FATTY ACIDS AS CANCER PREVENTIVE TOOLS IN THE DIETARY MODULATION OF ALTERED LIPID PROFILES ASSOCIATED WITH HEPATOCARCINOGENESIS

by

Stèfan Abel

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Promoter: **Prof. M. de Kock, University of the Western Cape.**

Co-Promoter: **Dr. W.C.A. Gelderblom, Medical Research Council, Tygerberg.**

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DECLARATION

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

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S. Abel   **Date**

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DEDICATION

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To my parents and family: thank you for your patience.

To Jok, Molly and Mickey:
Thank you for helping me through the ruff stuff!

ACKNOWLEDGEMENTS

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ABBREVIATIONS

	alpha
γ	gamma
κ	kappa
Δ	delta
μ	micro
ω	omega
Δ6 S/P	Delta-6 desaturase substrate/product ratio
ω6/ω3	omega-6 to omega-3 polyunsaturated fatty acid ratio
AA	arachidonic acid, C20:4ω6
AAF	2-acetylaminofluorene
AFB	aflatoxin B
AHA	American Heart Association
ALA	alpha(α)-linolenic acid, C18:3ω3
ANOVA	analysis of variance
ATPase	adenosine triphosphatase
BHT	butylated hydroxytoluene
cAMP	cyclic AMP
cdk	cyclin-dependent kinase
Chol	cholesterol
Chol/PL	cholesterol to phospholipid molar ratio
CM	chloroform/methanol
CMS	chloroform/methanol/saline
CoA	co-enzyme A
CoA-IT	co-enzyme A-independent transacylase(
COX	cyclooxygenase(s)
DAG	diacylglycerol
DEN	diethylnitrosamine
DGLA	dihomo-gamma(γ)-linolenic acid, C20:3ω6
DHA	docosahexaenoic acid, C22:6ω3
DNA	deoxyribonucleic acid
DPH	1.6-diphenyl-1.3.5-hexatrine
EDTA	ethylenediaminetetraacetic acid
EFA	essential fatty acid(s)

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ABBREVIATIONS

EFAD	essential fatty acid deficiency
EGFR	epidermal growth factor receptor
EPA	eicosapentaenoic acid, C20:5ω3
FA	fatty acid(s)
FAME	fatty acid methyl esters
FDA	Food and Drug Administration
FO	fish oil
GGT	γ-glutamyl transpeptidase
GLA	gamma(γ)-linolenic acid, C18:3ω6
GSH	glutathione (reduced)
GSSG	glutathione (oxidised)
GSTP$^+$	glutathione-S-transferase (placental) positive
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCl	hydrochloric acid
HCV	hepatitis C virus
HDL	high density lipoprotein
HETE	hydroxylated eicosatetraenoic acid(s)
HETrE	hydroxylated eicosatrienoic acid
HIV	human immune-deficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A reductase
HNF-4α	hepatic nuclear factor-4α
HPEPE	hydroperoxyeicosapentaenoic acid
HPETE	hydroperoxyeicosatetraenoic acid
IDL	intermediate density lipoprotein(s)
IGF-IR	insulin-like growth factor I receptor
iNOS	inducible nitric oxide synthase
IP3	inositol-1,4,5-triphosphate
ISSFAL	International Society for the Study of Fatty Acids and Lipids
LA	linoleic acid, C18:2ω6
LC	long-chain
LCAT	lecithin cholesterol acyltransferase
LCPUFA	long chain polyunsaturated fatty acids

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ABBREVIATIONS

LDL	low density lipoprotein(s)
LOX	lipoxygenase(s)
LXR	liver X receptor(s)
MAPK	mitogen-activated protein kinase
MAP3K/MEKK	mitogen-activated protein kinase kinase kinase
MDA	malondialdehyde
Mic	microsomes
Mn-SOD	manganese superoxide dismutase
mM	milli moles
MOX	monooxygenase(s)
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid(s)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NF-κB	nuclear factor-kappa B
N/S	nodule to surrounding ratio
NSAIDs	nonsteroidal anti-inflammatory drugs
NO	nitric oxide
OA	oleic acid, C18:1ω9
PAUSET	Public Awareness and Understanding of Science and Technology
P/S	polyunsaturated to saturated fatty acid ratio
PC	phosphatidylcholine
PC/PE	phosphatidylcholine to phosphatidylethanolamine phospholipid ratio
PE	phosphatidylethanolamine
PEMT2	N-methyltransferase-2
PG	prostaglandin(s)
PI	phosphatidylinositol
PI3-K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PL	phospholipid(s)
PLA$_2$	phospholipase A$_2$
PLC	phospholipase C

ABBREVIATIONS

PM	plasma membrane
PPAR	peroxisome proliferator-activated receptors
PS	phosphatidylserine
P/S	polyunsaturated to saturated fatty acid ratio
PUFA	polyunsaturated fatty acid(s)
RB	retinoblastoma
RNS	reactive nitrogen species
ROS	reactive oxygen species
RXR	retinoid X receptor(s) (
SATS	saturated fatty acid(s)
SDS	sodium dodecyl sulphate
SFO	sunflower oil
SM	sphingomyelin
SOY	soybean oil
SREBP	sterol regulatory element-binding proteins
TBARS	thiobarbituric acid reacting substances
TG	triglyceride(s)
TLC	thin layer chromatography
TNF-α	tumour necrosis factor-alpha
UCP-2	uncoupling protein-2
VLDL	very low density lipoproteins

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CHAPTER 1

boxes show specific examples. Membrane receptors bind to active ligands such as growth factors resulting in enzymatic activity, such as kinases, associated with the intracellular part of the receptor.   This can affect the association of the receptor with intracellular mediators or the localization or function of those mediators. The mediators alter the activity of 'effector' enzymes, some of which can move to the nucleus and control gene expression or can induce other proteins to affect gene expression.   Other effector enzymes target small molecules, either generating further signaling mediators (second messengers) or controlling the metabolic state of the cell.   Certain signaling pathways may bypass some of the steps described or may have several components working either in series or parallel.   …………………..………………………….…… 19

Figure 5:   Several signalling pathways may be involved in stimulating cancer growth and this has been attributed to cross-talk between these pathways.   Cross talk between the signalling molecules PI3-K, Akt and MAPK can occur in suppressing apoptosis and increasing cell survival.   ………………………...…………….. 21

Figure 6:   Signal transduction pathways involved in the regulation of cellular processes such as proliferation, gene expression and apoptosis.   Progress in elucidating signaling pathways has established the involvement of certain genes in these signaling events. This diagram depicts the complexity of protein interactions with phosphates and lipids. Some of the genes known to be functionally altered in cancer are highlighted in red.   …………..……..………………………….……….. 22

Figure 7:   The acquired characteristics of cancer cells. Deregulated tumour growth displays distinct characteristics such as increased proliferation of cells, loss of cell differentiation, decreased apoptotic rate, formation of new blood vessels by angiogenesis, alterations in signaling pathways, invasion and the movement of cancerous cells to other body organs termed metastasis. …………..………....... 23

Figure 8:   The digestion and transport of lipids in the body. Dietary fat is digested in the intestine and broken down to form complexes, called micelles, in the presence of bile salts and which are absorbed into the intestinal cell. In the enterocytes, the FA are re-esterified and together with cholesterol and apolipoproteins form chylomicrons. These are eventually discharged into the blood, broken down to

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PREFACE

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When considering the causes of death worldwide, cancer ranks second after cardiovascular disease (1). It is estimated that 30 to 35% of all cancer cases are affected by the diet, implicating diet as major role player in cancer aetiology and prevention (2, 3). The American Institute for Cancer Research and the World Cancer Research Fund estimate that 30 to 40 percent of all cancers can be prevented by appropriate diets, physical activity and maintenance of appropriate body weight (4). Although inconsistencies exist across studies, there is a basic assertion that dietary factors influence cancer risk (5). Research from several sources provide strong evidence that vegetables, fruits, whole grains, dietary fiber, certain micronutrients, certain fatty acids (FA) and physical activity protect against certain forms of cancer. In contrast, other factors, such as obesity, alcohol, some FA and food preparation methods may increase risks (5). Studies examining the relationship between specific genotypes and the risk for developing diet-related diseases, particularly regarding cancer, diabetes and vascular disease, have also illustrated the importance of individual gene typing. This field of study has been referred to as nutrigenomics and nutrient-gene interactions may explain why certain individuals respond more favorably to dietary interventions (6). Amongst dietary factors influencing cancer prevention, the role of polyunsaturated fatty acids (PUFA) of the ω3 family has gained prominence. This largely resulted from the pioneering studies by Dyerberg, Bang and Hjorne (7) on the low cardiovascular and cancer incidence rate amongst Greenland Eskimos which was associated with a high intake of dietary marine ω3 FA. Since then various studies in cell culture, animal cancer models and in humans have shown the effectiveness of ω3 FA in preventing or slowing down the development of cancer via various mechanisms. These mechanisms include alterations in cell membranes effecting membrane-bound enzymes, regulation of eicosanoid production, alterations in immune response, signal transduction pathways and expression of genes involved in proliferation and apoptosis (5).

Mechanistically, under normal physiological conditions, an equilibrium exists regarding growth, differentiation and apoptosis in the renewal and removal of cells. However, disruption of these processes plays a vital role in the establishment and survival of the malignant phenotype (8). Several studies have indicated the importance of regulating the cell cycle and processes such as apoptosis and cell proliferation in the prevention

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and treatment of cancer. Investigating cancer growth and development provides an opportunity for gaining insight into the functional regulatory mechanisms involved in cell survival. By understanding the processes involved in cancer, the function of normal signaling and cell interactions can also be elucidated.

Research has indicated that the amount and type of dietary fat can alter cellular responses by affecting membrane composition and intracellular signaling. In this regard, the liver plays a central role in the metabolism of dietary FA and transport to other body organs. Manipulation of the dietary PUFA, especially of the ω3 family in a certain ratio with the ω6 FA, will become increasingly important in the management of health and certain diseases. Initial studies in this thesis examined the kinetics of dietary FA replacement in rat liver utilising different fat sources with varying ω6/ω3 FA ratios, followed by an investigation of the lipid alterations associated with the development of hepatocyte nodules. Information obtained from these studies was utilised to investigate the modulating role of dietary fats with different ω6/ω3 FA ratios on the development of hepatocyte nodules in rat liver. The main aim was to define a dietary fat with a specific ω6/ω3 ratio which could effectively modulate cancer development in the liver. Subsequently, changes in membrane lipids associated with liver cancer development in humans were investigated in order to characterise specific changes in lipid metabolism as compared to that occurring in the animal model with the aim of subsequent modulation.

Following this Preface, Chapter 1 provides a general overview on cancer and various processes involved in the initiation and development of cancer. Aspects concerning the role of the ω6 and ω3 FA families in cancer development, their interaction in regulating certain signaling pathways and gene expression relevant to carcinogenesis are also discussed. The importance of the dietary ω6/ω3 ratio and type of FA constituting the ratio will also be included. FA replacement and effect on membrane characteristics, such as fluidity in rat liver membranes when feeding rats low and high ω6/ω3 FA dietary ratios, will be discussed in Chapter 2. The focus of the study was to examine the kinetics and threshold effects of dietary ω6 and ω3 FA interactions in liver cell membranes. Alterations observed in the FA status and relevant membrane lipid parameters of preneoplastic nodules in rat liver with respect to cancer development are

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presented in Chapter 3. Information obtained from these studies was utilised to investigate the modulating role of fat sources with different dietary ω6/ω3 FA ratios on the development of hepatocyte nodules in rat liver (Chapter 4). Changes regarding the oxidative status and FA replacement in the nodule environment were critically evaluated in relation to the interaction between the different FA families. To assess the relevance and possible extrapolation of the current findings to humans, detailed lipid parameters associated with development of hepatocellular carcinoma (HCC) in humans were evaluated. The relevant lipid and oxidative parameters were highlighted and similarities to the rat liver cancer model discussed. Chapter 6 is a summary and conclusion of the results with relevance to developing cancer chemopreventive strategies.

REFERENCES

1. Serfontein, W. 2002. Beating cancer: Healing from within. Tafelberg Publishers, Cape Town, South Africa.

2. Doll, R. and Peto, R. 1981. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. J. Natl. Cancer Inst. 66, 1191-1308.

3. Lewis, C.J. and Yetley, E.A. 1999. Health claims and observational human data: relation between dietary fat and cancer. Am. J. Clin. Nutr. 69, 1357S-1364S.

4. WCRF/AICR: Food, nutrition and the prevention of cancer: a global perspective. World Cancer Research Fund / American Institute for Cancer Research 1997.

5. Greenwald, P., Clifford, C.K. and Milner, J.A. 2001. Diet and cancer prevention. Eur. J. Cancer 37, 948-965.

6. Müller, M. and Kersten, S. 2003. Nutrigenomics: goals and strategies. Nat. Rev. Genet. 4, 315-322.

7. Dyerberg, J., Bang, H.O. and Hjorne, N. 1975. Fatty acid composition of the plasma lipids in Greenland Eskimos. Am. J. Clin. Nutr. 28, 958-966.

8. Simopolous, A.P. 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed. Pharmacother. 56, 365-379.

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1.   INTRODUCTION

Research into cell biology provided evidence that all living beings consist of individual cells whose activities, inheritance and ability to assemble into organisms can be understood in logical biochemical terms (1). This demonstrated that however miraculous the existence of life on earth might be, life's mechanisms can be understood. Cell biology can be divided into a number of branches which are becoming progressively intertwined as research advances are made (2). The study of biological membranes is the branch most responsible for elucidating cell function as cellular membranes are the very boundaries within which life exists. A membrane separates the vast array of biochemical reactions that define a living cell from the extracellular world. Within the cell, membranes also organize and separate these biochemical reactions from each other, generating compositionally and morphologically distinct compartments.

For the past century, epidemiological evidence has indicated that the frequency of diet-related cancers has increased, especially in Westernized populations (3, 4, 5). In the 1970's focus on the causes of cancer shifted from environmental to the diet (6). Many epidemiologic and experimental studies were undertaken to investigate the possible association between dietary factors and cancers of the bowel, stomach, upper alimentary and respiratory tract, lung, breast, prostate and pancreas amongst others (7, 8). Subsequently, information regarding diet and cancer were reviewed indicating that evidence existed to support a dietary association with the development of certain cancers (6, 8). Doll and Peto (9) established that 35% of cancer deaths in the USA could be attributed to the diet. Also of interest was the change in cancer incidence amongst populations emigrating to different countries or even regions (4).

Dietary components such as vegetables, fruits, grains, fibre, certain micronutrients and certain fatty acids (FA) have been associated with a protective effect against the development of certain cancers (10). Amongst these, interest in polyunsaturated fatty acids (PUFA) of the ω3 family has gained prominence. This largely resulted from the pioneering studies by Dyerberg, Bang and Hjorne (11) on the low cancer incidence rate amongst Greenland Eskimos associated with a high intake of dietary ω3 FA. Since then studies utilising various cell culture systems and cancer models in animals,

indicated the effectiveness of ω3 FA in preventing or slowing down the development of cancer through various mechanisms.

This literature review consists of a brief description on cancer, carcinogenesis, the changes in the type and level of dietary fat available in our diet(s) over time and association with the development of certain diseases. The main focus of this overview will be on the ω6 and ω3 essential fatty acids (EFA) and their interaction with regards to carcinogenesis.

2. CANCER

In healthy tissue, cell differentiation, proliferation and programmed cell death (apoptosis) are closely regulated processes in maintaining organ homeostasis (12). Tumour growth is the breakdown or dysfunction of these finely controlled processes and the regulatory feed-back mechanisms involved (13, 14). Tumour cells have six important characteristics which contribute to their survival, i) loss of regulated cellular proliferation, ii) loss of regulated cell differentiation, iii) sustained cell division, iv) evasion or inhibition of apoptosis, v) invasion of other tissue and organs (metastases) and vi) angiogenesis (15).

Although great advances in cancer research have been made, a complete understanding of the causes is not yet fully elucidated. The current model for carcinogenesis stipulates that cancer is initiated by a mutagenic event due to various factors (16). This implies that there is no single cause and that cancer results from the interaction of many factors. The factors involved may be genetic, environmental or constitutional characteristics of the individual. The model states that cancer is initiated by DNA damage which can occur via external factors such as chemicals, viruses and irradiation or by endogenous elements such as an overproduction of reactive oxygen (ROS) or nitrogen species (RNS) generated by normal cellular processes (17). To counteract this, various systems exist in the body to protect and repair DNA damage. These include genes which monitor and repair DNA damage amongst which is the well-known p53 gene which regulates cell cycle progression.

Tumour cells can be defined as either being benign or malignant. Benign tumours are characteristically slow growing and retain the specialization of the originating tissue (18). Characteristically, malignant tumours are fast growing, poorly differentiated with the ability to metastasize to other organs. Non-invasive tumours are termed benign because they do not spread to other organs and can easily be removed from the organ of origin, in contrast to malignant or invasive tumours (9). Malignant growth is the invasion of tumour cells beyond their point of origin into the general circulation of the body and the formation of secondary tumours. This process is called metastases. The characteristic of malignant cancer is the invasion and metastases of tumour cells, which ultimately can lead to death. This fatal character of malignant tissue is due to the disruption and interference of cellular processes important for the maintenance of normal cell growth (13).

2.1. Gene Mutations

Certain cancers contain some type of genetic alterations which can be inherited or sporadic. There are three main types of genes that can affect cell growth and which are altered or mutated (18) in certain cancer types:

2.1.1. Oncogenes

These genes regulate the normal growth of cells. Malfunctioning causes uncontrolled growth of cells subsequently leading to the genesis of a cancer cell.

2.1.2. Tumour suppressor genes

Tumour suppressor genes control abnormal growth and replication of damaged cells and can interrupt their replication until the defect is corrected. If the tumour suppressor genes are mutated and they do not function properly, tumour growth may occur.

2.1.3. Mismatch-repair genes

These genes help recognize errors in DNA. If the DNA does not "match" perfectly, these genes repair the mismatch and correct the error. Incorrect functioning of these genes allows DNA errors to be transmitted to new cells, causing the error to be "fixed".

2.2. Carcinogenesis

Cancer development is a multistage process whereby a normal cell is transformed or altered into cells expressing the malignant phenotype. The occurrence and evolution of cancer provides important insight into the process of malignancy. The occurrence of

cancer is relatively rare, with an estimated mutation rate of 1 in 2 x 10^7 per gene during cell division in an average human with a population of 10^{14} target cells (12). In South Africa the lifetime risk (aged 0 to 74 years) for developing cancer is 1 in 4 for males and 1 in 5 for females (19), whereas in the USA it is 1 in 2 and 1 in 3, respectively for males and females (20). The rarity of cancer therefore illustrates the effectiveness of potent antitumorigenic mechanisms residing in normal cells, and cancer only occurs when these protective mechanisms have failed.

The transformation of normal cells into malignant cells can occur via various pathways. For a specific cancer type, the mutation of particular target genes such as Ras or p53 may occur in only a subset of histological identical tumours. Mutations in certain oncogenes and tumour suppressor genes can also occur early in some tumour progression pathways, but late in others. The appearance of events such as resistance to apoptosis, sustained angiogenesis and unlimited replicative potential can appear at different times during these various progressions. Therefore, the sequence in cancer acquiring events can vary widely among tumours of the same type and between tumours of different types. However, irrespective of how the steps in the genetic pathways are arranged, the biological endpoints ultimately reached are common to all types of tumours (12).

The process of cancer development (carcinogenesis) commences by the alteration of a normal cell into a genetically altered or "initiated" cell involving DNA damage (13). If not repaired, the damaged or alteration in DNA becomes permanent during cell replication, resulting in transformed or mutated cells. Alterations usually occur in growth control genes, proto-oncogenes, tumour suppressor genes as well as the regulatory genes for the tumour suppressor genes resulting in the deregulation of cell growth (21). Promotion of the altered cells is characterized by the clonal expansion of these cells by cell proliferation and/or inhibition of apoptosis, resulting in the formation of an identifiable focal lesion. This results in neoplastic (new growth) development whereby the preneoplastic cell proliferates faster than the surrounding normal cells. The promotion stage requires the continuous presence of the promoting stimuli, and can therefore be reversed. Further mutations can be fixed by continued proliferation, resulting in a population or foci of cells with a selective growth advantage (21, 22). A unique characteristic of cancer cells is the ability to divide and proliferate under conditions in which normal healthy cells do not (23). Progression is the final stage in

the carcinogenic process and is characterized by the accumulation of additional genetic damage, leading to the transition of cells from a benign to malignant phenotype, and is considered an irreversible process. The process from initiated cell to cancer is long with the latter manifesting only after 30 to 50 years in humans (22).
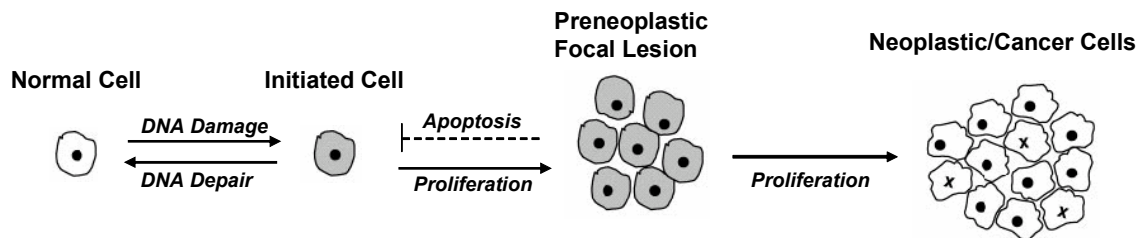
Figure 1: The stages of carcinogenesis. Initiation results in the mutation of the DNA in a cell nucleus producing an altered cell. At least one round of DNA synthesis is required to "fix" the genetic DNA damage. Promotion is characterized by the clonal expansion of initiated cells by cell proliferation and/or inhibition of apoptosis, resulting in the formation of preneoplastic focal lesions. The promotion stage requires the continuous presence of the promoting stimuli and is a reversible process. Progression of these focal lesions is characterized by the accumulation of additional genetic damage, resulting in the malignant phenotype. This is considered to be an irreversible process (14).

However, not all initiated or genetically altered cells develop into cancer as a large percentage of initiated cells are removed. Amongst the systems which detect genetic mutations affecting cell growth and proliferation, is the tumour suppressor gene p53 which initiates the removal of altered cells by apoptosis (24).

Tumours are diverse and heterogeneous, but all share the ability to proliferate beyond the constraints limiting growth in normal tissue. Aberrations in the regulation of key pathways that control cell proliferation and survival are compulsory for the establishment of all tumours (25). Deregulated cell proliferation, together with suppressed apoptosis, represents the minimal common platform upon which all neoplastic transformation occurs. An important issue is to identify how tumour cells differ from normal cells and how those differences can be exploited in developing chemopreventive strategies. Key aspects in the transformation of normal cells to cancer have been identified and are currently being investigated worldwide. These include the regulation of the cell cycle, cell proliferation, apoptosis and signal transduction pathways.

2.2.1. The Cell Cycle

The cell cycle is a process by which cells reproduce, underlying growth and development of all living organisms. The cell cycle consists of four phases: G1, S, G2, and M (Figure 2). During the G1 phase, the cell prepares for DNA synthesis which takes place in the S-phase. During G2, the cell prepares for mitosis during which the cell divides into two daughter cells. The most important events of the cell cycle involves the copying and partitioning of hereditary material, i.e. replicating the chromosomal DNA during the S-phase and separating the replicated chromosomes during mitosis (26). The precision with which the cell cycle is executed ensures the survival of living organisms, while loss of this precision increases genomic instability, an important factor in the formation of cancer. At certain points in the cycle, checkpoints are in place determining whether an earlier event, such as the S-phase, has been properly executed before proceeding to a later event, such as mitosis. These checkpoints also cover other situations such as blocking mitosis after DNA damage until the damage is repaired (26). The main regulatory points and safeguards of the cell cycle are the checkpoints © and the restriction point ® (Figure 2). In the presence of mitogens and when other requirements, such as the presence of adequate nutrients, are met, the cell will pass the restriction point and commit itself to another round of DNA synthesis. The checkpoints, which assure that the integrity of the genome is maintained, include the G1 and G2 checkpoints which arrest the cell cycle if DNA damage is detected, the S-phase checkpoint which arrests the cell cycle if an error with DNA replication occurs, and the M-phase checkpoint which arrests the cell cycle if an error with mitotic spindle assembly occurs. When the cell cycle arrests at a checkpoint, the cell either corrects the defect detected (e.g., by repairing damaged DNA) or it undergoes apoptosis (27).
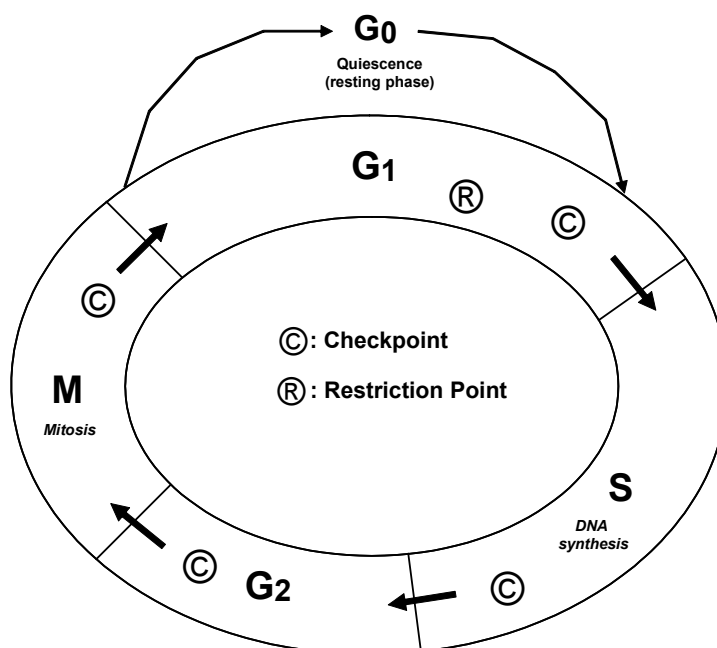
Figure 2: Diagram of the phases of the cell cycle depicting the restriction and checkpoints for cell repair or deletion from the cycle. The cell cycle consists of four phases: G1, S, G2, and M. During G1, the cell is prepared to enter DNA synthesis. The S phase is characterized by the cell undergoing DNA synthesis. In the G2 phase, the cell prepares for mitosis. Mitosis occurs in the M phase in which the cell divides into two daughter cells. The major regulatory points and safeguards of the cell cycle include the restriction point ® and the checkpoints ©. Under normal conditions the cell cycle is under strict control. In the presence of mitogens, the cell will pass the restriction point and is committed to another round of DNA synthesis. The checkpoints, which assure that the integrity of the genome is maintained, include the G1 and G2 checkpoints that arrest the cell cycle if DNA damage is detected, the S phase checkpoint that arrests the cell cycle if a problem with DNA replication occurs and the M phase checkpoint that arrests the cell cycle if a problem with mitotic spindle assembly occurs. When stopped at a checkpoint, the cell either corrects the defect detected (by repairing damaged DNA) or it undergoes apoptosis (adapted from 27).

Therefore, the cell cycle plays a central role in the operation and development of cellular homeostasis. Cancer can also be referred to as a disease of the cell cycle, involving deregulation of the cell cycle (23).

In malignant tissue, tumour growth is basically regulated by 2 mechanisms; (i) the rate of tumour cell proliferation and (ii) the rate at which tumour cells die (25, 28). The rate of tumour cell death is largely determined by apoptosis. Cellular signaling pathways regulate all the critical phases of cell growth including cell proliferation and apoptosis. In malignant cells the apoptotic process is impaired resulting in an imbalance between cell proliferation and apoptosis, with a net effect favouring proliferation leading to the characteristic rapid growth of cancer cells (29). Both proliferation and apoptotic cell death are complex processes that involve the participation of many genes (30).

