crypts. With respect to the S only diet, C18:2ω-6 is the main dietary FA and likely the main driving force behind the modulation of cell proliferation by regulating the level of C20:4ω-6 and the production of PGE$_2$. The latter prostanoid has been demonstrated to stimulate enhanced proliferation in colon cancer cells (Castellone et al., 2005). However, a critical level of C20:4ω-6 seems to determine the balance between cell proliferation and apoptosis. Animal experiments indicated that a minimum amount of C18:2ω-6 is required to promote the growth of carcinogen-induced tumours in rodents and that a threshold exists above which the FA did not appear to exhibit a specific tumour-promoting effect (Zock and Katan, 1998). A similar response is noticed in the current study where specific FA ratios exhibited opposite responses with respect to the crypt cells proliferative index in the carcinogen-induced lesions in the colon. Regarding the SF oil mixture, the dietary intake FA profile, as well as the level of FA incorporation in the colon mucosa, the ω-3 FA content was significantly increased, which decreased the ω-6/ω-3 ratio in both PC and PE phospholipids (Abrahams, 2009 – Addendum 4). The increased incorporation of the ω-3 FA significantly increased the level of lipid peroxidation (LPO) in the colon mucosa, which is related to an increased level of oxidative stress and the induction of apoptosis. This is in accordance with the literature where an increased dietary PUFA intake and associated LPO plays a determining role in the induction of apoptosis (Das, 1999; Valko et al., 2007; Serini et al., 2009). Clearly a critical balance between cell proliferation and the level of apoptosis will determine the effect of a specific oil mixture on the growth of altered pre-neoplastic lesions in the colon. In the current study, the SB and SBF oil mixtures are likely to favour the induction of apoptosis at the cost of cell proliferation. This effect results in a reduction in the multiplicity of ACF lesion, while the opposite effect is noticed with the SF and S oils.
The reference oils provided a very low supply of PUFA compared to the SB and SBF oil diets, thereby reducing the potential for initiating LPO. Furthermore, the high intake of FA C18:1ω-9, major constituents of olive and canola oil, exerts a protective affect against oxidative stress (Bonanome et al., 1992; Duval et al., 2002; Lee et al., 1998). In addition, the polyphenolic content of the reference oils may also be a contributing factor in protecting the initiated condition against oxidative stress (Kuwahara et al., 2004; Manna et al., 1997). However, the polyphenolic content of the oils has also demonstrated anticancer affects. Canolol or 4-vinyl-2, 6-dimethoxyphenol, a novel and potent antioxidant in canola oil has been shown to reduce the level of COX-2 expression that enhances PGE\textsubscript{2} induced cell proliferation in gastric cancer cells (Jiang et al., 2013). Similarly, the anticancer effects of hydroxytyrosol, a major polyphenol in olive oil, has been linked to its ability in arresting the cell cycle in the G2/M phase, thus preventing cell proliferation and inducing apoptosis (Corona et al., 2007; Elamin et al., 2013). Therefore, the underlying mechanisms required to suppress colon carcinogenesis by these natural oils may be related to specific polyphenol and FA interactions that modulate the cell growth parameters associated with cell proliferation and apoptosis.

### 3.4.3 Modulation of membrane lipids

Apart from changes in the FA content of membranes, the relative distribution of membrane lipids including cholesterol and the major phospholipids, PC, PE and the sphingolipid, sphingomyelin are also key determinants of cell growth indices as well as membrane fluidity. These membrane constituents have been reported to be changed by DMH, which reduced the cholesterol and the cholesterol:phospholipid ratio in the colon mucosa (Kanwar et al., 2011). In addition, the phospholipids PC and PE were also increased, although separate analyse of the individual concentrations were not conducted. A comparative study in rats indicated that the SF oil diet significantly decreases PE, resulting in an increase in the PC/PE ratio (Abrahams, 2009 - Addendum 4). The SB diet significantly decreases the ratio due to a marked decrease and increase in PC and PE, respectively. No clear effect was noticed on the cholesterol membrane level, although the SBF oil diet showed a marked increase. It was suggested that an increase in membranes PC content in the DMH treated rats was associated with an increased proliferative capacity (Kanwar et
Therefore, changes in the cholesterol, PC and PE levels in the mucosal membrane by the different dietary fat mixtures could be indicative of the diverse responses related to the induction of cell proliferative and apoptotic indices and the development of ACF in the colon.

3.5 CONCLUSIONS

Colon tissue biomarkers from the carcinogen treated animals displayed varied responses relative to the intake of the different FA constituents from the dietary fat mixtures supplied. The oil mixtures containing borage oil (SB and SBF), as well as canola and olive oil reduced crypt multiplicity and cell proliferation in the colon. These responses are likely a consequence of specific FA interactive effects with dietary antioxidants on signal transductions pathways related to cell survival indices. Conversely, fish oil (SF oil diet) and to some extent sunflower oil (S diet), appears to have an enhancing effect on crypt multiplicity during cancer promotion, although interactive effects between ω-3 PUFA (SF diet) and ω-6 PUFA (SB diet) in the combined SBF diet provide interesting chemopreventive research alternatives. A plausible explanation is that the pre-neoplastic lesions may be resistant to the oxidative stress environment effected by fish oil. Hereby, the survival of the pre-existing cancerous lesions is enhanced. Therefore, to allow for the clonal expansion of the transformed cells, protective mechanisms are likely activated that deters the diet’s potential oxidative stress induced removal of damaged cells (Hardman et al., 2002). Studies demonstrate that an increased endogenous antioxidant capacity plays a significant role in protecting neoplastic cells against oxidative stress-induced cell death, allowing these cells to survive and proliferate (Hardman et al., 2002; Sarotra et al., 2010). In addition, threshold effects and specific ω-6/ω-3 FA interactions, related to the production of prostanoids with differential effects on cell proliferation and the induction of apoptosis, also seems to be the main underlying driving force in determining the altered growth patterns of pre-neoplastic lesions in the colon. Of interest is the potential mechanism involving oxidative stress as well as the roles of C20:5ω3, C20:3ω-6 and C20:4ω-6 FA and their respective prostanoids in the regulation of cell survival regulatory pathways related to cell proliferation and apoptosis.
Antioxidants supplied by the diet also have a considerable effect in protecting cells against oxidative stress (Lee and Lee, 2006). Therefore, the polyphenolic constituents of the dietary oils, particularly those of the canola and olive oils, likely exert anti-proliferative properties as noted by the significant decrease in ACF multiplicity and its associated proliferative activity. Further investigation into these eluded mechanisms may also provide a more comprehensive perspective of the possible interactive effects of selected dietary fat mixtures and its bioactive polyphenolic constituents.

3.6 REFERENCES


CHAPTER 4

MODULATION OF THE EXPRESSION OF OXIDATIVE STRESS AND ANTIOXIDANT DEFENCE GENES BY DIETARY FAT DURING 1, 2-DIMETHYLHYDAZINE INDUCED COLON CARCINOGENESIS IN RATS.
ABSTRACT

Cancer cells have a highly adaptive nature that enables their survival under abnormal cellular conditions. During cancer promotion, pre-neoplastic cells demonstrate resistance to oxidative stress that plays a significant role in cell survival. Dietary components can modify cells oxidative status by influencing the redox balance associated regulatory responses that include altering gene expressions. In the current investigation, the effect dietary fat mixtures with varying ω-6/ω-3 fatty acid compositions have on colon carcinogenesis was evaluated by screening the expression of genes associated with changes in cells redox balance. Male Fischer rats (150g) feeding on a modified diet containing sunflower oil (5% w/w) as a fat source, was administered with 1, 2-dimethylhydrazine (20 mg/100 grams body weight subcutaneously) for 5 consecutive weeks. One week following cancer initiation rat were fed diets with the following dietary fat sources: sunflower oil only (S), S + borage oil (SB), S + fish oil (SF), SBF and canola and olive oils as reference. After 4 months, RNA was isolated from the colonic mucosa epithelium according to standard protocols, and the expression of 84 genes associated with oxidative stress and antioxidant defence mechanisms were assessed by quantitative real-time PCR microarrays. The expression of 36 genes was significantly altered relative to the sunflower oil diet, with canola and olive oil based diets having the majority of effects. Of the genes analysed, the Gpx family of antioxidant enzymes was significantly (p<0.05) up-regulated by the SB, SF, canola and olive oil based diets, demonstrating a protective response against increased ROS levels in the colon. Other genes up-regulated by canola and olive oils included those involved in DNA repair mechanisms, maintenance of cell membrane integrity and immune responses. The canola and olive oil induced modulation of redox associated regulatory mechanisms related to the expression of genes that mediate cell proliferation and apoptotic pathways. These effects may potentially serve as a chemopreventive measure against the development of colon cancer with major emphasises on interactions between the FA and polyphenolic constituents.

Key words: colon carcinogenesis, fatty acids, microarrays, gene expression, oxidative stress, antioxidants
4.1 INTRODUCTION

The development of colon cancer is a multistage process involving genetic and epigenetic events that result in the gradual transformation of altered pre-neoplastic cells into tumours (Fearon and Vogelstein, 1990). Identifying the mechanisms that triggers these sequential events has been a major research focus in the development of intervention strategies (Greenwald et al., 1995; Sharma et al., 2001). Of the numerous mechanisms identified influencing this process, modulation of oxidative stress parameters has contributed significantly to the aetiology of the disease (Perše, 2013; Reuter et al., 2010).

Oxidative stress refers to the imbalance of pro-oxidants and antioxidants favouring the former, which can lead to the depletion of the antioxidant capacity of a cell and resulting in the damage of critical macromolecules, e.g. DNA, protein and lipids. Under normal physiological conditions, cells generate pro-oxidants or free radicals such as reactive oxygen species (ROS) as a by-product of numerous aerobic regulatory processes (Dröge, 2002). Simultaneously, antioxidant systems are active to maintain cellular homeostasis and integrity (Valko et al., 2007). Inadequate scavenging by antioxidants or high levels of ROS generates a skewed redox balance that leads to oxidative stress, which contributes towards both the initiation and promotion stages of malignant transformation (Bakalova et al., 2013; Jrah-Harzallah et al., 2013). An increase in ROS is commonly observed during carcinogenesis induction in the colon of rats treated with mutagenic agents such as 1, 2-dimethylhydrazine (DMH) (Arutiunian et al., 1997). In addition, changes in gene expression mediated by ROS can either lead to the activation/inactivation of specific signal transduction pathways that greatly influence the promotion of tumour development (Allen and Tresini, 2000; Aw, 1999). Under normal conditions, ROS play a significant role in activating signals for cell proliferation (Chiu and Dawes, 2012), as wells as influencing apoptotic responses (Dröge, 2002). Both pathways are significantly altered during cancer development, with the process favouring increased cell proliferation and a reduction/inhibition of apoptosis (Chen et al., 2005; Hanahan and Weinberg, 2011).
Environmental factors and diet have a major effect on the redox balance within a cell. In humans, increased levels of oxidative stress in whole blood and urine have been noted after intake of dietary components such as the polyunsaturated fatty acids (PUFA) (Jenkinson et al., 1999). Similar effects have been observed in the colon and liver (Hong et al., 2002; Song and Miyazawa, 2001). The PUFA components of cell membranes are highly susceptible to oxidation with the resultant lipid radicals leading to multiple events that influence cell survival indices (Barrera et al., 2008; Das, 1999). Metabolites derived from certain PUFA, eicosanoids such as the 2-series prostaglandin E₂ (PGE₂), for example, are important facilitators of inflammatory processes (Tapiero et al., 2002). Consequently, the development of cancer has strongly been linked as a direct result of inflammation (Chapkin et al., 2007a; Multhoff et al., 2011; Ramos-Nino, 2013) that arise from oxidative insults (Reuter et al., 2010). Studies show that dietary intakes of ω-6 PUFA, arachidonic acid (C20:4ω-6), enhances disease development while ω-3 PUFA such as eicosapentaenoic acid and (C20:5ω-3) docosahexaenoic acid (C22:6ω-3) reduces the risk due to their opposing effects on inflammation (Chapkin et al., 2007b; Jones et al., 2003). It is of importance to note that intakes of both classes of fatty acids (FA) are critical for normal regulatory functioning. However, the quantity and type of FA consumed determines its effects on health and disease development (Simopoulos, 1999).

As mentioned, antioxidants also play a critical role in regulating oxidative stress. Cellular systems have developed an array of endogenous antioxidant enzymes that control the effects of free radicals (Birben et al., 2012). However, dietary antioxidants also provide a significant measure of protection against oxidative onslaughts (Pandey and Rizvi, 2009). Due to the potential genotoxic and epigenetic insults caused by excessive oxidative stress, increased ROS has been identified as a major risk factor for colon cancer development, while an added supply of dietary antioxidants is beneficial in minimizing the effects generated by these harmful oxidation products. Therefore, identifying measures that could modify the cellular redox status to attenuate the regulatory outputs that sustain cancer growth, may serve as a feasible intervention strategy. In the present study, different fat sources comprised of varying ratios of ω-6 and ω-3 PUFA were evaluated for their potential as modulators of oxidative stress during colon carcinogenesis. The influence of the
varying dietary FA contents on the post-initiation stage, with respect to the extent of gene expression of 84 genes associated with pro- or anti-oxidant functions was assessed in the colon utilizing a real-time polymerase chain reaction (PCR) microarray.

4.2 MATERIALS AND METHODS

4.2.1 Materials and chemicals
Colon carcinogen 1,2- dimethylhydrazine (DMH) RNA stabilizing reagent Trizol, absolute ethanol, isopropanol, sodium carbonate (Na$_2$CO$_3$), HPLC grade methanol, hexane and chloroform were all purchased from Sigma-Aldrich (St Louis, USA). Agilent technologies provided the RNA 6000 Nano LabChip for RNA quality determination. Folin-Ciocalteau was supplied by Merck (Darmstadt, Germany). Kits provided by Qiagen/SABiosciences included the RNeasy mini columns, RT$^2$ First Strand, RT$^2$ SYBR Green Mastermix and the RT$^2$ PCR profiler oxidative stress and antioxidant defence array kits. RNase-free water was supplied by Whitehead Scientific (Cape Town, South Africa).

4.2.2 Animals and ethical clearance
The use of animals for experimentation was cleared by the South African Medical Research Council’s Ethics Committee for Research on Animals (ECRA). Male Fischer rats, strain 344 (n= 30) were used for all experimentation. Weanlings with body weights (BW) of ± 50 grams (g) were fed the standard AIN-76A containing sunflower oil as a fat source (5% w/w).

4.2.3 Experimental design
A similar experiment design described in Chapter 3 (page 54) was used. The FA profile of the experimental dietary oil mixtures was assessed by gas chromatography, and summarized in Chapter 3 (Table 3.2, page 58). In addition, the total polyphenol content was analysed as described below (section 4.2.4). The animals were fed the experimental diets for a period of 4 months with their body weights (BW) and feed intakes (FI) being monitored every 2nd week. Colons (N = 3 per dietary group) were removed, sliced open and cleaned in a phosphate buffer solution. Mucosal epithelial cells were collected by scraping the distal end,
approximately 1-2 centimetres in length, of the colon with a glass slide. Samples were weighed and preserved in 500 μl of the RNA stabilizing reagent Trizol (Sigma-Aldrich, Germany) and stored at -20 °C until total RNA extraction was performed.

4.2.4 Total polyphenol content determination
The total polyphenol (TTP) content of the experimental dietary oil mixtures was determined in triplicate according to the method adapted from Haiyan et al. (2007). Three gram of each oil, sunflower, SB, SF, SBF, canola and olive, comprising a specific dietary fat ratio, were dissolved in 15 ml hexane and extracted by shaking with 5 ml methanol for 10 minutes followed by centrifugation at 500 rpm for 15 minutes. The resultant bottom methanol layer was collected and the extraction repeated twice more, and the combined samples stored at 4 °C overnight. The combined methanol extract, was extracted by 25 ml hexane by vortexing (1 minute) followed by centrifugation at 500 rpm for 15 minutes. An aliquot of the resultant methanol extract (0.5 ml) was used for TTP determination. A stock solution of gallic acid (1 mg/ml) was prepared in distilled water (dH₂O) to obtain a standard series of 10-100 μg/ml in a final reaction volume. In a test tube, the standard, blank (dH₂O) or oil methanol extract (0.5 ml), were combined with 10% Folin-Ciocalteau reagent (2.5 ml) and 7.5% sodium carbonate (Na₂CO₃) (2 ml) in a total volume of 5 ml. Samples were mixed by inversion and incubated for 2 hours in a water bath at 37 °C. The absorbance was determined at 765 nm, and TTP expressed as mg gallic acid equivalents per kilogram oil (Addendum 2A, Table 4.1).

4.2.5 Total RNA extraction from colon epithelial tissue
Trizol stabilized samples were defrosted on ice and homogenized for 1 minute with a single stainless steel bead (Qiagen 5 mm) using a tissue lyser (Retsch MM400, Haan, Germany) for 1 minute. Samples were kept on ice for 1 minute and the homogenizing process repeated another two times to ensure uniform sample dispersion. Chloroform (i.e. 100 μl) was added to each tissue aliquot (i.e. 500 μl), and mixed by inversion. Samples were incubated at room temperature (RT) for 5 minutes, and then centrifuged at 12 000x g for 10 minutes at 4 °C. The upper aqueous phase containing the RNA was transferred to a clean Eppendorf tube, and precipitated with 250 μl isopropanol (trizol:isopropanol ratio equals 2:1). RNA
samples were incubated at RT for 10 minutes and centrifuged at 10 000 x g for 10 minutes at 4 °C. Supernatants were discarded, and the pellets were suspended in 75% ethanol to remove the residual isopropanol. Samples were then centrifuged at 7500 x g for 5 minutes, and the subsequent pellets air dried for 5 minutes and suspended in adequate volumes of RNase-free water (50-100 μl). Further RNA clean-up was employed using RNeasy mini columns (Qiagen, Valencia, CA, USA). RNA samples were quantified using a UV spectrophotometer (NanoDrop; A<sub>260</sub>/A<sub>280</sub>) and the quality of the RNA was assessed using the Agilent RNA 6000 Nano kit on a sample bioanalyzer (Agilent Technologies, Waldbronn, Germany). Only high quality RNA with a RIN above 7 was used for subsequent steps.

4.2.6 RNA digestion and precipitation
To ensure the removal of genomic DNA, 5 µg aliquots of total RNA was digested with 5 µl DNase (Fermetas, Thermo Scientific, Wilmington, DE, USA). Briefly, DNase treated samples were incubated at 37 °C for 30 minutes on a PCR thermal cycler (Eppendorf). The digestion process was stopped by adding 5 µl EDTA (50 mM) at 65 °C on the thermal cycler for 10 minutes. The RNA was further concentrated by adding ⅟10 volumes of sodium acetate (3 M, pH 5.5) and 2.5 volumes absolute ethanol followed by gently mixing by pipetting. Samples were stored overnight at -20 °C, and subsequently centrifuged at 12000 x g for 10 minutes. The supernatant was discarded and pellets washed with 100-500 μl 75% ethanol, and centrifuged at 10 000 x g for 5 minutes. The supernatant was carefully discarded and residual ethanol being removed by pipette. The pellet was allowed to air dry for 10 minutes, suspended in 20 μl RNase-free water and the RNA allowed to completely dissolve by heating for 5 minutes at 55 °C. RNA concentration was quantified by UV spectrophotometry (Nano Drop Thermo Scientific, Wilmington, DE, USA).

4.2.7 cDNA synthesis and real-time PCR microarray
cDNA was prepared according to specifications in the RT<sup>2</sup> First Strand kit (Qiagen). Briefly, cDNA was synthesized from 400 ng of the precipitated RNA, which was incubated at 42 °C for 5 minutes with a volume of genomic DNA elimination mix. This was followed with incubation at 42 °C for exactly 15 minutes with an equivalent volume of the reverse transcriptase mix provided in the kit. The reaction was stopped
by incubating at 95 °C for 5 minutes. RNase-free water was added to a total cDNA volume of 111 μl. A volume of cDNA (102 μl) was combined with 550 μl RT² SYBR Green Mastermix (Qiagen/SABiosciences) that contains HotStart DNA Taq Polymerase. This reaction was then set up to a final volume of 1100 μl with RNase-free water (i.e. 448 μl). For the analyses, 5x384-well PCR Array plates (Qiagen/SABiosciences) embedded with primers encoding for 84 genes (Addendum 2B, Fig. 4.1 and Addendum 2C, Table 4.2) involved in oxidative stress and antioxidant defence mechanisms were used. Sample volumes (10 μl) were transferred to the 384-well plate that contains 4 replicates of 96 assays that can be used for the analysis of 4 samples. The real-time cycle conditions set on an Applied Biosystems cycler (model 7900HT) were as follows: PCR initial activation step at 95 °C for 10 minutes and a total of 40 cycles for melting (95 °C, 15 seconds), and annealing (60 °C, 1 minute). The relative fold change in gene expression with the experimental dietary fat ratio diets were determined by using the SA Biosciences web-based software that automatically performs quantification using the ΔΔC_T method.

4.2.8 Statistical analyses

(a) Body weight gain, feed, fatty acid and total polyphenol intakes
The results presented are expressed as the mean±standard deviation (i.e. tables or graphs). Normal distribution amongst all the dietary groups means were determined by the Kolmogorov-Smirnov test, with variances homogeneity being tested as well (Bartlett’s test). One way analysis of variance (1-ANOVA) was used to investigate any differences between the control diet sunflower oil only and the experimental dietary oil mixtures (SB, SF, SBF), as well as canola and olive oils. A post hoc Tukey-Kramer test determined the significant differences between these comparison groups. Analyses were determined with GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com. Significant values were set at p<0.05, p<0.001 or p<0.0001 where indicated.

(b) PCR array data normalization and analysis
All pathway targeted genes were normalized against the assays housekeeping genes. The comparative CT (ΔΔC_T) method used calculated the relative amounts of
transcripts in the sunflower oil (reference) and experimental dietary fat ratio groups (SB, SF, SBF, canola and olive oils). The fold change in gene expression for the latter groups relative to the sunflower oil group was calculated using the formula $2^{-\Delta\Delta Ct}$. Statistical analysis was determined using the Student's t-test comparing the $\Delta Ct$ values for each gene in the sunflower oil group versus that of those in the experimental dietary fat ratio groups. Significant fold changes observed were set at p-values less than 0.05 (p<0.05).

4.3 RESULTS

4.3.1 Body weight gain and feed intake
The animals showed no significant differences in body weight gain throughout the exposure period to the different diets (Table 4.3). However, feed intake was significantly influenced by these diets. Relative to the sunflower oil only diet, animals feeding on the olive oil based diet demonstrated the highest level of feed intake at 4.70 grams feed/100 grams body weight (p<0.001).

4.3.2 Selective fatty acid intake
The intake of select fatty acids provided by the experimental oil mixture diets (SB, SF, and SBF), the reference diets with canola and olive oils, were compared to that of the sunflower oil only diet (Table 4.3).

4.3.2.1 C18:1ω-9 (oleic acid)
Intake of the monounsaturated FA, C18:1ω-9, increased significantly with the canola oil (3-fold) and olive oil (5-fold) based diets (p<0.0001) (Table 4.3), with the latter providing the highest intake (Chapter 3, Table 3.2). The oil mixture diets, SB, SF and SBF demonstrated no significant increase of C18:1ω-9 relative to the sunflower oil only diet.

4.3.2.5 C18:3ω-3 (alpha-linolenic acid)
Compared to the sunflower oil only diet, the inclusion of borage oil with the SB and SBF diets significantly (p<0.0001) increased C18:3ω-3 intake (Table 4.3). A similar increasing (p<0.05) effect is observed with olive oil. The inclusion of fish oil (SF diet)
only supplied trace levels of the FA. Canola oil, however, provided the highest (p<0.0001) intake of C18:3ω-3.

4.3.2.6 C20:5ω-3 (eicosapentaenoic acid)
The fish oil based diets, SF and SBF, provided the highest intake of C20:5ω-3 (p<0.0001) relative to the control diet containing only sunflower oil as the fat source (Table 4.3). The sunflower oil only diet, together with the SB, canola and olive oils diets only provided trace levels of the FA.

4.3.2.7 C22:6ω-3 (docosahexaenoic acid)
Compared to the sunflower oil only diet intake of C22:6ω-3 was significantly increased (p<0.0001) with the SF and SBF diets (Table 4.3). The inclusion of fish oil with the SF diet supplied a higher intake, while only trace levels of C22:6ω-3 was observed with the SB, canola and olive oil based fat diets.

4.3.2.2 C18:2ω-6 (linoleic acid)
Inclusion of fish oil in the sunflower oil (SF oil diet) significantly increased (p<0.05) the intake relative to the sunflower oil only diet while a marked increase was also noticed with the SB oil diet (Table 4.3). The canola and olive oil containing diets, however, significantly reduced (p<0.0001) C18:2ω-6 intake, especially the olive oil where reduction of up to 5 times was noticed.

4.3.2.3 C18:3ω-6 (gamma-linolenic acid)
Animals’ consumption of the borage oil based diets, SB and SBF, significantly increased the intake of C18:3ω-6 (p<0.0001) when compared to the sunflower oil only diet that contains negligible amounts (Table 4.3). Low levels of C18:3ω-6 intake was observed with the inclusion of fish oil (SF diet), as well as with the canola and olive oil diets.

4.3.2.4 C20:4ω-6 (arachidonic acid)
A significant increase (p<0.0001) in this FA’s intake (Table 4.3) was only observed with the SBF oil diet compared to the control sunflower oil only diet. The SF, SB, canola and olive oil based diets only provided trace levels of this FA.
Table 4.3: Average body weight gain, feed and selected fatty acid intake parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diet</th>
<th>S</th>
<th>SB</th>
<th>SF</th>
<th>SBF</th>
<th>Canola</th>
<th>Olive</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW</td>
<td>90.67±14.57</td>
<td>97.00±16.85</td>
<td>90.67±4.93</td>
<td>89.00±21.91</td>
<td>69.50±5.00</td>
<td>83.33±14.01</td>
<td></td>
</tr>
<tr>
<td>Feed intake</td>
<td>3.54±0.38</td>
<td>4.06±0.53</td>
<td>3.83±0.48</td>
<td>4.19±0.33</td>
<td>4.11±0.13</td>
<td>4.70±0.56**</td>
<td></td>
</tr>
</tbody>
</table>

**Fatty acid intake**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>C18:1ω-9</th>
<th>C18:3ω-3</th>
<th>C20:5ω-3</th>
<th>C22:6ω-3</th>
<th>C18:2ω-6</th>
<th>C18:3ω-6</th>
<th>C20:4ω-6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37.53±4.07</td>
<td>44.00±5.79</td>
<td>38.55±4.91</td>
<td>40.53±3.24</td>
<td>95.47±2.99***</td>
<td>161.40±8.71***</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.27±0.03</td>
<td>2.13±0.28***</td>
<td>&lt;0.01</td>
<td>2.41±0.32***</td>
<td>18.14±1.05***</td>
<td>1.35±0.07*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>11.92±1.51***</td>
<td>8.96±0.71***</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>2.07±0.26***</td>
<td>1.47±0.12***</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2ω-6</td>
<td>96.18±8.08</td>
<td>106.40±11.50</td>
<td>108.20±12.89</td>
<td>102.70±7.74</td>
<td>62.33±1.84***</td>
<td>15.87±0.76***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:3ω-6</td>
<td>&lt;0.01</td>
<td>13.44±1.77***</td>
<td>0.08±0.01</td>
<td>13.56±1.09***</td>
<td>0.23±0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C20:4ω-6</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.63±0.05</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent the mean ± standard deviations (n = 3/diet) of body weight (BW) gain in grams and feed intake (grams/100 g BW). Fatty acid intake is expressed in mg FA/100 grams BW. Dietary fat sources: Sunflower = sunflower oil, SB = sunflower oil + borage oil; SF = sunflower oil + fish oil; SBF = sunflower oil + borage oil + fish oil. Significant differences compared to the sunflower oil diet set at p<0.05 (*), p<0.001 (**) and p<0.0001 (***)

4.3.3 Total polyphenol intake

The dietary intake of total polyphenols (TTP) provided by the animals feed intake via the different diets is illustrated in Fig. 4.2. Relative to the sunflower oil only control diet, the experimental dietary fat ratios (SB, SF, and SBF) and the reference diets canola and olive oils provided significantly different TPP intake levels. Olive oil provided the highest (p<0.0001) intake, whilst canola oil the lowest amount (p<0.001). Both the inclusion of borage and fish oils (SB and SF diets) increased TPP intake significantly (p<0.05). The combination of these two oils (SBF diet) further increased animals TPP intake (p<0.0001).
Figure 4.2: Total polyphenol intake expressed as mg gallic acid per 100 gram body weight. Animal feed diets contained the following oil mixtures: S = sunflower oil only, SB = sunflower oil + borage oil, SF = sunflower oil + fish oil, SBF = sunflower oil + borage oil + fish oil, Canola = canola oil and Olive = olive oil. Significant differences compared to the sunflower oil only (control) diet set at p<0.05 (*), p<0.001 (**) and p<0.0001 (***)..

