The Modulating Effect of Fatty Acids on the Lipid Profile in Colon Epithelial Mucosa *In Vivo*

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DECLARATION

I declare that the work contained in this thesis is my own, and has not previously in its entirety, or in part, been submitted for any degree or examination at any other university. All sources I have used or quoted have been indicated and acknowledged by complete references.

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Celeste H Abrahams **Date**

DEDICATION

This thesis is dedicated in loving memory to my dad, who always encouraged this “homo sapien” to learn and share knowledge for the greater good!

“Knowledge is like money,
To be of value it must circulate
And in circulating,
It can increase in quantity
And hopefully in value”

~ Louis Dearborn L’Amour
(1908-88)

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ABBREVIATIONS

AOM	azoxymethane
BBOT	2,5-bis-(5'-tert-butylbenz-oxazolyl-[2'])thiophene
BHT	butylated hydroxytoluene
cAMP	cyclic adenomonophosphate
C18:2n-6	linoleic acid
C18:3n-3	α-linolenic acid
C18:3n-6	γ-linolenic acid
C20:3n-6	dihomo-γ-linolenic acid
C20:5n-3	eicosapentaenoic acid
C20:4n-6	arachidonic acid
C20:4n-6/C20:5n-3	arachidonic acid/eicosapentaenoic acid ratio
C22:6n-3	docosahexaenoic acid
CD	conjugated dienes
CDKs	cyclin dependent kinases
chol	cholesterol
chol/PL	cholesterol/phospholipids ratio
CM	chloroform:methanol
COX	cyclooxygenase
DNA	deoxyribose nucleic acid
EFA	essential fatty acids
FA	fatty acid (s)
FAP	familial adenomatous polyposis
GSH	glutathione
HETEs	hydroxylated eicosatetranoic acids
i.p	intraperitoneal
LC	long chain
LCPUFA	long chain polyunsaturated fatty acids
LOX	lipoxygenases
LPO	lipid peroxidation
NSAIDs	non steroidal anti-inflammatory drugs
Mn-SOD	manganese superoxide dismutase

MUFA	monounsaturated fatty acid (s)
n-3	omega-3
n-6	omega-6
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PC/PE	phosphatidylcholine/ phosphatidylethanolamine ratio
PG	prostaglandin (s)
PI	phosphatidylinositol
PI3K	phosphotidylinositol-3-kinase
PKC	protein kinase C
PL	phospholipid (s)
PLA_2	phospholipase A_2
PLC	phospholipase C
polyp/surr	polyp/surrounding tissue ratio
PPAR	peroxisome proliferators activated receptor
pRB	phosphorylated retinoblastoma
PS	phosphatidylserine
P/S	polyunsaturated fatty acids/saturated fatty acids ratio
PUFA	polyunsaturated fatty acid (s)
RBC	red blood cell (s)
ROS	reactive oxygen species
S	sunflower oil diet
SATS	saturated fatty acid (s)
SB	sunflower-borage oils diet
SBF	sunflower-borage-fish oils diet
SCD	stearol-CoA desaturase
SD	standard deviation
SF	sunflower-fish oil diet
SM	sphingomyelin
SOD	superoxide dimutase
TAG	triacylglycerol
UCP-2	uncoupling proteins-2

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WESTERN CAPE

PREFACE

Preface

Diet supplies the human body with the nutrients and energy sources that are required to maintain and sustain normal cell function. Apart from physical activity, a balanced diet is a key factor to maintain optimal health. The problem with Western diets is that there has been a major change in dietary habits since the beginning of the Agricultural Revolution (Cordain *et al*., 2005). With the greater availability of processed foods, the consumption of food that is high in fat has increased and an intake of vegetables and fibres has been reduced.

Prior to the start of the agricultural revolution, the intake of polyunsaturated fatty acids (PUFA) n-6 and n-3 was maintained at a ratio of approximately 1:1 (Simopoulos, 2006). However, with the modernization of cultures and the availability of processed foods the ratio has shifted in favour of n-6 fatty acids (FA), with an n-6/n-3 ratio of 15-20:1 (Simopoulos, 2006). These dietary FA imbalances have had a great influence on the increase of several diseases that includes cardiovascular disease, diabetes and some cancers (Das, 1995; Holmes *et al.,* 1999).

Diets high in fats such as the saturated and trans-fatty acids and low in n-3 PUFA in particular have been associated with these diseased states (Simopoulos, 1999; Hughes-Fulford *et al.,* 2001; Kromhout, 2001). Generally, FA such as the PUFA play an important role in maintaining cellular homeostasis with respect to optimising cell structure (i.e. lipid membrane fatty acid content) and function (i.e. secondary cell signalling messengers) (Benatti *et al.*, 2004). FA, particularly the n-6 and n-3 PUFA affect cell structure and influence cellular processes such as proliferation, apoptosis and differentiation (Hilakivi-Clarke *et al*., 2004). These processes are regulated either directly by the FA or through conversion to metabolites such as the eicosanoids (Benatti *et al.*, 2004). Furthermore, the oxidation of membranes PUFA by free radicals can also affect cell growth and/or development due to changes to the cellular oxidative status (Gago-Dominguez *et al*., 2007). This is of particular importance for the survival of cancer cells as their oxidative status is very low (Cheeseman *et al.,* 1988).

Excessive amounts of n-6 PUFA and a high n-6/n-3 ratio, present in Western diets, have been found to enhance tumour growth in some cancers. Diets with a low n-6/n-3 PUFA content, however, could protect against further neoplastic development (Larsson *et al.*, 2004). Studies have shown that diets supplemented with fish oil, which is high in n-3 PUFA eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3), reduces cell proliferation in some cancers (Aronson *et al.*, 2001). One of the mechanisms by which n-3 PUFA exert their protective effect might be through the cyclooxygenase-2 (COX-2) pathway. The COX-2 enzyme is involved in the formation of prostanoids from free PUFA and converts n-6 PUFA such as arachidonic acid (C20:4n-6) into the 2-series prostanoids, while γ-linolenic acid (C18:3n-6) and the C20:5n-3 are converted into the 1- and 3-series prostanoids, respectively (Tapiero *et al.,* 2002). The 2-series prostanoids promote cell proliferation and decrease apoptosis and the immune response, while the 1- and 3-series prostanoids inhibits tumourigenesis. Several mechanisms have been proposed to explain how n-3 PUFA especially fish FA C20:5n-3 and C22:6n-3 could protect against cancer development. One mechanism is the competition between n-3 and n-6 FA for COX-2 activity, leading to decreased production of the 2-series prostanoids and an increase in 3-series products, inhibiting tumourgenesis (Hardman, 2002). Inclusion of C18:3n-6 in the diet also leads to the production of anti-inflammatory 1-series prostanoids that lead to further tumour cell growth inhibition (Fan and Chapkin, 1998). The n-3 and n-6 PUFA also vie for the desaturases and elongases that convert the precursors linoleic acid (C18:2n-6) into C20:4n-6 and α-linolenic acid (C18:3n-3) into C20:5n-3 (Horrobin, 1993). Therefore a low ratio of n-6 to n-3 FA may lead to decreased production of C20:4n-6 and 2-series prostanoids. This mechanism can also account for the antitumourigenic effect of a high ratio of fish FA (C20:5n-3 and C22:6n-3) to C20:4n-6 in animal models (Dommels *et al.,* 2002).

With the membrane lipid pool being affected by the dietary intake of n-6 and n-3 PUFA, it has been suggested that the mechanisms by which high n-6 FA enhances tumour development can be modulated or manipulated through dietary intake of specific fatty acid combinations (Abel *et al.,* 2004). Therefore,

lowering the n-6/n-3 fatty acid ratio could potentially reduce the risk of further cancer development by influencing cell survival.

Research has shown that FA has both a detrimental and beneficial effect on the carcinogenesis process, depending on the saturation of the FA. Dietary fat intake impacts on the level of PUFA present in the cell membrane, and thus affect cellular structure and function. Membrane PUFA content affects the regulation of cell proliferation and apoptosis, and impacts on the oxidative status of a cell. As mentioned, all these parameters play an important role in neoplastic progression. Thus establishing a balance between n-6 and n-3 PUFA content may determine the final outcome of cancer development.

The current project evaluated the lipid profiles in colon polyps and surrounding mucosa. In addition the effect of different dietary fats with varying n-6/n-3 FA ratios was monitored on the lipid profiles of colon mucosa and red blood cell membranes in normal rats. In Chapter 1 the current knowledge pertaining to colon cancer, PUFA and their association with carcinogenesis as well as their role in cancer chemoprevention is reviewed. Differences in the lipid profiles of colon polyps and surrounding mucosa and the role of a high n-6/n-3 ratio diet in the development of colon polyps will be critically discussed in Chapter 2. The incorporation and modulating effect of different dietary n-6/n-3 FA ratios on the lipid profile of rat colon mucosa and red blood cell (RBC) membranes are presented in Chapter 3. The modulating effect of the different dietary n-6/n-3 FA ratios will be assessed on the membrane cholesterol, phospholipids and FA content, as well as its effect on the extent of lipid peroxidation. As numerous studies utilise RBC as a biomarker for dietary fat intake, the lipid profile of colon mucosa and RBC membranes were compared to determine whether the membranes reflect a similar lipid pattern with respect to dietary fat intake. The main findings of the study will be integrated and summarized in Chapter 4, while supportive experimental procedures will be summarized in the Addendum section.

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CHAPTER 1

LITERATURE REVIEW

1. Introduction

In all multicellular organisms maintenance of cellular homeostasis is vital for an individual to remain alive and healthy. Several factors impact on this dynamic equilibrium, one of which is diet. Our daily diet provides a source of energy and nutrients that assist in regulating cellular metabolic processes. Scientific evidence supports the view that alterations in diet have strong effects, both positive and negative, on health throughout life (WHO, 2003). Dietary adjustment may thus not only influence present health, but may determine whether or not an individual will develop diseases such as diabetes, cardiovascular disease and cancer much later in life.

Research has shown that dietary fat in particular, plays an important role in human health and disease. Fat is a source of energy, but it is also a vital component of the cellular membrane structure, and is involved in many cell signalling pathways (Green and Tzagoloff, 1966; http://www.lipidlibrary.co.uk/Lipids/whatdo/index.htm, accessed 11 August 2009). Mammals, including humans, can synthesize saturated and monounsaturated fatty acids (FA), but cannot synthesize the principle omega-6 and omega-3 polyunsaturated fatty acids (PUFA), linoleic acid and α-linolenic acid respectively (Hardman, 2004). These two FA are termed essential fatty acids and must be obtained from the diet (Horrobin, 1986). Both FA undergo desaturation and chain elongation to yield metabolically functional 20- to 22-carbon FA. The omega-6 and omega-3 PUFA have a number of vital functions in the human body as structural lipids of cellular membranes modulating membrane fluidity, cellular interaction and signal transduction pathways (Hardman, 2004). Moreover, they play an important role in the regulation of inflammatory pathways and the immune system by acting as precursors for the synthesis of eicosanoids (Roynette *et al.*, 2004).

Carcinogenesis is regarded as a multi-step process, which is reflected by progressive genetic alterations that drive the transformation of normal cells into highly malignant derivatives (Grander, 1998). It has been well established that the characteristic features of cancer are accelerated cell proliferation, reduced apoptosis and the inhibition of cell differentiation, resulting from the

deregulation of signalling cascades. Stern *et al.* (1999) have postulated that the primary defect in carcinogenesis to be an architectural, organizational, and compositional change in the plasma membrane that results in the loss of homeostasis. Therefore, the suggested mechanisms by which dietary fat in the form of PUFA influence cancer development may be through its direct influence on lipid content in cell membranes, thereby affecting cell function. As tumours are dependent on host circulation for the type and amount of PUFA available, it should be possible to alter FA composition by dietary means. Thus dietary modulation of tumour membrane FA could alter the growth of tumour cells by influencing cell replication by interfering with components of the cell cycle, or by increasing cell death, either by cell necrosis or apoptosis (Field and Schley, 2004).

One of these modulation mechanisms relates to the pattern of FA composition and their impact on eicosanoid production. Eicosanoids are the instigators of cellular inflammatory responses, with their production dependent on the PUFA composition in membranes (James *et al*., 2000). Therefore, the consumption of dietary PUFA impacts on the spectrum of eicosanoids produced by the cell, and thereby attenuates the biological effects of growth factor stimuli (Thies *et al.*, 2001). Dysfunctions in eicosanoid metabolism have been associated with the development of specific cancers linked with an imbalanced PUFA intake, related to diets with a high n-6 and/or low n-3 PUFA content (Bartsch *et al.*, 1999).

An additional mechanism by which PUFA impacts on the development of cancer is through its generation of free oxygen radicals and lipid peroxides. Data supports the notion that increased formation of reactive oxygen species (ROS) may play an important role in carcinogenesis (Waris and Ahsan, 2006). Numerous *in vitro* experiments show that ROS damages DNA, inducing premutagenic modifications of nucleotides and promoting oxidation of protein and lipids resulting in lipid peroxidation (Gago-Dominguez *et al*., 2005). Lipid peroxidation is a well known example of oxidative damage, a degenerative process that perturbs structure and/or function of the target system, often with cytopathological consequences. According to Özdemirler Erata *et al.* (2005),

oxidative stress plays an important role in the molecular mechanism of colorectal cancer. Degradation products formed may interfere with intracellular signalling cascades involved in cell replication and cell death (Udilova *et al.*, 2003). Lipid compositions of cellular membranes are associated with the sensitivity of cells towards lipid peroxidation. Hence, alterations in the FA composition may contribute to the peroxidative effect of FA such as docosahexaenoic acid (C22:6n-3) (Song *et al.*, 2000). Various studies have shown that tumour development is promoted by a decrease in PUFA and lipid peroxidation (Galeotti *et al.*, 1986; North *et al.*, 1994). Research by Abel *et al.* (2001) indicated that decreased levels of long-chain PUFA in hepatocyte nodules are associated with decreased lipid peroxidation, impairing apoptosis and enhancing proliferation in these lesions.

It is evident that PUFA have a profound effect on cellular function with regards to cell membrane regulation of metabolic processes. A full understanding of its regulatory capacity is therefore essential to elucidate their role on the development of cancer. This review aims to briefly debate the association between dietary fat and colon cancer development. The current knowledge on dietary PUFA and its biological functions in mammalian cells and its association with cancer development and chemoprevention will also be addressed.

2. Cancer

In healthy tissue, growth regulatory mechanisms endeavour to maintain cellular homeostasis. Homeostasis within a cell is regulated by the balance between proliferation, growth arrest and apoptosis (Evan and Vousden, 2001). When homeostasis is disturbed, either by an increased proliferation rate or a decrease in cell death, initiated or preneoplastic cells might progress into a tumour.

Cancer is thus the product of malfunctions within the regulation of the cell cycle, such that injured or mutated cells that are normally killed are allowed to progress through the cell cycle, accumulating mutations (Foster, 2007). At least three important classes of genes play key roles in tumour initiation:

proto-oncogenes, tumour suppressor genes, and genes involved in DNA repair mechanisms. Mutations, amplifications or deletions in these genes may lead to decoupling of biological mechanisms involved in the regulation of normal cell growth and differentiation (Tysnes and Bjerkvig, 2007). Proto-oncogenes normally act at different levels of cell proliferation, but can promote tumour growth when mutated. Similarly, mutation of tumour suppressor genes (e.g. p53) would impair the inhibition of the cell cycle progression, thus facilitating abnormal growth (Foster, 2007).

2.1 Cell cycle

The cell cycle is a fundamental process in mammalian cells that enables them to grow and replicate (Fig. 1). Regulation of cell cycle progression is controlled by external factors, amongst which are nutrients and growth factors (Paul *et al.*, 1978; Bohnsack and Hirschi, 2004).

The cell cycle governs the fate of the cell and is the mechanism that interprets the growth-regulating signals received, deciding whether the cycle proceeds. The cell cycle consists of four phases: G_1, S, G_2 and M. Cells that are committed to replication move from G_1 phase (the first gap) of the cycle, into the S (stationary) phase for DNA synthesis. The S phase is followed by the G_2 (the second gap) phase, where cells prepare for duplication in the M (mitosis) phase (Fig. 1). Progression through the cell cycle is dependent upon the integration of a large number of intra- and extra-cellular signals that integrate also with intrinsic genetic controls, resulting in several checkpoints in different phases of the cycle (Boonstra and Post, 2004). These checkpoints include the DNA damage checkpoints: S-checkpoint at G_1/S and M-checkpoints at G_2/M. At these checkpoints the progression through the cell cycle can be arrested to enable the cells to repair DNA upon damage caused by irradiation or a chemical carcinogen. Specific cellular mechanisms that control cell cycle progression and checkpoint progression through the intermitotic phases are deregulated in cancer (Golias *et al*., 2004).

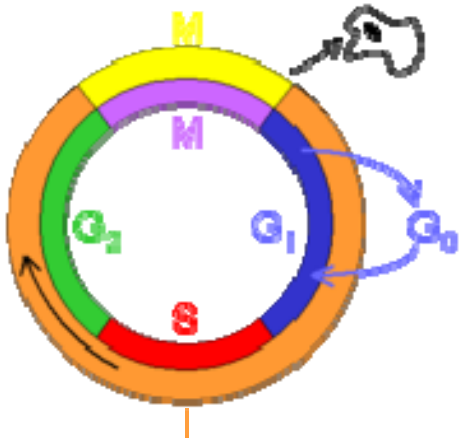

Fig. 1 Cell cycle: the complete series of events from one cell division to the next. It is usually divided into the phase when DNA is replicated (S phase), the phase when the cell actually divides into two cells (M phase), the two intervening gap phases (G_1 and G_2), and a nondividing state called "quiescence" (G_0).
(http://en.wikipedia.org/wiki/Image:Cell_cycle.png, accessed 28 January 2008).

2.2 Cell cycle control mechanisms disrupted in cancer

2.2.1 Cell proliferation

Regulatory pathways that control the proliferation response in normal cells are altered in most cancers. One class of mutations required for tumour development acts by short-circuiting the normally obligate requirement of somatic cells for external mitogenic signals (Evan and Vousden, 2001). A second class of growth deregulating mutations comprises those that target the regulatory checkpoints, such as the principle late G_1-S checkpoint regulated by phosphorylated retinoblastoma (pRB) (Knudsen and Knudsen, 2006). Defects in this pathway include deletion of the RB genes itself and deregulation of the cyclin-dependent kinases (CDKs) that phosphorylate and functionally inactivate pRB. Uncontrolled cell proliferation associated with cancer depends on the dysfunction of at least one of these checkpoint pathways.

2.2.2 Apoptosis

Apoptosis is a form of cellular suicide used by multicellular organisms to eradicate cells in diverse physiological and pathological settings. This evolutionary conserved death process is important for normal tissue development and tissue homeostasis by regulating cell numbers and

eliminating damaged cells (Renehan *et al*., 2001). Deregulation of apoptosis is a key feature of cancer development. Defects in apoptosis allow damaged cells to live beyond their lifespan, accumulate genetic mutations and sustain growth under hypoxia and oxidative stress (Yu and Zhang, 2003). A mutation in the tumour suppressor gene p53, which is a critical regulator of apoptosis, is frequently observed in tumour cells. In the absence of p53, cells that have sustained DNA damage fail to arrest, and mutations are perpetuated, leading to genomic instability and cancerous promoting genetic alterations. Many of the signals that inactivate p53 are important during cancer initiation and promotion, because a loss of p53 creates a permissive environment in which there is inappropriate proliferation and survival (Zamzami and Kroemer, 2005).

2.2.3 Differentiation

In most tissues, cells go through a process of terminal differentiation. This process serves as a transition stage between proliferation and cell death. By definition, differentiation is the process by which a cell matures and becomes capable of performing specific functions (Rudolph *et al*., 2001). Fully differentiated cells in constantly renewing tissue often lose their proliferative capability and have a defined relatively short life span.

Rapid tissue renewal by terminal differentiation ensures effective elimination of damaged cells before they become neoplastic (Ding *et al*., 2001). Cells committed to terminal differentiation undergo a genetically programmed death process that exhibits features of apoptosis such as nuclear DNA fragmentation and increased Bax expression (Cai *et al*., 2004). In colon cancer, for example, the failure of intestinal epithelial cells to undergo normal differentiation, results in continuous DNA synthesis and cell proliferation (Lipkin, 1974).

2.3 Colon cancer

Cancer of the bowel is one of the leading causes of death in both men and women in industrialised Western countries (Dommels *et al*., 2002). Parkin *et al*. (2002) reported that colon and rectum cancer accounted for 1 million new

cases, approximately 9.4% of the total world population, with numbers not varying significantly between men and women (ratio 1.2:1). In terms of incidence, colorectal cancer ranks fourth in men and third in women globally.

The complexity of colon cancer development involves both genetic and environmental factors. Genetic dispositions, such as Familial Adenomatous Polyposis (FAP), increase the risk of developing colon cancer by the age of 21 (Roynette *et al*., 2004). However, the prevalence of such predisposition is low and 90% of cases are due to exogenous factors, from which 50-80% are environmental (Bartsch *et al*., 1999). Among the environmental factors, dietary habits play a major role. Numerous reports have shown associations between diet and colon cancer (Ferguson, 2002; Le Marchand *et al*., 2002). A relation commonly found in epidemiological studies is an increase in risk associated with a high-fat, low fibre diet pattern (Singh and Fraser, 1998).

2.3.1 Colon cancer development

Colorectal cancer, commonly called colon cancer or bowel cancer, includes cancerous growths in the colon, rectum and appendix (Fig. 2). As with all cancers, colon cancer development is a multistage process involving genetic and epigenic defects that normally occur over a large part of the individual's lifespan (Johnson, 2004).

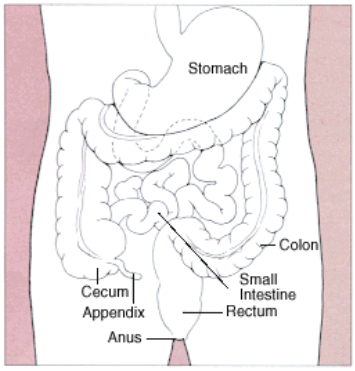

Fig. 2 Anatomy of the lower digestive tract - comprises the colon, small intestine and rectum (http://www.heartspring.net/images/colon01.gif, accessed 21 November 2007).

Colon cancer originates from the epithelial cells (colonocytes) that line the intestinal tract. The inner surface of the colon is lined with crypts; the fold of colonic epithelium that are continuously renewed by stem cell division (Komarova and Cheng, 2006). A dynamic balance between proliferation, differentiation and apoptosis maintains the steady state of cell growth of normal colonic epithelium. Stem daughter cells proliferate in the lower part of the crypt, move in tight cohort toward the upper crypt regions, gradually lose their capacity to divide and acquire the differentiated phenotype (Lamprecht and Lipkin, 2002). In normal colonic epithelium the size of the various functional compartments along the crypt axis are maintained within precise boundaries by multiple homeostatic signals. Disruption of these processes, for example by diet, can lead to colorectal carcinogenesis.

The disease progresses through a series of clinical and histopathological stages ranging from single crypt lesions through small benign tumours (adenomatous polyps) to malignant cancers (carcinomas) (Fig. 3). The model of colorectal tumourigenesis includes several genetic changes that are required for cancer initiation and progression (Michor *et al*., 2005).

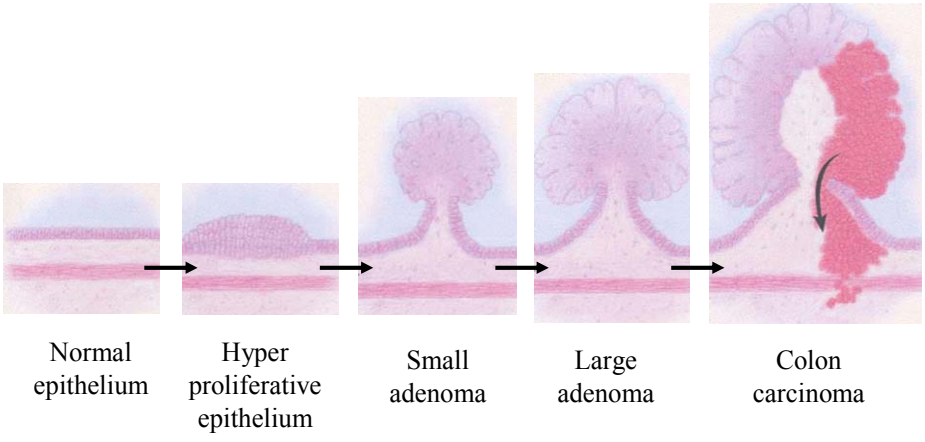

Fig. 3 Sequential histopathological changes in the colon that transform normal epithelium to invasive carcinoma during the development of colon cancer (adapted form Jänne and Mayer, 2000).

2.3.2 Dietary risk factors associated with colon cancer development

Several factors impact on the development of colon cancer. Genetic predispositions account for only a small percentage of colon cancer occurrences (Johnson, 2004). Research has shown that the prevalence of the disease's development is largely related to dietary habits.

2.3.2.1 Body fat and energy intake

Energy intake among individuals is dependent on physical activity level, body size, metabolic efficiency and energy balance (Willet and Stampfer, 1986). The maintenance of a healthy body weight is determined by the ratio of energy intake to energy expenditure (Gunter and Leitzmann, 2006). An excess of energy input over energy output results in positive energy balance and leads to weight gain.

For cancer of the colon, the increase in risk associated with excess body weight or increase in abdominal fat may be mediated by alterations in the metabolism of insulin and insulin-like growth factor-1 (IGF-1) (Bianchini *et al*., 2002). A metabolic consequence of obesity, and specifically the accumulation of intra-abdominal fat, is the development of insulin resistance, which leads to an increase in secretion of insulin from the pancreas (Bianchini *et al*., 2002). Insulin resistance is believed to be the causal factor in metabolic perturbation. Specifically, insulin stimulates proliferation and reduces apoptosis in colorectal cancer cell lines (Koenuma *et al*., 1989), and promotes colon carcinogenesis in animal models (Tran *et al*., 1996).

The relation between excess body weight and cancer risk suggest that excess energy is an important risk factor for development of cancer. Several physiological mechanisms have been postulated to explain the effects of energy balance and adiposity in cancer risk. These include increases in the endogenous production of reactive oxygen species (ROS) and oxidative DNA damage, alterations in carcinogen-metabolising enzymes, tissue homeostasis and alterations in endogenous hormone balance (Bianchini *et al*., 2002).

Increased energy availability may also contribute to colon carcinogenesis by stimulating reactive oxygen species production. An intracellular lipolytic environment rich in oxidizable substrates may result in the generation of lipid peroxidation products, depleted levels of antioxidants and an overall environment of oxidative stress (Draper & Bettger, 1994). High levels of ROS exert a toxic effect on biomolecules such as DNA, proteins and lipids, leading to the accumulation of oxidative damage in cells that deregulate redox-sensitive metabolic and signalling pathways resulting in pathological conditions (Schrader and Fahimi, 2006).

2.3.2.2 Dietary fat intake

A large body of epidemiological evidence, together with data from animal and *in vitro* studies, strongly supports relationships between dietary constituents and the risk of specific cancers (Dommels *et al*., 2002; Zhang *et al*., 1996; Nano *et al*., 2003). Generally, vegetables and fruits, dietary fibres, and certain micronutrients appear to be protective against cancer (Jenkins *et al*., 2001), whereas fat, excessive calories and alcohol seem to increase the risk (Boffetta and Hashibe, 2006; Ferguson, 2002).

Research suggests that a high intake of dietary fat plays a role in colorectal cancer development (Slattery *et al*., 1998; Salim *et al*., 2002). Studies demonstrate that not only the amount of fat, but also types of fat differing in FA composition are important determining factors in colon tumour development. Both saturated and unsaturated FA has been implicated in the carcinogenic process and has been shown to affect tumour growth, incidence and multiplicity (Rao *et al*., 2001, Reddy *et al*., 1991). A study links dietary n-6 PUFA to increased risks for cancer of the colon, particularly in combination with low intake of n-3 PUFA or monounsaturated FA (Nkondjock *et al*., 2003).

Several mechanisms have been proposed to explain the enhancement of colon cancer by n-6 PUFA, including the modulation of the immune response as a result of changes in the eicosanoid metabolism (Bartoli *et al*., 2000) and alterations of membrane structure and properties (Awad *et al*., 1996b). Laboratory studies have shown that high fat corn oil diets promotes colon

tumourigenesis in rats by up regulating cyclooxygenase-2 (COX-2), an enzyme that catalyzes the metabolism of n-6 and n-3 PUFA during prostaglandin biosynthesis (Singh *et al*., 1997). Increased intakes of n-6 FA such as linoleic acid and arachidonic acid elevate the biosynthesis of 2-series prostaglandins that enhances cell proliferation, a feature characteristic of cancer development (Benatti *et al*., 2004). Furthermore alterations to the membrane fatty content change the membrane-receptor and cell-to-cell communications resulting in abnormal and unregulated cell function. A study investigating the interaction of human colorectal carcinoma cells with Kupffer cells and extracellular matrix protein indicated that an increased membrane n-6 FA content decreased adherence to extracellular matrix protein, which may lead to increased cell motility and invasiveness (Meterissian *et al*., 1995). It has been shown that Kupffer cell binding precedes tumour cell phagocytosis and killing, thus decreased binding may improve tumour cell survival.

In contrast, n-3 PUFA-enriched diets have been shown to reduce the development of colon tumours by reducing signalling pathways that enhance cell proliferation (Calviello *et al*., 2004). A study by Chang *et al.* (1998) suggested that fish oil rich in n-3 FA blocks the progression of colon cancer by increasing cell differentiation and apoptosis. The mechanism by which n-3 FA may lower the risk of cancer is through their suppressing effect on the biosynthesis of arachidonic-derived eicosanoids. This effect is achieved at several levels. First, high intakes of n-3 FA result in their incorporation into membrane phospholipids, where they partially replace arachidonic acid (Crawford *et al*., 2000). By decreasing the availability of arachidonic precursors, this substitution suppresses the biosynthesis of arachidonic derived eicosanoids in favour of those derived from n-3 FA (Larsson *et al*., 2004). An increase in long chain n-3 FA such as eicosapentaenoic acid and docosahexaenoic acid in membranes may have antitumour effects by affecting gene expression or the activities of signal transduction molecules involved in the control of cell growth, differentiation and apoptosis (Larsson *et al*., 2004).

3. Polyunsaturated Fatty Acids (PUFA)

Amongst dietary fats, the essential fatty acids (EFA) have been identified as essential nutrients (Cunnane, 2003). An essential nutrient is one that is needed for normal development and function of mammalian cells throughout the life cycle. Most vertebrates are capable of synthesizing FA containing either no (saturated) or one (monounsaturated) carbon-carbon double bond per mole. Dietary PUFA linoleic acid and α-linolenic acid are the sole sources of FA that cannot be synthesized by many animal species, including humans.

The two principle dietary EFA linoleic acid (C18:2n-6) and α-linolenic acid (C18:3n-3) (Fig. 4) are the biosynthetic precursors of carbon-20 and carbon-22 PUFA with three to six double bonds that are formed via sequential desaturation and chain elongation steps.

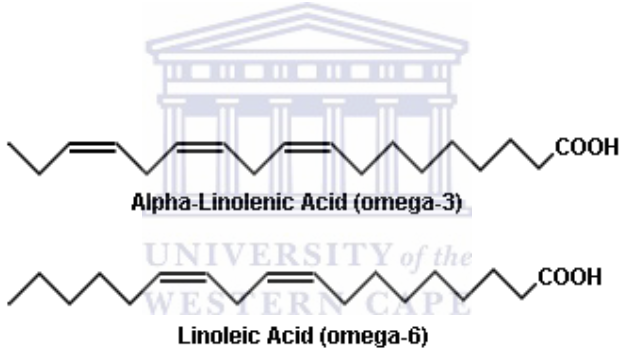

Fig. 4 Principle omega-3 and omega-6 EFA (http://www.scientificpsychic.com/fitness/fattyacids.html, accessed 26 March 2007).

3.1 Nomenclature

N-6 and n-3 FA are PUFA with two or more double bonds in the carbon atom chain. The distinction between omega-3 and omega-6 FA begins with structural dissimilarities. In designation of FA structure, numerical notation indicates the number of carbon atoms followed by the number of double bonds and an omega designation (ω- or n-) referring to the position of the first double bond from the methyl terminus (Jho *et al*., 2004). For example, C18:2n-6 has 18 C-atoms and two double bonds, with the first double bond at the 6th carbon atom counted from the methyl end. C18:3n-3 has 18 C-atoms

and three double bonds, with the first bond at the 3rd carbon atom counted from the methyl group. C18:2n-6 is the parent compound of the n-6 family, whereas C18:3n-3 is the parent compound of the n-3 FA family.

3.2 Dietary sources

Vegetables are the main source of n-6 FA. The parent n-6 FA, C18:2n-6 is found in vegetable seeds and oils such as safflower, soybean, corn and sunflower (Table 1) (http://www.benbest.com/health/essfat.html, accessed 13 June 2008). The marine food chain is based on the n-3 FA, which are present in plankton and algae on which the fish feed (Bartsch *et al*., 1999). Several species of marine fish, which include fatty fish such as salmon, tuna, herring, mackerel and anchovy, offer a rich dietary source of eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22;6n-3). However, fish as a food source is not regularly included in the Western diet. Therefore, for the majority of the population, the alternative dietary source of long chain n-3 FA may be their precursor, C18:3n-3. This FA is found mainly in foods including perilla oil from the Asian beefsteak plant (Perilla frutescen), linseed oil, grapeseed oil, blackcurrent oil, flaxseed oil, borage oil, walnuts and green leafy vegetables such as purslane and spinach (Bartsch *et al*., 1999; Kurowska *et al*., 2003).

3.3 Metabolism of PUFA

Diet primarily provides EFA in the form of C18:2n-6 and C18:3n-3. In humans these 18-carbon precursors can be desaturated and elongated to more highly unsaturated members of their family, principally, arachidonic acid (C20:4n-6) and C20:5n-3. The liver is the primary site for EFA metabolism, although it does take place in other tissue as well (Pearce, 1983).

The n-3 and n-6 FA are not metabolically interconvertible but share the same the same enzymes when desaturated and elongated (Cho *et al*., 1999). The first part of the metabolic pathway leading to the synthesis of metabolically functional PUFA of the n-3 and n-6 series takes place in the endoplasmic reticulum (Benatti *et al*., 2004). C18:2n-6 and C18:3n-3, undergo sequential alternating desaturation and elongation steps that are catalyzed by Δ^6- and Δ^5-desaturase and FA elongase, (Fig. 5) (Benatti *et al*, 2004).

Table 1: FA percentages of commercially available dietary oils

Food	Palmitic acid C16:0	Stearic acid C18:0	Oleic acid C18:1n-9	Linoleic acid C18:2n-6	α-Linolenic acid C18:3n-3
Perilla oil	6	2	17	15	61
Flaxseed oil	3	7	21	16	53
Menhaden herring oil (1)	19	4	13	1	1
Canola oil	5	2	53	22	10
Walnut oil	7	2	15	60	10
Soybean oil	11	4	23	51	7
Butter (2)	25	11	26	2	2
Beef fat	29	20	42	2	0
Palm oil	45	5	38	10	0
Olive oil	14	3	71	10	0
Corn oil	11	2	25	55	0
Sunflower seed oil	6	4	24	65	0
Borage oil (3)	11	4	16	39	0
Evening primrose oil (4)	6	1	11	72	1
Safflower oil	7	3	15	75	0

(1) menhaden herring oil contains 1% EPA and 9% DHA
(2) 30% of butter is saturated fat of chain length less than 16 (butyric acid. C4:0 has a "butter" flavour)
(3) Borage oil contains 24% C18:3n-6
(4) Evening primrose oil contains 10% C18:3n-6
(Adapted from http://www.benbest.com/health/essfat.html, accessed 13 June 2008)

C18:3n-3 is desaturated and elongated to C20:5n-3 and C22:6n-3. C18:2n-6 is metabolized by Δ^6-desaturation to form γ-linolenic acid (C18:3n-6), which is rapidly elongated to dihomo-γ-linolenic acid (C20:3n-6). C20:3n-6 can be further desaturated to C20:4n-6. However, due to the limited activity of Δ^5-desaturase in rodents and humans, only a small fraction of C20:3n-6 is converted to C20:4n-6 (Johnson *et al*., 1997). The synthesis outcome is dependent on competition between C18:3n-3, C18:2n-6 and some of their longer chain products for Δ^6-desaturation, the influence of various hormones on desaturation activity, and negative feed-back regulation of long chain (LC)PUFA on both the Δ^6- and Δ^5-desaturases (Nakamura and Nara, 2004).