2.2.2. Cellular Proliferation

Altered control of cellular proliferation is one of the main characteristics of a neoplastic cell population. Normal somatic cells have a finite replicative lifespan that restricts cell division by a process known as replicative or cellular senescence (30). Normal cells proliferate for a finite number of cell divisions after which they withdraw irreversibly from the cell cycle, entering a senescent state. Senescent cells remain viable indefinitely but do not initiate DNA replication in response to mitogens. The molecular mechanisms that underlie loss of proliferative capacity as cells withdraw from the cell cycle are largely unknown. The ability to arrest growth in quiescence, terminal differentiation and cellular senescence reflects fundamental growth regulatory mechanisms present in untransformed cells but which are defective in tumour cells (30). The discovery that phosphorylation of selected proteins by cyclin-dependent kinases is the main driving force behind the cell cycle, provided important information relating to the control of proliferation and its deregulation. Therefore, the control of proliferation relies on initiating or stopping the mechanisms which drive cells through the cycle (30). Crucial components of these pathways are proteins encoded by checkpoint genes which evaluate the balance of mitogenic and antimitogenic pathways preventing temporarily cycle initiation or its progression by inhibiting the corresponding cyclin-dependent kinases. Uncontrolled cell proliferation associated with cancer always depends on the dysfunction of at least one of these checkpoint pathways. The checkpoint or tumour suppressor protein p53 is one of these proteins and mutations in the gene encoding p53 are present in more than half of all human tumours (30). Restoration of checkpoint pathways either prevents further proliferation of cells with damaged genome until repair is completed or, alternatively, the dismantling of these checkpoints direct those cells to apoptosis.

2.2.3. Apoptosis

Apoptosis or programmed cell death plays an important part in the maintenance of tissues and organ systems by providing a control over cell deletion to counterbalance cell proliferation (31). Apoptosis is a physiological process whereby defective or potentially neoplastic cells are removed and is distinguished from necrosis by the absence of an inflammatory response (29, 32). Various studies have shown that a dysfunctional apoptotic process is a contributing factor and that regulation thereof is important during carcinogenesis (33, 34). The deregulation of apoptosis has been linked to all facets of cancer development such as hyperplasia, neoplastic

transformation, tumour expansion, neovascularization and metastasis (35). Research indicates that certain dietary factors can either inhibit or activate the apoptotic process (24). Therefore, the diet can play an important role in affecting processes involving deregulated cell growth and disease.

Apoptosis has been shown to play a major role in chemically-induced hepatocarcinogenesis (22, 28). In the classical Farber and Solt chemically-induced rat liver cancer model (22), the increased proliferation of preneoplastic cells is initially compensated for by a higher apoptotic index, approximately 70-90% of the initiated preneoplastic cells are eliminated in this way (36). Therefore, alteration or inhibition of the apoptotic pathway can influence the survival and growth of preneoplastic cells and ultimately cancer development. Manipulation of the apoptotic pathway has therefore been a growing research field with regards to controlling tumour growth and development (37, 38).

Apoptosis is mediated by the activation of the caspases cascade (Figure 3) which can be activated by two different paths, i.e. the extrinsic path by death receptor signaling at the plasma membrane involving tumour necrosis factor (TNF) or via the mitochondria (intrinsic pathway) with the release of cytochrome *c* (39). However, the two pathways can be cross-linked at different levels creating a feedback loop (27).

Cell Membrane (extrinsic pathway)	Mitochondria (intrinsic pathway)
↓	↓
Death receptor	**Cytochrome *c* release**
↓	↓
Activation of Caspase-8	**Activation of Caspase-9**

Activation of caspases cascade (caspase 3/7)

↓
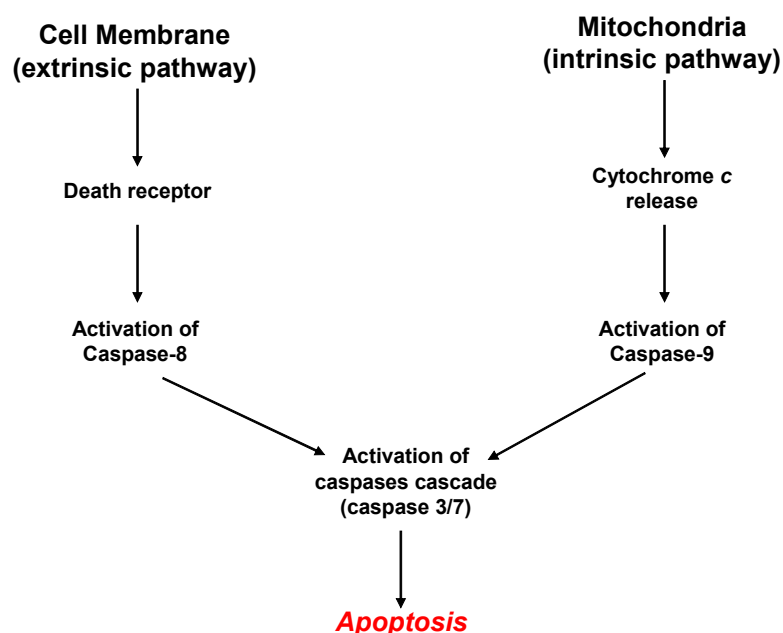
Apoptosis

Figure 3: Apoptosis may be initiated by the release of cytochrome *c* from mitochondria (intrinsic pathway) or by the activation of death receptors in the cell membrane (extrinsic pathway). These pro-apoptotic events result in the activation of the initiating proteases, caspase 8 and caspase 9, which activate other caspases (effector caspases 3 or 7) which then carry out the process of cell elimination (adapted from 27).

Apoptosis is regulated by two component classes, i.e. sensors and effectors (25). The sensors are responsible for monitoring the extracellular and intracellular environment for conditions determining whether a cell should live or die. These signals regulate the second class of components, which function as effectors of apoptotic death. The sentinels include cell surface receptors that bind survival or death factors. Intracellular sensors monitor the cell's well-being and activate the death pathway in response to detecting abnormalities, including DNA damage, signaling imbalance provoked by oncogene action, survival factor insufficiency or hypoxia. Many of the signals which elicit apoptosis converge on the mitochondria, which respond to pro-apoptotic signals by releasing cytochrome *c*, a catalyst of apoptosis. The ultimate effectors of apoptosis include an array of intracellular proteases termed caspases. Of these, two initiating caspases, -8 and -9, are activated by death receptors or by cytochrome *c* released from mitochondria (25). In some cancers, the apoptotic pathway is compromised by decreased expression of the pro-apoptotic proteins Bax and Bak, which induce mitochondria to activate the caspase pathway (40). The overexpression of certain anti-apoptotic proteins, such as Bcl-2, Akt and nuclear factor-kappa B (NF-κB), has also been observed in many types of human tumours (41).

The resistance to apoptosis, typical in many cancer cells, can be acquired by cancer cells through a variety of strategies. The most commonly occurring loss of a pro-

apoptotic regulator through mutation involves the p53 tumour suppressor gene (25). The resulting functional inactivation of its product, the p53 protein, is seen in more than 50% of human cancers and results in the removal of a key component in the detection of DNA damage which can induce the apoptotic effector cascade. The p53 tumour suppressor protein causes either cell cycle arrest to allow DNA repair or apoptosis if the damage is excessive (25). Moreover, a growing number of other genes involved in sensing and repairing DNA damage or in assuring correct chromosomal segregation during mitosis are found to be lost in different cancers, labeling these caretakers as tumour suppressors (42). Their loss of function is envisioned to allow genome instability and variability and the generation of consequently mutant cells with a selective growth advantage.

In cancer development the balance between the rate of cell proliferation and apoptosis is important (43). The deregulation of cell growth and suppression of apoptosis are defining characteristics of neoplastic or cancer tissue and can be affected by changes in signal transduction pathways which regulate cell growth (12, 43).

2.2.4. Signal Transduction

Signal transduction plays a central role in cell proliferation, differentiation and apoptosis (25). At the level of the whole body, signaling controls growth and development, as well as aspects of metabolism and behaviour. Signaling mechanisms which result in cell proliferation are closely associated with tumour growth and progression.

Many diseases involve malfunctions in signaling pathways. In particular, research on the basic functioning on the regulation of cell proliferation has been carried out to obtain a better understanding of cancer. Once a cell suffers an inactivating mutation in a growth inhibitory pathway, such as in a tumour-suppressor gene, or an activating mutation in a growth promoting pathway, i.e. in an oncogene, it displays a competitive proliferative advantage over its neighbouring/surrounding cells (25). This advantage can eventually lead to the manifestation of malignant growth and ultimately, cancer. To avoid cancer, living organisms have developed several signaling pathways which prevent uncontrolled cell proliferation. The study of oncogenes led to the identification of several signaling proteins which are involved in cell growth, including growth factors, their receptors, intracellular mediators and transcription factors. The study of tumour-suppressor genes led to the identification of proteins which negatively regulate

cell-cycle progression, cell survival and the ability to invade surrounding tissue. Various other non-infectious diseases are caused by defects in signaling pathways. Diabetes, for example, results from defects in the insulin-signaling pathway used to control blood glucose levels (44, 45, 46).

Typically, signal transduction pathways encompass a receptor by which the signal is received, the cell membrane, cytosolic proteins such as kinases and phosphatases which convey the signal and transcription factors such as NF-κB, which down- or up-regulate the expression of specific genes (43, 47).
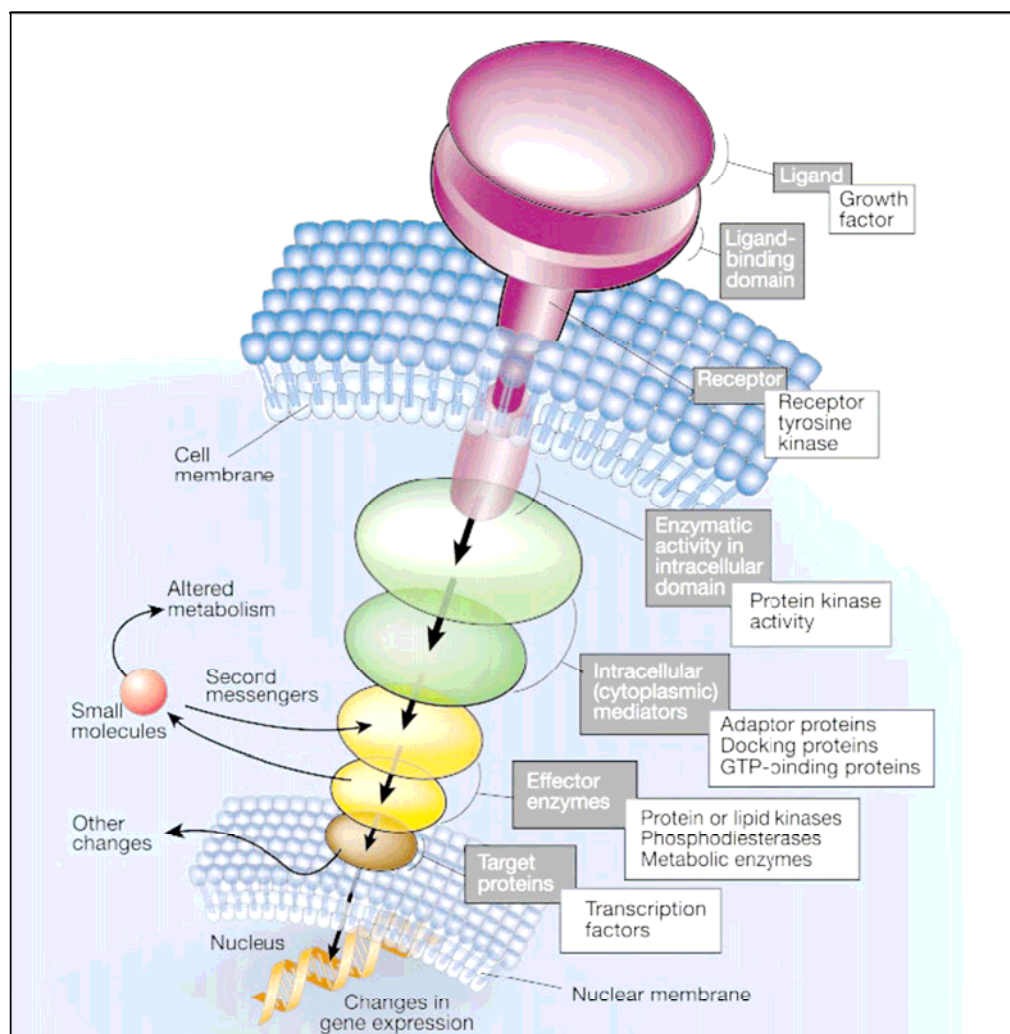
Figure 4: Typical elements involved in a generic signaling pathway. The grey boxes indicate typical elements constituting a generic signaling pathway and the white boxes show specific examples. Membrane receptors bind to active ligands such as growth factors resulting in enzymatic activity, such as kinases, associated with the intracellular part of the receptor. This can affect the association of the receptor with intracellular mediators or the localization or function of those mediators. The mediators alter the activity of 'effector' enzymes, some of which can move to the nucleus and control gene expression or can induce other proteins to affect gene expression. Other effector enzymes target small molecules, either generating further signaling mediators (second messengers) or controlling the metabolic state of the cell. Certain signaling pathways may bypass some of the steps described or may have several components working either in series or parallel (48).

A generic signaling pathway is depicted in Figure 4. The grey boxes indicate general components of signaling pathways, while the white boxes show specific examples. On the outer cell membrane, receptors bind to ligands such as growth factors resulting in the activation of certain intracellular enzymes. This can affect the association of the receptor with intracellular mediators or the localization or function of those mediators. These in turn alter the activity of effector enzymes such as the kinases. Some effectors can move to the nucleus and control gene expression or induce other proteins related to gene expression. Other effectors target small molecules generating further signaling mediators, such as second messengers, or control the metabolic state of the cell. Signaling pathways may bypass entire classes of these molecules or may have several components working either in series or in parallel (48).

Normal cells require mitogenic growth signals before they can move from a quiescent state into an active proliferative state (25). Normal cells cannot proliferate without these stimulatory signals. Many of the oncogenes in cancer cells function by mimicking normal growth signaling (25). The dependence on growth signaling can be demonstrated by normal cells in culture, which typically proliferate only when supplied with appropriate mitogenic factors. In contrast, tumour cells exhibit a reduced/lack of dependence on exogenous growth stimulation. It appears that tumour cells generate many of their own growth signals, reducing their dependence on stimulation from their normal tissue environment. This independence from exogenously derived signals disrupts critically important homeostatic mechanisms which normally ensure the correct functioning of the various cell types.

During carcinogenesis, it appears that certain classes of signaling proteins and pathways are targeted more with a resultant increase in alterations involving growth, differentiation and development. An example is mutations in the phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathway which can regulate cell survival, growth and proliferation (43). Cross talk between various signaling molecules has also been shown to affect cell survival (49). Figure 5 illustrates the interactive pathways between PI3-K, Akt and mitogen activated protein kinase (MAPK) in suppressing apoptosis and promoting cell survival.
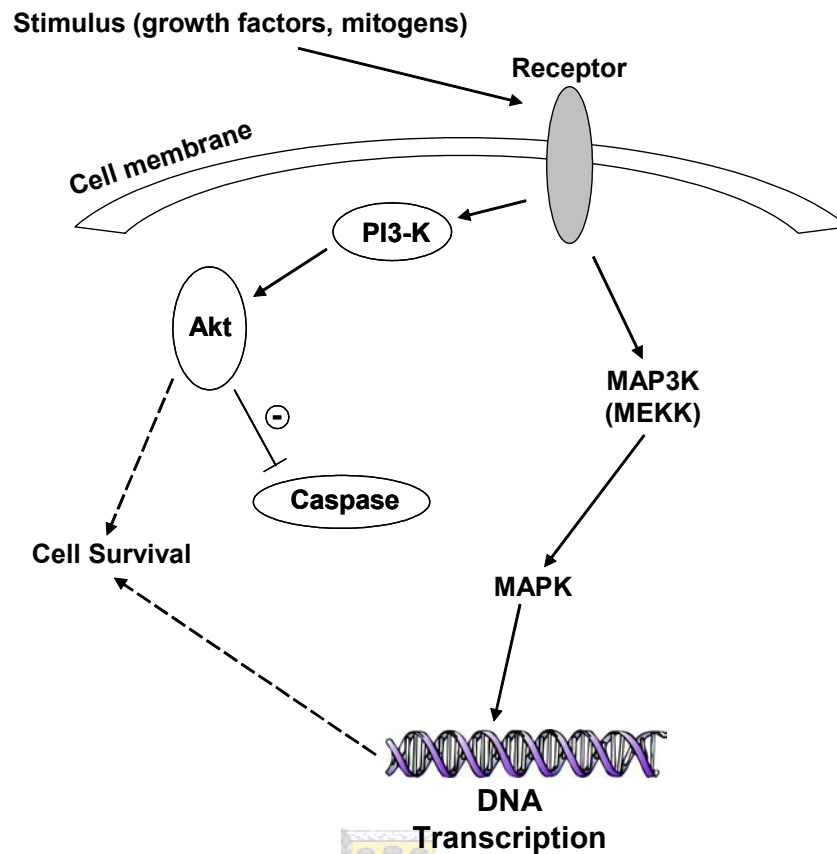
Figure 5: Several signaling pathways may be involved in stimulating cancer growth and this has been attributed to cross-talk between these pathways. Cross talk between the signaling molecules PI3-K, Akt and MAPK can occur in suppressing apoptosis and increasing cell survival. Θ, inhibition; PI3-K, phosphatidylinositol 3-kinase; MAP3K/MEKK, mitogen-activated protein kinase kinase kinase; MAPK, mitogen-activated protein kinase (adapted from 49).

The control in translocation of signaling proteins, such as Ras and Akt, can also alter signaling pathways by changing the interacting target (43).

2.3. Summary

It is clear from the previous sections that the development of cancer is a complex process. This complexity involves the correct functioning and interaction of various signal transduction pathways, as illustrated in Figure 6. These signal transduction pathways involve the regulation of cellular processes such as proliferation, gene expression and apoptosis.
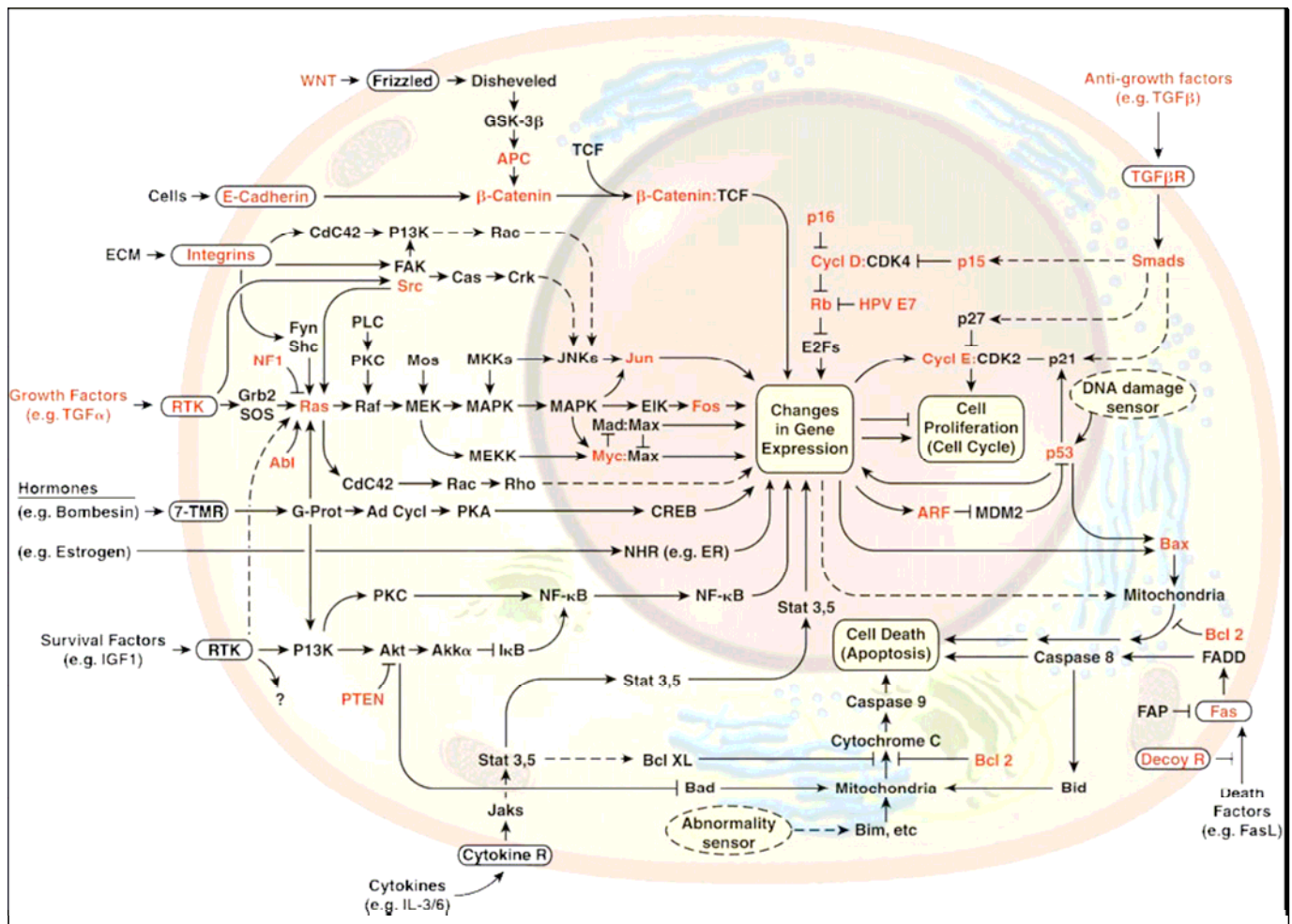
Figure 6: Signal transduction pathways involved in the regulation of cellular processes such as proliferation, gene expression and apoptosis. Progress in elucidating signaling pathways has established the involvement of certain genes in these signaling events. This diagram depicts the complexity of protein interactions with certain phosphates and lipids. Some of the genes known to be functionally altered in cancer are highlighted in red (25).

The deregulated growth of tumours, eventually resulting in cancer, also displays certain distinct characteristics (Figure 7) which provide these cell types with a growth advantage and survival as compared to normal cells (25). These characteristics result in cancer and involve alterations in processes such as a decreased apoptotic index, increased cell proliferation, loss of cell differentiation, alterations in normal cellular signaling pathways leading to angiogenesis, invasion and ultimately to the movement of the cancer cells away from the point of origin to other organs. This process is called metastasis.
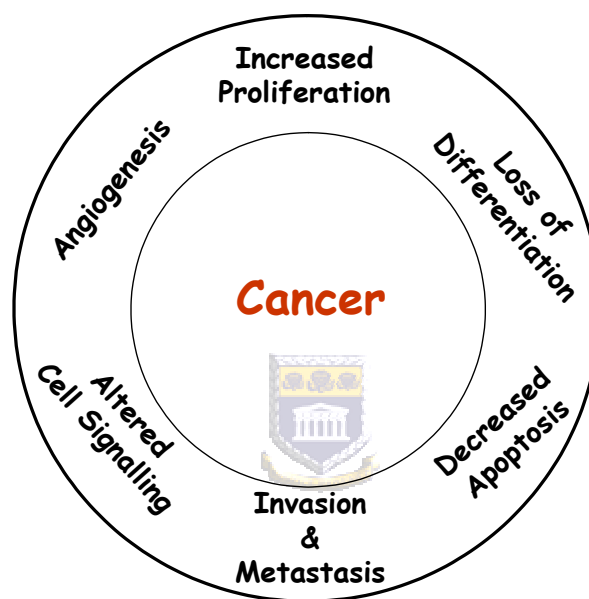
Figure 7: The acquired characteristics of cancer cells. Deregulated tumour growth displays distinct characteristics such as increased proliferation of cells, loss of cell differentiation, decreased apoptotic rate, formation of new blood vessels by angiogenesis, alterations in signaling pathways, invasion and the movement of cancerous cells to other body organs termed metastasis (adapted from 25).

3.   DIETARY FAT AND CANCER

Dietary fat, including triglycerides (TG), phospholipids as well as FA in the form of saturated FA (SATS), monounsaturated FA (MUFA) and PUFA, is an important macronutrient for the growth and development of all living organisms. Fat is a substrate for energy metabolism and membrane structural components, as well as signaling molecules like ceramide and the eicosanoids. Changes in the quantity of fat ingested or its composition impacts several physiological systems, many of which are cell and tissue-specific (50). Excessive levels of dietary fat or an imbalance of saturated fat versus unsaturated fat or ω6 to ω3 (ω6/ω3) PUFA ratio have been implicated in the onset and progression of several chronic diseases, such as coronary artery disease, atherosclerosis, diabetes, obesity and cancer. Therefore, considerable clinical and scientific research has been aimed at understanding the basis of FA effects on physiological systems impacting human health.

The association of dietary fat with cancer was amongst the most prominent early findings in nutritional causes of cancer. Evidence from rodent studies showed that by manipulating the dietary fat composition, the rate of tumour development as well as the number of tumours could be modified (51). Data from epidemiological studies also indicated that differences in international cancer rates were strongly associated with differences in fat intake (6, 52).

Evidence from cardiovascular studies, together with data associating dietary fat and cancer, prompted the National Academy of Sciences to recommend a reduction in dietary fat in the Principal Guidelines for the prevention of cancer in its 1982 report on Diet, Nutrition and Cancer (53). The evidence was strongest for an association of dietary fat with breast, large bowel and prostate cancers and was suggestive for other cancers (51). At that time, very few epidemiologic studies of dietary fat and cancer had been published and virtually no data from prospective cohort studies or clinical trials were available. However, since then numerous studies have examined the association of dietary fat and cancer, including a number of prospective studies (51). Further international reports and data from animal studies provided compelling evidence that lifestyle factors, such as dietary fat, play an important role in the etiology of these cancers (51).

The Western type lifestyle, of little exercise and fast foods with a high dietary saturated fat content, is associated with an increased risk for the development of certain diseases such as heart disease, diabetes, arthritis and cancer (3, 54). Epidemiological data supports an inverse relationship between cancer risk and intakes of vegetables, fruits, whole grains, dietary fibre, certain micronutrients and certain types of fat, i.e. ω3 FA, as well as physical activity (6). The discovery of the essential nature of dietary fat by George and Mildred Burr in 1929 (55, 56) generated interest into the nature of fats in our diet. Since then research has highlighted the significance and role of particular types of FA in health. A recent report on cancer published by the World Health Organisation emphasized the importance of nutrition in counteracting the expected increase in global cancer incidence (57). With regards to dietary fat, the degree of risk for diseases can vary according to various factors such as the type and level of FA intake, percentage of energy from total fat, dietary cholesterol, dietary fibre, physical activity level and general health status (58).

Another link in the diet-cancer relationship are studies which show that a traditional Mediterranean diet is associated with a low cancer incidence (59). This diet is considered to consist of a high MUFA/SATS ratio, moderate alcohol intake, high intake of legumes, cereals such as bread, fruit, vegetables, low consumption of meat and meat products and a moderate intake of milk and dairy products. Specifically, this dietary effect is attributed to the high intake of MUFA in the form of olive oil, natural antioxidants found in the fruit and vegetables and a low ω6/ω3 FA ratio, i.e. high ω3 content (60). Olive oil is rich in the MUFA oleic acid (OA, C18:1ω9) and also contains phenolic antioxidants which may have potential for inhibiting carcinogenesis (6). Studies conducted in Spain and Greece showed a reduced risk of breast cancer in women who consumed high levels of olive oil (61, 62).