4.3.4 Oxidative stress and antioxidant defences related genes expression

The influence of the experimental dietary fat ratios (SB, SF, SBF, canola and olive oils) on the relative expression of 84 genes involved in oxidant stress and antioxidant defences was evaluated. The effect on gene expression of all the experimental diets was compared using the sunflower oil only diet as control. The number of genes differentially influenced by the dietary treatments is illustrated in Figure 4.3. Genes affected are summarised in Table 4.3.

4.3.4.1 Number of genes affected in the colon mucosa

Animals’ exposure to olive and canola oils demonstrated the highest number of genes affected at 26 and 22, respectively (Fig. 4.3). The SB and SF diets altered the expression of only 2 genes each, whilst the SBF diet only changed the expression of a single gene.
4.3.4.2 Expression of genes affected according to functional classification.

Out of the 84 genes evaluated per dietary treatment, the expression of only 36 genes in total was significantly (p<0.05) altered relative to the sunflower oil diet (Table 4.4).

Antioxidant defence genes

(i)  Glutathione peroxidases

Several sub-types of this class of potent antioxidants were significantly up-regulated. Gpx 2 expression was up-regulated 32.90 fold (p = 0.003) with the canola oil based diet, whilst olive oil increased the expression of Gpx5 (p = 0.003) and Gpx8 (p = 0.01). The SB, SF, canola and olive dietary oils increased Gpx4 expression significantly (p<0.05), with olive oil presenting the highest fold increase at 185.03.

(ii) Peroxiredoxins

Ehd2 encoding the EH-domain containing 2 protein, was significantly up-regulated by both canola (p value = 0.008) and olive oil (p = 0.0001), with the former having a greater enhancing effect.
(iii) **Other peroxidases**

The expression of several other peroxidase encoding genes mutually affected by canola and olive oils included the up-regulation (p<0.05) of serine (or cysteine) peptidase inhibitor, clade B, member b1 (*Seprpin1b*) and tropomodulin (*Tmod1*) encoding genes. Individually, canola oil resulted in the significant 33.36 fold increase (p =0.04) in *Mpo*, encoding for myeloperoxidase, expression. A similar increasing effect is observed for the recombination activating gene 2 (*Rag2* , p = 0.02). A significant up-regulation (p<0.05) in several other peroxidase encoding genes expression was observed with olive oil: adenomatous polyposis coli (*Apc*), cathepsin B (*Ctsb*), eosinophil peroxidise (*Epx*) and prostaglandin-endoperoxide synthase 2 (*Ptgs2*).

(iv) **Other antioxidants**

Canola and olive oils mutually affected the expression of sulfiredoxin 1 homolog *S. cerevisae* (*Srxn1*) by a fold change of 23.81 and 28.31, respectively. Canola oil up-regulated (p = 0.01) the expression of thioredoxin 1 (*Txnrd1*), whilst olive oil increased (p = 0.001) the expression of glutathione reductase (*Gsr*) by a fold change of 21.16, and also thioredoxin 2 (*Txnrd2*, p =0.04) by a 60.97 fold.

**Genes involved in ROS metabolism**

(i) **Genes involved in superoxide metabolism**

The expression of the Ncf2 gene, encoding for the neutrophil cytosolic factor 2, was mutually up-regulated by the canola and olive oil based diets with a respective fold change of 24.82 (p = 0.03) and 27.22 (p = 0.02).

(ii) **Oxidative stress responsive genes**

In the SB dietary group a down-regulation of -0.36 fold (p = 0.04) was observed in the Ercc2 gene, which encodes for the excision repair cross-complementing rodent repair deficiency, complementary group 2. The SF diet, as well as the canola oil based diet resulted in an increase in *Nudix* (*nucleoside diphosphate linked moiety X*)-type motif 1 (*Nudt1*) expression by 45.78 (p = 0.04) and 187.84 (p = 0.01) fold, respectively. The SBF diet significantly up-regulated (p = 0.02) xeroderma pigmentosum, complementation group A (*Xpa*) gene expression by 166.96 fold. Canola and olive oil mutually up-regulated (p<0.05) the following genes: Parkinson
disease (autosomal recessive, early onset) 7 (Park7), peroxiredoxin 1 (Prdx1) and thyroid peroxidise (Tpo). An increase of 55.72 fold in peroxiredoxin 2 (Prdx2) expression was also observed with canola oil exposure. Olive oil up-regulated the expression of the isocitrate dehydrogenase (Idh1), protein phosphatise 1, regulatory subunit 15b (Ppp1r15b) and prion protein (Prnp) encoding genes by fold changes of 43.01, 19.61 and 1110.25, respectively.

**Oxygen transporters**

The canola and olive oil based diets mutually affected the expression of genes encoding for proteins similar to serine/threonine protein kinase ATR (LOC367198) and cytoglobin (Cygb) by up-regulating (p<0.05) their expression. Canola oil increased the expression of the Fanconi anemia, complementation group C (Fancc) and neuroglobin (Ngb) genes by 51.39 (p = 0.01) and 11.63 (p = 0.001) fold, respectively. Dynamin 2 (Dnm2) expression increased significantly (p =0.02) with olive oil (103.51 fold).
Table 4.4: Select genes expression significantly affected in the colon of carcinogen treated Fisher 344 rats by the experimental dietary oils relative to the control, sunflower oil only.

<table>
<thead>
<tr>
<th>Functional gene grouping and name</th>
<th>Gene symbol</th>
<th>SB Fold change</th>
<th>SB p-value</th>
<th>SF Fold change</th>
<th>SF p-value</th>
<th>SBF Fold change</th>
<th>SBF p-value</th>
<th>Canola Fold change</th>
<th>Canola p-value</th>
<th>Olive Fold change</th>
<th>Olive p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANTIOXIDANTS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.  <strong>Glutathione peroxidases (GPX)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase 2</td>
<td>Gpx2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+32.90</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione peroxidase 4</td>
<td>Gpx4</td>
<td>+38.76</td>
<td>0.04</td>
<td>+48.39</td>
<td>0.03</td>
<td></td>
<td></td>
<td>+164.59</td>
<td>0.05</td>
<td>+185.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Glutathione peroxidase 5</td>
<td>Gpx5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+31.05</td>
<td>0.003</td>
</tr>
<tr>
<td>Glutathione peroxidase 8</td>
<td>Gpx8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+17.07</td>
<td>0.01</td>
</tr>
<tr>
<td>ii. <strong>Peroxiredoxins (TPX)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EH-domain containing 2</td>
<td>Ehd2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+29.79</td>
<td>0.008</td>
<td>+25.69</td>
<td>0.0001</td>
</tr>
<tr>
<td>iii. <strong>Other peroxidases</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenomatous polyposis coli</td>
<td>Apc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+30.70</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cathepsin B</td>
<td>Ctsb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+50.56</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophil peroxidase</td>
<td>Epx</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+20.53</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>Mpo</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+33.36</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostaglandin-endoperoxide</td>
<td>Ptgs2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+16.06</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synthase 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recombination activating gene 2</td>
<td>Rag2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+36.67</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serine (or cysteine) peptidase</td>
<td>Serpinb1b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+17.11</td>
<td>0.03</td>
<td>+19.88</td>
<td>0.001</td>
</tr>
<tr>
<td>inhibitor, clade B, member b1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tropomodulin</td>
<td>Tmod1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+35.10</td>
<td>0.04</td>
<td>+33.51</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Data presented is the average fold change with its respective p-value normalised to the sunflower oil treated animals (n = 3 per dietary treatment). Values are significant at p<0.05. + indicates up regulation; - indicates down regulation.
Table 4.4 (cont.)

<table>
<thead>
<tr>
<th>Functional gene grouping and name</th>
<th>Gene symbol</th>
<th>SB Fold change</th>
<th>SB p-value</th>
<th>SF Fold change</th>
<th>SF p-value</th>
<th>SBF Fold change</th>
<th>SBF p-value</th>
<th>Canola Fold change</th>
<th>Canola p-value</th>
<th>Olive Fold change</th>
<th>Olive p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANTIOXIDANTS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iv. Other antioxidants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>Gsr</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+21.16</td>
</tr>
<tr>
<td>Sulfiredoxin 1 homolog (S. cerevisiae)</td>
<td>Srxn1</td>
<td>+23.81</td>
<td>0.01</td>
<td>+28.31</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thioredoxin reductase 1</td>
<td>Txnrd1</td>
<td>+67.65</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+60.97</td>
</tr>
<tr>
<td>Thioredoxin reductase 2</td>
<td>Txnrd2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GENES INVOLVED IN ROS METABOLISM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i. Genes involved in superoxide metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil cytosolic factor 2</td>
<td>Ncf2</td>
<td>+24.82</td>
<td>0.03</td>
<td>+27.22</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ii. Oxidative stress responsive genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)</td>
<td>Als2</td>
<td>+43.41</td>
<td>0.01</td>
<td>+42.22</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apolipoprotein E</td>
<td>Apoe</td>
<td>+41.39</td>
<td>0.008</td>
<td>+58.62</td>
<td>0.0003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dual oxidase 1</td>
<td>Duox1</td>
<td>+36.59</td>
<td>0.02</td>
<td>+37.10</td>
<td>0.0003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excision repair cross-complementing rodent repair deficiency, complementary group 2</td>
<td>Erc2</td>
<td>-0.36</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data presented is the average fold change with its respective p-value normalised to the sunflower oil treated animals (n = 3 per dietary treatment). Values are significant at p<0.05. + indicates up regulation; - indicates down regulation.
### Table 4.4 (cont.)

<table>
<thead>
<tr>
<th>Functional gene grouping and name</th>
<th>Gene symbol</th>
<th>SB Fold change</th>
<th>SB p-value</th>
<th>SF Fold change</th>
<th>SF p-value</th>
<th>SBF Fold change</th>
<th>SBF p-value</th>
<th>Canola Fold change</th>
<th>Canola p-value</th>
<th>Olive Fold change</th>
<th>Olive p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GENES INVOLVED IN ROS METABOLISM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ii. Oxidative stress responsive genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isocitrate dehydrogenase 1 (NADP+), soluble</td>
<td><em>Idh1</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+43.01</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Nudix (nucleoside diphosphate linked moiety X)-type motif 1</td>
<td><em>Nudt1</em></td>
<td>+45.78</td>
<td>0.04</td>
<td>+187.84</td>
<td>0.01</td>
<td>+106.89</td>
<td>0.004</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parkinson disease (autosomal recessive, early onset) 7</td>
<td><em>Park7</em></td>
<td>+43.11</td>
<td>0.01</td>
<td>+19.61</td>
<td>0.0007</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein phosphatase 1, regulatory (inhibitor) subunit 15b</td>
<td><em>Ppp1r15b</em></td>
<td>+55.72</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peroxiredoxin 1</td>
<td><em>Prdx1</em></td>
<td>+19.79</td>
<td>0.03</td>
<td>+25.11</td>
<td>0.0009</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peroxiredoxin 2</td>
<td><em>Prdx2</em></td>
<td>+55.72</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prion protein</td>
<td><em>Prnp</em></td>
<td></td>
<td></td>
<td>+111.25</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid peroxidase</td>
<td><em>Tpo</em></td>
<td>+11.42</td>
<td>0.03</td>
<td>+14.69</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xeroderma pigmentosum, complementation group A</td>
<td><em>Xpa</em></td>
<td>+166.96</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>OXYGEN TRANSPORTERS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytoglobin</td>
<td><em>Cygb</em></td>
<td></td>
<td></td>
<td>+140.06</td>
<td>0.05</td>
<td>+71.18</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dynamin 2</td>
<td><em>Dnm2</em></td>
<td></td>
<td></td>
<td>+51.39</td>
<td>0.01</td>
<td>+103.51</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fanconi anemia, complementation group C</td>
<td><em>FancC</em></td>
<td>+42.32</td>
<td>0.01</td>
<td>+59.16</td>
<td>0.0003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Similar to serine/threonine protein kinase ATR</td>
<td><em>LOC367198</em></td>
<td></td>
<td></td>
<td>+11.63</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuroglobin</td>
<td><em>Ngb</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data presented is the average fold change with its respective p-value normalised to the sunflower oil treated animals (n = 3 per dietary treatment). Abbreviations: NADP⁺ = oxidized nicotinamide dinucleotide phosphate, ATR = ataxia telangiectasia and Rad3-related. Values are significant at p<0.05. + indicates up regulation; - indicates down regulation.
4.4 DISCUSSION

To ensure their survival, cancer cells have adapted their cellular environment to enable the aberrant control of critical biochemical pathways (DeBerardinis et al., 2008). Alterations to tissues redox balance are considered a major adaptation of cancer cells for their survival. Cancer cells have evolved mechanisms to protect themselves from intrinsic oxidative stress and have developed a sophisticated adaptation system that essentially involves the rearrangement of the antioxidant functions and up-regulation of pro-survival molecules (Bakalova et al., 2013; Fiaschi and Chiarugi, 2012; Gibellini et al., 2010). Studies show that diet has a significant effect on cells redox balance due to its supply of oxidation responsive components (e.g. ascorbic acid, vitamin E, polyphenols, FA) (Bouayed and Bohn, 2010; Rahman et al., 2006; Sies et al., 2005). Of interest is the research on dietary FA, particular the mono- and polyunsaturated FA families. Considerable experimental evidence demonstrates their beneficial protective effects against the development of cancer (Gerber, 1997; Llor et al., 2003; Simopoulos, 1999; Stephenson et al., 2013). Olive oil, best known for its protective properties against an array of pathologies, has a high quantity of the monounsaturated FA oleic acid (C18:1ω-9), in addition to many phenolic antioxidant compounds (Waterman and Lockwood, 2007). Similarly, eicosapentaenoic acid (C20:5ω-3) and docosahexaenoic acid (C22:6ω-3) sourced from fish and fish oil, as well as the high α-linolenic acid (C18:3ω-3) content of canola oils, are known for their anti-cancer properties (Bhatia et al., 2011; Chapkin et al., 2008; Deschner et al., 1990; Hong et al., 2005; Lin et al., 2013; Vaughan et al., 2013). Borage or primrose oils with high levels of γ-linolenic acid (C18:3ω-6) has also demonstrated numerous therapeutic and anticancer benefits (Horrobin, 1992).

The purpose of the current study was to determine whether a varied supply of dietary oils, each with a defined ω-6/ω-3 FA ratio, modulates the expression of genes responding to oxidative stress that occurs as a result of an imbalanced redox status. Although an increase in ROS can lead to cell death via apoptosis or necrosis, these reactive molecules can also stimulate cancer cell growth by altering gene expression, particularly those encoding growth factors and proto-oncogenes (Klaunig et al., 2011). Utilizing the dietary oils mentioned above, the expression of genes responding to diet induced changes in the redox balance was assessed within a
cellular environment during the cancer promoting phase of colon tumour development.

4.4.1 Modulation of genes expression in response to oil-induced changes to the redox balance

Metabolism of the colon carcinogen DMH yields highly reactive molecules that elicit oxidative stress due to the imbalance of ROS and endogenous antioxidants (Arutiunian et al., 1997). This increase in ROS is mainly responsible for the DNA damaging effects of DMH in the colon (Arutiunian et al., 1997). Colon cells that have been genetically altered have an adapted microenvironment highly resistant to the potential damaging effects caused by oxidative stress, which allows for their selective clonal expansion into focal lesions during the promotion stage of development (Fiaschi and Chiarugi, 2012). External factors such as diet may either further promote the survival of this adapted phenotype (Erdelyi et al., 2009; Wu et al., 2004), or result in the delay or removal of the altered pre-neoplastic cells via apoptosis (Latham et al., 1999).

The PUFA and MUFA constituents of dietary oils can differentially influence oxidative stress in biomembranes (Udilova et al., 2003; Wiseman, 1996). In liver nodules, it was demonstrated that an increase in lipid peroxidation (LPO), a biomarker of oxidative stress, with diets composed of a low ω-6/ω-3 FA ratio with a specific FA content, has a reducing modulating effect on cancer development (Abel et al., 2004). Other studies utilizing diets with an enhanced ω-3 PUFA intake show similar effects (Bartsch et al., 2002; Sarotra et al., 2010; Song and Miyazawa, 2001). PUFA are highly susceptible to damaging, free radical initiated LPO, especially when intakes of antioxidants, such as vitamin E, are low (Lord and Bralley, 2002). In the current investigation, animals’ exposures to the different dietary fat mixtures (SB, SF, SBF, canola and olive oils) provided increased intakes of oxidative prone PUFA, as well as antioxidants (i.e. TPP, C18:1ω-9). When oxidative stress occurs, cells function by countering the oxidant effect and to restore redox balance by resetting critical homeostatic parameters. Such cellular activity leads to activation or silencing of genes encoding defence enzymes, transcription factors and structural proteins (Scandalios, 2005). The dietary supply of redox responsive components is likely to
influence the oxidative status in the colon by affecting the up/down-regulation of selected genes related to antioxidants and oxidative stress.

4.4.1.1 Antioxidant defence related genes

Escalated rates of ROS generation are a primary component of highly proliferative cancer cell environments (Grek and Tew, 2010). Thus, to delay this accumulation of ROS and maintain a higher level of tolerance, tumour cells initiate vast up-regulation of multiple antioxidant systems (Mates et al., 1999). Increases in the expression of subtypes of the glutathione peroxidase (Gpx) family are a major indication of the cell’s response to oxidative stress (Toussaint et al., 1993). As a defence response against oxidative onslaught, the Gpx enzymes catalyse the reduction of hydrogen peroxide ($H_2O_2$) to water at the expense of oxidising glutathione (Brigelius-Flohé and Maiorino, 2013). Insufficient Gpx activity in the colon mucosal epithelium may trigger oxidative stress resulting in acute and chronic inflammation that is linked to cancer development (Chu et al., 2004).

$Gpx2$, expressed in the epithelium of the gastrointestinal tract, is largely localised in the colonic crypt bases and plays a role in the continuous self-renewal and differentiation of intestinal cells via the Wnt pathway, thus influencing mucosal homeostasis (Brigelius-Flohé and Maiorino, 2013). Canola oil up-regulated the expression of $Gpx2$ in the colon mucosa of rats following cancer initiation. It has been suggested that this increase in expression may be a consequence of the constitutive activation of a proliferative state regulated by the Wnt pathway, which is a distinct feature in colon cancer cells (Brigelius-Flohé and Maiorino, 2013; Kipp et al., 2012).

The expression of the gene coding for the selenocysteine protein $Gpx4$, was significantly up-regulated by the SB, SF, canola and olive oil based diets. $Gpx4$ plays an important role in the reduction of lipid membrane peroxides and protects cells from hydroperoxides derived from cholesterol and cholesterol esters (Imai and Nakagawa, 2003). Furthermore, $Gpx4$ mediates oxidative stress induced apoptosis in the colon (Fan et al., 2011), and also impacts on eicosanoids synthesis (Heirman et al., 2006). Olive oil eliciting an apparent hyper up-regulation of the gene suggests to an elevated level of oxidative stress in the tissue. This hyper up-regulated state of
Gpx4 has demonstrated anti-apoptotic effects in mice (Fan et al., 2011). Simultaneously, the significant intake of exogenous antioxidants (i.e. C18:1ω-9 and TPP) possibly act synergistically with this up-regulated state to re-establish redox homeostasis (Bouayed and Bohn, 2010). Canola oil produced a similar hyper response. However, here the interactive input is likely due to the intake of C18:1ω-9, which is also known for its antioxidant potential (Lee et al., 1998). The fish and borage oil containing diet (SF and SB) also elicited an up-regulated response in Gpx4 expression, but not to the same extent as the other two diets containing the reference oils. The oil mixture diets significant supply of C20:5ω-3 (SF diet) and C18:3ω-6 (SB diet) enhances the level of stress in the tissue due to the FA susceptibility to oxidative damage.

Both Gpx5 and Gpx8 were up-regulated only by the olive oil diet. Gpx5 is specific to the mammalian epididymis, while Gpx8 is a membrane protein of the endoplasmic reticulum (Brigelius-Flohé and Maiorino, 2013). Up-regulation in their expression is indicative of oxidative stress present in the colon. Besides acting in an antioxidant capacity, however, their additional function in the colon is unknown.

Increases in the expression of the peroxiredoxin EH-domain 2 (Ehd2), as well as several peroxidises was exerted by both the canola and olive oil based diets. The Ehd2 gene encodes for a plasma membrane associated protein that regulates internalisation, and plays a role in membrane reorganization and endocytic transport in response to nucleotide hydrolysis (Benjamin et al., 2011). Ehd2 protein mobilized to the nucleus functions as a transcriptional co-repressor (Pekar et al., 2012). The protein down-regulates the transcriptional activity of the Krüppel-like factor 7 gene that stimulates cyclin dependent kinase inhibitors, thus contributing to the proliferative state of cells (Pekar et al., 2012). Ehd2 has also been implicated as a tumour suppressor gene candidate mapping to a 1.6 Mb 19q region of deletion in glioma tumors (Smith et al., 2000). The proliferative nature of normal cells is affected by the level of oxidative stress generated by ROS, with low levels stimulating key cell surviving processes, whilst high ROS has damaging effects (Dröge, 2002). Transformed cells appears to adapt to increased ROS levels that promotes an enhanced proliferative nature (Klaunig and Kamendulis, 2004; Matés and Sánchez-Jiménez, 2000; Schumacker, 2006). Thus a diet induced up-regulation of the anti-
proliferative *Ehd2* gene suggests towards a protective effect by the canola and olive oils against possible oxidative stress induced proliferation that is characteristic of cancer cells.

Canola oil up-regulated the expression of peroxidises *Mpo* and *Rag2*, whilst olive oil increased *Apc*, *Ctbs*, *Epx* and *Ptgs2* expression. *Apc* (adenomatous polyposis coli) is a tumour suppressor gene and loss of its function leads to malignant transformations in the gut (Fodde, 2002). The olive oil effect resulted in an increase in its expression as a protective response, hereby possibly preventing the maintenance of damaged/ altered mucosal epithelium through the activation of apoptosis (Morin *et al*., 1996). *Apc* also impacts on cell turnover by inhibiting COX-2 expression (Lew *et al*., 2002). The lysosomal protease cathepsin B (*Ctbs*) functions in normal cellular degradation and turnover (Turk *et al*., 2012), regulating cell numbers by activating initiator caspases, thus increasing apoptosis (Vancompernolle *et al*., 1998; Wang *et al*., 2008). This up-regulated state is a further indication of oxidative stress.

The eosinophil peroxidase (*Epx*) genes encodes a 77,000 Da, 2-subunit protein, and is the predominant granular protein of eosinophils, which are specialized phagocytes that play a beneficial role in eliminating tissue invasive agents (Acharya and Ackerman, 2014). *Epx* catalyzes the formation of cytotoxic oxidants, leading to tissue injury, thus adding to oxidative stress levels present in tissue. Up-regulation of *Epx* by olive oil thus may be indicative of increased oxidative stress in the colon mucosa, which is contrary to the oil’s reported protective effects against oxidation (Kasdallah-Grissa *et al*., 2008). It is plausible to reason that the dietary supply of antioxidants in this case may be insufficient to stem the inherent oxidative stress in the transformed state during the promotion phase of development. The protective effect of olive oil and its bioactive components (antioxidants) is dependent on its bioavailability in cells/tissue, and not just dietary intake thereof (Fitó *et al*., 2007).

Olive oil’s up-regulation of prostaglandin endoperoxide synthase (*Ptgs2*) expression could also be determined by its protective components actual concentration within the tissue (i.e. bioavailability). The increased expression of *Ptgs2*, also known as cyclooxygenase 2 (COX-2) has a significant function in the process of inflammation,
by metabolizing C20:4ω-6 into eicosanoids such as prostaglandin E₂ that increases colon cancer risk (Hendrickse et al., 1994; Marks et al., 2000). Feeding with olive oil has been shown to decrease the availability of C20:4ω-6, thus reducing the potential for inflammation (Bartoli et al., 2000), as well as reducing oxidative stress (Moreno et al., 2001). However, in the current investigation the expression of Ptgs2 was up-regulated with olive oil consumption despite only trace levels of C20:4ω 6 being noted. Up-regulation in this inducible enzyme’s gene may be a consequence of the initiated condition wherein the transformed colonic cells display enhanced inflammation due to the effect of the carcinogen treatment (Greenhough et al., 2009; Huang et al., 2015). Nevertheless, this inflammatory effect is likely counteracted by action of the up-regulated expression in Apc noted earlier.

Myeloperoxidase (Mpo) is an abundant lysosomal enzyme produced by neutrophilic leucocytes, and catalyzes the formation of hypochlorous acid that is cytotoxic to microorganisms. The enzyme influences the initiation of LPO under conditions of oxidative onslaught (Zhang et al., 2002), and also contributes towards oxidative stress mediated apoptosis (Nakazato et al., 2007). Canola oil’s induction of Mpo expression suggests to a cellular environment experiencing an oxidative onslaught, likely due to the provision of FA C18:3ω-3, whose metabolism generates long chain PUFA highly susceptible to free radical attack. High levels of the enzyme are suggested to be a good prognosis for colorectal cancer in terms of initiating the removal of oxidatively damaged cells (Droeser et al., 2013).

The gene products of recombination activating gene 2 (Rag2) and Rag1 are crucial for the rearrangement of recombining immunoglobulin and T cell molecular receptor genes (Kallenbach et al., 1992), thus influencing the adaptive immune response in cells. Studies utilizing Rag2 knockouts reveal the lack of functional immune responsive cells, resulting in enhanced inflammation (Erdman et al., 2009; Maggio-Price et al., 2009), a major risk factor for the development of colon cancer. Canola oil’s induction of the gene possibly then infers an anti-inflammatory response.

The Serpinb1b and Tmod1 genes were mutually up-regulated by the canola and olive oils based diets. Serpinb1b is a gene that encodes for a protein that inhibits neutrophil serine proteases that are important for terminating microbes (Benarafa,
By blocking the activity of these proteases, Serpinb1b, which is found in high concentrations in the cytoplasm of neutrophils, has an anti-inflammatory effect (Benarafa et al., 2007). Tropomodulin protein, of which there are 4 isoforms, regulates the organization of actin filaments (Fischer and Fowler, 2003), thus impacting on cell cytoskeleton structure and motility. The product of gene Tmod1 regulates actin filament length and stability in cytoskeletal structures (Gokhin et al., 2010).