The n-3 and n-6 FA are competitive inhibitors of each other's metabolism. The competition is apparent at the desaturation step (Fig. 5), where a large amount of one type of FA will interfere with the metabolism of the other. With regards to FA metabolism, the rate limiting Δ^6-desaturase enzyme is important in regulating the level of LCPUFA (Horrobin, 1993). It has been shown that the

Δ^6-desaturase enzyme is compromised by a decrease in activity in cancer cells and tumours (Horrobin, 1993). In effect, this results in low levels of LCPUFA in the cancer tissue.

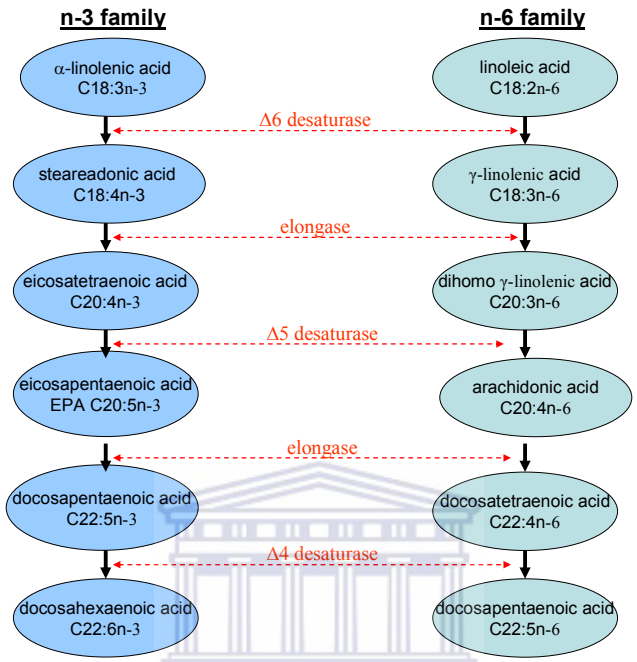

Fig. 5 Metabolism of EFA α-linolenic acid (n-3 FA) and linoleic acid (n-6 FA) to metabolically functional PUFA. (Adapted from Sprecher, 2002).

3.4 Biological and functional effects of PUFA in the mammalian cell

Membranes are of fundamental importance to cell structure and function. FA are the defining constituents of membrane lipids and are therefore responsible for their distinctive physical and metabolic properties. Any PUFA produced in the endoplasmic reticulum (ER) may be removed from the biosynthetic cascade and used for membrane lipid synthesis by enzymes localized in the ER (Sprecher, 2002).

3.4.1 Components of membrane structure

PUFA are important constituents of the phospholipids, where they appear to confer distinctive properties to the membrane, in particular by decreasing their rigidity. Phospholipids are the dominant lipids in all cell membranes. These glycerol based lipids contain side chains with variable FA contents namely,

phosphatidylserine (PS), phosphotidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) (Juanéda *et al*., 1990)

The phospholipids bilayer forms the basic structure of all membranes. Membrane phospholipids distribution is asymmetrical, with PC and sphingomyelin (SM) located in the outer leaflet, and PE and PS located in the inner leaflet (Deveaux, 1991). Most phospholipids contain a PUFA at the second carbon atom (sn-2 position) of the glyceride backbone and a saturated FA or monounsaturated FA at the sn-1 position. The presence of saturated and monounsaturated FA ensure that there is a correct balance between rigidity and flexibility.

The FA composition of phospholipids determines biophysical (and functional) characteristics of membranes (e.g. membrane fluidity) (Burns *et al*., 1979), and influence the conformation and activity of many membrane associated enzymes and receptors involved in the second messenger system and cell signalling (Woutersen *et al*., 1999; Wiseman, 1996). With diet being a major source of FA, intake can modulate membrane FA composition and alter membrane associated functions (Yehuda et al, 2005).

3.4.2 Functional properties of PUFA

The importance of PUFA to health has been highlighted by studies reviewing FA deficiencies (Ruggieri *et al*., 1976; Holman *et al*., 1991). PUFA and their metabolites have two main physiological functions. Firstly PUFA are structural components of cellular membranes. In the retina and the brain, C20:5n-3 and C22:6n-3 is essential structural components (Crawford *et al*., 1978; SanGiovanni and Chew, 2005). PUFA confer distinctive properties to the membrane, in particular by decreasing their rigidity. Without the availability of PUFA, membranes will incorporate more saturated FA resulting in less fluid and unstable membranes.

In addition, PUFA are 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 PUFA of the n-6 series have been shown to lower plasma cholesterol (Horrobin and Manku, 1983).

3.4.2.1 Eicosanoid production by PUFA substrates

The role of EFA such as C18:2n-6 and C18:3n-3 obtained from plant ingredients in the diet is crucial. Without a source of these precursory FA, the production of eicosanoids would be compromised. In the mammalian organism eicosanoids are synthesized by every tissue and cell type and seem to be involved in the majority of physiological events. At the cellular level, they may act in a typical hormone-like manner by playing a role in normal metabolic processes (Tapiero *et al.*, 2002). Metabolic activities influenced by eicosanoids include platelet aggregation, inflammation, haemorrhage, vasoconstriction, blood pressure and immune function (Benatti *et al.*, 2004).

Fig. 6 Arachidonic acid
(http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/prostag.htm, accessed 28 February 2007)

Most eicosanoids are derived from C20:4n-6 (Fig. 6) (http://www.rpi.edu/dept/bcbp/molbiochem/MBWeb/mb2/part1/prostag.htm, accessed 28 February 2007). In unstimulated cells, this 20-carbon FA is found to be sequestered in phospholipids from which it is released upon stimulation. As a consequence, the eicosanoids are produced only on demand. Thus, availability of these mediators is tightly controlled by the cellular concentrations and activities of the enzymes involved in their biosynthesis. These enzymes may be classified into four major groups, phospholipases for the signal induced release of C20:4n-6 from membrane phospholipids; prostaglandin endoperoxide synthases or cyclooxygenases (COX) for the metabolism of C20:4n-6 to prostanoids, i.e. prostaglandins, prostacyclins, and

thromboxanes; lipoxygenases (LOX) for the formation of hydroxylated eicosatetranoic acids (HETEs), leukotrienes etc. and cytochrome P450-controlled monooxygenases for epoxidation and hydrolation of C20:4n-6 (Fig. 7) (Marks *et al*., 2000).

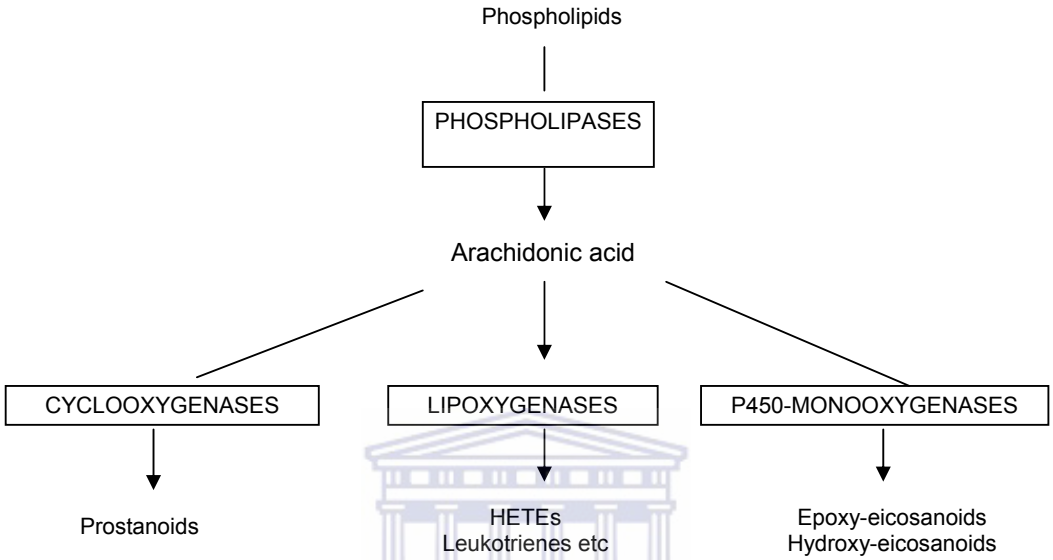

Fig. 7 Major enzymatic pathways involved in the formation of eicosanoids from lipid FA (Marks *et al*., 2000).

Other 20-carbon PUFA, such as C20:3n-6 and C20:5n-3 is also substrates for eicosanoid production cascades that parallel and compete with the C20:4n-6 cascade. C20:5n-3 is a precursor of the prostaglandin of the 3-series, with three double bonds. The n-3 FA is converted by the same COX enzyme to the 3-series endoperoxide PGH_3, by way of PGG_3, which can be further metabolised to PGE_3 (Rose and Connolly, 1999). C20:3n-6 provides a third, less prominent cascade and yields prostaglandins of the 1-series. Although C20:3n-6 and C20:5n-3 is generally poorer substrates for prostaglandin synthase than C20:4n-6, they both compete for the enzyme binding site and can reduce the production of C20:4n-6-derived prostaglandins (Tocher *et al*., 1997). Therefore, the supply of precursor PUFA for eicosanoid synthesis is directly related to the FA composition of membrane phospholipids which in turn is influenced by dietary PUFA intake and metabolism

Prostaglandins occur at very low levels in nearly all-mammalian tissue. Prostaglandins have been found to exert a wide variety of pharmacological

effects on humans and animals: PGE_2 contraction and relaxation of smooth muscles of the uterus, PGE_1 has vasodilatory properties, PGI_2 reduces blood pressure and inhibits platelet aggregation by reducing calcium concentrations (Pai *et al*., 2002; Tapiero *et al*., 2002). These PUFA derivatives have also been shown to regulate cell processes such as mitosis, cell proliferation and cell adhesion (Rishikesh and Sadhana, 2003).

3.4.2.2 PUFA modulates signal transductions

PUFA released from membrane phospholipids by cellular phospholipases or exogenous sources are important signalling molecules (La et al., 2003). Research has shown that FA can act as second messengers or regulators of signal-transducing molecules, hereby regulating the transmission of signals from the extracellular environment (Sumida *et al*., 1993). The effects of PUFA on gene expression can be sustained for a time period dependent on its availability through the diet. In these cases, the PUFA acts like a hormone to control the activity or abundance of key transcription factors (Jump, 2004).

PUFA effects on gene expression are cell specific and influenced by FA structure and metabolism. The molecular mechanism by which PUFA interact with the genome through several mechanisms is not fully understood. Studies suggest that these effects might be mediated by ligand-activated transcription factors. The peroxisome proliferators activated receptor (PPAR), a member of nuclear receptor supergene family of transcription factors, is suggested to be activated by PUFA. PPAR monitors the levels of nonesterified FA (Pawar and Jump, 2003). As such, FA binding to PPAR leads to changes in the transcription of many genes involved in lipid metabolism and storage.

PUFA can cause protein phosphorylation, which is important for the activation or inhibition of enzymes involved in receptor mediated signal transduction pathways. Protein kinase C (PKC) phosphorylates target proteins in cells that regulate cellular proliferation and differentiation (Pajari *et al*., 1997). The activation of PKC is dependent on the lipid and FA composition of membrane phospholipids (Nishizuka, 1992).

3.4.2.3 Oxidative stress inducers

Oxidative stress occurs when the production of oxidising agents, free radicals and reactive oxygen species (ROS) exceeds the antioxidant capacity of cellular antioxidants in a biological system (Schrader & Fahimi, 2006). This imbalance leads to tissue injuries and to the progression of degenerative diseases in humans such as cancer (Wijeratne & Cuppett, 2006).

PUFA in membrane lipids containing two or more bonds, are opportune targets for free radicals. PUFAs create oxidative stress in biological systems as they under go lipid peroxidation, forming free radicals such as peroxyl and alkoxyl radicals. Although these lipid hydroperoxides are short-lived, their breakdown results in the formation of secondary products of lipid peroxidation (aldehydes such as malondialdehyde and 4-hydroxyalkenals) that are longer-lived and can attack a variety of cellular targets (http://findarticles.com/p/articles/mi_m0FDN/is_1_7/ai_83582815/, accessed 24 September 2008).

Lipid peroxidation is initiated by the attack on a FA or fatty acyl side chain of any chemical species that has sufficient reactivity to abstract a hydrogen atom from a methylenes carbon in the side chains. The greater the number double bonds in a FA side chain, the easier is the removal of a hydrogen atom, which is why PUFAs are particularly susceptible to peroxidation. The removal of a hydrogen atom from a PUFA double bond produces a reactive species that can propagate further reactions (Fig. 8).

Products from this oxidative process may in turn react with DNA to form ethno- or malonaldehyde-derived DNA adducts that may cause misreplication during DNA synthesis (De Kok *et al*., 2003). The affected cell can try to repair this damage, which may involve exiting from the cell cycle and hence an anti-proliferative effect, or if damage is too severe cell death can occur (Diggle, 2002). Cell death can occur by necrosis, but lipid peroxidation can trigger the process of apoptosis, activating the intrinsic suicide pathway present within all cells (Gago-Dominguez *et al*., 2005).

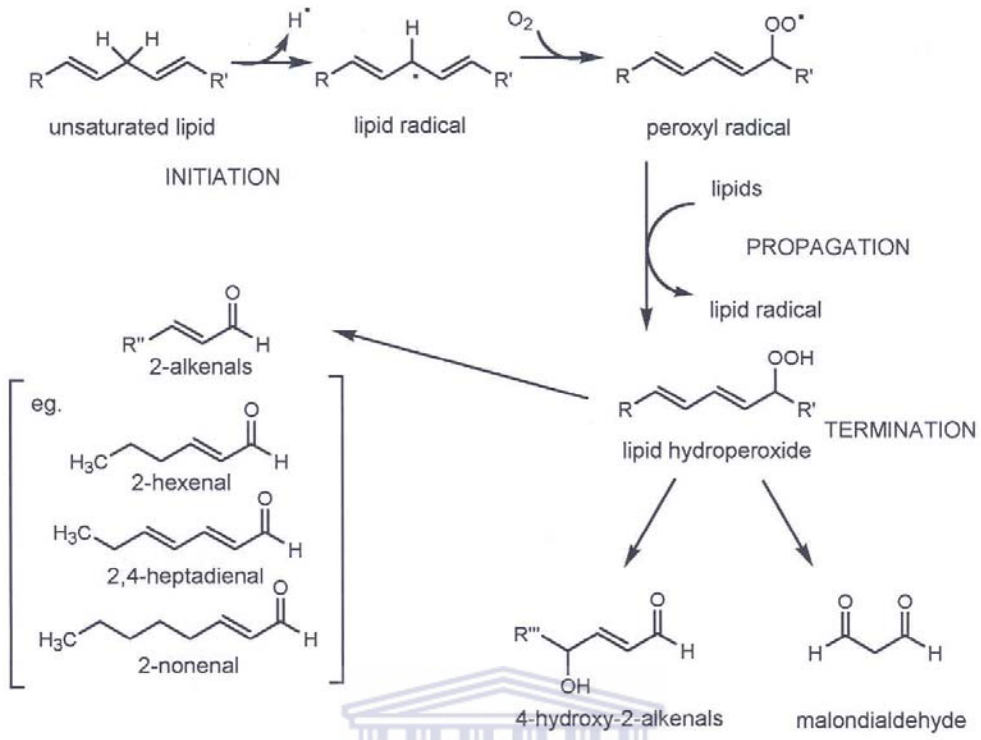

Fig. 8 Schematic view of lipid peroxidation. Free radicals attack membrane lipids unsaturated FA, setting in motion a chain reaction which generates reactive species with the potential to cause cellular damage. (http://www.library.wur.nl/wda/dissertations/dis3424.pdf, accessed 7 March 2006).

3.5 PUFA and cancer development

With the FA content of membranes playing an important role in normal cell function, clearly changes can have a profound effect on cellular functions such as signal transductions and membrane trafficking. Abnormalities in FA metabolism have been suggested to play a critical role in cancer development. Studies show that the impairment of Δ^6-desaturase activity to be a characteristic feature of cancer cells (Horrobin, 1990). Impaired enzyme activity reduces the synthesis of the biologically functional LCPUFA. As a result, key membrane associated functions linked to membrane LCPUFA availability is affected. Consequently, these alterations play an important role in the abnormal cellular growth, which prevails in preneoplastic lesions.

A causal relationship between cancer development and an abnormal over expression of eicosanoid forming enzymes has been implicated with a wide

variety of tumours (Accioly *et al*., 2008). After the release of C20:4n-6 from membrane phospholipids by phospholipases, it can be enzymatically oxidised to PGG_2 and PGH_2, the parent compounds of prostaglandins by cyclooxygenase (COX) (Whelan and McEntee, 2004). Formation of PGH_2 is the committed step in the biosynthetic pathway. There are two COX isoforms, COX-1 and COX-2. COX-1 is constitutively expressed at low levels in most tissues and is thought to have cellular housekeeping function. COX-2 is not normally expressed in most tissues and is considered to be the inducible form of the enzyme. COX-2 has been detected in virtually all cancers, and its expression is variable and dependent on the stage of the neoplasia (Prescott and Fitzpatrick, 2000). Key events of tumour promotion such as cellular hyper proliferation and inhibition of programmed cell death have been found to be related to the pathological over expression of the pro-inflammatory enzyme COX-2. Genotoxic by-products of COX-2 catalyzed FA metabolism (such as active oxygen species, free radicals etc.) are suspected to contribute to genetic instability and thus to malignant progression of tumour cells.

Two PGs, PGE_2 and PGI_2, derived from C20:4n-6 has been linked to colorectal cancer development. When PGs are produced, they act locally on cell-surface receptors in paracrine or autocrine manner to mediate their pathological effects (Cao and Prescott, 2000). PGE_2 mediated tumourigenesis via 4 G-protein coupled receptors (EP 1-4) that modify intracellular calcium or cAMP (Hansen-Petrik *et al*., 2002). Cytoplasmic fluxes in either cAMP or calcium can have broad downstream effects on colorectal neoplasia; however the precise molecular mechanisms have yet to be clarified. PGI_2 appears to mediate its protumourigenic effects via the activation of peroxismal proliferator-activated receptor-γ, which is up regulated in colorectal tumours, promoting tumourigenesis and inhibiting apoptosis (Whelan and McEntee, 2004).

It has been shown that several signal transduction systems including the PKC system are involved in the regulation of cell proliferation and this may play a role in fast growing cells. A key enzyme in this system is phospholipase C (PLC) which is a membrane bound enzyme that provides the second messengers for activation of PKC. In the human colon cell line, HT-29

adenocarcinoma cells, it has been shown that alterations of the membrane lipid content by the addition of EFA influences cell proliferation by modulating the activity of phospholipases (Awad *et al.*,1996a). PLC activity was shown to be much lower in cancer cells exposed to n-6 FA (C18:3n-6) compared to n-3 FA (C18:3n-3, C20:5n-3 and C22:6n-3).

Oxidative stress is considered to play a major role in carcinogenesis, with diet having a significant effect on the production of ROS (Kondo *et al*., 1994; Rainis *et al*., 2007). The unregulated or prolonged production of ROS has been linked to mutation (induced by oxidant-induced DNA damage), as well as modification of gene expression, which are characteristic of carcinogenesis (Bartsch and Nair, 2002). Owing to their structure, which is rich in double bonds, PUFA render cellular membranes vulnerable to damage from free radicals, resulting in a lipid peroxidation cascade. Under normal cellular conditions excessive ROS damage to lipid membranes can be prevented by cellular natural defences (Hiraishi *et al*., 1991). However, the role of oxy-radicals during the initiation and promotion stages of carcinogenesis has been suggested to resulting in a decreased PUFA content in the tumour subcellular membranes (Masotti *et* al., 1988; Dudeja and Brasitus, 1990).

A reduction in tumour membranes degree of unsaturation is the most relevant rate-limiting factor of peroxidation. Membranes decrease in PUFA content has been linked to the activities of the FA metabolising enzymes such as Δ^6-desaturase, which is much lower in tumours (Hrelia *et al*., 1994). In Morris hepatoma cells, alterations to the membranes lipid composition, particularly regarding the degree of unsaturation and the loss of protective enzymes against oxygen radicals, such as superoxide dismutase (SOD), increased microsomal membranes resistance to lipid peroxidation (Galeotti *et al*., 1984). Increasing cellular peroxidation by enhancing membranes PUFA content can play a role in the control of cell proliferation by inhibiting cell growth and stimulating or enhancing apoptosis by (Bartsch *et al*., 1999). Thus, increasing membranes resistance to lipid peroxidation may play an important role in promoting neoplastic growth.

4. Chemoprevention of cancer using PUFA

Chemoprevention by definition is the use of agents to slow the progression of, reverse, or inhibit carcinogenesis, thereby lowering the risk of developing invasive or clinically disease (Sun *et al*., 2004). The goal of any therapeutic strategy is to impact on the target cell within limited detrimental effect to normal cell function (Kasibhatia and Tseng, 2003). Suggested mechanisms of cancer prevention at a cellular level have been reported to be the promotion of differentiation, induction of apoptosis and inhibition of proliferation in tumour cells (Latham *et al*., 1999).

As previously discussed, PUFA plays a critical role in cellular regulatory processes. Modulation of the FA content in membranes through dietary intake could thus be a plausible mechanism by which cancer progression could be delayed.

4.1 n-3 PUFA

Animal experiments and *in vitro* studies have highlighted that long chain, marine n-3 PUFA C20:5n-3 and C22:6n-3 is able to suppress the development of colon cancer (Davidson *et al*., 2004; Takahashi *et al*., 1997; Chen and Istfan, 2001). C20:5n-3 and C22:6n-3 ingested through the diet, enrich the membrane phospholipids pool, resulting in competition with n-6 PUFA such as C20:4n-6 for Δ^6-desaturase and COX enzymes, reducing eicosanoid biosynthesis (Larsson *et al*., 2004; McEntee and Whelan, 2002). Therefore, n-3 FA inhibits tumour development by competitive inhibition of C20:4n-6 metabolism, as well as by the unique opposing effects of n-3 derived metabolites.

The incorporation of n-3 FA into cells membrane phospholipids can alter membrane fluidity and modulate cell signalling (Mahèo *et al*., 2005). Due to low PUFA content in tumour cells, lipid peroxidation is low, providing a good environment for growth. Another possible mechanism by which dietary fish oil rich in n-3 LCPUFA such as C20:5n-3 and C22:6n-3 inhibit carcinogenesis is that n-3 PUFA may increase the accumulation of lipid peroxidation products in tumour cells, which may decrease cell proliferation and increase cell death

(Latham *et al*., 1999; Woutersen *et al*., 1999). Hong *et al*. (2002) showed that fish oil increases the oxidative stress levels in rat colonocytes. With the elevation of mitochondrial phospholipid unsaturation, an increase in ROS initiated an apoptotic cascade, which is suggested to inhibit colon cancer development.

Overall, it appears that the n-3 FA achieve their anticancer effects by competitive inhibition of n-6 FA metabolism, which suggests that it is not the total amount of n-3 FA, but the balance of n-3 FA and n-6 FA that is important.

4.2 n-6 PUFA: γ-linolenic acid (C18:3n-6)

C18:3n-6 is metabolized to C20:3n-6, a precursor for the anti-inflammatory 1-series prostaglandins. Dietary supplementation with C18:3n-6 has been shown to influence the production of eicosanoids by its interference with the metabolism of C20:4n-6 to bioactive metabolites (Johnson *et al*., 1997). Research suggests a link between the irritation caused by an inflammatory response and colon cancer development (Nakanishi and Rosenberg, 2006). Thus, as a preventive measure to the biosynthesis of tumour promoting C20:4n-6 metabolites, an increase in tissue C18:3n-6 attenuates their production. As C18:3n-6 bypasses the Δ^6-desaturase, a key regulatory rate-limiting enzymatic step controlling the formation of PUFA, it may alleviate any decrease in the resultant long chain desaturated FA as noticed in cancer cells (Galeotti *et* al., 1984).

In vitro studies have shown that C18:3n-6 can selectively kill tumour cells while causing little or no harm to normal cells. Seegers *et al.* (1997) have shown that C18:3n-6 causes inhibition of cell progression and induces apoptosis in transformed cells. C18:3n-6 has the ability to inhibit both motility and invasiveness of human colon cancer cells by increasing the expression of E-cadherin, a cell-to-cell adhesion molecule that acts as a suppressor of metastasis (Jiang *et al*., 1997).

4.3 n-6/n-3 FA ratios

The balance of EFA is important for good health and normal development. In Western industrialised countries diets contain levels of fat high in n-6 PUFA (e.g. corn oil), which are associated with tumour promotion (Wu *et al*., 2004). The development of breast cancer, for example, has been associated with the consumption of high n-6/n-3 PUFA ratios (Capone *et al*., 1997). It has been shown that dietary n-6 FA influences cancer development by affecting biochemical events that occur after the initiation of a cancerous cell, whereas the addition of n-3 FA to the diet can block the promoting effects of n-6 FA (Cave, 1997). It is therefore necessary to reduce the adverse effects of n-6 PUFA and its metabolic products through dietary supplementation with n-3 PUFA from fish and fish oils.

However, studies suggest that the dietary n-6/n-3 PUFA ratio, rather than the quantity of n-3 PUFA is a key factor in the anti-tumour effect (Rose and Connolly, 1999). Intake of fish or fish oil FA C20:5n-3 and C22:6n-3 partially replaces the n-6 FA, especially C20:4n-6, in the membrane of all cells (Simopoulos, 2002). It is therefore possible that the ratio of n-6/n-3 PUFA entering the cellular pool from dietary sources can alter the ratio of eicosanoid precursor FA in membrane phospholipids.

It is important to note that although the therapeutic strategy is to reduce the n-6 FA content in tumour membranes through supplementation with n-3 rich food, n-6 FA intake needs to be maintained in order for normal cell function to continue. Therefore, a balanced n-6/n-3 FA ratio is an important factor in terms of metabolic effects. Historically, before the advent of the agricultural revolution, the n-6/n-3 FA ratio was maintained at approximately 1:1. However, with the modernization of cultures and the availability of processed foods the ratio has shifted in favour of n-6 FA, with a n-6/n-3 ratio of 15-20:1 (Simopoulos, 2002). Thus strategies have been proposed that a lower n-6/n-3 PUFA ratio is more desirable in reducing the risk of chronic diseases such as cancer.

When proposing a dietary intervention strategy for cancer therapies, the type of FA constituting the ratio is the critical factor to be considered. Cancer modulation studies indicate that low fat diets containing fish oil significantly decreases the n-6/n-3 PUFA ratio in plasma and gluteal adipose tissue in men with prostate cancer (Aronson *et al*., 2001). Administration of high doses of fish oil inhibits Δ^6-desaturation and reduces the n-6 FA content in tumour membranes. In association with dietary C18:3n-6, the reduction by fish oil of the levels of n-6 derived FA can be maintained at an optimal level to compensate for normal cell functions. In cancer cells Δ^5- and Δ^6-desaturase activity is impaired, thus C18:3n-6 is elongated to C20:3n-6 without further desaturation to C20:4n-6. This could explain why C18:3n-6 supplementation may attenuate C20:4n-6 metabolism in some cells (Johnson *et al*., 1997). Thus C20:5n-3 given with C18:3n-6 raises the levels of the desirable C20:5n-3 and C20:3n-6, without raising C20:4n-6 (Nassar *et al*., 1986; Horrobin *et al.,* 1984).

Abnormal EFA metabolism, such as the inability of Δ^6-desaturase to synthesize C18:3n-6 and C20:3n-6, or an overproduction of the undesirable 2-series eicosanoids from C20:4n-6, has been linked to the development of many chronic degenerative diseases including cancers. This defect can thus be potentially overcome by dietary supplementation with a mixture of C18:3n-6-rich oil (e.g. borage oil) and C20:5n-3-rich fish oil. Research has shown that C18:3n-6 supplementation can bypass the pathologically defective Δ^6-desaturase activity and elevate the formation of C20:3n-6 and subsequently the synthesis of the desirable 1-series eicosanoids (Fan and Chapkin, 1998). C20:5n-3 supplementation meanwhile increases tissue C20:5n-3 levels, which not only suppress the C20:4n-6 levels, but also reduces the synthesis of 2-series eicosanoids with their undesirable effects. In addition, C20:5n-3 also suppresses the conversion of C20:3n-6 to C20:4n-6, increases the ratio of C20:3n-6 to C20:4n-6 in tissue and plasma phospholipids, and hence increases the formation of 1-series eicosanoids. Thus dietary supplementation with the proper combinations of C18:3n-6 and C20:5n-3 could modulate the synthesis of 1-, 2- and 3-series eicosanoids.

Subsequently, a balanced eicosanoid metabolism may reduce the development of cancer.

In addition to modulating eicosanoid production, dietary intakes of low n-6/n-3 FA ratios increase membranes PUFA content (Abel *et al.,* 2004). Due to the relative increase of PUFA, particularly the n-3 LCPUFA C20:5n-3 and C22:6n-3, membranes are more susceptible to lipid peroxidation (Song *et al.,* 2000). An increase in lipid peroxidation increases the oxidative stress levels within cells due to the production of lipid oxidative products, which have deleterious effects due to stimulating apoptosis (Lotem *et al.,* 1996).

In cancer cells the level of lipid peroxidation is low due to a reduction in membrane PUFA content (Cheeseman *et al.,* 1988). Thus, by specifically increasing membranes n-3 LCPUFA through dietary fat intake, cancer cells are made more susceptible to oxidative damage and are therefore stimulated to undergo apoptosis due to an increase in oxidative stress (Narayanan *et al*., 2001). The study by Abel *et al.* (2004) illustrated that low dietary n-6/n-3 FA ratios not only alter membranes FA profile in hepatocyte nodules, but also increased susceptibility to lipid peroxidation. By increasing the oxidative stress within cancer cells, processes regulating apoptosis could be stimulated (Latham *et al*., 1999). This strategy utilizing low dietary n-6/n-3 FA ratios to modulate membranes lipid profile could be useful tool in the chemoprevention of cancer.

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CHAPTER 2

LIPID PROFILE OF COLON POLYPS AND NORMAL MUCOSA TISSUE IN RATS FED A DIET HIGH IN N-6 POLYUNSATURATED FATTY ACIDS

Abstract

Several abnormal conditions, including some cancers, have been associated with changes in the membrane lipid and FA composition. Dietary fat serves as a major source of lipids and FA, particularly the polyunsaturated fatty acids (PUFA), n-6 and n-3. High intakes of n-6 PUFA have been linked to the development of colon cancer in association with low n-3 PUFA intake. Therefore understanding the differences in the lipid and FA profiles between cancer and normal cells in the colon, and the role diet plays in these factors may be invaluable in understanding their role in carcinogenesis. This study compares the lipid profile of azoxymethane (AOM) induced colon polyps to that of the surrounding mucosa tissue in rats fed a diet high in n-6 PUFA. Male Fischer rats were fed the AIN-76A diet containing sunflower oil that has high n-6 PUFA content for a period of nine months. Results indicate that the lipid and FA content of the colon polyps differs significantly from the surrounding mucosa. Colon polyps had an increase in membrane phopholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE). Changes in membrane fluidity were indicated by the decrease ($p<0.05$) in the PC/PE and cholesterol/phospholipids (chol/PL) ratios, and increase ($p<0.05$) in the polyunsaturated FA/saturated FA (P/S) ratio. Metabolism of FA was significantly altered in the polyps favouring n-6 FA metabolism and the production of prostaglandin E_2. No clear indication of impaired Δ^6-desauturase enzyme activity was noticed. Increases in the n-6 PUFA content could be a reflection of the dietary FA intake that increases FA incorporation in the polyps. Changes in the FA parameters of the polyps, particularly an increase in C20:4n-6 and the n6/n3 ratio have been shown to contribute to the rapid growth of cancer tissue. These lipid changes associated with the development of colon polyps could provide unique targets for developing strategies in chemoprevention by dietary manipulation.

Keywords: colon polyps, membrane lipid profile, n-6 polyunsaturated fatty acids

2.1 Introduction

Fatty acids (FA) as integral components of cell membranes play an important role in normal cell function. Membrane FA profiles influences cell structure with regards to membrane phospholipid composition, membrane fluidity and the activity of membrane enzymes and receptors (Goldberg and Zidovetzki, 1997; Lund *et al., 1999*). In addition, FA act as signalling molecules involved in cell proliferation and/or apoptosis (Hwang and Rhee, 1999; Artwohl *et al.*, 2004).

Studies have indicated that the altered lipid content of tumour tissue appears to play an important role in the process of carcinogenesis (Eriksson and Andersson, 1992). With tissue phospholipids being metabolically active and providing a dynamic reservoir of long-chain polyunsaturated fatty acids, alterations of the membrane FA content of tumour cells may play an important role in their altered growth characteristic (Jones *et al.,* 2003).

Although the role of individual FA in human cancer development is still poorly understood, animal studies have shown that diets containing high levels of n-6 polyunsaturated fatty acids (PUFA) increase the incidence of chemically induced colonic tumours in rats (Reddy *et al*., 1991). Several studies indicate that diets high in lard, beef tallow, or corn oil, fat sources that have a high saturated fat and n-6 PUFA content increase the concentration of colonic luminal secondary bile acids (Craven *et al.,* 1987; Chapkin *et al.,* 1993). Secondary bile salts have been shown to induce cell proliferation and to act as promoters in colon carcinogenesis (Bull *et al.,* 1993). In humans the high consumption of n-6 PUFA have been shown to increase the formation of exocyclic DNA adducts in white blood cells (Nair *et al.,* 1997). These biomarkers of lipid peroxidation-derived DNA damage have been utilized to identify the links between increased intakes of dietary n-6 PUFA, DNA damage and the elevated risk for cancers of the breast, colon and prostate.

Several mechanisms have been proposed to implicate n-6 PUFA involvement in the promotion of cancer development. One of these mechanisms is associated with the production of eicosanoids derived from PUFA substrates

(Tapiero *et al.*, 2002). With the dietary intake and metabolism of dietary n-6 PUFA such as linoleic acid, the availability of the metabolically active long chain PUFA, arachidonic acid (C20:4n-6) increases (Nicholson *et al.,* 1991). C20:4n-6 derived metabolites serve as precursors for the synthesis of the short-lived 2-series prostaglandins (PG) that act in an autocrine or paracrine manner to convey their biological effects. Evidence linking the effects of dietary fat on mammary carcinogenesis to PG synthesis derives mainly from the observation that non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the promoting effects of diets rich in n-6 PUFA (Carter *et al.,* 1983*;* Cunningham *et al.,* 1997*)*. PG synthesis, particularly of the 2-series, also suppresses the immune response and may thereby promote cancer development (Marnett, 1992).

With the lipid FA content of cell membranes playing an important role in maintaining cellular structure and function, the characterization of lipid alterations associated with tumour development would be of importance. The present investigation evaluated the differences in the lipid profiles of cancerous tissue and surrounding tissue in a colon cancer animal model with rats fed a diet high in n-6 PUFA.

2.2 Methods and materials

2.2.1 Chemicals

Azoxymethane (AOM), sodium pentobarbital, perchloric acid, methanol and chloroform, fluorescing agent 2,5-bis-(5'-tert-butylbenz-oxazolyl-[2'])thiophene (i.e. BBOT), butylated hydroxytoluene (BHT), sodium hydroxide (NaOH), malachite green and the phospholipid standards were obtained from Sigma-Aldrich. Acetic acid, petroleum ether, boric acid, sodium carbonate (Na_2CO_3), sodium tartrate, copper sulphate ($CuSO_4.5H_2O$) and silica thin layer chromatography plates were supplied by Merck (Darmstadt, Germany). Sulphuric acid was obtained from BDH/Merck (Poole, England), sodium dodecyl sulphate from Fluka (Darmstdat, Germany) and bovine serum albumin (BSA) from Mees Laboratories (South Africa). The sunflower oil was supplied by RFS Catering Supplies (Milnerton, South Africa).