The effect of certain dietary factors on cancer prompted Sporn *et al.* (63) to coin the term "chemoprevention" in the 1970's to describe the pharmacological ability of dietary components to prevent cancer by either arresting or reversing initiation or progression of premalignant cells and therefore delay or prevent the development of cancer. This definition contributed to the interest in the diet as a modifiable risk factor for cancer. As cancer chemoprevention research has progressed, interest has focused on the investigation of natural products, a number of which have been found to exhibit

chemoprotective properties in animal models when administered at levels that appear to lack systemic toxicity. Prominent among these compounds are nutrients, including vitamins, the ω3 FA, food additives such as curcumin and food-associated natural products, such as indole 3-carbinol and lycopene (64).

Overall, findings suggest that the link between fat and cancer risk depends on the type of fat consumed rather than, or in addition to, total fat intake (6). Some evidence suggests that consumption of olive oil may reduce breast cancer risk. Epidemiological and clinical data support a possible inverse relationship between consumption of fish and long-chain (LC) ω3 PUFA and risk for breast and colorectal cancers (60). A review on epidemiological and experimental studies suggested that LC ω3 PUFA may retard disease progression in prostate cancer (65).

However, many questions still need to be resolved with regards to which specific dietary factors are most closely linked to cancer prevention and the mechanisms by which food components exert their anti-cancer effects. A compounding effect is inter-individual variations in susceptibility arising from common polymorphisms in genes governing the metabolism of exogenous substances which can modify the carcinogenic or anti-carcinogenic effects of food components and, thus, add an extra level of difficulty to the interpretation of studies (6).

4. LIPID DIGESTION

Dietary fat in the form of TG are digested in the intestine where they are emulsified, and digested by removing the FA from the TG backbone (Figure 8).
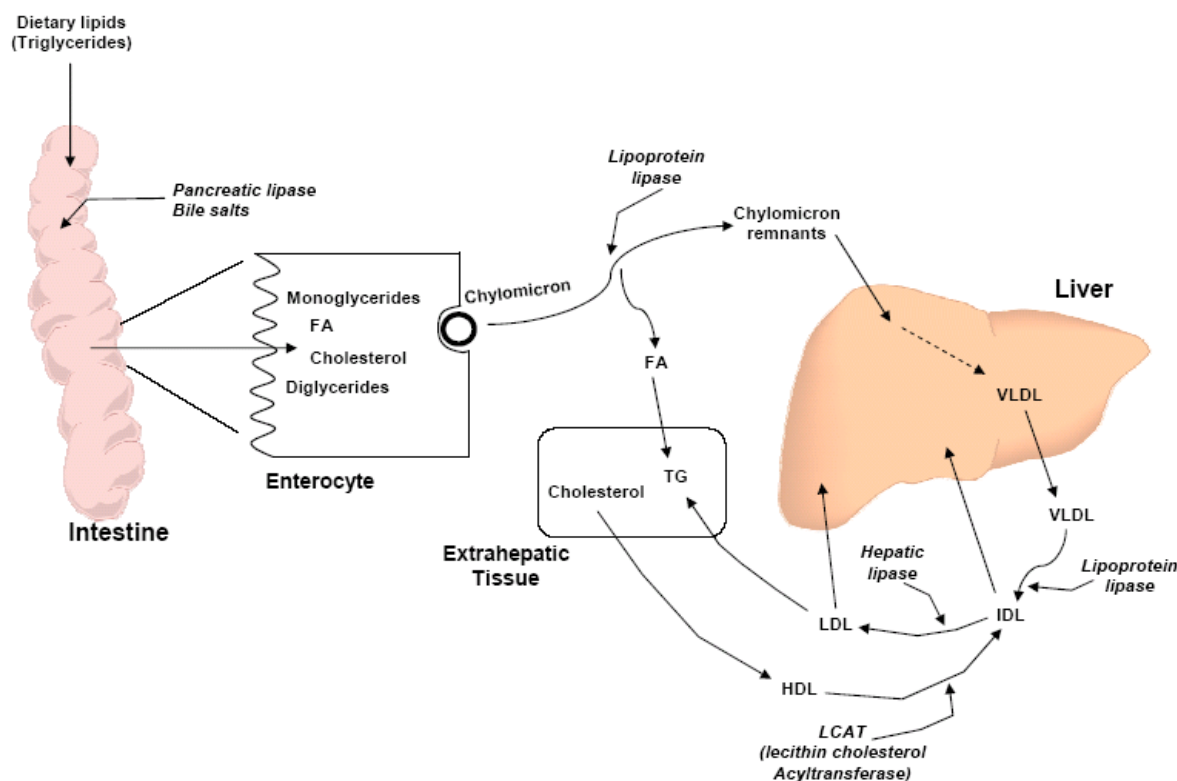
Figure 8: The digestion and transport of lipids in the body. Dietary fat is digested in the intestine and broken down to form complexes, called micelles, in the presence of bile salts and which are absorbed into the intestinal cell. In the enterocytes, the FA are re-esterified and together with cholesterol and apolipoproteins form chylomicrons. These are eventually discharged into the blood, broken down to chylomicron remnants which are transported to the liver. In the liver VLDL is formed for the transport of cholesterol and FA to other extrahepatic cells. FA, fatty acid(s); VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; HDL, high density lipoprotein; LDL, low density lipoprotein; LCAT, lecithin cholesterol acyltransferase; TG, triglyceride (adapted from 66).

The FA are cleaved by the enzyme lipase from the sn-1 and sn-3 positions of the TG backbone to generate free FA and a monoglyceride remnant with one FA in the sn-2 position (66). Monoglycerides can be further broken down enzymatically into free FA and glycerol. FA and monoglycerides form complexes with bile salts called micelles, which are absorbed passively into the intestinal cell (68). FA can also be actively transported by a carrier-mediated process across the intestinal membrane by FA binding proteins (69, 70). Inside the enterocyte, FA are re-esterified into TG and packaged with cholesterol and lipoproteins into large particles called chylomicrons (68,

71). These particles are eventually discharged into the blood circulation via the lymph system. The shorter chain free FA (10 to 12 carbon atom length) are transported directly to the liver via the portal vein (66).

Once in the blood stream, chylomicrons interact with the enzyme lipoprotein lipase found in endothelial cells lining the blood vessels (72, 73). This enzyme removes some of the FA, which are then taken up by extra-hepatic tissues and resynthesized into TG for storage, export, oxidation or further metabolism. The chylomicron remnants are taken up by the liver, while the remaining lipids and proteins are transferred to high density lipoproteins (HDL) in the blood (73).

The liver is a primary site for FA metabolism. FA are removed from the circulation, synthesized into TG, and packaged with proteins, cholesterol, and other lipids into very low density lipoproteins (VLDL) in the liver. The VLDL particles are discharged back into the circulation and are the primary vehicle for delivering TG to tissues, particularly adipose tissue and skeletal muscle. In the circulation, TG are removed from VLDL through the action of lipoprotein lipase, leaving intermediate-sized particles called intermediate density lipoproteins (IDL), as well as low density lipoproteins (LDL). LDL are rich in cholesterol which is made available for membrane structure and intracellular storage (74). HDL transports cholesterol from cells and via the action of the enzyme lecithin cholesterol acyltransferase (LCAT), transfers cholesterol esters back to LDL via IDL.

5. LIVER FUNCTION

The main types of functional cells in the liver are the hepatocytes which are structured into lobes and lobule functional units. From the liver, nutrients enter the venous bloodstream and are transported to their final destinations. Bile canaliculi, situated between the hepatocytes, transport the bile that is secreted by the hepatocytes to the common bile duct, via the terminal bile ducts and hepatic bile duct. From there the bile either flows directly into the duodenum, required for lipid absorption, or is stored in the gallbladder.

The liver has multiple functions ranging from the storage of energy, synthesis of important substances, such as proteins and lipids, regulation of digestion and elimination of waste products, drugs, and toxins (66). Amongst its various functions, the liver is essential in maintaining lipid homeostasis through its central role in lipid synthesis and catabolism. Many lipoproteins, phospholipids and cholesterol are also synthesized by the liver (66). Due to the rich blood supply from the abdominal organs to the liver via the portal vein, the liver is a common site of metastases from a variety of organs such as lung, breast, colon and rectum (75).

6.   FATTY ACIDS

FA are integral components of cellular membranes which function to maintain cellular integrity and regulate the activities of many membrane enzymes. In human physiology, FA play a role by i) functioning as a source of energy and structural components for cells, ii) regulation of gene expression which can affect lipid, carbohydrate and protein metabolism as well as cell growth and differentiation. The FA composition of cell membranes can be influenced by dietary fat (76). FA affect many structural, metabolic, and regulatory components of cells. Many of these effects can be linked to changes in membrane lipid composition affecting cell signaling mechanisms. The effects of FA on gene expression are cell-specific and determined by the FA type and metabolism.

6.1.   Nomenclature

FA consist of long chains of carbon atoms and usually contain even numbers of carbon atoms (Figure 9). They consist of three major classes, (i) the saturated fatty acids (SATS) containing no carbon-carbon double bonds, (ii) the monounsaturated fatty acids (MUFA) containing one carbon-carbon double bond, and (iii) polyunsaturated fatty acids (PUFA) containing two or more carbon-carbon double bonds. The unsaturated FA, i.e. the MUFA and PUFA, can be further defined as belonging to certain families such as the $\omega 9$, $\omega 7$, $\omega 6$ and $\omega 3$. Various naming schemes are in use when describing the FA families. These names, such as omega (ω) or n, are derived from the position of the first carbon-carbon double bond counting from the methyl end (-CH_3 group) of the FA carbon chain and also indicate the number of carbon atoms and double bonds (unsaturation level) in the FA (64). The carbon atoms number 2 and 3 from the

carboxyl group (COOH) are referred to as the α and β carbons, respectively, and the last carbon is the ω or n -carbon. For example C18:1ω9 has a single double bond located between the 9th and 10th carbon atoms (Figure 9) from the methyl end and is designated an ω9 (or n-9) MUFA.
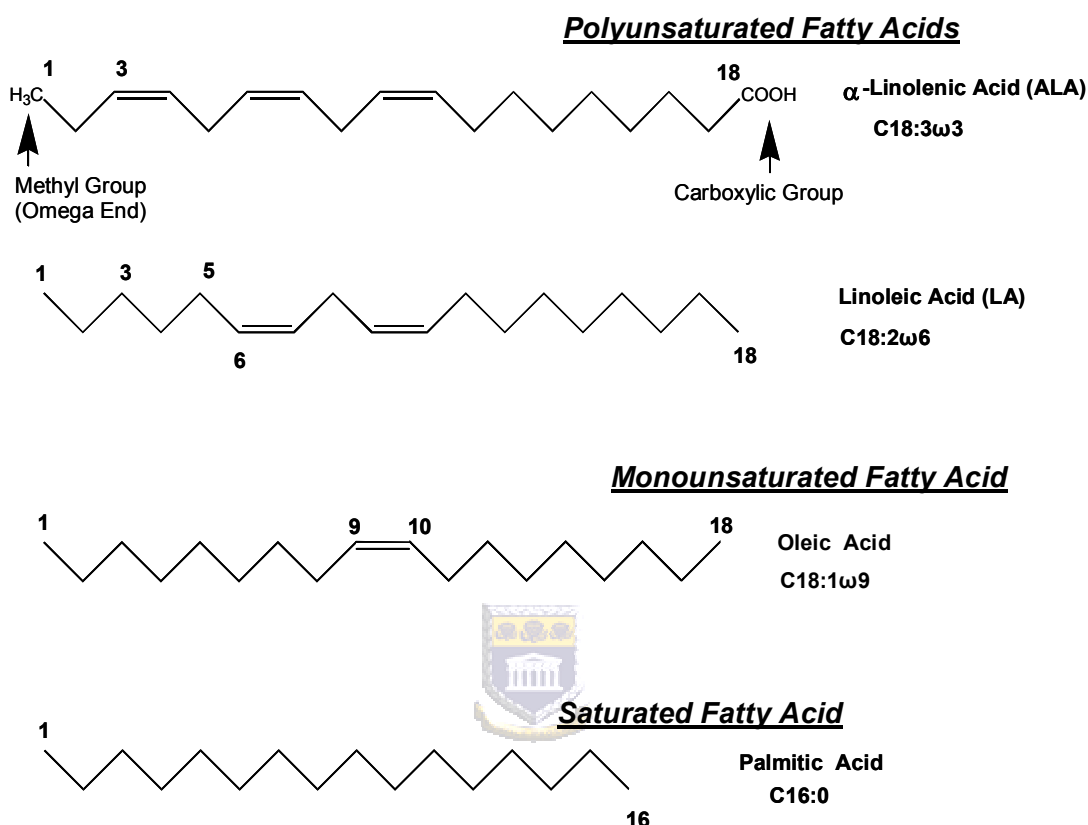
Figure 9: Examples of the three classes of FA, i.e. the polyunsaturated fatty acids, α-linolenic acid (C18:3ω3); linoleic acid (C18:2ω6); monounsaturated fatty acid, oleic acid (C18:1ω9); and saturated fatty acid, palmitic acid (C16:0).

Humans can synthesize most FA, such as the ω9 and ω7 families, except for linoleic acid (LA, C18:2ω6) and α-linolenic acid (ALA, C18:3ω3), precursors for the ω6 and ω3 families, respectively, which must be obtained through the diet (3, 64). These two FA are also known as EFA since mammals cannot introduce a double bond beyond the delta-9 position in the FA carbon chain (77). Once ingested, these EFA can then be further desaturated and elongated to varying degrees depending on delta-6 and delta-5 desaturase and elongase enzyme activity as well as tissue location (78, 79).

6.2. Sources

The majority of FA in the Westernized human diet are of the C18-chain length, such as stearic acid (C18:0), C18:1ω9 and C18:2ω6, as well as C16-chain FA, such as palmitic acid (C16:0) (68). However, regions with a high fish intake consume higher levels of the C20- and C22-chain FA, while population groups consuming a Mediterranean based diet have a high intake of C18:1ω9 present in olive oil (80). The ω6 FA are found mainly in maize, certain nuts and vegetable seed oils such as sunflower, sesame and evening primrose (81). Most green leafy vegetables and certain vegetable oils such as linseed (flaxseed), rapeseed (canola) and walnut oil contain ω3 FA such as C18:3ω3 (81). The FA content of certain seed oils and animal products are shown in Table 1. The ω3 LCPUFA, i.e. eicosapentaenoic acid (EPA, C20:5ω3) and docosahexaenoic acid (DHA, C22:6ω3), are formed in marine algae, plankton and planktonic crustacea at the bottom of the marine food chain. They are then passed up the food chain into the higher fish, i.e. herring, mackerel, sardines, and ultimately to humans. C20:5ω3 and C22:6ω3 are also referred to as the fish oil FA, because of their high content in these oils (82). Fish found in the cold northern oceans generally have higher levels of ω3 FA than fish in the warmer southern oceans, which contain higher levels of the ω6 LCPUFA, arachidonic acid (AA, C20:4ω6) (83). This is due to the higher C20:4ω6 content along the food chain (83). This fact is important to consider when using fish sources in advocating a higher dietary intake of ω3 FA.

Table 1: Fatty acid content (percentage of total fat) of certain common seed oils and animal products (84).

Food	Fatty acid content as a % of total fat for selected foods				
	Palmitic acid (C16:0)	Stearic acid (C18:0)	Oleic acid (C18:1ω9)	Linoleic acid (C18:2ω6)	α-Linolenic acid (C18:3ω3)
Perilla oil	6	2	17	15	61
Flax/Linseed oil	3	7	21	16	53
Menhaden oil[a]	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/Milk fat[b]	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 oil	6	4	24	65	0
Borage oil[c]	11	4	16	39	0
Evening primrose oil[d]	6	1	11	72	1
Safflower oil	7	3	15	75	0

[a]Menhaden oil contains 11% EPA and 9% DHA. [b]Butter consists of 30% saturated fat with chain lengths less than 16 carbons. [c]Borage oil contains 24% γ-linolenic acid (GLA). [d]Evening primrose oil contains 10% γ-linolenic acid (GLA).

Alternative sources of ω3 FA are being studied for commercial viability (85). Perilla, containing C18:3ω3, is already available in some countries and krill, abundant in the Antarctic Ocean, are being targeted as a future ω3 PUFA source. Genetic modification is also being explored, with the insertion of genes which will produce ω3 LCPUFA into existing oilseed varieties. ω3-producing algae are also being investigated as an alternative source.

6.3.   Metabolic Pathways

Mammals are capable of desaturating stearic acid (C18:0) to C18:1ω9 and palmitic acid (C16:0) to palmitoleic acid (C16:1ω7) by a delta-9 desaturase enzyme. However, mammals generally lack the delta-12 and delta-15 desaturases (present in plants), which are required for the production of C18:2ω6 and C18:3ω3 (78). After ingestion, the first members or "parents" of the ω6 and ω3 FA families, C18:2ω6 and C18:3ω3 respectively, are desaturated and elongated by the desaturase and elongase enzymes to produce important 20- and 22-carbon LCPUFA. The desaturation step adds a double bond and elongation adds two carbon atoms to the FA chain. The first desaturation step is catalysed by the delta-6 desaturase enzyme resulting in γ-linolenic acid (GLA, C18:3ω6) and stearidonic acid (C18:4ω3) for the ω6 and ω3 FA families, respectively (Figure 10). This is followed by elongation, then desaturation by the delta-5 desaturase enzyme, further elongation followed by desaturation with delta-6 desaturase again and finally one cycle of β-oxidation, resulting in the ω6 and ω3 LCPUFA, C22:5ω6 and C22:6ω3, respectively (78, 86). The desaturation step tends to be slow in contrast to elongation which occurs rapidly, therefore the C18:3ω6 and stearidonic acid levels are very low in tissue, being rapidly elongated to longer-chain FA (87).

Figure 10: The metabolic pathways of the ω9, ω6 and ω3 fatty acids involving increased chain-length by the elongase, unsaturation by the desaturase enzymes and β-oxidation of C24:5ω6 and C24:6ω3 to form C22:5ω6 and C22:6ω3, respectively. Also shown are the substrates for eicosanoid synthesis, i.e. C20:3ω6, C20:4ω6 and C20:5ω3 (78, 86).

6.4. Delta-6 Desaturase

Twenty years after the discovery of the EFA, the desaturation and elongation pathway of the ω6 and ω3 FA was identified by Holman, Klenk and associates (88, 89) by studying the conversion of C18:2ω6 and C18:3ω3 into longer-chain FA. Delta-6 desaturase is a microsomal enzyme (90) and catalyses the desaturation of C18:2ω6 to C18:3ω6 and C18:3ω3 to stearidonic acid. The human delta-6 desaturase contains various membrane-spanning domains, such as a NADPH cytochrome b_5 and three conserved histidine-rich domains (91). In mammals, the activity of this enzyme may vary, depending on specie and tissue type (90). It was commonly thought that the activity of delta-6 desaturase is low in most tissues except for the liver and central nervous system of the foetus and young animals (92). Consequently, the liver has been regarded as the primary site for the production of PUFA (93, 94). However, delta-6 desaturase activity has been observed in brain, testis, kidney and several types of primary cell cultures and cell lines (90). Molecular studies indicated that many

human tissues also express delta-6 desaturase mRNA, which are most abundant in the liver, brain, heart, and lung (91). The human brain may contain several times more desaturase mRNA than in other tissues, including the liver.

The ω9, ω7, ω6 and ω3 FA families share the same desaturation and elongation metabolic path. However, the FA families cannot be interconverted in mammals and the metabolism of one FA family can partially inhibit metabolism of the other (3). Although the desaturase enzyme has a higher affinity for the ω3 FA (95), high dietary ω6 FA levels can monopolize the activity of this enzyme and cause the levels of the ω3 FA to decrease, especially the LCPUFA, C20:5ω3 and C22:6ω3 (87, 96). The desaturase enzyme is therefore an important regulation or rate-limiting step in determining the type and quantity of LCPUFA present in the body. The activity of the enzyme can be affected by changes in the membrane content of cholesterol, phospholipids, as well as the level of FA unsaturation (97). Other factors which can affect the activity of the enzyme are *trans* FA, fasting, hormones such as insulin and glucocorticoids, and alcohol (93).

The synthesis of C22:6ω3 from C22:5ω3 and C22:5ω6 from C22:4ω6 was believed to occur by desaturation via a delta-4 desaturase enzyme, although in mammals the existence of this microsomal enzyme was never proven (98, 99). However, Sprecher and co-workers (86) demonstrated that the delta-6 desaturase enzyme is also involved in the synthesis of C22:5ω6 and C22:6ω3 by the retro-conversion of 24-carbon FA via β-oxidation in peroxisomes. A recent report by Cunnane (100) recalled that rats can synthesize C18:2ω6 and C18:3ω3 from the 14-carbon precursors, C14:2ω6 and C14:3ω3 (101, 102).

6.5.   *Structural and Functional Properties of Fatty Acids*

As components of cellular phospholipids, FA are the main structural building blocks of all cell membranes (76, 93). Cell membranes control the transport of substances in or out of the cell as well as communication (signaling) across the membrane by affecting receptors and enzymes. FA bound to cholesterol esters in HDL in the plasma play a role in the removal of cholesterol from cells to the liver and finally for excretion. Cholesterol bound to C18:2ω6 and C18:3ω3 are more easily removed from tissues for transport to the liver than cholesterol oleate, a mechanism whereby EFA may decrease cholesterol (103). Certain FA are also substrates for the synthesis of

eicosanoids which are involved in the regulation of cell growth, cell activity and cell function (104). Functionally, certain FA also affect gene expression by binding to and regulating the activity of nuclear receptors controlling major regulatory networks that impact cell metabolism and signaling mechanisms (50). Several transcription factors have been reported to be regulated, either directly or indirectly, by FA. FA effects on gene expression are not, however, directed exclusively at the control of transcription factor activity or abundance. Effects on mRNA turnover and protein abundance have also been observed leading to changes in cellular metabolism, growth, and differentiation (50, 105, 106).

6.5.1. Structural Properties

6.5.1.1.   The Cell Membrane

The basic membrane structure is composed of a bilayer in which amphiphilic lipids are arranged with the hydrophobic moieties oriented towards the center of the membrane and the hydrophilic groups at the two surfaces (Figure 11).
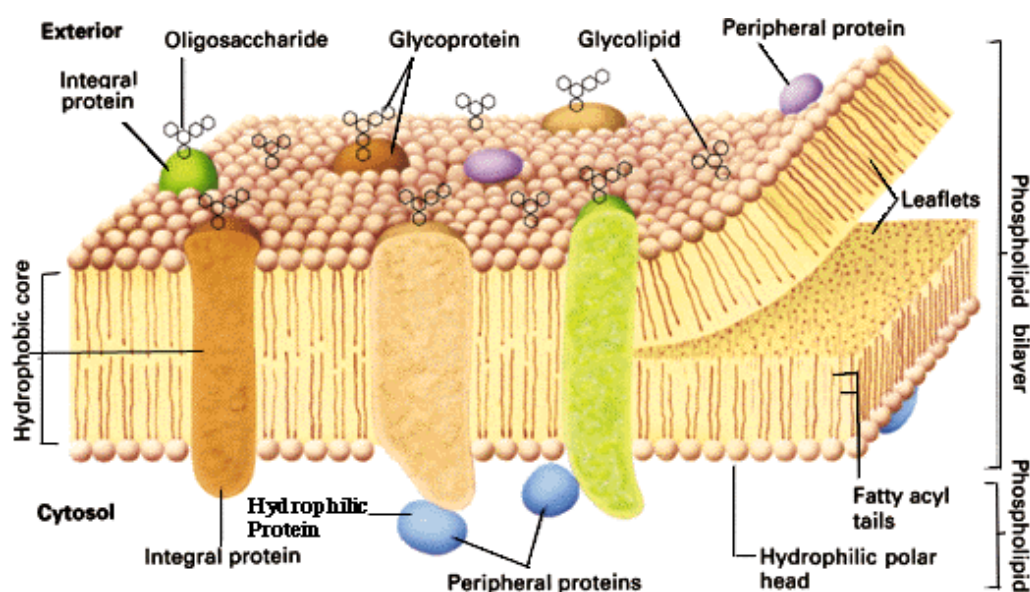
Figure 11: A modified version of the classical fluid-mosaic cell membrane model showing the phospholipid bilayer, fatty acids and other membrane related molecules such as membrane-bound proteins (2, 107).

Cell membranes are mainly composed of phosphoglycerides, cholesterol and proteins; the ratio of which may vary towards each other in different cell types. The membrane is stabilized by interactions between the lipids and proteins.

The proteins in the membrane may be on either surface or may extend completely through with intracytoplasmic, intramembrane, and extramembrane portions (77). Generally, the proteins function as receptors, ion channels or as surface enzymes. When ligands bind to their membrane receptors, the ligand-receptor complex is often internalized after which the receptor may be recycled to the membrane. Membrane bound proteins are sensitive to their FA environment and can therefore be affected by the lipid content. The properties of the cell membrane depend on the actual FA composition which may be influenced by several factors, including genetic make-up, but also by dietary intake. Amongst the FA, the SATS, MUFA and PUFA as well as interaction between these classes play important roles in maintaining the integrity and functioning of the cell membrane.
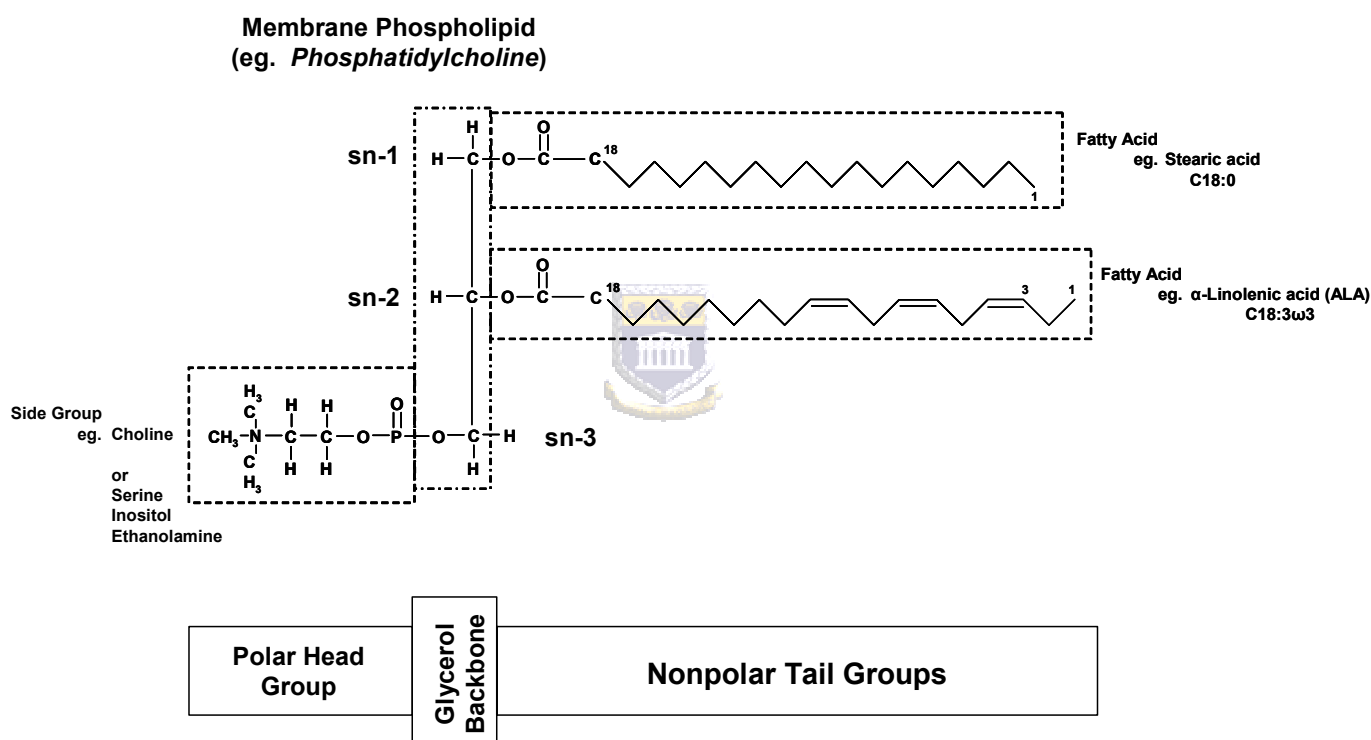
Figure 12: Phospholipid structure. Illustrated is phosphophatidylcholine (PC) containing the fatty acids C18:0 (stearic acid) at the sn-1 position of the glycerol backbone, C18:3ω3 (α-linolenic acid) at the sn-2 position and a choline group at the sn-3 position of the polar head group.