The enzyme glutathione reductase (Gsr) catalyzes the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of glutathione disulfide (GSSG) to glutathione (GSH) (Dröge, 2002). In DMH induced rats, Gsr activity reduces significantly when compared to untreated animals (Sengottuvelan et al., 2006). With the current study, the up-regulated expression of the antioxidant gene Gsr reflects a protective effect against any harmful oxidants generated by the altered environment in response to exposure to the olive oil diet, which likely acts in synergy with the diet supplied antioxidants to restore the redox balance.

Sulfiredoxin (Srxn) catalyzes the energy dependent reduction of cysteine sulfinic acid of hyperperoxidized peroxiredoxin (Jeong et al., 2012), and also catalyzes the removal of glutathione from modified protein (Findlay et al., 2005). Furthermore, it has a functional role in protecting cells from apoptosis under low levels of H$_2$O$_2$ due to its ability to inhibit the accumulation of hyper-oxidized 2-cysteine peroxiredoxins by catalysing the reversal of over oxidation in these scavenging molecules (Baek et al., 2012; Woo et al., 2005). Hereby, the inactivation of peroxiredoxins is prevented. The sulfiredoxin 1 homolog gene (Srxn1), derived from yeast, was mutually up-regulated by canola and olive oils, suggesting towards a possible enhanced mobilization of peroxiredoxins that actively reduce H$_2$O$_2$, which is toxic to cells.

The thioredoxin system, composed of thioredoxin reductase (Txnrd), thioredoxin and NADPH, plays a central role in regulating cellular redox homeostasis and signalling pathways (Arnér and Holmgren, 2000). The Txnrd enzymes are selenium dependent flavoproteins with broad substrate specificity that also reduce non-disulfide substrates such as hydroperoxides, vitamin C or selenite (Arnér and Holmgren, 2000). The translated product of the Txnrd1 gene, up-regulated by canola oil’s
exposure, is noted for its increased activity in numerous malignant tumours, and together with Gpx2 has been shown to have a protective effect against oxidative stress mediated apoptosis (Barrera *et al*., 2012). Similarly, increased expression of Ttxrd2 was observed with the olive oil diet. This increase in thioredoxin activity, in cells with higher than normal oxidative stress levels, may relate to its essential role in facilitating transcription in an environment where cytosolic oxidant stress signalling is required for stimulation of proliferation (Schumacker, 2006).

4.4.1.2 Genes involved in ROS metabolism

Under pathological conditions, ROS are critical modulators affecting the activation of various signalling mechanisms that lead to the development of cancer (Clerkin *et al*., 2008). The expressions of genes (Ncf2, Duox1) whose proteins form subunits of the NADPH oxidase complex were mutually up-regulated by both canola and olive oils. NADPH oxidase, produced by neutrophils, initiates the innate immune response that results in an inflammatory response (Gougerot-Pocidalo *et al*., 2002). Diet induced up-regulation of the neutrophil cytosolic factor 2 (Ncf2) gene, which encodes the protein p67phox, is mobilized to the cell membrane, where it activates the NADPH oxidase complex to generate NADP+ and superoxide from molecular oxygen (El-Benna *et al*., 2005). Activation of the Ncf2 gene is suggested to be a significant factor in p53 mediated apoptosis (Italiano *et al*., 2012). The protein produced by gene dual oxidase 1 (Duox1) is another subunit of the NADPH oxidase complex, and generates ROS that initiate invading microbes death via phagocytosis (Grandvaux *et al*., 2007). Als2 (amyotrophic lateral sclerosis 2 homolog) protein, functions as a guanine nucleotide exchange factor for the small GTPase RAB5, and acts as a modulator for endosomal dynamics (Otomo *et al*., 2003). Expression of this gene (Als2) was up-regulated by both canola and olive oils in the current investigation. Its role in colon cancer development is not fully understood, since it is largely associated with neurodegenerative diseases (Hadano *et al*., 2007).

Apolipoprotein E (*Apoe*) is a glycoprotein that functions in lipoprotein metabolism and cellular lipid transport (Mahley, 1988). Both the canola and olive oil diets induced the up-regulation of Apoe. A deficiency in the gene promotes increased oxidative stress (Shea *et al*., 2002), and has been shown to elicit an apoptotic response in macrophages (Elliott *et al*., 2008). The protein Apoe is a potent inhibitor of tumour
cell proliferation due to its competitive interaction with growth factors for binding to proteoglycans, as well as its anti-adhesive activity (Vogel et al., 1994). Therefore, the canola and olive oil diets likely have a suppressive effect on the transformed colon cells proliferative state, as demonstrated by other studies as well (Hardman, 2007; Llor et al., 2003).

In humans the expression of Ercc2 generates a protein that is a subunit of TFIIH, a protein factor required for transcription initiation by RNA polymerase II (Schaeffer et al., 1994). Ercc2, also referred to as xeroderma pigmentosum complementary group D, is an 87 kDa ATP-dependent helicase that unwinds DNA in the 5'-3' direction (Schaeffer et al., 1994). Thus, the protein has a significant influence on the process of genomic material replication necessary for the production of new daughter cells. The SB diet down-regulated the expression of Ercc2, suggesting a reduction in cell proliferation. Intake of the FA C18:3ω-6 was significantly higher compared to the SF, canola and olive oil based diets. This increase in C18:3ω-6 has been shown to have an anti-proliferative effect in cancer cells (Hrelia et al., 1996; Itoh et al., 2010).

Isocitrate dehydrogenase (Idh) is a reversible enzyme that catalyzes the NADP+-dependent oxidative decarboxylation of isocitrate (ICT) to α-ketoglutarate (αKG) and the NADPH/CO2-dependent reductive carboxylation of αKG to ICT (Leonardi et al., 2012). Due to the enzyme’s metabolizing effect of generating energy source NADPH, which is critical for detoxification processes, Idh is required for the efficient recycling of GSH, thus affecting cells response to oxidative stress (Lee et al., 2002). Olive oil resulted in the up-regulation of the Idh1 gene that also plays a role in FA metabolism and glucose sensing. Cells with highly over expressed Idh display enhanced resistance against oxidative stress, whilst with low levels, cells become more sensitive to oxidative damage by H2O2 (Lee et al., 2002). Mutations in genes Idh1 and Idh2 are prevalent in cancers (Molenaar et al., 2014; Reitman and Yan, 2010).

The protein encoded by the Nudt1 gene, nucleoside diphosphate linked moiety X-type motif 1, hydrolyzes oxidized purine nucleoside triphosphates, such as 8-oxo-dGTP and 2-hydroxy-dATP to monophosphates. Hence it plays a role in DNA repair
by preventing base misincorporation (Garre et al., 2011). Expression of the gene is significantly up-regulated during conditions of elevated oxidative stress (Garre et al., 2011), as demonstrated by the current study with the SF and canola oil based diets. The SF diet provided a significant intake of ω-3 PUFA C20:5ω-3, and to a lesser extent, C22:6ω-3, that are prone to oxidation. Therefore, the SF diet likely increases the level of oxidative stress present in the colon tissue, as seen with other tissue (Song and Miyazawa, 2001). Canola oil induced a much higher response in Nudt1 expression as a consequence of the high level of C18:3ω-3 intake, also a highly susceptible FA to oxidation.

_Park7_ encodes for the protein DJ-1 that provides cells with protection against oxidative stress induced damage (Xu et al., 2010; Yasuda et al., 2013). Canola and olive oils mutual up-regulation of the gene thus indicates a protective response against the diet induced oxidative stress. _Ppp1r15b_ is a gene that encodes for CReP (constitutive repressor of eIF2α phosphorylation), which together with the protein GADD34 repress the phosphorylation of the translation initiation factor 2α (eIF2α). Phosphorylation of eIF2α inhibits translation initiation promoting a cytoprotective process referred to as the integrated stress response (Harding et al., 2009). _Ppp1r15b_ expression was up-regulated by olive oil, suggesting a protective effect against oxidative stress was activated.

Peroxiredoxins are a family of thiol peroxidises that degrade H₂O₂ to water. Furthermore, oxidation of these enzymes indicates towards a disturbance in redox homeostasis (Poynton and Hampton, 2014). The expression of peroxiredoxin _Prdx1_ was mutually up-regulated by the canola and olive oils based diets, whilst the expression of _Prdx2_ only increased with canola oil. Both enzymes are located in the cytosol. The enzyme produced by _Prdx1_ appears to play a role in cell death as knockouts of the gene results in the activation of apoptosis signal-regulating kinase 1 (ASK1) in response to H₂O₂ (Kim et al., 2008). Enzyme Prdx 2 has been demonstrated to contribute towards cell growth in colon cancer cells by influencing the Wnt/β-catenin signalling pathway, with its absence inhibiting the process (Lu et al., 2014).
The prion protein (gene \textit{Prnp}) acts as a free radical scavenger and/or sensor molecule for oxidative stress in tumour cells, thus contributing towards the antioxidant defence (Sauer \textit{et al.}, 2003). Up-regulation in the gene’s expression by olive oil indicates that protection against oxidative stress is initiated. Thyroid peroxidase (Tpo) is mainly responsible for the synthesis of hormones in the thyroid gland, and belongs to the NADPH oxidase family that function in the immune response (Ruf and Carayon, 2006). Tpo requires H$_2$O$_2$ to initiate hormone synthesis (De Deken \textit{et al.}, 2002). Therefore, up-regulation of the gene \textit{Tpo} by canola and olive oils indicate toward increased oxidative stress levels likely due to the presence of H$_2$O$_2$.

Xeroderma pigmentosum, complementary group A protein has a critical function in the nucleotide excision repair process (Pascucci \textit{et al.}, 2011). Cells that are deficient in this protein have abnormal responses to oxidative DNA damaging agents such as increased H$_2$O$_2$ genotoxicity, a lower threshold for H$_2$O$_2$-induced cell cycle arrest, and reduced repair of H$_2$O$_2$ generated DNA lesions (Low \textit{et al.}, 2008). Up-regulation in the expression of gene \textit{Xpa} by the SBF diet suggest DNA repair is being initiated in response to oxidative damage to nucleotide bases. The supply of FA (C20:4ω-6, C20:5ω-3 and C22:6ω-3) from the combination of borage and fish oil increases the possibility of oxidative stress and its damaging effects on macromolecules such as DNA. Hence repair mechanisms (i.e. \textit{Xpa} up-regulation) are activated.

\textbf{4.4.1.3 Oxygen transporters}

The expression of five genes (\textit{Cygb, Dnm2, Fancc, LOC367198, Ngb}) involved in oxygen transport was significantly up-regulated by the canola and olive oil diets. Cytoglobin (\textit{Cygb}) is ubiquitously expressed in all vertebrates and is considered a hypoxia responsive molecule due to its mRNA expression being augmented under hypoxia seen in fibroblast cell lineages and rat brain (Li \textit{et al.}, 2006). In addition, \textit{Cygb} displays tumour suppressor gene function (Shivapurkar \textit{et al.}, 2008). This gene was mutually up-regulated by canola and olive oil, suggesting hypoxic conditions was present in the tissue. Another globin gene, neuroglobin (\textit{Ngb}), was up-regulated by canola oil. Neuroglobin acts as a scavenger of ROS (Ascenzi \textit{et al.}, 2004), thus likely protects against diet induced oxidative stress.
The protein dynamin 2 functions in membrane remodelling and endocytosis (Kasai et al., 1999; Shpetner and Vallee, 1989). Also, the protein can function as an upstream regulator of p53-dependent apoptosis in dividing cells (Fish et al., 2000). Olive oil’s up-regulation of the gene Dnm2 expression may therefore be mobilized for the induction of the p53 mediated apoptotic pathway in response to oxidative stress (Johnson et al., 1996). The Fancc gene encodes the protein fanconi anaemia, complementary group C that acts as an intracellular antioxidant (Kruyt et al., 1998). In addition, it participates in redox signalling and repair of oxidative DNA damages (Zunino et al., 2001). Therefore, oxidative stress induced by canola oil likely resulted in the up-regulation of Fancc expression. Proteins ataxia telangiectasia and Rad3-related (ATR), encoded by the gene LOC367198, plays an essential role in the maintenance of genome integrity (Shiloh, 2003), being activated in response to DNA damage (Helt et al., 2005). Therefore, up-regulation of the gene may be a consequence of the diet (canola and olive oils) induced oxidative stress injury. This process has been shown to activate p53, an important regulator of apoptosis (Das and Dashnamoorthy, 2004). Interestingly, as noted previously, it appears that the anticancer effects of the dietary oils is likely determined by the level of oxidative stress within the transformed cells, which serves as an important deciding factor for the cellular responses elicited.

4.5 SUMMARY AND CONCLUSIONS
Under the current study’s experimental conditions, modulation of redox responsive genes expression was dominated by canola and olive oils influence, with the dietary oil mixtures (SB, SF and SBF) having minimal effects. Exposure to the latter experimental dietary fats appears to induce some protection against oxidative damage by up-regulating antioxidant defence (Gpx4 – SB and SF) and DNA repair (Nudt1 – SF; Xpa – SBF). In addition, an anti-proliferative response was induced by the SB oil diet as indicated with the down regulation of Ercc2 expression that reduces the occurrence of DNA replication. These responses suggest that these oil mixtures bioactive components, particularly its FA supply, marginally stimulate mechanisms that alter the oxidative status of the cells, likely by increasing LPO, which affects cell survival. Of interest, in a previous study, the oil mixtures (SF and SBF) increased membranes incorporation of long chain ω-3 FA, C20:5ω-3 and C22:6ωFA in rat colon mucosa that was associated with an increased LPO.
This effect significantly enhanced oxidative stress in the mucosa due to an increase in LPO in the colon, known to affect cell survival indices (Barrera et al., 2008).

The effects of the canola and olive oil based diets during the post-initiation phase in the colon, provide evidence of an anti-cancer response and hence their chemopreventive properties. These included mechanisms involving the regulation of important genes associated with oxidative stress and antioxidant defence. The modulation of a subset of these genes are important in mediating cell proliferation and apoptotic pathways, two critical processes affected during carcinogenesis (summarised in Fig. 4.4).

![Summary of possible anticancer properties of canola and olive oils resulting from the up-regulation (+) of redox sensitive genes expression. Canola oil induced effects on gene expressions are underlined; olive oil in bold.](image)

The anticancer effects of canola and olive oils are likely related to its FA constituents, as well as dietary antioxidants (polyphenolic) content. A study in epithelial oesophageal cancer cells has shown that C18:3ω-3 and C18:1ω-9, found in high quantities in canola oil, can suppress cell proliferation (Moon et al., 2014). Furthermore, although fairly low relative to the other experimental diets TTP content, canola oil's phenolic constituents has also demonstrated anti-proliferative effects by reducing the potential of PGE₂ induced cell division by down-regulating COX-2.
expression (Jiang et al., 2013). Apart from its rich C18:1ω-9 content, olive oil’s bioactive polyphenols may also induce anti-proliferative and apoptotic effects in colon cancer cells (Corona et al., 2007; Notarnicola et al., 2011). These results, together with the present study’s diet induced redox modulation of regulatory mechanisms illustrate the benefits of utilizing canola and olive oils as a chemopreventive measure against the development of colon cancer.

4.6 REFERENCES


CHAPTER 5

ALTERATIONS IN MEMBRANE FATTY ACID PROFILE BY DIETARY FAT EMULSIONS AND THE EFFECT ON SURVIVAL INDICES OF HUMAN COLON CANCER CELLS \textit{IN VITRO}
ABSTRACT

The growth and survival of cancer cells can be affected by fatty acid (FA) mediators that participate in cell regulatory mechanisms. ω-3 FA obtained from dietary sources such as fish oil has demonstrated numerous anti-cancer effects, whilst certain ω-6 FA appears to enhance neoplastic developments. Studies suggest that manipulating the dietary ω-6/ω-3 FA ratio may be a critical factor in determining cancer cell survival. This study evaluated the effects of different oil emulsions (sunflower oil: S, S + borage oil: SB, S + fish oil: SF and SBF) each with a distinct FA composition and ω-6/ω-3 FA ratio (S = 249:1; SB = 28:1; SF = 12:1 and SBF = 12:1) on cell viability (ATP production) and the proliferative state of HT-29 and Caco-2 human colon cancer cells. Changes in lipid content and the level of FA incorporation associated with apoptotic cell death indices were also characterised. Results show that the two colon cancer cell lines exhibited differential sensitivity towards the effects of the FA supplied and incorporated into membranes. Fish oil containing emulsions (SF and SBF) reduced (p<0.05) the HT-29 cells energy status (ATP production) and proliferative (BrdU incorporation) ability, but were resistant to the induction of apoptosis. The Caco-2 cells were less susceptible with respect to the reduction in cell viability, however, apoptosis was increased (p<0.05) by the S, SF and SBF emulsions in a dose dependent manner. At the highest concentration, ATP-dependent apoptosis was decreased, which was associated with a significant (p<0.05) decrease in cell viability. The induction of apoptosis in Caco-2 cells was associated with specific changes in membrane lipid and FA composition effected by the different oil emulsions. A decrease (p<0.05) in total membrane SATS and MUFA as well as the membrane ω-6/ω-3 FA ratio, resulted from an increased C20:5ω-3 and C22:6ω-3 content in the phospholipid phosphatidylethanolamine (PE) that appears to selectively favour the induction of apoptosis by borage oil (SB and SBF). Similarly, an increase (p<0.05) in the PC/PE ratio by the all oil emulsions, and a decrease (S and SB) and increase (SF and SBF) (p<0.05) in the chol/PL ratio appears to facilitate apoptosis. A different threshold of the FA and lipid composition parameters elicits the inhibition of cell proliferation utilising lower oil emulsion concentrations. Therefore, the dietary supply of fats characterised by a defined low ω-6/ω-3 FA ratio can selectively modulate the growth indices of cancer. Specific oil ratio combinations by incorporating borage oil and fish oil, provides a selective strategy for chemoprevention in the colon.

Key words: colon cancer, oil emulsions, fatty acids, membranes lipid parameters, cell survival indices
5.1 INTRODUCTION

Colon cancer accounts for one of the top 3 causes of cancer related deaths in developed countries (Ferlay et al., 2015). Numerous epidemiological studies suggest that diet plays a significant role in the development of the disease, particularly regarding fat intake (Arem et al., 2013; Bruce et al., 2000; Dixon et al., 2004; Giovannucci and Goldin, 1997; Slattery et al., 1998). Investigations reveal that demographically the incidence of colon cancer is greatly reduced in regions where dietary patterns are characterized by low fat intakes with unique dietary compositions. This fact is evident in regions consuming a Mediterranean based diet, which is composed of high intakes of olive oil rich in beneficial phenolic and monounsaturated fatty acid (MUFA), oleic acid (C18:1ω-9), content (Cicerale et al., 2010; Escrich et al., 2007; La Lastra et al., 2001; Trichopoulou et al., 2000). However, with most Western diets the intake of saturated fatty acids (SATS) and ω-6 polyunsaturated fatty acids (PUFA) are very high relative to low ω-3 PUFA consumption (Simopoulos, 1999). This high ω-6 and low ω-3 fatty acid (FA) intake, resulting in a high ω-6/ω-3 FA ratio, has been shown to be positively associated with cellular mechanisms sustaining the survival of cancer cells (Bartsch et al., 1999; Williams et al., 2011).

PUFA are important macromolecules required by cells to regulate their membrane structure and associated metabolic functions. These macromolecules are critical components of cell membrane phospholipids, affecting cell structure and membrane fluidity (Ibarguren et al., 2014). In addition, PUFA participate in cellular homeostatic regulation by influencing signalling cascades, gene expressions and cellular oxidative status (Alexander-North et al., 1994; Jump, 2004; Sumida et al., 1993). Unlike endogenously synthesized SATS and MUFA, the cells PUFA supply are largely dependent on dietary intake since mammals cannot synthesize the parent essential fatty acids (FA) linoleic acid (C18:2ω-6) and α-linolenic acid (C18:3ω-3). Obtained mainly in the triglyceride form from plants and dietary oils, these parent precursors are digested and absorbed by intestinal cells to where they can be metabolized into longer carbon chain and more bioactive, functional molecules (Beguin et al., 2013; Iqbal and Hussain, 2009). The cellular responses elicited by long chain (LC) ω-6 PUFA arachidonic acid (C20:4ω-6) and ω-3 PUFA
Eicosapentaenoic acid (C20:5ω-3) and docosahexaenoic acid (C22:6ω-3) appear to vary in numerous cancer cell studies due to different cell types and function (Dommels et al., 2002; Nano et al., 2003; Di Nunzio et al., 2011). Of particular importance are C20:5ω-3 and C22:6ω-3, sourced mainly from fish and fish oils, which have demonstrated anticancer effects by retarding the growth of malignant cells via inhibiting cell proliferation which could lead to the induction of apoptosis (Bégin et al., 1986; Chamras et al., 2002; Giros et al., 2009; Hawkins et al., 1999). In contrast, C20:4ω-6 derived from the metabolism of C18:2ω-6, which is obtained mainly from vegetable oils, appears to favour cancer cells survival (Cabral et al., 2013; Whelan and McEntee, 2004). However, γ-linolenic acid (C18:3ω-6), a product of Δ6-desaturase metabolism of C18:2ω-6, has also exhibited anticancer effects by effectively blocking cell proliferation in cancer cells, as well as by causing cell death by inducing apoptosis (Hrelia et al., 1996; Kong et al., 2006).

Most of the effects mentioned above, however, are the result of isolated exposure to individual PUFA. Dietary intake of PUFA is complex due to interaction with endogenous synthesis and competitive interaction of ω-6 and ω-3 FA. Consequently, their resultant differential reactions may potentially have a significant impact on disease progress. In a previous investigation, it has been demonstrated that interaction of specific dietary ω-6 and ω-3 PUFA can impact significantly on FA associated cellular characteristics (Abrahams, 2009). It was shown that a reduced dietary ω-6/ω-3 FA ratio intake resulted in the selective incorporation of LC ω-3, which altered the lipid and FA composition of colon mucosa. Hereby the level of oxidative stress in the colon was enhanced due to an increase in lipid peroxidation. Therefore, it has been suggested that by manipulating the type of PUFA consumed the incidence of disease development can be modulated (Simopoulos, 2008). The objective of this investigation was to evaluate the effect of dietary oils with varying ω-6/ω-3 FA ratios on the survival of colon cancer cells by monitoring cell viability, cell proliferation and apoptosis. The level of FA incorporation in cellular membrane and associated lipid changes was also assessed to confirm PUFA uptake and their role in the modulation of cell growth indices.
5.2 MATERIALS AND METHODS

5.2.1 Chemicals and reagents
Culture media McCoy’s 5A was purchased from Sigma-Aldrich, Germany. Eagle’s Minimal Essential medium, fetal bovine serum (FBS), L-glutamine, sodium pyruvate and non-essential amino acids were supplied by Whitehead Scientific (South Africa). L-alpha phosphatidylcholine from eggs yolks, butylated hydroxytoluene (BHT), 2,5-bis-(5’-tert-butylbenz-oxazolyl-[2’])thiopene (BBOT), sodium chloride, staurosporine, bovine serum albumin (BSA), perchloric acid and all organic solvents (chloroform, methanol, hexane) was purchased from Sigma-Aldrich. Silica thin layer chromatography plates, acetic acid, boric acid, sulphuric acid and diethyl ether was obtained from Merck. Petroleum ether was supplied by Fluka (Germany) and peroxide free Triton-X100 by Roche Applied Science (Randburg, South Africa).

5.2.2 Preparation of oil emulsions
Oils utilized for the cell culture experiments were as follows: sunflower oil only (S), sunflower + borage oil (SB), sunflower + fish oil (SF) and sunflower + borage + fish oil (SBF). Oil in water emulsions, constituting various ω-6/ω-3 FA ratios, was prepared by the method adapted from Fox and DiCorleto (1988). Briefly, the respective oils (0.1-1.0 grams) were dissolved in chloroform to which 5% (wt/wt) L-alpha phosphatidylcholine from eggs yolks and 0.03% (wt/wt) butylated hydroxytoluene (BHT = antioxidant) was added. The solvents were dried under vacuum for 15 minutes, with intermittent washes with a few drops of diethyl ether. Samples were then further dried under a nitrogen gas stream for 5-10 minutes, and resuspended in phosphate-buffered saline (PBS) to a lipid concentration of 15 mg/ml. The lipid content was emulsified by sonication for 10 minutes in a water bath sonicator at 37 °C. Samples were filter sterilized and diluted with culture media as required.

5.2.3 Cell culture maintenance and oil emulsion treatments
The human colon adenocarcinoma cell line HT-29 (ATCC, Rockville, MD, USA) was maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine. Caco-2 human colon adenocarcinoma cells (ATCC, Rockville, MD, USA) were maintained in Eagles’ minimal essential medium (EMEM)
supplemented with FBS (10%), L-glutamine (1%), sodium pyruvate (1%) and non-essential amino acids (1%). Incubation parameters were set at 5% CO\textsubscript{2}/95% O\textsubscript{2} for both epithelial cancer cell lines. Cells were sub-cultured every 2-3 days at 80% cell confluence. For all 96-well microtitre plate experimentations, cells were seeded at densities 10 000 cells/well (HT-29 cells, passages 151-158) and 5000 cells/well (Caco-2 cells, passages 53-62). The colon cancer cell lines were exposed to oil emulsions for 48 hours ranging from 0-12.5 mg/ml by dilution in 0.5% FBS culture media to the exact concentrations required.

5.2.4 Cell viability

The cell viability of the colon cancer cell lines were assessed by quantifying ATP production as an indication of metabolically active cells. Cells seeded in opaque white 96-well microtitre plate (HT-29 = 10 000 cells/well; Caco-2 cells = 5000 cells/well) were incubated with the oil emulsions (0-10 mg/ml) for 48 hours. Thereafter cell cultures were removed from the incubator and cells were equilibrated to RT for 30 minutes. Utilizing a luminescent assay kit for ATP content quantification (CellTitre-Glo, Promega, Madison, WI, USA), 100 μl of the CellTitre-Glo reagent was added in equal volumes to each well (i.e. 1:1; cell media:reagent). Samples were mixed on a plate shaker for 2 minutes to induce cell lysis and to stabilize the luminescent signal, the plates were incubated at RT for 10 minutes. The luminescent signal was recorded on a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA, USA). Data was quantified as a percentage of viable cells relative to the control (i.e. 0 mg/ml oil emulsion) representing 100% ATP production. From this data the inhibitory concentration at 50% ATP production or viability (IC\textsubscript{50}) was also calculated for each oil emulsion and cancer cell line.

5.2.5 Cell proliferation

Cell proliferation was quantified based on the measurement of 5-bromodeoxyuridine (BrdU) incorporation during DNA synthesis in dividing cells utilizing a chemiluminescent ELISA assay (Roche Applied Sciences, Randburg, SA). The cancer cell lines (HT-29 = 10 000 cells/well; Caco-2 cells = 5000 cells/well) were seeded in 100 μl media in opaque 96-well microtitre plates and after a 48 hour incubation with the oil emulsions (0-7 mg/ml), the plates were processed according to manufacturer’s specifications. Briefly, 10 μl Brdu labelling solution (i.e. diluted in
0.5% culture media – 1:100; BrdU labelling solution: 0.5% culture media) was added to every 100 μl cells, and incubated at 37 °C for 2 hours. The labelling solution and cell media was removed by vacuum suction and cells were fixed with 200 ul FixDenat solution per well, and incubated at RT for 30 minutes. The FixDenat solution was removed by vacuum suction, and a working solution of 100 μl anti-BrdU-POD (i.e. POD = monoclonal antibody from mouse-mouse hybrid cells conjugated with peroxidase) was added per well. Plates were incubated at RT for 90 minutes. The antibody conjugate was removed and cells were washed in 200 μl washing buffer solution for 5 minutes. This step was repeated twice more. The washing buffer was discarded and 100 μl substrate solution added per well. Microtitre plates were incubated at RT on a plate shaker for 3 minutes. The chemiluminescent signal was quantified within 10 minutes after adding the substrate solution on a Veritas microplate luminometer. The response was quantified as a percentage of BrdU incorporating (proliferating) cells relative to the control (i.e. 0 mg/ml oil emulsion) representing 100% cell proliferation. The inhibitory concentration at 50% BrdU incorporation (IC50) was determined for each oil emulsion and cancer cell line from the aforementioned response profile.