2.2.2 Induction of colon polyps

The Ethics Committee of the Medical Research Council of South Africa approved the use of laboratory animals in this study. Starting at 7 weeks of age (approximately 150 g body weight), male Fischer rats (n=13) were injected i.p. with 15 mg/kg azoxymethane (AOM) once a week for three consecutive weeks. The rat body weights were determined three times weekly.

2.2.3 Animals and treatment

The animals were housed individually in wire-bottomed cages under controlled lighting (12 hr cycles) and temperature (23-25°C) with free access to water. All rats were fed the AIN-76A diet (AIN, 1977) containing sunflower oil as a fat source for 9 months following the AOM treatment. The FA profile of sunflower oil is summarized in Table 1. Nine (9) months after initiation, all the rats were euthanized by injection (i.p.) with sodium pentobarbital (0.15 ml /100 g body weight). The colon from each rat was removed and opened longitudinally. The macroscopic polyps were excised and the surrounding epithelial mucosa collected by scraping the inner surface with a microscope glass slide. The polyps and surrounding mucosa samples were frozen in liquid nitrogen and stored at -80 °C until analyzed.

2.2.4 Lipid extraction

For the colon polyp and surrounding mucosa lipid extraction, the tissue was ground to a fine powder in liquid nitrogen. A sub fraction of the mucosa powder (10-30 mg) was taken for protein analyses while the remaining sample (50-100 mg) mucosa samples were used for the lipid extractions. Lipids were extracted from the colon polyps and mucosa with chloroform/methanol (CM; 2:1; v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant. Briefly, the method adapted from Folch *et al.* (1957): polyp and surrounding mucosa tissue previously ground to a fine powder in liquid nitrogen, were weighed (80-100 mg) and resuspended in 0.5 ml saline and the lipids were extracted with 12 ml CM. The CM mixture was filtered (sinterglass filters using Whatman glass microfibre filters, Cat N, 1820 866, Whatman International, Ltd, Maidstone, England), and the filtrate was evaporated to dryness in vacuo

at 40 °C. The extract was, transferred to glass culture tubes (5 x 2 ml CM), washed with saline saturated with CM (1 ml), and stored at 4 °C under nitrogen until analyzed.

Table 1: Fatty acid content (%of total FA) of sunflower oil.

Fatty acid	% of total FA
C14:0	0.07±0.01
C16:0	6.68±0.18
C17:0	0.04±0.02
C18:0	4.45±0.07
C20:0	0.29±0.02
C22:0	0.75±0.06
C24:0	0.25±0.01
SATS total	12.53±0.37
C16:1	0.06±0.02
C18:1	25.17±0.50
C20:1	0.21±0.01
C22:1	0.02±0.01
C24:1	0.05±0.01
MUFA total	25.50±0.55
C18:2n-6	62.25±0.76
C18:3n-6	<0.01
C20:2n-6	0.02±0.01
C20:3n-6	<0.01
C20:4n-6	<0.01
C22:4n-6	<0.01
C22:5n-6	<0.01
n-6 total	62.31±0.77
C18:3n-3	0.25±0.01
C20:3n-3	<0.01
C20:5n-3	<0.01
C22:5n-3	<0.01
C22:6n-3	<0.01
n-3 total	0.25±0.01
n-6/n-3	249.24±1.64
PUFA	62.56±0.78
P/S ratio	4.99±2.11

n=2; SATS = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; P/S = PUFA/SATS.

2.2.5 Thin layer chromatography (TLC)

The lipid extracts were fractionated by thin layer chromatography (TLC). Lipid extracts (i.e. 30 μl spot per sample) were applied on 2 separate silica (10 x 20 mm) TLC plates for fatty acids (FA) and phospholipids analyses, respectively. The plates were developed using the solvent chloroform: methanol: petroleum ether: acetic acid: boric acid (40:20:30:10:1.8; v/v/v/v/w) containing the fluorescent agent 2,5-bis-(5'-tert-butylbenz-oxazolyl-[2'])thiophene (i.e. BBOT), for 90 minutes (Addendum 1). The plates were dried under nitrogen

gas and phospholipids visualized under UV light. The lipid fractions PC and PE were identified against phospholipid standards.

2.2.6 Fatty acid analyses

For FA analyses 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 hrs (Benadé *et al.,* 1988), and the resultant FA methyl esters (FAME) extracted with hexane and distilled water (v/v, 2:1). The hexane (top layer) was removed and dried under nitrogen gas in a water bath (37 °C) and the samples dissolved in hexane prior to analyses by gas chromatography. The FAME were analyzed by gas chromatography on a Varian 3300 gas Chromatograph equipped with 30 m fused silica BPX-70 capillary columns (SGE, USA) of 0.32 mm internal diameter (Addendum 2). The individual FAME were identified by comparison of the retention times to those of a standard FA mixture containing C14:0 to C24:1 (Nu-Chek-Prep, MN, USA) (Addendum 3). 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 (Addendum 3).

2.2.7 Phospholipid determination

The phospholipids fractions PC and PE were determined colorimetrically based on the method according to Itaya and Ui (1966). Briefly, PC and PE fractions were separated by TLC (see section 2.2.5), and digested in perchloric acid (i.e. PC: 400 μl; PE: 300 μl) on a 170 °C heating block for two hours. After digestion, distilled water was added to the samples at a ratio of 5: 1. Samples were vortexed and centrifuged at 500 rpm for 15 minutes and 250 μl of the samples were transferred to clean tubes. A volume of 1 ml of phospholipid reagent (Addendum 4) containing malachite green, ammonium molbydate and tween at a ratio of 15:5:1 was added to each sample, and incubated at room temperature for 20 minutes, and the colour development measured spectrophotometrically at 660 nm and referenced against a phosphate standard curve.

2.2.8 Cholesterol determination

The cholesterol content of the polyps and surrounding mucosa samples was determined by an enzymatic iodide method using cholesterin-oxidase and cholesterin-esterase (Richmond, 1973). Briefly, to 20 μl lipid CMS extract (see section 2.2.4) a volume of 50 μl chloroform:methanol (2:1 v/v) was added per sample and vortexed. Peroxide free Triton X-100 (300 μl) was added, vortexed and evaporated to clearness under nitrogen gas. Cholesterol reagent (1.7 ml) (Addendum 5) was added to each sample and vortexed. The cholesterol reagent B (20 μl) was added to 1 ml of the lipid sample solution transferred into clean glass tubes, and vortexed. To the remaining 1 ml cholesterol sample solution 20 μl 3 M NaCl was added and vortexed. After a 30-minute incubation at room temperature, the absorbance was measured at 365 nm with samples being referenced against the corresponding blank. Cholesterol content of the polyp and surrounding mucosa were quantified from a cholesterol standard curve. The cholesterol/phospholipid molar ratio (chol/PL) was calculated by using the combined PC and PE concentrations representing the main membrane phospholipids.

2.2.9 Protein determination

Powdered polyp and colon mucosa preparations (10-15 mg) from the liquid nitrogen homogenization step (see section 2.2.4) were solubilised in a 0.5 N NaOH 5% sodium dodecyl sulphate (SDS) solution at 37 °C for 48 hrs in a shaking water bath. The protein content was determined by using a modified Lowry method (Markwell *et al*., 1978; Addendum 6A). Briefly, solution 1 containing 5% SDS + 0.5 N NaOH; solution 2 containing Na_2CO_3 (2%), sodium tartrate (0.16%) and solution 3, $CuSO_4.5H_2O$ (4%), was prepared. Solubilised polyps and surrounding mucosa protein sample volumes ranging from 75-200 μl were dissolved with distilled water to a total volume of 200 μl. Solution 1 (300 μl) was added to each sample and vortexed. Solution 4 (1.5 ml) consisting of 100 parts of solution 2 and 1 part of solution 3 was added to each sample then vortexed. Folin-Ciocalteu reagent (150 μl) diluted to a 1:1 ratio with distilled water was added to each sample, vortexed, and samples incubated in a 37 °C water bath for 45 minutes. The absorbencies of the

samples were determined spectrophotometrically at 660 nm. Polyps and surrounding mucosa protein content were quantified against a BSA (0.5 mg/ml) standard curve.

2.2.10 Statistical analyses

Statistical analyses (ANOVA) of the colon polyps and surrounding mucosa tissue mean fatty acid profile, phospholipids and cholesterol contents were conducted. For the FA the qualitative (i.e. percentage of total FA) and quantitative (μg FA/mg protein) value was determined as well. All the statistical analyses were carried out using the analyses of variance. The Students-T distribution Test was used to determine the significant differences between the means of the control (i.e. surrounding tissue/colon mucosa) and experimental (i.e. colon polyps) groups. Values were considered significantly different if $p<0.05$.

2.3 Results

2.3.1 Dietary oil fatty acid content (Table 1)

The sunflower oil diet's FA content consisted mainly of C18:1n-9 (25.17%) and C18:2n-6 (62.25%), with an n-6/n-3 FA ratio of approximately 250:1. The total PUFA content was (62.56%) with a PUFA/SATS (P/S) ratio of 4.99.

2.3.2 Phospholipids and cholesterol content

The PC and PE phospholipid fractions (Fig. 1A) increased significantly in the polyp tissue when compared to the surrounding mucosa. However, the increase in PE was much greater than PC, resulting in a significantly decreased ($p<0.05$) PC/PE ratio in the polyp tissue (Fig. 1B). The cholesterol content (Fig. 1C) of the polyp tissue decreased significantly ($p<0.05$) in comparison to the surrounding with a concomitant decrease ($p<0.05$) in the cholesterol/phospholipid ratio (Fig. 1D).

2.3.3 Fatty acids

The qualitative (i.e. % of total FA) and quantitative (i.e. μg FA/mg protein) fatty acid composition of the polyp vs. surrounding tissues in the PC and PE fractions are summarized in Table 2.

2.3.3.1 Saturated FA (SATS)

Qualitative: (i) PC: The total SATS level was significantly reduced ($p<0.05$) in the colon polyps. This notable change was mainly because of decreases in C14:0, C16:0, C20:0 and C22:0. Polyp C18:0 and C24:0 content increased significantly ($p<0.05$) when compared to surrounding tissue. (ii) PE: A significant decrease in total SATS in the polyp PE fraction occurred as a result of reduced levels of C14:0, C16:0, C22:0 and C24:0, while no effect was noticed for C18:0.

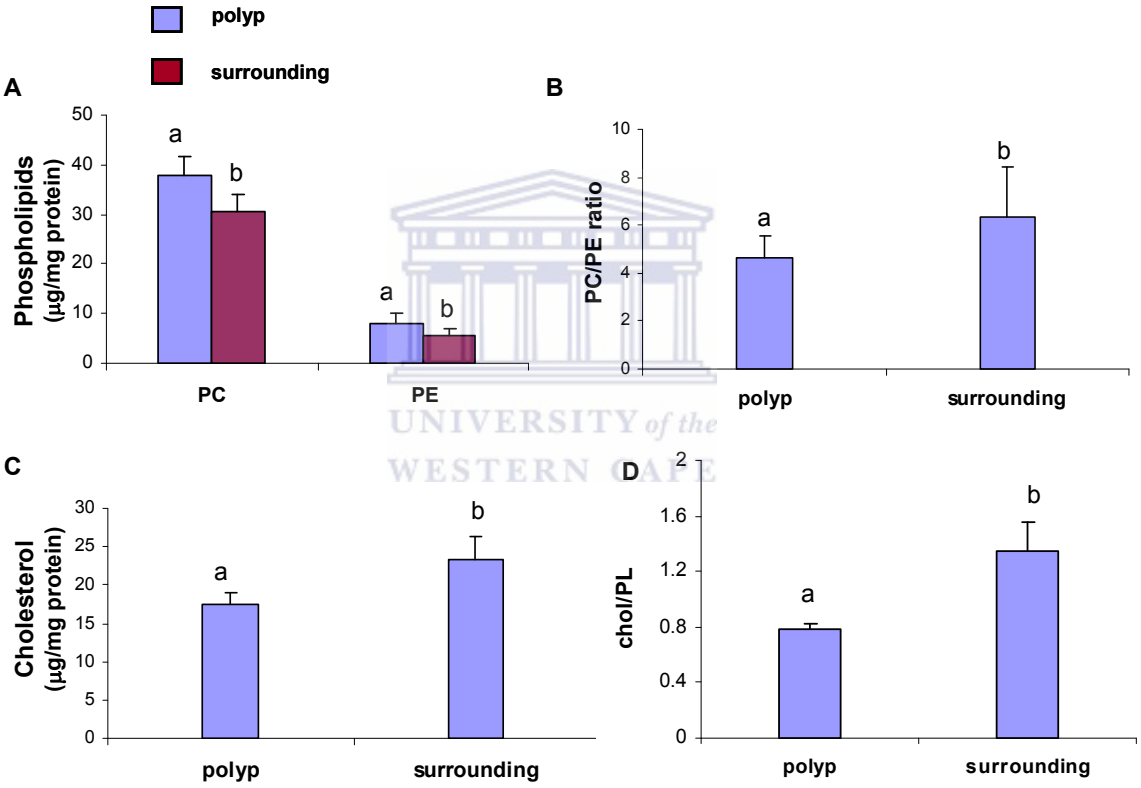

Fig. 1 Comparative phospholipids and cholesterol (i.e. µg/mg protein) analyses of colon polyps and surrounding mucosa tissue. Results are the means (n=13) ± standard deviation. (A) Membrane phospholipids content, (B) PC/PE ratio, (C) Cholesterol content and (D) Cholesterol/Phospholipid (chol/PL) ratio. If letters (lowercase) on columns differ, then $p<0.05$. The chol/PL molar ratio was calculated from the sum of PC and PE which constitute the two major phospholipids fractions in rat tissue. PC = phosphatidylcholine, PE = phosphatidylethanolamine.

Quantitative: (i) PC: The total SATS content was significantly increased ($p<0.05$) in the polyp tissue largely due to an increase in C16:0, C18:0, C22:0 and C24:0. The fatty acid C20:0 content decreased significantly when

compared to the surrounding tissue. (ii) PE: In the polyps there was a significant increase in SATS levels as a result of an increase in C18:0, C20:0 and C24:0. Levels of C14:0 was significantly reduced ($p<0.05$) in the polyp tissue.

2.3.3.2 Monounsaturated FA (MUFA)

Qualitative: (i) PC: The total MUFA increased in the polyps largely due to significant increases ($p<0.05$) in C16:1, C20:1 and C24:1. The C22:1 content decreased significantly in comparison to the surrounding tissue. A marginal increase ($p<0.1$) in C18:1 was observed in the polyps when compared to the surrounding mucosa. (ii) PE: Total polyp MUFA decreased only due to a significant decrease ($p<0.05$) in C16:1 and C18:1.

Quantitative: (i) PC: In comparison to the surrounding tissue, the total MUFA content in polyps increased significantly due to increased levels of C16:1, C18:1, C20:1 and C24:1 ($p<0.05$). (ii) PE: Total MUFA increased due to a significant increase in C24:1, while C18:1 and C20:1 was only marginally ($p<0.1$) increased.

2.3.3.3 Polyunsaturated FA (PUFA)

(a) n-6 PUFA

Qualitative: (i) PC: The total n-6 PUFA content in the polyp tissue increased significantly with increases in C18:2n-6, C20:2n-6, C20:4n-6, C22:2n-6, C22:4n-6 and C22:5n-6 ($p<0.05$). (ii) PE: Significantly increased levels of C20:4n-6 and C22:4n-6 resulted in a higher total n-6 PUFA content in the polyps ($p<0.05$). However, the C18:2n-6 content was significantly decreased ($p<0.05$) in comparison to the surrounding tissue, while C20:3n-6 was marginally ($p<0.1$) decreased.

Quantitative: (i) PC: Significant increases ($p<0.05$) in polyp C18:2 n-6, C18:3n-6, C20:2n-6, C20:3n-6, C20:4n-6, C22:4n-6 and C22:5n-6 content increased the total n-6 PUFA. (ii) PE: Comparative to the surrounding tissue, the polyps n-6 PUFA composition increased in C18:2n-6, C20:2n-6, C20:4n-6,

C22:2n-6 and C22:4n-6 content significantly ($p<0.05$), resulting in a significant increased total n-6 PUFA level.

(b) n-3 PUFA

Qualitative: (i) PC: The total n-3 PUFA content increased significantly due to an increase in C20:5n-3 ($p<0.05$) in the polyps. (ii) PE: In the polyps C22:5n-3 increased significantly ($p<0.05$), whereas C20:5n-3 decreased significantly resulting in no change in the total n-3 PUFA level.

Quantitative: (i) PC: Levels of C20:5n-3, C22:5n-3 and C22:6n-3 increased significantly in the colon polyps ($p<0.05$), thereby increasing membrane total n-3 PUFA content. (ii) PE: The total n-3 PUFA composition of polyps increased mainly due to increased levels of C22:5n-3 ($p<0.05$) and C22:6n-3 ($p<0.1$).

(c) Total PUFA and total long chain (LC) PUFA

Qualitative: In both PC and PE phospholipid fractions the total PUFA content increased significantly in the colon polyps when compared to the surrounding tissue. The n-6 and n-3 long chain (LC) PUFA levels also increased significantly ($p<0.05$) within the polyp, hereby increasing the total LC PUFA content.

Quantitative: Polyps had a much higher total PUFA content ($p<0.05$) in comparison to the surrounding tissue in both PC and PE fractions. Significant increases ($p<0.05$) in n-3 and n-6 LC PUFA increased the levels of total LC PUFA in polyp membranes.

Table 2: Rat colon polyp and surrounding mucosa fatty acid content (% and μg FA/m protein)

Fatty Acid	% of total fatty acids				μg FA/mg protein			
	PC		PE		PC		PE	
	Polyp	Surrounding	Polyp	Surrounding	Polyp	Surrounding	Polyp	Surrounding
C14:0	0.40±0.19a	0.99±0.44b	0.25±0.11a	1.29±0.89b	0.85±0.34	0.89±0.44	0.26±0.12a	0.52±0.18b
C16:0	32.92±3.18a	46.15±7.62b	12.93±2.82a	21.89±6.98b	54.79±7.99a	41.95±8.42b	13.02±2.71	13.00±2.71
C18:0	28.76±3.69a	21.65±4.18b	21.85±1.36	22.72±1.83	48.57±11.26a	19.54±3.16b	24.78±3.15a	15.33±5.50b
C20:0	1.74±0.48a	5.55±1.71b	2.24±0.77	2.59±0.38	2.69±0.91a	4.88±1.23b	2.34±1.07#	1.62±0.60#
C22:0	1.26±0.18a	1.61±0.44b	0.75±0.08a	1.26±0.26b	2.03±0.44a	1.34±0.31b	0.81±0.16	0.77±0.27
C24:0	1.46±0.24a	1.00±0.26b	0.57±0.16a	0.77±0.27b	2.24±0.36a	0.88±0.25	0.60±0.15a	0.45±0.16b
SATS Total	66.43±2.60a	76.71±6.63b	38.46±3.42a	50.09±8.71b	102.12±10.58a	63.21±6.70	42.62±4.58a	31.06±6.83b
C16:1	0.57±0.21a	0.38±0.17b	0.50±0.19a	0.79±0.34b	1.07±0.38a	0.30±0.13b	0.52±0.15	0.50±0.15
C18:1	16.31±1.67#	13.60±4.23#	14.32±1.65a	17.24±1.64b	25.03±4.48a	13.37±3.57b	17.34±3.16#	13.69±4.75#
C20:1	0.75±0.13a	0.32±0.12b	0.51±0.21	0.56±0.16	1.21±0.27a	0.28±0.11b	0.56±0.23#	0.38±0.11#
C22:1	0.12±0.04a	0.28±0.07b	0.12±0.04	0.15±0.04	0.19±0.08	0.24±0.06	0.14±0.04	0.13±0.05
C24:1	0.68±0.20a	0.31±0.11b	0.23±0.09	0.20±0.07	1.13±0.32a	0.28±0.10b	0.22±0.05a	0.13±0.05b
MUFA Total	18.24±1.76#	15.51±3.75#	15.56±1.81	18.96±1.21	28.40±4.92a	14.35±3.62b	18.70±3.32a	14.65±5.01b
C18:2n-6	4.40±0.72a	2.52±0.79b	5.65±0.58a	6.26±0.67b	8.02±2.08a	2.21±0.74b	6.15±1.02a	3.90±1.50b
C18:3n-6	0.23±0.09	0.23±0.13	0.22±0.02	0.38±0.22	0.33±0.08a	0.21±0.12b	0.24±0.02	0.18±0.07
C20:2n-6	0.83±0.25a	0.52±0.16b	0.77±0.21	0.80±0.15	1.29±0.32a	0.46±0.15b	0.83±0.24a	0.54±0.17b
C20:3n-6	0.87±0.43	0.66±0.22	1.45±0.59#	1.88±0.53#	1.52±0.94a	0.59±0.20b	1.66±0.82	1.44±0.74
C20:4n6	5.58±0.90a	2.02±1.13b	23.51±2.46a	14.29±4.92b	9.80±2.99a	1.60±0.85b	28.03±4.30a	14.75±6.74b
C22:2n-6	0.24±0.13a	0.37±0.06b	0.29±0.07	0.29±0.06	0.38±0.20	0.33±0.07	0.31±0.08a	0.18±0.08b
C22:4n-6	0.98±0.22a	0.30±0.12b	11.44±1.82a	2.84±0.98b	1.50±0.36a	0.26±0.10b	13.36±2.49a	2.43±0.97b
C22:5n-6	0.33±0.05a	0.22±0.08b	1.46±0.24	1.73±0.51	0.49±0.09a	0.19±0.08b	1.65±0.06	1.25±0.61
n-6 Total	13.77±1.37a	6.70±1.73b	44.61±3.92a	28.10±7.79	23.19±3.78a	5.87±1.47b	52.69±7.15a	28.26±6.75b
C18:3n-3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
C20:5n-3	0.54±0.10a	0.25±0.08b	0.22±0.04a	0.33±0.07b	0.88±0.25a	0.22±0.07b	0.24±0.05	0.27±0.08
C22:5n-3	0.21±0.10	0.22±0.08	0.60±0.10a	0.23±0.03b	0.44±0.13a	0.20±0.06	0.68±0.15a	0.21±0.07b
C22:6n-3	0.25±0.08#	0.19±0.07#	0.95±0.17	1.13±0.33	0.39±0.12a	0.17±0.06b	1.01±0.34	0.87±0.41
n-3 Total	0.98±0.14a	0.65±0.15b	1.70±0.34	1.64±0.43	1.66±0.57a	0.56±0.15b	1.85±0.60a	1.23±0.62b
PUFA	14.75±1.38a	7.37±1.77b	46.31±4.10a	29.59±7.94b	24.85±4.24a	6.44±1.52b	54.75±7.35a	29.90±6.71b
n-6 LC PUFA	8.78±1.24a	4.17±1.51b	38.80±3.84a	21.17±7.77b	14.93±3.45a	3.49±1.08b	46.02±6.83a	24.00±6.90b
n-3 LC PUFA	0.98±0.14a	0.65±0.15b	1.70±0.34	1.64±0.43	1.66±0.57a	0.56±0.15b	1.85±0.60a	1.23±0.62b
Total LC PUFA	9.77±1.26a	4.85±1.56	40.50±4.01a	23.20±7.95b	16.68±3.38a	4.08±1.13b	48.08±7.03a	25.64±6.90b

Values (n=13) are means ± SD of the relative and absolute fatty acid contents of colon polyps and surrounding tissue. Means followed by the same letter (lowercase) do not differ. If letters differ, then $p<0.05$, while marginal effects are represented by #. PC=phosphatidylcholine; PE=phosphatidylethanolamine; SATS= saturated fatty acids; MUFA=monounsaturated fatty acids; PUFA= polyunsaturated fatty acids; LC PUFA= long-chain PUFA.

2.3.3.4 Membrane FA ratios

PC: The polyunsaturated/saturated (P/S) FA ratio increased significantly ($p<0.05$) when compared to the surrounding tissue (Table 3). The C20:4n-6/C20:5n-3 and n-6/n-3 ratios also tended to increase, although not significantly.

PE: The C20:4n-6/C20:5n-3, n-6/n-3, P/S and delta-6 desaturase substrate/product (Δ6 S/P) ratios increased significantly ($p<0.05$) in the PC and PE phospholipid fractions of the colon polyps (Table 3).

The C20:4n6 PC/PE ratio (Fig. 2A and 2B) significantly ($p<0.05$) increased in the colon polyps. The qualitative (%) and quantitative (μg) content of C20:4n-6 tended to increase in the PC phospholipid fraction of the polyp compared to the surrounding mucosa (Table 4). In phospholipid PE, the C20:4n-6 content was much higher in the polyps, with both percentage of total FA and μg FA/mg.

Table 3: Quantitative (μg FA/mg protein) fatty acid ratios of rat colon polyps and surrounding mucosa tissue.

FA Ratios	PC		PE	
	polyp	surrounding	polyp	surrounding
C20:4n-6/C20:5n-3	11.85±2.13	8.54±4.88	112.46±25.41a	48.41±27.37b
n-6/n-3	14.26±3.38	10.33±3.03	26.08±3.61a	20.83±4.28b
P/S	0.22±0.03a	0.10±0.03b	1.24±0.17a	0.76±0.18b
Δ6 S/P (%)*	6.67±1.88a	4.79±2.22b	7.75±2.29a	3.25±0.89b

Values (n=13) are means ± SD of the fatty acid ratios of colon polyps and surrounding tissue. Means followed by the same letter (lowercase) do not differ. If letters differ, then $p<0.05$. C20:4n-6/C20:5n-3= arachidonic acid/eicosapentaenoic acid ratio; P/S= polyunsaturated fatty acid/saturated acid ratio; Δ6 S/P =delta-6-desaturase substrate/product ratio. * Relative FA values were used to calculate ratio.

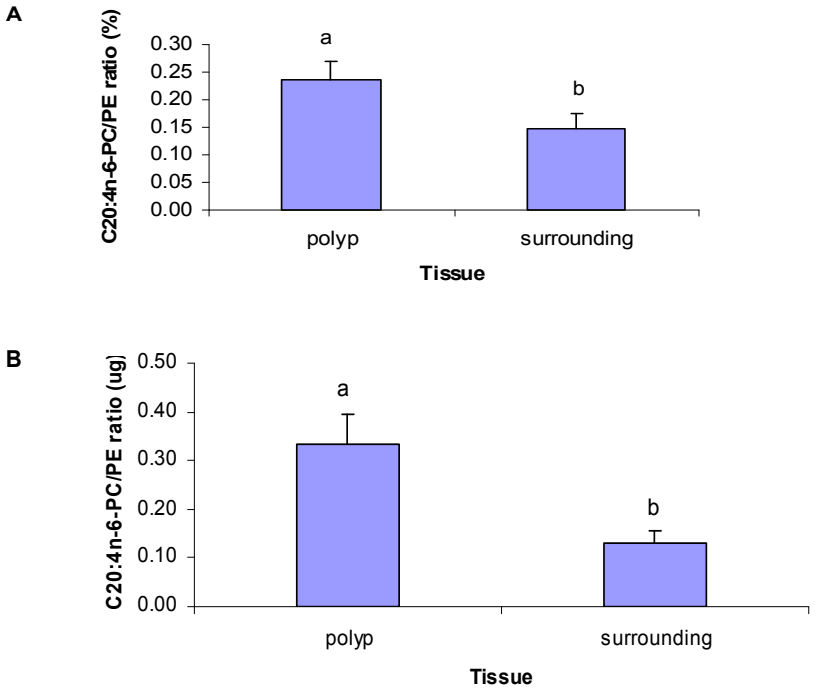

Fig. 2 PC/PE ratio of C20:4n-6 content in the polyp and surrounding mucosa tissues. If letters (lowercase) on columns differ, then $p<0.05$.

Table 4: Qualitative (% of total FA) and quantitative (μg FA/mg protein) content of the comparative C20:4n-6 content in the PC and PE phospholipid fractions of the polyp versus surrounding tissue ratio.

Phospholipid	C20:4n-6-polyp/surr ratio
PC (% of total FA)	1.94±0.69
PE (% of total FA)	1.47±0.28
PC (μg FA/mg protein)	5.19±1.75
PE (μg FA/mg protein)	2.28±1.02

Values (n=13) are means ± SD of the colon polyp/surrounding tissue ratio of the C20:4n-6 qualitative (% of total FA) and quantitative (μg FA/mg protein) content. PC= phosphatidylcholine and PE= phosphatidylethanolamine.

2.4 Discussion

FA form the building blocks of lipids that provide cell structure and regulate membrane functions that include mediating receptor signalling cascades, cell-to-cell interactions and membrane fluidity (Clandinin *et al*, 1991, Lund *et al*., 1999). Changes in the FA compositions of cells have been attributed to several pathological conditions including some cancers (Dommels *et al.*, 2002). Thus understanding the differences in FA profiles between cancer and healthy cells may be invaluable in understanding their role in carcinogenesis. The aim of this investigation was therefore to compare the lipid profile of colon polyps and surrounding mucosa tissue in rats fed a diet high in n-6 polyunsaturated FA.

Results from the current study show that the development of colon polyps is associated with a distinctly different lipid pattern. In terms of membrane phospholipid content, the colon polyps display a significant increase in the levels of PC and PE when compared to the surrounding mucosa. Increases in PC and PE content could be a prerequisite for the process of cell growth and division (Pasternak and Bergeron, 1970). In normal colonic mucosa, epithelial cells proliferate rapidly within the basal proliferative zone of the crypts (Lipkin, 1973). Within this zone cells divide providing a constant source of new cells every 4-8 day. The increase in polyp phospholipid content could thus reflect the need for membrane constituents, such as phospholipids, to generate more cells. Increased proliferation and expansion of the basal proliferative zone in colon mucosa have been associated with a high risk of developing colon cancer (Newmark *et al.*, 1991). Thus, the differences in the phospholipids profiles between the polyps and surrounding mucosa phospholipids content could be associated with increased cell turnover due to alterations in cell growth regulatory mechanisms.

Changes in the PC/PE ratio, the cholesterol/phospholipid molar ratio (chol/PL) and the degree of unsaturation (P/S ratio) are important in maintaining membrane fluidity (Owen *et al.*, 1982). Membrane fluidity plays a determining role in membrane functions such as activity of membrane-associated enzymes and receptors involved in a wide range of functions regarding cell

signaling pathways (Peck, 1994). As PC and PE constitute almost 90% of the total phospholipids, the ratio of PC to PE provides a sensitive indicator of changes in the phospholipids content of membranes (Williams *et al.*, 1998). Both PC and PE increased significantly in the polyps, while the PC/PE ratio decreased significantly in the colon polyps due to a higher increase in PE. An increase in PC content has a “fluidizing” effect on membranes due to the large choline head group reducing the tight packaging between the phospholipid head groups (Soulages and Brenner, 1989; Eskin *et* al., 2006; Hamai *et al*., 2006). Furthermore, the reduction of the polyp cholesterol content and concomitant increase in phospholipids resulted in lowering the chol/PL ratio. As a result, the polyp membranes are less rigid due to the loss of cholesterol-associated inhibition of the overall motion of the acyl (hydrocarbon) chains (Petrache *et al*., 2005). An increase in membrane unsaturation with respect to long chain PUFA content was indicated by the elevated P/S ratio, also suggest an increase in membrane fluidity. Alterations in the above mentioned membrane fluidity parameters could potentially change the cell growth kinetics in order to sustain the altered growth pattern in the polyps.

Tissue membrane phospholipids maintain a consistent pattern of fatty acid composition, yet within a limited range, exhibit responsiveness to changes in the availability of circulating FA (Lands, 1991), and therefore the diet. In terms of fatty acid content, the alterations observed were significantly different between the two phospholipid fractions, PC and PE, in the polyp tissue. Quantitatively (μg FA/mg protein) the total SATS content increases significantly in both PC and PE when compared to the surrounding mucosa tissue. In PC the SATS levels are much higher due to increases in C16:0 and C18:0, while only C18:0 increased in PE. Despite the quantitative increase in SATS, qualitatively (% of total FA) C16:0 decreased in both phospholipid fractions, while increasing C18:0 in PC. The SATS content have been implicated to play an important role in tumourigenesis. In primary mouse embryonic fibroblasts Zeng *et al.* (2008) observed that C16:0 and C18:0 compromised the expression of the pro-apoptotic genes p21 and Bax in

response to DNA damage. With the resultant decrease and increase in C16:0 and C18:0, respectively, it could be hypothesized that they, by negatively regulating the DNA damage response pathway may promote cell transformation. In this context, it appears that the increased availability of SATS in precancerous/cancer cells may contribute to tumour growth and potentially stimulate polyp development. Saturated FA also appears to modulate cancer cells programmed death. In MDA-MB-231 breast cancer cells, free fatty acid palmitate (C16:0) induces apoptosis by reducing the mitochondrial membrane potential that allows for the release of the cytosolic pro-apoptotic factor cytochrome c (Hardy *et al.,* 2003). Therefore, it appears that depending on cell type, changes in C16:0 and C18:0 membrane levels influences apoptosis and cell survival.

MUFA increased significantly in both PC and PE phospholipids in the polyps mainly due to an increase in C16:1n-7, C18:1n-9 and C24:1n-9. Of these FA, C18:1n-9 has been implicated to play a role in activating key cellular functions that promote the progression of the neoplastic phenotype. In this regard the C18:1n-9 membrane content seems to play a role in the activation of protein kinase C (PKC), an enzyme that promotes cellular proliferation in tissues (Craven and DeRubertis, 1987; Khan *et al*., 1992). It would appear that one of the mechanisms utilized to promote polyp development may be through increased C18:1n-9 stimulation of PKC. Incidentally, increases in this enzyme's activity has been linked the increased risk of colon cancer (Craven *et al.,* 1987; Blobe *et al.*, 1994).

When comparing the differences in polyp FA content with the surrounding mucosa, the total PUFA content increased significantly in both the PC and PE lipid fractions. The differences between the surrounding mucosa and polyps PUFA content could therefore be linked to changes in the mechanisms regulating the metabolism of the essential FA. The metabolism of FA is a critical factor for the cell to maintain its membrane structure and function. Cells are dependent on Δ^6- and Δ^5- desaturases for the synthesis of long chain polyunsaturated FA (LCPUFA), which are mainly esterified into phospholipids and contribute to maintaining membrane fluidity. Δ^6-desaturase

is the rate-limiting enzyme that catalyzes the first reaction of both n-3 and n-6 PUFA synthesis. In rat liver, Cho *et al.* (1999) observed that Δ^6-desaturase expression increases when animals are fed diets deficient in FA, while PUFA added to the diet decreases the enzyme activity. In the colon polyps, the Δ^6-desaturase-substrate/product (Δ6 S/P) ratio, which is a reflection of Δ^6-desaturase activity, is increased significantly in both PC and PE, hereby suggesting a decrease in enzyme activity. However when considering the high level of LCPUFA in both PC and PE it would appear that the Δ^6-desaturase is not altered or impaired in the polyps and that the tentative increased substrate level could be related to negative product feedback regulation of the enzyme (Nakamura *et al*., 2000). This is also in agreement with the high level of the n-3 and specifically the n-6 LCPUFA in the PC and PE phospholipid fractions of the polyps. Of interest is the significantly increase in C20:4n-6 in both PC and PE fraction which again argued against an impaired Δ^6-desaturase. In intestinal tumourigenesis, Hansen-Petrik *et al*. (2002) indicated that selective Δ^6-desaturase inhibition resulted in 36-37% fewer tumours in Apc(Min/+) mice and a 35% decrease in primary tumour size in nude mice bearing HT-29 colon cancer cell xenographs.