6.5.1.2.   Membrane Phospholipids

Phospholipids are classified according to the attached group present at the sn-3 (carbon-3) position of the glycerol backbone (Figure 12) such as choline, inositol, ethanolamine and serine (87). Also present in the membrane are sphingomyelin and cardiolipin. Phospholipids are not symmetrically distributed between the outer and inner membrane leaflets. Studies involving the red blood cell membrane indicate that most of the phosphatidylethanolamine (PE) and phosphatidylserine (PS) are situated within the inner or cytoplasmic membrane leaflet (Figure 13), while most of the phosphatidylcholine (PC) and sphingomyelin (SM) are found in the outer leaflet (108, 109). Most phospholipids contain a PUFA at the sn-2 position of the glyceride backbone and a SATS or MUFA at the sn-1 position. Changes in the FA content, especially with regards to PUFA and SATS, can affect the fluidity of cell membranes, ultimately affecting membrane function (110).
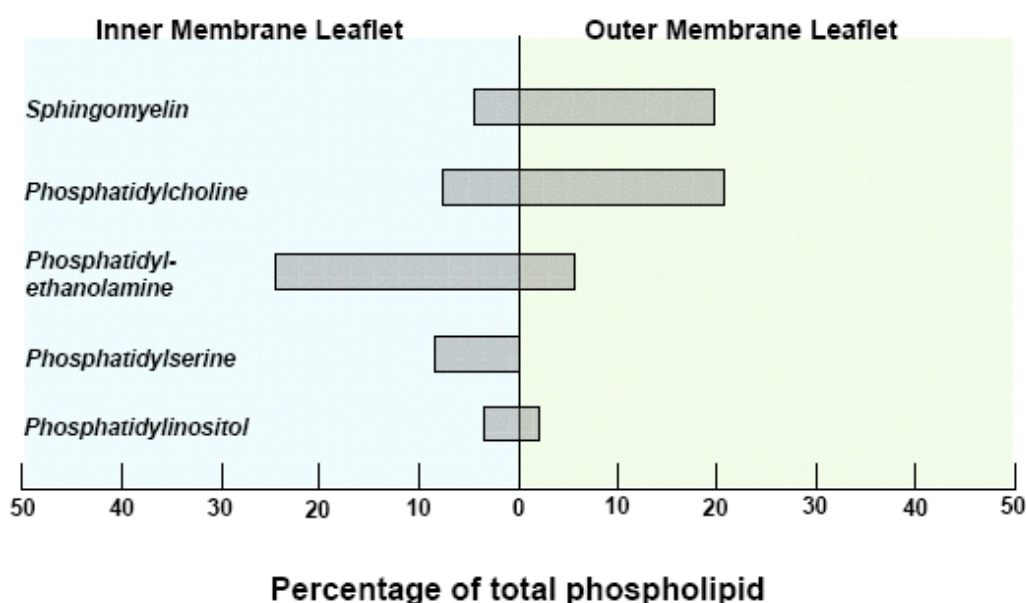
Figure 13: Lipid asymmetry in the red blood cell membrane bilayer depicting the distribution of the different phospholipid types in the inner and outer cell membrane leaflets (111).

The characteristic asymmetric lipid distribution of cell membranes is important in the physiological functioning of the membrane. Events such as translocation of PS from the inner to the outer membrane leaflet of red blood cells or vascular endothelium promotes blood coagulation and translocation of PS and PE to the outer membrane leaflet is a signal for apoptosis and removal of an injured or damaged cell (112, 113). Therefore, disruption of the membrane phospholipid distribution or the PC/PE ratio can change the responsiveness of the cell to normal physiological control mechanisms.

6.5.1.3. Membrane Cholesterol

Another important membrane component is cholesterol which is usually situated into the curve of the phospholipid-bound PUFA with the cholesterol 3-hydroxyl group next to the phosphate group of the phospholipid (103). This characteristic position of cholesterol has a structural organizational effect on the FA acyl chains (Figure 14) and can play a role in altering membrane fluidity, thereby affecting membrane function (114). The major site of cholesterol synthesis is the liver and cholesterol is the precursor for various molecules such as vitamins, steroid hormones, lipoproteins and bile acids (115). In proliferating cells, there is a higher rate of cholesterol synthesis implying that increased cholesterol levels are involved in cell growth (115, 116).
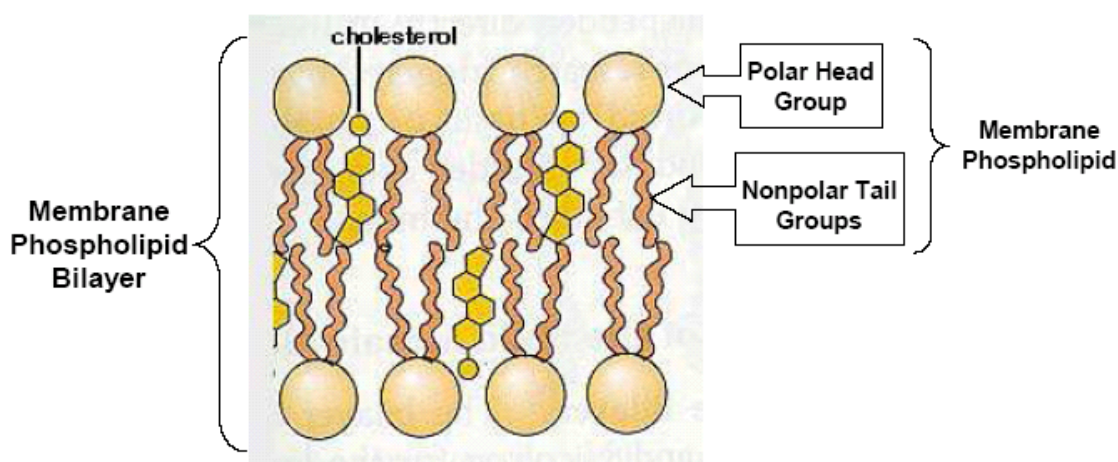
Figure 14: Position of cholesterol in the plasma membrane. Cholesterol is situated into the curve of the membrane phospholipid-bound PUFA and structurally organises the FA acyl chains. Cholesterol can also affect membrane fluidity, thereby influencing membrane function. FA, fatty acid; PUFA, polyunsaturated fatty acid (114).

6.5.1.4. Membrane Fluidity

The various membrane components, such as cholesterol, PUFA and phospholipid content are also important in determining the fluidity state of the membrane. Biological membranes usually exist in a fluid liquid crystalline state, the maintenance of which is important for the normal functioning of the membrane (117). A consequence of the asymmetrical arrangement of the phospholipids in the membrane as well as cholesterol and FA unsaturation level, is that any disruption in these components can affect membrane function. Indicators of membrane fluidity with regards to membrane lipid parameters include the cholesterol/phospholipid molar ratio, the PC/PE phospholipid ratio, the polyunsaturated/saturate (P/S) ratio and the type and level of LCPUFA present in the membrane (117).

6.5.1.5.   Membrane Lipid Rafts

Adding complexity to the membrane structure is the cellular distribution of some lipids compartmentalized into highly organized structures which are unevenly distributed into distinct microdomains called *lipid rafts* (118).   These regions appear to play an important role with regards to membrane structure and function.   The content and interaction between the different membrane lipid components is important for the existence and function of these lipid rafts which are enriched with cholesterol, SM, PS, and plasmenylethanolamine (119, 120).   Alterations in the lipid content of these lipid rafts could be important in controlling certain cell signaling events (121, 122, 123).

6.5.2. Functional Properties

6.5.2.1.   Fatty Acids and Eicosanoids

Certain FA serve as substrates for the synthesis of hormone-like signaling molecules collectively called eicosanoids consisting of prostacyclins, prostaglandins, thromboxanes and leukotrienes.   These metabolites regulate cell growth, activity and cell functions such as inflammation, immunoregulation, communication between and within cells, blood flow and pregnancy, amongst others (104).   The eicosanoids are synthesized by all cell types in the body and are involved in most physiological events (124).   The stimuli for the release of FA precursors for eicosanoid synthesis can either be physiological or pathological of origin (50, 125, 126).   Physiological stimuli include hormones such as angiotensin II, bradikinin and epinephrine, and proteases such as thrombin.   The pathological stimuli include mechanical damage, ischemia, Ca^{2+} ionophores and tumour promoters.

6.5.2.2.   Sources for Eicosanoid Synthesis

The 20-carbon LCPUFA dihomo-γ-linolenic acid (DGLA, 20:3ω6), C20:4ω6 and C20:5ω3 are important precursors for these metabolites and compete for the same enzyme systems in the synthesis of eicosanoids (127).   Prior to eicosanoid synthesis, the FA precursors are released from the phospholipids in cell membranes by the action of phospholipase enzymes, such as phospholipase A_2 (PLA_2) (104).   As with the desaturase enzyme, high levels of the one FA family can inhibit the synthesis of eicosanoids from the other family.   There are four major groups of enzymes responsible in the biosynthesis of eicosanoids (Figure 15), (i) phospholipases for the release of C20:4ω6, C20:3ω6 and C20:5ω3 from membrane phospholipids, (ii) the cyclooxygenases (COX) for synthesis of the prostanoids i.e. prostaglandins,

prostacyclins and thromboxanes, (iii) the lipoxygenases (LOX) for production of hydroxylated eicosatetraenoic acids (HETEs) and leukotrienes, and (iv) the cytochrome P450-controlled (CYP) monooxygenases (MOX) for the epoxydation and hydroxylation of the FA precursors (128, 129, 130). The COX are key enzymes in the regulation of prostanoids of which there are two isoforms. The COX-1 isoform is constitutively expressed by various cell types and plays an important role in cell homeostasis, while COX-2 is induced by various cytokines, growth factors and mitogens (130, 131, 132).
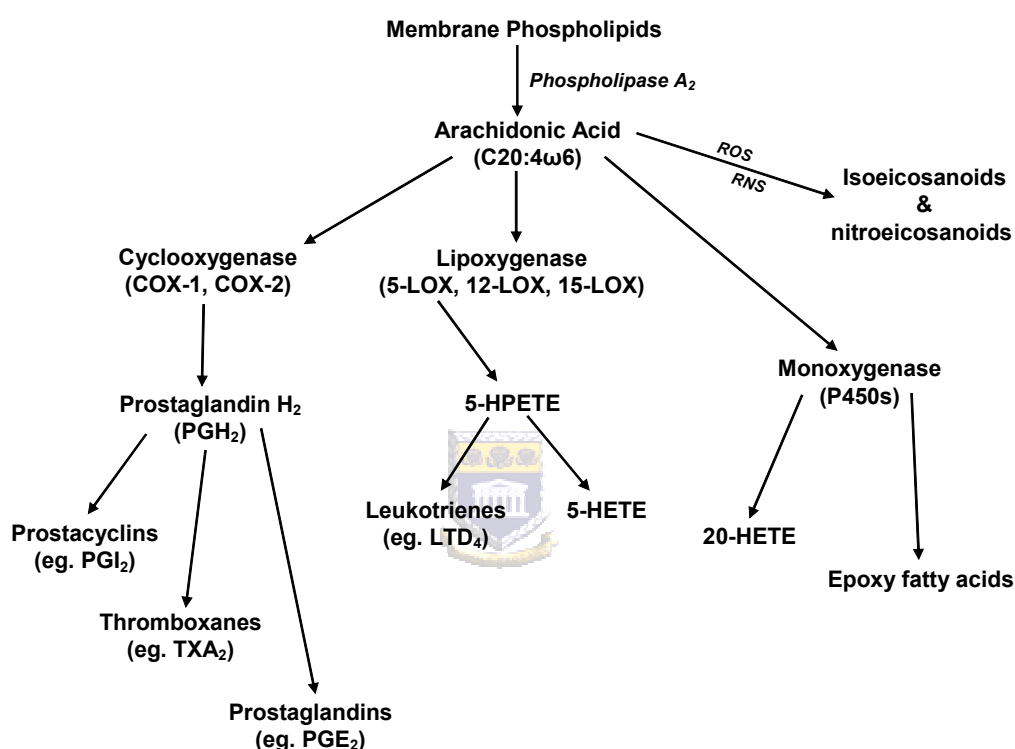
Figure 15: Metabolic pathways depicting generation of eicosanoid metabolites from arachidonic acid. Arachidonic acid (C20:4ω6) is released from the sn-2 position of inner leaflet phospholipids by phospholipase A_2 and serves as a substrate for cyclooxygenases (COX-1 and COX-2), lipoxygenase (5-LOX, 12-LOX, 15-LOX) or the P450 monooxygenases. Arachidonic acid can also converted by non-enzymatic, free radical-mediated oxidation involving reactive oxygen (ROS) and reactive nitrogen species (RNS) resulting in the formation of iso-eicosanoids and nitro-eicosanoids (50, 118).

With regards to the prostaglandins (PG), there are 3 families or series, depending on their FA precursor. Series 1 (i.e. PGE_1) are derived from C20:3ω6, series 2 (i.e. PGE_2) are derived from C20:4ω6 and series 3 (i.e. PGE_3) are from C20:5ω3 (87, 95). In humans consuming a typical Western diet with a high ω6 FA content, C20:4ω6 is the main source for eicosanoid synthesis (3, 129, 133). In general, eicosanoids derived from C20:4ω6 are potent mediators of inflammation, whereas those from C20:3ω6 and C20:5ω3 are less potent (91). A high dietary intake of ω6 FA at the cost of ω3 FA

favours a predominant synthesis of the 2-series prostanoids and 4-series leukotrienes derived from C20:4ω6, creating a pro-inflammatory and pro-aggregatory environment. Increased intake of the ω3 FA stimulates the production of less inflammatory and aggregatory 3-series prostanoids and 5-series leukotrienes from C20:5ω3. Prostaglandin E_1, derived from C20:3ω6, also possesses some anti-inflammatory properties, therefore dietary studies utilising C18:3ω6 as the precursor for C20:3ω6 have also been investigated in modulating inflammatory responses (134).

The microsomal cytochrome P450-linked MOX are members of a large superfamily of enzymes that catalyzes the NADPH-dependent oxidation of a diverse array of lipophilic compounds including FA (105). MOX-mediated oxidation of C20:4ω6 yields a variety of eicosanoids, such as epoxides, midchain hydroxy FA, α-hydroxy FA and dihydroxy FA. Of these oxidized lipids, the epoxy derivatives of C20:4ω6 are reported to modulate calcium signaling, channel activity, transporter function, mitosis and affect hypertension. ω3 PUFA are also converted to both epoxy and hydroxy FA by the MOX. Currently, there is little information available on the bioactivity of C20:5ω3-derived MOX products on biological systems (105). Another separate and distinct pathway exists consisting of the non-enzymatic, free radical-mediated oxidation of eicosanoids involving ROS and RNS resulting in the formation of iso-eicosanoids and nitro-eicosanoids (118).

C20:5ω3 and C22:6ω3 have also been established as the precursors of a series of endogenous antiinflammatory mediators in human blood, leukocytes, murine brain and human glial cells, called resolvins and docosatrienes (135). These mediators are biosynthesized via enzymatic oxygenation by COX-2 in the presence of aspirin and are potent regulators of both leukocytes infiltration and glial cells by blocking cytokine production, such as TNF-α.

6.5.2.3.   Eicosanoid Function

Classically because of its abundance in cell membranes, the eicosanoids derived from C20:4ω6 have been studied the most. Over 400 lipids derived from C20:4ω6 oxidation have been structurally characterized using mass spectrometry (118). Overall, C20:4ω6 is oxidized to at least 30 structurally different primary eicosanoids with each having unique biological origin and properties. Typically, eicosanoids are synthesized in subnanomolar concentrations and function as signaling molecules through specific receptors. These metabolites play important roles in various functions of the body and as mediators of inflammation, asthma, fever, pain, hypertension, stroke and many other pathologies). Some of the most important antiinflammatory drugs (aspirin, naproxen, ibuprofen, acetaminophen, coxibs, lukasts) block either the formation or the effects of eicosanoids (118). Several eicosanoids or their analogs have also been found to have applications as therapeutic agents. Eicosanoids can regulate cell signaling by controlling the release of second messengers such as cAMP, diacylglycerol (DAG), inositol-1,4,5-triphosphate (IP3), phosphokinase C (PKC) and activators of the peroxisome proliferator-activated receptors (PPAR) transcription factors (129). Another important C20:4ω6 metabolite is HETE which is synthesized via the enzyme LOX (136, 137). Metabolites of LOX are reported to be important regulators in proliferation and apoptosis (138, 139). Therefore, the analysis of eicosanoids has been an important part of understanding their role in health and disease and in drug development.

6.5.3. Fatty Acid Signal Transduction

In response to extracellular signals, cells can up- or down-regulate the expression of certain genes, resulting in altered metabolism, proliferation, differentiation or apoptosis (Figure 16). Research in cell signaling pathways has demonstrated that FA can modulate many receptor-mediated signal-transduction pathways. Growing evidence suggests that FA, in addition to their roles as structural components of membrane lipids and as precursors of eicosanoids, can act as second messengers or regulators of signal-transducing molecules.
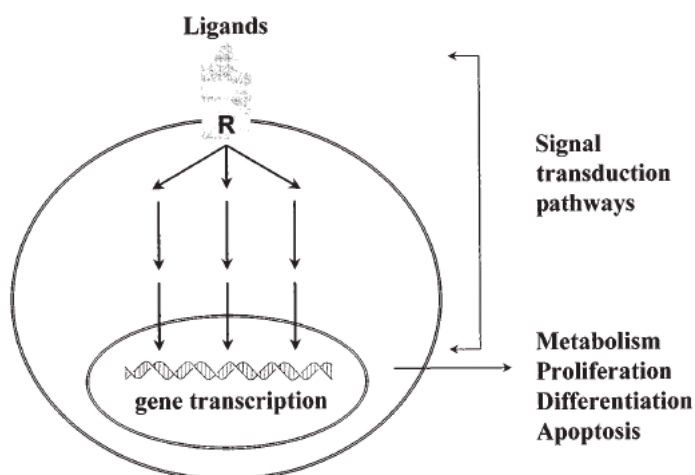
Figure 16: Ligand binding of a receptor (R) and the subsequent activation of downstream signaling pathways, resulting in the transcription of specific genes, which in turn lead to various cellular responses (140).

FA can affect signaling pathways directly by binding to specific transcription factors or indirectly by the effect of their metabolites, the eicosanoids (50).

6.5.3.1.   *Fatty Acid Regulation of Gene Expression*

PPAR-α was one of the first transcription factors identified as a FA receptor (105). Four PPAR subtypes have been described, namely α, β, γ1 and γ2 (50). PPAR-α plays a role in the regulation of genes involved in glucose and lipid metabolism including FA transport, FA-binding proteins, fatty acyl-CoA synthesis and microsomal, peroxisomal, and mitochondrial oxidation. PPAR-β is suggested to play a role in development, myelination, lipid metabolism, epidermal cell proliferation and inflammation, while PPAR-γ is involved in cell differentiation, glucose and lipid storage as well as inflammation (50). Several apolipoproteins, i.e. apoCII and -CIII, are also influenced by PPAR-α.   Activated PPAR-α induces lipoprotein lipase and FA transporters and enhances adipocyte differentiation as well as inhibiting NF-κB function and cytokine and COX-2 expression (63, 96). In addition, several other transcription factors have been identified as targets for FA regulation, such as hepatic nuclear factor-4α (HNF-4α), sterol regulatory element-binding proteins (SREBP-1c), the liver X receptors (LXR-α and -β), retinoid X receptors (RXR) and NF-κB (105). Figure 17 depicts the sequence of events involved in the direct activation of gene expression via PPAR.
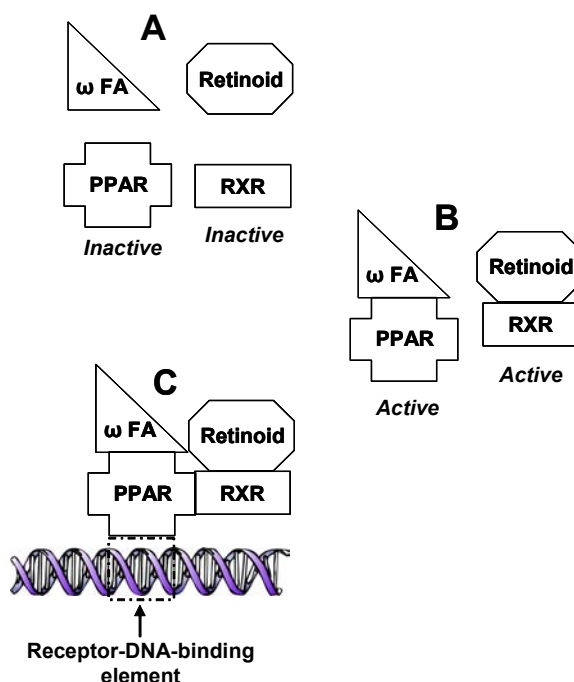
Figure 17: Sequence of events involved in the direct gene regulation of PUFA on PPAR-binding. Several steps must occur before PPAR can bind to the receptor response element and induce gene transcription. A: The transcription factors PPAR and RXR are inactive in the unbound state. B: Transcription factor activation of PPAR and RXR is accomplished by PUFA-specific binding. C: The activated transcription factor-PUFA complexes bind to each other and the subsequent heterodimeric structure binds to the receptor binding element of DNA through PPAR. PPAR, peroxisome proliferation-activated receptors; RXR, retinoid X receptor; ωFA, ω3 or ω6 PUFA (141).

6.6.   *Fatty Acid Deficiency*

In classical nutrition, a nutrient is considered essential if its removal from the diet leads to symptoms of deficiency, which often include impaired growth and development. C18:2ω6, the "parent" of the ω6 FA family, deficiency is associated with slower growth and reduced tissue accumulation of C20:4ω6. C18:2ω6 is essential as the precursor of C20:4ω6 which is the precursor for the production of eicosanoids, potent regulatory and inflammatory substances. A lack of C18:2ω6 in the diet is also associated with the loss of dermal integrity (142) as it is an essential component of complex lipids such as the sphingolipids in skin (143, 144).

Scientific consensus on the question of the essentiality of ω3 FA was achieved only recently. Initially, because C18:3ω3 did not fully improve the symptoms of EFA deficiency (EFAD) in laboratory animals, it was not considered an EFA. Studies by Bjerve *et al*. (145, 146) and Holman *el al*. (147) provided evidence for the essentiality of ω3 FA in humans. C18:3ω3 is considered to be essential as it is a precursor of

C20:5ω3, from which the ω3 eicosanoids are derived, and C22:6ω3, an important FA in the structural lipids of brain and other nervous tissues.

The symptoms associated with a lack of EFA in the diet were originally described by Burr *et al*. (55, 56). However, smaller deficits may not result in acute and overt symptoms, but may culminate in disease over a period of several years or decades. In EFAD, the body attempts to replenish and restore PUFA by utilizing an endogenous synthesis pathway, the ω9 pathway, as a source of PUFA (134, 147). This pathway results in the formation of Mead's acid (20:3ω9) which is classically used as a marker for EFAD (147). However, this measurement is skewed towards indicating a deficiency in ω6 FA due to the customary use of the C20:3ω9/C20:4ω6 ratio (triene to tetraene ratio) as a marker for deficiency which does not take the level of the ω3 PUFA into consideration (100).

6.7. SUMMARY
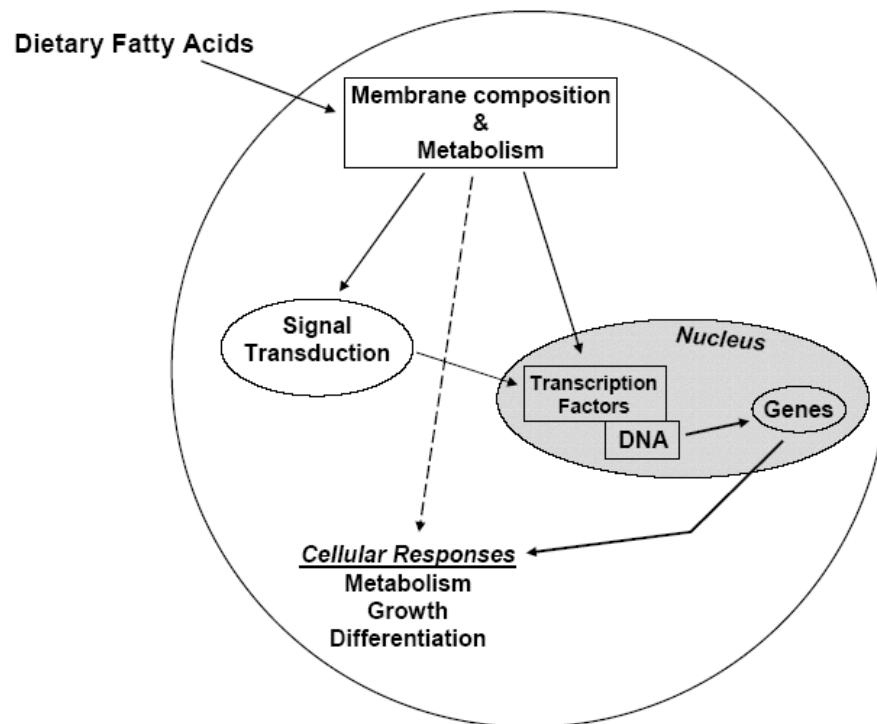
Figure 18: A summary of the multiple roles and effects which dietary fatty acids have on cell structure and function. Fatty acids regulate various transcription factors by direct binding or they regulate gene transcription by controlling mechanisms that affect nuclear abundance or activity of the transcription factor. The cellular levels of fatty acids, or their metabolites, affect transcription factor activity or abundance which can regulate gene transcription (50).

The multiple effects of PUFA on cell structure and function are summarized in Figure 18. Dietary FA affect cell membrane composition and structure and the release and metabolism of membrane FA can regulate signal transduction pathways affecting transcription factors. These events play a central role in regulating gene transcription which control cellular metabolism, growth and differentiation.

7.   DIETARY FAT: ASSOCIATION WITH DISEASE

Many recognized health problems are associated with excessive intake of dietary fat, such as obesity, insulin resistance, coronary heart disease and certain types of cancer (3, 54).   Diets in the Westernized world are very different from those before the agricultural revolution 10 000 years ago, particularly since the industrial revolution during the eighteenth century.   With regards to dietary fat, the Western diet is characterised by an increased energy intake and decreased energy expenditure, increased SATS, ω6 FA and *trans* FA with decreased intake of ω3 FA, vegetables and fruit (3, 54).   Since the industrial revolution, processed oils in the form of seed oils and the frying of foodstuffs has dramatically changed the dietary fat which formed a large part of the daily caloric intake (148).   Food processing, the need for long shelf-life products and the emergence of intensive livestock farming using artificially enriched diets, has also resulted in the replacement of natural EFA by their *trans*-isomers and the SATS (3).
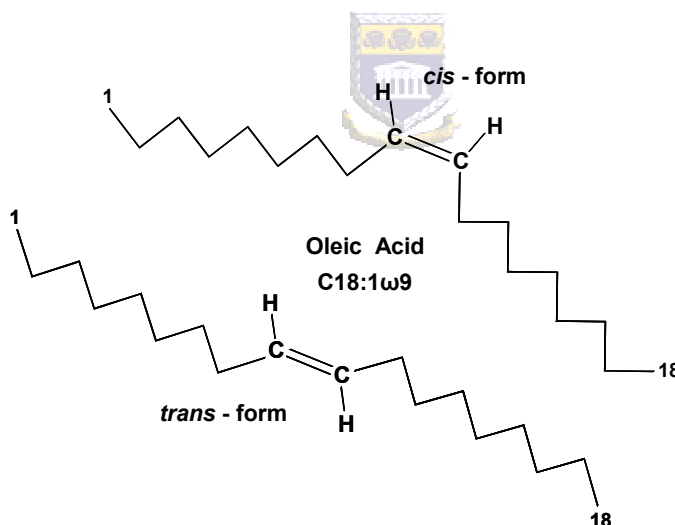
Figure 19: The *cis* and *trans* forms of oleic acid (149, 150).