5.2.6 Cell cytotoxicity
The potential cytotoxic effects of the oil emulsions were evaluated by determining cellular release of lactose dehydrogenase (LDH). After 48 hours exposure to the oil emulsions (0-12.5 mg/ml), a volume of cell culture supernatant (25 μl) representative of spontaneous/experimental LDH release, was transferred to clean 96-well plates. To the original translucent microtitre plates, 15 μl of 5% Triton-X100 (1% in media) was added to each well, and shaken for 3 minutes. Plates containing the remaining cell lysates were frozen at -20 °C for 30 minutes and thawed on a 37 °C heating block. This step was repeated twice to ensure total LDH release. Plates were then spun at 250 g for 5 minutes at 4 °C. A volume (25 μl) of total cell lysates representing total LDH release was transferred to clean wells. The kit provided LDH substrate (Cytotox96-non radioactive cytotoxicity assay, Promega, Madison, WI, USA) was added to the wells containing the spontaneous/experimental and total LDH release lysates (i.e. Total volume/well = 50 μl), and incubated at room temperature (RT), protected from light, for 30 minutes. To impede further reaction, 25 μl stop solution
was added per well. The LDH activity was measured spectrophotometrically at 340 nm on a plate reader (Synergy Mx, BioTek). The proportion of LDH release (i.e. necrotic cell death) induced by the oil emulsions, relative to the totals, was expressed as a percentage of the viable cells present.

5.2.7 Apoptosis
The effect of cell exposure to the oil emulsions on induction of cell death via apoptosis was evaluated with the luminescent Caspase-Glo 3/7 assay (Promega, Madison, WI, USA). Both colon cancer cell lines were seeded in 96-well microtitre plates and exposed to a gradient concentration of oil emulsions (HT-29 = 0-12.5 mg/ml and Caco-2 = 0-11.5 mg/ml) for 48 hours. Staurosporine (100 nM) was used as a positive stimulator of apoptosis. A concurrent run for ATP production was included in the investigation due to the process of cell death’s known requirement for energy (Zamaraeva et al., 2005). Post the incubatory period, 20 μl of the cell suspension was transferred to clean opaque white 96-well microtitre plates. A volume of the kit supplied reagent (20 μl) was added to each well. Mix the contents of the wells on a plate shaker for 30 seconds, and incubate at RT for 30 minutes. Plates were read on a luminometer to measure the luminescent signal generated by the cells reactions. Cell death via apoptosis was quantified calculating the treated colon cancer cell lines fold change in caspase 3/7 activities relative to the control (0 mg/ml oil emulsion) set at 1.

5.2.8 Fatty acid incorporation
The Caco-2 cells were selected for treatment with the 4 oil emulsions and subsequent FA analysis due to its susceptibility to apoptosis. Cells were seeded at 8.6x10^5 per 100 mm petri dish and maintained in 10% FBS supplemented EMEM culture media overnight. Media was discarded and replaced with 0.5% FBS supplemented EMEM containing the respective oil emulsions at concentrations inducing apoptosis and incubated for 48 hours. The FA profiles of the oil mixtures used to make the emulsions were assessed by gas chromatography (Table 5.1) as described in Chapter 3 (section 3.2.2).
Table 5.1: Fatty acid profile of oil-in-water emulsions expressed as a percentage of total content.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Oil-in-water emulsions (% of total fatty acids)</th>
<th>S</th>
<th>SB</th>
<th>SF</th>
<th>SBF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>6.68±0.18</td>
<td>7.47±0.15</td>
<td>6.22±0.03</td>
<td>6.80±0.41</td>
</tr>
<tr>
<td>C16:0</td>
<td></td>
<td>4.45±0.07</td>
<td>4.90±0.01</td>
<td>5.87±0.01</td>
<td>4.53±0.01</td>
</tr>
<tr>
<td>Total SATS</td>
<td></td>
<td>12.53±0.37</td>
<td>13.66±0.39</td>
<td>13.63±0.28</td>
<td>12.56±0.55</td>
</tr>
<tr>
<td>C18:0</td>
<td></td>
<td>25.17±0.50</td>
<td>19.72±0.05</td>
<td>18.85±0.61</td>
<td>18.83±0.08</td>
</tr>
<tr>
<td>Total MUFA</td>
<td></td>
<td>25.50±0.55</td>
<td>22.45±0.13</td>
<td>19.23±0.71</td>
<td>20.67±0.50</td>
</tr>
<tr>
<td>C18:1ω-9</td>
<td></td>
<td>0.25±0.01</td>
<td>1.27±0.01</td>
<td>0.13±0.05</td>
<td>1.19±0.25</td>
</tr>
<tr>
<td>C18:2ω-6</td>
<td></td>
<td>62.25±0.76</td>
<td>54.63±0.25</td>
<td>61.25±0.14</td>
<td>54.28±3.31</td>
</tr>
<tr>
<td>C18:3ω-6</td>
<td></td>
<td>&lt;0.01</td>
<td>6.70±0.08</td>
<td>&lt;0.01</td>
<td>5.97±0.41</td>
</tr>
<tr>
<td>C20:3ω-6</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C20:4ω-6</td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.22±0.01</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>Total ω-6 PUFA</td>
<td></td>
<td>0.25±0.01</td>
<td>2.21±0.05</td>
<td>5.06±0.47</td>
<td>5.36±0.48</td>
</tr>
<tr>
<td>C18:2ω-3</td>
<td></td>
<td>249.24±1.64</td>
<td>28.03±7.40</td>
<td>12.20±0.36</td>
<td>11.54±1.24</td>
</tr>
<tr>
<td>Total PUFA</td>
<td></td>
<td>62.56±0.78</td>
<td>64.15±0.42</td>
<td>66.79±0.64</td>
<td>67.20±5.25</td>
</tr>
</tbody>
</table>

Values represent the mean ± standard deviation of the water-in-oil emulsions fatty acids profile expressed as a percentage of total content (N = 2/treatment group). Dietary oils: S = sunflower oil only, SB = sunflower oil + borage oil; SF = sunflower oil + fish oil; SBF = sunflower oil + borage oil + fish oil. SATS = saturated fatty acids, MUFA = monounsaturated, PUFA = polyunsaturated fatty acids.

5.2.8.1 Cell Harvesting

After 48 hours of emulsion treatment, the culture media was discarded and cells washed three times with ice cold saline (0.9% NaCl). Petri dishes (i.e. 2 plates combined representing one sample; 5 samples in total/oil emulsion) were pooled and cell suspensions harvested in saline, and centrifuged at 500 rpm for 10 minutes. Supernatants were discarded and the pellets were suspended with 1.1 ml saline. An aliquot (100 μl) was removed and stored at -20 for protein determination, and the remaining cell suspension (1 ml) was used for lipid extraction.

5.2.8.2 Lipid Extraction

Lipids were extracted from the 1 ml saline cell suspension with chloroform:methanol (CM; 2:1 v/v) containing 0.01% BHT according to the method of Folch et al. (1957), with the cell suspension:CM ratio of 20:1. The solvent and saline cell mixture was shaken for 30 minutes, where after saline saturated with chloroform/methanol/saline (CMS, 86:14:1, v/v/v, 0.01% BHT) was added, vortexed (1 minute) and then...
centrifuged at 500 rpm for 15 minutes. The bottom chloroform rich layer containing the lipids was removed and transferred into a round bottom flask. This extraction step was repeated twice by adding CMS (10 ml) to the initial extraction sample. The collected chloroform rich extracts were evaporated and dried under vacuum utilizing a rota-vapour set at 40 °C. The dried lipid residues were transferred quantitatively into glass tubes with 5 x 2 ml CMS. Saline saturated CMS (1 ml) was added to the samples, vortexed and then centrifuged at 500 rpm for 15 minutes. The top aqueous layer was removed, and the solvent lipid extracts stored at 4 °C under nitrogen gas until further processed.

5.2.8.3 Thin layer chromatography
The lipid extracts from above were fractionated by thin layer chromatography (TLC) according to the method of Gilfillan et al. (1983). Briefly, the lipid extract were dried at 40 °C under nitrogen gas, resuspended in 100 μl CMS and aliquot (i.e. 30 μl per sample) was applied on silica TLC (10 x 20 mm) plates to separate the major phospholipid classes. The plates were developed for 90 minutes using the running solvent chloroform: methanol: petroleum ether: acetic acid: boric acid (40:20:30:10:1.8; v/v/v/v/wt) containing the fluorescent agent 2, 5-bis-(5'-tert-butylbenz-oxazolyl-[2'])thiopene (BBOT). Plates were dried under nitrogen gas and the major phospholipid fractions (phosphatidylcholine, PC and phosphatidylethanolamine, PE) were visualized under ultra violet light by identification against a standard mixture of known phospholipids.

5.2.8.4 Fatty acid analysis
For FA analysis the phospholipid fractions, PC and PE, were scraped off the TLC plates and transmethylated with 2 ml methanol/18 M sulphuric acid (95:5; v/v) at 70 °C for 2 hours (Smuts et al., 1994), and the resultant FA methyl esters (FAME) extracted with hexane (2 ml) and distilled water (1 ml). The hexane (top layer) was removed and dried under nitrogen gas in a 37 °C water bath, and the samples dissolved in hexane (PC = 20 μl; PE = 10 μl) of which 1 μl was injected into a gas chromatograph (GC). The FAME were analysed by a dual FID channel GC (Thermo Scientific Trace GC Ultra, SGE Analytical Science) equipped with two 30 metre column of 0.32 mm in diameter and a 0.25 μm film (BPX-70, SGE). Hydrogen was
used as the carrier gas. Individual FAME was identified by comparison of the retention times to those of a FA standard mixture containing C14:0 to C24:1 (Nu-Check-Prep, MN, USA). Results for individual FA were quantified using an internal standard (C17:0) and expressed both quantitatively (i.e. μg FA/mg protein) and qualitatively as a percentage of the total FA.

### 5.2.8.5 Phospholipid determination

The inorganic phosphate content of the phospholipid fractions, PC and PE, were determined colorimetrically using the malachite green phosphate detection kit (R&D Systems). Briefly, PC and PE fractions were separated by TLC (see above), and digested in perchloric acid (i.e. PC = 400 µl and PE = 300 µl) on a 170 °C heating block for 2 hours. After digestion, 6 N potassium hydroxide (PC = 775 µl and PE = 581 µl) and distilled water (PC = 2 ml and PE = 1.5 ml, 5:1 v/v, water:perchloric acid). Samples were vortexed and centrifuged at 1000 rpm for 15 minutes, and 80 µl of the samples were aliquoted to translucent 96-well microtitre plates in duplicate. A volume of kit reagent A (10 µl) was added to each sample well, mixed and incubated at RT for 10 minutes. Thereafter, reagent B (10 µl) was added and mixed with the samples, and left at RT for a further 20 minutes. Thereafter, colour development was measured at 620 nm on a plate reader (Synergy Mx, BioTek), and samples were quantified against a phosphate standard curve.

### 5.2.8.6 Cholesterol determination

The cholesterol content of Caco-2 cells was determined by an enzymatic iodide method using cholesterin-oxidase and cholesterin-esterase (Richmond, 1973). Briefly, 20 µl of the lipid extract in a glass tube (5.2.8.2) was dried under a nitrogen gas stream in a 37 °C water bath. CM (20 µl) was added to the sample, tubes vortexed and then placed on a 50 °C heating block for 30 minutes. Thereafter, 50 µl of 1% Triton-X100 (peroxide-free) solution was added, vortexed and centrifuged at 1000 rpm for 1 minute. Aliquots of 20 µl per sample were removed and transferred to a translucent UV-grade 96-well microtitre plate in duplicate. Cholesterol reagent (108 µl) and cholesterol enzyme (20 µl) was added to each sample and incubated at RT for 30 minutes. Sample absorbencies were measured at 365 nm in a plate reader (Synergy Mx, BioTek) and quantified against a cholesterol standard curve.
5.2.8.7 Protein determination

The 100 μl aliquots removed from the cell suspensions prior to lipid extraction and reserved for protein analysis were centrifuged at 2500 x g for 10 minutes. The supernatants were discarded and the resultant pellets resuspended with 100 μl M-PER mammalian protein extraction reagent (Thermoscientific, Rockford, Illinois, USA). Samples were agitated gently for 10 minutes at 37 °C, and then centrifuged at 14 000 x g for 15 minutes to remove all cell debris. Sample supernatants were used for analysis. The protein content of the supernatants was assessed in 96-well microtitre plates using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules CA, USA). A stock solution of bovine serum albumin (BSA; 1.44 mg/ml) was prepared in PBS to obtain a standard series in the final reaction volume. PBS was used as a blank control. Aliquots of the sample/blank/standards (5 μl/well) were added to translucent 96-well microtitre plates and a volume of the assay reagent A (25 μl) and reagent B (200 μl) was added to each well. Plates were covered and mixed on a plate shaker for 1 minute at medium speed, and further incubated at room temperature for 15 minutes. Sample/blank/standards absorbencies were measured spectrophotometrically at 630 nm on a plate reader (Synergy Mx, BioTek) and quantified against the BSA standard curve and expressed in mg/ml protein.

5.2.9 Statistical analyses

One way analysis of variance (1-ANOVA) was performed on all the variables to compare all group means (i.e. control, S, SB, SF and SBF). A post hoc Tukey-Kramer multiple comparisons test determined the significant differences between the means. Where applicable, main effects by the individual oils (FISH/BORAGE) were performed using pooled data, followed by 2-ANOVA if the initial analysis indicated significant interactive effects. Significant effects were determined using the NCSS Statistical analysis package (NCSS 9 Statistical software, Kaysville, Utah, USA). Statistical significant values were considered at p<0.05 and p<0.0001.
5.3 RESULTS

5.3.1 Modulation of cell viability (ATP production)

The ATP production of the 2 colon cancer cell lines were monitored as an indicator of metabolically active cells. The inhibitory oil emulsion concentrations required to produce 50% inhibition (IC50) were determined (Table 5.2). Distinct differences were noted when comparing the IC50’s between the HT-29 and Caco-2 cells. The fish oil emulsions significantly (p<0.0001) reduced cell viability (main effect) in HT-29 cells, whilst borage oil had a lesser reducing effect (p<0.05). Significant interactions indicated that fish oil containing emulsions (SF and SBF) significantly reduced (p<0.05) cell viability with the SBF emulsion exhibiting the highest effect. In the Caco-2 cells, however, both the borage oil and fish oil emulsions (SB, SF and SBF) reduced (P<0.05) cell viability when considering the main effects (Table 5.2). Significant interactions indicated that the S emulsion was the least active in inhibiting Caco-2 cell viability relative to SB, whilst the SBF emulsions was the most (p<0.05) effective.

Table 5.2: Oil emulsion concentrations in mg/ml required to produce 50% cell activity (IC 50) as indicated by ATP release and BrdU incorporation in the colon cancer cells after 48 hour exposure.

<table>
<thead>
<tr>
<th>Cancer cell line</th>
<th>Assay</th>
<th>Oil emulsions (IC50 – mg/ml)</th>
<th>F/B Main effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S</td>
<td>SB</td>
</tr>
<tr>
<td>HT-29 ATP</td>
<td>4.77&lt;sup&gt;a&lt;/sup&gt; (0.45)</td>
<td>4.47&lt;sup&gt;ab&lt;/sup&gt; (0.27)</td>
<td>4.03&lt;sup&gt;b&lt;/sup&gt; (0.11)</td>
</tr>
<tr>
<td>BrdU</td>
<td>8.35&lt;sup&gt;a&lt;/sup&gt; (0.85)</td>
<td>8.16&lt;sup&gt;b&lt;/sup&gt; (0.78)</td>
<td>9.75&lt;sup&gt;c&lt;/sup&gt; (1.69)</td>
</tr>
<tr>
<td>Caco-2 ATP</td>
<td>6.69&lt;sup&gt;a&lt;/sup&gt; (0.43)</td>
<td>5.65&lt;sup&gt;b&lt;/sup&gt; (0.03)</td>
<td>4.96&lt;sup&gt;c&lt;/sup&gt; (0.17)</td>
</tr>
<tr>
<td>BrdU</td>
<td>5.54&lt;sup&gt;a&lt;/sup&gt; (0.25)</td>
<td>5.13&lt;sup&gt;b&lt;/sup&gt; (0.31)</td>
<td>4.77&lt;sup&gt;c&lt;/sup&gt; (0.35)</td>
</tr>
</tbody>
</table>

Values represent the means ± standard deviations of the inhibitory concentration set at 50% (IC50) in mg/ml of 4 independent assay runs. Oil emulsions: S = sunflower oil, SB = sunflower + borage oils, SF = sunflower + fish oils and SBF = sunflower + borage + fish oils. Values in a row are significantly different following different letters (superscript). Values in a column representing the corresponding assay variable between the two cell lines (i.e. ATP IC50 vs. BrdU IC50) within the same oil emulsion treatments are significantly different at p<0.05 (uppercase subscript). Main FISH (F) or BORAGE (B) effects illustrated by an ↑ (increase) and ↓ (decrease) at p<0.05 (*) and p<0.0001 (#).
5.3.2 Modulation of cell proliferation (BrdU incorporation)
Fish oil had a significant (<0.05) increasing main effect on the HT-29 cells proliferation response (Table 5.2). However, no significant interactions were observed for any of the oil emulsions to effect the IC50 concentrations. In the Caco-2 cells, however, borage and fish oil reduced (p<0.05) proliferation (main effect; Table 5.2). Significant interactions indicated that the SB, SF and SBF emulsions decreased (p<0.05) proliferation with the fish oil emulsions having the greater (p<0.0001) effect.

5.3.3 Modulation of cell cytotoxicity (LDH release)
Data were expressed as a function of cell viability by subtracting the % dead cells as determined by LDH release. A significant difference (p<0.05) between the 2 colon cancer cell lines response to the cytotoxic effects of the oil emulsions was observed with the HT-29 cells being more sensitive (p<0.05) when compared to the Caco-2 cells (Table 5.3). Furthermore, the HT-29 cells displayed a significant dose response with increasing concentrations in the S, SB and SBF emulsions decreasing (p<0.05) cell viability. In the Caco-2 cells, however, a distinct interaction between dose and reduction in cell viability was observed, with borage oil exhibiting a reducing (p<0.05) effect when compared to the effects of S and SF which only have a marginal effect (Fig. 5.1).

5.3.4 Induction of apoptosis
In the colon cancer cell lines no significant apoptotic response was noted with any of the oil emulsions at the IC50 values for ATP and BrdU (data not shown). Hence, a dose response of the oil emulsions at higher concentrations 2 times above the ATP IC50 was used to induce apoptosis (Table 5.3). The HT-29 cells showed resistance to the induction of apoptosis at any doses of the oil emulsions utilised (Table 5.3). However, separate analysis on cell cytotoxicity (LDH release) and cell viability (ATP content) showed that all the oil emulsions increased and decreased these cytotoxic parameters respectively, in a dose response manner while it tended to decrease apoptosis. Of interest is that the SB oil emulsion decreased (p<0.05) apoptosis at the two lower concentrations, while no effect was noticed at the highest concentration. A similar effect was noticed in the Caco-2 cells where the lowest concentration also
Table 5.3: Modulation of cytotoxicity, induction of apoptosis and cell viability by oil emulsions in different colon cancer cells.

<table>
<thead>
<tr>
<th>Oil emulsion</th>
<th>S (mg/ml)</th>
<th>SB (mg/ml)</th>
<th>SF (mg/ml)</th>
<th>SBF (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HT-29 cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LDH cytotoxicity</strong></td>
<td>79.31a (8.68)</td>
<td>83.87a (1.84)</td>
<td>88.54a (1.02)</td>
<td>84.75a (1.39)</td>
</tr>
<tr>
<td>(% viable cells)</td>
<td>80.49a (4.39)</td>
<td>83.10a (4.09)</td>
<td>70.63b (2.26)</td>
<td>69.95b (3.25)</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>1.00a (0.22)</td>
<td>0.51b (0.02)</td>
<td>0.79 (0.06)</td>
<td>0.56b (0.08)</td>
</tr>
<tr>
<td>(caspase-3 fold)</td>
<td>0.85a (0.11)</td>
<td>0.70b (0.06)</td>
<td>0.82 (0.05)</td>
<td>0.49b (0.05)</td>
</tr>
<tr>
<td>Cell viability</td>
<td>100.00a (5.13)</td>
<td>79.12b (2.58)</td>
<td>76.81b (2.48)</td>
<td>73.28b (2.76)</td>
</tr>
<tr>
<td>(%ATP)</td>
<td>38.13c (3.22)</td>
<td>54.38c (3.92)</td>
<td>43.76c (3.92)</td>
<td>66.48b (8.42)</td>
</tr>
</tbody>
</table>

| **Caco-2 cells** |           |            |            |             |
| **LDH cytotoxicity** | 84.42a (3.94) | 88.41b (3.19) | 89.44b (1.37) | 88.35 (0.55) |
| (% viable cells) | 85.30ab (2.01) | 85.01a (2.53) | 85.88 (2.46) | 83.85 (2.66) |
| Apoptosis | 1.00a (0.09) | 1.93b (0.11) | 0.77b (0.14) | 1.42b (0.20) |
| (caspase-3 fold) | 2.04b (0.12) | 1.45c (0.06) | 2.14c (0.22) | 1.85d (0.11) |
| Cell viability | 100.00a (5.67) | 77.28b (8.21) | 58.51b (3.26) | 41.90b (1.10) |
| (%ATP) | 58.47c (4.48) | 43.89c (0.92) | 48.37c (4.43) | 48.44b (4.49) |

Mean and standard deviation (in brackets) of duplicate runs (N = 10 per concentration). Oil emulsions: S = sunflower oil, SB = sunflower + borage oils, SF = sunflower + fish oils and SBF = sunflower + borage + fish oils. LDH cytotoxicity expressed as a function of cell viability by subtracting the % dead cells as determined by LDH release.
Figure 5.1: Caco-2 cells dose response relative to borage oil.

reduced apoptosis. All the other oil emulsions increased (p<0.05) apoptosis in a dose dependent manner, with the S emulsion increasing (p<0.05) apoptosis at the lower concentrations while it tended to decrease (p<0.05) at the higher concentration. The SF and SBF oil emulsions increased (p<0.05) apoptosis at higher dose levels, which was associated with an increased cell cytotoxicity (Table 5.3).

5.3.5 Modulation of lipid metabolism of Caco-2 cells

The Caco-2 cells were selected for subsequent studies based on their sensitivity of the different oils emulsion regarding the modulation of cell proliferation and the induction of apoptosis. Alterations in the Caco-2 cell membrane lipid content were compared to the untreated control cells following treatment with the different oil emulsion constituting varying ω-6/ω-3 FA ratios. Furthermore, for each oil emulsion two different concentrations were used which was associated with: (i) the induction of apoptosis (S = 8.5 mg/ml; SB = 7.5 mg/ml; SF = 8.9 mg/ml; SBF = 6 mg/ml) which was2 times above the IC50 values in reducing cell viability (ATP), and (ii) the BrdU IC50 (S = 5.5 mg/ml; SB = 5.10 mg/ml; SF = 4.8 mg/ml; SBF = 4.8 mg/ml) that reduced cell proliferation by 50%. Changes associated with specific dose response
effects associated with apoptosis in relation to the inhibition of cell proliferation were also highlighted.

5.3.5.1 Cholesterol
With the induction of apoptosis in the Caco-2 cells, cholesterol content (Fig. 5.2A) was significantly increased (p<0.05) by the SF emulsion (8.9 mg/ml) when compared to the control and other emulsions (S, SB, SBF) at these treatment concentrations. The cholesterol was significantly (p<0.05) increased by the S, SB and SF emulsions at the BrdU IC50 emulsion concentrations relative to the untreated control.

Dose response effects: The S and SB emulsion had a significant reducing (p<0.05) effect on membranes cholesterol content with the induction of apoptosis when compared to the lower BrdU IC50 treatments.

5.3.5.2 Phosphatidylcholine (PC)
Compared to the control, Caco-2 cells membrane phospholipid content of PC (Fig. 5.2B) increased (p<0.05) with the induction of apoptosis by the oil emulsions. The SF emulsion significantly (p<0.05) increased PC more effectively than the S emulsion and control. All the emulsions increased (p<0.05) the PC content to the same extent at the respective BrdU IC50 when compared to the control.

Dose response effects: With the induction of apoptosis membranes PC content was significantly (p<0.05) higher with the SB, SF and SBF emulsions when compared to the lower dose (BrdU IC50).

5.3.5.3 Phosphatidylethanolamine (PE)
When compared to the control, no change in PE content (Fig. 5.2C) was noted with the induction of apoptosis. However at the BrdU IC50 treatments, PE was significantly (p<0.05) increased by the S, SB and SF emulsions with the S emulsion exhibiting a higher response than the SB and SF emulsions.

Dose response effects: PE content was significantly (p<0.05) decreased with the induction of apoptosis by the S, SB and SBF emulsion when compared to the lower BrdU IC50 treatments.
5.3.5.4 **PC/PE ratio**

The PC/PE ratio (Fig. 5.2D) in the membrane of the Caco-2 cells increased (p<0.05) significantly with the induction of apoptosis by the oil emulsions when compared to the control, with SB, SF and SBF exhibiting the largest increasing effects. At a lower dosage (BrdU IC50), only the SF and SBF emulsions increased (p<0.05) the PC/PE ratio compared to the control.

*Dose response effects*: The PC/PE ratio increased (p<0.05) significantly with the induction of apoptosis by the SB, SF and SBF emulsions when compared to the lower BrdU IC50 treatments.

5.3.5.5 **Cholesterol/phospholipid (chol/PL) ratio**

The chol/PL ratio (Fig. 5.2E) in the Caco-2 cells membrane was decreased (p<0.05) by the S, SB and SF emulsions with the induction of apoptosis when compared to the control. Including the SBF emulsion, this same reducing (p<0.05) effect was noted at the BrdU IC50 treatment.

*Dose response effects*: Membranes chol/PL ratio was significantly (p<0.05) increased with the induction of apoptosis by the SF and SBF emulsions, whilst S and SB resulted in the ratio’s decrease (p<0.05) when compared to the lower BrdU IC50 treatments.

5.3.6 **Modulation of the Caco-2 cells fatty acid composition**

To evaluate the level of FA incorporation, two concentrations were used per oil emulsion, (i) high concentration associated with the induction of apoptosis and (ii) a low concentration (IC50 for BrdU) related to the reduction of cell proliferation. The FA content of the phospholipids PC and PE was expressed qualitatively (%) and quantitatively (μg) (Tables 5.5-5.9 in Addendum 3). The percentage reflected the distribution of the fatty acid in the respective phospholipid, while the concentration (μg FA/mg protein) is the level which is also dependent on the concentration of the respective phospholipid. All the data were compared relative to the untreated control cells.
Figure 5.2: Cholesterol and phospholipids content in the Caco-2 cells membranes after 48 hours exposure to different oil emulsion concentrations (apoptosis inducing and BrdU IC50). (A) Cholesterol, (B) Phosphatidylcholine (PC) content, (C) Phosphatidylethanolamine (PE) content, (D) PC/PE ratio and (E) cholesterol/phospholipids (chol/PL) ratio. Significant differences between groups set at p<0.05 are indicated by different letters: lowercase – differences between oil emulsions within assay (ATP IC50 or BrdU IC50); uppercase - corresponding lipid variable compared at different concentrations (i.e. apoptosis inducing vs. BrdU IC50) within the same oil emulsion treatment.
5.3.6.1 Modulation of membranes saturated fatty acids (SATS) composition

Qualitative (%)

**Apoptosis**: Compared to the control, the total SATS (summarized in Fig. 5.3) was not changed in PC, although C18:0 was reduced (p<0.05) by the S emulsion. In PE, a decrease (p<0.05) in SATS was noted with the SF and SBF emulsions. This was mainly due to the SF emulsion decreasing (p<0.05) the C16:0 content. The SB emulsion also decreased C16:0, while the S and SBF decreased 16:0 marginally.