C20:4n-6 is an important FA in maintaining normal cellular homeostasis because of the multiple roles it plays, (i) structurally as part of membrane phospholipids, (ii) functionally as a precursor to the 2-series eicosanoids and (iii) as an intermediate involved in signal transduction pathways regulating cell proliferation and apoptosis (Khan *et al*., 1995). In this study, the colon polyps had a significantly higher C20:4n-6 content in both lipid fractions compared to the surrounding tissue. An increase in the C20:4n-6 content of rat colonic mucosal phospholipids has been shown to be associated with increased rates of cell proliferation (Lee *et al*., 1993). FA such as C20:4n-6 may differentially influence carcinogenesis via their metabolism into various prostaglandins (PGs) as it has been reported that PGE_2 protects colonocytes from programmed cell death by activating anti-apoptotic genes (Wang *et al*., 2004). This is significant because apoptosis is progressively suppressed during the development of colon cancer (Bedi *et al.,* 1995). Therefore, modifications of

membrane phospholipid FA may play a critical role in the regulation of several cell signalling pathways related to the development of cancer.

The polyps have a much higher level of n-6 PUFA (C18:2n-6, C20:4n-6, C22:4n-6, C22:5n-6) than n-3 FA in both phospholipid fractions resulting in a significant increase in the n-6/n-3 ratio, specifically in PE. In the colon polyps the relative C20:5n-3 content was lower than C20:4n6, as indicated by a significant ($p<0.05$) increased C20:4n-6/C20:5n-3 ratio. Competitive effects exist between the C20:4n-6 and C20:5n-3 fatty acid precursor substrates of COX-2. Thus, increasing membrane C20:5n-3 could lead to competitive inhibition of C20:4n-6 metabolism as well as the production of metabolites such as PGE_3 (Rose and Connolly, 1999). These metabolites are less biologically active than the corresponding C20:4n-6 derived metabolites such as PGE_2 (Fischer, 1997). Since C20:4n-6 synthesis and its subsequent conversion to prostaglandins contribute significantly to the growth of intestinal tumours, modulation of the C20:4n-6 metabolic cascade should inhibit carcinogenesis.

The increase in the polyp C20:4n-6/C20:5n-3, C20:4n-6 PC/PE and C20:4n-6-polyp/surrounding ratios suggest that in connection with high n-6 PUFA dietary intake, the production of 2-series prostaglandins stimulus could be increased. Therefore, regulation of C20:4n-6 metabolism as well as membrane content plays an important role in determining cell survival parameters. The increased C20:4n-6-polyp/surr ratio showed that there was a shift in the concentration of C20:4n-6, towards the polyps. When considering the C20:4n-6 membrane distribution or gradient a significant shift is noticed towards PC as indicated by the increased C20:4n-6 PC/PE ratio despite the fact that C20:4n-6 content is significantly higher in the PE phospholipid fraction. The shift in membrane content of C20:4n-6 has implications with regards to the metabolic availability of C20:4n-6 that may affect cell survival parameters associated with the promotion of tumour growth (Chen and Hughes-Fulford, 2000). Of interest is the finding in rat liver carcinogenesis where the C20:4n-6 PC/PE ratio was decreased in hepatocyte nodules due to an increased level of PE (Abel *et al.*, 2001). This altered pattern was

implicated in the altered growth characteristics of these lesions. The differences between the two tissue compartments could be related to the proliferative status with the liver being the more quiescent in nature (Mabuchi *et al.,* 2004). Despite these differences, C20:4n-6 seems to be a major factor determining the altered growth properties of cancerous lesions.

The FA content of the sunflower oil diet used in the present study consisted mainly of the essential FA C18:2n-6, which is the precursory n-6 FA substrate that undergoes rapid elongated and desaturation to produce C20:4n-6 (Sprecher and Chen, 1999). Consequently, the increase in membrane n-6 LCPUFA, particularly C18:2n-6 and C20:4n-6 in the polyps could be a reflection of the dietary fat. Although C20:4n-6 is essential for normal cell functioning (Johnson *et al*., 1986; Martin-Venegas *et al*., 2006), a high dietary level of C18:2n-6 has been implicated in colon carcinogenesis. Therefore, dietary strategies to reduce excessive C18:2n-6 intake needs to be adopted. In this regard dietary intake of n-3 PUFA has been shown to reduce the availability of C18:2n-6 in membrane phospholipids (Liou *et al*., 2007). Therefore, with increased intake of n-3 FA, the availability of the elongation-desaturation product C20:4n-6 may be reduced. N-3 LCPUFA, have been suggested to play a protective role in colorectal cancer development by inhibiting the cyclooxygenase-2 (COX-2) enzyme and the production of C20:4n-6 derived eicosanoids (Singh *et al*., 1997). This suggests that n-3 FA may play an important role in the prevention of polyp development by reducing the differences in the C20:4n-6 PC/PE and polyp/surrounding ratios.

2.5 Conclusions

The present study suggests that alterations in lipid metabolism in the colon are likely to play an important role in its neoplastic development. The dynamic state of the lipid bilayer would allow the manipulation of the lipid content by dietary means. Hereby, the type of FA available for the maintenance of membrane fluidity and the synthesis of prostaglandins, for example, may be determining factors required for persistent cellular transformation into the malignant phenotype. Changes in the colon polyp's cholesterol, phospholipid

and FA profiles could provide unique targets for developing strategies in chemoprevention by means of dietary manipulation.

2.6 References

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CHAPTER 3

LIPID MODULATION OF RAT COLON MUCOSA AND RED BLOOD CELL MEMBRANES BY DIETARY FAT WITH VARYING 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 phosphatidylethanoamine (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 Δ^6-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.

Keywords: dietary n-6/n-3 ratios, modulation, lipid peroxidation, colon mucosa, red blood cells, membrane

3.1 Introduction

Dietary intakes of n-6 and n-3 polyunsaturated fatty acids (PUFA) have received considerable attention as key modulators of health (Sumida *et al.,* 1993; Sessler and Ntambi, 1998). Both PUFA families are readily incorporated into cell membrane phospholipids, where they influence the physical and functional properties of membranes. In western cultures the consumption of foods rich in n-6 PUFA (e.g. plant based fats: vegetable oils) exceeds that of n-3 rich foods such as fish and fish oils (Simopoulos, 2006). Studies suggest that this dietary pattern facilitates the development of cancers of the breast, colon and prostate (Reddy and Sugie, 1988; http://www.eurekalert.org/pub_releases/2006-02/uoc--ofc012706.php, accessed 21 October 2008; Thiébaut *et al.,* 2009). It has been suggested that a critical balance between membrane n-6 and n-3 PUFA content and their interactions determines whether cells are transformed into an abnormal state (Simopoulos, 2006). Therefore, with diet playing a key role in neoplastic transformations, it is reasonable to assume that exposure to specific dietary n-6/n-3 fatty acid (FA) ratios could potentially modulate or prevent the development of colon cancer (Campos *et al.,* 2005).

Health benefits associated with the intake of fish oil and borage oil have been extensively studied (Horrobin, 1992; Engler *et al.,* 1998*;* Belch and Hill, 2000; Kris-Etherton *et al.,* 2002*;* Das, 2008). Fish oils, specifically from fish such as mackerel and salmon, are rich in the n-3 long chain (LC) PUFA, eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3) (Covington, 2004). In contrast, borage oil is rich in the n-6 PUFA, γ-linolenic acid (C18:3n-6). Both classes of PUFA derived from these oils are readily incorporated into the phospholipids of cell membranes of all tissue types, such as the colon (Gee *et al.,* 1999) and liver (Palombo *et al.,* 1993). Alterations in membrane FA composition as a result of PUFA incorporation may lead to changes in membrane fluidity and membrane mediated function and cell signaling e.g. eicosanoids, lipid secondary messengers, signalling proteins (Benatti *et al.,* 2004).

Intakes of n-3 LCPUFA through the consumption of fish or fish oil reduces the risk of colon cancer by inducing apoptotic pathways that suppress further cell growth (Narayanan *et al.,* 2001; Courtney *et al.,* 2007*).* Many of the clinical effects of C18:3n-6 supplementation has been attributed to its capacity to block the metabolism of arachidonic acid (C20:4n-6) to bioactive eicosanoids (Johnson *et al.,* 1997). The problem, however, is that the metabolism of C18:3n-6 by elongase and Δ^5-desaturase activities also generates dihomo-γ-linolenic acid (C20:3n-6), which is a potential precursor of C20:4n-6 (Sprecher, 2000). Studies suggest using combined ratios of fish oil and borage oil (i.e. C20:5n-3 and C18:3n-6) to minimize the production of C20:4n-6 (Barham *et al.,* 2000). Hereby, pro-inflammatory processes involving C20:4n-6 derived metabolites, the eicosanoids, which may potentially lead to disease development (e.g. cancer), may be reduced by n-3 LCPUFA intake (Takahashi *et al.,* 1987).

In addition to modulating eicosanoid production, membrane incorporation of LCPUFA, particularly the n-3 FA, enhances susceptibility to free radical damage that leads to oxidative stress (Song and Miyazawa, 2001). Lipid peroxidation (LPO) is a cellular process utilized by cells to regulate cell numbers by stimulating mechanisms that lead to growth inhibition and cell death (apoptosis) (Girotti, 1998). With regard to cancer, the level of LPO is very low due to reduced membrane PUFA content (Horrobin, 1990). The reduced membrane susceptibility to LPO is likely to play an important role in the abnormal growth, which prevails in preneoplastic lesions (Biasi *et al.,* 2002). Therefore, dietary intakes of specific FA, such as the n-3 LCPUFA, have been suggested to inhibit further cancer development through the enhancement of LPO induced cell growth inhibition (Chen and Istfan, 2001; Abel *et al*., 2004).

The objective of the present study was to investigate the effect of the intake of dietary fats with specific n-6/n-3 FA ratios on the lipid profiles of normal rat colon mucosa and red blood cell membranes. The effect of membrane FA changes on the susceptibility to LPO was also monitored.

3.2 Materials and methods

3.2.1 Chemicals

N-3 FA rich fish oil (EPA-55) was obtained from Equazen UK Ltd (St Petersburg Place, London, UK). Borage oil, rich in C18:3n-6 was obtained from Créde Natural Oils (Strand, South Africa). Sunflower oil was supplied by RFS Catering Supplies (Milnerton, South Africa). Perchloric acid, methanol and chloroform, fluorescing agent 2,5-bis-(5'-tert-butylbenz-oxazolyl-[2'])thiophene (i.e. BBOT), butylated hydroxytoluene (BHT), sodium hydroxide (NaOH), sodium phosphate monobasic monohydrate (NaH_2PO_4), sodium phosphate dibasic anhydrous (Na_2HPO_4), sodium chloride (NaCl) and malachite green were supplied by Sigma-Aldrich. Silica TLC plates, acetic acid, petroleum ether, boric acid, sodium carbonate (Na_2CO_3), sodium tartrate and copper sulphate ($CuSO_4.5H_2O$) was obtained from Merck (Darmstadt, Germany). Sulphuric acid was supplied by BDH/Merck (Poole, England). Sodium dodecyl sulphate (SDS) was supplied by Fluka (Germany), bovine serum albumin (BSA) by Mees Laboratories (South Africa) and BCA (bicinchoninic acid) by Separations (South Africa).

3.2.2 Preparation of diets

A rat diet (AIN-93) (Reeves *et al.*, 1997) 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 all the diets. For the experimental ratio diets, the AIN-93 diet fat content was modified with fish oil and borage oil to obtain fats with varying n-6/n-3 FA ratios for the duration of the experiment. The experimental fat sources consisted of: (1): sunflower oil only (S diet; n-6/n-3=250:1), (2): sunflower oil supplemented with fish oil (SF diet; n-6/n-3= 12:1), (3): sunflower oil supplemented with borage oil (SB diet; n-6/n-3= 30:1) and (4): sunflower oil supplemented with fish oil and borage oil (SBF diet; n-6/n-3 = 12:1). The FA profile of the dietary oil mixtures were analyzed using gas chromatography (Varian 4600) and the exact n-6/n-3 FA ratios (Table 1, page 89) calculated. The diets were kept at -20 °C under nitrogen until use.

3.2.3 Animals and treatment

The Ethics Committee of the Medical Research Council of South Africa approved the use of laboratory animals in this study. At weaning (body weight 50 g), male Fischer rats (n=48) 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 fed *ad libitum* the AIN-93 diet (Reeves *et al.*, 1997), with sunflower oil as a fat source (5%) for a period of three weeks.

After the three weeks, at a body weight of 150 g, the animals were divided into four groups (n=9 per dietary group), and placed on their respective dietary groups (diet S, SF, SB and SBF). Body weights were measured once a week, while feed intakes were recorded bi-weekly. Animals were terminated by sodium pentobarbital injection i.p after four months exposure to the experimental ratio diets. The colon was excised, opened longitudinally and the epithelial mucosa collected by scraping the inner surface with a microscope glass slide. The mucosa sample were frozen in liquid nitrogen and stored at -80 °C until analysed. Blood samples were collected from the abdominal aorta into heparin-coated blood vials.

3.2.4 Preparation of RBC membranes

Immediately after termination, packed RBC was obtained by centrifugation of the blood at 3000 rpm for 10 min at 4 °C. After the removal of the plasma and the buffy coat by aspiration, cells were washed three times with a 0.9% NaCl solution. For the preparation of hemoglobin-free ghosts, a slightly modified method of Dodge et al (1963) was followed. Hemolysis of the washed RBC was performed in 5 mM sodium phosphate buffer (pH 8.0) and pellets were obtained by centrifugation at 15 000 rpm for 20 min at 4 °C. The hemolysis process was repeated 3 times until the pellet was a pale pink colour. The RBC membranes were finally suspended in 2 ml of the buffer and stored at -80 °C until analyzed.

3.2.5 Lipid extraction

Lipids were extracted from the phosphate buffer RBC membrane solution (≥2 mg protein) with chloroform/-methanol (CM; 2:1; v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant (Folch *et al.,* 1957). The tissue:CM ratio was approximately 20:1. The samples were extracted using a rotary shaker for 20 minutes. Saline saturated chloroform-methanol-saline (CMS 86:14:1 v/v/v) was added to the RBC solution, vortexed (60s) and centrifuged at 500 rpm for 15 minutes. The bottom CM layer was removed and transferred into a round bottom flask. The CM (10 ml) extraction step was repeated twice. The collected CM extracts were evaporated on a rota-vapor at 40 °C, and transferred with 5x2ml CMS to glass culture-tubes. Saturated CMS (1 ml) was added, vortexed and centrifuged at 500rpm for 15 min. The top layer (saline) was removed, and samples stored under nitrogen at 4 °C until analyzed.

For the colon mucosa lipid extraction, the tissue was ground to a fine powder in liquid nitrogen. A sub fraction of the mucosa powder (10-30 mg) was taken for protein analyses. The remaining mucosa samples were weighed (15-80 mg) and resuspended in 0.5 ml saline and the lipids were extracted with 12 ml CM. The sample was filtered (sinterglass filters using Whatman glass microfibre filters, Cat N, 1820 866, Whatman International, Ltd, Maidstone, England) and the filtrate evaporated to dryness *in vacuo* at 40 °C, and transferred to glass culture tubes. The extract was treated with saline saturated with CMS as described above, and stored at 4 °C under nitrogen until analysed.

3.2.6 Thin layer chromatography (TLC)

The lipid extracts from the mucosa and RBC ghosts were fractionated by thin layer chromatography (TLC) as described in Chapter 2. The major phospholipids fractions PC and PE were collected for phospholipid and FA analysis. Phospholipids were identified against a phospholipid standard mixture containing PC, PE, sphingomyelin (SM), phosphatidylserine (PS) and phosphatidyl inositol (PI).

3.2.7 Fatty acid analysis

For FA analyses the phospholipid fractions, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were collected and transmethylated with 2 ml methanol/18 M sulphuric acid (95:5; v/v) at 70 °C for 2 hrs (Benadé *et al.,* 1988). The resultant FA methyl esters (FAME) extracted with hexane and distilled water (v/v, 2:1). The hexane (top layer) was removed and dried under nitrogen gas in a water bath (37 °C). The dried samples were dissolved in hexane and analyzed by gas chromatography on a Varian 3300 gas Chromatograph equipped with 30 m fused silica BPX-70 capillary columns (SGE, USA) of 0.32 mm internal diameter (Addendum 2). The individual FAME were identified by comparison of the retention times to those of a standard FA mixture containing C14:0 to C24:1 (Nu-Chek-Prep, MN, USA) (Addendum 3). Individual FA was quantified using C17:0 as an internal standard and expressed as μg FA/mg protein as well qualitatively as a percentage of the total fatty acids (Addendum 3).

3.2.8 Cholesterol and phospholipid determination

Cholesterol and phospholipids contents were determined as described in Chapter 2. Briefly, the cholesterol content of the rat mucosa and RBC samples (i.e. 20 μl of CMS lipid extracted samples) was determined by an enzymatic iodide method using cholesterin-oxidase and cholesterin-esterase (Richmond, 1973). The phospholipid concentrations of PC and PE were determined colorimetrically based on the method according to Itaya and Ui (1966).

3.2.9 RBC membranes and mucosa protein determination

The protein content of the RBC membranes was determined using a bicinchoninic acid reaction (Kaushal and Barnes, 1986; Addendum 6B). Briefly, RBC membrane proteins were solubilized in a 2% SDS 20 mM $NaCO_3$ 2 mM EDTA solution at a ratio of 1:10. Solubilised RBC protein sample volumes ranging from 40-80 μl were diluted in distilled water to a final volume of 600 μl. A protein determining reagent, containing bicinchoninic acid (BCA) detection reagent and 4% $CuSO_4.5H_2O$ (50:1), was added to the samples at a

volume of 400 μl. The samples were vortexed and incubated in a 60 °C water bath for 1 hr and the absorbance measured at 562 nm. Samples were quantified against a BSA (0.1 mg/ml) standard curve. The protein content of the mucosa samples was determined according to a modified Lowry method (Markwell *et al*., 1978) as described in Chapter 2.

3.2.10 Determination of conjugated dienes

The extent of lipid peroxidation was determined by measuring the levels of conjugated dienes (CD) according to the method of Hu *et al.* (1989). Firstly for the tissue homogenate preparation, mucosa samples were homogenized on ice in 19 volumes of 1.15% potassium chloride in 0.01 mM phosphate buffer (pH 7.4) using Teflon motorized Dounce (i.e. dounce approx. 10x). A sample volume of 100 μl was removed for protein determination and the remaining volume (1.9 ml) stored at -80 °C until required. The protein content of the mucosa homogenates was determined according to the Kaushal and Barnes (1986) method as described above. Mucosa homogenate and RBC ghost membranes containing approximately 2 mg of protein were used for the assay. For sample preparation, 2.5 mM ferrous sulphate (200 μl) was added to the reactions and incubated in a shaking water bath at 37 °C for one hour. Chloroform-methanol (CM, 1:2, v/v) (vortex 60 sec), then chloroform (vortex 30 sec), and finally saline saturated CM (vortex 30 sec) were added to the samples at a ratio of 3:1:1 and samples centrifuged at 1000xg for 10 minutes at 4 °C. The bottom layer was transferred to clean glass culture-tubes Samples were re-extracted with chloroform. The extracts were dried under N_2 gas in a 37 °C water bath, hexane (1 ml) was added, and absorbencies read at 233 nm. Lipid peroxidation was expressed as nmol/mg protein CD equivalents, using the molar extinction coefficient 27000 $M^{-1}cm^{-1}$ (Hu *et al.,* 1989).

3.2.11 Statistical analyses

(a) Feed intake and body weight gain

Feed intake

A mixed effects regression model was used with FISH, BORAGE and MONTH (time of measurement) as the fixed effects covariates. The covariance structure of the repeated measurements residuals within each rat was modelled by a first order autoregressive process. Interaction terms between the various fixed effects were introduced and they were kept based on their improvement of the model fit. The FISH*BORAGE and also the FISH*MONTH interaction (difference in slopes) were tested since this would indicate an intervention (FISH) effect.

- The Satterthwaite degrees of freedom specification was used for the fixed effects testing.
- Proc Mixed in SAS Version 9.1 was used for the regression modeling.

Least Squares Means (LSMeans) were used to estimate treatment (FISH) effects the time (WEEK) effect at weeks 2, 4, 6, 8, 10, 12 and 14 based on the mean response model. Time (WEEK) comparisons and 95% confidence intervals for the effects and differences were obtained.

Body weight gain

A mixed effects regression model was used with FISH, BORAGE, WEEK (time of measurement) and the baseline weight as the fixed effects covariates. The random effects model within each rat was based on the time of measurement and the baseline response. The covariance structure of the repeated measurements residuals within each rat was modelled by a first order autoregressive process. After starting with only linear time effects in both the fixed and random components of the model the introduction of a quadratic time effect in both produced a dramatic improvement in the model fit based on Akaike Criterion (AIC). Interaction terms between the various fixed effects were introduced (especially FISH*BORAGE) and they were kept based on their improvement of the model fit. The FISH*WEEK and BORAGE*WEEK interactions (difference in slopes) were tested since this would indicate an

intervention (FISH and /or BORAGE) effect since the randomization ensured that the four groups started at the same mean baseline weight.

- The Satterthwaite degrees of freedom specification was used for the fixed effects testing.
- Proc Mixed in SAS Version 9.1 was used for the regression modeling.

Least Squares Means (LSMeans) were used to estimate treatment (FISH and/or BORAGE) effects at weeks 1 to 18 based on the mean response models. This was done to capture the time dependent FISH and/or BORAGE effects. Treatment (FISH and/or BORAGE) comparisons and 95% confidence intervals for the effects and differences were obtained.

(b) Lipid profile and extent of lipid peroxidation

The data was analyzed using a 2-way ANOVA as the data had a 2 x 2 factorial design. The GLM procedure (SAS, Version 9.1) was used to test for interactions and main effects. Where the interaction effect was significant (tested first), confidence limits for the differences of LS-Means were used to measure the effects of all the multiple comparisons in the interaction term (different combinations of factors with each-other). Each of the main effects was tested separately in a 1-way ANOVA using the F-test. Where no interaction effect was observed the main effect was directly obtained from the 2-way ANOVA. Statistical significance was considered at $p<0.05$.

3.3 Results

The statistical analyses of the data examined determined whether any significant interaction (2-way ANOVA) between the fish and borage oil contents of the diets influenced the lipid parameters and the extent of lipid peroxidation present in the rat mucosa and RBC membranes. Where fish/borage oil interaction occurred, the confidence limits ($p<0.05$) were determined to measure the effects of all the multiple comparisons in the interaction term for interactions between diets: (a) S and SB, (b) S and SF, (c) S and SBF, (d) SB and SF, (e) SB and SBF, and lastly (f) SF and SBF. In addition, the main effect of the fish and borage oils were also determined to

indicate whether the fish oil or borage oil content in the diets had a significant ($p<0.01$ or $p<0.05$) effect overall, not specific to any diet.

3.3.1 Fatty acid content of dietary oils (Table 1)

The sunflower oil (S) diet consisted mainly of C18:1n-9 (25.17%) and C18:2n-6 (62.25%), with an n-6/n-3 FA ratio of approximately 250:1. The total PUFA content was (62.56%) with a PUFA/SATS (P/S) ratio of 4.99. Fish oil (F) contained C18:1n-9 (2.63%), C18:2n-6 (1%), C18:3n-3 (0.86%), C20:5n-3 (73.01%) and C22:6n-3 (11.65%), with a total PUFA content of 89.29%. The fish oil had an n-6/n-3 ratio of 0.02 and a P/S ratio 38.32. Borage oil consisted mainly of C18:1n-9 (17.9%), C18:2n-6 (33.76%) and C18:3n-6 (19.32%), with a total PUFA percentage of 57.51. The borage oil had an n-6/n-3 ratio of 14.98 and a P/S ratio of 3.77.

The sunflower and fish oil (SF) diet contained C18:1n-9 (18%) C18:2n-6 (61.25%), C18:3n-3 (0.13%), C20:5n-3 (4.40%) and C22:6n-3 (0.53%), with an n-6/n-3 FA ratio of 12:1. SF had a P/S ratio of 4.90 and a total PUFA of 66.85%. The SB diet contained C18:1n-9 (19%), C18:2n-6 (54.63%), and C18:3n-6 (6.70%), with an n-6/n-3 FA ratio at approximately 30:1. SB consisted of 64.15% PUFA, and a P/S ratio of 4.7. The combination diet containing both fish and borage oil (SBF), predominantly consisted of C18:1n-9 (18.8%), C18:2n-6 (56.63%), C18:3n-6 (4.98%), C18:3n-3 (1.01), C20:5n-3 (3.62%) and C22:6n-3 (0.42%). The SBF diet had an n-6/n-3 ratio of approximately 12:1, a total PUFA 67.05% and P/S ratio of 5.4.

Table 1: FA content of the oils (% of total FA) used in the experimental diets.

Fatty Acids	Dietary oils					
	S	F	B	SF	SB	SBF
C14:0	0.07±0.01	0.21±0.03	0.09±0.01	0.22±0.04	0.12±0.04	0.08±0.01
C16:0	6.68±0.18	0.98±0.12	9.86±0.25	6.22±0.03	7.47±0.15	6.80±0.41
C17:0	0.04±0.02	<0.01	0.26±0.08	0.23±0.07	0.13±0.06	0.09±0.01
C18:0	4.45±0.07	0.75±0.10	4.48±0.09	5.87±0.01	4.90±0.01	4.53±0.01
C20:0	0.29±0.02	<0.01	0.39±0.02	0.34±0.01	0.32±0.03	0.31±0.01
C22:0	0.75±0.06	<0.01	0.23±0.07	0.56±0.08	0.54±0.09	0.59±0.09
C24:0	0.25±0.01	0.39±0.01	<0.01	0.16±0.04	0.18±0.01	0.16±0.01
SATS total	12.53±0.37	2.33±0.26	15.31±0.52	13.63±0.28	13.66±0.39	12.56±0.55
C16:1	0.06±0.02	0.70±0.03	0.11±0.04	0.15±0.03	0.10±0.01	0.12±0.01
C18:1	25.17±0.50	2.63±0.41	17.90±0.55	18.85±0.61	19.72±0.05	18.83±0.08
C20:1	0.21±0.01	0.30±0.02	4.34±0.54	0.23±0.07	1.45±0.07	1.23±0.38
C22:1	0.02±0.01	<0.01	<0.01	<0.01	<0.01	<0.01
C24:1	0.05±0.01	<0.01	2.15±0.28	<0.01	0.83±0.27	0.49±0.03
MUFA total	25.50±0.55	3.63±0.46	24.50±1.41	19.23±0.71	22.45±	20.67±0.50
C18:2n-6	62.25±0.76	1.00±0.01	33.76±0.84	61.25±0.14	54.63±0.25	54.28±3.31
C18:3n-6	<0.01	0.67±0.01	19.32±0.14	<0.01	6.70±0.08	5.97±1.41
C20:2n-6	0.02±0.01	<0.01	0.83±0.26	0.26±0.02	0.61±0.04	0.08±0.03
C20:3n-6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
C20:4n-6	<0.01	<0.01	<0.01	0.22±0.01	<0.01	0.18±0.02
C22:4n-6	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
C22:5n-6	<0.01	0.39±0.01	<0.01	<0.01	<0.01	<0.01
n-6 total	62.31±0.77	2.06±0.03	53.91±1.24	61.73±0.17	61.94±0.37	61.84±4.77
C18:3n-3	0.25±0.01	0.86±0.11	3.60±0.01	0.13±0.05	1.27±0.01	1.19±0.25
C20:3n-3	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
C20:5n-3	<0.01	73.01±0.41	<0.01	4.40±0.24	<0.01	3.52±0.12
C22:5n-3	<0.01	1.71±0.39	<0.01	<0.01	0.33±0.04	0.16±0.01
C22:6n-3	<0.01	11.65±0.65	<0.01	0.53±0.18	<0.01	0.49±0.10
n-3 total	0.25±0.01	87.23±1.56	3.60±0.01	5.06±0.47	2.21±0.05	5.36±0.48
n-6/n-3	249.24±1.64	0.02±0.01	14.98±3.88	12.20±0.36	28.03±7.4	11.54±1.24
PUFA	62.56±0.78	89.29±1.59	57.51±1.25	66.79±0.64	64.15±0.42	67.20±5.25
P/S ratio	4.99±2.11	38.32±6.12	3.76±0.17	4.90±0.89	4.70±1.08	5.35±0.25

Value are means ±SD of two determinations. SATS = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; P/S = PUFA/SATS. S = sunflower oil; F = fish oil; B = borage oil; SF = sunflower oil + fish oil; SB = sunflower oil + borage oil; SBF = sunflower oil + borage oil + fish oil.

3.3.2 Feed intake and weight gain

(a) Daily feed intake

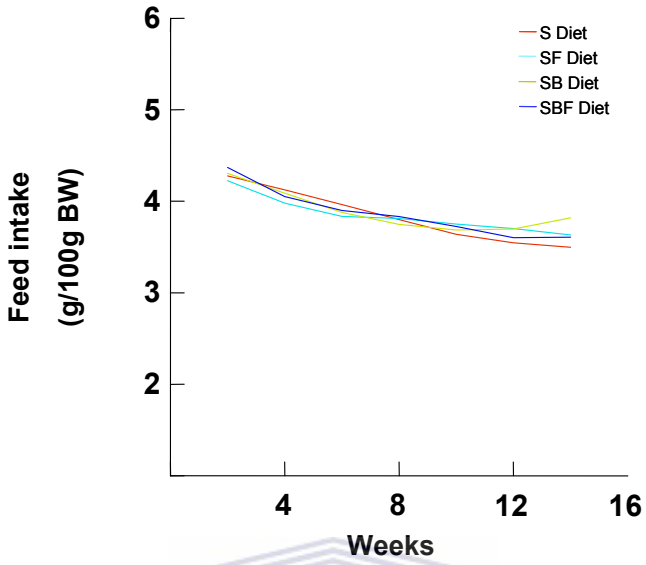

Fig. 1 Daily feed intake profiles over a period of 14 weeks

No significant fish/borage oil interaction was observed. A significant time effect (WEEK) was observed. Rats of all four dietary groups significantly ($p<0.01$) decreased their feed intake from week 2 to week 12, except for the group on the SB diet that was increased between week 12 to week 14.

(b) Daily fatty acid intake

The intake of specific dietary n-6 and n-3 FA quantities correlated with the dietary oils FA composition (Fig 2). All the diets provided a high percentage of the n-6 FA, C18:2n-6 (Fig 2A), With the S diet, rats consumed between 0.5 mg C18:3n-3/100 g BW, with trace quantities (<0.1 mg/100 g BW) of C18:3n-6, C20:4n-6, C20:5n-3 and C22:6n-3 (Fig 2B). The SF diet predominantly provided the animals with n-3 FA, EPA (C20:5n-3), in quantities of approximately 9 mg/100 g body weight (BW) with minor quantities (<1 mg/100 g BW) of C20:4n-6 and C22:6n-3 (Fig 2C). Fat intake with the SB diet mainly provided the rats with n-6 FA C18:3n-6 (approximately 13 mg/100 g BW) and n-3 FA C18:3n-3 (approximately 2 mg/100 g BW) (Fig 2D). Consumption of the SBF diet increased the rats' intake of C18:3n-6, C20:5n-3 and C18:3n-3 predominantly with low levels of C22:6n-3 (Fig 2E).

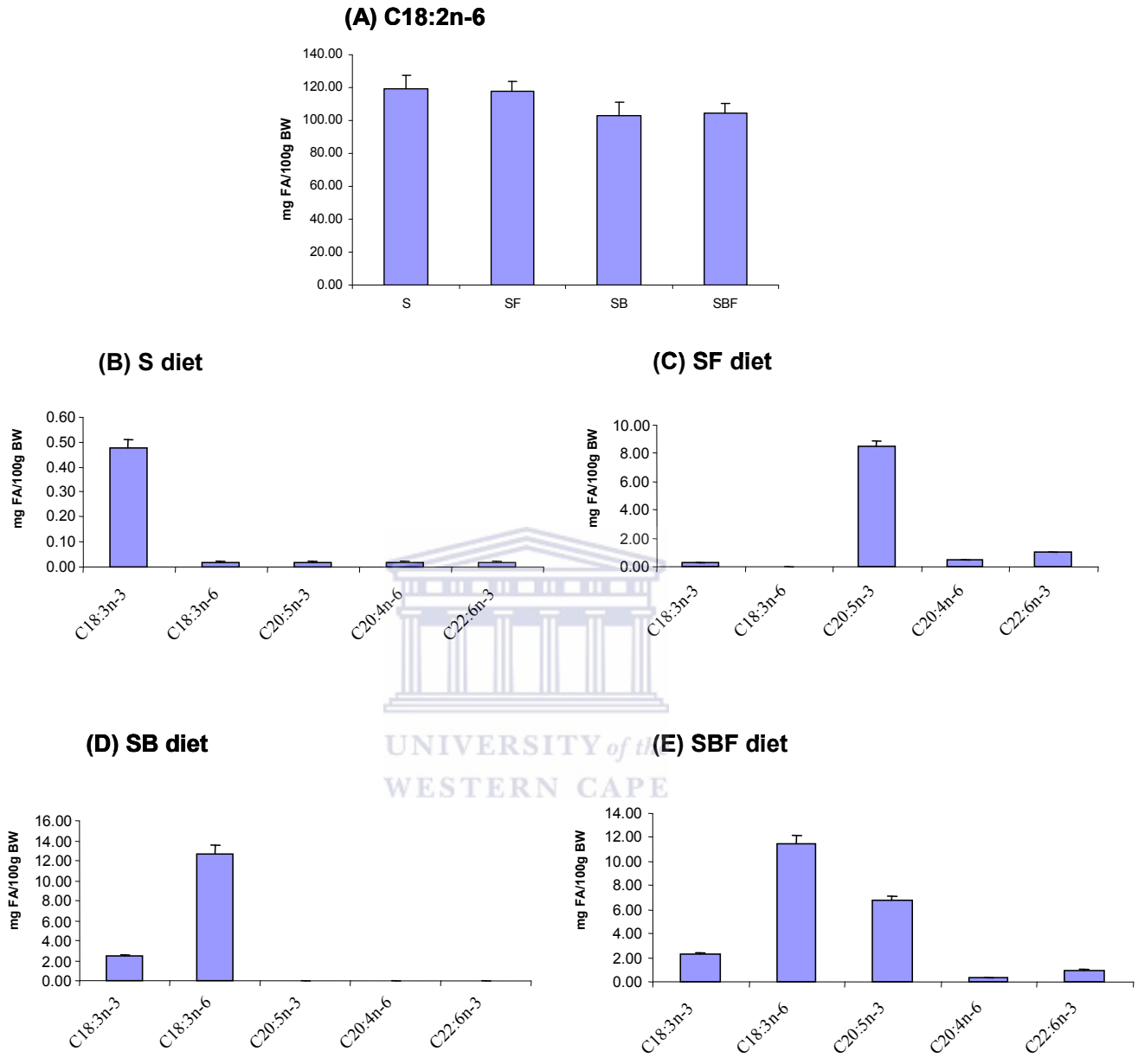

Fig. 2 Fatty acid intake (mg/100 g BW/day) in relation to the different n-6/n-3 FA ratio diets.

(c) Body weight gain

No interaction was observed indicating that the body weight gain profile between fish and borage oils were similar (Fig.3). However, individually both fish and borage oils had significant main effects over time. Fish oil resulted in significantly ($p<0.01$) higher weight gain from week 12 onwards as compared to the control and this effect increased linearly over time (Fig. 4A). Although a significant main effect for the borage oil (Fig.4B) was observed resulting in a higher body weight gain, this effect decreased linearly over time. The linear decrease was only significant ($p<0.05$) from week 15 onwards.

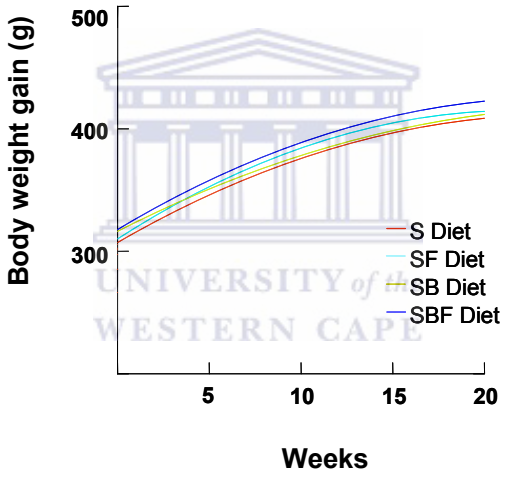

Fig. 3 Body weight gain of rats fed diets supplemented with fish oil and/or borage oil to obtain varying n-6/n-3 FA ratios.