During food manufacturing processes the naturally occurring *cis* form of FA may be changed to *trans* FA (Figure 19).   These *trans* FA can replace as well as block the conversion of the natural-occurring *cis* FA to their active metabolites by interfering with the desaturation, elongation and incorporation into membranes of the ω6 and ω3 PUFA, further complicating the risk for certain diseases (149, 150).   Figure 20 is a graphical depiction of the alteration in dietary fat intake over historical times.   Changes

in fat intake are reflected by an increase in total dietary fat comprised of SATS, ω6 FA and *trans* FA. This has been associated with a decreased intake of the ω3 FA in the 20th century resulting in an increased dietary ω6/ω3 FA ratio intake (151).
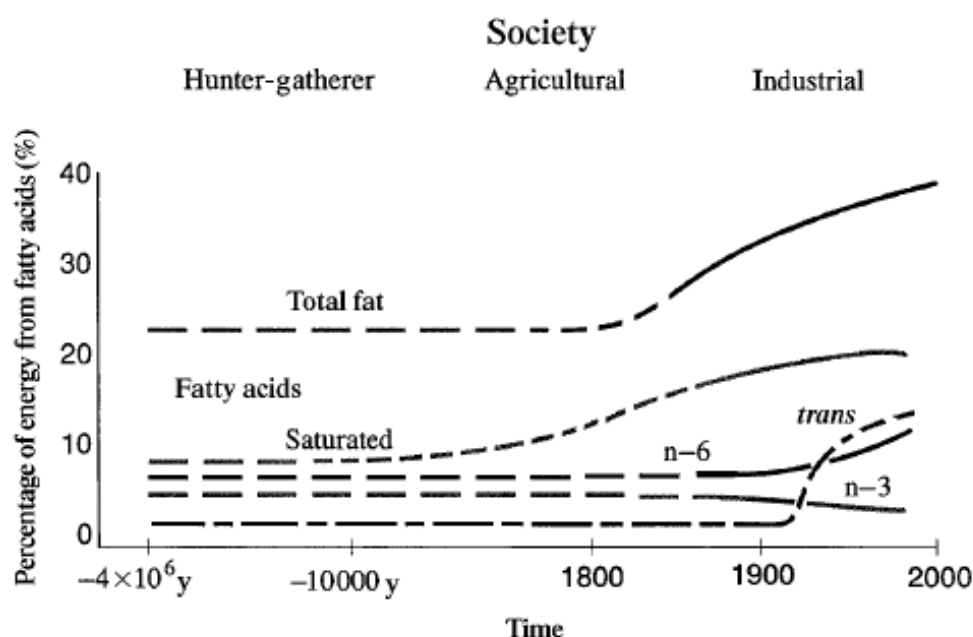
Figure 20: Hypothetical scheme of the changes in percentages of fat and different fatty acid families in human nutrition depicted extrapolated from cross-sectional analyses of contemporary hunter-gatherer populations and from longitudinal observations and their putative changes during the preceding 100 years. The introduction of hydrogenation to produce stable fat-containing products has dramatically increased the content of *trans* fatty acids in the food supply during this century. n-6, ω6 fatty acids; n-3, ω3 fatty acids (152).

Research indicate that changes in the fat component of the Western diet food supply, especially regarding FA, may not be optimal for the physiology of the human body (3). Studies suggest that humans evolved on a diet containing an ω6/ω3 FA ratio of approximately 1:1 (3, 153). A typical Westernized diet has a high ω6/ω3 FA ratio ranging from 20:1 to 50:1. This resulted largely from the health recommendations to replace saturated animal fat with vegetable oils (high in ω6 FA) to reduce serum cholesterol levels in the battle against cardiovascular disease (3, 148, 154). In response, more vegetable oils like sunflower oil, high in ω6 FA, were included in our diets (3, 148) which led to the increased usage of ω6 containing foodstuffs, such as margarines and cooking oils.

Until the early 1950s, the predominant dietary fats were hard fats such as butter, lard or hydrogenated fats in hard margarines and cooking fats. Epidemiological studies noted that countries with a higher intake of PUFA had low incidences of heart disease,

therefore recommendations were formulated by health experts to increase the dietary PUFA content to reduce the risk for heart disease. It was not fully realised at that time that two different PUFA family types existed. Research by Ahrens *et al*. (155) and Keys *et al*. (156) firmly established the importance of the $\omega 6$ FA in the prevention of cardiovascular disease, to the detriment of $\omega 3$ FA. Further, studies on determining the apparent required intake for EFA was solely based on dietary levels of $\omega 6$ PUFA (00). This has led to a decreased dietary $\omega 3$ FA content resulting in a significant change in the balance of the $\omega 6$ and $\omega 3$ PUFA. The tissue ratio of $\omega 6/\omega 3$ FA as well as the specific C20:4ω6/C20:5ω3 ratio, an indicator for prostaglandin type synthesis, is associated with mental illness attributed to an increased synthesis of the pro-inflammatory type of eicosanoids (157). A shift towards a high $\omega 6/\omega 3$ FA ratio, i.e. high $\omega 6$ dietary levels, can increase the susceptibility to certain diseases, such as an increased risk of stroke or heart attack, greater incidence of asthma, eczema and other allergic disorders, greater incidence of depression and schizophrenia, more dyslexia, more hyperactivity in children, inflammatory diseases and increased incidences of cancer (3, 54).
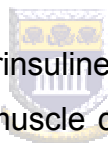
Borkman *et al*. (158) showed that hyperinsulinemia and insulin resistance was inversely associated with the level of PUFA in muscle cell membrane phospholipids in patients with coronary heart disease and normal volunteers. The decrease in PUFA could result from a high dietary intake of *trans* FA, genetic defects in the delta-6 and delta-5 desaturases, genetic defects which interfere with the transport or binding of PUFA. Figure 21 depicts the effect on diseases resulting from alterations in dietary PUFA intake.

Diet low in PUFA

| Diet high in *trans* FA interferes with desaturation and elongation of EFA. | → | Decrease in PUFA in skeletal-muscle membrane phospholipids. | ← | Impaired delta-6 and delta-5 desaturation |

Insulin resistance, hyperinsulinemia ← Diet high in linoleic acid (C18:2ω6)

Decreased FA oxidation

Type 2 diabetes

Hypertension

Low HDL High TAG

Lipogenesis

Coronary heart disease

Obesity

Figure 21: The effect of alterations in dietary PUFA on PUFA content in skeletal-muscle and the resultant interaction in insulin resistance, hyperinsulinemia and chronic diseases such as heart disease and obesity. EFA, essential fatty acids; FA, fatty acids; PUFA, polyunsaturated fatty acids; HDL, high density lipoprotein; TAG, triacylglycerol (adapted from 152).

While intakes of saturated and *trans* FA and C20:4ω6 have been linked to the development of chronic disease, research shows ω3 FA, specifically fish oils, are essential in the prevention and treatment of disease. Attention was drawn to health benefits of the ω3 FA in the 1970s when studies in Greenland Eskimos indicated that their incidence of cardiovascular disease is very low. Investigations by Danish medical researchers, Dyerberg, Bang and Hjorne (11) resulted in the conclusion that this was associated with the high level of marine ω3 PUFA in their diet. Subsequent studies in Japan, Sweden, and Denmark confirmed that the higher the consumption of ω3-rich fish, the lower the incidence of heart disease (148). Since then investigations all over the world have shown the potentially beneficial effects of ω3 FA in various diseases (Table 2).

Table 2: Diseases associated with beneficial effects after ω3 FA supplementation (3).

Schizophrenia
Attention Deficit Hyperactivity Disorder (ADHD)
Inflammatory diseases (rheumatoid arthritis, ulcerative colitis, Crohn's disease)
Reduction of blood pressure / Dilation of blood vessels
Important role in pregnancy
Importance in growth and development of infants and young children
Cardiovascular Disease
Depression
Diabetes
Skin disorders (eczema and psoriasis)
Cancer

8. FATTY ACIDS AND CANCER

As research in chemoprevention progresses, interest in the potential inhibitory or preventative properties of natural products has increased. A number of these products have been found to be effective in animal cancer models when administered at levels that appear to lack toxicity (64). Amongst these products, the ω3 FA found in fish and fish oils have gained great interest.

FA may influence various steps in carcinogenesis through numerous mechanisms such as; alterations in cell membranes and membrane fluidity affecting membrane structure and function, effects on membrane-bound enzymes which regulate xenobiotic metabolism, FA regulation of eicosanoid production, FA activation of nuclear transcription factors leading to cell differentiation, modulation of signal transduction pathways by FA leading to altered gene expression and effects on cell proliferation and apoptosis (58, 60, 65, 148, 159, 160).

As mentioned, dietary fat has been associated with an increased risk in the development of certain cancers. However, the type of fat as well as the ratio of dietary fat intake has been shown to be important. In this regard, the associated increased cancer risk has been linked to a high dietary intake of ω6 FA together with a low intake of the ω3 FA (60, 159). Epidemiological and experimental data implicate the ω6 FA as stimulators and LC ω3 PUFA as inhibitors in the development and progression of a range of human cancers (27, 81, 159, 161, 162). Generally, ω6 FA appear to enhance the promotional phase of carcinogenesis in preclinical models for breast, colon and prostate cancers, whereas ω3 PUFA appear to exert inhibitory effects (60, 95).

In vitro studies demonstrated that certain FA have a cytotoxic and/or anti-proliferative effect on cancer cells which is tumour specific with little or no effect on normal cells (87). These results imply that tumour/cancer cells are more sensitive to the metabolic effects of PUFA than normal cells. The anti-cancer effect (cytotoxicity and/or anti-proliferative effect) of PUFA on cell cultures *in vitro* has been shown to be dependent on the cell type used, the culture conditions as well as the type and dose of the individual PUFA (159). These studies have shown that the level of unsaturation or number of double bonds in the FA chain influences the effect of the FA on the cell (159). SATS such as palmitic acid (16:0) and stearic acid (18:0) are least able to induce a cytotoxic effect, closely followed by the MUFA such as C18:1ω8 (159). Generally, this pattern also holds true for the PUFA. The ω6 PUFA, C18:2ω6 containing 2 double bonds, is the least effective ω6 PUFA (159, 163). Within the ω3 PUFA family, C18:3ω3 with three double bonds, was generally not as effective as the LC ω3 PUFA, C20:5ω3 and C22:6ω3. Differences also exist when the cytotoxic effects of PUFA are compared between normal and cancer cells. C18:3ω6 and C20:3ω6 do not appear to induce harmful effects on normal cells at a dose which kills cancer cells (163). C20:5ω3 exhibited a less harmful effect in normal cells when compared to C20:4ω6, indicating differences between the two FA families.

8.1. Fatty Acid Metabolism and Cancer

8.1.1. Delta-6 Desaturase

In human liver, the activity of delta-6 desaturase is low compared to a higher activity in rats and mice, but which still contributes significantly to the metabolism of PUFA (93, 164, 165, 166). With regards to FA metabolism, the rate-limiting delta-6 desaturase enzyme is important in regulating the level of LCPUFA in tissue (163). The impaired

functioning of this enzyme has been observed in BL6 melanoma and Morris hepatoma 9618A cell lines and in various types of liver cancer with different origins (134, 167, 168). It has been shown that the delta-6 desaturase enzyme is compromised by a decrease in activity in cancer cells and tumours (169). In effect, this results in low levels of LCPUFA in the cancer tissue.

The activity of this enzyme can be affected by fasting, glucose levels, C18:2ω6 and C20:4ω6 membrane level, alcohol, *trans* FA, cholesterol levels and hormones such as insulin, catecholamines and corticosteroids (87, 93, 170). Leikin and Shinitzky (97) observed that the rat delta-6 desaturase enzyme is associated with a surrounding lipid environment consisting of PC and cholesterol in a 4:1 ratio. This ratio, including the FA acyl chains of the lipid components, appears to be important for optimal enzyme activity. Therefore, the activity might be influenced by the type of FA incorporated into the surrounding lipid environment.

8.1.2. Lipid Peroxidation

One of the main processes whereby PUFA mediate tumour cell cytotoxicity is through increased lipid peroxidation (159). Cancer cells have low levels of lipid peroxidation, partly due to unusually high levels of anti-oxidants such as vitamin E and C18:1ω9, which displays possible anti-oxidant properties (171). The main reason, however, appears to be due to the lack of or low levels of PUFA which have undergone delta-6 desaturation. This could be due to the increased requirement for cell proliferation in addition to the impaired functioning of the delta-6 desaturase enzyme in tumour cells (134). LCPUFA, which are key substrates for lipid peroxidation, can play a role in the control of cell proliferation by inhibiting cell growth and stimulating or enhancing apoptosis by increased cellular lipid peroxidation (60, 172). Therefore, a low oxidant status may be associated with neoplastic growth.

Several *in vitro* studies have shown that, in cell culture, ω6 and ω3 PUFA are cytotoxic and inhibit the growth of cancer cells via the production of lipid peroxides and aldehydes (173, 174, 175, 176). Through the process of lipid peroxidation, PUFA form free radicals such as peroxyl and alkoxyl radicals and aldehydes such as malondialdehyde, products which can affect cancer growth and cell viability (27). Aldehydes can affect the cell cycle by reducing the rate of cell proliferation. This occurs by inhibiting cells from entering the G1 phase, prolonging the G1 phase, slowing down

progression through the S phase by inhibition of the DNA polymerases and inhibition of the cell cycle due to a checkpoint arrest (27, 177, 178).

8.1.3. Signal Transduction

FA are known to induce changes in genes which affect cell survival (159). One of the mechanisms whereby PUFA induce cell death in cancer cells, *in vitro*, has been described to occur through a p38 MAPK pathway involving PPAR-α, a FA binding transcription factor (159). However, a p38 independent path can also be involved through the release of cytochrome *c*, eventually leading to apoptosis. PUFA may also effect changes in the phosphorylation of retinoblastoma (RB) and cyclin-dependent kinase-2 (cdk2) and the relocalisation of Ras, preventing cells from continuing in the cell cycle. ω3 FA have been demonstrated to suppress Ras activation in carcinogen-induced colon cancer with a concomitant decrease in DAG, thereby affecting PKC iso-enzyme activity (174, 180, 181). There are numerous isoforms of PKC, which may affect the interaction of various signal transduction pathways and influence the formation of cytokines, hormones, growth differentiation pathways and other cellular activities, including gene expression (182). Changes in membrane structure can also alter the activity of the enzyme PKC (183). In particular, the phospholipid PC and the FA unsaturation level of this phospholipid is associated with the regulation of PKC with regards to cell differentiation and proliferation (184, 185). Therefore alterations in the cell membrane PC/PE ratio could also affect PKC activity with resultant effects on signaling paths.

LCPUFA have been shown to induce apoptosis by suppressing Bcl-2 expression, an anti-apoptotic signaling molecule (186). Interestingly, Bcl-2 has been shown to have an anti-oxidant property and it is suggested that a balance exists between PUFA levels and the expression of Bcl-2 to regulate apoptosis by controlling lipid peroxidation (187, 188). Phosphorylation appears to be one of the control mechanisms in regulating Bcl-2 and phosphorylated Bcl-2 has been shown to no longer prevent lipid peroxidation (180). Another factor involved in apoptosis is FA synthase (Fas) via the caspases cascade (180). Fas is known to stimulate apoptosis and to be stimulated by increased levels of lipid peroxidation (189). Therefore, it appears that there is a close link between PUFA, Bcl-2 and Fas in controlling apoptosis.

With regards to lipid peroxidation, PPAR-α activators such as fish oil can alter antioxidant gene expression profiles by negative feedback to defend against excessive PPAR-α activation and ROS production (190). Feeding with fish oil has been shown to upregulate antioxidant genes, such as glutathione transferase in the cytosol and the uncoupling protein-2 (UCP-2) and manganese superoxide dismutase (Mn-SOD) in the mitochondria, to counteract increased ROS production (190). A paper by Fujimoto *et al*. (191) indicated that ROS and RNS also appear to play a role in the synthesis of prostaglandins by regulating the activity of COX-1 and -2.

8.1.4. Cholesterol

Alteration in the membrane cholesterol content has been implicated in carcinogenesis (108). An increase in membrane cholesterol is associated with malignancy involving a disruption in the regulation of cholesterol synthesis (115, 122) presumably due to loss in feedback control (115). This process involves the of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA), a key enzyme in mevalonate synthesis (192, 193). In cancer cells, as well as normal proliferating cells, there is a higher rate of cholesterol synthesis (115, 194), suggesting that cholesterol synthesis plays a role in cell growth. In this regard, ω3 FA can lower the level of cholesterol in tissue, suggesting a potential therapeutic role influencing cellular proliferation (195).

8.2.   ω6 and ω3 PUFA in Cancer

8.2.1. ω6 PUFA

Diets containing high levels of the ω6 FA, such as in corn oil, have been shown to enhance the development of tumours (196, 197). Most of these effects have been attributed to the metabolism and cell signaling properties of C20:4ω6. The control over C20:4ω6 release and turnover may be an important factor in controlling cellular proliferation and apoptosis. The deregulation or disturbance in C20:4ω6 metabolism has been connected to a large number of pathological disorders, indicating the need for tight control over the metabolism of this FA (129). However, another member of the ω6 PUFA family, namely C18:3ω6, has been shown to ameliorate the effects of C20:4ω6 and to cause apoptosis of cancer cells in culture and decreased tumour growth in animal models (134, 163, 167, 173, 198, 199, 200, 201).

8.2.1.1.   *Arachidonic Acid and Phospholipase A$_2$*

Phospholipase A$_2$ is an important enzyme, whose activity is responsible for the release of FA from the sn-2 position of membrane phospholipids (202, 203).   Increased expression of PLA$_2$ has been implicated in carcinogenesis and tumour growth (204). The release of membranal C20:4ω6 by PLA$_2$ has also been implicated in the cytotoxicity of TNF-α and Fas in several cancer cell lines as well as in apoptotic signaling via ceramide (205, 206, 207).   Cell lines exhibiting a decrease in PLA$_2$ activity, are shown to be resistant to TNF-α-mediated cytotoxicity associated with low levels of free or unesterified C20:4ω6 (188, 208).   Other studies confirmed that the suppression or increased activity of PLA$_2$ leads to a decrease or increase in cell death, respectively, suggesting that blocking the release of C20:4ω6 results in cellular resistance to TNF-α cytotoxicity (209, 210).   Resistance to TNF-α cytotoxicity has also been associated with a defect in the delta-6 desaturase enzyme which, indirectly, regulates the level of free/unesterified C20:4ω6, thereby affecting apoptosis (211).

8.2.1.2.   *Arachidonic Acid and Cyclooxygenase*

The COX prostanoid pathway is known to play an important role in inflammation, carcinogenesis and various liver diseases (130, 212).   COX-2 has been found to be increased in animal and human colon cancer tissue and is associated with increased PGE$_2$ levels (212, 213, 214, 215).   Elevated COX-2 expression has been found in familial adenomatous polyposis and sporadic colorectal cancer, as well as in cancer of the stomach, lung, oesophagus, liver, bile duct, pancreas and breast (129).   COX-2 is also thought to be involved in head and neck cancers (216).   Overexpression of COX-2 in different malignant tissue has been suggested to control cell proliferation by regulating the cell cycle progression (130, 217).   In this regard, COX-2 overexpression has been associated with increased cell proliferation, decreased apoptotic activity and the facilitation of angiogenesis (130, 212, 218, 219).   Using an inhibitor of the enzyme, COX-2 has been shown to control the G0 to G1 phase in the cell cycle (130).   Another regulator of COX-2 expression is the Ras signaling pathway, of which the Ras oncogene is often mutated in cancer and is associated with increased COX-2 levels (220).   COX-2 has also been implicated in tumour invasion and metastasis and appears to have a synergistic effect with matrix metalloproteinases in facilitating metastasis (221).

C20:4ω6, the main precursor for prostaglandins of the 2-series, is almost exclusively found as an ester at the sn-2 position of membrane phospholipids (222). After PLA$_2$-catalysed release from membrane phospholipids, oxygen is inserted into free C20:4ω6 by a reaction catalysed by COX to form PGG$_2$, an unstable intermediate, which is rapidly converted to PGH$_2$ by the peroxidase activity of COX. Specific isomerases then convert PGH$_2$ into the different prostaglandins and thromboxanes. Two isoforms of the COX enzyme exist, of which COX-1 is expressed constitutively in most tissues and appears to mediate the synthesis of prostaglandins that regulate normal physiological functions such as the maintenance of gastric mucosa and renal blood flow. COX-2 is however practically undetectable in normal tissue and is induced by pro-inflammatory and mitogenic stimuli (222). The main difference between the two isoforms is that COX-1 is normally present in most cell types and is considered to be a constitutive housekeeping enzyme. In contrast, COX-2 is normally not found in most cells but will rapidly appear in large amounts in a range of pathological, often inflammatory, situations (2223). Increased expression of COX-2 has been found in premalignant tissues as well as in malignant tumours (224-233). However, it appears that COX-1 may also be involved in carcinogenesis (234). The evidence that overexpression of COX-2 is mechanistically linked to the development of cancer has been shown by pharmacological as well as gene research by inhibition of the enzyme or with the use of gene knockout studies in rodents (212, 222). The pharmacological studies have shown that selective COX-2 inhibitors suppress the growth of tumours (222). These studies suggest that the COX-2 enzyme could be a potential target for preventing and controlling cancer growth and development.

Studies involving cell culture, animal experiments, therapeutic trials as well as epidemiological data indicate that nonsteroidal anti-inflammatory drugs (NSAIDs) block colon cancer at an early stage (212, 235). Inhibition of COX-2 by NSAIDs, such as aspirin and indomethacin, has indicated a decrease of colon polyps, precursors for colon cancer (129). The mechanism responsible is a block on COX-2 expression with a concomitant decrease in prostaglandin synthesis (235, 236, 237). COX-2 overexpression also appears to be involved in decreasing the activity of the enzymes involved in apoptosis, such as caspase-3, -8 and -9 (238).

8.2.1.3.   *Arachidonic Acid and Apoptosis*

It appears that the importance of C20:4ω6 in carcinogenesis is whether unesterified C20:4ω6 is metabolized further, i.e. by COX-2 to produce eicosanoids linked to tumour growth or is diverted to apoptosis via ceramide (188). As mentioned above, several studies have shown that high levels of free C20:4ω6, associated with TNF-α cytotoxicity and ceramide signaling, can stimulate apoptosis whereas increased levels of COX-2 can lower free C20:4ω6 levels, thereby inhibiting apoptosis (239, 240, 241, 242). In certain cancers, especially colon, the COX-2 enzyme is overexpressed which could decrease levels of free or unesterified C20:4ω6 (Figure 22), thereby removing or inhibiting the free C20:4ω6-mediated pro-apoptotic signal.
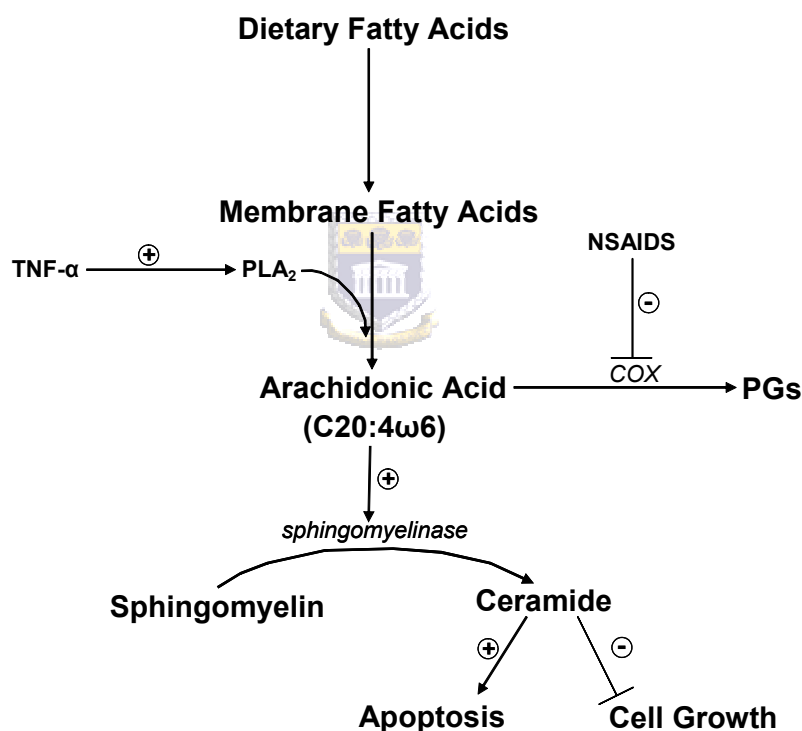
Figure 22: Arachidonic acid (C20:4ω6)-induced ceramide synthesis. Tumour necrosis factor-α (TNF-α) can interact with certain membrane receptors which in turn may activate PLA$_2$. C20:4ω6 is released from membrane phospholipids by phospholipase A$_2$ (PLA$_2$), which hydrolyzes plasma membrane lipids or lipids derived from the diet. C20:4ω6 is utilised as a substrate by the cyclooxygenase (COX) enzymes to produce eicosanoids such as prostaglandins (PGs). Non-steroidal antiinflammatory drugs (NSAIDs) inhibit the activity of the COX enzymes, which increases the cellular pool of free C20:4ω6. Free C20:4ω6 stimulates sphingomyelinase activity, which catalyzes the hydrolysis of sphingomyelin to generate ceramide. Ceramide acts as a second messenger activating apoptosis. Θ, inhibition; ⊕, stimulation (240, 243).

Inhibition of the enzyme CoA-independent transacylase (CoA-IT), which controls the remodeling of C20:4ω6 from choline containing phospholipids to ethanolamine-phospholipids without changing the PE/PC ratio, has been shown to block cell proliferation and induce apoptosis in HL-60 cells (239). The inhibition of CoA-IT has been shown to lead to the accumulation of free C20:4ω6 causing an increase in ceramide levels (239). The induction of apoptosis by NSAIDs in colorectal cells is due to an accumulation of free C20:4ω6 which stimulates the conversion of sphingomyelin to ceramide (240). The inhibition of COX and subsequent decrease in prostaglandin synthesis, may lead to increased C20:4ω6 levels resulting in higher ceramide production, thereby promoting apoptosis (181). In this regard, C20:4ω6 is known to stimulate sphingomyelinase which catalyzes the conversion of SM to ceramide (243). The effect of C20:4ω6 on apoptosis appears to depend on the exchange of the FA between the different phospholipid fractions within the cell membrane (1113). After the addition and incorporation of free C20:4ω6 into cell membranes, the FA moves from the PC, PI and PS phospholipid fractions into the PE fraction. Inhibition of this migration, which is dependent on the enzyme CoA-IT, by CoA-IT inhibitors results in apoptosis (241). Blocking of the enzyme also decreased cell proliferation and increased ceramide levels (239, 241). Therefore, increased C20:4ω6 levels in PC, PI or PS may be associated with C20:4ω6-induced apoptosis.

Utilising COX-2 inhibitors, Cao *et al*. (188) demonstrated that an increased level of unesterified C20:4ω6 stimulates apoptosis. The block in apoptosis is attributed to an increased expression of Bcl-2, which can be overridden by COX-2 inhibitors by down-regulating Bcl-2 expression. PE_2 has also been shown to inhibit apoptosis by inducing the expression of Bcl-2 and increasing the level of cAMP, thereby stimulating cell survival (244, 245). Therefore, important events for carcinogenesis are an over-expressed COX-2, up-regulated Bcl-2, increased PGE_2, decreased levels of unesterified C20:4ω6 and decreased apoptosis.