**BrdU IC50**: No significant change in the total SATS was noted in PC when compared to the control, whilst in PE the SB emulsion resulted in a significant (p<0.05) decrease. This effect was mainly due to a decrease (p<0.05) in C16:0.

**Dose response effects**: With the induction of apoptosis, membranes total SATS was significantly (p<0.05) decreased by the SBF emulsion in PE when compared to the lower BrdU IC50 treatments.

Quantitative (µg)

**Apoptosis**: In PC and PE, the total SATS (Fig. 5.3) was significantly (p<0.05) decreased by the S, SB and SF emulsions. The emulsions mainly decreased (p<0.05) C16:0, whilst the S emulsion only decreased (p<0.05) C18:0 in PC and SF having this same reducing (p<0.05) effect in PE.

**BrdU IC50**: In PC, the total SATS remained unchanged compared to the control, although a decrease (p<0.05) in C16:0 was noted with the SB emulsion. The S and SF emulsions increased (p<0.05) total SATS in PE, whilst the SB emulsion resulted in a significant (p<0.05) decrease. This latter effect was due to a decrease (p<0.05) in C16:0, whereas the S and SF emulsions increased (p<0.05) C18:0. An increase (p<0.05) in C16:0 was also noted with the S emulsion in PE.

**Dose response effects**: The S and SF emulsions significantly (p<0.05) decreased total SATS in PC and PE with the induction of apoptosis when compared to the BrdU IC50 treatment. This effect was mainly due to a decrease (p<0.05) in C16:0 and C18:0.
Figure 5.3: Summary of the modulation of Caco-2 cells SATS composition by the oil emulsions when compared to the control. The dose responses indicated are the apoptosis inducing concentrations effects on the FA relative to the BrdU IC50 treatments.

5.3.6.2 Modulation of membranes monounsaturated fatty acids (MUFA) composition

Qualitative (%)

Apoptosis: Compared to the control, no significant change in total MUFA (summarized in Fig. 5.4) was noted in PC and PE. However, in both phospholipid fractions the SB and SF emulsions decreased (p<0.05) C16:1ω-7 and C18:1ω-9. In PC the S and SBF emulsions also decreased (p<0.05) C16:1ω-7, whilst only SBF resulted in its decrease (p<0.05) in PE.
**BrdU IC50**: In PC and PE, no significant change in total MUFA was noted compared to the control. A decrease \((p<0.05)\) in \(C16:1\omega-7\) and \(C18:1\omega-9\), however, was observed with the SF emulsion in PC.

**Dose response effects**: In PC, the S emulsion decreased \((p<0.05)\) total MUFA, whilst in PE the SB and SF emulsions resulted in a decrease \((p<0.05)\) with the induction of apoptosis. The S emulsion reduced \((p<0.05)\) \(C16:1\omega-7\) in PC, whereas the SB and SF emulsions decreased \((p<0.05)\) \(C16:1\omega-7\) and \(C18:1\omega-9\) in PE when compared to the BrdU IC50 treatments.

**Quantitative (µg)**

**Apoptosis**: No significant change in the total MUFA (Fig 5.4) was noted in PC, although the SF emulsion decreased \((p<0.05)\) \(C18:1\omega-9\) when compared to the control. In PE the SF emulsion resulted in a decrease \((p<0.05)\) in total MUFA due to a reduction \((p<0.05)\) in \(C16:1\omega-7\) and \(C18:1\omega-9\).

**BrdU IC50**: In PC and PE, the total MUFA was significantly \((p<0.05)\) increased by the S emulsion, whilst only being increased \((p<0.05)\) by the SF and SBF emulsions in PE compared to the control. The latter effect was due to an increase \((p<0.05)\) in \(C16:1\omega-7\), \(C18:1\omega-9\) and \(C18:1\omega-7\) by SF, whereas the SBF emulsion only increased \((p<0.05)\) \(C18:1\omega-9\).

**Dose response effects**: Total MUFA decreased \((p<0.05)\) significantly in PC and PE with the S and SF emulsions inducing apoptosis. A similar effect was noted with the SBF emulsion in PE. The S and SF emulsions decreased \((p<0.05)\) \(C16:1\omega-7\), \(C18:1\omega-9\) and \(C18:1\omega-7\) in both phospholipid fractions, whilst SBF reduced \((p<0.05)\) \(C16:1\omega-7\) in only PE when compared to the BrdU IC50 treatments.
Figure 5.4: Summary of the modulation of Caco-2 cells MUFA composition by the oil emulsions when compared to the control. The dose responses indicated are the apoptosis inducing concentrations effects on the FA relative to the BrdU IC50 treatments.
5.3.6.3 Modulation of membranes polyunsaturated fatty acids (PUFA) composition

(a) ω-3 PUFA

Qualitative (%)

**Apoptosis:** Total ω-3 FA (Fig. 5.5) was significantly (p<0.05) increased in PC and PE by the SF and SBF emulsions, whilst only increasing (p<0.05) with the S and SB emulsions in PE when compared to the control. The SF emulsion increased (p<0.05) only C20:5ω-3 in PC, whilst both the SF and SBF emulsions increased (p<0.05) C20:5ω-3, C22:5ω-3 and C22:6ω-3 in PE. The S emulsion increased (p<0.05) C22:5ω-3 in PE, whereas SB increased (p<0.05) C22:5ω-3 and C22:6ω-3.

**BrdU IC50:** Compared to the control, the total ω-3 FA increased (p<0.05) in PC and PE with the SB, SF and SBF emulsions. The S emulsion also resulted in the total’s increase (p<0.05) in PC only due to an increase (p<0.05) in C22:5ω-3 and C22:6ω-3, whilst decreasing (p<0.05) C18:3ω-3. In PC and PE, the SF emulsion increased (p<0.05) C20:5ω-3 and C22:5ω-3.

**Dose response effects:** In PC, the oil emulsions decreased (p<0.05) the total ω-3 FA, whilst being significantly (p<0.05) increased in PE with the induction of apoptosis. The reduction in PC was due to the oil emulsions decreasing (p<0.05) effect on C22:6ω-3, with the S and SF emulsions also decreasing (p<0.05) C20:5ω-3 and C22:5ω-3, and SB reducing (p<0.05) C22:5ω-3. The total increase in PE was the result of the oil emulsions increasing (p<0.05) effect on C22:5ω-3 and C22:6ω-3, whilst the S, SF and SBF emulsions also increased C20:5ω-3 when compared to the BrdU IC50 treatments.

Quantitative (µg)

**Apoptosis:** The total ω-3 FA was significantly (p<0.05) increased in PC by the SBF emulsion, whilst the SF and SBF emulsions increased the content in PE when compared to the control. In PC, the SBF emulsion resulted in an increase (p<0.05) in C18:3ω-3 and C22:5ω-3. The SF and SBF emulsions increased (p<0.05) C20:5ω-3 and C22:6ω-3 in PE.

**BrdU IC50:** Compared to the control, the total ω-3 FA increased (p<0.05) with all the oil emulsions in PC, whilst in PE content was only increased (p<0.05) by the SF and
SBF emulsions and decreased by SB. The change in PC was due to an increase (p<0.05) in C22:5ω-3 and C22:6ω-3. An increase (p<0.05) in C18:3ω-3 and C20:5ω-3 was also noticed with the SF and SBF emulsions and only C18:3ω-3 with SB. In PE, the SF and SBF emulsions significantly (p<0.05) increased C20:5ω-3 and C22:5ω-3, whilst only SF increased (p<0.05) C18:3ω-3 and C22:6ω-3. The SB emulsion decreased (p<0.05) C22:5ω-3 and C22:6ω-3 in PE, whereas an increase (P<0.05) in C18:3ω-3 and C22:5ω-3 was noted with the S emulsion.

**Dose response effects:** In PC, the total ω-3 content significantly (p<0.05) decreased due to a reduction (p<0.05) in C20:5ω-3 and C22:6ω-3. Also, membrane content in C18:3ω-3 decreased with the SB and SF emulsions, whilst C22:6ω-3 decreased (p<0.05) with S, SB and SF. In PE, however, the total ω-3 FA increased (p<0.05) as the membrane content in C20:5ω-3 and C22:6ω-3 increased (p<0.05) with the SB and SBF emulsions as apoptosis was induced. The SB emulsion also increased (p<0.05) C22:5ω-3, whilst SF decreased (p<0.05) the total FA content in PE by reducing (p<0.05) C18:3ω-3, C20:5ω-3, C22:5ω-3 and C22:6ω-3 when compared to the BrdU IC50 treatments. The S emulsion also decreased (p<0.05) the total ω-3 FA content by reducing (p<0.05) C18:3ω-3 and C22:5ω-3.
Figure 5.5: Summary of the modulation of Caco-2 cells $\omega$-3 FA composition by the oil emulsions when compared to the control. The dose responses indicated are the apoptosis inducing concentrations effects on the FA relative to the BrdU IC50 treatments.
(b) ω-6 PUFA

Qualitative (%)

**Apoptosis**: In PC and PE, a significant (p<0.05) increase in total ω-6 FA (Fig. 5.6) was noted with the oil emulsions due to an increase (p<0.05) in C18:2ω-6 and C20:4ω-6 when compared to the control. In PC, the SB and SBF emulsions increased (p<0.05) C20:3ω-6, whilst also being increased (p<0.05) by all the oil emulsions in PE.

**BrdU IC50**: Changes in membrane total ω-6 FA composition was similar to that of the cells in which apoptosis was induced.

**Dose response effects**: With the induction in apoptosis, membranes total ω-6 FA decreased (p<0.05) in PC with the SF emulsion due to a reduction (p<0.05) in C18:2ω-6. A decrease (p<0.05) in C20:4ω-6 was also noted with the S and SB emulsions, whilst SBF reduced (p<0.05) C20:3ω-6. In PE, the SBF emulsion increased (p<0.05) the total ω-6 FA due to an increase (p<0.05) in C20:4ω-6, which was also increased (p<0.05) by the S and SF emulsions. The SB and SF emulsions decreased (p<0.05) C18:2ω-6 when compared to the BrdU IC50 treatments.

Quantitative (μg)

**Apoptosis**: Compared to the control, in PC and PE the total ω-6 FA content increased (p<0.05) due to a significant (p<0.05) increase in C18:2ω-6. In PC the S, SB and SBF emulsions increased (p<0.05) C20:3ω-6, whilst C20:4ω-6 was reduced (p<0.05) by S, SB and SF. All the oil emulsions increased (p<0.05) C20:3ω-6 in PE, but only SB, SF and SBF resulted in an increase (p<0.05) in C20:4ω-6.

**BrdU IC50**: Total ω-6 FA content was significantly (p<0.05) increased in PC and PE due to an increase in C18:2ω-6, C20:3ω-6 and C20:4ω-6 when compared to the control.

**Dose response effects**: To induce apoptosis the total ω-6 content in PC was reduced (p<0.05) by the S, SB and SF emulsions. These effects were due to a decrease (p<0.05) in C20:4ω-6, but only the SF and SBF emulsions reduced C20:3ω-6. In PC, the content of C18:2ω-6 was decreased (p<0.05) by the S and SF emulsions, whilst being increased (p<0.05) by SBF. The total ω-6 FA decreased (p<0.05) in PE due to a reduction (p<0.05) in C18:2ω-6. An increase (p<0.05) C20:4ω-6 was noted with
the SB and SBF emulsions, whilst being decreased (p<0.05) by SF in PE when compared to the BrdU IC50 treatments.

<table>
<thead>
<tr>
<th></th>
<th>PC</th>
<th>PE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S</td>
<td>SB</td>
</tr>
<tr>
<td>% C18:2 ω-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>BrdU IC50</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Dose response</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>% C20:3 ω-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>−</td>
<td>↑</td>
</tr>
<tr>
<td>BrdU IC50</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Dose response</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>% C20:4 ω-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>BrdU IC50</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Dose response</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>% total ω-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>BrdU IC50</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Dose response</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>µg C18:2 ω-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>BrdU IC50</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Dose response</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>µg C20:3 ω-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>BrdU IC50</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Dose response</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>µg C20:4 ω-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>BrdU IC50</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Dose response</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>µg total ω-6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>BrdU IC50</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Dose response</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

No effect: −; increase: ↑; decrease: ↓

Figure 5.6: Summary of the modulation of Caco-2 cells ω-6 FA composition by the oil emulsions when compared to the control. The dose responses indicated are the apoptosis inducing concentrations effects on the FA relative to the BrdU IC50 treatments.
5.3.6.4 Modulation of membranes total PUFA and FA ratios composition

Qualitative (%)

**Apoptosis:** The oil emulsions increased (p<0.05) the Caco-2 cells total PUFA and the Δ6 substrate/product (S/P) ratio in PC and PE (Fig. 5.7). Membranes ω-6/ω-3 FA and PUFA/SATS ratios increased (p<0.05) in PC, whilst being decreased (p<0.05) in PE. The C20:4ω-6/C20:5ω-3 ratio increased with the S and SB emulsions in PC and PE, but the C20:4ω-6 ratio decreased (p<0.05) with all the oil emulsions.

**BrdU IC50:** Compared to the control, in PC and PE the total PUFA, ω-6/ω-3 FA and Δ6 S/P ratios were increased (p<0.05) by the oil emulsions. In both PC and PE the PUFA/SATS ratio increased (p<0.05) with the SB and SF emulsions, whilst increasing (p<0.05) only with S and SBF in PC. The C20:4ω-6/C20:5ω-3 ratio was increased (p<0.05) by the S and SB emulsions in PC and PE, but only increased (p<0.05) in PE by SBF.

**Dose response effects:** To induce apoptosis, the total PUFA was decreased (p<0.05) in PC by the S, SB and SF emulsions in PC, whilst being increased (p<0.05) in PE by the S and SBF emulsions. The oil emulsions increased (p<0.05) the ω-6/ω-3 FA ratio in PC, but resulted in a decrease (p<0.05) in PE. A similar decrease (p<0.05) in membranes Δ6 S/P ratio was noticed in PE, whilst the SB, SF and SBF emulsions increased (p<0.05) the ratio in PC. The PUFA/SATS ratio was decreased (p<0.05) by the SB and SF emulsions in PC, but increased (p<0.05) with S, SF and SBF in PE. Membranes C20:4ω-6/C20:5ω-3 ratio decreased (p<0.05) with only the S emulsion in PE, but the 20:4ω-6 PC/PE ratio decreased with all the emulsions.

Quantitative (µg)

**Apoptosis:** The total PUFA content was significantly (p<0.05) increased by the oil emulsions in PC, whilst only SB, SF and SBF increased the total in PE. In PC and PE the Δ6 S/P ratio increased (p<0.05) with the SB, SF and SBF emulsions. All oil emulsions increased the ω-6/ω-3 FA ratio in PC, whilst an increase (p<0.05) by only S and SB was noted in the ratio. The PUFA/SATS ratio increased (p<0.05) with the SF and SBF emulsions in PC and PE, but only increased (p<0.05) with S in PC. In PC an increase (p<0.05) in the C20:4ω-6/C20:5ω-3 ratio was noted with S and SB
emulsions, but only SB increased (p<0.05) the ratio in PE. The C20:4ω-6 PC/PE ratio decreased (p<0.05) with the oil emulsions.

*BrdU IC50:* Compared to the control, all the oil emulsions increased (p<0.05) the total PUFA in PC and PE, whilst the SB, SF and SBF emulsions increased (p<0.05) the PUFA/SATS ratio. The ω-6/ω-3 FA ratio was increased (p<0.05) in PC by only the S, SB and SF emulsions, but increased (p<0.05) by all the emulsions in PE. An increase (p<0.5) in membranes Δ6 S/P ratio with the SB, SF and SBF emulsions was noted in PC, while all the oil emulsions increased (p<0.05) the ratio in PE. The C20:4ω-6/C20:5ω-3 ratio increased (p<0.05) in both PC and PE due to the effects of the S and SB emulsions, but increased (p<0.05) with SF in PE. Membrane C20:4ω-6 PC/PE ratio decreased (p<0.05) with the SF emulsion.

*Dose response effects:* To induce apoptosis, the total PUFA content was significantly (p<0.05) decreased in PC by the S, SB and SF emulsions, whilst in PE the S and SF emulsions decreased (p<0.05) the content and SB resulted in an increase (p<0.05). The membrane ω-6/ω-3 FA ratio was increased (p<0.05) by all the oil emulsions in PC, but decreased (p<0.05) in PE. A similar decrease (p<0.05) in the C20:4ω-6 PC/PE ratio was noted. The PUFA/SATS ratio was decreased (p<0.05) by the SB and SF emulsions in PC, and the C20:4ω-6/C20:5ω-3 ratio decreased (p<0.05) by S in PE. In PC the SB, SF and SBF emulsion increased (p<0.05) the Δ6 S/P ratio, but was decreased (p<0.05) in PE by S, SF and SBF.
Figure 5.7: Modulation of Caco-2 cells total PUFA and FA ratios composition by the oil emulsions when compared to the control. The dose responses indicated are the apoptosis inducing concentrations effects on the FA relative to the BrdU IC50 treatments.
5.4 DISCUSSION

In the current study, the survival of two human colon cancer cells were assessed and compared after exposure to certain oil ratio emulsions. This was done to establish whether the use of dietary fats with well-defined FA compositions exhibited chemopreventive potential against the survival of cancer cells. Numerous studies show that ω-3 FA possess anti-cancer properties (Bartsch et al., 1999; Larsson et al., 2004), whereas certain ω-6 FA appear to enhance the altered phenotype of cancer cells (Cabral et al., 2013; Whelan and McEntee, 2004). With neither class of FA being consumed in isolation, it has been suggested that manipulating the balance of ω-6 and ω-3 of FA (i.e. ω-6/ω-3 ratio) may be an important strategy in inhibiting the survival of cancerous cells (Simopoulos, 2008). With dietary intake providing a major source of these unsaturated carbon chains, such an approach is feasible. A key observation made in this investigation was the differing cellular responses between the HT-29 and Caco-2 colon cancer cell lines that were elicited by exposure to different oil emulsions with distinctive FA content and ω-6/ω-3 ratio. In addition specific changes in membrane lipid and FA composition, associated with apoptosis and the inhibition of cell proliferation were characterised using the Caco-2 cancer cells.

5.4.1 Modulation of colon cancer cells survival by the oil emulsions

The two colon cancer cell lines displayed differential sensitivity to the effects of the different oil emulsions and its respective FA constituents. This has been reported to occur in other cancer cells as well (Ding et al., 2004; Mengeaud et al., 1992; Schønberg et al., 2006). In the HT-29 and Caco-2 cancer cells, initial analyses showed that the reduction of ATP production (cell viability) and inhibition of BrdU incorporation (cell proliferation) was dependent on the dose of the oil emulsion treatment. Regarding reduction in cell viability, the HT-29 cells exhibited greater sensitivity to the modulating effects of the fish oil containing emulsions (SF and SBF), whilst borage oil (SB) was less active. However, in the HT-29 cells a higher amount of the different oil emulsions was required to elicit an inhibitory response on cell proliferation when compared to its effect on the inhibition of ATP production. A similar dose was required in the Caco-2 cells. This response could be related to an increased resistance of the HT-29 cells to cellular oxidative stress triggered by LC
PUFA. In this regard, the SF and SBF emulsions were more effective in reducing the cell growth parameters in both cell types. This is in accordance with the literature as fish oil has been shown to increase mitochondrial membranes level of unsaturation, which enhances cells level of ROS (Hong et al., 2002). This is due to the supply of LC ω-3 PUFA, C20:5ω-3 and C22:6ω-3 (SF and SBF) that are highly susceptible to lipid peroxidation (LPO) and has been shown to inhibit cancer cell growth (Das, 1999; Larsson et al., 2004). In contrast, in the Caco-2 cells borage oil and fish oil required similar IC50 concentrations for the inhibition of cell viability and proliferation. Borage oil also exhibited a greater reducing effect when compared to the S emulsion, though the SF and SBF emulsions were still more active. The borage oil containing emulsions (SB and SBF) increased the supply of C18:3ω-6 to the cells, which has demonstrated anti-proliferative properties due to modifying membranes FA pattern, decreasing the mitochondrial membrane potential and increasing oxidative stress (Hrelia et al., 1996; Itoh et al., 2010).

Further investigation showed that interesting associations seems to exist when considering the oil emulsions effect on ATP production, cytotoxicity (necrosis) and the induction of apoptosis. It would appear that a threshold with respect to the level of cellular ATP determines whether the colon cancer cells are removed via apoptosis or necrosis (Tsujimoto, 1997). Apoptosis is widely accepted as an efficient, energy dependent and stepwise process ensuring the removal of damaged cells. Conversely, necrosis is an accidental form of cell death lacking any underling signalling events and results from exposure to severe stress conditions or toxic agents. In addition, necrotic cell death occurs in the absence of ATP (Eguchi et al., 1997). As noticed with the HT-29 cells, the significant reduction of ATP and ATP-dependent apoptosis by the S, SB and SBF emulsions suggests that the uncontrolled degenerative form of cell removal was obtained, particularly at the highest cytotoxic concentrations. Interestingly, it has been shown that the HT-29 cells have a highly apoptosis-resistant phenotype due to its ability to thrive under conditions of micro environmental stresses (Suzuki et al., 1998). This suggests that the cell line has adapted mechanisms to maintain its survival.

The dietary FA-induced depletion of the energy status within the cancer cell line is indicative of metabolic reprogramming centred on the mitochondria that play a critical
role in initiating a necrotic or apoptotic response in cells (Eguchi et al., 1997). In normal cells, the mitochondria play a pivotal role in mediating apoptosis and regulating the cellular energy supply via oxidative phosphorylation. However, in cancer cells, both these processes are defective due to mitochondrial dysfunction (Costa et al., 2014). Therefore, cancer cells are characterised by a loss in apoptosis and a shift in energy production occurring via glycolysis (Warburg effect), which supports its proliferative nature (Lunt and Vander Heiden, 2011). To further supplement its energy reserves, cells also utilize FA to increase the energy supply via mitochondrial beta-oxidation (Nakamura et al., 2014). Regardless, with the decrease in ATP, this supplementary effect is not evident here with the HT-29 cells at the specified emulsion concentrations. This is likely due to cancer cells preferentially using FA for the biosynthesis of membranes and signalling molecules to generate more daughter cells (Baenke et al., 2013). Consequently, the level of ATP is likely insufficient to induce apoptosis despite the increased supply of FA energy sources and associated substrates susceptible to free radical attack. In this regard, cancer cells are known to contain highly effective antioxidant defence mechanisms to combat adverse effects mediated by excessive levels of free radicals (Trachootham et al., 2009).

In contrast, the Caco-2 cells responded to the apoptotic inducing effects of all the oil emulsions with increasing concentration. The induction of apoptosis was also decreased as low levels in ATP production was reached, effecting marginal cytotoxic effects. As mentioned, the induction of apoptosis relies on mitochondrial metabolism, which supplies energy to activate the cell death executing cysteine protease caspase-3 (Budihardjo et al., 1999). With a reduction in mitochondrial ATP production being apparent, it is plausible that as a result of the mitochondrial energy crisis, the glycolytic route likely contributes sufficient intracellular ATP to induce apoptosis (Zheng, 2012). At low cytotoxicity, particularly with borage oil (SB), the induction of alternate mechanisms, such as cell cycle arrest may also be a possible reason for the reduction in energy status. Inhibition of mitochondrial ATPase, the enzyme that catalyzes the formation of ATP from ADP, has been shown to block cell cycle progression in the G1 phase, thus inhibiting an increase in cell numbers (Gemin et al., 2005). Meanwhile, the increase in apoptosis at the higher concentrations in S, SF and SBF is presumably due to the cancer cells enhanced
level in cellular oxidative stress arising from the oil emulsions FA supply and cellular metabolism. Consequently, only the Caco-2 cell line was selected to investigate the level of FA incorporated into membranes associated with the induction of apoptosis and inhibition of cell proliferation.

5.4.2 Caco-2 cells membranes lipid content and FA incorporation and distribution facilitating the induction of apoptosis and inhibition of cell proliferation

In cancer cells, the continuous biosynthesis of cell membrane building components plays a critical role in sustaining its abnormal proliferative state. As a result, alterations in lipid and FA metabolism have been identified as a major adaptation of cancer cells to ensure its survival (Zhang and Du, 2012). The oil emulsions utilised in the current study resulted in significant modification of membranes qualitative and quantitative FA composition in the Caco-2 cells, hereby altering their structure and function (Kremmyda et al., 2011; Tvrzicka et al., 2011). As a consequence of the membrane alterations, either apoptosis was induced or cell proliferation was inhibited.

5.4.2.1 Modulation of membranes total PUFA and ω-6/ω-3 FA ratio composition

PUFA have been identified for having both chemopreventive and promoting effects on cancer development due to its effects on various cell regulatory processes (Serini et al., 2009; Tapiero et al., 2002; McEntee and Wheelan, 2002). Mammalian cells are inefficient at synthesizing PUFA due to a lack in Δ12- and Δ15- desaturases (Pereira et al., 2003). Therefore, the supply of PUFA is largely dependent on dietary intake, particularly of parent FA C18:2ω-6 and C18:3ω-3 that is further metabolized into the longer chain PUFA or is consumed directly.

The total percentage of PUFA increased in the Caco-2 cell membranes in response to the S and SBF emulsions in PE when compared to the inhibitory effect of the oil emulsions on cell proliferation. However, PUFA content (μg) decreased with the S and SF emulsions in both PC and PE, but increased with SB (PE). The decrease of PUFA content in membranes has been linked to the activity of the FA metabolising enzyme Δ6-desaturase, which is much lower in tumours (Hrelia et al., 1994).
Although not physically measured, a decrease in this enzyme’s activity was indicated by the emulsions enhancement of the Δ6 substrate/product ratio in PC and PE (% and μg), which showed that more Δ6 substrates such as C18:2ω-6 was incorporated into the membranes. However, a decrease in the ratio with respect to the dose required for the induction of apoptosis was also observed in PE (μg). Therefore, the Δ6 substrate/product ratio may not be an efficient indicator for this process. Also, apart from an increase in protective antioxidant responses, tumour cells reduction in membrane unsaturation contributes towards resistance to oxidative stress, which reduces the opportunity to initiate apoptosis via LPO (Horrobin, 1990). Although the Caco-2 cells total PUFA content decreased, the emulsions dose response resulted in a decreased ω-6/ω-3 FA ratio in PE. This effect enhanced membranes LC ω-3 FA (C20:5ω-3 and C22:6ω-3) content with the SB and SBF emulsions. Of importance, the increase of Δ6-desaturase product C22:6ω-3 was supplied by exposure and uptake, and not FA metabolism. This increase in ω-3 FA enhances membranes susceptibility to LPO and likely contributed to the induction of apoptosis (Das, 1999; Hofmanová et al., 2005; Song and Miyazawa, 2001).