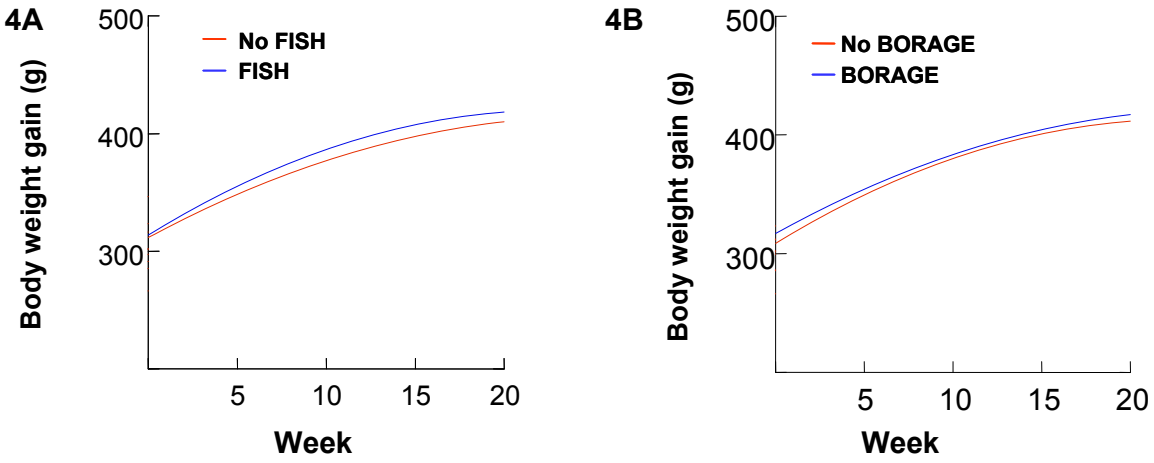

Fig. 4 Effect of fish oil (A) and borage oil (B) on rat weight gain.

3.3.3 Cholesterol and phospholipid content (Table 2)

3.3.3.1 Cholesterol

The fish and borage oil diets had no significant interaction or main effects on the cholesterol content in neither the colon mucosa nor the RBC membranes. In the colon mucosa, however the SBF diet tended to increase the level.

3.3.3.2 PC and PE phospholipid content

Mucosa membranes: No significant fish/borage interaction was noticed. PC tended to be decreased by the SF and SB diets while PE tended to be decreased and increased by the SF and SB diets respectively. The decrease in PC and PE by the SBF diet was similar to the SF diet. Overall, main effects showed a decrease ($p<0.05$) in PE by fish oils.

RBC membranes*:* No significant fish/borage oil interaction was observed. PC and PE phospholipid content tended to increase with all the ratio diets SF and SBF. The SBF diet increased the RBC PC, while the PE content was similar to the level observed with the SF diet. Overall, the fish oil main effect resulted in an increase ($p<0.01$) in the PC and PE phospholipids.

3.3.3.3 PC/PE ratio

Mucosa membranes: No significant fish/borage oil interaction was noticed. The SF and SB diets tended to increase and decrease the ratio, respectively, while the SBF diet tended to counteract the SB diet's reducing effects. Overall, fish and borage oil exhibited significant main effects by increasing ($p<0.05$) and decreasing ($p<0.01$) the ratio, respectively.

RBC membranes: No significant fish/borage oil interaction was observed. The SF diet tended to increase the ratio, with the SBF diet having a greater increasing effect. As a main effect fish oil increased the PC/PE ratio.

3.3.3.4 Chol/PL ratio

Mucosa membranes: No significant fish/borage oil interaction or any main effects were noticed. However, the ratio tended to decrease with the SB diet, while increasing with the SBF diet.

RBC membranes: Significant ($p<0.01$) fish/borage oil interaction was noticed. Both the SF and SB diets significantly decreased ($p<0.05$) the chol/PL ratio. Overall, the fish and borage oils have significant main effects by reducing ($p<0.05$) the chol/PL ratio in the RBC membranes.

Table 2: Comparative effect of the varying n-6/n-3 FA ratio oil diets on the cholesterol and phospholipids (PC and PE) content of the rat colon mucosa and red blood cell membranes.

Lipid Profile	Colon mucosa					Red blood cell membranes				
	S	SF	SB	SBF	Main Effects	S	SF	SB	SBF	Main Effects
Cholesterol (μg chol/mg protein	54.24±13.43	55.53±15.12	48.22±16.23	72.45±7.25	–	0.003±0.001	0.003±0.001	0.003±0.001	0.003±0.001	–
PC	335.67±75.89	280.40±71.70	214.75±63.68	318.21±59.42	–	2.00±0.64	3.37±0.50	2.29±0.84	3.64±0.67	F * ↑
PE	216.18±65.31	140.57±40.41	260.29±33.11	168.94±28.86	F * ↓	0.66±0.17	1.02±0.22	0.76±0.30	0.96±0.37	F * ↑
(μg Pi/mg protein)										
PC/PE (Molar)	1.97±0.38	2.56±1.03	1.18±0.57	1.78±0.04	F $^{\#}$ ↑, B * ↓	2.73±0.48	3.24±0.76	2.93±0.46	3.80±0.66	F * ↑
chol/PL (Molar)	0.31±0.19	0.28±0.13	0.22±0.08	0.32±0.07	–	0.003±0.001^{abc}	0.001±0.001^{b}	0.002±0.001^{a}	0.001±0.001^{c}	F * ↓, B $^{\#}$ ↓

Values are means (n =4 -9) ± SD of the cholesterol and phospholipid content expressed in μg (cholesterol or phosphate) per mg protein. Diets: S = sunflower oil only; SF = sunflower oil + fish oil; SB = sunflower oil + borage oil; SBF = sunflower oil + borage oil + fish oil. Phospholipids: PC = phosphatidylcholine; PE = phosphatidylethanolamine; chol/PL = cholesterol/phospholipids ratio. Significant fish/borage oil interaction at $p<0.05$ (superscript) specified by the same letter. Main effects due to the presence of fish oil (F) or borage oil (B) on the lipid profiles: increase =↑, decrease =↓, no effect = –. Significant main effects of F/B indicated by * = $p<0.01$ and # = $p<0.05$.

3.3.4 Fatty acids

The FA content of the phospholipids was expressed qualitatively (%) and quantitatively (μg).

3.3.4.1 Saturated fatty acids (SATS) (Table 3)

Mucosa PC (%): No significant fish/borage oil interaction or main effects were observed.

Mucosa PC (μg): No significant fish/borage oil interaction or overall main fish or borage oil effects were observed.

Mucosa PE (%): No significant fish/borage oil interaction was observed. However, the SF diet tended to increase total SATS, in contrast to a decrease with the SB diet. The SBF diet reduced the total SATS. Overall, main effects produced by fish oil increased ($p<0.01$) the total SATS due to a significant increase ($p<0.01$) in C18:0, as well as a marked increase in C14:0, C16:0 and C20:0. Borage oil, however, counteracted this effect by reducing ($p<0.01$) the total SATS as a result of a decrease ($p<0.01$) in C16:0. Borage oil significantly ($p<0.05$) increased C20:0.

Mucosa PE (μg): Significant fish/borage oil interaction was only observed for C18:0. The SB diet reduced ($p<0.05$) C18:0 significantly when compared to both the S and SF diets. The SBF diet counteracted these effects, with the fish oil increasing C18:0 above the level observed with the SB diet. Overall, fish oil had a significant main effect ($p<0.05$) resulting in an increase in total SATS content due to increases in C14:0, C16:0 and C20:0. However, borage oil countered these effects by significantly reducing ($p<0.01$) the levels of C14:0, C16:0 and total SATS.

Table 3: Saturated fatty acid composition (% total fatty acids and μg fatty acid/mg protein) of the membrane phospholipids PC and PE in rat colon mucosa and red blood cell membranes.

Fatty Acids	Qualitative (% of total fatty acids)					Quantitative (μg FA/mg protein)				
	S	SF	SB	SBF	Main effects	S	SF	SB	SBF	Main effects
Mucosa PC										
C14:0	0.85± 0.38	0.43± 0.18	0.39± 0.22	0.18± 0.01	–	2.10±1.24	0.85± 0.75	0.62± 0.47	<0.01	–
C16:0	25.33± 1.57	23.29± 2.92	22.20± 4.44	22.62± 3.33	–	45.83± 7.82	33.35± 8.17	34.01± 18.45	41.29± 11.48	–
C18:0	8.31± 1.55	9.16± 1.75	11.07± 3.77	8.58± 1.04	–	17.39± 6.83	14.47± 3.98	12.82± 5.30	15.40± 2.72	–
C20:0	0.71± 0.25	0.76± 0.24	0.65±0.07	0.90± 0.33	–	1.41± 0.32	2.89± 2.19	1.28± 0.45	1.59± 0.56	–
C22:0	0.54± 0.12	0.56± 0.04	0.49±0.11	0.58± 0.13	–	1.14± 0.24	0.77± 0.03	0.79± 0.51	1.09± 0.45	–
C24:0	0.31± 0.09	0.44± 0.10	0.29±0.10	0.33± 0.05	–	0.68± 0.11	0.68± 0.17	0.52± 0.40	0.62± 0.15	–
Total SATS	34.38± 5.09	34.31± 3.98	34.48±1.67	32.83± 4.16	–	67.84± 14.54	50.58± 12.16	36.62± 19.36	59.52± 13.97	–
Mucosa PE					–					
C14:0	0.20± 0.07	0.38± 0.27	0.16± 0.00	0.07± 0.01		0.26± 0.09	1.07± 0.54	0.17± 0.07	0.08± 0.01	B * ↓
C16:0	9.60± 1.58	11.89± 2.45	5.82± 2.10	6.90± 1.67	B * ↓	12.36± 2.43	14.11± 4.57	4.14± 2.80	7.99± 2.14	B * ↓
C18:0	14.54± 0.56	17.74± 3.65	12.72± 2.95	16.49± 1.15	F * ↑	20.54± 6.26^{a}	20.69± 7.32^{b}	5.29± 1.76^{abc}	19.32± 5.37^{c}	–
C20:0	0.44± 0.14	0.49± 0.09	0.65± 0.10	0.64± 0.32	B # ↑	0.68± 0.35	0.99± 0.79	0.46± 0.27	0.74± 0.49	–
C22:0	0.69± 0.23	0.64± 0.15	0.82± 0.15	0.66± 0.11	–	0.97± 0.42	0.63± 0.20	0.55± 0.24	0.80± 0.29	–
C24:0	<0.01	<0.01	<0.01	<0.01	–	<0.01	<0.01	<0.01	<0.01	
Total SATS	25.39± 1.30	30.97±5.87	20.07± 3.35	24.74± 1.95	F * ↑, **B * ↓**	34.74± 9.18	36.68± 12.54	8.73± 3.36	28.91± 6.92	**F # ↑**, B * ↓
RBC PC										
C14:0	<0.01	0.36±0.11	0.25±0.19	0.33±0.07	–	0.07±0.06^{ab}	0.02±0.01^{b}	<0.01^{a}	0.03±0.02	–
C16:0	12.05±1.54^{a}	19.34±4.18^{ab}	16.54±2.44	12.74±4.22^{b}	–	0.30±0.07^{a}	0.80±0.19^{ab}	0.50±0.13^{bc}	0.37±0.11^{c}	F # ↑
C18:0	10.27±1.12^{ab}	17.10±3.63^{b}	14.94±2.08^{a}	13.13±3.54	–	0.29±0.11^{a}	0.65±0.26^{ab}	0.43±0.15	0.32±0.05^{b}	F # ↑
C20:0	0.37±0.30	0.58±0.17	0.24±0.09	0.40±0.22	–	0.03±0.01	0.20±0.01	0.01±0.01	0.04±0.05	–
C22:0	0.11±0.02	0.16±0.08	0.32±0.19	0.25±0.11	B # ↑	<0.01	<0.01	<0.01	<0.01	–
C24:0	0.33±0.23	0.25±0.04	0.22±0.07	0.21±0.05	–	0.02±0.00^{a}	0.01±0.01	<0.01^{a}	0.01±0.00	B # ↓
Total SATS	21.33±3.26^{abc}	38.36±4.60^{bd}	29.71±2.72^{a}	28.16±6.39^{cd}	F * ↑	0.55±0.12	1.48±0.35	0.92±0.29	1.38±0.93	F * ↑
RBC PE										
C14:0	0.34±0.03	0.91±0.82	0.49±0.35	0.67±0.37	–	<0.01	0.02±0.02	<0.01	0.02±0.02	–
C16:0	5.49±1.19	6.18±2.31	7.98±2.75^{a}	3.30±0.71^{a}	–	0.06±0.01	0.10±0.04	0.11±0.06	0.08±0.05	–
C18:0	7.63±2.41	10.54±5.36^{a}	7.80±1.93	4.82±1.92^{a}	–	0.07±0.02	0.22±0.15	0.09±0.03	0.24±0.15	F * ↑
C20:0	0.58±0.14^{a}	0.20±0.04^{a}	0.24±0.12	0.27±0.10	B # ↓	<0.01	<0.01	<0.01	<0.01	–
C22:0	0.41±0.02	0.51±0.24	0.53±0.29	0.40±0.04	–	<0.01	<0.01	<0.01	0.03±0.03	–
C24:0	<0.01	0.28±0.14	0.29±0.22	0.28±0.22	–	<0.01	<0.01	<0.01	<0.01	–
Total SATS	14.19±4.12	22.07±11.38^{a}	17.73±5.75	9.06±2.08^{a}	–	0.15±0.03	0.21±0.13	0.20±0.07	0.25±0.17	–

Values are means (n = 4-9) ± SD of the qualitative (%) and quantitative (μg FA/mg protein) fatty acid content of rat colon mucosa and RBC membranes. Diets: S = sunflower oil only; SF = sunflower oil + fish oil; SB = sunflower oil + borage oil; SBF = sunflower oil + borage oil + fish oil; PC= phosphatidylcholine; PE = phosphatidylethanolamine; SATS= saturated fatty acids. Significant fish/borage oil interaction indicated by significant differences between 2 dietary groups at p<0.05 (superscript) as shown by the same letters. Main effects due to the presence of the fish (F) or borage (B) oils indicated by: increase = ↑, decrease = ↓, no effect = –. Significant main effects specified by * = p<0.01 and # = p<0.05. Where both F and B have significant main effects at p<0.01 or p<0.05, the value highlighted in bold has the highest main effect.

RBC PC (%): Significant fish/borage oil interaction affected the total SATS, C16:0 and C18:0. The SF and SB diets increased ($p<0.05$) C16:0, C18:0 and total SATS, with the SF diet having the greater effect. The SBF diet decreased ($p<0.05$) C16:0 and total SATS in comparison to the level observed with the SB diet, where borage oil tended to counter the effect of the SF diet. Overall, the presence of fish oil had a significant main effect ($p<0.01$) resulting in an increased total SATS, due to increases in C16:0 and C18:0, while borage oil significantly ($p<0.05$) increased the level of C22:0.

RBC PC (μg)*:* Significant fish/borage oil interaction influenced the membrane content of C14:0, C16:0, C18:0 and C24:0. The SF diet significantly increased ($p<0.05$) C16:0 and C18:0, while decreasing ($p<0.05$) C14:0. The SB diet significantly reduced ($p<0.05$) C14:0 and C24:0 compared to the S, while C16:0 decreased ($p<0.05$) to a level below that observed with the SF diet. The SBF diet decreased ($p<0.05$) C16:0 and C18:0 due to borage oil countering the increasing effects caused by the fish oil. Overall, fish oil significantly increased the total SATS by increasing C16:0 ($p<0.05$) and C18:0 ($p<0.05$), while borage oil significantly reduced ($p<0.05$) C24:0.

RBC PE (%): Significant fish/borage oil interaction influenced the total SATS, C16:0, C18:0 and C20:0. The SF diet tended to increase C14:0, C18:0 and C24:0, while significantly ($p<0.05$) decreasing C20:0, resulting in an increase in total SATS. The SB diet significantly ($p<0.05$) increased C16:0 in comparison to the SBF diet. The SBF diet significantly ($p<0.05$) decreased the total SATS largely due to reductions in C18:0 ($p<0.05$). Overall, only borage oil had a significant main effect ($p<0.05$) resulting in a decrease in C20:0.

RBC PE (μg): No significant fish/borage oil interaction was observed. Fish oil, overall, had a significant main effect ($p<0.01$) that resulted in an increase in C18:0.

3.3.4.2 Monounsaturated fatty acids (MUFA) (Table 4)

Mucosa PC (%): The MUFA was not affected by any significant fish/borage oil interaction. The SF and SBF diets tended to increase the level of C18:1n-9. Overall, no significant fish oil or borage oil main effects were observed.

Mucosa PC (μg): No significant fish/borage oil interaction influenced the MUFA content. The SB diet tended to increase C18:1n-9 while the SBF diet favoured an increase in C18:1n-9, mainly due to the influence of the borage oil. Overall, borage oil significantly increased the total MUFA as a main effect due to the increased ($p<0.01$) C18:1n-9 level.

Mucosa PE (%): No significant fish/borage oil interaction influenced the MUFA. Overall, fish oil had a significant main effect by increasing ($p<0.05$) C18:1n-9, whilst borage oil resulted in a decrease ($p<0.01$) in C16:1 and an increase ($p<0.01$) in C20:1.

Mucosa PE (μg): Fish/borage oil interaction significantly influenced the total MUFA and C18:1n-9 content. The SB diet significantly decreased ($p<0.05$) the total MUFA due to a decrease ($p<0.05$) in C18:1n-9. With the combination diet SBF, the total MUFA and C18:1n-9 increased ($p<0.05$) significantly due to the fish oil countering the reducing effect observed with the SB diet. Overall, the fish and borage oils did not have any significant main effects on the MUFA content.

RBC PC (%): Significant ($p<0.05$) fish/borage oil interaction altered the total MUFA. Individually, both the SF and SB diets significantly ($p<0.05$) increased the total MUFA mainly due to a marked increase in C18:1n-9. Overall, no fish or borage oil main effects were observed.

RBC PC (μg): Significant fish/borage oil interaction was observed to alter the levels of 18:1n-9, with the SF diet significantly ($p<0.05$) increasing the content in relation to the S diet. Overall, main effects produced by fish oil significantly increased C18:1n-9 ($p<0.05$).

Table 4: Monounsaturated fatty acid composition (% total fatty acids and μg fatty acid/mg protein) of the membrane phospholipids PC and PE in rat colon mucosa and red blood cell membranes.

Fatty Acids	Qualitative (% of total fatty acids)					Quantitative (μg FA/mg protein)				
	S	SF	SB	SBF	Main effects	S	SF	SB	SBF	Main effects
Mucosa PC										
C16:1	1.23±.0.28	0.91± 0.12	0.86± 0.58	1.01± 0.26	–	2.09± 0.83	0.91± 0.38	1.43± 1.34	1.95± 0.89	–
C18:1n-9	12.72± 1.09	13.01± 1.17	12.11± 2.24	14.07± 1.12	–	19.22± 6.73	14.18± 4.09	24.94± 5.89	26.18± 7.60	B *↑
C20:1	0.45± 0.09	0.48± 0.12	0.49± 0.12	0.51± 0.09	–	0.83± 0.48	1.20± 1.11	0.84± 0.45	0.96± 0.34	–
C22:1	0.20±.0.07	0.21± 0.05	0.15± 0.04	0.20± 0.08	–	0.48± 0.23	0.86± 0.68	0.33± 0.13	0.41± 0.23	–
C24:1	0.20± 0.12	0.23± 0.01	0.14± 0.12	0.06± 0.03	–	0.41± 0.25	1.09± 1.11	0.13± 0.02	0.10± 0.02	–
Total MUFA	20.45± 1.27	20.00± 1.24	20.01± 2.77	21.88± 1.59	–	23.74± 16.84	23 .32± 4.78	36.49±4.31	36.81±9.89	B $^{\#}$↑
Mucosa PE										
C16:1	0.42± 0.13	0.50± 0.13	0.27± 0.14	0.25± 0.07	B *↓	0.51± 0.11	0.91± 0.72	0.24± 0.30	0.31± 0.09	–
C18:1n-9	7.92± 0.69	9.09± 1.57	7.61± 1.98	9.24± 0.35	F $^{\#}$↑	11.03± 3.43^{a}	9.69± 3.36	2.45± 0.99^{ab}	11.03± 2.86^{b}	–
C20:1	0.19± 0.03	0.15± 0.04	0.36± 0.04	0.30± 0.13	B *↑	0.27± 0.08	0.15± 0.02	0.24± 0.13	0.40± 0.17	–
C22:1	0.24± 0.14	0.30± 0.16	0.23± 0.03	0.15± 0.02	–	0.35± 0.24	0.58± 0.48	0.58± 0.48	0.18± 0.08	–
C24:1	0.31± 0.10	<0.01	0.17± 0.07	<0.01	–	0.44± 0.19	<0.01	0.17± 0.01	<0.01	–
Total MUFA	11.22± 1.68	12.23± 1.54	9.15±5.19	13.01± 0.72	–	16.48± 4.90^{a}	13.17± 3.79	6.11± 0.87^{ab}	15.71± 4.03^{b}	–
RBC PC										
C16:1	0.23±0.17	0.26±0.08	0.21±0.06	0.36±0.23	–	0.01±0.01	0.01±0.00	<00.1	0.01±0.01	–
C18:1n9	3.00±0.85	4.15±1.13	3.57±0.51	3.73±0.96	–	0.07±0.01^{a}	0.16±0.04^{a}	0.11±0.04	0.11±0.04	F $^{\#}$↑
C20:1	<0.01	0.19±0.06	0.11±0.04	0.14±0.05	–	<0.01	<0.01	<0.01	<0.01	–
C22:1	0.15±0.07	0.04±0.03^{a}	0.07±0.00	0.17±0.06^{a}	–	<0.01	<0.01	<0.01	<0.01	–
C24:1	<0.01	<0.01	<0.01	<0.01	–	<0.01	<0.01	<0.01	<0.01	–
Total MUFA	4.09±0.68^{ab}	5.83±0.81^{b}	5.64±0.70^{a}	5.40±1.51	–	0.18±0.11	0.22±0.07	0.17±0.06	0.27±0.17	–
RBC PE										
C16:1	<0.01	0.29±0.07	0.22±0.10	0.33±0.14	–	<0.01	<0.01	<0.01	0.01±0.00	–
C18:1n-9	3.06±0.73^{a}	5.54±2.50^{a}	4.69±0.97	4.10±1.76	–	0.03±0.01	0.11±0.08	0.07±0.04	0.09±0.06	F $^{\#}$↑
C20:1	0.93±0.54	0.22±0.00	0.21±0.11	0.64±0.54	–	0.02±0.02	<0.01	<0.01	<0.01	–
C22:1	<0.01	<0.01	<0.01	<0.01	–	<0.01	<0.01	<0.01	<0.01	–
C24:1	<0.01	<0.01	0.96±0.22	2.10±0.62	–	<0.01	<0.01	0.01±0.00	0.03±0.02	–
Total MUFA	5.37±1.37	6.43±2.03	7.34±1.41	7.09±2.83	–	0.06±0.02	0.11±0.05	0.10±0.04	0.15±0.07	B *↑

Values are means (n = 4-9) ± SD of the qualitative (%) and quantitative (μg FA/mg protein) fatty acid content of rat colon mucosa and RBC membranes. Diets: S = sunflower oil only; SF = sunflower oil + fish oil; SB = sunflower oil + borage oil; SBF = sunflower oil + borage oil + fish oil; PC=phosphatidylcholine; PE=phosphatidylethanolamine; MUFA= monounsaturated fatty acids. Significant fish/borage oil interaction indicated by significant differences between 2 dietary groups at p<0.05 (superscript) as shown by the same letters. Main effects due to the presence of the fish (F) or borage (B) oils indicated by: increase =↑, decrease = ↓, no effect = –.

RBC PE (%): Only C18:1n-9 was significantly affected by a fish/borage oil interaction. The SF diet increased ($p<0.05$) C18:1n-9. Diets SF, SB and SBF tended to increase the total MUFA mainly due to increases in C18:1n-9. However, neither fish nor borage oil exhibited any overall main effects.

RBC PE (μg): The RBC membranes MUFA content was not influenced by any fish/borage oil interaction. However, overall borage oil significantly ($p<0.01$) increased the total MUFA, while fish oil significantly ($p<0.05$) increased C18:1n-9.

3.3.4.3 Polyunsaturated fatty acids (PUFA)

(a) n-6 PUFA (Table 5)

Mucosa PC (%): No significant fish/borage interactions were observed to influence the total membrane n-6 PUFA content. The SF diet tended to increase total n-6 PUFA, C18:2n-6 and C20:3n-6 while reducing C22:5n-6. The SB diet tended to reduce C18:2n-6, while increasing C20:3n-6 and C20:4n-6 in comparison to the S diet. C20:4n-6 tended to be increased by the SBF diet due to borage oil countering fish oil's decreasing effect. Overall, fish oil significantly increased C18:2n-6 ($p<0.01$) and the total n-6 PUFA ($p<0.05$).

Mucosa PC (μg): No significant fish/borage oil interactions were observed to influence the n-6 PUFA content. However, the SF diet tended to increase C18:3n-6, C20:2n-6 and C22:2n-6, while reducing C18:2n-6 and C20:4n-6, compared to the S diet. A similar result was detected with the SB diet, with the diet also reducing C18:2n-6 and C22:4n-6, while increasing C20:4n-6 and total n-6 PUFA. The SBF diet tended to decrease C18:3n-6, C20:3n-6 and C20:2n-6 by cancelling the individual increasing effects of the two oils. C18:2n-6 and C20:4n-6 was increased with the SBF diet, due to the enhancing effect of borage oil. No fish oil neither borage oil main effects were observed.

Mucosa PE (%): Significant fish/borage oil interaction was observed to influence the n-6 fatty acids. The SB diet tended to increase C18:3n-6, C20:3n-6 and C20:4n-6, while the SBF diet had a similar effect. Overall main

borage oil effects significantly increased ($p<0.01$) C20:3n-6, C20:4n-6 and C22:4n-6 resulting in an increase ($p<0.05$) in total n-6 PUFA, while C20:2n-6 and C22:2n-6 was reduced ($p<0.05$). The fish oil mainly reduced ($p<0.01$) C22:4n-6 and C22:5n-6.

Mucosa PE (μg): C20:2n-6 and C22:5n-6 content was significantly altered by fish/borage oil interactions. The SF diet increased C20:2n-6 significantly ($p<0.05$), but lowered ($p<0.05$) C22:5n-6 with a resultant decrease in total n-6 PUFA compared to the S diet. SF tended to decrease C18:2n-6, C20:4n-6, C22:4n-6 and C22:5n-6 while 18:3n-6 was increased. The SB diet significantly ($p<0.05$) reduced C20:2n-6 and C22:5n-6 in comparison to the SF and S diets, respectively, while C18:3n-6 and C20:4n-6 tended to increase, with C18:2n-6 and C22:4n-6 decreasing as well. The SBF diet reduced C20:2n-6 and C22:5n-6 significantly ($p<0.05$) in comparison to the SF and S diets, respectively. Overall, main effects exerted by borage oil reduced C20:2n-6 content ($p<0.01$), whereas fish oil reduced C22:5n-6 content ($p<0.05$).

RBC PC (%): No significant fish/borage interaction affected the total n-6. The SF diet tended to increase C18:2n-6 and decrease C20:3n-6, C20:4n-6, C22:4n-6 and C22:5n-6 compared to the S diet. The SB diet tended to increase the total n-6 PUFA, and C20:4n-6. With the SBF diet, C20:3n-6 was increased due to SB diet countering the reducing effects of the SF diet. An opposite effect was noticed when considering the C20:4n-6 level. Overall, significant main effects exerted by fish oil reduced ($p<0.01$) C20:4n-6 and C22:5n-6, whereas borage oil increased ($p<0.01$) C20:4n-6.

RBC PC (μg): No significant fish/borage oil interactions were noted. The SF diet tended to increase the total n-6 PUFA while the SB diet tended to increase C20:4n-6 which was also reflected with the SBF diet. Overall, fish oil main effects significantly increased ($p<0.05$) total n-6 PUFA content, while borage oil increased ($p<0.05$) C20:4n-6.

Table 5: Polyunsaturated fatty acid (n-6) composition (% total fatty acids and μg fatty acid/mg protein) of the membrane phospholipids PC and PE in rat colon mucosa and red blood cell membranes.

Fatty Acids	Qualitative (% of total fatty acids)					Quantitative (μg FA/mg protein)				
	S	SF	SB	SBF	Main effects	S	SF	SB	SBF	Main effects
Mucosa PC										
C18:2	11.00± 1.72	14.45± 1.68	9.61± 2.54	12.58± 1.36	F * ↑	16.72± 5.89	14.59± 6.07	14.18± 9.55	22.86± 7.19	–
C18:3	0.29± 0.07	0.23± 0.04	0.33± 0.09	0.29± 0.05	–	0.56± 0.29	0.97± 0.76	0.60± 0.41	0.53± 0.18	–
C20:2	0.76± 0.14	1.04± 0.25	0.80± 0.09	0.77± 0.11	–	1.66± 0.64	3.75± 3.73	1.75± 0.18	1.41± 0.49	–
C20:3	1.74± 0.32	2.28± 0.41	2.15± 0.74	2.61± 0.61	–	3.14± 2.20	3.08± 0.72	3.69± 2.29	4.85± 2.24	–
C20:4	8.00± 0.91	7.89± 0.90	8.81± 2.47	9.59± 2.18	–	11.36± 6.61	8.05± 3.89	15.27± 8.95	20.10± 6.76	–
C22:2	0.19± 0.06	0.21± 0.05	0.22± 0.09	0.12± 0.02	–	0.38±0.09	0.83± 0.98	0.47± 0.31	<0.01	–
C22:4	0.64± 0.13	0.51± 0.04	0.66± 0.12	0.65± 0.13	–	1.25± 0.60	1.14± 1.12	1.04± 0.50	1.09± 0.25	–
C22:5	0.32± 0.16	<0.01	0.16± 0.03	<0.01	–	0.67± 0.16	<0.01	0.27± 0.17	<0.01	–
Total n-6 PUFA	22.81± 2.40	26.64± 2.95	22.63± 5.77	28.41± 1.65	F # ↑	28.53± 15.79	21.26± 11.68	39.02± 22.04	45.97± 21.08	–
Mucosa PE										
C18:2	5.30± 1.12	4.69± 1.14	4.72± 1.45	6.24± 0.62	–	7.60± 2.05	4.42± 0.68	4.38± 4.71	7.39± 1.85	–
C18:3	0.12± 0.06	0.17± 0.13	0.58± 0.48	0.25± 0.08	–	0.16± 0.05	0.68± 0.83	0.51± 0.48	0.28± 0.12	–
C20:2	0.69± 0.24	1.17± 0.74	0.54± 0.14	0.49± 0.06	B # ↓	1.03± 0.43^{a}	2.86± 0.73^{abc}	0.37± 0.19^{b}	0.58± 0.15^{c}	B * ↓
C20:3	1.93± 0.47	1.87± 0.71	2.65± 0.83	3.17± 0.37	B * ↑	2.75± 1.24	1.75± 0.62	3.27± 1.71	3.82± 1.31	–
C20:4	20.36± 5.44	19.69± 5.77	29.62± 4.71	30.43± 2.03	B * ↑	25.52± 7.22	18.87± 3.05	32.23± 16.41	36.39± 11.03	–
C22:2	0.63± 0.22	<0.01	0.79± 0.31	0.85± 0.44	B # ↓	<0.01	<0.01	0.57± 0.14	1.22± 0.64	–
C22:4	3.31± 0.73	2.01± 0.73	4.36± 0.89	2.85± 0.42	**F * ↓**, B * ↑	3.67± 0.12	1.92± 0.62	2.46± 1.75	3.25± 0.63	–
C22:5	1.51± 0.42	0.51± 0.15	1.62± 0.63	0.61± 0.09	F * ↓	2.58± 0.69^{abc}	0.51± 0.18^{b}	0.96± 0.48^{a}	0.73± 0.26^{c}	F # ↓
Total n-6 PUFA	29.05± 9.44^{ab}	30.76±7.20^{c}	44.07±5.31^{a}	44.71± 1.85^{bc}	B * ↑	41.46± 6.50	29.69±1.26	45.16± 22.41	59.95± 9.22	–
RBC PC										
C18:2	3.77±0.72	4.57±0.79	4.72±0.77	4.52±1.79	–	0.12±0.03	0.17±0.04	0.13±0.05	0.13±0.06	–
C18:3	<0.01	<0.01	0.21±0.08	0.20±0.08	–	<0.01	<0.01	0.01±0.01	<0.01	–
C20:2	0.21±0.10	0.59±0.05	0.45±0.34	1.76±2.19	–	0.04±.0.01	0.02±0.01	<0.01	0.09±0.10	–
C20:3	0.32±0.15	0.19±0.03	0.22±0.04	0.29±0.10	–	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	–
C20:4	4.69±1.50	4.11±0.39	7.36±1.44	4.94±1.74	F * ↓, **B * ↑**	0.13±0.04	0.16±0.03	0.19±0.05	0.25±0.15	B # ↑
C22:2	<0.01	<0.01	<0.01	<0.01	–	<0.01	<0.01	0.01±0.01	<0.01	–
C22:4	0.41±0.08	0.26±0.10	0.44±0.07	0.42±0.42	–	0.02±0.01	0.01±0.01	0.02±0.01	0.02±0.01	–
C22:5	0.56±0.22	0.09±0.06	0.39±0.12	0.10±0.03	F * ↓	0.02±0.02	<0.01	0.01±0.01	<0.01	–
Total n-6 PUFA	9.24±2.63	10.04±1.56	12.88±2.14	10.53±3.72	–	0.32±0.09	0.40±0.08	0.29±0.06	0.39±0.08	F # ↑
RBC PE										
C18:2	1.81±0.35	1.85±0.46	1.78±0.15	1.38±0.54	–	0.03±0.02	0.03±0.01	0.02±0.01	0.03±0.01	–
C18:3	6.66±4.81	<0.01	0.23±0.06	0.23±0.07	–	<0.01	<0.01	<0.01	<0.01	–
C20:2	0.39±0.09	1.52±1.08	0.52±0.31	1.11±0.61	F # ↑	<0.01	0.02±0.02	<0.01	0.02±0.01	–
C20:3	0.88±0.13^{abc}	0.25±0.16^{b}	0.19±0.07^{a}	0.33±0.13^{c}	B# ↓	0.01±0.01	<0.01	<0.01	0.01±0.01	–
C20:4	8.45±0.60	7.11±2.17	9.50±2.18^{a}	5.87±1.20^{a}	–	0.10±0.02	0.11±0.03	0.12±0.03	0.09±0.03	–
C22:2	<0.01	<0.01	<0.01	<0.01	–	<0.01	<0.01	<0.01	<0.01	–
C22:4	3.39±0.57^{ab}	2.08±0.94^{ac}	4.07±0.96^{cd}	1.13±0.41^{bd}	F * ↓	0.05±0.01	0.03±0.01	0.06±0.05	0.06±0.05	–
C22:5	0.75±0.20	0.63±0.21	1.02±0.45	0.40±0.25	F * ↓	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01	–
Total n-6 PUFA	14.48±3.59	11.89±4.14	15.24±3.92	9.85±1.30	F * ↓	0.20±0.05	0.20±0.03	0.20±0.06	0.19±0.10	–

Values are means (n = 4-9) ± SD of the qualitative (%) and quantitative (μg FA/mg protein) fatty acid content of rat colon mucosa and RBC membranes. Diets: S = sunflower oil only; SF = sunflower oil + fish oil; SB = sunflower oil + borage oil; SBF = sunflower oil + borage oil + fish oil; PC=phosphatidylcholine; PE = phosphatidylethanolamine; PUFA=polyunsaturated fatty acids. Significant fish/borage oil interaction indicated by significant differences between2 dietary groups at p<0.05 (superscript) as shown by the same letters. Main effects due to the presence of the fish oil (F) or borage oil (B) indicated by: increase =↑, decrease =↓, no effect –. Significant main effects specified by * = p<0.01 and # = p<0.05. Where both F and B have main effects at p<0.01 or p<0.05, the value highlighted in bold has the highest main effect.