8.2.1.4.   *Arachidonic Acid and Lipoxygenase*
Another important C20:4ω6 metabolic enzyme is LOX, which has been found to be overexpressed in cancer (212). Metabolites of LOX are reported to be involved in tumour growth by stimulating cell proliferation, inflammation and angiogenesis and decreasing apoptosis (138, 139, 212, 246). LOX exhibits region specificity when

interacting with a substrate, such as C20:4ω6, and is designated as arachidonate 5-, 8-, 12-, 15-lipoxygenase or 5-LOX, 8-LOX, 12-LOX and 15-LOX (247). These four enzymes insert oxygen at carbon 5, 8, 12 or 15 of C20:4ω6 and the products formed are 5S-, 8S-, 12S- or 15S-hydroperoxyeicosatetraenoic acid (5-, 8-, 12- or 15-HPETE), which can be further reduced by glutathione peroxidase to the hydroxy forms 5-, 8-, 12-, 15-HETE. The metabolite 5-HETE has been observed to stimulate tumour cell invasion and metastases and to inhibit apoptosis (136, 137). Evidence indicates that 12-LOX is involved in cancer cell proliferation as inhibition of 12-LOX induces apoptosis and decreases cell proliferation in carcinosarcoma cells (247). In human prostate cancer, the degree of 12-LOX expression correlates with the tumour grade and stage and is also associated with tumour cell metastasis and stimulation of prostate cancer cell migration (246, 247). The roles of 8- and 15-LOX in carcinogenesis are not yet clear, with conflicting data being reported. More studies need to be performed to elucidate the effect of these enzymes in carcinogenesis.

8.2.1.5.   *Arachidonic Acid and iNOS*

An excessive production of nitric oxide (NO) is also linked to the genesis of many pathological conditions, including cancer (248). NO is an important bioactive signaling molecule that mediates a variety of normal physiological functions such as vasodilatation, neurotransmission, host defense and iron metabolism. NO is endogenously produced by a family of enzymes known as nitric oxide synthase (NOS). This enzyme exists in constitutive and inducible forms. In the stomach of rats, the constitutive form has been shown to result in the production of physiological levels of NO important for gastric mucosal functions, such as regulation of vascular tone and neurotransmission (249). NO can also be cytoprotective in the gastrointestinal mucosa by interacting with sensory neuropeptide and endogenous prostaglandins (249). Inducible nitric oxide synthase (iNOS) is synthesized in a variety of cell types from multiple mammalian species and can produce high concentrations of NO which may have pathobiological effects (250).

The increased expression of both iNOS and COX-2 has been demonstrated in intestinal inflammation in certain animal models and human diseases (251, 252). A study examining rat gastric mucosa indicated that endogenous NO released after induction of iNOS also stimulates COX-2 in the mucosa (249). The involvement of iNOS and COX-2 in human carcinogenesis has been well supported by data obtained

in tumours of different organ systems (250). Increased iNOS activity and protein expression in both colorectal adenomas and carcinomas has been recently reported (253). A study by Franco *et al*. (254) demonstrated the overexpression of iNOS and COX-2 in pancreatic cancer as compared to non-tumour tissue, suggesting that both iNOS and COX-2 are involved. *In vitro* studies showed that both iNOS and COX-2 have mutagenic and tumourigenic activity and that iNOS can regulate COX-2 expression and the production of prostaglandins (254, 255, 256, 257, 258).

8.2.1.6. *Arachidonic Acid and Cell Growth*

Dietary PUFA may regulate the growth and progression of cancer through the modulation of epidermal growth factor receptor (EGFR). EGFR is a membrane bound protein involved in the growth stimulation of cells and activates a signal transduction cascade inducing the activity various kinases, such as GTP-bound Ras, Raf-1, MAPK kinase (MEK) and MAPK (251). The role of MAPK in this pathway is to modulate enzymes and gene transcription involved in cellular proliferation and mitogenesis (259).

In humans, it has been demonstrated that increased expression of EGFR is directly related to the invasiveness of tumours such as in mammary cancer (260). The activation of Ras, an upstream effector of MAPK, can up-regulate Bcl-2, a suppressor of apoptosis (261), suggesting that MAPK may play a role in the progression of mammary cancer through both growth stimulation and decreased apoptosis. Inhibition of MAPK can lead to inhibition of mammary cancer cell growth and enhanced killing of mammary cancer cells by cytotoxic compounds (259, 262). Inhibition of MAPK has also been shown to increase the induction of apoptosis in tumour cells (263). Several mechanisms have been proposed by which PUFA may regulate the EGFR/MAPK-induced growth of cancer cells (Figure 23). ω6 PUFA, such as C20:4ω6, can inhibit GTPase-activating proteins (264), which are involved in the hydrolysis of GTP-bound (active) Ras protein in the EGFR/MAPK cascade. By inhibiting the GTPase-activating protein, C20:4ω6 prolongs the signal transduction of EGFR to the nucleus leading to an increased growth stimulus. LOX metabolites derived from ω6 PUFA such as C20:4ω6, have been implicated in the activation of several isoforms of PKC (265, 266), which are effectors of MAPK signaling. *In vivo*, PKC has been shown to activate Raf-1 and MEK and subsequently MAPK (267). This provides evidence that ω6 PUFA, i.e. C20:4ω6, may influence the MAPK mitogenesis of cells through a variety of mechanisms.

C18:1ω9 has also been shown to activate EGFR as well as PI3-K, a second messenger for pathways relating to cell proliferation and apoptosis (268, 269).
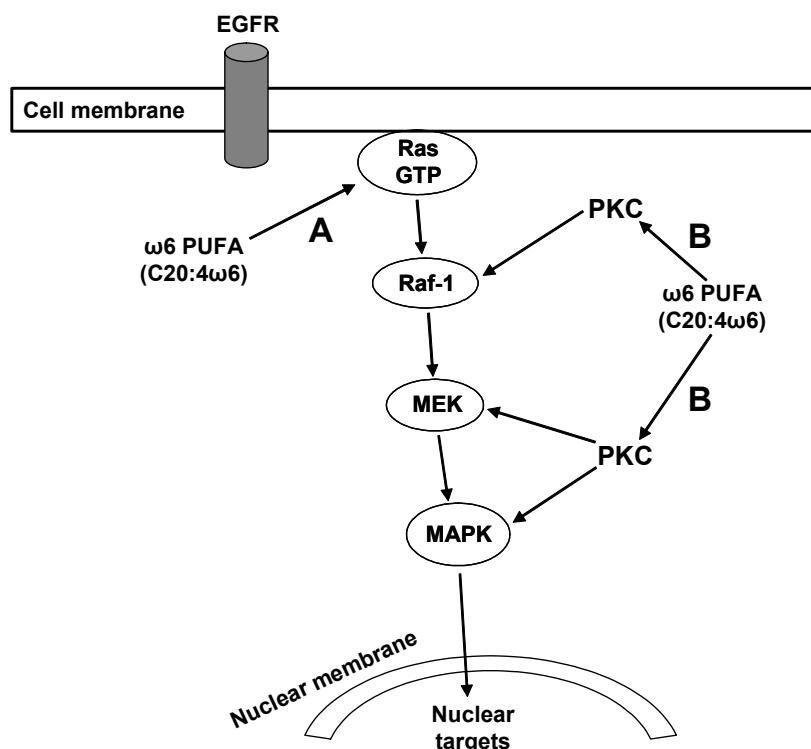
Figure 23: ω6 Polyunsaturated fatty acids (PUFA), such as arachidonic acid (C20:4ω6), stimulation of the epidermal growth factor receptor/mitogen-activated protein kinase (EGFR/MAPK) growth stimulatory cascade. A: Inactivation of GTPase activating protein by ω6 PUFA leads to continual mitogenic and proliferation activities of MAPK. B: ω6 PUFA stimulate protein kinase C (PKC) which then activates components of the MAPK signaling cascade (adapted from 259).

Another growth factor receptor associated with carcinogenesis is the insulin-like growth factor I receptor (IGF-IR) in the development, maintenance and progression of cancer (270). The IGF-IR is over-expressed in many tumour types and is an important signaling molecule for tumour growth and survival by stimulating cell proliferation and inhibiting apoptosis. The receptor ligands, IGF-I and IGF-II, have been shown to be mitogens for a variety of cancer cell lines such as prostate, breast, colon, myeloma, melanoma, ovary and lung and are mediated through the IGF-IR. The main signaling pathways for the IGF signal involve MAPK and PI3-K/Akt (270).

8.2.2. γ-Linolenic Acid

γ-Linolenic acid (GLA, C18:3ω6) is an 18-carbon PUFA belonging to the ω6 FA family and, by elongation to C20:3ω6, is a precursor to the 1-series prostaglandins (PGE$_1$) and is also metabolized by 15-LOX into 15-(S)-hydroxy-8,11,13-eicosatrienoic acid (15-HETrE) (134). These metabolites are less inflammatory than eicosanoids derived from

C20:4ω6 (271, 272). A number of studies show the potential of C18:3ω6 to suppress tumour growth and metastasis by affecting various processes such as decreased cell invasion, inhibition of cell growth, cell cytotoxicity, angiogenesis and decreased cell proliferation (273, 274). Many of the clinical effects of C18:3ω6 supplementation have been associated with its capacity to block the metabolism of C20:4ω6 to eicosanoids (134).

Early studies using hepatoma cells indicated that C18:3ω6 induced the appearance of fragmented DNA, an indicator of apoptosis (273). A study by De Kock *et al.* (275) in HeLa cells demonstrated that C18:3ω6 decreased MAPK activity and reduced cellular levels of c-Jun but increased c-Myc levels, resulting in apoptosis. *In vitro*, C18:3ω6 inhibits 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 (276). C18:3ω6 also reduces tumour-endothelium adhesion, a key factor in the establishment of distant metastases, partly by improving gap junction communication within the endothelium (278). These observations were corroborated by Kokura *et al.* (279) who demonstrated that dietary C18:3ω6 is effective in suppressing tumour growth *in vivo*.

C18:3ω6 is also toxic to tumour cells in culture with little or no effect on the survival of normal cells. Mechanistically, evidence suggests that C18:3ω6 suppresses the expression of the oncogene Ras and Bcl-2 and enhances the activity of p53, thereby promoting apoptosis (180). This effect by C18:3ω6 is due to the limited or low activity of delta-5 desaturase in rodents and humans, whereby only a small fraction of dietary C20:3ω6 is converted to C20:4ω6 (280). Research indicates that supplementation with C18:3ω6 in many cell types leads to increased levels of C20:3ω6 and does not result in increased C20:4ω6 levels. The increase in C20:3ω6 relative to C20:4ω6 is capable of decreasing the biosynthesis of C20:4ω6 metabolites, such as the 2-series prostaglandins and 4-series leukotrienes (280). In addition, because C18:3ω6 bypasses the delta-6 desaturase, a key regulatory rate-limiting enzymatic step controlling the formation of PUFA, it may alleviate any decrease in the resultant LC desaturated FA. Such a reduced capacity to convert C18:2ω6 to C18:3ω6 has been associated with various physiologic/pathophysiologic states, including aging, diabetes, alcoholism, atopic dermatitis, premenstrual syndrome, rheumatoid arthritis, cancer and cardiovascular disease (87, 281). The impairment of delta-6 desaturase activity,

observed in cancer cells (282), is an important factor when considering supplementation with C18:3ω6. Therefore, bypassing the delta-6 desaturase and the formation of less active inflammatory eicosanoids when compared to C20:4ω6, may be an important factor in the anti-cancer effect of C18:3ω6.

Another C18:3ω6-anticancer mechanism, proposed by Nwankwo (201) and expanded on by Menendez *et al.* (274), involves the inhibition of Fas overexpression in tumour cells. Fas is a key enzyme involved in the *de novo* synthesis of FA and has been found to be overexpressed in various human malignancies (274, 283). Inhibition of Fas has been implicated in decreasing cell proliferation and cancer cell survival (284, 285). In this instance, C18:3ω6 toxicity to tumour cells appears to be dose dependent, with low physiological levels resulting in cell death by lipid peroxides. At high physiological levels, C18:3ω6-induced inhibition of Fas leads to accumulation of the Fas substrate malonyl-CoA which inhibits FA oxidation. This inhibition may deprive tumour cells of energy metabolism via FA oxidation, resulting in an energy deficit in proliferating tumour cells leading to the inhibition of proliferation and ultimately causing apoptotic cell death (274).

8.2.3. *ω3 PUFA*

Numerous studies have highlighted the beneficial health effects of the ω3 PUFA (58, 64, 95, 286). The classic studies by Dyerberg, Bang and Hjorne (11) on the Eskimos in Greenland demonstrated the rare incidence of cardiovascular disease amongst this population group. This protective effect was attributed to the ω3 FA content of their diet. Further research studies such as the Chicago Western Electric study, the GISSI heart study, the Health Professional Study and the US Physicians' Health Study also showed the potential protective effects of ω3 FA in cardiovascular disease (286, 287, 288, 289, 290). The management of diabetes has also shown promising results due to increased intake of ω3 FA (46). The ω3 FA also play an important role in the management and prevention of inflammatory and immune disorders such as asthma, Crohn's disease and rheumatoid arthritis where control over increased PGE$_2$ levels appears to be a major factor (104). Data from different studies have shown an inverse association with seafood intake and the rate of major depression (77, 291). In major depression the plasma and red blood cell content of ω3 FA, especially C20:5ω3 and C22:6ω3, was found to be relatively low (292). Intervention studies with ω3 FA, such as C20:5ω3, have also shown promising results in the treatment of schizophrenia

(293). ω3 FA also appear to play an important role in maternal health as well as the healthy development of the foetus and newborn infant (294, 295, 296).

8.2.3.1.   *ω3 PUFA and Cancer*

The effect of ω3 FA in the fight against cancer is one of the most exciting developments in FA research. In particular, it is the ω3 LCPUFA, C20:5ω3 and C22:6ω3, found in fish and fish oils that have gained importance in the fight against cancer and diet-related diseases. Epidemiological and experimental data implicate the ω6 PUFA as stimulators and ω3 PUFA as inhibitors for the development and progression of certain cancers in humans (27, 81). Certain epidemiological studies show that cancer is less common in communities that consume large amounts of fish, such as the traditional Eskimos, and this is thought to be due to the presence of the ω3 PUFA in fish (3, 11). Studies such as the prospective Women's Health Study, New York University (297), showed that consumption of fish could protect against the development of colorectal cancer. Findings from studies such as this have been confirmed experimentally in various animal colon cancer studies where fish oil prevented the development of cancer (161). In Japan, where the average dietary fish intake is high, there is a very low rate of breast cancer amongst the women and Japanese men also have a lower risk for prostate cancer. However, this trend appears to be changing to a more Western pattern of disease due to an increase in Western lifestyle, especially amongst the younger generations (298, 299). In Europe and the Far East, stomach and intestinal cancers also appear to be less common in people with a high dietary fish intake. C20:5ω3 and C22:6ω3 supplementation in the form of fish oil, have also been found to suppress both breast and colon cancer tumour growth and metastasis in nude mice (300).

Studies indicated that the antitumour effects of C20:5ω3 and C22:6ω3 appear to be distinctly different from each other. The effects induced by C20:5ω3 is mainly related to the suppression of cell proliferation, whereas C22:6ω3-induced effects appear to be related to its ability to induce apoptosis (301, 302). These differences may be related to the manner in which C20:5ω3 and C22:6ω3 inhibit C20:4ω6-derived eicosanoids. C20:5ω3 inhibits both the COX and LOX pathways, whereas C22:6ω3 only inhibits the COX path (303, 304, 305). *In vitro* studies by Stillwell *et al*. (305, 306, 307) demonstrated that C22:6ω3 in the sn-2 position of the phospholipid, 1-stearoyl-1,2-docosahexaenoyl phosphatidylcholine (C18:0, C22:6ω3-PC), is cytotoxic to tumour

cells, whereas a similar phospholipid containing C20:5ω3 was not. It also appears that the cytotoxicity of C22:6ω3 is due to being a good substrate for lipid peroxidation (87).

The heath benefits attained by the ω3 LCPUFA appear to be mainly due to the following mechanisms; (i) their structural incorporation into cell membranes changing various parameters such as receptor and enzyme activity, (ii) alteration in the peroxidation status, especially in cancer tissue, (iii) competitive inhibition of C20:4ω6-derived eicosanoid or prostaglandin synthesis, (iv) modulation of cell signaling, gene expression and apoptosis, and v) an increase in anti-angiogenic activity (50, 64, 104, 105, 308).

8.2.3.2. *ω3 PUFA and Lipid Peroxidation*

Studies involving animal cancer models showed that the effectiveness of the ω3 FA as anti-cancer agents also depends on the peroxidation status of the cancer tissue (309). All healthy cells have a natural background of peroxidation, due to normal metabolic processes, while cancer cells have low peroxidation levels providing a good environment for growth. ω3 FA, especially C20:5ω3 and C22:6ω3, appear to increase the susceptibility of cancer cells to peroxidation and may inhibit cancer growth by inducing apoptosis. The addition of vitamin E, a natural protector against peroxidation, to cancer cell tissue cultures decreases the anti-cancer effect of the ω3 FA (27). However, *in vitro* and *in vivo* studies showed that the addition of anti-oxidants did not prevent the growth inhibitory effect of fish oils in all cases (27). This indicated that an additional mechanism, besides lipid peroxidation, was responsible for the inhibition of tumour cell growth by PUFA (27). In addition, studies in cell cultures have shown that the addition of and membrane incorporation of C20:5ω3, C22:6ω3 and C18:3ω6 can affect cancer growth and metastasis by eicosanoid-independent mechanisms such as altering membrane fluidity, membrane receptors, initiating cell cycle arrest by apoptosis, alteration of cell adhesion and regulating tight junction function (278, 310, 311).

8.2.3.3. *ω3 PUFA and Eicosanoids*

Numerous studies over the last 25 years have suggested that dietary supplementation with ω3 PUFA has a beneficial effect on human disease. The beneficial effects of ω3 PUFA on prostaglandin biosynthesis has been proposed to occur by i) preventing the conversion of C20:4ω6 to pro-inflammatory eicosanoids, and ii) to be used as an

alternative substrate for less potent prostaglandins and leukotrienes (128). The inhibition of C20:4ω6-derived eicosanoids by ω3 FA was first proposed by Lands *et al*. (312) and has been shown to act similarly to NSAIDs such as ibuprofen (312, 313). These include the inhibition of delta-6 desaturase and COX activities and reduction of C20:4ω6 tissue content by enriching the phospholipids with ω3 PUFA (314). Due to the predominantly high ω6 FA in our diet, most prostaglandins produced by the human body tend to be derived from C20:4ω6 (3). Increasing the dietary intake of the ω3 PUFA alters this balance and this is thought to be in part responsible for their beneficial health impact. The ω3-derived prostaglandins have more moderate biological effects than the ω6-derived prostaglandins and PGE_3, derived from C20:5ω3, is metabolically less active than PGE_2. This implies that a higher intake of ω3 FA can effect anti-inflammatory, anti-thrombotic and vasodilatory properties. These beneficial effects may protect against heart disease, hypertension, diabetes and a wide variety of inflammatory conditions such as rheumatoid arthritis and ulcerative colitis as well as cancer (3).

8.2.3.4.   *ω3 PUFA, Signal Transduction and Apoptosis.*

The ω3 FA can change the encoding of certain mRNA and which remain in effect as long as the levels of these FA are maintained (105). Some of the gene transcription factors which can be affected are NF-κB, TNF-α and PPAR (104, 315, 316). NF-κB also plays a role in the control of cellular proliferation and growth (317, 318) and in the development of cancer and metastasis (319). Several studies using human derived solid tumour cell lines show increased nuclear levels of NF-κB in comparison to non-transformed control cell lines (320, 321, 322). The inhibition of NF-κB in head and neck squamous cell carcinoma has been shown to inhibit cell survival and tumour growth (322). Activators of NF-κB (Figure 24) are Ras, Raf, PI3-K, Akt and TNF (318, 319). Mechanisms for NF-κB functioning is via suppression of apoptosis and promotion of cell growth through the transcriptional upregulation of cyclin D1 (323). NF-κB is also known to regulate expression of cell adhesion molecules (324) and cell surface proteases (325). COX-2 is also known to be transcriptionally activated by NF-κB (319). The ω3 FA have been shown to decrease transcription of TNF-α, thereby modulating intercellular signal transduction pathways (326). The mechanism of NF-κB inhibition by ω3 FA involves decreased IκB phosphorylation and consequent decrease in the ability of NF-κB to bind to TNF-α resulting in the inhibition of the NF-κB signal transduction

cascade (316). The decrease in NF-κB activity is translated into a concomitant decrease in TNF-α mRNA transcription (316, 327).

Ras, Raf, PI3-K, Akt, TNF

Activators

NF-κB ⊣ ┌─────────────┐
╎ **NSAIDS** ╎
╎ **ω3 Fatty Acids** ╎
└─────────────┘

Apoptosis ⊣ ⊖

NF-κB

Cell Growth
Cell Proliferation
Metastasis
Angiogenesis

Nucleus
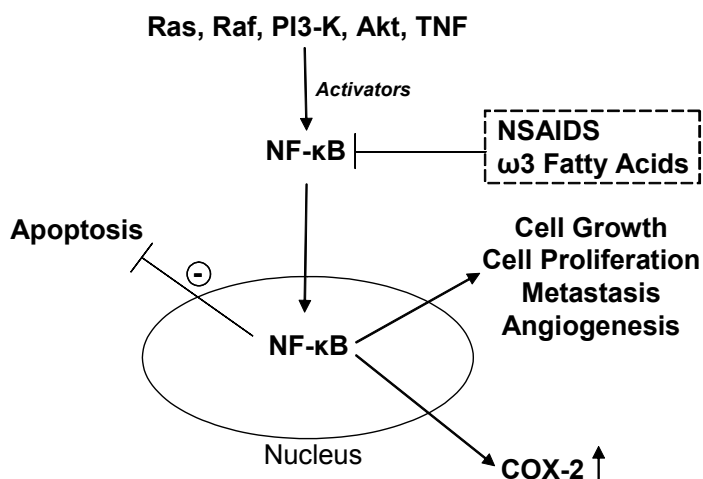
COX-2 ↑

Figure 24: Inhibition of NF-κB signaling cascade by NSAIDS and ω3 FA, affecting various signaling pathways involved in cell proliferation, apoptosis, angiogenesis and metastasis. Θ, inhibition; COX-2, cyclooxygenase-2; PI3-K, phosphatidylinositol 3-kinase; TNF, tumour necrosis factor; NF-κB, nuclear factor-kappa B; NSAIDS, nonsteroidal anti-inflammatory drugs (319, 326, 328).

A study by Plümpe *et al*. (328) demonstrated that during normal liver regeneration, such as after partial hepatectomy, the TNF-α-dependent activation of NF-kB is essential to trigger cell proliferation of the hepatocytes and restoration of liver mass. This study suggested the importance of NF-kB in triggering cell proliferation under normal circumstances.

Additional pathways altered by ω3 PUFA involve the Ras oncogene and the suppression of mitosis. ω3 FA decrease the activity of Ras and AP-1 (326) oncogenes that are frequently activated in cancers and which stimulate mitosis (329). This effect was associated with a suppression of colonic tumour development. Dietary fish oil modulates the intracellular localization of Ras by reducing its level at the plasma membrane in colonocytes (326). This has been linked to the ω3 PUFA, C22:6ω3, which can disrupt Ras-dependent signal transduction by suppressing its plasma membrane localization. Ras can activate the MAPK/ERK kinase pathway. Membrane GTP-bound Ras can also interact with and activate the enzyme PI3-K which in turn results in the activation of Akt, a serine/threonine kinase that promotes cell survival (156, 288). The ω6 FA, C18:2ω6 and C20:4ω6, have also been associated with the

activation of PKC and induction of mitosis in colonocytes (64, 329). In contrast, the ω3 PUFA, C20:5ω3 and C22:6ω3, reverse the PKC activity changes associated with colon carcinogenesis. Other kinases which are inhibited by C20:5ω3 and C22:6ω3 include protein kinase A (PKA) and MAPK such as ERK1 and -2 (330).

8.3.   Modulation   of   Arachidonic   Acid   Metabolism: implications   for carcinogenesis.

Various studies have shown the involvement of C20:4ω6 metabolism and C20:4ω6-derived eicosanoids in tumour growth and metastasis (64, 331, 332, 333).   In this regard, the interaction of ω3 PUFA, particularly C20:5ω3, and C18:3ω6 are of importance in counteracting or modulating the C20:4ω6 mediated effects.

8.3.1.   ω3 PUFA

Studies in which rodents were fed a ω6 based diet, showed an increase in C20:4ω6-derived eicosanoid levels associated with cancer promotion, tumour cell invasion, angiogenesis and metastasis.   These effects have been linked with growth factors, oncogenes and protein kinases (64, 3331, 334, 335, 336). The conversion of C20:4ω6 to the active eicosanoids affects cell behavior by acting as ligands for specific receptors (337).   *In vitro* studies have demonstrated that the most prominent mechanism by which the ω3 FA may lower the risk of cancer is through their suppression/inhibition of C20:4ω6-derived eicosanoids (95).   This effect is mediated at several levels.   A high intake of the ω3 FA leads to their incorporation into membrane phospholipids where they partially replace C20:4ω6.   The ω3 PUFA also compete with ω6 PUFA for the desaturase and elongase enzyme systems, with the ω3 PUFA having a higher enzyme affinity (95).   Therefore, a higher intake of the ω3 PUFA reduces the desaturation and elongation of C18:2ω6 to C20:4ω6 (64).

Prostaglandin synthesis from C20:4ω6 can be regulated in four ways; modulation of the enzymatic activity of delta-6 desaturase, PLA_2 and COX and by changes in tissue levels of C20:4ω6 itself (337).   Modification of tissue C20:4ω6 content via competitive inhibition or inactivation of the desaturase is associated with corresponding changes in prostaglandin levels.   ω3 PUFA were shown to decrease the activity of PLA_2, phospholipase C (PLC) and COX-2 in colon cancer thereby decreasing the availability of C20:4ω6 and PGE_2 levels (214, 338). The replacement of C20:4ω6 suppresses the biosynthesis of C20:4ω6-derived eicosanoids as C20:5ω3 suppresses COX-2 activity and competes with C20:4ω6 for the COX enzymes to form the C20:5ω3-derived 3-

series prostanoids, such as PGE$_3$ (95). C20:5ω3 is also the preferred substrate for LOX in relation to C20:4ω6, therefore an increased C20:5ω3 dietary intake leads to an increased synthesis of C20:5ω3-derived LOX products, the 5-series leukotrienes (339). Finally, ω3 PUFA can enhance eicosanoid catabolism which is postulated to be mediated through induction of peroxisomal enzymes (340). The formation of C20:4ω6-derived eicosanoids is not only influenced by the ω3 PUFA alone, but also by C20:5ω3-derived eicosanoids and some of these eicosanoids (eg, 15-hydroperoxyeicosapentaenoic acid; 15-HPEPE) exhibit a greater inhibitory effect than C20:5ω3 itself (341).

8.3.2. γ-Linolenic Acid

The ω6 FA, C18:3ω6, has also demonstrated anticancer effects and able to modulate C20:4ω6 metabolism. In addition, C18:3ω6 and the ω3 FA, C20:5ω3, exhibit synergistic effects as the C18:3ω6 metabolite, C20:3ω6, appears to enhance the synthesis of PGE$_3$ from C20:5ω3, while C20:5ω3 enhances the synthesis of PGE$_1$ from C20:3ω6 (87, 134, 342). In C18:3ω6 and C20:5ω3 treated tumour cells, caspases activation and DNA fragmentation were observed, events which are linked to apoptotic cell death (196, 199, 200).