Borage oil’s (SB and SBF) increasing effect on membranes C20:4ω-6 content in PE of the Caco-2 cells appears to also favour the induction of apoptosis. Elevated levels of C20:4ω-6 is considered a risk factor associated with colon cancer development due to its metabolites amplifying cancer cells proliferative nature (Cathcart et al., 2012). However, dependent on its concentration in cells, C20:4ω-6 participates in apoptosis upon its release from membranes acyl backbone by phospholipase A₂ (PLA₂), and subsequently acting as a secondary messenger activating the process (Cao et al., 2000).

Evidently, different thresholds with regards to modification of membranes PUFA composition appears to determine the oil emulsions anti-cancer effect (apoptosis vs. inhibition of proliferation). To inhibit proliferation, membranes PUFA composition differed significantly at the lower emulsion concentrations, favouring an increase in PUFA and the ω-6/ω-3 FA ratio in both phospholipid fractions. With the increase in membrane C20:3ω-6, C20:5ω-3 in PC, an anti-inflammatory effect likely occurred due to the incorporated PUFA being metabolized into prostanoids (prostaglandins PGE₁ and PGE₃) that results in the reduction of cancer cells proliferation (Larsson et
al., 2004; Wang and Gu, 2012). Although membranes content in C20:4ω-6 and the C20:4ω-6/C20:5ω-3 ratio increased as well, the PUFA metabolizing enzyme cyclooxygenase-2 preferentially catalyzes the 20-carbon substrates initiating an anti-inflammatory response (Larsson et al., 2004). Thus, any cancer promoting effects by PGE$_2$, a product of C20:4ω-6 metabolism (Cabral et al., 2013), is counteracted by these aforementioned FA.

### 5.4.2.2 Modulation of membranes SATS and MUFA composition

To further facilitate the induction of apoptosis in the Caco-2 cells, the oil emulsions altered membranes SATS and MUFA content. In cancer cells, the relative amount of SATS and MUFA increases due to a high rate of de novo synthesis (Rysman et al., 2010). A significant decrease in total SATS, C16:0 and C18:0 (S and SF) and total MUFA, C16:1ω-7, C18:1ω-7 and C18:1ω-9 (S, SF and SBF) in PC and PE was observed in the Caco-2 cells due to preferential incorporation of PUFA. Increased incorporation of SATS like C16:0 into cancer cell membranes appears to have a role in enhancing the synthesis of structural and signalling lipids (Louie et al., 2013). The MUFA C18:1ω-9 displays antioxidant properties, providing additional protection to cancer cells against oxidative damage (Schuhr, 2008). Therefore, its reduction in PC and PE probably assists in the Caco-2 cancer cells removal through LPO-induced apoptosis. Opposite effects was observed in the inhibition of cell proliferation, with an increase in SATS and MUFA in PE. In response to the dose of emulsions S and SF, the cancer cells increase in C16:0 appears to have an inhibitory effect on proliferation. Hardy and colleagues demonstrated in MDA-MB breast cancer cells that the FA significantly reduces the activity of phosphatidylinositol 3-kinase, which phosphorylates inositol lipids that act as secondary messengers for several pathways related to cell proliferation (Hardy et al., 2000).

### 5.4.2.3 Modulation of membranes lipid components

Membranes lipid bilayers are mainly composed of phospholipids of various classes that are distributed asymmetrically. PC and PE are the predominant lipids, with PC dominating the outer leaflet of plasma membranes, whereas PE in localised to the inner leaflet (Devaux, 1991). Both phospholipids serve as reservoirs for FA, and their content in membranes can be affected by dietary fat intake (Khuu Thi-Dinh et al., 1990).
With the induction of apoptosis, in the Caco-2 cells, membranes PC content was increased as a result of the SB, SF and SBF emulsions dose response. This increase likely reflects the incorporation of the emulsifier L-alpha-phosphatidylcholine, which was used to enhance FA incorporation into membranes and is not indicative of cell requirement for phospholipids to produce more cell (Jackowski, 1996). Cells usually maintain a strict level of PC homeostasis to ensure for optimal functioning in terms of its role of maintaining membrane integrity and participating in signal transduction pathways (Dennis et al., 1991; Ehelt al., 2010). Therefore, an abnormal accumulation of PC could compromise the integrity of the membrane and alter signalling responses. In liver cancer cells, it has been demonstrated that enhanced uptake of PC results in an apoptotic response via the death ligands (Fas and/or TNF-alpha) pathway followed by caspase-8 and -3 inductions (Sakakima et al., 2009). A decrease in PE content was also noted in response to S and SB emulsions. The phospholipids role in apoptosis is linked to its distribution between the inner and outer leaflet of membranes. Externalisation of PE and a loss of membranes asymmetric distribution of aminophospholipids during the early stages of apoptosis appear to have a significant role in the process, although the exact mechanism is not fully understood (Emoto et al., 1997; Wang et al., 2004). Of interest, exogenous PE has been shown to induce apoptosis in a dose dependent manner by reducing the mitochondrial transmembrane potential, and resulting in increased caspase-3 activity (Yao et al., 2009). Evidently, the decrease of the C20:4ω-6 PC/PE ratio adds to the emulsions modulating effect on the Caco-2 cells lipid membrane profile that predisposes it to apoptosis.

With regards to the inhibition cell proliferation, an opposite response relating to an increase in PE content was in effect. The increase in PC was much lower to that required to initiate apoptosis. This again highlights the dependence of a lipid threshold required to elicit a specific cellular response (apoptosis vs. inhibition of proliferation). An accumulation of PC in cells has been shown to inhibit proliferation, presumably through its interaction with PLA₂, which mediates the formation of C20:4ω-6 derived metabolites that participate in the regulation of cell proliferation and apoptosis (Baburina and Jackowski, 1999; Chiu and Jackowski, 2001).
5.4.2.4 Modulation of membrane fluidity

The distribution of FA within the PC and PE lipid fractions were greatly influenced by the oil emulsions variation in concentration and FA content, with each phospholipid displaying preferential incorporation of certain FA classes. These compositional changes greatly affect membranes fluidity, influencing embedded protein receptors and its associated functionality (Wiseman, 1996). Changes in the PC/PE, PUFA/SATS and chol/PL ratios and cholesterol content have been utilized as indicators of membrane fluidity (Abel et al., 2001).

With the induction of apoptosis, an increase in the PC/PE ratio and the PUFA/SATS ratio in PE in response to both borage oil and fish oil occurred. This effect suggests that the Caco-2 cells membrane fluidity is increased. This is due to the lipid fractions increased PUFA content, whose double bond, kinked chemical structure reduces the tight packaging of the lipid components (Eyster, 2007). Membranes increase in fluidity by PUFA may affect specialized lipid micro domains or lipid rafts that participate in signal transduction pathways regulating cell survival (Patra, 2008). It has been demonstrated that increasing lipid rafts ω-3 FA enhances the apoptotic response in human breast cancer cells by deactivating epidermal growth factor receptor associated signalling (Schley et al., 2007). Therefore, dependent on the level of PUFA and type of unsaturated chains that are being incorporated into membranes, the cellular response is swayed towards either apoptosis or inhibition of cell proliferation as determined by the signalling pathways activated.

Cholesterol is an integral and stabilizing component of membranes and lipid rafts. In the Caco-2 cells, the emulsions (S and SB) resulted in a decrease in cholesterol content of the cancer cell membranes to initiate an apoptotic response. Depletion of cholesterol results in the destabilization of membranes and lipid rafts, thus affecting critical signalling responses (Li et al., 2006). Consequently, the relative decrease in cholesterol as compared to the phospholipids (chol/PL ratio) will elicit an increase in the fluidity as it stabilizes the fatty acyl groups in the lipid bilayer (Ohvo-Rekila et al., 2002). A decrease in lipid rafts cholesterol content by cholesterol depleting agents has been shown to activate the apoptotic process by increasing caspase-3 activity and also resulting in the deactivation of Akt/protein kinase B, which phosphorylates pro-apoptotic proteins and decreases their activities (Li et al., 2006). To sustain its
proliferative nature, cancer cells tend to maintain a high content of cholesterol (Sun et al., 2014) Therefore, a decrease in cholesterol resulted in the inhibition of cell proliferation, but at a different content threshold in the Caco-2 cell membranes compared to the induction of apoptosis.

5.5 CONCLUSIONS

The oil emulsions elicited variable responses in the two colon cancer cell lines, demonstrating their differential sensitivity to the FA supplied, with the HT-29 cells displaying resistance to apoptosis. Meanwhile, the Caco-2 cancer cells showed a greater responsiveness to the oil emulsions. Evidently, the concentration and level of FA incorporated into the cancer cell membranes determines the cellular response: apoptosis versus cell proliferation inhibition. The induction of apoptosis by both fish oil and borage oil in the cell line is predetermined by a decreased content in membranes ω-6/ω-3 FA ratio and preferential incorporation of LC ω-3 FA (C20:5ω-3 and C22:6ω-3) in PE. This modulatory effect likely resulted in an anti-cancer response through enhanced oxidative stress reactions such as LPO. Alterations in the lipid and FA composition influences membrane fluidity, which has a great effect on the execution of signalling cascade regulating cell survival. In addition, the membranes altered PUFA composition may determine the production of specific eicosanoid effectors. Overall, this study shows that the supply of variable dietary FA content, with a defined ω-6/ω-3 ratio can modulate specific cancer cells development, particularly those provided by fish oil and borage oil, thus providing selective strategies for chemoprevention.

5.6 REFERENCES


CHAPTER 6
GENERAL DISCUSSION AND CONCLUSIONS
UNIVERSITY of the WESTERN CAPE
6.1 GENERAL DISCUSSION

Over the last few decades numerous lifestyle factors, including diet, have been identified as being major contributors in the development of numerous non-communicable diseases that includes colon cancer (Huxley et al., 2009). Regarding diet, food intake patterns characterised by high saturated and ω-6 fatty acids (FA) and low fibre are considered as risk factors associated with the development of colon cancer (Giovannucci and Goldin, 1997; Slattery et al., 1998). In contrast, dietary ω-3 FA are associated with several beneficial health effects, including cancer prevention (Larsson et al., 2004; Rose and Connolly, 1999).

FA, which include the saturated, mono- and polyunsaturated classes, are important macromolecules required by cells, but especially the ω-6 and ω-3 polyunsaturated FA (PUFA) play an essential role in normal cellular function (Ibarguren et al., 2014). With its supply being diet dependent, however, research suggests that a balanced intake of these two PUFA families is the critical factor determining the physiological and biochemical processes within cells. It has been demonstrated that an imbalanced intake, i.e. high ω-6/ω-3 FA ratio, may ultimately influence the growth kinetics of cancer cells allowing them to persist (Simopoulos, 2008). Some of these mechanisms include the FA modulating effects on signal transduction pathways that results in an uninhibited proliferative activity, affecting redox homeostasis, as well as enhancing their resistance to apoptosis that favours their survival (Bartsch et al., 1999). Consequently, as a preventive measure against malignancy, the selective management of the amount and type of dietary FA intake is advocated to modulate and inhibit the progress of colon cancer development (Reddy, 1994; Shike, 1999).

6.1.1 Studies utilising an animal colon cancer model

A reduction in hepatocyte nodules was observed in laboratory animals consuming dietary fats constituting a low ω-6/ω-3 FA (12:1) ratio (Abel et al., 2004). Furthermore, this dietary pattern increased membrane incorporation of ω-3 FA, altering the animals’ tissue oxidative status by enhancing the level of lipid peroxidation (LPO), which favoured the reduction in the pre-neoplastic liver lesions. Similar dietary FA ratio dependent anti-cancer effects have also been noted in other cancer studies (Kobayashi et al., 2006; Sarotra et al., 2010).
In the current study, the effect of different dietary fats with varying $\omega$-6/$\omega$-3 FA ratios and reference dietary oils (canola and olive oils) on carcinogen-induced rat colon aberrant crypt foci (ACF) development during cancer promotion was monitored. The modulation of proliferative and apoptotic indices were assessed in the colon crypt epithelium. In addition, alterations in the cells redox homeostasis was elucidated in the colon by monitoring the expression of oxidative stress and antioxidant defence genes. Specific combinations of fats normally associated with a human diet (MacKenzie, 2007; Meyer et al., 2003) were included and consisted of sunflower (S), borage (B) and fish (F) oils, each with a distinct FA profile. These fats were combined to generate different $\omega$-6/$\omega$-3 FA ratios designated as SB, SF and SBF for dietary treatment regimens. Canola and olive oils were used as comparative reference oils. No toxic effects on the liver and kidney were noticed when utilising serum biochemical chemistry analyses, which is a key characteristic for any chemopreventive approach (Kelloff et al., 1995).

The development of ACF, the earliest biomarkers for cancer in the colon tissue appears to be enhanced by the fish oil containing diet, SF (Table 6.1). Furthermore, ACF multiplicity was differentially altered in response to the fat diets with the SF and the S oils, favouring an increased development of larger lesions containing 7 or more crypts/focus, that are known to progress into a more aggressive cancer phenotype (Raju, 2008; Srivastava et al., 2001). Relative to the other combined fats (SB and SBF) and the two reference oils, canola and olive, the SF and S diets seem to enhance both cell proliferation and apoptosis in the colon crypt epithelium. The latter result is of interest as the development of ACF and crypt multiplicity is determined by a critical balance between cell proliferation and apoptosis. Evidently, in early precursor lesions the balance is disrupted and cell proliferation seems to dominate resulting in their outgrowth (Roncucci et al., 2000). This occurred despite the increased dietary supply of C20:5$\omega$-3 and C22:6$\omega$-3, and subsequent elevation in cellular oxidative stress effected by long chain (LC) $\omega$-3 FA known to enhance apoptosis in the colon (Hong et al., 2005).

A previous study in rat colon mucosa utilizing the same oil mixtures (S, SB, SF and SBF) illustrated that the fish oil containing diets significantly altered membranes content of LC $\omega$-3 PUFA due to an increase in C20:5$\omega$-3 and C22:6$\omega$-3 (Abrahams,
2009, Addendum 4). This effect enhanced the membranes susceptibility to LPO, which increased the level of oxidative stress in the colon mucosa. Dependent on the level of oxidative stress, signalling pathways promoting cell division or apoptosis can be activated (Schumacker, 2006), which essentially determines the survival of the pre-neoplastic lesions. Consequently, an altered apoptotic response relative to the cell proliferative response contributes significantly to cancer cells growth and survival (Hanahan and Weinberg, 2011), as noted with the SF diet under the current experimental conditions. This effect is contrary to numerous studies demonstrating the anti-cancer effects of fish oil (Rao et al., 2001; Reddy and Sugie, 1988; Reddy et al., 1991), and may be related to the stage of intervention and the resistance of pre-neoplastic lesions to oxidative stress resulting in the differential stimulation of their growth.

Table 6.1: Effects of the dietary oil ratios on ACF development and cell survival indices.

<table>
<thead>
<tr>
<th>Tissue response</th>
<th>Dietary oils</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SB</td>
</tr>
<tr>
<td>Total ACF</td>
<td></td>
</tr>
<tr>
<td>Multiplicity (≥7 crypts/focus)</td>
<td></td>
</tr>
<tr>
<td>Proliferation</td>
<td></td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
</tr>
</tbody>
</table>

ACF = aberrant crypt foci. Tissue responses: no effect = –; increased =↑, decreased =↓.

Cancer cells display escalated rates in generating ROS that aids in sustaining its proliferative nature (Grek and Tew, 2010). This is usually counterbalanced by an increase in cells antioxidant activities, which maintain ROS accumulation to a level that bypasses oxidative stress induced apoptosis (Valko et al., 2007). In the colon, the expression of Nudt1 and Gpx4 (see Chapter 4) was up-regulated due to the increased level of oxidative stress induced by the SF diet. The enhancement of ACF development and increased crypt multiplicity of larger lesions through the up-regulated expression of Nudt1 may be associated with its subsequent translation and
supply of protein that eliminates the accumulation of oxidised DNA bases such as 8-oxo-dGTP, thus protecting cells from oxidative stress induced apoptosis (Garre et al., 2011; Nakabeppu, 2014). As a result, the repair of damaged DNA is enhanced, favouring the pre-neoplastic cells growth and survival. Up-regulation in the cells antioxidant capacity with Gpx4 (Table 6.2) likely reduced the SF diet induced oxidative stress, thus minimizing the apoptotic response in relation to cell proliferation and hereby sustaining the development of the higher level of crypt multiplicity. Thus, in this case the SF diet appears to have a protective effect on existing pre-neoplastic lesions in the colon (Landriscina et al., 2009).

In contrast to the SF diet, crypt multiplicity was significantly reduced by the borage oil containing diets (SB and SBF) (Table 6.1). The latter diets decreased both the proliferative and apoptotic activity in the animals’ colon mucosa. It would appear that these cell growth indices followed a similar pattern and that induction of apoptosis seems to be dominant in the large ACF, hence the decrease in size. Down-regulation of Errc2 expression by the SB oil diet was noted earlier in this study (Chapter 4). This effect results in the reduction of DNA replication due to inhibiting the activity of RNA polymerase, which is a critical initiating factor for DNA transcription (Schaeffer et al., 1994). Hereby, the proliferative response in the transformed colonic cells is reduced.

The SB diet’s up-regulation of Gpx4 (Table 6.2) protects the aberrant crypts against the eliminating effects of hydrogen peroxide (H$_2$O$_2$) (Imai and Nakagawa, 2003). A similar response in enhanced antioxidant capacity was noted with the canola and olive oil diets, with the latter displaying the greater effect. Increased levels of H$_2$O$_2$ have been linked to promoting cancer development by enhancing signal transductions mediating a proliferative response and resistance to apoptosis (del Bello et al., 1999; Burdon, 1995; Polytarchou et al., 2005). The combination of borage and fish oil with diet SBF also resulted in a decreased apoptotic response, mainly due to the increased DNA repair effect exerted by the up-regulation in the expression of Xpa (Pascucci et al., 2011). This occurred despite an increase in LC FA, C20:4ω-6, C20:5ω-3 and C22:6ω-3 that are potent inducers of LPO and ultimately apoptosis (Brash, 2001; Das, 1999). Also, animals increased intake of total polyphenols (TTP: olive>SBF>SB>SF>canola) may be an additional factor
contributing to the diet’s reduction in controlled cell death normally initiated by an increase in cellular oxidative stress. Dietary polyphenols are noted for their antioxidant capacity and subsequent reduction in free radical induced damage in cells (Fresco et al., 2006).

Table 6.2: Dietary oil ratios protective effects against increased oxidative stress during the promotion.

<table>
<thead>
<tr>
<th>Cellular response</th>
<th>SB</th>
<th>SF</th>
<th>SBF</th>
<th>Canola</th>
<th>Olive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased protection against oxidative stress (i.e. antioxidant capacity)</td>
<td>+Gpx4</td>
<td>+Gpx4</td>
<td>+Gpx4</td>
<td>+Gpx4</td>
<td>+Gpx4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+Srxn1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+Gsr</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+Ngb</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+Srxn1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+Idh1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+Ppp1r15b</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+Prnp</td>
<td></td>
</tr>
</tbody>
</table>

Genes expression: up-regulated (+)

The canola and olive oil diets provided the highest protection against the continued survival of the pre-neoplastic lesions in the colon mucosa as noted with the decline in crypt multiplicity (Table 6.1). This effect resulted from the oils decreasing modulation of both proliferation and apoptosis in the aberrant crypts similar to the SB and SBF diets that favoured a reduction in the larger lesions, thus inhibiting colon cancer development. The reference oils up-regulated the expression of several genes that functioned in enhancing the colon crypt cells protection against oxidative stress induced apoptosis (Table 6.2). Both oils mutually up-regulated the expression of Gpx4 and Srxn1, which lead to the reduction of cells H₂O₂ levels (Li et al., 2013). Oxidative stress induced apoptosis was further reduced by canola oil’s up-regulation of Ngb, who’s translated protein acts as a scavenger of ROS (Ascenzi et al., 2004). Similarly, olive oil reduced cells influx of ROS through the up-regulated antioxidant activity of Gsr, and increased the expressions of Idh1, Ppp1r15b and Prnp that mediate cellular responses that increases cells resistance against oxidative stress (Harvey et al., 2009; Reitman and Yan, 2010; Sauer et al., 2003). Hereby, modulating cells oxidative status can affect the induction of apoptotic mediators regulated by cells increased level of ROS (Chandra et al., 2000). The reduction of
cell proliferation and apoptosis by the SB, SBF and canola and olive oil, therefore could be related to the expression of these genes. The disruption of these growth indices in the colon crypts could then selectively reduce the outgrowth of the aberrant crypt lesions.

Increased intake of C18:1ω-9, a FA recognized for its antioxidant activity (Diplock et al., 1988; Schuhr, 2008), was also noted with the canola and olive oils diets (Chapter 3). This provided additional protection against ROS accumulation that has a modulating effect on the apoptotic and proliferative responses in the pre-neoplastic colonic cells. Relating to cells proliferative response, the expression of Ehd2 and Apoe (Table 6.3) was enhanced by both the canola and olive oil diets. Both these genes encodes for proteins that are potent inhibitors of tumour cell proliferation (Niemi et al., 2002; Shi et al., 2015). Although the oils resulted in a decreased apoptotic response, their mutual up-regulation of pro-apoptotic genes (Table 6.3) Cygb and LOC36198 (Helt et al., 2005; Shivapurkar et al., 2008) was indicative of the diets modulation of crypts cells level of oxidative stress that favoured the reduction of crypt multiplicity. Canola oil enhanced the expression of Mpo that also contributes towards oxidative stress mediated apoptosis (Nakazato et al., 2007), while olive oil up-regulated Apc, Ctbs and Dnm2 (Table 6.3). Up-regulated expression of Apc impacts on cell turnover by inhibiting COX-2 expression and functions as a tumour suppressor. (Fodde, 2002; Lew et al., 2002). Protein translated from the up-regulated expression of Dnm2 can function as an upstream regulator of p53-dependent apoptosis in dividing cells (Fish et al., 2000; Johnson et al., 1996). Similarly, up-regulated expression of Ctbs by the olive oil diet facilitates crypt cell regulation by activating initiator caspases, thus increasing apoptosis (Vancompernolle et al., 1998; Wang et al., 2008). Furthermore, the marginal apoptotic response in effect appears to be enhanced by canola and olive oil’s up-regulation of Tpo, Duox1 and Ncf2, whose protein mediate an increased oxidative stress response (Grandvaux et al., 2007; Harvey et al., 2009; Ruf and Carayon, 2006), thus effecting the crypts survival parameters. As a result of its high C18:3ω-3 content, canola oil appears to exert an anti-inflammatory effect by suppressing inflammatory mediators that impact on proliferation and apoptosis (Larsson et al., 2004). This was also noted with the diet’s up-regulation in the expression of immune response regulator, gene Rag2, who’s translated protein function as an anti-
inflammatory mediator (Erdman et al., 2009). In this study the anti-cancer properties of canola and olive oils appears to be exerted primarily through a polypenolic effect, which protected cells against oxidative stress. A similar change in the expression of genes regulation of the oxidative status in the liver was noted in response to tea polyphenols enhancing the cellular antioxidant defences (van der Merwe, 2012). In the aberrant crypts, the cell proliferation and apoptosis balance is likely to shift in favour of the latter response with the noticeable reduction in crypt multiplicity suggesting that a certain level of oxidative stress (threshold) was reached to suppress ACF development.

Table 6.3: The anti-cancer effects of canola and olive oil relating to its modification of oxidative stress and antioxidant defence genes expression in ACF.

<table>
<thead>
<tr>
<th>Cellular response</th>
<th>Canola</th>
<th>Olive</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Redox responsive genes expression effects</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cancer inhibition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-proliferative</td>
<td>+Ehd2</td>
<td>+Ehd2</td>
</tr>
<tr>
<td></td>
<td>+Apo</td>
<td>+Apo</td>
</tr>
<tr>
<td></td>
<td>+Mpo</td>
<td>+Apc</td>
</tr>
<tr>
<td></td>
<td>+Cygb</td>
<td>+Ctbs</td>
</tr>
<tr>
<td></td>
<td>+LOC367198</td>
<td>+Cygb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+Dnm2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+LOC367198</td>
</tr>
<tr>
<td>Pro-apoptotic</td>
<td>+Tpo</td>
<td>+Tpo</td>
</tr>
<tr>
<td></td>
<td>+Duox1</td>
<td>+Duox1</td>
</tr>
<tr>
<td></td>
<td>+Ncf2</td>
<td>+Ncf2</td>
</tr>
<tr>
<td><strong>Increases oxidative stress</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Anti-inflammatory</strong></td>
<td>+Rag2</td>
<td></td>
</tr>
</tbody>
</table>

Genes expression: up-regulated (+) or down regulated (-)

Regulation of cell proliferative indices by ω-6 and ω-3 fatty acids
An increased intake of C18:3ω-6 provided by borage oil’s intake with both the SB and SBF diets increases the incorporation of C20:3ω-6 as well as C20:5ω-3 in the mucosal membrane phospholipids (Abrahams, 2009 – Addendum 4) known for its anti-proliferative and anti-inflammatory effects via the effect of the PGE₁ type of prostaglandins (Kapoor and Huang, 2006; Wang et al., 2012). Interesting
interactions seems to exist regarding the role of the SF and S diets, which stimulate ACF multiplicity, and the reduction effect induced by the SBF. It would appear that thresholds of specific membranal FA seem to exist when considering the levels of C20:3ω-6, C20:4ω-6 and C20:5ω-3 as detailed in Chapter 3. The respective roles of their prostanoid metabolites in the regulation of cell growth indices appear to be critical in the differential stimulation and/or inhibition of the development of ACF in the colon. Hence various combinations of oils, as indicated by the chemopreventive effect of the SBF oil diet, provide interesting futuristic research opportunities in the regulation of colon cancer development in humans.

6.1.2 Investigations utilising human colon cancer cells

Colon cancer is a complex disease, and animal carcinogenesis models (in vivo) provides invaluable information regarding the multistage nature of the disease and the effects potential chemopreventive agents have on integrated cellular systems (Dommels et al., 2002). Cell culture or in vitro studies, however, are isolated cellular systems that serve as a useful tool in evaluating the mechanistic pathways mediating cancer development and the different ways natural or synthetic agents/compounds can interfere with the process under controlled conditions (Grajek and Olejnik, 2004). In the current study, utilizing oil ratio emulsions of defined ω-6/ω-3 FA ratio resulted in differential effects in two human adenoma colon cancer cell lines (HT-29 and Caco-2) with respect to their survival indices.

Modulation of colon cancer cells survival indices

The HT-29 cells displayed greater sensitivity to the effects of the fish oil containing emulsions (SF and SBF) that resulted in its reduction of cell viability and cell proliferation. It was suggested that the oil’s supply of polyunsaturates, C20:5ω-3 and C22:6ω-3 increases the level of oxidative stress due to its susceptibility to LPO, which in turn affects cell growth regulatory signals (Catalá, 2009). This effect at higher oil emulsion concentrations, however, was insufficient to elicit an apoptotic response despite a decrease in the energy status (ATP production), which is a critical determinant of this cellular process. Consequently, the HT-29 cells undergo necrotic cell death, which occurs when the level of ATP fall beyond a certain
threshold inside the cell (Eguchi et al., 1997), indicating the pivotal role mitochondrial metabolism plays in cell death processes related to necrosis and apoptosis.