RBC PE (%): Significant fish/borage oil interaction modulated C20:3n-6, C20:4n-6 and C22:4n-6. The SF diet decreased C20:3n-6 and C22:4n-6 significantly ($p<0.05$) compared to the S diet, while the SB diet decreased ($p<0.05$) C20:3n-6. Compared to the SB diet, the SBF diet significantly reduced ($p<0.05$) C20:4n-6 and C22:4n-6 due to the countering effect of SF. Overall, fish oil significantly reduced ($p<0.01$) total n-6 PUFA, C22:4n-6 and C22:5n-6, while increasing C20:2n-6 ($p<0.05$). C20:3n-6 was reduced significantly ($p<0.05$) due to a borage oil main effect.

RBC PE (μg): No significant fish/borage oil interaction was observed nor any main effects on the total n-6 content in the RBC membrane phospholipids.

(b) n-3 PUFA (Table 6)

Mucosa PC (%): No significant fish/borage interaction was noted. The SF and SB tended to increase C18:3n-3, C20:5n-3, C22:5n-3 and C22:6n-3 in comparison to the S diet. The SBF diet tended to further increase C20:5n-3, C22:5n-3 and C22:6n-3. Overall, fish oil had a significant main effect by increasing total n-3 PUFA, 20:5n-3 and C22:6n-3, while borage oil resulted in a significant increase ($p<0.05$ and $p<0.01$, respectively) in C20:5n-3 and total n-3 PUFA.

Mucosa PC (μg): No significant fish/borage oil interaction was observed. The results indicate that the SF diet tended to increase total n-3 PUFA, C18:3n-3, C20:5n-3, C22:5n-3 and C22:6n-3 compared to the S diet. The SB diet tended to decrease total n-3 PUFA, due to a decrease in C22:5n-3. The SBF diet had a similar effect than the S diet implying that the SB diet countered the effect of SF. Only fish oil had a significant overall main effect that resulted in an increase ($p<0.05$) in total n-3 fatty acid levels.

Mucosa PE (%): No significant fish/borage oil interaction affected the n-3 levels. Significant fish oil main effects include an increased ($p<0.01$) total n-3 PUFA as a result of increased levels of C20:5n-3 and C22:6n-3. Borage oil increased ($p<0.01$) the total n-3 PUFA, due to a significant increase ($p<0.05$) in C20:5n-3.

Table 6: Polyunsaturated fatty acid (n-3) composition (% total fatty acids and μg fatty acid/mg protein) of the membrane phospholipids PC and PE in rat colon and mucosa and red blood cell membranes.

Fatty Acids	Qualitative (% of total fatty acids)					Quantitative (μg FA/mg protein)				
	S	SF	SB	SBF	Main effects	S	SF	SB	SBF	Main effects
Mucosa PC										
C18:3	0.07± 0.00	0.12± 0.03	0.19± 0.17	0.09± 0.04	–	0.17± 0.06	0.60± 0.49	0.19± 0.06	0.33± 0.01	–
C20:5	0.09± 0.00	0.27± 0.05	0.15± 0.00	0.35± 0.09	F * ↑, B # ↑	0.22± 0.06	1.07± 0.81	0.35± 0.01	0.70± 0.26	–
C22:5	0.18± 0.04	0.21± 0.03	0.30± 0.20	0.37± 0.03	–	0.91± 0.61	1.78± 1.49	0.16± 0.01	1.04± 0.39	–
C22:6	0.18± 0.01	0.36± 0.09	0.26± 0.09	0.43± 0.12	F* ↑	0.39± 0.13	0.48± 0.14	0.42± 0.28	0.77± 0.28	–
Total n-3 PUFA	0.31± 0.14	0.89± 0.16	0.60± 0.22	1.23± 0.30	**F * ↑,** B* ↑	1.66± 0.90	3.35± 2.06	1.14± 0.87	2.50± 1.13	F # ↑
Mucosa PE										
C18:3	<0.01	0.33± 0.08	<0.01	<0.01		<0.01	0.38± 0.10	<0.01	<0.01	
C20:5	0.20± 0.06	0.69± 0.25	0.31± 0.10	1.01± 0.13	F * ↑, B #↑	0.29± 0.17	0.57± 0.21	0.27± 0.20	1.26± 0.41	F * ↑
C22:5	0.76± 0.63	0.66± 0.39	0.65± 0.30	0.93± 0.17	–	1.41± 0.87	2.01±0.01	0.42± 0.25	1.06± 0.31	B # ↓
C22:6	1.07± 0.11	1.75± 0.26	1.22± 0.53	1.94± 0.21	F*↑	1.51± 0.29	1.87± 0.16[a]	0.78± 0.60[ab]	2.25± 0.43[b]	F * ↑
Total n-3 PUFA	2.03± 0.56	2.80± 0.66	2.39± 0.62	4.10± 0.18	**F * ↑,** B * ↑	2.30± 0.73[a]	2.67± 0.54[c]	1.80± 1.12[b]	4.83±1.03[abc]	F # ↑
RBC PC										
C18:3	<0.01	0.08±0.04	0.24±0.07	0.16±0.08	–	<0.01	<0.01	<0.01	<0.01	–
C20:5	<0.01	0.12±0.02	0.14±0.04	0.18±0.06	–	<0.01	<0.01	<0.01	<0.01	–
C22:5	0.21±0.12	0.31±0.07	0.44±0.31	1.23±0.96	B # ↑	0.17±0.23[AB]	0.01±0.01[B]	0.01±0.01[A]	0.04±0.02	–
C22:6	0.28±0.15	0.55±0.16	0.38±0.11	0.84±0.38	F * ↑, B # ↑	0.04±0.05	0.02±0.01	0.02±0.01	0.02±0.01	–
Total n-3 PUFA	0.45±0.20	1.30±0.32	1.44±0.67	1.62±0.84	F# ↑, **B# ↑**	0.02±0.02	0.05±0.01	0.05±0.03	0.09±0.06	F # ↑, **B # ↑**
RBC PE										
C18:3	<0.01	0.32±0.05	0.10±0.01	0.30±0.16	–	<0.01	<0.01	<0.01	<0.01	–
C20:5	<0.01	0.23±0.07	<0.01	0.46±0.50	–	<0.01	0.01±0.01	<0.01	0.02±0.02	–
C22:5	1.23±0.37	2.00±0.39	0.86±0.20	1.86±0.65	F* ↑	0.02±0.01	0.04±0.02	0.02±0.02	0.03±0.01	F * ↑
C22:6	<0.01	4.00±1.32	1.37±0.71	0.64±0.20	–	<0.01	0.04±0.02	0.02±0.01	0.03±0.03	–
Total n-3 PUFA	1.11±0.26[a]	6.64±1.52[abc]	2.67±0.93[b]	2.11±1.33[c]	F * ↑, B # ↓	0.01±0.01[a]	0.13±0.04[abc]	0.03±0.02[b]	0.05±0.02[c]	F * ↑

Values are means (n = 4-9) ± SD of the qualitative (%) and quantitative (μg FA/mg protein) fatty acid content of rat colon mucosa and RBC membranes. Diets: S =sunflower oil only; SF = sunflower oil + fish oil; SB = sunflower oil + borage oil; SBF = sunflower oil + borage oil + fish oil; PC=phosphatidylcholine; PE = phosphatidylethanolamine; PUFA = polyunsaturated fatty acids. Significant fish/borage oil interaction indicated by significant differences between 2 dietary groups at p<0.05 (superscript) as shown by the same letters. Main effects due to the presence of the fish oil (F) or borage oil (B) indicated by: increase = ↑, decrease = ↓, no effect = –. Significant main effects specified by * = p<0.01 and # = p<0.05. Where both F and B have main effects at p<0.01 or p<0.05, the value highlighted in bold has the highest main effect.

Mucosa PE (μg): Significant fish/borage oil interactions influenced the total n-3 PUFA as well as C22:6n-3. The SF diet significantly increased ($p<0.05$) C22:6n-3 in comparison to the SB diet. The SB diet significantly decreased ($p<0.05$) C22:6n-3 in comparison to the SBF diet, thus reducing the total n-3 PUFA. With the SBF diet, total n-3 PUFA and C22:6n-3 was significantly increased due to the fish oil countering the reducing effects observed with the SB diet. Overall main effects produced by fish oil increased total n-3 PUFA due to the significant ($p<0.01$) increases in C20:5n-3 and C22:6n-3. Borage oil significantly decreased ($p<0.05$) C22:5n-3.

RBC PC (%): n-3 PUFA was not influenced by any significant fish/borage oil interaction. Overall, fish oil main effects significantly increased total n-3 PUFA ($p<0.05$) and C22:6n-3 ($p<0.01$). Borage oil main effects also resulted in an increase ($p<0.05$) in total n-3 PUFA, C22:5n-3 and C22:6n-3.

RBC PC (μg): Significant fish/borage oil interaction was noticed. The SF and SB diets significantly reduced C22:5n-3 ($p<0.05$) in comparison to the S diet. Overall fish oil and borage oil significantly increased the total n-3 PUFA.

RBC PE (%): Significant fish/borage oil interaction was noticed. The SB diets significantly reduced ($p<0.05$) total n-3 PUFA in comparison to the SF diet mainly due to a decrease in C18:3n-3, C22:5-3 and C22:6n-3. The SB diet, however, tended to increase C22:6n-3 and the total n-3 PUFA as compared to sunflower oil. The SBF diet significantly reduced ($p<0.05$) total n-3 PUFA below the level observed with the SF diet. Overall fish oil significantly ($p<0.01$) increased C22:5n-3 and the total n-3 PUFA while borage oil, countered ($p<0.05$) the fish oil effect.

RBC PE (μg): Significant fish/borage oil interaction was observed to increase the total n-3 PUFA. The SF diet significantly increased total n-3 PUFA. The SBF diet reduced ($p<0.05$) the total n-3 PUFA with SB countering the effect of SF. The fish oil content of the SBF diet increased the C22:6n-3 slightly above

that observed with SB. Overall, fish oil significantly ($p<0.01$) increased C22:5n-3 and the total n-3 PUFA.

(c) Total PUFA and LCPUFA (Table 7)

Mucosa PC (%): No significant fish/borage oil interactions were observed. The main effects exerted by fish oil resulted in increased ($p<0.05$) n-3 LCPUFA, as well as the total PUFA. Borage oil increased ($p<0.05$) the total LCPUFA and n-3 LCPUFA. The SBF diet reflected a combined effect of the SF and SB regarding the increase of the different FA parameters.

Mucosa PC (μg): No significant fish/borage oil interactions were noted. SF also tended to decrease total PUFA, LCPUFA and the n-6 LCPUFA. SBF closely mimicked SB with respect to the total PUFA, LCPUFA and n-6 PUFA and the n-3 PUFA similar to the SF diet. Main effects exerted fish oil resulted in a significant increase ($p<0.05$) n-3 LCPUFA. Borage oil containing diets produced significant main effects that resulting in the increase ($p<0.05$) in total PUFA and LCPUFA and n-6 LCPUFA.

Mucosa PE (%): Significant fish/borage interaction modulated the total PUFA, LCPUFA and n-6 LCPUFA. Diets SF, SB and SBF significantly increased ($p<0.05$) total PUFA and LCPUFA in comparison to the S diet. The SBF diet significantly ($p<0.05$) increased the n-3 LCPUFA content in comparison to the S, SF and SB diets. Both fish and borage oil significantly, overall, increased the total PUFA ($p<0.05$) and n-3 LCPUFA ($p<0.01$), with fish oil having a greater effect. Borage oil also significantly increased ($p<0.01$) the total LC PUFA and n-6 LCPUFA.

Mucosa PE (μg): Fish/borage oil interactions significantly altered ($p<0.05$) the total n-3 LCPUFA content. The SF diet tended to increase the total n-3 LCPUFA, while the SB diet significantly ($p<0.05$) reduced the content. The SBF diet significantly increased ($p<0.01$) the total n-3 LCPUFA, due to the fish oil content countering the reducing effects of borage oil. Main effects exerted

by the borage oil increased ($p<0.05$) the total LCPUFA content largely due to an increase ($p<0.05$) in the n-6 LCPUFA.

RBC PC (%): Fish/borage interactions significantly modulated the total n-6 LCPUFA. In comparison to the SF and SBF diets, the SB diet significantly increased ($p<0.05$) n-6 LCPUFA and tended to increase the other PUFA parameters. SF tended to decrease and increase n-6 and n-3 LCPUFA, respectively. The SBF diet decreased ($p<0.05$) the total n-6 LCPUFA, due to the SF countering the SB effect. Main effects exerted by the fish oil significantly increased ($p<0.05$) n-3 LCPUFA and decreased total n-6 LCPUFA. Borage oil main effects include an increase ($p<0.05$) in all the PUFA parameters.

RBC PC (μg): No significant fish/borage oil interactions were indicated to have an effect on any of the parameters. However, both the SF and SB diets tended to increase the LCPUFA and n-3 LCPUFA. The SBF diet tended to increase all lipid parameters. Overall, both fish and borage oil had a significant ($p<0.05$) main effect by increasing the total n-3 LCPUFA content, with borage oil having the greater increasing effect.

RBC PE (%): Significant fish/borage oil interactions were noticed. The SF diet significantly ($p<0.05$) increased the total n-3 LCPUFA. The SBF diet decreased the total LC PUFA ($p<0.01$) and n-3 LCPUFA ($p<0.05$). No overall main effects were observed.

RBC PE (μg): Significant fish/borage oil interactions were observed. The SF diet significantly ($p<0.05$) increased the n-3 LCPUFA resulting in an increase in the total LCPUFA. The SB diet also tended to increase the n-3 LCPUFA. Overall, fish oil exerted a significant main effect that resulted in an increase ($p<0.05$) in the total LCPUFA mainly due to an increase in total n-3 LCPUFA.

Table 7: Total PUFA and different long chain (LC) PUFA parameters (% total fatty acids and μg fatty acid/mg protein) of the membrane phospholipids PC and PE in rat colon mucosa and red blood cell membranes.

Fatty Acid	Qualitative (% of total fatty acids)					Quantitative (μg FA/mg protein)				
	S	SF	SB	SBF	Main effects	S	SF	SB	SBF	Main effects
Mucosa PC										
PUFA	23.12± 2.50	27.53± 2.95	23.23± 5.67	29.64± 1.93	F # ↑	34.77± 13.93	25.04± 13.85	50.13± 13.00	48.47± 22.00	B # ↑
LC PUFA	11.80± 1.18	12.75± 1.27	13.24± 3.15	16.08± 1.49	B * ↑	16.29± 7.48	11.40± 8.22	28.67± 7.70	24.93± 15.01	B # ↑
n-6 LCPUFA	11.53± 1.12	11.96± 1.33	12.69± 3.30	14.94± 1.23	–	15.43± 7.47	11.90± 6.47	27.36± 7.72	22.57± 14.12	B # ↑
n-3 LCPUFA	0.27±0.10	0.80± 0.13	0.51± 0.23	1.14± 0.28	**F # ↑**, B # ↑	1.57± 0.97	2.90± 1.73	1.05± 0.86	2.80± 0.60	F # ↑
Mucosa PE										
PUFA	31.09± 9.55^{abc}	47.10± 5.17^{b}	46.46± 5.39^{a}	48.81± 1.67^{c}	**F # ↑**, B # ↑	43.96± 7.98	34.45± 13.32	46.51± 22.61	65.26± 9.63	–
LC PUFA	29.35± 5.55^{abc}	41.77± 4.87^{b}	42.13± 5.00^{a}	42.32± 2.11^{c}	B # ↑	37.25± 8.33	29.70± 12.70	48.18± 12.73	56.64± 9.74	B # ↑
n-6 LCPUFA	27.24± 5.56^{ab}	25.50±6.50^{cd}	39.74± 5.22^{ac}	38.22± 2.27^{bd}	B * ↑	34.75± 6.96	27.06± 12.62	39.50± 17.68	51.33± 9.35	B # ↑
n-3 LCPUFA	2.03±0 .56^{a}	2.67± 0.75^{c}	2.39± 0.62^{b}	4.09± 0.17^{abc}	F * ↑, B # ↑	2.30±073^{a}	2.48± 0.35^{c}	1.80± 1.12^{ab}	4.82± 1.05^{bc}	–
RBC PC										
PUFA	10.98±2.48	12.77±3.49	15.89±2.83	14.23±3.21	B * ↑	0.34±0.11	0.38±0.05	0.41±0.13	0.84±0.63	–
LC PUFA	6.18±1.32	5.73±0.90	9.16±2.08	11.18±4.17	B * ↑	0.14±0.06	0.32±0.18	0.29±0.09	0.37±0.22	–
n-6 LCPUFA	5.35±1.38^{a}	5.00±0.49^{b}	8.73±2.55^{abc}	5.78±1.49^{c}	F # ↓, B * ↑	0.19±0.06	0.21±0.05	0.22±0.07	0.27±0.16	–
n-3 LCPUFA	0.53±0.17	1.25±0.33	1.38±0.66	1.88±0.74	F # ↑, B * ↑	0.02±0.02	0.04±0.01	0.05±0.03	0.09±0.07	F # ↑, **B # ↑**
RBC PE										
PUFA	15.85±4.16	17.33±4.00	17.50±4.87	16.21±10.39	–	0.21±0.04	0.32±0.07	0.21±0.05	0.33±0.08	–
LC PUFA	13.07±2.24	15.38±3.97	15.32±4.89^{a}	8.64±3.24^{a}	–	0.18±0.04	0.35±0.15	0.19±0.04	0.20±0.11	F # ↑
n-6 LCPUFA	10.31±3.44	10.11±2.96	13.29±3.71	8.18±1.43	–	0.17±0.04	0.18±0.03	0.16±0.04	0.16±0.10	–
n-3 LCPUFA	1.22±0.49^{a}	5.74±2.10^{ab}	4.08±3.78	1.00±0.71^{b}	–	0.01±0.0^{a}	0.11±0.05^{a}	0.07±0.01	0.04±0.03	–

Values are means (n = 4-9) ± SD of the qualitative (percentage) and quantitative (μg FA/mg protein) fatty acid content of rat colon mucosa and RBC membranes. Diets: S = sunflower oil only; SF = sunflower oil + fish oil; SB = sunflower oil + borage oil; SBF = sunflower oil + borage oil + fish oil; PC=phosphatidylcholine; PE = phosphatidylethanolamine; PUFA= polyunsaturated fatty acids; LC PUFA= long-chain PUFA. Significant fish/borage oil interaction indicated by significant differences between 2 dietary groups at p<0.05 (superscript) as shown by the same letters. Main effects due to the presence of fish oil (F) or borage oil (B) indicated by increase =↑, decrease =↓, no effect = –. Significant main effects specified by * = p<0.01 and # = p<0.05. Where both F and B have main effects at p<0.01 or p<0.05, the value highlighted in bold has the highest main effect.

3.3.5 Membrane fatty acid ratios

(a) P/S; n-6/n-3; ∆6 S/P; C20:4n-6/C20:5n-3 (Table 8)

Mucosa PC (%): A significant ($p<0.05$) fish/borage interaction only affected the C20:4n-6/C20:5n-3 ratio. The SF and SB diets significantly ($p<0.05$) decreased the C20:4n-6/C20:5n-3 ratio with the SF diet having the greater effect. The SBF diet decreased ($p<0.05$) the ratio to the same extent as the SF diet compared to the SB diet. Overall, fish oil decreased the n-6/n-3 ($p<0.01$) and C20:4n-6/C20:5n-3 ratios while increasing, the P/S ($p<0.01$) and ∆6 S/P ($p<0.05$) ratios.

Mucosa PC (μg): Significant fish/borage interaction only affected the C20:4n-6/C20:5n-3 ratio. The C20:4n-6/C20:5n-3 ratio was significantly decreased with both the SF and SB diets. Diet SBF decreased ($p<0.05$) the ratio significantly compared to the SB diet, while to a similar extent as the SF diet. Overall, the fish oil decreased the n-6/n-3 ($p<0.05$) and C20:4n-6/C20:5n-3 ($p<0.01$) ratios, while borage oil increased ($p<0.01$) the n-6/n-3 ratio.

Mucosa PE (%): Significant ($p<0.05$) fish/borage oil interaction was noticed. The SB diet significantly ($p<0.05$) decreased the ∆6 S/P ratio compared to the SBF diet and to some extend the S diet. The SF and SBF diets decreased the C20:4n-6/C20:5n-3. Overall, borage oil increased ($p<0.01$) the P/S ratio, while fish oil decreased ($p<0.01$) the C20:4n-6/C20:5n-3 ratio.

Mucosa PE (μg): No significant interactions were observed, although the SB diet tended to increase the n-6/n-3 and P/S ratios. Overall, fish oil decreased ($p<0.01$) the C20:4n-6/C20:5n-3 ratio, as well as the n-6/n-3 ratio ($p<0.05$) while increasing the delta 6 S/P ratio.

RBC PC (%): Significant fish/borage oil interaction was observed for the n-6/n-3 and C20:4n-6/C20:5n-3 ratios. Both ratios were decreased ($p<0.05$) by the SF and SB with SF having the greater effect. The SBF diet mimicked

the effect of the SF diet. The SF diet tended to decrease the P/S ratio. Overall, fish oil decreased ($p<0.05$) the n-6/n-3 and C20:4n-6/C20:5n-3 ratios, but increased ($p<0.05$) the Δ6 S/P ratio. Borage oil, overall, increased ($p<0.05$) the P/S ratio, while decreasing the C20:4n-6/C20:5n-3 ratio.

RBC PC (μg): Fish/borage oil interaction was noticed. The C20:4n-6/C20:5n-3 ratio was decreased ($p<0.05$) by the SF and SB with SF having the greater effect. The SBF diet mimicked the effect of the SF diet. Overall, fish oil decreased ($p<0.05$) the n-6/n-3 ratio, while borage oil increased the P/S ($p<0.05$) ratio.

RBC PE (%): Significant ($p<0.01$) fish/borage oil interactions occurred. Both the SF and SB diets decreased ($p<0.01$) the n-6/n-3 and Δ6 S/P ratios, with a greater decrease effected by the SF diet. The SBF diet closely mimics the reduction in both ratios obtained with the SB diet while countering the major reduction noticed with the SF diet. Overall, the fish oil decreased ($p<0.01$) the n-6/n-3 and C20:4n-6/C20:5n-3 ratios, while borage oil increased ($p<0.05$) the P/S ratio.

RBC PE (μg): Significant ($p<0.01$) fish/borage oil interactions were observed. Both the SF and SB diets decreased the n-6/n-3 ($p<0.01$) and Δ6 S/P ($p<0.05$) ratios, respectively, with a greater effected noticed by the SF diet. The SBF diet increased ($p<0.05$) the n-6/n-3 ratio similar to the SB diet. Overall, the fish oil decreased the n-6/n-3 ($p<0.01$), C20:4n-6/C20:5n-3 ($p<0.01$) and Δ6 S/P ($p<0.05$) ratios.

Table 8: Fatty acid ratios (% of total FA and μg FA/mg protein) of the PC and PE phospholipid fractions of the rat colon mucosa and red blood cell membranes.

	Qualitative (% of total fatty acids)					Quantitative (μg FA/mg protein)				
	S	SF	SB	SBF	Main effects	S	SF	SB	SBF	Main effects
Mucosa PC										
P/S	0.65±0.12	0.85±0.12	0.66±0.16	0.95±0.19	F *↑	0.53±0.24	0.63±0.41	1.58±1.34	0.82±0.35	–
n-6/n-3	74.22±25.26	30.73±6.83	42.95±22.25	23.71±3.78	F *↓	12.09±6.03	5.42±2.82	34.11±13.94	19.86±5.77	F $^{\#}$↓, B *↑
Δ6 S/P	16.57±6.57	21.82±4.10	12.59±3.15	18.48±4.96	F $^{\#}$↑	29.54±7.83	28.28±24.93	30.96±13.96	37.92±11.24	–
C20:4n-6/C20:5n-3	101.51±7.29^{abc}	29.93±3.06^{b}	57.68±24.73^{ad}	28.41±9.84^{cd}	F *↓	101.51±7.29^{abc}	29.93±3.06^{b}	57.68±24.73^{ad}	28.41±9.84^{cd}	F *↓
C20:4n-6 PC/PE	0.35±0.07	0.43±0.14	0.30±0.06	0.31±0.06	B $^{\#}$↓	0.42±0.13	0.51±0.07	0.47±0.19	0.54±0.01	–
Mucosa PE										
P/S	1.36±0.27	1.67±0.36	2.30±0.44	1.98±0.18	B *↑	1.44±0.28	1.06±0.58	4.67±3.49	2.05±0.21	–
n-6/n-3	14.97±6.21	15.97±4.91	19.55±5.74	10.93±0.92	–	15.70±7.38	11.07±4.77	23.10±4.71	11.32±0.92	F $^{\#}$↓
Δ6 S/P	3.48±0.33	2.92±0.44	2.72±0.70^{a}	3.61±0.39^{a}	–	5.48±1.11	6.68±1.26	3.70±0.75	6.65±0.27	F *↑
C20:4n-6/C20:5n-3	148.72±8.44	28.20±3.59	109.84±40.43	30.32±3.48	F *↓	148.72±8.44	28.20±3.59	109.84±40.43	30.32±3.48	F *↓
RBC PC										
P/S	0.50±0.05	0.36±0.13	0.54±0.16	0.54±0.10	B $^{\#}$↑	0.47±0.08	0.36±0.13	0.54±0.16	0.53±0.20	B $^{\#}$↑
n-6/n-3	17.34±5.95^{abc}	8.10±2.83^{b}	9.52±3.73^{a}	9.16±3.76^{c}	F $^{\#}$↓	17.50±6.27	8.10±2.83	11.23±6.61	9.16±3.76	F $^{\#}$↓
Δ6 S/P	5.76±1.01	8.40±2.33	6.01±1.38	6.55±1.27	F $^{\#}$↑	9.66±3.45	12.93±5.43	9.95±2.43	9.20±2.53	–
C20:4n-6/C20:5n-3	468.72±150.32^{abc}	39.28±10.02^{b}	50.94±22.21^{a}	29.09±5.60^{c}	F *↓, B $^{\#}$↓	468.72±150.32^{abc}	39.28±10.02^{b}	50.94±22.21^{a}	29.09±5.60^{c}	F *↓, B $^{\#}$↓
C20:4n-6 PC/PE	0.58±0.22	0.66±0.13	0.78±0.38	0.66±0.21	–	1.50±0.23	1.63±0.25	1.82±0.77	2.16±0.70	–
RBC PE										
P/S	1.08±0.49	0.89±0.58	1.73±0.41	2.13±2.09	B $^{\#}$↑	1.02±0.38	1.03±0.67	1.47±0.63	1.60±1.46	–
n-6/n-3	14.65±3.13^{abc}	1.41±059^{bd}	6.72±1.42^{ad}	4.97±3.93^{c}	F *↓	13.44±4.02^{abc}	1.41±0.59^{bd}	6.72±1.42^{Aad}	4.97±3.94^{c}	F *↓
Δ6 S/P	7.62±1.52^{abc}	1.91±0.63^{bd}	2.87±098^{a}	4.07±1.51^{cd}	–	8.67±4.18^{abc}	4.00±2.45^{bd}	4.73±1.41^{a}	6.34±2.93^{cd}	F $^{\#}$↓
C20:4n-6/C20:5n-3	657.71±229.82	34.80±4.91	881.04±270.23	31.46±15.31	F *↓	657.71±229.82	34.80±4.91	881.04±270.23	31.46±15.31	F *↓

Values are means (n= 4-9) ±SD of the ratios of the total fatty acid percentage and μg FA/mg protein in the Pc (phosphatidylcholine) and PE (phosphatidylethanolamine) lipid fractions of the rat colon mucosa and RBC membranes. Diets: S = sunflower oil only; SF = sunflower oil + fish oil; SB = sunflower oil + borage oil; SBF = sunflower oil + borage oil + fish oil. P/S = polyunsaturated/saturated fatty acid ratio; C20:4n-6/C20:5n-3 = arachidonic acid/eicosapentaenoic acid ratio; Δ6 S/P = delta-6 desaturase substrate/product ratio. Significant fish/borage interaction indicated by significant differences between two dietary groups at $p<0.05$ (superscript) as shown by the same letters. Main effects due to the presence of fish oil (F) or borage oil (B) on the lipid ratios indicated by: increase = ↑, decrease = ↓, no effect = –. Significant main effects of F/B indicated by * = $p<0.01$ and # = $p<0.05$.

(b) C20:4n-6 PC/PE ratio (Table 8)

No significant fish/borage oil interactions for the C20:4n-6 PC/PE ratio was observed in any of the membranes.

Mucosa (%): The C20:4n-6 PC/PE ratio tended to decrease with the SB diet. The SBF diet reduced the ratio to the same level observed with the SB diet. Overall, borage oil exerted significant main effects that resulted in a decrease ($p<0.05$) in the C20:4n-6 PC/PE ratio.

Mucosa (μg): The C20:4n-6 PC/PE ratio content tended to increase with the SF and SBF diets. No overall main effects were observed.

RBC (%): The C20:4n-6 PC/PE ratio tended to increase with all three ratio diets, with the SB diet exerting a greater increasing effect. No overall main effects were observed.

RBC (μg): The C20:4n-6 PC/PE ratio tended to increase with all three ratio diets in the following order, SF<SB<SBF. No overall main effects were observed.

3.3.6 Extent of lipid peroxidation (Table 9)

Mucosa: No significant fish/borage oil interactions on the CD levels were observed with iron incubation. However, overall, fish oil increased ($p<0.05$) the CD level significantly.

RBC: A significant fish/borage oil interaction ($p<0.05$) altered the oxidative status as determined by the conjugated dienes level (CD). Both the SF and SB diets significantly increased ($p<0.05$) the CD levels in comparison to the S diet, with the SF diet having the greater increasing effect. The SBF diet tended to increase the extent of lipid peroxidation above the levels observed with the individual SF and SB diets. Main effects significantly increasing the CD levels were caused by both fish oil ($p<0.01$) and borage oil ($p<0.05$), with fish oil having the greater increasing effect.

Table 9: Lipid peroxidation in rat colon mucosa and red blood cell membranes as measured by the level of conjugated dienes.

Tissue	Diets				Main effects
	S	SF	SB	SBF	
Mucosa	5.35±0.26	6.53±0.23	5.16±1.16	6.35±1.71	F $^{\#}\uparrow$
RBC	3.22±0.72^{abc}	4.86±0.71^{b}	4.62±0.43^{a}	5.03±1.17^{c}	F $^{*}\uparrow$, B $^{\#}\uparrow$

Values are means ± SD of the levels of conjugated dienes (measured in nmol per mg protein) in rat red blood cell membranes (n=9) and colon mucosa (n=4). Diets: S = sunflower oil only; SF = sunflower oil + fish oil; SB = sunflower oil + borage oil; SBF = sunflower oil + borage oil + fish oil. Significant fish/borage oil interaction indicated by significant differences between 2 dietary groups at $p<0.05$ (superscript) as shown by the same letters. Main effects due to the presence of fish oil (F) or borage oil (B) on the lipid profiles: increase =↑, decrease =↓. Significant main effects indicated by $p<0.01$ = * and $p<0.05$ = #.

3.4 Discussion

Due to the physiological effects of dietary FA, a critical balance between n-6 and n-3 PUFA is required to maintain normal cellular homeostasis (Sumida *et al*., 1993; Sessler and Ntambi*,* 1998). With dietary fat intake playing a key role in the supply of these FA, alterations to the ratio of n-6 and n-3 FA have the potential to manipulate biochemical and physiological cellular responses (Jump and Clark, 1999; Collett *et al.,* 2001). These alterations may influence the risk of developing diseases such as cancer that have been shown to be associated with specific FA intakes (Nkondjock *et al*., 2003). The present study demonstrates that dietary fat intakes with specific n-6/n-3 FA ratios modulated the lipid profile of rat colon mucosa and red blood cell (RBC) membranes.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the two main phospholipids in eukaryotic cells comprising 50% and 25% of the phospholipid mass, respectively (Henneberry *et al.,* 2002). In normal cells, PC and sphingomyelin dominate the outer leaflet of the plasma membrane, whereas the inner leaflet contains PE and phosphatidylserine (PS) (Van Hoeven and Emmelot, 1972). Alterations in these lipid components have an impact on membrane structure and function (Hulbert *et al.,* 2005). Thus, with diet being an important source of the lipid components, such as fatty acids, membranes phospholipids composition can readily be manipulated. Borage oil reduced the mucosa membrane PC content, while increasing PE, which resulted in a decrease in the PC/PE ratio. Fish oil reduced PE, thereby increasing the mucosa PC/PE ratio. In the RBC membranes the opposite effect was observed with the fish oil containing diets SF and SBF increasing the content of both phospholipid fractions while borage oil had no effect. From this data it is evident that dietary FA intake played an important role in either the distribution of membrane phospholipid and/or the synthesis thereof which varies depending on the specific tissue compartment.

The intestinal mucosa serves as an interface between the environment and mammals acquiring water, nutrients and other bioactive compounds, whilst the mucosa also serves as a barrier against toxins and infective agents

(Jankowski *et al.,* 1994). As a result cells are constantly renewed every 2-6 days (depending on species and intestinal site) (Lipkin, 1973) with the production of new cells in the crypts equal to the rate at which cells are lost from the villus tip by extrusion (Madara, 1990) or by apoptosis (Hall *et al.,* 1994). Therefore, the maintenance of homeostasis in normal tissue requires adequate balance and active interplay between these two regulatory processes. In the present study, the n-6/n-3 FA ratio diets provided a pool of FA that are utilized for the synthesis and distribution of membrane lipids thereby altering the membrane structure. In the colon mucosa, membrane phospholipids, PC and PE, decreased or increased depending on the n-6/n-3 FA ratio and hence the specific oil used in the diet. Consequently, changes in the membrane phospholipid composition may incur different metabolic responses in the cell that may stimulate cell proliferation (Pajari and Mutanen, 1999). In terms of phospholipids synthesis, cells mainly double their phospholipid mass in order for cell growth to occur (Jackowski, 1996).

The membrane PE content has been linked to the control of cell proliferation, with reduced levels resulting in the inhibition of cell proliferation (Kano-Sueka and King, 1988). Similarly, PC is also an important cellular component required by signalling molecules that regulate cell numbers (Tercé *et al*., 1994; Cui and Houweling, 2002). Dietary fat intake is capable of modifying the phospholipid content in the membranes depending on the amount and type of fat consumed (Khuu Thi-Dinh *et al.*, 1990; Parrish *et al.*, 1997). Due to dietary modifications to the membranes phospholipid profile, the biochemical systems regulating cell production may be affected significantly. In a study by Anti *et al.* (1992), consumption of fish oil that is rich in the n-3 FA C20:5n-3 and C22:6n-3 modified the proliferative pattern of the rectal crypt epithelial cells in patients with sporadic adenomatous polyps. Similarly, the FA C20:5n-3 and C22:6n-3 has been shown to modify cell turnover in normal colonic mucosa (Calviello *et al.,* 1999). These results suggest that diets with specific n-6/n-3 FA ratios play an important role in the biosynthesis of phospholipid membrane constituents.

As mature RBC cannot synthesize lipids, they rely on a continuous exchange of lipids between their membranes and plasma lipoproteins (Gold and Philips, 1990). The RBC membrane phospholipid turnover with the plasma is a passive process that occurs very slowly (London *et al.*, 1949; Ganzoni *et al,* 1971). Changes in plasma lipid concentration are a net result of the balance between two processes, firstly by both dietary intakes, as well as endogenous synthesis, and secondly by the utilization for energy, membrane incorporation and storage (Visiolo *et al.,* 2006). In the present study, the increase in membrane phospholipid concentrations and distribution ratio could be a consequence of altered plasma lipids affected by the different n-6/n-3 FA ratio diets.