Figure 25 depicts the synergistic interaction between C18:3ω6, C20:3ω6 and C20:5ω3. PGE$_1$ synthesized from C20:3ω6 increases cAMP levels which inhibits the C20:4ω6-phospholipid releasing action of PLA$_2$. In addition, C20:3ω6 and C20:5ω3 inhibit the formation of eicosanoids from C20:4ω6 by acting as competitive substrates for the COX and LOX enzymes (134, 271, 343). These effects reduce the C20:4ω6-derived eicosanoids at different levels which are associated with inflammation and carcinogenesis.
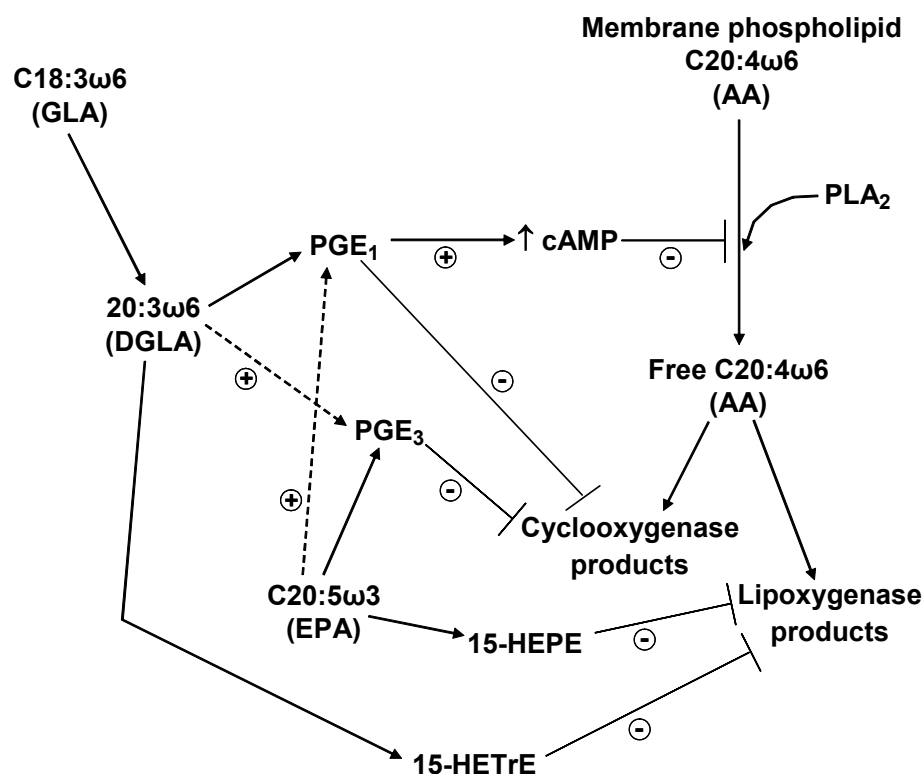
Figure 25: The synergistic interactions between DGLA (C20:3ω6, Dihomo-γ-linolenic acid), GLA (C18:3ω6, γ-Linoleic acid) and EPA (C20:5ω3, Eicosapentaenoic acid) and competitive inhibition of AA (C20:4ω6, Arachidonic acid) metabolism. C20:3ω6 and C20:5ω3 can synergistically inhibit the formation of eicosanoids from C20:4ω6 by acting as alternate substrates for the conversion into biologically less potent eicosanoids. These effects reduce the C20:4ω6-derived eicosanoids at different levels which are associated with inflammation and carcinogenesis. PGE1 derived from C20:3ω6 may also inhibit the release of C20:4ω6 from membrane phospholipids. Θ, inhibition; ⊕, stimulation; PGE_1, prostaglandin E_1; PGE_3, prostaglandin E_3; PLA_2, phospholipase A_2; HEPE, hydroxylated eicosapentaenoic acid; HETE, hydroxylated eicosatetraenoic acid(s); HETrE, hydroxylated eicosatrienoic acid. (134, 271, 343).

8.4. *Multiple Modulatory Mechanisms of Carcinogenesis by PUFA*

Figure 26 depicts the multiple competitive interactions between the ω6 and ω3 PUFA in the stimulation or prevention of cancer. Dietary PUFA have a wide ranging effect on cellular structure and function, ranging from effects on cellular phospholipid composition and membrane structure, as substrates for signaling metabolites influencing gene expression pathways involved in cellular proliferation and apoptosis. Important enzymes involved in FA release and their subsequent transformation into active prostanoids metabolites are PLA_2, COX-2 and LOX. The overexpression of any one of these enzymes may affect cellular processes such as proliferation and apoptosis. PUFA may also regulate gene transcription factors which can affect cellular metabolism, growth and differentiation. In tumour cells, PLA_2, COX-2 and LOX are often overexpressed leading to overproduction of C20:4ω6-derived eicosanoids that

enhance inflammation, tumour cell proliferation, and inhibit apoptosis. Dietary ω3 PUFA, such as C20:5ω3 and C22:6ω3 and the ω6 FA C18:3ω6, counteract these pro-tumourigenic effects of C20:4ω6 by reducing the conversion of C18:2ω6 to C20:4ω6, regulating the incorporation of C20:4ω6 into membranes, decreasing the biosynthesis of C20:4ω6-derived eicosanoids, thereby suppressing inflammation, decreasing angiogenesis, stimulating apoptosis and up-regulating the expression of genes coding for antioxidant enzymes, thereby inhibiting tumour growth and cancer development (95). In this regard, the metabolic regulation of free C20:4ω6 is an important determinant of whether a cell undergoes proliferation, such as by a stimulatory signal via COX-2 over-expression and PGE_2 synthesis, or is directed into the apoptotic pathway via ceramide generation. The multiple regulating mechanisms of dietary PUFA, such as C18:3ω6 and the ω3 PUFA, in the control of cellular proliferation and apoptosis is therefore an important issue for health and disease.

Figure 26: Interactions between ω6 and ω3 FA in the stimulation or inhibition of cancer development. The ω3 FA can regulate the membrane content of C20:4ω6, as well as the availability of C20:4ω6 for conversion to eicosanoids by competitive inhibition as an alternate substrate in the synthesis of eicosanoids. C20:3ω6 can also inhibit the conversion of C20:4ω6 to eicosanoids and serve as an alternate substrate to less potent eicosanoid metabolites. The regulation of free C20:4ω6 metabolism is also important in directing cells to undergo (i) proliferation by stimulatory signaling via COX-2 over-expression and PGE$_2$ synthesis, (ii) or directing towards apoptosis via ceramide synthesis. The regulation of C20:4ω6 metabolism is important for suppressing inflammation, decreasing angiogenesis, stimulating apoptosis, gene expression, thereby inhibiting tumour growth and cancer development. Θ, inhibition; ⊕, stimulation; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; GLA, gamma(γ)-linolenic acid; AA, Arachidonic acid; PLA2, phospholipase A2; COX-2, cyclooxygenase-2; LOX, lipoxygenase, PUFA, polyunsaturated fatty acid(s) (adapted from 25 and 95).

9.   ω3 PUFA AND MODULATION OF CANCER

9.1.   Studies in Cell Cultures

There are many reports in the literature where the cytotoxic and/or anti-proliferative effects of PUFA have been identified in cell cultures. The anti-cancer effects of PUFA are specific for tumour cells with negligible or no effects on normal cells with the effectiveness of individual PUFA dependent on their carbon chain length and number of double bonds (159). These studies provided many answers as to the effectiveness and mechanisms by which ω3 PUFA affect cancer development. The value is that *in vitro* events can be monitored under controlled conditions and permits the investigation of interaction between specific cell types and dietary components.   However, direct extrapolation of information from cell culture to studies in animals and humans must be handled with care as the effects monitored may be too simplistic. *In vitro* studies involve cells which are removed from their natural environment, i.e. the whole organism as a unified system, which could exclude mechanisms not measurable in a culture environment, i.e. an isolated system. Another reason for the discrepancy between the *in vitro* and *in vivo* results may be the lack of interaction *in vitro* with other cell systems and integrative functions involved in the process of carcinogenesis. In general, cell lines can be useful to study mechanistic pathways and physiological processes, but care should be taken in interpretation of the results.

In vitro, a wide variety of cell types are affected and supplementation with both ω6 and ω3 PUFA are effective in killing cancer cells such as the LCPUFA, C18:3ω6, C20:4ω6, C20:5ω3 and C22:6ω3 (95, 159, 163, 174, 278). The effectiveness of the PUFA can be influenced by various factors listed in Table 3.

Table 3: *In vitro* conditions which may affect the growth and survival of cells in culture (159, 278).

Cell culture conditions	The exclusion or inclusion as well as concentration of serum or albumin in the culture medium.
Concentration range of PUFA	Opposite effects on cell proliferation have been observed, depending on a high or low concentration.
Saturation of PUFA	Generally, the more unsaturated a PUFA the more effect is observed.
Cell type	Varying effects have been noticed in different cell types in culture.
Cellular growth rate	Certain cell types may be more or less vulnerable to PUFA induced-toxicity than quiescent cells.

The varying effects observed in different cell types could be ascribed to differences in the metabolic status of the cell. For instance, due to the key role of the liver in FA metabolism, cultures derived from liver cells may respond more readily than other cell types due to specific FA transporters in the membrane. The mechanisms involved in PUFA induced cytotoxicity appear to be an alteration in oxidative stress by increased lipid peroxidation, a change in the type of eicosanoid synthesised, activation and induction of apoptosis and changes in membrane composition affecting membrane function and signaling pathways (159, 278). Whether the same mechanisms of cell death are effective *in vivo* is not yet clear.

9.2.   *In Vivo* Studies in Experimental Animals

The use of animal cancer models in representing the development and prevention of certain types of cancers relevant to humans, have aided the understanding of mechanisms involved in carcinogenesis. The most commonly used animals are rodents such as mice and rats because their physiological characteristics have been well documented. However, the use of experimental animals also has certain disadvantages (159). The different strains of rats and mice are genetically similar due to inbreeding as compared to the diversity found in humans. Although there are certain physiological similarities between rodents and humans, differences in metabolism can

result in different responses when exposed to the same carcinogen or dietary treatment. The carcinogen dosage or the dietary dose levels used are far removed from both rodent and human dietary normality, thereby creating additional problems in the interpretation of the rodent systems and the subsequent extrapolation to the human situation. However, animal cancer models still provide valuable insight into the effects of diet and cancer and have several advantages over *in vitro* models in studying carcinogenesis. They can for example be used to test hypotheses about mechanisms in a physiological context as well as the effectiveness of a substance in preventing carcinogenesis. Another advantage is the possibility to quantify the development of tumours in a short time.

With regards to liver cancer one of the frequently utilised animal models is the resistant hepatocyte rat model developed by Solt and Farber (344). This model is based on the induction of "resistant" hepatocytes during initiation by the carcinogen diethylnitrosamine (DEN) which develop into foci after a selective promotion step by 2-acetylaminofluorene (2-AAF) and partial hepatectomy as a growth or mitogenic stimulus. These foci eventually develop into preneoplastic nodules. Liver foci, visualized histologically by alteration in the enzymes γ-glutamyl transpeptidase (GGT) and/or glutathione-S-transferase placental form (GSTP), usually develop after 7 to 10 days randomly throughout the liver (22). The reference to a "resistant" hepatocyte or "resistant phenotype" refers to the resistance of an initiated altered liver cell to the growth inhibitory effect of another carcinogen, such as 2 acetylaminofluorene (2-AAF), thereby instilling an inherent growth advantage to the mutated cell compared to normal unaltered cells. Blumberg and London (345) suggested that hepatitis B infected liver may also initially induce "resistant" hepatocytes leading to the development of hepatocellular carcinoma. The multi-step nature of this model therefore lends itself to examining carcinogenesis at various stages.

A literature review conducted by Fay *et al.* (346) from studies involving 97 data sets from experimental animals *in vivo*, found that the ω6 FA had a strong tumour promoting/enhancing effect, MUFA such as C18:1ω9 had no significant effect, whereas the ω3 PUFA had a small protective effect. Studies in which the diet of tumour-bearing rodents were supplemented with ω3 oils containing C20:5ω3 and/or C22:6ω3 showed a reduced growth of various cancer types such as lung, colon, mammary and prostate (347). Black *et al.* (348) showed that UV-induced cancer in mice was enhanced when

fed a ω6 oil based diet, whereas an ω3 FA based diet negated the UV effect. In a study on tumour-bearing mice with cachexia-inducing colon cancer, C20:5ω3 inhibited weight loss, tumour growth rate, delayed tumour progression and survival rate was doubled (349). The growth of human colon tumour cells transplanted into mice was shown to be retarded when fed a ω3 FA rich diet, together with reduced C18:2ω6 and C20:4ω6 levels (350). The growth of breast tumours in rats and mice have also been shown to be inhibited by ω3 FA based diets and associated with increased lipid peroxidation and decreased prostaglandin synthesis (351, 352, 353). In rodent colon carcinogenesis models, dietary C20:5ω3 and C22:6ω3 inhibited the formation and growth of aberrant crypt foci, a marker for colon carcinogenesis, and a fish oil-based diet resulted in a lower incidence of adenocarcinoma compared to a corn oil diet by increasing apoptosis (161, 197, 354, 355). A study by Okuno *et al*. (356) demonstrated a decrease in the multiplicity of liver adenoma when rats were fed a perilla oil-based diet, rich in C18:3ω3. The ethyl ester forms of C20:5ω3 and C22:6ω3, supplemented into the diet, also demonstrated growth inhibition of Morris hepatoma cells transplanted into rats by decreased cell proliferation and increased apoptosis (301). These studies tend to confirm epidemiological evidence that ω3 PUFA are protective, whereas the ω6 PUFA promote cancer formation.

9.3. ω3 PUFA and Cancer in Humans

Various epidemiological and experimental studies have linked a high intake of ω3 PUFA with a decrease in incidences of cancer amongst humans (64). Most of the data are derived from studies investigating the relationship of fat and cancer of the colon, breast and prostate (60, 159, 196). The interest in ω3 PUFA started with studies by Dyerberg, Bang and Hjorne (11) in Greenland Eskimos/Inuits which indicated low incidence rates for cardiovascular disease as well as cancer amongst this population group. However, this trend appears to be shifting towards a Western pattern due to changes in lifestyle (357). Further studies looking at cancer incidence amongst, largely, high fish intake populations such as in Denmark and Japan, have supported the epidemiological link of a decreased incidence of cancer with a high dietary intake of ω3 PUFA (298, 299). Japanese immigrants to the USA who change their traditional diet to a more Western dietary pattern, i.e. changing from a low to a high ω6/ω3 FA dietary ratio, show an increase in the incidence of colon cancer (358).

The association of dietary fat with cancer was amongst the most striking early findings in the study of nutritional causes of cancer. An early and optimistic 1982 recommendation by the National Academy of Sciences, based on the best available evidence at that time, suggested that breast, colorectal, and prostate cancers were associated with dietary fat intake and that decreased fat intake may result in decreased rates of those cancers (53). The strong international correlations and animal studies of fat and carcinogenesis that formed the basis for this optimism still provide compelling evidence that lifestyle factors probably play an important role in the etiology of these cancers. However, results from analytic epidemiologic studies conducted since that time, have generally failed to reinforce these initial findings. In the case of breast cancer, there appears to be some evidence that olive oil or other sources of MUFA may modestly decrease risk (51).

Short-term intervention studies indicate that fish oil supplementation may protect against carcinogenesis in humans and that $\omega 6$ PUFA intake may increase the risk (160). The prospective New York University Women's Health Study demonstrated that certain fish types may be protective against developing colorectal cancer in women (297). A case-control study by Simonsen *et al.* (359) demonstrated the importance in considering the background dietary level of $\omega 6$ FA in relation to $\omega 3$ FA in breast cancer. When considering total $\omega 3$ PUFA, no significant inverse relationship was found for the risk of developing breast cancer. However, an inverse relationship was found when considering the ratio of $\omega 6$ to total $\omega 3$ FA. In South Africa, a high intake of fish was observed to protect fishermen from colorectal cancer compared to urban dwellers (360). A randomized clinical trial study by Gogos *et. al* (361) involving human patients with generalized solid tumours, demonstrated that the fish oil supplementation increased the survival time of all patients. In patients with sporadic adenomatous colorectal polyps, supplementation with C20:5$\omega 3$ and C22:6$\omega 3$ for 30 days decreased cell proliferation in the colon, associated with increased tissue levels of C20:5$\omega 3$ and C22:6$\omega 3$ and decreased C20:4$\omega 6$ (362). A recent case-control study by Maillard *et al.* (350) observed that $\omega 3$ PUFA exhibited a protective effect on breast cancer risk, depending on the $\omega 6/\omega 3$ FA ratio in breast adipose tissue (363).

The efficacy of dietary $\omega 3$ PUFA intervention can depend on the levels of dietary C20:4$\omega 6$ and the baseline patterns of cell proliferation (215). Epidemiological studies suggest a reduction in epithelial cell proliferation and PGE_2 biosynthesis in the colon,

associated with a low ω6/ω3 FA dietary ratio (337, 364). Earlier studies only considered total marine FA intake when examining effects on cancer in humans. The types of fish used in the studies were also not identified. However, later studies have started to address these shortfalls in specifying the type of marine FA as well as the level of ω6 FA, such as C20:4ω6 (364). The types of questionnaires also take in to account the various cooking methods. Animal studies and moreover, data from short-term human biomarker studies indicate a promising beneficial effect of fish oil supplementation, with high amounts of ω3 PUFA, on carcinogenesis. However, more human dietary intervention studies are needed to evaluate the real implication of ω3 PUFA supplementation, especially in relation to ω6 PUFA (ω6/ω3 FA ratio) with regards to tumour development in humans.

9.4.   ω6/ω3 Fatty Acid Dietary Ratios

Studies indicate that the dietary ω6/ω3 FA ratio, rather than the quantity of ω3 FA, appears to be the principle factor in the anti-cancer effect of these PUFA (3, 64). A study by Bartram *et al*. (365) found that a relatively low ω6/ω3 FA ratio was required to suppress PGE_2 synthesis in human rectal mucosa. Simonsen *et al*. (358) reported an inverse association with the ω6/ω3 FA ratio and the risk of breast cancer in adipose tissue biopsies. Rose *et al*. (366) reported that the incidence of breast cancer in Japanese women has increased over the last 40 years which correlates with an increased dietary ω6/ω3 FA ratio. The importance of the dietary ω6/ω3 FA ratio also appears to be important in the modulation eicosanoid biosynthesis from C20:4ω6 (64).

In addition to the ω6/ω3 FA ratio, the type of FA constituting this ratio needs to be considered. Different combinations of FA such as C18:3ω3/C18:2ω6, C18:3ω3/C18:3ω6, C20:5ω3 + C22:6ω3/C18:2ω6 and C20:5ω3 + C22:6ω3/C18:3ω6 have been suggested for the treatment of various diseases (170). However, certain metabolic effects make the use of some of these combinations unsuitable. These effects include the competitive inhibition of delta-6 desaturase by FA, level of dietary cholesterol and hormones such as catecholamines, insulin and corticosteroids affecting the delta-6 desaturase activity, as well as the impaired functioning of the desaturase enzyme in cancer tissue (169, 170). In this regard, the C20:5ω3 + C22:6ω3/C18:3ω6 combination appears to be the most effective FA mixture in overcoming these negative effects. All three FA comprising this combination bypass any inhibition or impaired function of the delta-6 desaturase.

As mentioned previously, both C20:5ω3 and C18:3ω6, via C20:3ω6, are precursors to beneficial eicosanoids and inhibit the conversion of C20:4ω6 to its eicosanoids (95, 170).

Although there is ample experimental evidence that ω3 FA can inhibit the progression of tumours in various organs and animal cancer models, the evidence from epidemiologic studies is less clear. The reason for this may be that the intake or tissue concentrations of specific marine FA did not account for the type of fish consumed which hampered the strength, consistency, and dose response effects regarding marine FA intake and human cancer prevention (160). Although most of the studies did not show an association between fish consumption or marine FA intake and the risk of hormone-related cancers, the results of the few studies from populations with a generally high intake of marine FA are encouraging.   Future epidemiologic investigations will benefit from the assessment of specific FA in the diet, including C20:5ω3 and C22:6ω3, the background tissue content of C20:4ω6 as well as specific ω6/ω3 FA ratios that have been examined infrequently in humans (160).

10. CONCLUSION

In today's societies, the increased availability of dietary fat and calories and the ever-decreasing rates of physical activity predispose our bodies to the increased risk of cardiovascular disease, diabetes, some types of cancer, hypertension, obesity etc. Worldwide there is a tendency to reintroduce or increase the level of ω3 FA in our diets because of the associated health benefits. The availability of ordinary foods fortified with small amounts of fish oils has been introduced commercially and varies from one country to another, but in general is increasing all the time, as awareness of the health benefits of the ω3 PUFA become known. Food products enriched with ω3 FA include milks, yoghurts, fruit juices, margarines, breads and eggs. However, it is important to maintain a balanced intake of ω6 FA, especially C20:4ω6, due to their importance for growth and development.   This raises the question of what the optimal balance between ω6 and ω3 dietary FA intake should be. Due to the competitive metabolism and incorporation into cell membranes of these two FA families, it is important to

balance their intake without depleting FA of the one type. Studies imply that the ideal ω6/ω3 FA ratio is in the order of 2:1 to 5:1 (3, 153). Suggested means of increasing dietary ω3 FA intake is by changing cooking oils from ω6 based, such as corn and sunflower oils, to ω3 based, such as perilla, flaxseed (linseed) and rapeseed oils. Eating fish two to three times a week would also increase ω3 FA intake (148, 153).

The increasing number of clinical and experimental studies on the ω3 FA reflects the growing awareness and importance of these components in our diet. The ω3 FA, in a specific ratio with ω6 FA, are proving to be not only important for normal growth and development but also for the prevention and treatment of certain diseases. In this regard, the wide influence range that dietary PUFA may exert on cells, structurally and functionally, is important. Structurally, PUFA may exert alterations in cell behaviour by their incorporation into membranes affecting cholesterol content and receptor activity. Functionally, as substrates for signaling metabolites, PUFA may affect cellular processes such as proliferation and apoptosis by influencing cellular signaling. However, much remains to be learned about the actions of PUFA in health and disease and many questions remain unanswered, such as what constitutes an optimum range of intake for ω3 FA? Does this range remain the same in different background diets with different amounts of ω6 and ω3 FA? Is the optimum range a reflection of absolute requirements or optimum FA ratios? How are requirements affected by different levels and types of dietary fat? These and many other questions motivate research to improve our fundamental understanding of biochemistry, health promotion and disease prevention.

REFERENCES

1.   Yeagle, P.L. 1989. Lipid regulation of cell membrane structure and function. FASEB J. 3, 1833-1842.

2.   Robertson, J.D. 1981. Membrane structure. J. Cell Biol. 91, 189s-204s.

3.   Simopolous, A.P. 2002. The importance of the ratio of omega-6/omega-3 essential fatty acids. Biomed. Pharmacother. 56, 365-379.

4.   Willett, W.C. 2000. Diet and cancer. The Oncologist 5, 393-404.

5.   Walker, A.R. and Segal, I. 1988. Colorectal cancer. Some aspects of epidemiology, risk factors, treatment, screening and survival. S. Afr. Med. J. 73, 653-657.

6.   Greenwald, P. 2001. From carcinogenesis to clinical interventions for cancer prevention. Toxicol. 166, 37-45.

7.   Reddy, B.S., Cohen, L.A., McCoy, D., Hill, P.B., Weisburger, J.H. and Wynder, E.L. 1980. Nutrition and its relationship to cancer. Adv. Cancer Res. 32, 237-345.

8.   National Academy of Sciences, National Research Council, Committee on Diet, Nutrition and Cancer. 1982. Diet, Nutrition and Cancer, National Academy Press, Washington, DC.

9.   Doll, R. and Peto, R. 1981. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. J. Natl. Cancer Inst. 66, 1191-1308.

10.   Greenwald, P., Clifford, C.K. and Milner, J.A. 2001. Diet and cancer prevention. Eur. J. Cancer 37, 948-965.

11.   Dyerberg, J., Bang, H.O. and Hjorne, N. 1975. Fatty acid composition of the plasma lipids in Greenland Eskimos. Am. J. Clin. Nutr. 28, 958-966.

12.   Evan, G.I. and Vousden, K.H. 2001. Proliferation, cell cycle and apoptosis in cancer. Nature 411, 342-348.

13.   Mareel, M. and Leroy, A. 2003. Clinical, cellular and molecular aspects of cancer invasion. Physiol. Rev. 83, 337-376.

14.   Klaunig, J.E. and Kamendulis, L.M. 2004. The role of oxidative stress in carcinogenesis. Annu. Rev. Pharmacol. Toxicol. 44, 239-267.

15.   Hanahan, D. and Weinberg, R.A. 2000. The hallmarks of cancer. Cell 100, 57-70.

16.   Schneider, B.L. and Kulesz-Martin, M. 2004. Destructive cycles: the role of genomic instability and adaptation in carcinogenesis. Carcinogenesis 25, 2033-2044.

17.   Wogan, G.N., Hecht, S.S., Felton, J.S., Conney, A.H. and Loeb, L.A. 2004. Environmental and chemical carcinogenesis. Sem. Cancer Biol. 14, 473-486.

18.   Mellors, R.C. 1999. Neoplasia. Cornell University Medical College. http://edcenter.med.cornell.edu/CUMC_PathNotes/Neoplasia/Neoplasia_01.html

19.   Incidence of histologically diagnosed cancer in South Africa, 1996-1997. National Cancer Registry of South Africa, National Health Laboratory Service, Johannesburg, 2003.

20.   American Cancer Society. Cancer facts and figures 2005. Atlanta: America Cancer Society; 2005.

21.   Williams, G.M. 2001. Mechanisms of chemical carcinogenesis and application to human cancer risk assessment. Toxicol. 166, 3-10.

22.   Farber, E. 1996. The step-by-step development of epithelial cancer: from phenotype to genotype. Adv. Cancer Res. 70, 21-48.

23.   Clurman, B.E. and Roberts, J.M. 1995. Cell cycle and cancer. J. Natl. Cancer Inst. 87, 1499-1501.

24.   Watson, W.H., Cai, J. and Jones, D.P. 2000. Diet and apoptosis. Annu. Rev. Nutr. 20, 485-505.

25.   Hanahan, D. and Weinberg, R.A. 2000. The hallmarks of cancer. Cell 100, 57-70.

26.   Nurse, P. 2000. A long twentieth century of the cell cycle and beyond. Cell 100, 71-78.

27.   Conklin, K.A. 2002. Dietary polyunsaturated fatty acids: impact on cancer chemotherapy and radiation. Alt. Med. Rev. 7, 4-21.

28.   Vermes, I., Haanen, C. and Reutelingsperger, C.P.M. 1998. Molecular biology of apoptosis and programmed cell death. In *Molecular Biology of free radicals in human diseases.* Aruoma, O.I. and Halliwell, B. eds. OICA International, Chelsea, London, UK. pp 225-286.

29.   Renehan, A.G., Booth, C. and Potten, C.S. 2001. What is apoptosis, and why is it important? BMJ 322, 1536-1538.

30.   Stoeber, K., Tlsty, T.D., Happerfield, L., Thomas, G.A., Romanov, S., Bobrow, L., Williams, E.D. and Williams, G.H. 2001. DNA replication licensing and human cell proliferation. J. Cell Science 114, 2027-2041.

31.   Gerl, R. and Vaux, D.L. 2005. Apoptosis in the development and treatment of cancer. Carcinogenesis 26, 263-270.

32.   Chang, W.C.L., Chapkin, R.S. and Lupton, J.R. 1997. Predictive value of proliferation, differentiation and apoptosis as intermediate markers for colon tumorigenesis. Carcinogenesis 18, 721-730.

33.   McDonnel, S.J., Meyn, R.E. and Robertson, L.E. 1995. Implications of apoptotic cell death regulation in cancer therapy. Cancer Biol. 6, 53-60.