Apoptotic responses in cancer cells are usually blocked due to mitochondrial dysfunction and its reduced sensitivity to oxidative stress (Pelicano et al., 2004). Thus, cancer cells tend to favour the glycolytic pathway (Warburg effect) to maintain its energy status and are not dependent on mitochondrial production of ATP (DeBerardinis et al., 2008). The latter seems to apply to the HT-29 cells, which displayed resistance to apoptosis, and exhibited a reduction in cell viability through the inhibition of ATP production by the oil emulsions. As a result, the cancer cells were depleted via necrotic cell death. The HT-29 cells resistance to apoptosis is likely associated with an impaired glycolytic pathway (Graz and Cowley, 1997), which would further exhaust the ATP yield to levels that are insufficient to stimulate the process. Conversely, at high oil emulsion concentration, cell cytotoxicity in the Caco-2 cells was not overtly triggered despite the fact that cell viability is adversely affected. Apart from the inhibitory effect exerted on cell viability and cell proliferation, the fish oil (SF) and borage oil (SBF) emulsions also induced an apoptotic response dependent on the cells ATP production at higher concentrations presumably involving mitochondrial production. Borage oil (SB) tended to only increase the apoptotic response at the highest concentrations. The differences in the emulsion induced responses are likely indicative of the cells shift in energy production. Therefore, in the Caco-2 cells, as mitochondrial ATP is depleted the glycolytic pathway supplies sufficient ATP for apoptosis to be activated (Tsujimoto, 1997).

The anti-cancer effect (i.e. induction of apoptosis or inhibition of cell proliferation) of the oil emulsions was associated with modification of the FA and lipid composition of the Caco-2 cell membranes that are summarised in Fig. 6.1 and Fig. 6.2. As result of these alterations, specific cell signals were activated dependent on the FA and lipid content of the cellular membranes. Cells undergoing apoptosis displayed a significant decrease in membranes content of SATS and MUFA, which studies show are increased in cancer cells and provides protection against oxidative stress (Rysman et al., 2010). Although PUFA content tended to decrease, the reduced membrane ω-6/ω-3 FA ratio enhanced its LC ω-3 FA (C20:5ω-3 and C22:6ω-3) composition, that predisposes membranes to LPO and subsequent removal via
apoptosis (Falconer et al., 1994; Hawkins et al., 1999; Udilova et al., 2003). Borage oil’s (SB) increase of C20:4ω-6 in PE also appears to facilitate this process (Hofmanová et al., 2012; Seegers et al., 1997). Cancer cells increase in C20:4ω-6 is well known to have a stimulatory effect on proliferation due to the activity of prostanoid, PGE$_2$ (Castellone et al., 2005). However, excessive accumulation of the FA can result in an increase of ceramides that are potent stimulators of apoptosis (Cao et al., 2000; Surette et al., 1999). Alternate mechanisms of cancer cell removal likely involved the participation of eicosanoid signalling. The metabolism of membranes increased content of C20:3ω-6 and C20:5ω-3 (SB and SBF) results in an anti-inflammatory response that indirectly triggers apoptosis (Gillis et al., 2002; Kapoor and Huang, 2006; Roynette et al., 2004). In addition, changes in the Caco-2 cells lipid parameters also had a role in activating this process (Fig. 6.2).

**Figure 6.1:** Summary of the Caco-2 cell membranes altered FA content required to elicit apoptosis or inhibit cell proliferation in response to oil emulsion exposure.

In apoptotic cells, the respective increase and decrease of PC and PE reflected the disruption of membranes homeostatic balance of phospholipids content in response to the oil emulsions. PC increased mainly due to its increased incorporation via the synthetic PC molecule (L-alpha phosphatidylcholine), which enhanced the uptake
of the oil emulsions FA supply. Excessive PC incorporation can affect the integrity of the membrane structure and modify associated functions. This increased effect has been shown to enhance the apoptotic response via the death ligands (Fas and/or TNF-alpha) pathway followed by caspase-8 and -3 inductions in the liver (Sakakima et al., 2009). A decrease in PE (S and SB) was effected by the emulsions in the apoptotic response as well. Externalisation of PE and a loss of membranes asymmetric distribution of aminophospholipids during the early stages of apoptosis appear to have a significant role in the process, although the exact mechanism is not fully understood (Emoto et al., 1997; Wang et al., 2004).

**Figure 6.2:** Summary of the Caco-2 cell membranes altered lipid content required to elicit apoptosis or inhibit cell proliferation in response to oil emulsion exposure.

Furthermore, modulation of the lipid parameters affecting membrane fluidity also affected the survival of the Caco-2 cells. A decrease in cholesterol and subsequent increase in the cholesterol/phospholipids (chol/PL) ratio increased membranes fluidity affecting the function of membrane associated proteins and specialised microdomains (lipid rafts) (Baritaki et al., 2007). Lipid rafts, characterized by its high cholesterol content that provides greater structural stability than the surrounding membrane, are important co-ordinators of various signalling pathways, and is often dysregulated in cancer cells (Patra, 2008; Wassall and Stillwell, 2009). Higher incorporation of LC ω-3 FA such as C20:5ω-3 and C20:6ω-3 into PE in response to
the borage oil containing emulsions (SB and SBF), increased membranes fluidity due to the FA structural configuration. This change could result in acylated proteins being displaced from lipid rafts (Stulnig et al., 2001) and reduce its cholesterol content, as noted with the Caco-2 cells. Consequently, lipid rafts modified biophysical properties may result in apoptosis being induced.

To inhibit the proliferative activity of the Caco-2 cells, significant alterations of membranes FA and lipid composition was applied by the oil emulsions at much lower concentrations (Fig. 6.1 and 6.2). These changes were opposite of that required for the induction of apoptosis. Evidently, specific anti-cancer responses are dependent on a certain threshold being reached pertaining to FA incorporation into membrane compartments (Abel et al., 1997). A decline in generating daughter was cells were characterized by an increase in SATS and MUFA, and a decrease in PUFA. This effect reduced cancer membranes susceptibility to oxidative stress as a result of a decrease in membranes LC ω-3 FA content. Consequently, membranes C20:3ω-6 content increased with all the emulsions, promoting an anti-proliferative effect (Fan and Chapkin, 1998). Furthermore, the lipid components PC, PE and cholesterol were all increased by the oil emulsions. Hereby, the physical structure of the Caco-2 cell membranes were also altered, thus affecting membrane associated signal transductions regulating proliferative responses (Eling and Glasgow, 1994)

6.1.3 Animal cancer model versus cell culture studies

Regarding the effect of the oil ratio combinations (SB, SF and SBF), different responses was observed with respect to the rat colon carcinogenesis model and the human colon cancer cell lines. Borage oil (SB and SBF) appears to have protective effects against cancer development in both models. This effect was probably due to modulating proliferative signals that resulted in its inhibition, presumably through specific interactions and the anti-proliferative effects of C20:3ω-6, C20:4ω-6 and C20:5ω-5 as previously discussed.

The cell culture study demonstrated that the oil ratio combinations constituting different ω-6/ω-3 FA ratios significantly modified the cancer cells FA and lipid composition, which determined the anticancer response. Similar modulatory effects
have been observed in animals (Abel et al., 2004). A previous study in rat colon mucosa showed that borage oil (SB and SBF diet) enhanced membranes incorporation of C20:5ω-3, C20:3ω-6 and C20:4ω-6 (Abrahams, 2009), which are the precursory FA required for the biosynthesis of the 3-, 1- and 2- series eicosanoids, respectively having opposing effects regarding cell proliferation and inflammatory responses (Levin et al., 2002). Elevated C20:4ω-6 content appears to enhance the survival of cancer cells by increasing its proliferative response through prostanoid PGE2 stimulation (Whelan and McEntee, 2004). However, this effect can be counteracted by the SBF oil due to the resultant increase in membrane C20:5ω-3 and C20:3ω-6, which increases the availability of the anti-inflammatory eicosanoids (3- and 1- series prostanoid) (Barham et al., 2000). This effect reduces the potential for persistent cell proliferation promoted by C20:4ω-6 derived metabolites.

Fish oil (SF diet) and sunflower oil (S diet) enhanced cell proliferative indices as well as the development of ACF and its multiplicity of larger lesions when compared to the effects of the borage oil and fish oil combination diets (SB and SBF). The colon crypts homeostatic balance between apoptosis and cell proliferation shifted in favour of the latter with the S and SF diets, thereby resulting in the formation of the larger lesions (Yamashita et al., 1994). The increase and persistence of these aberrant crypts of high multiplicity appears to favour disease progression. In addition, it was indicated that an enhanced antioxidant response (up-regulated Gpx4) protected the aberrant crypt cells from oxidative stress induced removal.

In contrast, both colon cancer lines (HT-29 and Caco-2) were responsive to the effects of fish oil. The HT-29 cells favoured a reduction in cell proliferation, whilst fish oil induced apoptosis and inhibited cell proliferation in the Caco-2 cells. The apoptotic response exerted by fish oil was associated with its increasing effect on cancer cell membranes C20:5ω-3 and C22:6ω-3 content that predisposes cells to LPO and subsequent removal via apoptosis. Changes in the PUFA content in cellular membranes also resulted from an increase in PC and a reduction in PE. These changes in membrane phospholipid as well as the cholesterol content affect membrane fluidity parameters likely to further alter signal transduction pathways related to cell survival. A threshold level pertaining to the incorporation of FA and
these lipid parameters into the cancer cell membranes determines whether cells will be sensitised or exhibit resistance towards the induction of apoptosis.

6.2 Conclusions

The dietary FA ratios employed in this investigation displayed differential effects on the development of cancer in both the animal cancer and cell culture models. However, altering the $\omega$-$6/\omega$-$3$ FA ratio intake and cancer cell membranes FA and lipid profile demonstrated a favourable reducing effect on the survival indices of colon cancer pre-neoplastic lesions \textit{in vivo} and cancer cells \textit{in vitro}. Borage oil combinations (SB and SBF) consistently displayed anti-cancer effects by inhibiting cell proliferation through the modulation of cell growth indices, presumably due to specific ratios of $\omega$-6 and $\omega$-3 FA. Fish oil had opposing effects on the animal and \textit{in vitro} models, demonstrating threshold effects while specific combinations with borage oil exhibited a protective effect against the development of pre-neoplastic lesion (SBF diet). A reduction in cells level of oxidative stress effected by the SB and SBF oil diets demonstrated an enhanced antioxidant response through the up-regulation of $Gpx4$ in the colon, which facilitated the decrease in apoptosis by both diets. However, the fish oil only diet (SF) appears to impart a selective resistance against oxidative stress, thus favouring the increase in proliferation of pre-neoplastic lesions during the promotion stage of colon carcinogenesis. In contrast, the HT-29 and Caco-2 colon cancer cells showed differential anti-cancer effects in response to fish and borage oil dependent on the level of $\omega$-3 PUFA incorporated into membranes. Consequently, alteration in membrane FA and other lipid constituents, such as phospholipids and cholesterol, impacted on the survival indices. Overall, modulating colon cancer development with dietary fats constituting defined $\omega$-$6/\omega$-$3$ FA ratio is extremely complex but shows promise as a chemopreventive tool under specific defined conditions.

6.3 References


lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation. Cancer Res. 70, 8117–8126.


Van der Merwe, J.D. (2012). Exposure to polyphenol-enriched rooibos (Aspalathus linearis) and honeybush (cyclopia spp.) extracts: Implications of metabolism for the oxidative status in rat liver. (PhD thesis). Stellenbosch University, South Africa.


ADDENDUM 1 – CHAPTER 3

Ethical approval for all animal research done at the former PROMEC Unit, MRC.

Dr Stéfan Abel,
Promec Unit,
MEDICAL RESEARCH COUNCIL

Dear Dr Abel,

YOUR APPLICATION TO THE ECRA COMMITTEE : REF: 02/09 “Modulating of colon aberrant crypt foci development by diets with varying PUFA and n-6/n-3 fatty acid ratios”.

Thank you for your submission to the ECRA.

The Committee reviewed your application and found it admissible and it was approved. You may start with your experiment now. Please keep in mind that you have to submit every six months an interim report to the Committee. We will remind you regularly of the reports.

Only two comments on it:

1. Your Project Title should include at the end the words “in a rat model”.
2. Section 17 – “Administration of Schedules”, the RESPONSIBLE PERSON, should be the Veterinarian, Dr Parsons.

Please correct these two pages and either e-mail or fax it to Mrs Fourie.

The Committee hereby wish to congratulate and thank you for such a high standard and good completed application. It makes their work much easier.

Kind regards.

PROF D DU TOIT
Chairperson : ECRA
ADDENDUM 2 – CHAPTER 4

2A.

Table 4.1 Total polyphenol content of the dietary oils.

<table>
<thead>
<tr>
<th>Dietary oil</th>
<th>TTP (mg gallic acid/kg oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>11.37±1.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SB</td>
<td>12.65±1.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF</td>
<td>13.20±1.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SBF</td>
<td>13.64±2.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Canola</td>
<td>6.37±0.63&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Olive</td>
<td>19.20±2.46&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are the mean±standard deviation of the total polyphenol (TTP) content expressed in mg gallic acid/kg oil. Dietary oils: sunflower oil = S, sunflower + borage oil = SB, sunflower + fish oil (SF), sunflower + borage + fish oil = SBF, Canola = canola oil and Olive = olive oil. Values in column followed by different letters (superscript) are significantly different at p<0.05.
<table>
<thead>
<tr>
<th>Aass</th>
<th>Als2</th>
<th>Apoe</th>
<th>Aqr</th>
<th>Cat</th>
<th>Ccs</th>
<th>Xirp1</th>
<th>Ctsb</th>
<th>Duox2</th>
<th>Cygb</th>
<th>Dhcr24</th>
</tr>
</thead>
<tbody>
<tr>
<td>A01</td>
<td>A02</td>
<td>A03</td>
<td>A04</td>
<td>A05</td>
<td>A06</td>
<td>A07</td>
<td>A08</td>
<td>A09</td>
<td>A10</td>
<td>A11</td>
</tr>
<tr>
<td>Dnm2</td>
<td>Duox1</td>
<td>Ehd2</td>
<td>Epx</td>
<td>Errc2</td>
<td>Ercc6</td>
<td>Fanco</td>
<td>Fmo2</td>
<td>Gab1</td>
<td>Gpx1</td>
<td>Gpx2</td>
</tr>
<tr>
<td>B01</td>
<td>B02</td>
<td>B03</td>
<td>B04</td>
<td>B05</td>
<td>B06</td>
<td>B07</td>
<td>B08</td>
<td>B09</td>
<td>B10</td>
<td>B11</td>
</tr>
<tr>
<td>Gpx4</td>
<td>Gpx5</td>
<td>Gpx6</td>
<td>Gpx7</td>
<td>Gsr</td>
<td>Gstk1</td>
<td>Hba-a2</td>
<td>Hbz</td>
<td>Idh1</td>
<td>LOC367</td>
<td>Lpo</td>
</tr>
<tr>
<td>C01</td>
<td>C02</td>
<td>C03</td>
<td>C04</td>
<td>C05</td>
<td>C06</td>
<td>C07</td>
<td>C08</td>
<td>C09</td>
<td>C10</td>
<td>C11</td>
</tr>
<tr>
<td>Mpo</td>
<td>Mpp4</td>
<td>Ncf1</td>
<td>Ncf2</td>
<td>Ngb</td>
<td>Nos2</td>
<td>Nox4</td>
<td>Noxa1</td>
<td>Noxo1</td>
<td>Nqo1</td>
<td>Nudt1</td>
</tr>
<tr>
<td>D01</td>
<td>D03</td>
<td>D04</td>
<td>D05</td>
<td>D06</td>
<td>D07</td>
<td>D08</td>
<td>D09</td>
<td>D10</td>
<td>D11</td>
<td>D12</td>
</tr>
<tr>
<td>Nxn</td>
<td>Park7</td>
<td>Ppp1r15b</td>
<td>Prdx1</td>
<td>Prdx2</td>
<td>Prdx3</td>
<td>Prdx4</td>
<td>Prdx5</td>
<td>Prdx6</td>
<td>Prnp</td>
<td>Psmb5</td>
</tr>
<tr>
<td>E01</td>
<td>E02</td>
<td>E03</td>
<td>E04</td>
<td>E05</td>
<td>E06</td>
<td>E07</td>
<td>E08</td>
<td>E09</td>
<td>E10</td>
<td>E12</td>
</tr>
<tr>
<td>Ptgs2</td>
<td>Rag2</td>
<td>Gpx8</td>
<td>Serpinb1b</td>
<td>Klf9</td>
<td>Scd1</td>
<td>Ift172</td>
<td>Slc38a1</td>
<td>Slc38a4</td>
<td>Slc38a5</td>
<td>Slc41a3</td>
</tr>
<tr>
<td>F01</td>
<td>F02</td>
<td>F03</td>
<td>F04</td>
<td>F05</td>
<td>F06</td>
<td>F07</td>
<td>F08</td>
<td>F09</td>
<td>F10</td>
<td>F11</td>
</tr>
<tr>
<td>Sod2</td>
<td>Sod3</td>
<td>Srxn1</td>
<td>Tmod1</td>
<td>Tpo</td>
<td>Txnip</td>
<td>Txnrd1</td>
<td>Tnrd2</td>
<td>Ucp3</td>
<td>Vim</td>
<td>Xpa</td>
</tr>
<tr>
<td>G01</td>
<td>G02</td>
<td>G03</td>
<td>G04</td>
<td>G05</td>
<td>G06</td>
<td>G07</td>
<td>G08</td>
<td>G09</td>
<td>G10</td>
<td>G11</td>
</tr>
<tr>
<td>Rplp1</td>
<td>Hprt1</td>
<td>Rpl13a</td>
<td>Ldha</td>
<td>Actb</td>
<td>RGDC</td>
<td>RTC</td>
<td>RTC</td>
<td>RTC</td>
<td>PPC</td>
<td>PPC</td>
</tr>
<tr>
<td>H01</td>
<td>H02</td>
<td>H03</td>
<td>H04</td>
<td>H05</td>
<td>H06</td>
<td>H07</td>
<td>H08</td>
<td>H09</td>
<td>H10</td>
<td>H11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PPC</td>
</tr>
</tbody>
</table>

**Figure 4.11:** PCR microarray plate layout.
Table 4.2: Complete list of genes associated with oxidative stress and antioxidant defences in the RT² Profile™ PCR array (PARN-065). Presented as obtained from [www.sabiosciences.com](http://www.sabiosciences.com) on 18-11-2014.

<table>
<thead>
<tr>
<th>Position</th>
<th>Symbol</th>
<th>Description</th>
<th>Gene Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>A01</td>
<td>Aass</td>
<td>Aminoadipate-semialdehyde synthase</td>
<td>—</td>
</tr>
<tr>
<td>A02</td>
<td>Als2</td>
<td>Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)</td>
<td>—</td>
</tr>
<tr>
<td>A03</td>
<td>Apc</td>
<td>Adenomatous polyposis coli</td>
<td>RATAPC</td>
</tr>
<tr>
<td>A04</td>
<td>Apoe</td>
<td>Apolipoprotein E</td>
<td>APOEA</td>
</tr>
<tr>
<td>A05</td>
<td>Aqr</td>
<td>Aquarius homolog (mouse)</td>
<td>—</td>
</tr>
<tr>
<td>A06</td>
<td>Cat</td>
<td>Catalase</td>
<td>CS1, Cas1, Cs-1, RATCAT01, RATCATL</td>
</tr>
<tr>
<td>A07</td>
<td>Ccs</td>
<td>Copper chaperone for superoxide dismutase</td>
<td>—</td>
</tr>
<tr>
<td>A08</td>
<td>Xirp1</td>
<td>Xin actin-binding repeat containing 1</td>
<td>Cmya1</td>
</tr>
<tr>
<td>A09</td>
<td>Ctsb</td>
<td>Cathepsin B</td>
<td>—</td>
</tr>
<tr>
<td>A10</td>
<td>Duox2</td>
<td>Dual oxidase 2</td>
<td>Thox2</td>
</tr>
<tr>
<td>A11</td>
<td>Cygb</td>
<td>Cytoglobin</td>
<td>MGC95105, Staap, Stap</td>
</tr>
<tr>
<td>A12</td>
<td>Dhcr24</td>
<td>24-dehydrocholesterol reductase</td>
<td>—</td>
</tr>
<tr>
<td>B01</td>
<td>Dnm2</td>
<td>Dynamin 2</td>
<td>DYIIAAB</td>
</tr>
<tr>
<td>B02</td>
<td>Duox1</td>
<td>Dual oxidase 1</td>
<td>—</td>
</tr>
<tr>
<td>B03</td>
<td>Ehd2</td>
<td>EH-domain containing 2</td>
<td>MGEPS</td>
</tr>
<tr>
<td>B04</td>
<td>Epx</td>
<td>Eosinophil peroxidase</td>
<td>—</td>
</tr>
<tr>
<td>B05</td>
<td>Ercc2</td>
<td>Excision repair cross-complementing rodent repair deficiency, complementation group 2</td>
<td>—</td>
</tr>
<tr>
<td>B06</td>
<td>Ercc6</td>
<td>Excision repair cross-complementing rodent repair deficiency, complementation group 6</td>
<td>—</td>
</tr>
<tr>
<td>B07</td>
<td>Fancc</td>
<td>Fanconi anemia, complementation group C</td>
<td>Facc</td>
</tr>
<tr>
<td>B08</td>
<td>Fmo2</td>
<td>Flavin containing monoxygenase 2</td>
<td>—</td>
</tr>
<tr>
<td>B09</td>
<td>Gab1</td>
<td>GRB2-associated binding protein 1</td>
<td>—</td>
</tr>
<tr>
<td>B10</td>
<td>Gpx1</td>
<td>Glutathione peroxidase 1</td>
<td>GSHPx, GSHPx-1</td>
</tr>
<tr>
<td>B11</td>
<td>Gpx2</td>
<td>Glutathione peroxidase 2</td>
<td>GPX-GI, GSHPx-2, GSHPx-GI</td>
</tr>
<tr>
<td>B12</td>
<td>Gpx3</td>
<td>Glutathione peroxidase 3</td>
<td>GSHPx-3, GSHPx-P, Gpxp</td>
</tr>
<tr>
<td>C01</td>
<td>Gpx4</td>
<td>Glutathione peroxidase 4</td>
<td>Phgpx, gpx-4, snGpx</td>
</tr>
<tr>
<td>C02</td>
<td>Gpx5</td>
<td>Glutathione peroxidase 5</td>
<td>—</td>
</tr>
<tr>
<td>C03</td>
<td>Gpx6</td>
<td>Glutathione peroxidase 6</td>
<td>OBPII, Ry2d1</td>
</tr>
<tr>
<td>C04</td>
<td>Gpx7</td>
<td>Glutathione peroxidase 7</td>
<td>—</td>
</tr>
<tr>
<td>C05</td>
<td>Gsr</td>
<td>Glutathione reductase</td>
<td>—</td>
</tr>
<tr>
<td>C06</td>
<td>Gsk1</td>
<td>Glutathione S-transferase kappa 1</td>
<td>GST13-13, GSTkappa</td>
</tr>
<tr>
<td>C07</td>
<td>Hba-a2</td>
<td>Hemoglobin alpha, adult chain 2</td>
<td>HBAM, Hba-a1, Hba1</td>
</tr>
<tr>
<td>C08</td>
<td>Hbz</td>
<td>Hemoglobin, zeta</td>
<td>RGD1307486</td>
</tr>
<tr>
<td>C09</td>
<td>Idh1</td>
<td>Isocitrate dehydrogenase 1 (NADP+), soluble</td>
<td>—</td>
</tr>
<tr>
<td>C10</td>
<td>LOC367198</td>
<td>Similar to Serine/threonine-protein kinase ATR (Ataxia telangiectasia and Rad3-related protein)</td>
<td>—</td>
</tr>
<tr>
<td>C11</td>
<td>Lpo</td>
<td>Lactoperoxidase</td>
<td>—</td>
</tr>
<tr>
<td>C12</td>
<td>Mb</td>
<td>Myoglobin</td>
<td>—</td>
</tr>
<tr>
<td>D01</td>
<td>Mpo</td>
<td>Myeloperoxidase</td>
<td>—</td>
</tr>
<tr>
<td>Position</td>
<td>Symbol</td>
<td>Description</td>
<td>Gene Name</td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>D02</td>
<td>Mpp4</td>
<td>Membrane protein, palmitoylated 4 (MAGUK p55 subfamily member 4)</td>
<td>Dlg6</td>
</tr>
<tr>
<td>D03</td>
<td>Ncf1</td>
<td>Neutrophil cytosolic factor 1</td>
<td>Ncf-1, p47phox</td>
</tr>
<tr>
<td>D04</td>
<td>Ncf2</td>
<td>Neutrophil cytosolic factor 2</td>
<td></td>
</tr>
<tr>
<td>D05</td>
<td>Ngb</td>
<td>Neuroglobin</td>
<td></td>
</tr>
<tr>
<td>D06</td>
<td>Nos2</td>
<td>Nitric oxide synthase 2, inducible</td>
<td>Nos2a, iNos</td>
</tr>
<tr>
<td>D07</td>
<td>Nox4</td>
<td>NADPH oxidase 4</td>
<td></td>
</tr>
<tr>
<td>D08</td>
<td>Noxa1</td>
<td>NADPH oxidase activator 1</td>
<td></td>
</tr>
<tr>
<td>D09</td>
<td>Noxo1</td>
<td>NADPH oxidase organizer 1</td>
<td></td>
</tr>
<tr>
<td>D10</td>
<td>Nqo1</td>
<td>NAD(P)H dehydrogenase, quinone 1</td>
<td>Dia4, MGC93075</td>
</tr>
<tr>
<td>D11</td>
<td>Nud1</td>
<td>Nudix (nucleoside diphosphate linked moiety X)-type motif 1</td>
<td>Mth1</td>
</tr>
<tr>
<td>D12</td>
<td>Nud15</td>
<td>Nudix (nucleoside diphosphate linked moiety X)-type motif 15</td>
<td></td>
</tr>
<tr>
<td>D13</td>
<td>Nnn</td>
<td>Nucleoredoxin</td>
<td></td>
</tr>
<tr>
<td>E01</td>
<td>Park7</td>
<td>Parkinson disease ( autosomal recessive, early onset ) 7</td>
<td>CAP1, DJ-1, Dj1, SP22</td>
</tr>
<tr>
<td>E02</td>
<td>Ppr15b</td>
<td>Protein phosphatase 1, regulatory (inhibitor) subunit 15b</td>
<td></td>
</tr>
<tr>
<td>E03</td>
<td>Prdx1</td>
<td>Peroxiredoxin 1</td>
<td>Hbp23, MGC108617</td>
</tr>
<tr>
<td>E04</td>
<td>Prdx2</td>
<td>Peroxiredoxin 2</td>
<td>Tpdx1</td>
</tr>
<tr>
<td>E05</td>
<td>Prdx3</td>
<td>Peroxiredoxin 3</td>
<td>Prx3</td>
</tr>
<tr>
<td>E06</td>
<td>Prdx4</td>
<td>Peroxiredoxin 4</td>
<td>MGC2724</td>
</tr>
<tr>
<td>E07</td>
<td>Prdx5</td>
<td>Peroxiredoxin 5</td>
<td>Aceb166</td>
</tr>
<tr>
<td>E08</td>
<td>Prdx6</td>
<td>Peroxiredoxin 6</td>
<td></td>
</tr>
<tr>
<td>E09</td>
<td>Prnp</td>
<td>Prion protein</td>
<td>PrP, Prm</td>
</tr>
<tr>
<td>E10</td>
<td>Scd1</td>
<td>Proteasome (prosome, matroapain) subunit, beta type 5</td>
<td></td>
</tr>
<tr>
<td>E11</td>
<td>Sodb5</td>
<td>Prostaglandin-endoperoxide synthase 1</td>
<td></td>
</tr>
<tr>
<td>E12</td>
<td>Pts1</td>
<td>Prostaglandin-endoperoxide synthase 2</td>
<td></td>
</tr>
<tr>
<td>E13</td>
<td>Pts2</td>
<td>Prostaglandin-endoperoxide synthase 3</td>
<td></td>
</tr>
<tr>
<td>E14</td>
<td>Rag2</td>
<td>Recombination activating gene 2</td>
<td></td>
</tr>
<tr>
<td>E15</td>
<td>Gpx8</td>
<td>Glutathione peroxidase 8</td>
<td>RGD1307506</td>
</tr>
<tr>
<td>F01</td>
<td>Serpinb</td>
<td>Serine (or cysteine) peptidase inhibitor, clade B, member 1</td>
<td>RGD1560688</td>
</tr>
<tr>
<td>F02</td>
<td>Serpinb2</td>
<td>Serine (or cysteine) peptidase inhibitor, clade B, member 2</td>
<td></td>
</tr>
<tr>
<td>F03</td>
<td>Srxn1</td>
<td>Sulfiredoxin 1 homolog ( S. cerevisiae )</td>
<td>Ab2-390, Npn3</td>
</tr>
<tr>
<td>F04</td>
<td>Tmod1</td>
<td>Tropomodulin 1</td>
<td>E-Tmod, Tmod</td>
</tr>
<tr>
<td>F05</td>
<td>Tpo</td>
<td>Thyroid peroxidase</td>
<td></td>
</tr>
<tr>
<td>F06</td>
<td>Scd1</td>
<td>Stearoyl-Coenzyme A desaturase 1</td>
<td></td>
</tr>
<tr>
<td>F07</td>
<td>Slc8a1</td>
<td>Intradisellar transport 172 homolog ( Chlamydomonas )</td>
<td>Slb</td>
</tr>
<tr>
<td>F08</td>
<td>Slc8a4</td>
<td>Solute carrier family 38, member 4</td>
<td>Ata1, Gnt1, Sat1</td>
</tr>
<tr>
<td>F09</td>
<td>Slc8a5</td>
<td>Solute carrier family 38, member 5</td>
<td>Ata3</td>
</tr>
<tr>
<td>F10</td>
<td>Slc41a3</td>
<td>Solute carrier family 41, member 3</td>
<td>Sn2</td>
</tr>
<tr>
<td>F11</td>
<td>Sod1</td>
<td>Superoxide dismutase 1, soluble</td>
<td>CuZnSOD</td>
</tr>
<tr>
<td>F12</td>
<td>Sod2</td>
<td>Superoxide dismutase 2, mitochondrial</td>
<td></td>
</tr>
<tr>
<td>F13</td>
<td>Sod3</td>
<td>Superoxide dismutase 3, extracellular</td>
<td>ECSODPT</td>
</tr>
<tr>
<td>F14</td>
<td>Snx1</td>
<td>Sulfiredoxin 1 homolog ( S. cerevisiae )</td>
<td>Ab2-390, Npn3</td>
</tr>
<tr>
<td>F15</td>
<td>Tmod1</td>
<td>Tropomodulin 1</td>
<td>E-Tmod, Tmod</td>
</tr>
<tr>
<td>F16</td>
<td>Tpo</td>
<td>Thyroid peroxidase</td>
<td></td>
</tr>
<tr>
<td>F17</td>
<td>Tnnt1</td>
<td>Thioredoxin interacting protein</td>
<td>MGC94673, Vdup1</td>
</tr>
<tr>
<td>F18</td>
<td>Tnnt2</td>
<td>Thioredoxin reductase 2</td>
<td></td>
</tr>
<tr>
<td>F19</td>
<td>Ucp2</td>
<td>Uncoupling protein 2 ( mitochondrial, proton carrier )</td>
<td></td>
</tr>
<tr>
<td>F20</td>
<td>Ucp3</td>
<td>Uncoupling protein 3 ( mitochondrial, proton carrier )</td>
<td></td>
</tr>
<tr>
<td>F21</td>
<td>Vim</td>
<td>Vimentin</td>
<td></td>
</tr>
<tr>
<td>F22</td>
<td>Xpa</td>
<td>Xeroderma pigmentosum, complementation group A</td>
<td></td>
</tr>
<tr>
<td>F23</td>
<td>Zmynd17</td>
<td>Zinc finger, MYND-type containing 17</td>
<td></td>
</tr>
<tr>
<td>F24</td>
<td>Rplp1</td>
<td>Ribosomal protein, large, P1</td>
<td>MGC27935</td>
</tr>
<tr>
<td>F25</td>
<td>Hprt1</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
<td>Hgprtase, Hprt, MGC112554</td>
</tr>
<tr>
<td>F26</td>
<td>Rpl13a</td>
<td>Ribosomal protein L13A</td>
<td></td>
</tr>
<tr>
<td>F27</td>
<td>Ldh1</td>
<td>Lactate dehydrogenase A</td>
<td>Ldh1</td>
</tr>
<tr>
<td>F28</td>
<td>Actb</td>
<td>Actin, beta</td>
<td>Actx</td>
</tr>
<tr>
<td>F29</td>
<td>RGD1</td>
<td>Rat Genomic DNA Contamination</td>
<td>RGD1</td>
</tr>
<tr>
<td>F30</td>
<td>RTC</td>
<td>Reverse Transcription Control</td>
<td>RTC</td>
</tr>
<tr>
<td>F31</td>
<td>RTC</td>
<td>Reverse Transcription Control</td>
<td>RTC</td>
</tr>
<tr>
<td>F32</td>
<td>RTC</td>
<td>Reverse Transcription Control</td>
<td>RTC</td>
</tr>
<tr>
<td>F33</td>
<td>PPC</td>
<td>Positive PCR Control</td>
<td>PPC</td>
</tr>
<tr>
<td>F34</td>
<td>PPC</td>
<td>Positive PCR Control</td>
<td>PPC</td>
</tr>
<tr>
<td>F35</td>
<td>PPC</td>
<td>Positive PCR Control</td>
<td>PPC</td>
</tr>
</tbody>
</table>
### ADDENDUM 3 – CHAPTER 5