Changes in the relative proportions of different lipid species, such as phospholipids, cholesterol and fatty acid saturation, modulate membrane permeability, rigidity and fluidity (Maxfield and Tabas, 2005). The cholesterol/phospholipid (chol/PL) ratio, PC/PE and polyunsaturated/saturated FA (P/S) ratios are lipid parameters used to monitor changes in membrane fluidity. Alterations in these lipid parameters illustrated that the colon mucosa and RBC membranes responded differently to diets with varying n-6/n-3 FA ratios. In the colon mucosa, changes to the phospholipid parameters did not significantly alter the chol/PL ratio. Only the borage oil (SB diet) tended to decrease the ratio due to subtle changes in PE and PC, implying that a less rigid membrane structure could affect membrane function. Studies illustrate that increased membrane fluidity plays a role in regulating plasma lipoprotein interactions (Fernandez and West, 2005). As a result, reduction in colon membranes fluidity may adversely affect critical cellular processes that are mediated by the membrane lipid structure. Both the fish oil and borage oil reduced the chol/PL ratio in the RBC membranes. Hereby RBC membrane fluidity was increased mainly due to an increase in phospholipid concentrations. The relative decrease in cholesterol as compared to the phospholipids will elicit an increase in the fluidity as it stabilizes the fatty acyl groups in the lipid bilayer (Ohvo-Rekila *et al.,* 2002).

The different n-6/n-3 FA ratio diets also altered the PC/PE and P/S membrane ratios in both tissues. In the colon mucosa the SF and SB diets increased and decreased the PC/PE ratio, respectively. The SF and SBF diets increased the PC/PE ratio in the RBC membranes due to an increase in the PC phospholipid levels. A decrease in the PC/PE ratio is associated with a reduction in membrane fluidity while an increase elevates the viscosity of the membrane (Mahler *et al.*, 1988). Variability in the PC/PE ratio between the two tissue types could be related to the difference in phospholipid availability and distribution due to the incorporation of FA (Abel *et al.,* 1997).

Fish and borage oil intake increased the total PUFA and LCPUFA content of PC and PE phospholipids in both tissues. As a result the P/S ratio was selectively elevated in the colon with fish and borage oil increasing the ratio in PC and PE. In the RBC membranes only borage oil increased the P/S ratio in both compartments. The resultant increase in the P/S ratio is known to enhance membrane fluidity (Zhang *et al.,* 2007). This is ascribed to the presence of the double bonds that reduces the tight packaging of the lipid components due to a kink in the FA chain (Eyster, 2007).

Alterations to membrane fluidity significantly influence the activity of certain enzymes and the affinity of receptors to their ligands (Tappia *et al*., 1997) while cell signal transduction pathways that are regulated by lipid-protein interactions are altered (Yang *et al*., 2005). Plasma membranes contain specialized lipid microdomains such as the lipid rafts and caveolae that influence cell signaling (Kenworthy, 2002). Incorporation of n-3 FA such as C20:5n-3 and C22:6n-3 into the lipid rafts of membranes has been suggested to have beneficial health effects (Schley *et al.,* 2007). This is largely due to perturbations in membrane composition modifying the function of resident protein, and thus cellular responses. Therefore, alterations in the lipid FA contents of the microdomains may modulate their biochemical function significantly. With diet influencing phospholipid availability, changes in RBC membrane lipid composition is likely to modulate the membrane physical properties, hereby affecting RBC morphology, receptor activity and oxygen transport (Escudero *et al.,* 1998; Vajreswari *et al.,* 2002). As a result,

modifications to the phospholipid parameters maintaining fluidity influence membrane functions such as permeability and enzyme binding properties, oxygen transport and enzyme activities related to transmembrane transport (Cooper, 1977; Lande *et al.*, 1995; Pande *et al.*, 2005).

Both the colon mucosa and RBC membrane fluidity were modulated by the specific n-6/n-3 FA ratio diets, and depending on the lipid fractions and tissue compartment it could be selectively enhanced or reduced when considering a specific lipid parameter (See below Fig. 5). However, when considering the overall effect of fish and borage oil, the membrane fluidity was increased in both tissue compartments. Significant differences between the two tissue membrane lipid compositions suggest that in relation to the main function of the tissues, membrane stability is altered differently with fatty acid intake.

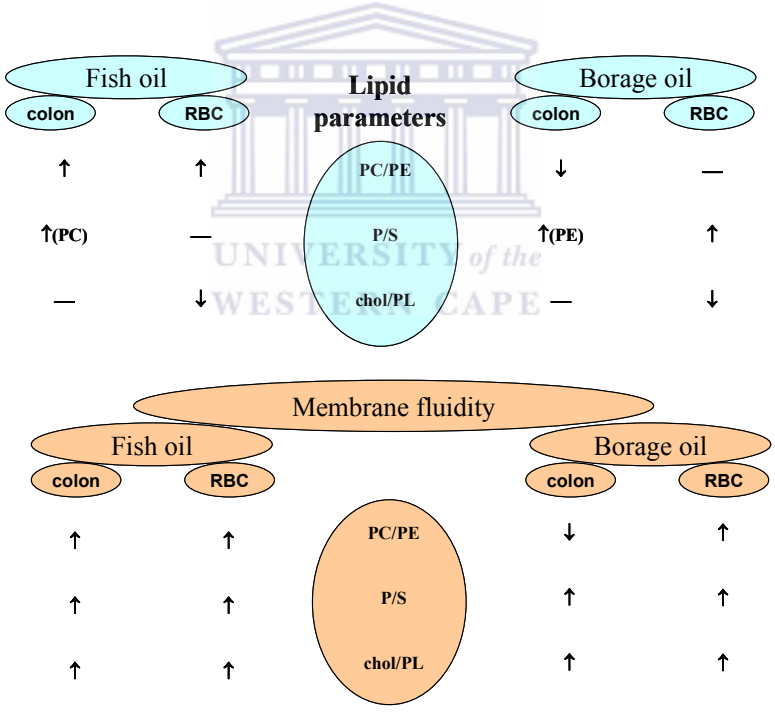

Fig. 5 Fish and borage oil effects on the lipid parameters influencing the membrane fluidity of the colon mucosa and RBC membranes. Results in brackets () indicate the phospholipids mainly affected by the dietary oil.

Differences in the mucosa and RBC phospholipids FA content is a reflection of (i) the intake of specific FA associated with the diets, (ii) differences in FA incorporation between the two tissue types and (iii) the relative ratio of the specific phospholipids in the membrane. When considering the lipid

parameters, cholesterol, PC and PE phospholipids, PC/PE and chol/PL ratios in the rats receiving the sunflower diet differed significantly between the two cellular compartments, which reflect the differences in their proliferative and functional properties. In general cholesterol is higher in the mucosa, as well as PC and PE with a lower PC/PE ratio due to a relative high PE component. This is likely to be associated with an increased proliferative status of the colon mucosa cells. With respect to the SATS and MUFA, higher levels are obtained in the PC fractions, while n-3 and n-6 FA levels are higher in PE. The only difference, however, is that in the RBC membranes MUFA levels are higher in the PE fraction. With respect to FA ratios the PE fractions contains more n-3 FA, therefore yielding a lower n-6/n-3 ratio. The C20:4n-6 level also dominates in the PE fractions resulting in a higher C20:4n-6 PC/PE ratio.

The incorporation of FA in a phospholipid will depend on interaction between n-6 and n-3 FA with respect to the substrate specificity of the Δ^6-desaturase enzyme (Rodriguez *et al.,* 1998) as well as the preferential incorporation of certain FA into phospholipids (Hatala *et* al., 1994). The objectives of this study was to determine (i) to what extend the FA patterns of the colon mucosa and RBC membranes would reflect the dietary FA composition, (ii) differences that exist between the tissue compartments and phospholipid fractions regarding the incorporation of specific fatty acids and (iii) the modulation of membrane susceptibility to oxidative damage.

These aspects were investigated by modulating sunflower oil with either fish or borage oil or a combination of the latter to obtain dietary fats with different n-6/n-3 FA ratios. With the SF diet specific interaction between C18:2n-6, the main FA in sunflower oil, and the major FA in fish oil (C20:5n-3 and C22:6n-3), were evaluated. When utilising the SB diet interaction between C18:2n-6 and the borage oil FA (C18:3n-3 and C18:3n-6) were monitored. The combination (SBF) diet monitored the use of an n-6/n-3 ratio diet similar to the SF diet where the interaction of C18:3n-6 and C20:5n-3 on the modulation of Δ^6-desaturase activity was determined by considering the substrate/product FA ratios for the enzyme. The effects induced by the individual fish (SF) and

borage (SB) oil diets on the different FA profiles of the selected tissue are summarised in Fig. 6 (page 122) and Fig. 7 (page 129).

In terms of the SATS composition, the fish oil content of the n-6/n-3 FA ratio diets exerted significant effects on the qualitative and quantitative composition of both tissue phospholipid fractions (see Fig. 6, page 122). The qualitative composition gave an indication of the percentage of total FA (%), whereas quantitatively defining the absolute FA levels (μg FA/mg protein) that are determined by the phospholipid content of the membrane. When considering the qualitative levels, fish oil increased, while borage oil decreased the total SATS in the PE fraction of the colon mucosa, respectively. This effect was reflected by the increase in C18:0 by fish oil and a decrease in C16:0 by borage oil. In contrast, fish and borage oil increased SATS in both phospholipid fractions in the RBC. Overall, the two tissue types exhibit distinct differences in terms of their levels of SATS incorporation. A similar pattern is noticed when considering the quantitative levels although differences do exist. The increased incorporation of SATS is likely to compensate for the increased phospholipid n-3 FA unsaturation by the fish oil in order to maintain the homeostatic membrane fluidity. A similar argument could be used for the reduction of SATS in colon PE as the level of n-3 FA is decreased by borage oil. In addition, it is known that PUFA decreases stearol-CoA desaturase (SCD) activity, a key regulator of membrane SATS and MUFA composition, resulting in the segregation of SATS for storage as triacylglycerols (TAG) to preserve the membrane physio-chemical homeostasis (Ntambi, 1999; Scaglia and Igal, 2005). As a result, the availability of SATS for phospholipid remodelling is decreased. The SATS, C16:0 and C18:0 have been implicated to play a role in controlling cell numbers by indirectly inducing apoptosis (Hardy *et al.,* 2003; Artwohl *et al*., 2004). It would appear that the reducing effect exerted by the SB diet on membrane C16:0 and C18:0 levels may influence the mechanisms or signalling pathways regulating apoptosis in the colon. In transformed human lung fibroblasts SCD depletion strongly correlates with growth inhibition, thus establishing a direct relationship

between Δ^9-desaturation, the accumulation of C16:0 and C18:0 and cell survival (Scaglai and Igal, 2005).

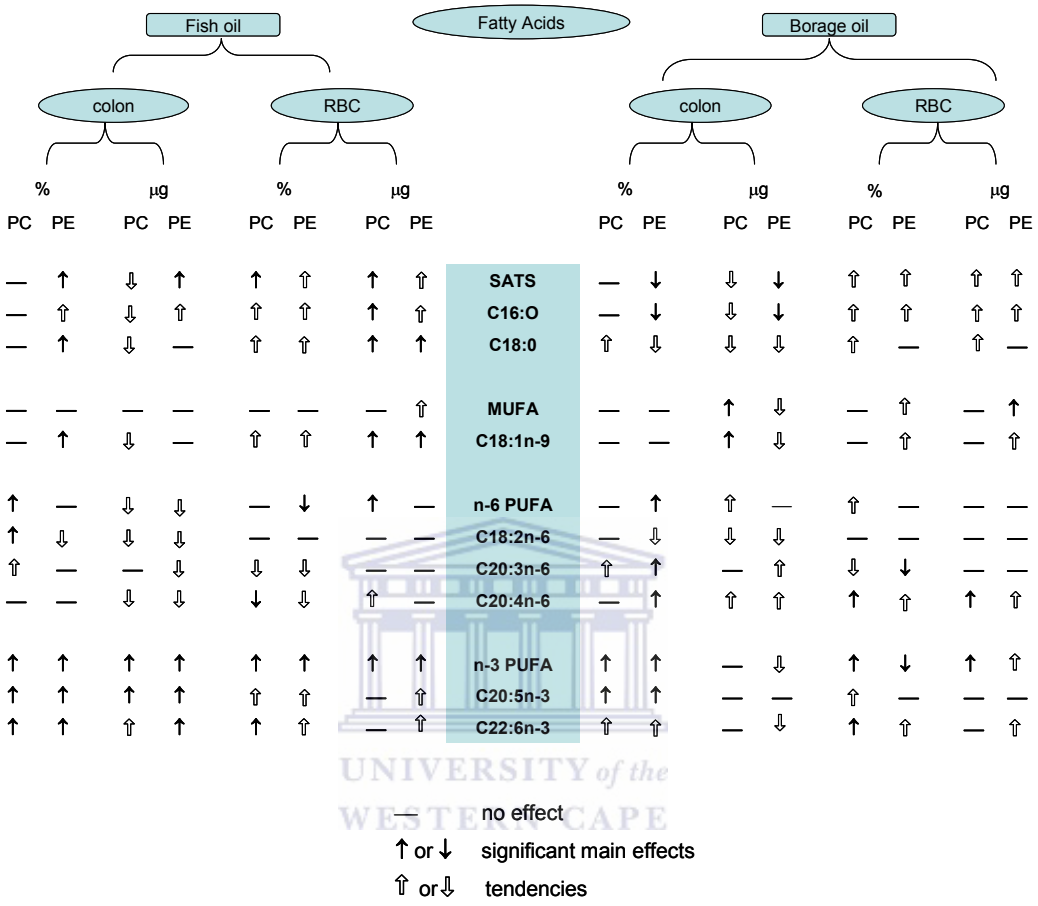

Fig. 6 Fish and borage oil effects on the fatty acid composition of the colon mucosa and RBC membranes.

In the RBC both fish and borage oils increased MUFA quantitatively in PE. In the colon borage oil increased MUFA in PC while it decreased in PE. Fish oil has no marked effect although it increased C18:1n-9 in PE. Alterations in the tissue MUFA profile were predominantly associated with the dietary modulation of the C18:1n-9 level. Studies have shown that diets high in PUFA, particularly C20:5n-3 and C22:6n-3 has an inhibitory effect on both Δ^6 - and Δ^9-desaturation (Raz *et al.*, 1997; Borgeson *et al.*, 1989). Thus, increases in membrane C18:1n-9 in both tissues is likely due to dietary FA intake particularly with the fish oil containing diets. C18:1n-9 and C16:0 have been implicated to play a role in the control of cellular proliferation and apoptosis. In MDA-MB231 breast cancer cells the release of low levels of C18:1n-9 by

phospholipase A_2 has been shown to stimulate cell proliferation by rapidly activating phosphatidylinositol 3-kinase (PI3-K) signalling pathways (Hardy *et al.,* 2000). C18:1n-9 is metabolized preferentially into TAG, while it also promotes the incorporation of the saturated FA, C16:0, thereby decreasing the apoptotic effect of the latter (Hardy *et al.,* 2003).

Dietary FA intakes (i.e. mg FA/100 g BW) gave a clear indication of the qualitative amount of n-6 and n-3 PUFA consumed. Consequently, significant changes in the phospholipid n-6 and n-3 PUFA composition were observed due to fish and borage oil dietary FA intake. Rats consumed a high proportion of C20:5n-3 with the SF diet relative to the other PUFA components. Fish oil significantly increased C20:5n-3 in the mucosa PC and PE phospholipids fraction, while in the RBC membranes, it tended to increase qualitatively in both PC and PE. Increases in the membrane C20:5n-3 content coincided with an increase in C22:6n-3, which is a product of the sequential elongation and desaturation of C20:5n-3 (Sprecher, 2000). In all the phosholipid parameters of the mucosa and RBC an increase in the total n-3 LCPUFA, particularly in PE, was significant due to the fish oil's supply of C20:5n-3 and C22:6n-3. Increases in membrane n-3 PUFA content could be a consequence of dietary n-3 FA being directly incorporated into the membranes rather than through the supply via FA metabolism (Vidgren *et al.,* 1997). Increases in the tissue n-6 LCPUFA, such as C20:3n-6 and C20:4n-6 was mainly elicited in response to borage oil increasing the membranes n-6 FA content. The SF diet, however, appears to have the opposite effect demonstrated with the SB diet, with the fish oil containing diet reducing n-6 LCPUFA content, particularly C20:4n-6 in the PC (%) phospholipid fraction of the RBC membranes. This is due to fish oil having a reducing effect on the metabolism of n-6 PUFA (Garg *et al.*, 1990).

Fish oil intake significantly influences FA metabolism due to it inhibitory effects on Δ^6-desaturase (Garg *et al., 1988*). The relative (%) Δ6 S/P ratio, which reflects the activity of the enzyme (Raz et al., 1997), showed that the SF diet increased the ratio in the mucosa phospholipids. In RBC a similar pattern is noticed in PC and as well as a decrease in PE. This result shows that more Δ^6-desaturase substrates than products were available in the PC fraction,

while in PE the opposite result was in effect. Of interest is that the RBC membrane PC phospholipid also reflects a similar Δ6 S/P ratio pattern. As it is known that fish oil inhibits Δ^6-desaturase, although RBC lacks the desaturases enzymes (Russo *et al.,* 1997), the membrane Δ6 S/P ratio is utilized as a biomarker for the fish oil induced alteration of Δ^6-desaturase activity (Fuhrman *et al.,* 2006). Borage oil tended to decrease the Δ^6-desaturase S/P ratio in PE and to some extent in PC implying an increase in the activity of the enzyme. The increased effect in PE could also be a reflection of the increased levels of C20:4n-6 due to the increased level of C18:3n-6 in borage oil that bypasses the Δ^6-desaturase enzyme. It has been postulated that the increase in enzyme substrates may rather be a consequence of direct FA incorporation into the membranes rather than Δ6-desaturase products (Cho *et al*., 1999).

Despite high intakes of C18:3n-6 with the SB diet the content in the mucosa and RBC membranes was not significantly altered in the PC and PE phospholipids fractions. However, trends suggest that borage oil increased the FA's qualitative (%) and quantitative (μg) content in both the PC and PE phospholipid fractions of the colon mucosa. However, qualitatively the C20:3n-6 content increased due to borage oil's effect on the PE phospholipid fraction of the mucosa. This increase in C20:3n-6 may be a consequence of the rapid elongation of the FA substrate C18:3n-6 (Johnson *et* al., 1997). With the increase in membrane C20:3n-6 in the mucosa, the FA is subject to desaturation by Δ^5-desaturase (Umeda-Sawada *et al.,* 2006). As a result, C20:4n-6 content tended to increase in both phospholipid fractions, with borage oil having a significant qualitative increasing effect in PE. In the RBC membrane borage oil tended to decrease the relative C20:3n-6 content in PE while increasing C20:4n-6 in both phospholipid fractions. The resultant increase in C20:4n-6 elicited by the SB diet may be a consequence of an increase in Δ^5-desaturase activity (Engler and Engler, 1998).

An important aspect of the current study was to monitor the effect of a specific n-6/n-3 dietary FA ratio by combining sunflower, borage and fish oil (SBF diet). When utilizing the SBF diet the borage oil countered the fish oil reducing effect

on PE resulting in a PC/PE ratio similar to the S diet in the colon mucosa (Table 2, page 95). In the RBC membrane the PE/PC ratio of the SBF diet reflects that of the SF diet. With respect to total SATS, the combination diet reflected the opposing effects of borage oil on fish oil (Table 3, page 97). In the mucosa PE (μg), the SBF diet maintained the effect observed with the fish oil on C18:0, while an opposite effect was noticed with C16:0. In the RBC membrane PC (μg) borage oil countered the increasing fish oil effect on C18:0 and C16:0, while in PE (μg) the fish oil increasing effect on C18:0 dominates. Fish oil countered the reducing effect of borage oil in the combined diet when considering the total MUFA level, specifically of C18:1n-9 (mucosa PE μg) (Table 4, page 100). Borage oil dominated in PC (μg) by increasing the total MUFA level countering an apparent reduction by fish oil on C18:1n-9. Of interest is a type of "antagonistic" effect of the two oils on C22:1 (PC % and μg) where the combined effect reduced the level despite an increasing effect of the individual oils. In RBC PC (%) an opposite increased effect was noticed for C20:1 with the SBF diet. When considering the mucosa PC and PE n-6 PUFA levels, a response similar to the increased SB diet effect was demonstrated with the SBF diet (Table 5, page 103). The increasing effect of fish oil in the mucosa PC (%) specifically on C18:2n-6 could be related to the inhibition of the Δ^6-desaturase. This is further demonstrated in the RBC PE (%) fraction with the reducing effect of fish oil on C22:4n-6, C22:5n-6 and total n-6 PUFA. Of interest is the increased SBF response on C20:3n-6 and C20:4n6 in the mucosa PE (% and ug) which reflected the effect of borage oil. A similar response was noticed in C20:4n-6 RBC PC (%) and PE (%) level with the borage oil although the SBF diet countered the effect presumably due to a reducing fish oil effect. With respect to the n-3 FA, the effects of the SBF diet reflected the increased additive effect of borage and fish oil with the latter making the greatest contribution (Table 6, page 105). This increase was mainly obtained due to fish oil enhancing the levels of n-3 LCPUFA C20:5n-3 and C22:6n-3 (Innis *et al*., 1995). The only exception is the apparent counter effect of borage oil with respect to the increase fish oil response on the total n–3 PUFA in RBC PE (%).

Similar type of interactions was noticed between fish and borage oil when considering the different FA parameters (Table 7, page 109). The SBF diet further enhanced the fish oil and borage oil increasing effect regarding the PUFA and n-3 LCPUFA in the mucosa PC and PE both qualitatively and quantitatively. Borage oil was more prominent in contributing to the increased effects of the SBF diet on LCPUFA and n-6 LCPUFA. Qualitatively the RBC membranes increase in n-6 LCPUFA content in PC by borage oil was countered by fish oil, while both oils increased the n-3 LCPUFA and PUFA content. Regarding the different FA ratios (Table 8, page 112) the SBF diet also reflects the differential effects of the dietary oils depending on the tissue compartment. With respect to the P/S ratio the SBF diet reflects the borage oil increase in the mucosa PE (%) and RBC PC (%) and PE (%) fractions thereby counteracting the fish decreasing effect. Both the fish and borage oils reducing effect contributed to the decreased n-6/n-3 ratio by the SF diet in mucosa PC (%) and RBC PC (%) and PE (%). A similar type of response was noticed in the mucosa PE (%) fraction. The borage oil tended to counteract the increasing effect of fish oil using the SBF diet as reflected in the Δ^6-desaturase S/P ratio in both the mucosa and RBC PC (%) fraction. The SBF diet also mimics the reducing effect of fish oil on the C20:4n-6/C20:5n-3 ratio in the mucosa PC (%) and PE (%) as well as the RBC PC (%) and PE (%) although the borage oil also reduced the ratio.

In order to preserve tissue homeostasis, the specific levels of certain FA in membranes needs to be maintained. The n-6 FA C20:4n-6 is a key regulator of normal cellular functions (Brash, 2001). In this study borage oil with a high level of 18:3n-6 resulted in an increase in the elongation product C20:3n-6, which is the substrate for Δ^5-desaturation, thus ensuring the availability of C20:4n-6. High membrane levels of C20:4n-6 has however been associated with the development of tumours (Nicholson *et al.,* 1991). Thus, dietary manipulations utilizing FA ratios containing both C20:3n-6 and C20:5n-3 has been proposed to modulate membrane C20:4n-6 content (Barham *et al*., 2000). Such ratios ensure that sufficient levels of C20:4n-6 is available to mediate processes such as eicosanoid production without raising the content

to levels that facilitate pathological conditions. The relative proportion of these FA also determines their availability after phospholipase cleavage as substrates for cycloxygenases, and hence regulates the production of eicosanoids (Li *et al.,* 1994).

The C20:4n-6/C20:5n-3 and C20:4n-6 PC/PE ratios have been used as indicators or biomarkers to determine the class of eicosanoid produced (Li *et al*., 1994; Watkins *et al*., 2000). Reductions in membrane n-6/n-3 and C20:4n-6/C20:5n-3 ratios were illustrated in both the colon mucosa and the RBC membranes, with the fish oil containing diets having a prominent effect (Fig 7, page 129). The reduced C20:4n-6/C20:5n-3 ratio suggests that C20:5n-3 levels are elevated, particularly with the inclusion of fish oil in the diets. A study by Calviello *et al.* (1999) indicates that the administration of C20:5n-3 and C22:6n-3 does not modify the homeostasis of normal rat colonic mucosa. However, C20:5n-3 inhibits cell proliferation while C22:6n-3 enhances cell differentiation and apoptosis. In addition, the increased availability of C20:5n-3 ensures the biosynthesis of anti-inflammatory eicosanoids such as the 3-series prostaglandins (Calder, 2006). Conversely C20:4n-6 metabolism gives rise to pro-inflammatory eicosanoids such as the 2-series prostaglandin PGE_2 which has been linked to the development of cancer (Chan, 2006).

Barham *et al.* (2000) demonstrated that the production of pro-inflammatory eicosanoids can be reduced by supplementation with a mixture of C18:3n-6 and C20:5n-3 rich foods. C18:3n-6 supplementation can bypass the pathologically defective Δ^6-desaturase activity and elevate the formation of C20:3n-6 and subsequently the synthesis of the desirable anti-inflammatory 1-series eicosanoids (Fan and Chapkin, 1998). An increase in tissue C20:5n-3 level suppresses membrane C20:4n-6 levels, and subsequently the synthesis of 2-series eicosanoids with their potentially adverse effects (Rose and Connolly, 1999). C20:5n-3 also suppresses the conversion of C20:3n-6 to C20:4n-6, increases the ratio of C20:3n-6 to C20:4n-6 in tissue and plasma phospholipids (Barham *et al*., 2000), and hence increases the formation of 1-series eicosanoids. Thus dietary supplementation with the proper

combinations of C18:3n-6 and C20:5n-3 could modulate the synthesis of 1-, 2- and 3-series eicosanoids that may reduce the development of cancer. As FA substrates compete for key metabolizing enzymes dietary manipulations of the eicosanoid precursors may determine which cellular responses are elicited.

Borage oil had a decreasing effect on the relative C20:4n-6-PC/PE ratio in the colon mucosa. This implies that the PE phospholipid fraction has a higher C20:4n-6 content relative to PC. This finding is in accordance with a study indicating that C20:4n-6 is mainly localized in the PE phospholipids of the inner leaflet of membrane (Mahadevappa and Holub*, 1987).* Localization of C20:4n-6 in PE is important, since it has been shown that this phospholipid serves as a precursor for the synthesis of eicosanoids after FA cleavage by phospholipase A_2 (Mahadevappa and Holub*,* 1987*).* This shift in membrane C20:4n-6 content has implications with regards to the metabolic availability of the FA that may affect various processes, such as prostaglandin synthesis and apoptosis changes of which have been associated with the promotion of tumour growth.

An increase in membrane PUFA content is dependent on both dietary fat intake and endogenous FA synthesis. Therefore, the activities of the FA metabolizing enzymes, Δ^6- and Δ^5-desaturases are regulated by membranes FA substrate availability, feedback regulation and level of PUFA product. Δ^6-desaturase is the rate-limiting enzyme that catalyzes the first reaction of both n-3 and n-6 PUFA synthesis (Sprecher, 2000). In most tissues the Δ^6-desaturase activity is low, except for in the liver, which is the primary site of FA metabolism (Brenner, 1989). The enzyme has a greater affinity for the n-3 FA, with enzyme preference increasing in the order: n-9<n-6<n-3 (Brenner and Peluffo, 1966). With dietary intakes of LCPUFA products such as C20:5n-3, feedback inhibition of Δ^6- desaturation is enhanced, hereby reducing the enzyme's affinity for n-6 FA substrates (Brenner, 1989).

Incidentally, these results agree with previous findings that indicate that fish oil inhibits Δ^6-desaturase activity (Raz *et al.,* 1997). The only exception was that

the SBF diet reduced the Δ6 S/P ratio in the PE (μg) phospholipid fraction of the RBC membranes. This reduction in the ratio was most likely due to the borage oil content of the diet countering the inhibitory effect of the fish oil on the enzyme activity. A study by Shimada et *al*. (2003) indicated that in the liver increases in PE FA concentration correlated significantly to a reduction in the level of Δ^6-desaturase activity.

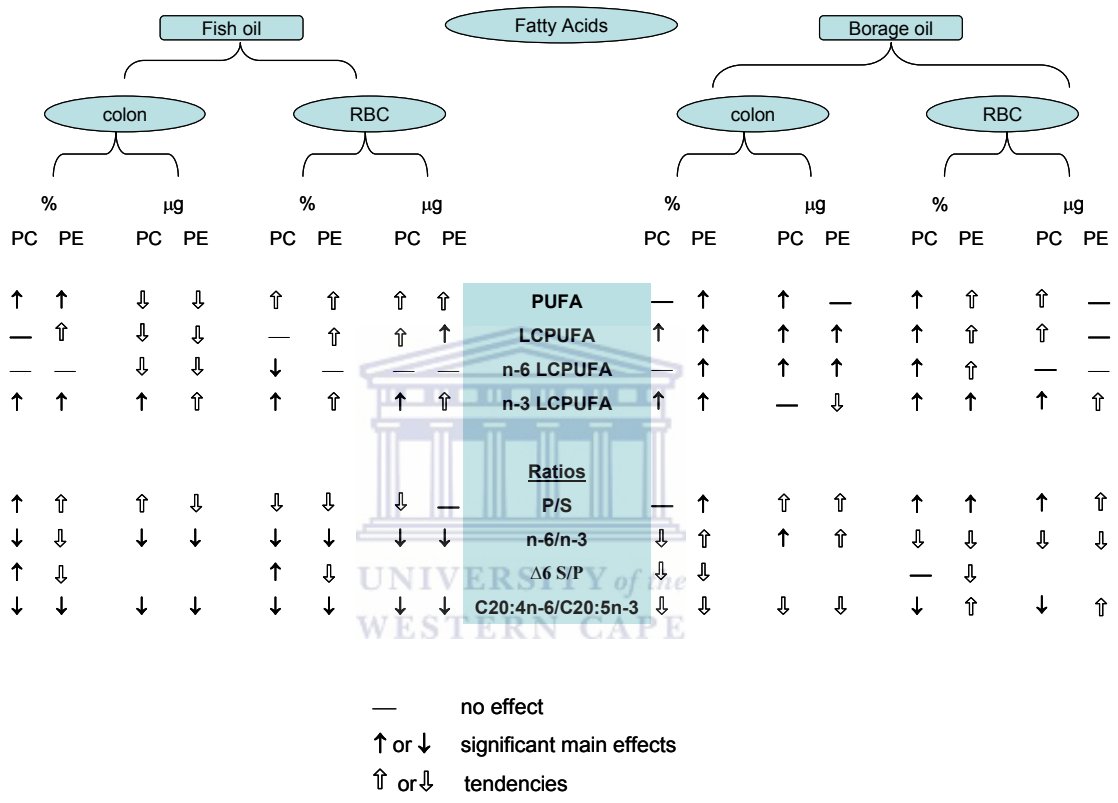

Fig. 7 Effect of fish and borage oil on PUFA parameters and FA ratios in colon mucosa and RBC membranes.

Dietary intakes of both fish oil and borage oil increased the mucosa and RBC membranes incorporation of n-6 and n-3 PUFA in the membrane phospholipids. The increase in membrane unsaturation, particularly with the n-3 LCPUFA enhances the membranes susceptibility to lipid peroxidation (Ando *et al*., 1998; Hong *et al*., 2002). Lipid peroxidation (LPO) is a tightly regulated process utilized by cellular systems to control cell numbers (Barrera *et al.,* 2008). Studies have shown that changes in fat intake and dietary FA composition modulates the mucosal cell membrane lipid content in the rat

large intestine, thus influencing membrane sensitivity to LPO (Turini *et al*., 1991). The incorporation of n-3 LCPUFA such as C20:5n-3 and C22:6n-3 has been widely attributed to increasing membrane sensitivity to free radical attack, therefore increasing LPO (Song *et al.,* 2000). These FA have a methylene (-CH_2) group between two double bonds that are susceptible to oxidation processes initiated by free radicals (Spiteller, 2003). These LCPUFA play a role in the control of cell proliferation by inhibiting cell growth and promoting apoptosis as a result of increased free radical damage due to LPO (Courtney *et al*., 2007).

Conjugated dienes (CDs), formed from the methylene bridges of FA with isolated double bonds, have been widely used as an index of LPO (Kappus, 1985). With the increase in membrane LCPUFA content, colon mucosa and RBC membranes were more susceptible to LPO. CD levels were more enhanced in the rat colon mucosa and RBC with the ratio diets containing fish oil (SF and SBF). The extent of LPO increased in the order of the diets SB<SBF<SF in the colon mucosa membranes, while increasing with the order SB<SF<SBF in the RBC membranes. FA incorporation into membranes enhances their susceptibility to lipid peroxidation, particularly with increased incorporation of n-3 LCPUFA such as C22:6n-3, which may have implications for colon cancer development by inducing free radical damage and apoptosis in cancer cells.

In healthy cells, LPO elevates the level of oxidative stress due to the generation of noxious LPO products that may stimulate the apoptotic pathway (Kruman *et al*., 1997). Endogenous (GSH, SOD and catalases) as well as exogenous antioxidant systems (vitamin E) reduce the oxidative stress induced by the oxidative degradation of membrane lipid (Fang *et al.,* 2002). Fish oil has been shown to up-regulate certain antioxidant genes, such as glutathione transferase, UCP-2 and Mn-SOD in the liver (Takahashi *et al.,* 2002). Presumably, this up-regulation counteracts excessive ROS production in normal cells and could explain the selective toxicity of PUFA to cancer cells (Jiang *et al.,* 1998). Studies show that in cancer cells the level of PUFA is decreased while membranes contain high levels of antioxidants such as

vitamin E and C18:1n-9 (Cheeseman *et al*., 1988; Horrobin, 1990). These factors play a role in reducing the extent of LPO in cancer cells, thus propagating the continuance of damaged cells. Studies in culture demonstrate that cancer cells produce more peroxidation products than normal cells when supplemented with PUFA (Das *et al.,* 1987; Bégin *et al.,* 1988). Normal cells are well equipped to withstand LPO-mediated oxidative stress through controlled antioxidant systems, while tumour cells are stimulated to undergo apoptosis due to oxidative damage (Stoll, 2002). Thus, dietary membrane modulations by increasing the n-3 LCPUFA content with fish oil based diets, as demonstrated in this study, may serve as a chemopreventive strategy for cancer research due to their availability enhancing LPO induced apoptosis.

3.5 Conclusions

The observations from this study indicate that the colon mucosa and RBC membranes responded differently to the fish and borage oil diets. Both fish oil and borage oil each have a distinct FA composition. Therefore, upon dietary intake, both individually (diets SF and SB) and in combination (diet SBF), the FA content of these oils elicit specific modifications to the membranes lipid and FA content in the two tissue compartments (see Fig. 6, page 122 and Fig. 7, page 129). Fish oil, while mostly having a significant effect on the level of membrane n-3 and n-6 PUFA, affected other lipid (i.e. cholesterol, phospholipids) and FA (i.e. SATS and MUFA) parameters as well. Similarly, borage oil modified the same parameters. These diet-induced alterations in membrane FA content may define or determine the specific functional responses related to the tissue membranes.

The colon mucosa and RBC membranes lipid profile can be altered by the intake of fish and borage oil diets with specific n-6/n-3 FA ratios. In this study the dietary modifications to the mucosa tissue and RBC membranes lipid and FA content altered their susceptibility to LPO differently. As previously highlighted, the individual diets, SF and SB, either increased or decreased the aforementioned features. In combination, however, diet SBF had varied effects where the diet maintained, countered or enhanced the effects of the individual diets. These responses are most likely related to the direct

incorporation of dietary FA within the specific tissue. Membranes readily incorporated the FA provided by the dietary pool, however, the level of FA incorporation varied with tissue. This suggests that RBC, which has frequently been used as biomarkers for dietary fat exposure, cannot solely be used to determine the level of dietary FA incorporation in other cellular compartments. The effects exerted by the fish oil diet SF and borage oil diet SB differed significantly from each other in both tissue types. Enhancement of membrane LCPUFA content, particularly with the n-6/n-3 FA ratio diets containing fish oil, increased the membranes susceptibility to LPO due to the increased availability of n-3 FA C20:5n-3 and C22:6n-3. Increasing these FA in membranes sensitizes cell membranes to oxidative damage that facilitates the induction of apoptosis, and thus could be utilized as a chemopreventive strategy for cancer growth inhibition.