#### Table 5.4: Cholesterol and phospholipids content in Caco-2 cell membranes after 48 hours exposure to oil emulsions.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Control</th>
<th>S</th>
<th>SB</th>
<th>SF</th>
<th>SBF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg/ml</td>
<td>8.5 mg/ml</td>
<td>7.5 mg/ml</td>
<td>8.9 mg/ml</td>
<td>6.0 mg/ml</td>
</tr>
<tr>
<td>Apoptosis inducing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.60±0.38^a</td>
<td>1.57±0.33^a</td>
<td>2.61±0.39^a</td>
<td>4.71±0.31^b</td>
<td>2.12±0.33^a</td>
</tr>
<tr>
<td>PC</td>
<td>61.11±5.22^a</td>
<td>128.3±14.61^b</td>
<td>258.50±12.05^c</td>
<td>225.80±11.41^c</td>
<td>197.00±19.91^c</td>
</tr>
<tr>
<td>PE</td>
<td>30.88±2.28</td>
<td>45.79±5.93^a</td>
<td>39.12±4.67^a</td>
<td>39.99±3.80</td>
<td>42.54±2.61</td>
</tr>
<tr>
<td>PC/PE</td>
<td>1.60±0.23^a</td>
<td>3.56±0.12^a</td>
<td>7.87±0.55^c</td>
<td>7.44±0.55^c_A</td>
<td>7.33±1.21^c_A</td>
</tr>
<tr>
<td>chol/PL</td>
<td>0.047±0.006^ad</td>
<td>0.020±0.009^b_A</td>
<td>0.017±0.004^b_A</td>
<td>0.032±0.003^b_A</td>
<td>0.048±0.002^d_A</td>
</tr>
<tr>
<td>BrdU IC50</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 mg/ml</td>
<td>5.5 mg/ml</td>
<td>5.1 mg/ml</td>
<td>4.8 mg/ml</td>
<td>4.8 mg/ml</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>1.60±0.38^a</td>
<td>4.30±0.88^b_B</td>
<td>3.49±0.18^b_B</td>
<td>4.91±0.91^b</td>
<td>2.07±0.19^a</td>
</tr>
<tr>
<td>PC</td>
<td>61.11±5.22^a</td>
<td>121.00±5.99^b</td>
<td>101.60±5.01^b_B</td>
<td>111.50±6.41^b_B</td>
<td>112.1±5.69^b_B</td>
</tr>
<tr>
<td>PE</td>
<td>30.88±2.28^a</td>
<td>80.35±6.00^b_B</td>
<td>66.73±0.72^b_B</td>
<td>46.69±4.61^c</td>
<td>42.93±3.65^c</td>
</tr>
<tr>
<td>PC/PE</td>
<td>1.60±0.23^a</td>
<td>1.39±0.10^b_B</td>
<td>1.88±0.24^b_c_B</td>
<td>2.76±0.18^b_d_B</td>
<td>2.27±0.23^c_d_B</td>
</tr>
<tr>
<td>chol/PL</td>
<td>0.047±0.006^a</td>
<td>0.032±0.002^b_B</td>
<td>0.038±0.003^b_B</td>
<td>0.019±0.002^c_B</td>
<td>0.024±0.001^c_B</td>
</tr>
</tbody>
</table>

Values represent the means ± standard deviations (N = 4-5) of the cholesterol (chol) and phospholipid (PL) content expressed in μg cholesterol or phosphate per mg protein in the Caco-2 cell membranes. Oil emulsions: S = sunflower oil, SB = sunflower + borage oils, SF = sunflower + fish oils and SBF = sunflower + borage + fish oils. PC = phosphatidylcholine and PE = phosphatidylethanolamine. Values in a row followed by different letters (superscript) are significantly different at p<0.05. Values in a column representing the corresponding lipid variable at different concentrations (i.e. apoptosis inducing vs. BrdU IC50) within the same oil emulsion treatments are significantly different at p<0.05 following different letters (uppercase subscript).
Table 5.5: Saturated fatty acids content of Caco-2 cell membranes after 48 hours exposure to oil emulsions.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Variable</th>
<th>PC fraction</th>
<th>PE fraction</th>
<th>Apoptosis inducing</th>
<th>BrdU IC50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>S</td>
<td>SB</td>
<td>SF</td>
<td>SBF</td>
</tr>
<tr>
<td></td>
<td>0 mg/ml</td>
<td>8.5 mg/ml</td>
<td>7.5 mg/ml</td>
<td>8.9 mg/ml</td>
<td>6.0 mg/ml</td>
</tr>
<tr>
<td>C16:0</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>μg</td>
<td>30.77±7.41a</td>
<td>12.09±1.80b_A</td>
<td>16.06±1.87b</td>
<td>13.87±3.99b_A</td>
<td>23.24±2.34ab</td>
</tr>
<tr>
<td>C18:0</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>μg</td>
<td>9.19±0.80ab</td>
<td>4.66±0.90b</td>
<td>7.93±1.39</td>
<td>6.04±1.41</td>
<td>7.03±1.86</td>
</tr>
</tbody>
</table>

| Total SATS | %    | 31.19±2.80 | 30.87±6.61 | 29.87±1.06 | 26.57±2.98 | 31.81±3.76 |
|            | μg   | 37.62±5.58a | 16.16±0.46b_A | 24.63±3.42b | 20.99±7.50b_A | 33.80±5.54ab |

| Apoptosis inducing | BrdU IC50 |
|                   |          |
|                   | 0 mg/ml  | 8.5 mg/ml | 7.5 mg/ml | 8.9 mg/ml | 6.0 mg/ml | 0 mg/ml | 8.5 mg/ml | 7.5 mg/ml | 8.9 mg/ml | 6.0 mg/ml |
| C16:0             | %        | 25.72±2.97 | 21.35±3.04 | 19.52±1.26 | 19.98±1.97 | 22.17±2.31 | 17.19±6.44a | 18.82±3.10a | 8.98±0.76b | 12.89±2.64ab | 15.27±3.98ab |
| μg                | 30.77±7.41a | 26.96±8.70b_ab | 17.36±2.35b | 25.33±3.85b_A | 30.25±8.40a | 16.15±2.39ad | 21.38±1.67b | 6.07±6.06c | 18.31±1.05ab | 12.66±2.43d | 20.86±4.32 |
| C18:0             | %        | 9.19±0.80 | 6.71±1.49 | 6.99±0.93 | 6.67±1.20 | 5.72±0.64 | 16.29±4.94 | 19.14±2.55 | 14.25±1.03 | 18.55±1.09 | 20.86±4.32 |
| μg                | 6.90±3.17 | 6.21±1.01b_A | 6.26±1.27 | 8.82±2.09 | 6.61±1.25 | 13.85±5.72ac | 26.64±4.45b | 9.83±0.33c_b | 33.34±2.96d | 17.39±3.80c |

| Total SATS | %    | 31.19±2.80 | 28.28±1.92 | 28.67±2.11 | 27.25±2.30 | 28.27±1.03 | 34.98±3.74c_ac | 39.17±3.18ac | 24.50±1.00b | 32.56±3.83c | 38.13±6.53c_b |
|            | μg   | 37.62±5.58 | 33.76±8.87 | 25.70±4.20 | 36.29±5.17_b | 34.72±5.03 | 33.01±6.95c | 46.17±8.90b | 16.50±0.51c | 52.76±2.74b | 31.79±6.32a |

Values represent the means ± standard deviations (N = 4-5) of the qualitative (% of total fatty acids) and quantitative (μg FA per mg protein) fatty acid content in Caco-2 cell membrane phospholipid fractions phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Oil emulsions: S = sunflower oil, SB = sunflower + borage oils, SF = sunflower + fish oils and SBF = sunflower + borage + fish oils. SATS = saturated fatty acids. Values in a row followed by different letters (superscript) are significantly different at p<0.05. Values in a column representing the corresponding fatty acid variable at different concentrations (i.e. apoptosis inducing vs. BrdU IC50) within the same oil emulsion treatments are significantly different at p<0.05 following different letters (uppercase subscript).
## Table 5.6: Monounsaturated fatty acids content of Caco-2 cell membranes after 48 hours exposure to oil emulsions.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Variable</th>
<th>PC fraction</th>
<th>PE fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>S</td>
<td>SB</td>
</tr>
<tr>
<td></td>
<td>0 mg/ml</td>
<td>8.5 mg/ml</td>
<td>7.5 mg/ml</td>
</tr>
<tr>
<td>C16:1ω-7</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.29±1.52ab</td>
<td>3.67±1.07ab</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>4.24±1.05</td>
<td>4.55±0.54</td>
</tr>
<tr>
<td>C18:1ω-9</td>
<td>%</td>
<td>18.22±2.37a</td>
<td>15.44±2.06ab</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>19.39±5.11a</td>
<td>18.44±0.66ab</td>
</tr>
<tr>
<td>C18:1ω-7</td>
<td>%</td>
<td>5.21±1.57</td>
<td>4.83±1.14</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>5.96±1.80</td>
<td>4.97±0.51</td>
</tr>
<tr>
<td>Total MUFA</td>
<td>%</td>
<td>28.93±3.15</td>
<td>22.99±4.89a</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>27.15±7.38</td>
<td>29.17±1.24a</td>
</tr>
</tbody>
</table>

Values represent the means ± standard deviations (N = 4-5) of the qualitative (% of total fatty acids) and quantitative (μg FA per mg protein) fatty acid content in Caco-2 cell membrane phospholipid fractions phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Oil emulsions: S = sunflower oil, SB = sunflower + borage oils, SF = sunflower + fish oils and SBF = sunflower + borage + fish oils. MUFA = monounsaturated fatty acids. Values in a row followed by different letters (superscript) are significantly different at p<0.05. Values in a column representing the corresponding fatty acid variable at different concentrations (i.e. apoptosis inducing vs. BrdU IC50) within the same oil emulsion treatments are significantly different at p<0.05 following different letters (uppercase subscript).
Table 5.7: Polyunsaturated fatty acid (ω-3) content of Caco-2 cell membranes after 48 hours exposure to oil emulsions.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Variable</th>
<th>control</th>
<th>S</th>
<th>SB</th>
<th>SF</th>
<th>SBF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 mg/ml</td>
<td>8.5 mg/ml</td>
<td>7.5 mg/ml</td>
<td>8.9 mg/ml</td>
<td>6.0 mg/ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Apoptosis inducing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:3ω-3</td>
<td>%</td>
<td>0.13±0.10</td>
<td>0.09±0.03</td>
<td>0.08±0.01</td>
<td>0.06±0.03</td>
<td>0.12±0.02</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>0.04±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04±0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.06±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.14±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:5ω-3</td>
<td>%</td>
<td>0.95±0.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.40±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.63±0.15&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.35±0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.06±0.09&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>1.56±0.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.38±0.14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.47±0.13&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.05±0.38&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.18±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:5ω-3</td>
<td>%</td>
<td>0.48±0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.51±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.70±0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.82±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.37±0.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>0.29±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.52±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.55±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.20±0.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:6ω-3</td>
<td>%</td>
<td>0.98±0.20</td>
<td>1.33±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.03±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.10±0.42&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>0.60±0.27</td>
<td>1.31±0.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90±0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.74±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.41±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total ω-3</td>
<td>%</td>
<td>2.58±0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.35±0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.55±0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.35±0.58&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>3.37±0.60&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>2.52±0.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.39±0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.01±0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.44±0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.04±0.63&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>BrdU IC50</th>
<th>control</th>
<th>S</th>
<th>SB</th>
<th>SF</th>
<th>SBF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg/ml</td>
<td>0.07±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.10±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.10±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>5.5 mg/ml</td>
<td>0.15±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.07±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.04±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.07±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>5.1 mg/ml</td>
<td>0.11±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.06±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.08±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>4.8 mg/ml</td>
<td>0.05±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.06±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.08±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values represent the mean ± standard deviations (N = 4-5) of the qualitative (% of total fatty acids) and quantitative (μg FA per mg protein) fatty acid content in Caco-2 cell membrane phospholipid fractions phosphatidycholine (PC) and phosphatidylethanolamine (PE). Oil emulsions: S = sunflower oil, SB = sunflower + borage oils, SF = sunflower + fish oils and SBF = sunflower + borage + fish oils. Values in a row followed by different letters (superscript) are significantly different at p<0.05. Values in a column representing the corresponding fatty acid variable at different concentrations (i.e. apoptosis inducing vs. BrdU IC50) within the same oil emulsion treatments are significantly different at p<0.05 following different letters (uppercase subscript).
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Variable</th>
<th>control</th>
<th>S</th>
<th>SF</th>
<th>SBF</th>
<th>control</th>
<th>S</th>
<th>SF</th>
<th>SBF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 mg/ml</td>
<td>8.5 mg/ml</td>
<td>7.5 mg/ml</td>
<td>8.9 mg/ml</td>
<td>6.0 mg/ml</td>
<td>0 mg/ml</td>
<td>8.5 mg/ml</td>
<td>7.5 mg/ml</td>
</tr>
<tr>
<td>C18:2ω-6</td>
<td>%</td>
<td>1.68±0.30^a</td>
<td>6.22±1.85^b</td>
<td>7.12±1.44^bc</td>
<td>9.31±0.94^c</td>
<td>9.34±2.27^d</td>
<td>1.27±0.08^a</td>
<td>4.22±0.32^b</td>
<td>3.04±0.48^c</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>1.85±0.31^a</td>
<td>5.51±0.57^A</td>
<td>5.95±1.09^A</td>
<td>7.29±1.22^A</td>
<td>11.60±0.71^A</td>
<td>1.07±0.08^a</td>
<td>3.30±0.21^A</td>
<td>3.79±0.52^c</td>
</tr>
<tr>
<td>C20:3ω-6</td>
<td>%</td>
<td>0.43±0.22^a</td>
<td>0.45±0.16^a</td>
<td>0.79±0.10^b</td>
<td>0.39±0.09^a</td>
<td>0.77±0.21^A</td>
<td>0.40±0.12^a</td>
<td>0.69±0.13^b</td>
<td>0.99±0.26^c</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>0.24±0.08^a</td>
<td>0.34±0.06^b</td>
<td>0.45±0.07^c</td>
<td>0.23±0.03^A</td>
<td>0.85±0.29^A</td>
<td>0.55±0.09^a</td>
<td>1.02±0.03^b</td>
<td>2.53±0.04^c</td>
</tr>
<tr>
<td>C20:4ω-6</td>
<td>%</td>
<td>1.04±0.34^b</td>
<td>1.78±0.46^b</td>
<td>2.28±0.08^A</td>
<td>2.27±0.16^c</td>
<td>2.37±0.64^c</td>
<td>2.55±0.87^b</td>
<td>6.81±0.71^b</td>
<td>7.51±1.79^f</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>2.47±0.05^b</td>
<td>1.57±0.38^bc</td>
<td>1.58±0.28^A</td>
<td>1.46±0.22^A</td>
<td>2.75±0.46^e</td>
<td>3.25±0.40^bc</td>
<td>3.64±0.41^ab</td>
<td>6.16±0.59^bc</td>
</tr>
<tr>
<td>Total ω-6</td>
<td>%</td>
<td>4.13±0.60^b</td>
<td>9.33±2.36^bc</td>
<td>11.55±2.64^bc</td>
<td>13.03±3.58^bc</td>
<td>13.90±3.52^bc</td>
<td>9.06±0.79^a</td>
<td>13.54±2.67^bc</td>
<td>13.64±2.74^bc</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>5.77±0.95^b</td>
<td>8.89±2.05^bc</td>
<td>8.97±2.24^bc</td>
<td>9.79±2.82^bc</td>
<td>16.90±4.39^c</td>
<td>7.57±1.00^a</td>
<td>9.66±1.49^bc</td>
<td>14.40±2.27^bc</td>
</tr>
</tbody>
</table>

Values represent the means ± standard deviations (N = 4-5) of the qualitative (% of total fatty acids) and quantitative (μg FA per mg protein) fatty acid content in Caco-2 cell membrane phospholipid fractions phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Oil emulsions: S = sunflower oil, SB = sunflower + borage oils, SF = sunflower + fish oils and SBF = sunflower + borage + fish oils. Values in a row followed by different letters (superscript) are significantly different at p<0.05. Values in a column representing the corresponding fatty acid variable at different concentrations (i.e. apoptosis inducing vs. BrdU IC50) within the same oil emulsion treatments are significantly different at p<0.05 following different letters (uppercase subscript).
Table 5.9: Total polyunsaturated fatty acid (ω-3 and ω-6) and ratios content of Caco-2 cell membranes after 48 hours exposure to oil emulsions.

<table>
<thead>
<tr>
<th>Fatty Acid Variable</th>
<th>PC fraction</th>
<th>0 mg/ml</th>
<th>S</th>
<th>8.5 mg/ml</th>
<th>SB</th>
<th>8.9 mg/ml</th>
<th>SF</th>
<th>6.0 mg/ml</th>
<th>Apoptosis inducing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>control</td>
<td>S</td>
<td></td>
<td>SB</td>
<td></td>
<td>SF</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total PUFA</td>
<td>%</td>
<td>6.71±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.06±1.55&lt;sup&gt;A&lt;/sup&gt;</td>
<td>14.29±0.92&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.98±1.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.3±0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.09±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.17±0.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>μg</td>
<td>8.29±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.28±0.51&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.91±0.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.26±0.57&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.94±1.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.16±1.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.11±0.36&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PUFA/SATS</td>
<td>%</td>
<td>1.60±0.12</td>
<td>3.97±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.57±0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.92±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.15±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.28±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.04±0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.82±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>2.29±0.01</td>
<td>3.72±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.49±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.04±0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.20±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.24±0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.64±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.98±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:4ω-6/C20:5ω-3</td>
<td>%</td>
<td>1.80±0.10</td>
<td>4.45±0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.72±0.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.85±0.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.23±0.45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.64±1.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.71±0.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.98±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>1.94±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.79±1.35&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.65±0.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.53±0.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.18±1.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.43±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.89±0.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.90±0.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Δ6 S/P</td>
<td>%</td>
<td>1.41±0.04</td>
<td>3.53±0.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.25±1.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.74±2.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.41±2.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.51±0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.83±0.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.01±0.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>1.86±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.65±0.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.54±2.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.19±4.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.53±1.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.97±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.97±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.97±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>%</td>
<td>0.78±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.26±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.27±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.82±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.31±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.26±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>μg</td>
<td>0.61±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.12±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.61±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.60±0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.91±0.63&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.61±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.88±0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.77±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values represent the means ± standard deviations (N = 4-5) of the qualitative (% total fatty acids) and quantitative (μg FA per mg protein) fatty acid content in Caco-2 cell membrane phospholipid fractions phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Oil emulsions: S = sunflower oil, SB = sunflower + borage oils, SF = sunflower + fish oils and SBF = sunflower + borage + fish oils. PUFA = polyunsaturated fatty acids, PUFA/SATS = polyunsaturated/saturated fatty acid ratio, Δ6 S/P = delta-6 substrate/product ratio, C20:4ω-6/C20:5ω-3 = arachidonic acid/eicosapentaenoic acid ratio. Values in a row followed by different superscript letters (a, b, c) are significantly different at p<0.05. Values in a column representing the corresponding fatty acid variable at different concentrations (i.e. apoptosis inducing vs. BrdU IC50) within the same oil emulsion treatments are significantly different at p<0.05 following different letters (uppercase subscript).
ADDENDUM 4

MSc Thesis reference

Website: http://etd.uwc.ac.za/xmlui/handle/11394/2802

Chapter 3 (pg. 77-143) referred to in current study: “Lipid modulation of colon mucosa and red blood cell membranes by dietary fat varying in n-6/n-3 fatty acid ratios”.

Abstract
This study investigated the effects of different dietary n-6/n-3 fatty acid (FA) ratios on the lipid profile of rat colon mucosa and red blood cell (RBC) membranes. Sunflower, fish and borage oils were utilized to vary the diets n-6/n-3 FA ratio. The lipid profiles of the colon mucosa and RBC membranes responded differently to the dietary FA presumably due to functional and proliferative properties. Fish oil reduced membrane phospholipid phosphatidylethanolamine (PE) in the mucosa, while increasing phosphatidylcholine (PC) and PE in the RBC membranes. Borage oil significantly reduced the PC/PE ratio in the mucosa, while fish oil increased the ratio in both membranes. The dietary FA significantly altered the membranes fluidity due to modifications to the fluidity lipid parameters including cholesterol/phospholipid ratio, PC/PE and polyunsaturated/saturated (P/S) ratios. Differential effects of fish and borage oil on the saturated and monounsaturated FA levels suggest that they may be involved in regulating cell survival responses. Modulation of the membrane lipid profiles by the different FA ratio diets particularly those using fish oil content, showed a reduction in the membrane n-6/n-3 ratio due to a higher n-3 long chain (LC) PUFA content. Borage oil exhibited a similar effect although to a lesser extent while it also increased in the n-6 FA level. Both dietary oils reduced the C20:4n-6/C20:5n-3 ratio suggesting a reduced production of the prostaglandin 2-series. The n-6/n-3 interactions are also reflected by variations in the membrane incorporation of LCPUFA, which is orchestrated by a reduced Δ⁶-desaturase activity as well as negative feedback regulation by FA product of the enzyme. The combination of fish and borage oil in the diet either counteracted or enhanced the individual effects
exerted by the oils. The intake of fish and borage oil was associated with an elevation in lipid peroxidation presumably due to an increase in LCPUFA. This study suggests that a low n-6/n-3 PUFA ratio diet could potentially be used as a cancer therapeutic tool due to its ability to alter prostaglandin synthesis and enhance the susceptibility to lipid peroxidation.
ADDENDUM 5

CONFERENCES AND PRESENTATIONS ASSOCIATED WITH THESIS CONTENT

Abrahams, C.H., Abel, S., de Kock, M., Swanevelder, S. and Gelderblom, W.C.A. Dietary polyunsaturated fatty acids modulate the lipid profile and oxidative status of rat colon mucosa. 15th World Congress of Food Science and Technology, IUFoST 2010, Cape Town, South Africa, 22-26 August 2010 (poster presentation).


Abrahams, C.H., Abel, S., de Kock, M., Swanevelder, S. and Gelderblom, W.C.A. Modulation of ACF development, cell proliferation and apoptosis by dietary FA ratios