3.6 References

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CHAPTER 4

GENERAL DISCUSSION AND CONCLUSIONS

4.1 General discussion

The continued increase in the incidence of several types of tumours in the Western world is of great concern (Parkin *et al*., 2002). Although strategies have been developed to detect and provide effective treatment, primary prevention is the most effective approach to decrease the incidences of highly fatal forms of cancer. Measures to prevent its occurrence can be achieved by identifying the factors associated with the risk of cancer development. Thereby, with this knowledge, strategies related to reducing the risk of cancer development can be adopted.

Studies have illustrated that diet plays a major role in the development of colon cancer (Reddy and Sugie, 1988; Lund, 2006). High intake of specific dietary FA, in particular, is suggested to facilitate its neoplastic development (Zock and Katan, 1999; Nkondjock *et al., 2003*). The mechanisms by which dietary FA enhance tumour development are linked to their involvement in cellular functions. In cells, the FA composition of cellular membranes plays a key role in maintaining cellular homeostasis by regulating structure and function (Pan and Storlien, 1993; Jump and Clarke, 1999). FA such as the PUFA influence membrane fluidity, signal transductions through lipid-protein interactions, as well as the immune response (Broughton and Wade, 2002; Duan, 2006). Changes in membrane FA composition could thus have a significant effect on cellular homeostasis by modulating important biochemical events that may result in abnormal cellular function. With the availability of these PUFA being largely dependent on dietary fat consumption, regulated intake of specific FA may determine which cellular responses are elicited.

Based on the principle of membrane FA composition having a significant effect on cellular function differences between the FA composition of cancer and healthy cells may be an important factor in determining their role in carcinogenesis. The present investigation showed that there are distinct differences in the membrane lipid profiles of precancerous and normal tissue associated with a high n-6 dietary FA intake. This dietary pattern, is associated with a low n-3 PUFA intake, characterizes a typical “western” diet associated with the development of cancer (Simopoulos, 2002). Based on

these findings, strategies to circumvent the occurrence of colon cancer can be adapted based on responses of specific dietary FA intakes.

The lipid profiles between polyps and surrounding tissue were compared to gain a better understanding of the differences associated with colon polyp development. The colon polyps exhibited a distinct lipid profile that differed significantly from the surrounding mucosa. The PC and PE phospholipid concentrations were significantly increased in the polyp membranes with high levels of the n-6 FA incorporation, particularly C18:2n-6 and C20:4n-6. The concomitant increase in the membrane phospholipids PC and PE in the polyps could be a reflection of an increase in cell turnover (Sakai *et al*., 1992). The chemical induction of colon polyps is linked with the accumulation of mutations in DNA (Perše and Cerar, 2005), which when expressed may lead to deregulated cellular functions that include unregulated cell proliferation which is likely to involve an altered lipid pattern. Unregulated cell proliferation has been well established to be a significant feature of carcinogenesis (Hanahan and Weinberg, 2000).

The fluidity of the membrane, which is determined by the membrane lipid composition, plays an important role in important cell regulatory processes. In the polyps, the results suggest that membrane fluidity was significantly increased due to alterations in the content of lipids such as the PC and PE phospholipids and cholesterol. Increased in levels of PC and PE, and low cholesterol have been linked to the development of colon cancer (Neugut *et al*., 1986; Dueck *et al.,* 1996; Li *et al.,* 2004). In the current study the cholesterol content decreased significantly in the polyps likely to compensate for the increased levels of the membrane phosholipids PC and PE in order to maintain the fluidity (Waheed *et al., 1998*). Cholesterol is known to stabilise fluidity in the membrane by aligning the acyl chains of FA (Petrache *et al*., 2005). The reduction of cholesterol is in agreement with the apparent increase in polyp membrane fluidity, illustrated by the decrease in the membrane biomarkers of fluidity, PC/PE and chol/PL ratios and an increased polyp P/S ratio. It would appear that due to changes in membrane fluidity, the cellular processes regulating the colon mucosa physical-chemical properties may be

adversely altered and therefore facilitate the neoplastic process to develop polyps.

Changes in the colon polyps membrane FA composition may be a key feature that promotes the continuance of uncharacteristic events that are usually absent or regulated under normal cellular conditions. Zeng *et al.* (2008) proposed that the increased levels of FA might modulate cell response to DNA damage, leading to defects in cell cycle checkpoints and apoptosis. Therefore, in terms of SATS promoting colon polyp development, it is likely that changes in the levels of C16:0 and C18:0 play a role in altering the mechanisms regulating apoptotic processes that ensure the removal of defective cells (Hardy *et al*., 2003). The altered colon polyp phenotype may also be promoted by an increase in MUFA such as C18:1 shown to be associated with the stimulation of cell proliferating pathways (Khan *et al.*, 1991; Nishizuka, 1995*).*

Increases of n-6 PUFA in membranes have been indicated to enhance cellular processes associated with the development of cancer (Rao *et al.,* 1996). In the present study the intake of the n-6 FA rich diet is likely to sustain the increased n-6 FA composition of PC and PE phospholipid fractions in the colon polyp membranes. In this regard the dietary supply of C18:2n-6 provides the substrate for further metabolism to longer chain FA via action of desaturases and elongases. Hereby, the levels of n-6 LCPUFA and specifically C20:4n-6, were significantly elevated in the polyps, in both the PC and PE phospholipid fractions. When considering the Δ6 substrate/product (Δ6 S/P) ratio, which is a reflection of Δ^6-desaturase activity, it was increased in both PC and PE in the colon polyps, suggesting a reduced enzyme activity. However, when considering the high level of LCPUFA in both PC and PE it would appear that the activity of the Δ^6-desaturase enzyme was not altered or impaired. This is in agreement with the high level of the n-3 and specifically the n-6 LCPUFA in the PC and PE phospholipid fractions. The increased Δ^6-desaturase substrate levels could also be related to negative product feedback regulation of the enzyme by LCPUFA products (Brenner, 1989).

Although the levels of n-3 PUFA increased significantly in the polyps, the membrane content was fairly low in comparison to the n-6 FA resulting in an increase in the membrane n-6/n-3 FA ratio. In this regard the increase in the C20:4n-6/C20:5n-3, C20:4n-6 PC/PE and C20:4n-6 polyp/surrounding ratios likely enhances tumour development through eicosanoid biosynthesis (e.g. prostaglandin PGE_2) that promotes neoplastic growth. It was suggested that the ratio of dietary n-6/n-3 FA might be an important factor that determines the progression of an initiated cell into cancer. A diet, utilizing low n-6/n-3 FA ratio, was shown to reduce the development of hepatocyte nodules (Abel *et al.*, 2004). Thus, by regulating the dietary intake of specific FA, cellular events associated with cancer development may potentially be modulated.

Dietary modulation of membrane lipid profiles may therefore be an important tool in the prevention of cancer development. Information regarding the effect of specific dietary FA ratios on the modulation of membrane lipid profiles in normal colon mucosa is therefore of relevance. As FA also play a determining role in the oxidative status of cancer tissue and hence cell survival, the effect of specific dietary FA is of relevance (Abel *et al*., 2004). One of the objectives of the present study was to determine the lipid profiles of normal colon mucosa with respect to the intake of specific FA and the impact it has on the oxidative status. Red blood cell membranes were also included to assess whether any similarities/differences exist between the tissue compartments regarding changes in the lipid profiles and oxidative status. The sources of fat used were sunflower oil (control diet) with an n-6/n-3 FA ratio of 250:1, sunflower plus fish oil (SF; n-6/n-3 FA ratio of 12:1), sunflower plus borage oil (SB; n-6/n-3 FA ratio of 30:1) and a combination SBF diet with an n-6/n-3 FA ratio of 12:1.

The lipid profiles between colon mucosa and RBC membranes lipid profile differed significantly. The RBC membrane phospholipids PC and PE content increased significantly, whilst the colon mucosa PE phospholipid content decreased with fish oil. Differences in the membrane phospholipid content may be indicative of the tissue type cell turnover relative to the specific tissue's function. The function of RBC is to primarily serve as a vehicle for the

transport of oxygen to body cells (http://www.helium.com/items/763669-the-functions-of-red-blood-cells, accessed 16 February 2009). Thus, as RBC is generated continuously to maintain a functional entity the fish oil diet plays a supportive role by increasing the PC and PE phospholipid constituents and PC/PE ratio. Due to the functional nature of the colon, mucosal membranes cells are constantly renewed to balance the removal of cells and those damaged by exposure to noxious elements. The PE phospholipid content and the PC/PE ratio of the colon mucosa was effectively reduced and increased, respectively, with the intake of fish oil containing diets. This is in contrast to the lipid content of polyps indicating that an increase in the PE phospholipid content relatively to PC and hence a reduced PC/PE ratio was associated with an increased proliferation potential and a diet high in n-6 FA. In the colon mucosa the PC/PE ratio is much lower compared to the RBC, which is indicative of the difference in proliferative status of the two cellular compartments. A similar response was also noticed in liver nodules, which was associated with an increased cell proliferative environment (Abel *et al*., 2004). Of interest is that, borage oil, with relative high n-6 FA content had an opposite effect on the PC/PE ratio due to a marked decrease in PC and increase in PE. Therefore, the fish oil containing diet induced changes influenced the colon mucosa cell turnover, thereby minimizing the potential occurrence of the proliferative events associated with the continuance of abnormal cellular growth.

As discussed for the polyps, changes in the lipid profile are likely to have a significant effect on the membrane fluidity of both the RBC and colon mucosa. The fish oil containing diets effectively increased the cholesterol content of the mucosal membranes, which as mentioned above is associated with a potentially more rigid cell structure (Petrache *et al*., 2005). However, this apparent increase in membrane rigidity was countered by a decrease in the chol/PL ratio an increase in the membrane unsaturation reflected by the increased P/S ratio and the PC/PE ratio. Changes in these parameters play a significant role in the regulation of critical cellular processes related to different cell growth parameters. In addition to the cholesterol and phospholipid constituents, the n-6/n-3 FA ratio diets had significant effects on the tissues

membrane FA composition in terms of the degree of FA incorporation into the PC and PE phospholipids. Fish oil increased SATS in the colon mucosa PE, while it was decreased by borage oil. In the RBC membranes SATS were increased in PC and PE by both oils. The differences in FA incorporation between the tissue compartments may be related to mechanisms that regulated membrane FA content. In this regard, fish oil increases n-3 LCPUFA resulting in a compensatory increase in SATS incorporation in order to maintain membrane fluidity. In contrast borage oil decreased SATS in the mucosa PE, presumably due to the preferential incorporation of the n-6 PUFA. Similarly to the fish oil effects, this may be a mechanism to maintain the homeostatic membrane fluidity.

Changes to the mucosa and RBC membranes MUFA composition were mainly associated with the dietary oils effect on C18:1n-9. Depending on the phospholipid both borage oil and fish oil increased MUFA in the colon. In the RBC both fish and borage oils increased MUFA in PE. The decrease in Δ^6-desaturase activity by fish oil could be responsible for the increased C18:1n-9 levels. As mentioned above, C18:1n-9 and C16:0 have opposite effects in terms of the regulation of cell proliferation and apoptosis with the latter promoting cell removal (Hardy *et al*., 2003).

Changes in membrane incorporation of dietary n-6 and n-3 have a significant effect on the functional regulation of cellular responses (Miles and Calder, 1998; Ng *et al.,* 2005; Shaikh and Edidin, 2006). Fish oil significantly increased the n-3 PUFA content, particularly C20:5n-3, in the PC and PE phospholipid fractions in both membrane compartments with a concomitant reduction of n-6 FA. It is known that n-3 FA are preferentially incorporated into membranes (Hwang *et al.,* 1987). Borage oil mainly increased the n-6 PUFA content, particularly C20:3n-6 and C20:4n-6, in the mucosa and RBC membranes due to the diet's supply of C18:3n-6 which is rapidly metabolised to functional n-6 LCPUFA. The fish and borage oil diets supply of PUFA and their subsequent incorporation into the membranes phospholipid compartments significantly reduced the activity of Δ^6-desaturase (Raz *et al*., 1997; Engler and Engler, 1998). This effect was illustrated by the qualitative

(%) increase in the Δ6 S/P ratio colon the PC fraction. Dietary intake of n-6 and n-3 PUFA thereby reduce the endogenous synthesis of these FA due to negative substrate feedback regulation of the Δ^6-desaturase enzyme, allowing for direct FA incorporation into membranes phospholipids (Brenner, 1989). Although the individual effects of fish and borage oil were evaluated (diets SF and SB), the response elicited by the combination diet (SBF) was of significant interest. The mucosa and RBC membranes level of FA incorporation in response to the SBF diet illustrated that the effects of the individual oils were maintained, countered or enhanced. Modulation of the membranes FA composition by the SBF diet may therefore promote a condition/environment that optimizes cellular responses that regulate the cell cycle (Calviello *et al*., 1999; Latham *et al*., 1999). Of specific interest is the regulation of C20:4n-6 levels by the introduction of dietary C18:3n-6 that bypass the Δ^6-desaturase which activity is down regulated by the n-3 FA (see paragraph below).

The ratio of n-6 and n-3 FA in the lipid compartments of the mucosa and RBC membranes have a significant effect on the synthesis of eicosanoids that elicit important cellular responses depending on the FA. With the SF diet, membranes n-3 PUFA content increases significantly, whereas with the SB diet, the n-6 PUFA content increased. C20:4n-6 and C20:5n-3 are the precursory FA required for the biosynthesis of the 2- and 3- series eicosanoid, respectively having opposing effects regarding cell proliferation and anti-inflammatory effects (Li *et al*., 1994). A high C20:4n-6/C20:5n-3 ratio elicits abnormal cellular responses, however, this effect can be countered by the incorporation of FA such as C20:5n-3 and C18:3n-6 that attenuate the effects C20:4n-6. This reaction can be achieved effectively by the SBF diet which makes provision for C20:4n-6, C20:5n-3 and C18:3n-6. Both C20:5n-3 and C18:3n-6 increases the availability of the anti-inflammatory eicosanoids (i.e. 3- and 1- series), and thus reduces the potential for persistent cell proliferation promoted by the C20:4n-6 derived metabolites. In the polyps the C20:4n-6/C20:5n-3, C20:4n-6 PC/PE and C20:4n-6 polyp/surrounding ratios were significantly increased which is associated with the altered growth patterns. The availability of C18:3n-6 makes provision for adequate C20:4n-6

levels required for normal cellular functions. However, the inclusion of C18:3n-6 and C20:5n-3 prevents the C20:4n-6 accumulation that sustains cell proliferation. The SBF diet may be an effective strategy against the promotion of colon carcinogenesis, by regulating the level of C20:4n-6 eicosanoid products.

Changes in the oxidative status of cells have a significant effect on carcinogenesis (Klaunig and Kamendulis, 2004). Membranes are prime targets for oxidation due to the chemical structure of the LC n-6 and n-3 PUFA that are rich in hydrogen bonds that are highly susceptible to damage from free radicals resulting in lipid peroxidation (Song *et* al., 2000; Kokura *et al.,* 2002; Spitellar, 2003). In cancer cells a low oxidative status promotes the survival of damaged cells presumably due to high vitamin E and C18:1n-9 levels (Horrobin, 1990). By increasing the level of oxidative stress in the cancer cells, pro-oxidant such ROS may be an effective mechanism to inhibit the continued survival of a neoplastic phenotype. ROS can initiate signal transductions that induce the removal of damaged and/or cancer cells via apoptosis (Simon *et al.,* 2000). In the colon mucosa and RBC membranes intakes of the dietary n-6/n-3 FA ratios increased the membranes PUFA content, particularly the n-3 LCPUFA C20:5n-3 with the inclusion of fish oil in the diet (SF and SBF diets). Elevated levels of C20:5n-3 and C22:6n-3 enhanced the membranes susceptibility to lipid peroxidation (LPO) and the level of oxidative stress within the tissue. This may be an effective way to reduce cancerous growth as was indicated by a recent study where enhanced the level of LPO in hepatocyte nodules by n-3 FA resulted in a reduction on growth (Abel et al. 2004). Therefore, the SF, SB and SBF diet can effectively increase the membrane content of n-3 LCPUFA content with concomitant increase in the oxidative status.

Although the colon polyps displayed a similar increase in LCPUFA, particularly with C20:4n-6, it is likely that increases in antioxidants such as C18:1n-9, GSH, vitamin E, which are known to be increased in cancer cells (Horrobin, 1990; Valko *et* al., 2006), will increase resistance to free radical attack. Therefore, with a reduction in oxidative membrane damage, the

decrease in oxidative stress by may play a significant role in sustaining polyp development. Utilizing fish oil based diets, particularly SBF, may be an effective strategy to initiate LPO in cancer cells. Hereby, responses that control cell proliferation by inhibiting cell growth and stimulating or enhancing apoptosis may be induced in neoplastic lesions to circumvent their further development.

4.2 Conclusions

Based on the findings from the present investigation, dietary fat intake plays a distinctive role in modifying the lipid composition of cellular membranes. As membrane structure and function are dependent on the lipid composition, diet induced alterations would impact significantly on the associated cellular features/characteristics. As a result, cellular functions that diverge from the norm may promote abnormal cellular states, such as the process of carcinogenesis, which may be modulated by specific dietary constituents. The development of colon polyps in the cancer model illustrated that a diet high in n-6 PUFA may play an underlying role. This result was conveyed by the significant differences between the lipid profiles of the polyps and surrounding mucosa, which was associated with a particular high n-6 FA diet. An interactive role of SATS, MUFA and C20:4n-6 was highlighted as the main driving force to sustain the altered growth characteristics of the polyps. The dietary FA modulation study indicated that colon mucosa and RBC membranes respond differently to dietary n-6/n-3 FA ratios in relation to their lipid profiles. Specific n-6/n-3 FA interactions exist which differs between the two cellular compartments. These interactions are directly or indirectly influenced by the effect of the different FA on the activity of the rate limiting Δ^6-desaturase. Hence, RBC membrane lipid profiles, which have been widely used as a biomarker for other tissues compartments (Vidgren *et al.,* 1997; Harris *et al.,* 2005; Kuriki *et al.,* 2006), are not a true reflection of alterations in the colon.

The selective incorporation of n-3 LCPUFA in both membranes increased their susceptibly to lipid peroxidation that may selectively sensitize cell survival through the induction of oxidative stress resulting in apoptosis, which could be

a strategy to adversely affect early neoplastic lesions. Modification to cell membrane lipids by specific dietary FA may be an ideal strategy to circumvent or prevent colon carcinogenesis.

4.3 References

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ADDENDUM

ADDENDUM 1

Analytical parameters for TLC

Solid phase	Silica Gel 60 plates 10x20 cm, activated 60 minutes at 100 °C	
Mobile Phase	Chloroform:petroleum benzene:methanol:acetic acid:boric acid (40:30:20:10:1.8 v/v/v/v/w), allowed to saturate tank atmosphere (45 min)	
Developing time	90 min	
Detection	2,5-bis-(5'tert-Butylbenzoxazoyl-[2']-thiophene (BBot) added to mobile phase, detection: viewing at λ= 366 nm	
Standard	mixture of PC, PI, PS, PE	
R_f values	PC	0.1
	PE	0.55

ADDENDUM 2

Set up parameters for GC analyses

gas chromatograph	Varian 3300, Varian Chromatography Systems, California, USA.	
columns	capillary columns, BPX-70, 30 m x 0.32 mm internal diameter (SGE, Texas, USA)	
column phase (0.25 µm)	70% Dicyanopropyl 30% Dimethyl Polysilphenylene-siloxane	
temperature programming	initial column temperature:	160°C
	final column temperature:	220°C
	gradient linear at	4°C/min
	injection temperature:	240°C
	detector temperature:	260°C
detector	FID (Flame Ionisation Detector)	
injection volume	1 µl	
solvent for sample/standards	hexane	
standards	mixture of fatty acid methyl esters C12:0 to C24:1 in heptane	
internal standard	C17:0	
gas flow rates	medical air (21% O_2 in N_2) 250 ml/min	
N_2	25 ml/min	
carrier gas H_2	25 ml/min	
split ratio	20:1	
column pressure	8 Psi	

ADDENDUM 3

Gas chromatograms

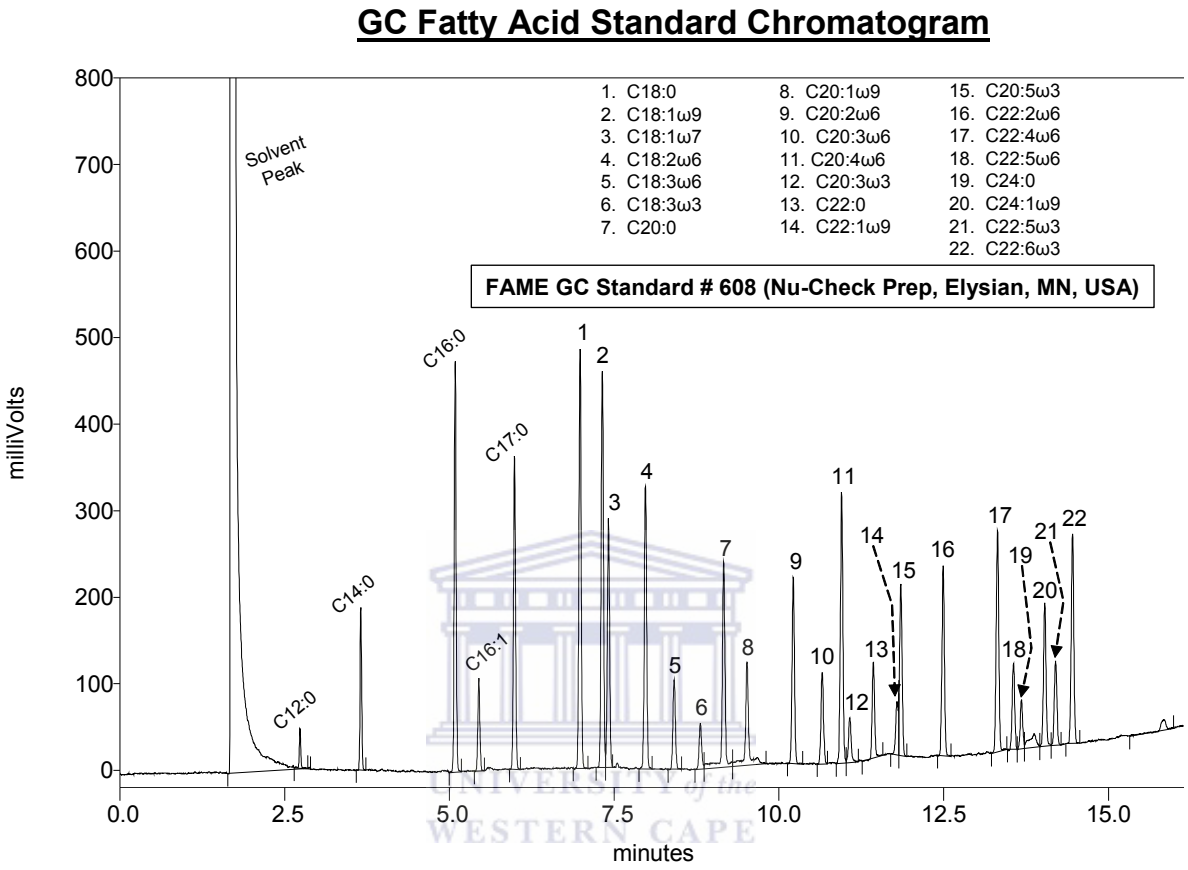

Fig. Chromatogram of a standard fatty acid mixture (C12:0 to C24:1)

Instrument:	Varian 3300 Gas Chromatograph
Column:	BPX70 (SGE, USA, cat. # 054616)
Length:	30 m
Internal Diameter:	0.32 mm
Phase thickness:	0.25 µm (70% Dicyanopropyl, 30% Dimethyl Polysilphenylene-siloxane)
Initial column temp.:	160°C
Rate:	4 °C/min.
Final column temp.:	220 °C
Final hold time:	10 min.
Injector temp.:	240 °C
Detector temp.:	FID, 250 °C
Carrier gas:	Hydrogen
Inlet pressure:	8 PSI
Average gas velocity:	1.85 ml/min.

FA calculations

Percentage total FA

$$\%FA = \frac{area_{FA} \cdot 100}{area_{TFA}}$$

$area_{FA}$ area of individual fatty acid (e.g C14:0)

$area_{TFA}$ sum of the total fatty acid areas

FA content µg per mg protein (i.e. m_{FA})

Colon polyps/mucosa

$$m_{FA} = \frac{area_{FA} \; m_{internal\ standard}[\mu g] \cdot f_{aliquot} \; x \; (^{100}/_{CLW})}{area_{internal\ standard} \; x \; m_{colon\_protein}[mg]}$$

RBC membranes

$$m_{FA} = \frac{area_{FA} \; m_{internal\ standard}[\mu g] \cdot f_{aliquot}}{area_{internal\ standard} \cdot C_{protein}}$$

$f_{aliquot}$ $V_{total\ CMS\ of\ sample}$ [µl]/ $V_{aliquot\ of\ CMS\ extract\ spotted\ for\ TLC}$ [µl]

$area_{FA}$ area of individual fatty acid (e.g C14:0)

$area_{internal\ standard}$ area of internal standard (i.e. I.S = C17:0)

$m_{internal\ standard}[\mu g]$ PC = 4.5 (50 µl I.S); PE = 2.25 (25 µl I.S)

$m_{colon\_protein}[mg]$ mg sample protein per 100 mg colon protein dry weight

CLW colon lipid weight (mg)

$C_{protein}$ RBC protein concentration (mg per ml)

unit: µg FA per mg protein

ADDENDUM 4

PHOSPHOLIPID DETERMINATION

Adapted from Itaya and Ui (1966). Clin. Chim. Acta, 14: 361-366.

Reagents

Malachite green:	0.2% in dH_2O (v/v) and filter before use
Ammonium molbydate:	4.2% in 5 N HCl
Tween 20:	0.5% in dH_2O

Ratio of working solution (MAT):

Malachite green	:	Ammonium molbydate	:	Tween
15	:	5	:	1

Leave the working solution at 4°C for at 30 minutes (background colour changes from green to brown).

Phospholipid fractions (PC and PE) identified from TLC:

- Scrape off into glass phospholipid tubes.
- Add perchloric acid (i.e. PC = 400 µl, PE = 300 µl).
- Place tubes on a 170 °C heating block for 2 hours until clear (NB: keep tops loosely fitted).
- Cool to room temperature.
- Add dH_2O at a ratio of 5:1 with the perchloric acid.
- Vortex and centrifuge at 500 rpm for 15 minutes

Standards: (in duplicate)

1) 20 µl of standard phosphate (Pi) solution (i.e. KH_2PO_4) = 1 µg of Pi
2) 40 µl
3) 60 µl
4) 80 µl
5) 100 µl

→ Evaporate standards on block (approx 20 min)

→ Cool to room temperature; add 400 µl perchloric acid + 2ml dH_2O

Blanks: 400 µl perchloric + 2 ml dH_2O (x3)

Loading of Samples:

- Load clean dry test tubes with 1 ml working solution (MAT)
- Add 250 µl of standard/blank/samples
- Vortex
- Incubate at room temperature for 20 minutes
- Read at 660 nm on spectrophotometer

Calculation of phospholipids phosphate (C_{Pi}) content:

Colon polyp/mucosa

$$C_{Pi} = \frac{f_{standard}\ [\mu g] \times f_{aliquot} \times f_{phospholipid} \times (^{100}/_{CLW}) \times f_{HCLO4} \times absorbance_{sample}}{m_{colon\_protein}\ [mg]}$$

RBC membranes

$$C_{Pi} = \frac{f_{standard}\ [\mu g] \cdot f_{aliquot} \cdot f_{phospholipid} \cdot f_{HCLO4} \cdot absorbance_{sample}}{C_{protein}}$$

$f_{standard}$	average of n values: $m_{standard}$ [µg]/ $absorbance_{standard}$
$f_{aliquot}$	$V_{total\ CMS\ of\ sample}$ [µl] / $V_{aliquot\ CMS\ extract\ spotted\ for\ TLC}$ [µl]
$f_{phospholipid}$	converting factor from phospholipids to P_i using the following M_r:

	$f_{phospholipid}$
PC	25.4
PE	23.2

CLW	colon lipid weight (mg)
f_{HClO4}	correction factor between dilution of standard and sample with perchloric acid $HClO_4$: $V_{standard}$ [µl] / V_{sample} [µl]
$m_{colon\_protein}$	mg sample protein per 100 mg colon protein dry weight
$C_{protein}$	RBC protein concentration (mg per ml)
unit:	µg P_i per mg protein

ADDENDUM 5

CHOLESTEROL DETERMINATION

Adapted from – Richmond (1973). Clin. Chem., 19: 1350-1356.
– Smuts *et al.* (1994). Cor. Art. Dis. 5: 331-338

Reagents:

(1) Cholesterol Colour Reagent:

To one liter volumetric flask add the following:

KH_2PO_4	22,1827 g
$K_2HPO_4.3H_2O$	8,4445 g
KI	19,9212 g
NaN_3	9,7515 g
Alkylbenzyldimethylammonium chloride	100 mg
Ammonium molbydate	12,4 mg
Triton X-100 (polyethylene glycol monether; peroxide free)	2 g

(2) Cholesterol Reagent B:

Cholesterol esterase	83.3 µl
Cholesterol oxidase	125 µl
3M NaCl	1792 µl

Method:

(1) Use 20 µl of the lipid sample from CMS extraction used for TLC spotting.
(2) Add 50 µl CM, vortex.
(3) Add 300 µl 1% Tritin-X100 (peroxide free), vortex.
(4) Evaporate emulsion under N_2, vortex until clear.
(5) Add 1.7 ml cholesterol reagent.
(6) Standards: - 10 µl from 4 different cholesterol standard concentrations
- 300 µl 1% Triton-X100
- Add 1.7 ml cholesterol reagent

(7) Carry 1 ml over from (5) and (6) into small sample tubes and add 20 µl cholesterol Reagent B.

(8) Add 20 µl 3 M NaCl to original tubes from (5) and (6).

(9) Incubate at room temperature.

(10) Read at 365 nm with both VIS and UV bulbs on. Read each sample together with its respective blank.

Cholesterol content calculation:

Colon polyps/mucosa

$$C_{cholesterol} = \frac{f_{standard} \times f_{aliquot} \times (^{100}/_{CLW}) \times absorbance_{sample}}{m_{colon\_protein}\ [mg]}$$

RBC membranes

$$C_{cholesterol} = \frac{f_{standard} \times f_{aliquot} \times absorbance_{sample}}{C_{protein}}$$

$f_{standard}$	average of n values: $m_{standard}$ [µg]/ $absorbance_{standard}$
$f_{aliquot}$	$V_{total\ CMS\ of\ sample}$ [µl]/ $V_{aliquot\ of\ CMS\ extract\ spotted\ for\ TLC}$ [µl]
$_{CLW}$	colon lipid weight (mg)
$m_{colon\_protein}$ [mg]	mg sample protein per 100 mg colon protein dry weight
$C_{protein}$	RBC protein concentration (mg per ml)
unit:	µg cholesterol per mg protein

ADDENDUM 6

PROTEIN DETERMINATION METHODS

(A) Lowry method: (colon polyps and mucosa)

Adapted from Markwell *et al.* (1978), Anal. Biochem., 87: 206-210.

To liquid nitrogen powdered colon polyp/mucosa sample in plastic protein tubes:

- Add 5 ml of a 5% SDS + 0.5 N NaOH solution. Incubate the protein solution for 48 hours at 37 °C
- Rinse content of each tube into measuring tubes with 0.5 ml 5% SDS + 0.5 N NaOH. Repeat a further two times.
- Centrifuge for 10 minutes at 500 rpm to remove foam.
- Fill to a final volume of 5 ml.
- Vortex.
- Stores in plastic blue capped tubes (LP5) at 4 °C until analyzed.

Solution 1: 5% SDS + 0.5 N NaOH

Solution 2: 2% Na_2CO_3 + 0.16% Na-K-Tartrate

Solution 3: 4% $CuSO_4.5H_2O$

Solution 4: Alkaline copper reagent which consists of 100 parts of solution 2 and 1 part of solution 3 (working solution).

Solution 5: Folin –Ciocalteu reagent diluted with dH_2O (1:1 v/v)

Table 1: Lowry protein determination method

Standards:	(i) Sample (vol)	(ii) H_2O	(iii) Solution 1	(iv) Solution 4
	µl	µl	µl	ml
Blank		200	300	1.5
BSA (0.5 mg/ml)	25	175	300	1.5
	50	150	300	1.5
	75	125	300	1.5
	100	100	300	1.5
	150	50	300	1.5
	200	–	300	1.5

Total volume of i+ii+iii= 500 µl

(colon polyps/mucosa)

Samples: X (100 µl) 200 µl 300 ul - X

1.5 ml

(done in triplicate)

Do as follows:

(i) + (ii) + (iii) → Vortex → (iv) → Vortex → (v) + 150 µl solution 5 (total volume 2150 µl) → Vortex. Incubate at 37 °C for 45 minutes. Read absorbance at 660 nm against water.

Calculation of colon polyp/mucosa protein content:

→ Determined from 0.5 mg/ml standard curve: $y = mx + c$

y : sample absorbance reading – blank

m : standard curve slope/gradient

x : sample protein concentration (mg/ml)

c : y-intercept

i.e $x = \frac{y - c}{m}$

units : mg protein/ml

Sample protein concentration (X) in 2150 ul:

X = (x * 2150 μl)/sample volume (μl)

units: mg protein/ml

mg sample protein per 5 ml protein solution (Y):

Y = X/5 ml

units: mg protein

mg sample protein per 100 mg colon protein dry weight (Z):

Z = 100/(colon dry protein weight (mg) * Y)

Note: Z value used for fatty acid μg content/mg protein content determination

(B) Pierce Method: (RBC membranes)

Adapted from Kaushal and Barnes (1986), Anal. Biochem. 157: 291-294.

Solutions Required:

(i) 2% SDS solution (containing 2% SDS 2 mM EDTA 20 mM $NaHCO_3$)

(ii) BCA protein assay reagent A

(iii) BCA protein assay reagent B; 4% $CuSO_4.5H_2O$ (Make up own solution)

(iv) BSA standard

(v) Distilled water (H_2O)

BSA standard: Make up 1 mg/ml BSA stock solution and verify concentration on spectrophotometer

<u>NB</u>: Dilute collected samples (RBC) with 2% SDS solution prior to protein determination.

Table 2: Pierce protein determination method

	(i)	(ii)	(iii)	(iv)	(v)	
Samples	**BSA from diluted stock (µl)**	**Volume of 2% SDS (µl)**	**Unknown sample volume (µl)**	**Dist. H_2O (µl)**	**Reagent A + B (µl)**	**Total Reaction Volume (µl)**
Standards						
Blank (x3)	0	80	–	520	400	1000
S-1 (x2)	10	80	–	510	400	1000
S-2 (x2)	20	80	–	500	400	1000
S-3 (x2)	40	80	–	480	400	1000
S-4 (x2)	60	80	–	460	400	1000
S-5 (x2)	80	80	–	440	400	1000
S-6 (x2)	100	80	–	420	400	1000
Unknown Samples						
U-1	–	–	40	560	400	1000
U-2	–	–	60	540	400	1000
U-3	–	–	80	520	400	1000

Reagent A + B: calculate how much is needed. Mix in a ratio 50:1 (A:B).

Vortex samples and standards after addition of Reagent A + B mixture.

Incubate in a water bath at 60 °C for 1 hr.

Cool to room temperature.

Vortex prior to reading on spectrophotometer.

Read at 562 nm. (Can use disposable PS or PMMA semi-micro cuvettes, approximately 1.5 ml)

Calculation of RBC membranes protein content:

→ Determined from 0.1 mg/ml standard curve: $y = mx + c$

y : sample absorbance reading – blank

m : standard curve slope/gradient

x : sample protein concentration (mg/ml)

c : y-intercept

i.e $x = \frac{y - c}{m}$

units : mg protein/ml

Sample protein concentration (X) in 1000 μl:

X = [(x * 1000 μl)/sample volume (μl)] * dilution factor (i.e. RBC dilution factor)

units: mg protein/ml