34.   Thompson, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267, 1456-1462.

35.   Fadeel, B., Orrenius, S. and Zhivotovsky, B. 1999. Apoptosis in human disease: a new skin for the old ceremony? Biochem. Biophys. Res. Commun. 266: 699-717.

36.   Schulte-Hermann, R., Timmerman-Trosiener, I., Barthel, G. and Bursch, W. 1990. DNA synthesis, apoptosis, and phenotypic expression as determinants of growth of altered foci in rat liver during phenobarbital promotion. Cancer Res. 50, 5127-5135.

37.   Trauth, B.C., Klas, C., Peters, A.M.J., Matzku, S., Möller, P., Falk, W., Debatin, K.M. and Krammer, P.H. 1989. Monoclonal antibody-mediated tumour regression by induction of apoptosis. Science 245, 301-305.

38.   Rosenberg, S.A., Lotze, M.T., Yang, J.C., Aebersold, P.M., Linehan, W.M., Seipp, C.A. and White, D.E. 1989. Experience with the use of high-dose Interleukin-2 in the treatment of 652 cancer patients. Ann. Surgery 210, 474-485.

39.   Debatin, K.M. 2004. Apoptosis pathways in cancer and cancer therapy. Cancer Immunol. Immunother. 53, 153-159.

40.   Tamm, I., Schriever, F. and Dörken, B. 2001. Apoptosis: implications of basic research for clinical oncology. Lancet Oncol. 2, 33-42.

41.   Yu, J. and Zhang, L. 2003. Apoptosis in human cancer cells. Curr. Opin. Oncol. 16, 19-24.

42.   Lengauer, C., Kinzler, K.W. and Vogelstein, B. 1998. Genetic instabilities in human cancers. Nature 396, 643-649.

43.   Blume-Jensen, P. and Hunter, T. 2001. Oncogenic kinase signaling. Nature 411, 35-365.

44.   Czech, M.P. and Corvera, S. 1999. Signaling mechanisms that regulate glucose transport. J. Biol. Chem. 274, 1865-1868.

45.   Heine, R.J. and Schouten, J.A. 1989. The role of fat in the treatment of diabetes mellitus. In *The role of fats in human nutrition.* Vergroesen, A.J. and Crawford, M. eds. 2nd edition. Academic Press, London, UK. pp 45-79.

46.   Montori, V.M., Farmer, A., Wollan, P.C. and Dinneen, S.F. 2000. Fish oil supplementation in type 2 diabetes: a quantitative systematic review. Diabetes Care 23, 1407-1415.

47.   Nerbert, D.W. 2002. Transcription factors and cancer: an overview. Toxicol.181-182, 131-141.

48.   Downward, J. 2001. The ins and outs of signaling. Nature 411, 759-762.

49.   Sarkar, F.H. and Li, Y. 2004. Cell signaling pathways altered by natural chemopreventive agents. Mutation Research 555 (2004) 53–64

50.   Jump, D.B. 2004. Fatty acid regulation of gene transcription. Crit. Rev. Clin. Lab. Sci. 41, 41-78.

51.   Kushi, L. and Giovannucci, E. 2002. Dietary fat and cancer. Am. J. Med. 113, 63S-70S.

52.   Prentice, R.L., Pepe, M. and Self, S.G. 1989. Dietary fat and breast cancer: a quantitative assessment of the epidemiologic literature and a discussion of methodologic issues. Cancer Res. 49, 3147-3156.

53.   National Research Council (US), Committee on Diet and Health. 1989. *Diet and Health: Implications for Reducing Chronic Disease Risk.* Washington, DC: National Academy Press.

54.   Trowell, H. 1981. Hypertension, obesity, diabetes mellitus and coronary heart disease. In *Western diseases: their emergence and prevention*. Trowell, H.C. and Burkitt, D.P. eds. Edward Arnold Ltd., London, UK. pp 3-32.

55.   Burr, G.O. and Burr, M.M. 1929. A new deficiency disease produced by the rigid exclusion of fat from the diet. J. Biol. Chem. 82, 345-367.

56.   Burr, G.O. and Burr, M.M. 1930. On the essential nature and role of the fatty acids essential in nutrition. J. Biol. Chem. 86, 587-621.

57.   World Health Organisation, World Cancer Report, International Agency for Cancer Research, Lyon, 2003.

58.   Dommels, Y.E.M., Alink, G.M., van Bladeren, P.J. and van Ommen, B. 2002. Dietary n-6 and n-3 polyunsaturated fatty acids and colorectal carcinogenesis: results from cultured colon cells, animal models and human studies. Environ. Toxicol. Pharmacol. 12, 233-244.

59.   Simopoulos, A.P. 2001. The Mediterranean diets: what is so special about the diet of Greece? The Scientific Evidence. J. Nutr. 131, 3065S-3073S.

60.   Bartsch, H., Nair, J. and Owen, R.W. 1999. Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. Carcinogenesis 20, 2209-2218.

61.   Martin-Moreno, J.M., Willett, W.C., Gorgojo, L., Banegas, J.R., Rodriguez-Artalejo, F., Fernandez-Rodriguez, J.C., Maisonneuve, P. and Boyle, P. 1994. Dietary fat, olive oil intake and breast cancer risk. In. J. Cancer 58, 774-780.

62.   Trichopoulou, A., Katsouyanni, K., Stuver, S., Tzala, L., Gnardellis, C., Rimm, E. and Trichopoulo, D. 1995. Consumption of olive oil and specific food groups in relation to breast cancer risk in Greece. J. Natl. Cancer Inst. 87, 110-116.

63.   Sporn, M.B., Dunlop, N.M., Newton, D.L. and Smith, J.M. 1976. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Fed. Proc. 35, 1332-1338.

64.   Rose, D.P. and Connolly, J.M. 1999. Omega-3 fatty acids as cancer chemopreventive agents. Pharmacol. Ther. 83, 217-244.

65.   Rose, D.P. 1997. Dietary fatty acids and cancer. Am. J. Clin. Nutr. 66, 998-1003.

66.   Ganong, W.F. 1987. Review of Medical Physiology, Appleton and Lange, Lange Medical Publications, Connecticut, USA.

67.   Tso, P. 1985. Gastrointestinal digestion and absorption of lipid. Adv. Lipid Res. 21, 143-179.

68.   Friedman, H.I. and Nyland, B. 1980. Intestinal fat digestion, absorption and transport. Am. J. Clin. Nutr. 33, 1108-1139.

69.   Stremmel, W., Lotz, G., Strohmeyer, G. and Berk, P.D. 1985. Identification, isolation, and partial characterization of a fatty acid binding protein from rat jejunal microvillus membranes. J. Clin. Invest. 75, 1068-1076.

70.   Schulthess, G., Lipka, G., Compassi, S., Boffelli, D., Weber, F.E. and Paltauf, F. 1994. Absorption of monoacylglycerols by small intestinal brush border membranes. Biochem. 33, 4500-4508)

71.   Assman, G. 1982. Lipid metabolism and atherosclerosis. FK Schattauer Verlag. Stuttgart, Germany.

72.   Kane, J.P., Chen, G.C., Hamilton, R.L., Hardman, D.A., Malloy, M.J. and Havel, R.J. 1983. Remnants of lipoproteins of intestinal and hepatic origin in familial dysbetalipoproteinemia. Arteriosclerosis 3, 47-56.

73.   Havel, R.J. 1985. Role of the liver in atherosclerosis. Arteriosclerosis 5, 569-580.

74.   Nelson, G.J. and Ackman, R.G. 1988. Absorption and transport of fat in mammals with emphasis on n-3 polyunsaturated fatty acids. Lipids 23, 1005-1014.

75.   Worman, H.J. 1999. Diseases of the Liver. In *The liver disorders sourcebook*. Worman, H.J. ed. Lowell House, NTC/Contemporary Publishing Group, Lincolnwood (Chicago), Illinois, USA. pp 67.

76.   Funari, S.S., Barceló, F. and Escribá, P.V. 2003. Effects of oleic acid and its congeners, elaidic and stearic acids, on the structural properties of phosphatidylethanolamine membranes. J. Lipid Res. 44, 567-575.

77.   Fenton, W.S., Hibbeln, J. and Knable, M. 2000. Essential fatty acids, lipid membrane abnormalities, and the diagnosis and treatment of schizophrenia. Biol. Psychiatry 47, 8-21.

78.   Pereira, S.L., Leonard, A.E. and Mukerji, P. 2003. Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. Prost. Leukot. Essent. Fatty Acids 68, 97-106.

79.   Leonard, A.E., Pereira, S.L., Sprecher, H. and Huang, Y.S. 2004. Elongation of long-chain fatty acids. Prog. Lipid Res. 43, 36-54)79

80.   Llor, X., Pons, E., Roca, A., Àlvarez, M., Mañe, J., Fernández-Bañares, F. and Gassull, M.A. 2003. The effects of fish oil, olive oil, oleic cid and linoleic acid on colorectal neoplastic processes. Clin. Nutr. 22, 71-79.

81.   Simopoulos, A.P. 1991. Omega-3 fatty acids in health and disease and in growth and development. Am. J. Clin. Nutr. 54, 438-463.

82.   Whelan, J. and McEntree, M.F. 2004. Dietary (n-6) PUFA and intestinal tumourigenesis. J. Nutr. 134, 3421S-3426S.

83.   Fogerty, A.C., Evans, A.J., Ford, G.L. and Kennett, B.H. 1986. Distribution of n-6 and n-3 fatty acids in lipid classes in Australian fish. Nutrition Reports International 33, 777-787.

84.   Best, B. Fats you need - Essential Fatty Acids. Online article, http://www.benbest.com/health/essfat.html.

85.   Omega-3 Information Service website. The fats of life. http://www.omega-3info.com/wheredoifind.html

86.   Sprecher, H. 2000. Metabolism of highly unsaturated n-3 and n-6 fatty acids. Biochim. Biophys. Acta 1486, 219-231.

87.   Horrobin, D.F. 1990. Gamma-linolenic acid: an intermediate in fatty acid metabolism with potential as an ethical pharmaceutical and as a food. Rev. Contemp. Pharmacother. 1, 1-45.

88.   Mohrhauer, H. and Holman, R.T. 1963. The effect of dose level of essential fatty acids upon fatty acid composition of the rat liver. J. Lipid Res. 4, 151-159.

89.   Holman, R.T.1964. Nutritional and metabolic interrelationships between fatty acids. Fed. Proc. 23, 1062-1067.

90.   Zhou, L. and Nilsson, Å. 2001. Sources of eicosanoid precursor fatty acid pools in tissues. J. Lipid Res. 42, 1521-1542.

91.   Cho, H.P., Nakamura, M.T. and Clarke, S.D. 1999. Cloning, expression, and nutritional regulation of the mammalian delta-6 desaturase. J. Biol. Chem. 274, 471-477.

92.   Bourre, J.M., Piciotti, M. and Dumont, O. 1990. Delta 6 desaturase in brain and liver during development and aging. Lipids 25, 354-356.

93.   Brenner, R.R. 1989. Factors influencing fatty acid chain elongation and desaturation. In *The role of fats in human nutrition*. Vergroesen, A.J. and Crawford, M. eds. 2nd edition. Academic Press, London, UK. pp 45-79.

94.   Scott, B.L. and Bazan, N.G. 1989. Membrane docosahexaenoate is supplied to the developing brain and retina by the liver. Proc. Natl. Acad. Sci. USA. 86, 2903-2907.

95.   Larsson, S.C., Kumlin, M., Ingleman-Sundberg, M. and Wolk, A. 2004. Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. Am. J. Clin. Nutr. 79, 935-945.

96.   Simopolous, A.P. 1991. Omega-3 fatty acids in health and disease and in growth and development. Am. J. Clin. Nutr. 54, 438-463.

97.   Leikin, A. and Shinitzky, M. 1995. Characterization of the lipid surrounding the Δ^6-desaturase of rat liver microsomes. Biochim. Biophys. Acta 1256, 13-17.

98.   Qiu, X. 2003. Biosynthesis of docosahexaenoic acid (DHA, 22:6-4, 7,10,13,16,19): two distinct pathways. Prost. Leukot. Essent. Fatty Acids 68, 181-186.

99.   Nakamura, M.T. and Nara, T.Y. 2003. Essential fatty acid synthesis and its regulation in mammals. Prost. Leukot. Essent. Fatty Acids 68, 145-150.

100.   Cunnane, S.C. 2003. Problems with essential fatty acids: time for a new paradigm? Prog. Lipid Res. 42, 544-568.

101.   Sprecher, H. 1968. The synthesis and metabolism of hexadeca-4,7,10-trienoate, eicosa-8,11,14-trienoate, docosa-10,13,16-trienoate and docosa-6,9,12,15-tetranoate in the rat. Biochim. Biophys. Acta 152, 519-530.

102.   Cunnane, S.C., Ryan, M.A., Craig, K.S., Brookes, S., Koletzko, B., Demmelmair, H., Singer, J. and Kyle, D.J. 1995. Synthesis of linoleate and alpha-linolenate by chain elongation in the rat. Lipids 30, 781-783)

103.   Sinclair, H.M. 1984. Essential fatty acids in perspective. Hum. Nutr. Clin. Nutr. 38C, 245-260.

104.   Calder, P.C. 2002. Dietary modification of inflammation with lipids. Proc. Nutr. Soc. 61, 345-358.

105.   Jump, D.B. 2002. The biochemistry of n-3 polyunsaturated fatty acids. J. Biol. Chem. 277, 8755-8758.

106.   Duplus, E., Glorian, M. and Forest, C. 2000. Fatty acid regulation of gene transcription. J. Biol. Chem. 275, 30749-30752.

107.   Singer, S.J. and Nicholson, G.L. 1972. The fluid mosaic model of the structure of cell membranes. Science 175, 720-731.

108. Gordesky, S.E. and Marinetti, G.V. 1973. The asymmetric arrangement of phospholipids in the erythrocyte membrane. Biochem. Biophys. Res. Comm. 50, 1027-1031.

109. Daleke, D.L. 2003. Regulation of transbilayer plasma membrane phospholipid asymmetry. J. Lipid Res. 44, 233-242.

110. Shohet, S.B. and Beutler, E. 1983. The red cell membrane. In *Hematology.* Williams, W.J., Beutler, E., Ersley, A.J. and Lichtman, M.A. eds. 3rd edition. McGraw-Hill Book Company, New York, USA. pp 345.

111. Rothman, J.E. and Lenard, J. 1977. Membrane asymmetry. Science 195, 743-753.

112. Pomorski, T., Hrafnsdóttir, S., Devaux, P.F. and van Meer, G. 2001. Lipid distribution and transport across cellular membranes. Sem. Cell Dev. Biol. 12, 139-148.

1113. Rudolph, I.L., Kelley, D.S., Klasing, K.C. and Erickson, K.L. 2001. Regulation of cellular differentiation and apoptosis by fatty acids and their metabolites. Nutr. Res. 21, 381-393.

114. Darnell, J., Lodisch, H. and Baltimore, D. 1986. Molecular Cell Biology, Scientific American Inc. pp 576-577.

115. Singh, R.P., Kumar, R. and Kapur, N. 2003. Molecular regulation of cholesterol biosynthesis: implications in carcinogenesis. J. Environ. Pathol. Toxicol. Oncol. 22, 75-92.

116. Rao, K.N. 1995. The significance of the cholesterol biosynthesis pathway in cell growth and carcinogenesis. Anticancer Res. 15, 309-314.

117. Mahler, S.M., Wilce, P.A. and Shanley, B.C. 1988. Studies on regenerating liver and hepatoma plasma membranes - II. Membrane fluidity and enzyme activity. Int. J. Biochem. 20, 613-619.

118. Balazy, M. 2004. Eicosanomics: targeted lipidomics of eicosanoids in biological systems. Prost. Lipid Mediat. 73, 173-180.

119. Pike, L.J. 2003. Lipid rafts: bringing order to chaos. J. Lipid Res. 44, 655-667.

120. Pike, L.J., Han, X., Chung, K-N. and Gross, R.W. 2002. Lipid rafts are enriched in arachidonic acid and plasmenylethanolamine and their composition is independent of caveolin-1 expression: a quantitative electrospray ionization/mass spectrometric analysis. Biochemistry 41, 2075-2088.

121. Zajchowski, L.D. and Robbins, S.M. 2002. Lipid rafts and little caves: Compartmentalized signaling in microdomains. Eur. J. Biochem. 269, 737-752.

122. Zhuang, L., Lin, J., Lu, M.L., Solomon, K.R. and Freeman, M.R. 2002. Cholesterol-rich lipid rafts mediate Akt-regulated survival in prostate cancer cells. Cancer Res. 62, 2227-2231.

123.   Simons, K. and Vaz, W.L.C. 2004. Model systems, lipid rafts, and cell membranes. Annu. Rev. Biophys. Struct. 33, 269-295.

124.   Marks, F. and Fürstenberger, G. 1999. Prostaglandins, Leukotrienes and Other Eicosanoids. From Biogenesis to Clinical Application. Wiley-VCH, Weinheim, New York, USA.

125.   Smith, W.L. and Borgeat, P. 1985. The eicosanoids: Prostaglandins, thromboxanes, leukotrienes, and hydroxyl-eicosanoic acids. In *Biochemistry of lipids and membranes.* Vance, D.E. and Vance, J.E. eds. The Benjamin/Cummings Publishing Co., Inc. Menlo Park, California, USA. pp 325.

126.   Groene, H.J. and Dunn, M.J. 1985. The role of prostaglandins in arterial hypertension: A critical review. Adv. Nephro. 14, 241-272.

127.   Lands, W.E.M. 1989. Differences in n-3 and n-6 eicosanoid precursors. In *Advances in Prostaglandins, Thromboxanes and Leukotriene Research.* Samuelsson, B., Wong, P.Y.K. and Sun, F.F. eds. Raven Press, New York, USA, 19. pp 602-605.

128.   Serhan, C.N. and Oliw, E. 2001. Unorthodox routes to prostanoid formation: new twists in cyclooxygenase-initiated pathways. J. Clin. Invest. 107, 1481-1489.

129.   Marks, F., Müller-Decker, K. and Fürstenberger, G. 2000. A causal relationship between unscheduled eicosanoid signaling and tumour development: cancer chemoprevention by inhibitors of arachidonic acid metabolism. Toxicol. 153, 11-26.

130.   Hu, K-Q. 2003. Cyclooxygenase 2 (COX2)-prostanoid pathway and liver diseases. Prostaglandins Leukot. Essent. Fatty Acids 69, 329-337.

131.   Williams, C.S., Mann, M. and DuBois, R.N. 1999. The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 18, 7908-7916.

132.   Fosslien, E. 2000. Biochemistry of cyclooxygenase (COX)-2 inhibitors and molecular pathology of COX-2 in neoplasia. Crit. Rev. Clin. Lab. Sci. 37, 431-502.

133.   Piomelli, D. 1996. Arachidonic acid in cell signaling. Springer, New York.

134.   Horrobin, D.F. 1992. Nutritional and medical importance of gamma-linolenic acid. Prog. Lipid Res. 31, 162-194.

135.   Hong, S., Gronert, K., Devchand, P.R., Moussignac, R-L. and Serhan, C.N. 2003. Novel docosatrienes and 17*S*-Resolvins generated from docosahexaenoic acid in murine brain, human blood, and glial cells. J. Biol. Chem. 278, 14677-14687.

136.   Gao, X. and Honn, K.V. 1995. Biological properties of 12(*S*)-HETE in cancer metastases. Adv. Protagl. Thrombox. Leukotr. Res. 23, 439-444.

137. Tang, D.G., Chen, J.Q. and Honn, K.V. 1996. Arachidonate lipoxygenases as essential regulators of cell survival and apoptosis. Proc. Natl. Acad. Sci. 93, 5241-5246.

138. Ghosh, J. and Myers, C.E. 1998. Inhibition of arachidonate 5-lipoxygenase triggers massive apoptosis in human prostate cancer cells. Proc. Natl. Acad. Sci. USA 95, 13182-13187.

139. Ghosh, J. and Myers, C.E. 1997. Arachidonic acid stimulates prostate cancer cell growth: critical role of 5-lipoxygenase. Biochem. Biophys. Res. Commun., 235, 418-423.

140. Hwang, D. and Rhee, S.H. 1999. Receptor-mediated signaling pathways: potential targets of modulation by dietary fatty acids. Am. J. Clin. Nutr. 70, 545-556.

141. Kauwell, G.P.A. 2005. Emerging concepts in nutrigenomics: a preview of what is to come. Nutr. Clin. Prac. 20, 75-87.

142. Wertz, P.W., Cho, E.S. and Downing, D.T. 1983. Effect of essential fatty acid deficiency on the epidermal sphingolipids of the rat. Biochim. Biophys. Acta 753, 350-355.

143. Gray, G.M., White, R.J. and Majer, J.R. 1978. 1-(3'-O-acyl)-beta-glucosyl-N-dihydroxypentatriacontadienoylsphingosine, a major component of the glucosylceramides of pig and human epidermis. Biochim. Biophys. Acta 528, 127-137.

144. Hansen, H.S. and Jensen, B. 1985. Essential function of linoleic acid esterified in acylglucosylceramide and acylceramide in maintaining the epidermal water permeability barrier. Evidence from feeding studies with oleate, linoleate, arachidonate, columbinate and alpha-linolenate. Biochim. Biophys. Acta 834, 357-363.

145. Bjerve, K.S., Mostad, I.L. and Thoresen, L. 1987. Alpha-linolenic acid deficiency in patients on long-term gastric-tube feeding: estimation of linolenic acid and long-chain unsaturated n-3 fatty acid requirement in man. Am. J. Clin. Nutr. 45, 66-77.

146. Bjerve, K.S., Thoresen, L. and Borsting, S. 1988. Linseed and cod liver oil induce rapid growth in a 7-year-old girl with N-3 fatty acid deficiency. J. Parenter. Enteral. Nutr. 12, 521-525.

147. Holman, R.T. 1960. The ratio of trienoic:tetraenoic acids in tissue lipids as a measure of essential fatty acid requirement. J. Nutr. 60, 405-410.

148. Simopoulos, A.P. 2003. Omega-3 fatty acids and cancer. Indoor Built. Environ. 12, 405-412.

149. Mensink, R.P. and Katan, M.B. 1990. Effect of dietary trans fatty acids on high-density and low-density lipoprotein cholesterol levels in healthy subjects. N. Engl. J. Med. 323, 439-445.

150. Larqué, E., Zamora, S. and Gil, A. 2001. Dietary *trans* fatty acids in early life: a review. Early Human Dev. 65, S31-S41.

151. Connor, W.E. 2000. Importance of n-3 fatty acids in health and disease. Am. J. Clin. Nutr. 71, 171S-175S.

152. Simopoulos, A.P. 1999. Essential fatty acids in health and chronic disease. Am. J. Clin. Nutr. 70(suppl), 560S-569S.

153. Simopoulos, A.P., Leaf, A. and Salem, N. Jr. 2000. Workshop statement on the essentiality of and recommended dietary intakes for omega-6 and omega-3 fatty acids. Prost. Leukot. Essent. Fatty Acids 63, 119-121.

154. Kris-Etherton, P.M., Harris, W.S. and Appel, L.J. 2002. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Circulation 106, 2747-2757.

155. Ahrens, E.H., Blankenhorn, D.H. and Tsaltas, T.T. 1954. Effect on human serum lipids of substituting plant for animal fat in the diet. Proc. Soc. Exp. Biol. Med. 86, 872-878.

156. Keys, A., Anderson, J.T. and Grande, F. 1957. Serum cholesterol response to dietary fat. Lancet i, 787.

157. Maes, M., Smith, R., Christophe, A., Vandoolaeghe, E., Van Gastel, A., Neels, H., Demedts, P., Wauters, A. and Meltzer, H.Y. 1997. Lower serum high-density lipoprotein cholesterol (HDL-C) in major depression and in depressed men with serious suicidal attempts: relationship with immune-inflammatory markers. Acta Psychiatr. Scand. 95, 212-221.

158. Borkman, M., Storlien, L.H., Pan, D.A., Jenkins, A.B., Chisholm, D.J. and Campbell, L.V. 1993. The relation between insulin sensitivity and the fatty acid composition of skeletal-muscle phospholipids. N. Engl. J. Med. 328, 238-44.

159. Diggle, C.P. 2002. In vitro studies on the relationship between polyunsaturated fatty acids and cancer: tumour or tissue specific effects? Prog. Lipid Res. 41, 240-253.

160. Terry, P.D., Rohan, T.E. and Wolk, A. 2003. Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: a review of the epidemiologic evidence. Am. J. Clin. Nutr. 77, 532-543.

161. Bougnoux, P. 1999. n-3 polyunsaturated fatty acids and cancer. Curr. Opin. Clin. Nutr. Metab. Care 2, 121-126.

162. de Deckere, E.A. 1999. Possible beneficial effect of fish and fish n-3 polyunsaturated fatty acids in breast and colorectal cancer. Eur. J. Cancer Prev. 8, 213-221.

163. Horrobin, D.F. 1994. Unsaturated lipids and cancer. In *New approaches to cancer treatment: unsaturated lipids and photodynamic therapy*. Horrobin, D.F. ed. Churchill Communications, Nordic House, London. pp 3-64.

164. Crawford, M.A, Doyle, W., Williams, G. and Drury, P.J. 1989. The role of fats and EFAs for energy and cell structures in the growth of fetus and neonate. In *The role of fats in human nutrition.* Vergroesen, A.J. and Crawford, M. eds. 2nd edition. Academic Press, London, UK. pp 45-79.

165. Tocher, D.R., Leaver, M.J. and Hodgson, P.A. 1998. Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. Prog. Lipid Res. 37, 73-117.

166. Burgess, J.R., Stevens, L., Zhang, W. and Peck, L. 2000. Long-chain polyunsaturated fatty acids in children with attention-deficit hyperactivity disorder. Am. J. Clin. Nutr. 71, 327S-330S.

167. Gardiner, N.S. and Duncan, J.R. 1991. Possible involvement of delta-6-desaturase in control of melanoma growth by gamma-linolenic acid. Prostaglandins, Leukot. Essent. Fatty Acids 42, 149-153.

168. Hrelia, S., Bordoni, A., Biagi, P.L., Galeotti, T., Palombini, G. and Masotti, L. 1994. The role of delta-6- and delta-9-desaturase in the fatty acid metabolism of hepatomas with different growth rate. Biochem. Mol. Biol. Int. 34, 449-455.

169. Horrobin, D.F. 1993. Fatty acid metabolism in health and disease - the role of delta-6-desaturase. Am. J. Clin. Nutr. 57, S732-S737.

170. Huang, Y.-S. and Nassar, B.A. 1990. Modulation of tissue fatty acid composition, prostaglandin production and cholesterol levels by dietary manipulation of n-3 and n-6 essential fatty acid metabolites. In *Omega-6 essential fatty acids: Pathphysiology and Roles in Clinical Medicine*. Horrobin, D.F. ed. Alan R. Liss, Inc, New York, USA. pp. 127-144.

171. Ladas, E.J., Jacobson, J.S., Kennedy, D.D., Teel, K., Fleischauer, A. and Kelly, K.M. 2004. Antioxidants and cancer therapy: a systematic review. J. Clin. Oncol. 22, 517-528.

172. Ji, C., Rouzer, C.A., Marnett, L.J. and Pietenpol, J.A. 1998. Induction of cell cycle arrest by the endogenous product of lipid peroxidation, malondialdehyde. Carcinogenesis 19, 1275-1283.

173. Begin, M.E., Das, U.N., Ells, G. and Horrobin, D.F. 1985. Selective killing of human cancer cells by polyunsaturated fatty acids. Prost. Leukot. Essent. Fatty Acids 19, 177-186.

174. Begin, M.E., Ells, G., Das, U.N. and Horrobin, D.F. 1986. Differential killing of human carcinoma cells supplemented with n-3 and n-6 polyunsaturated fatty acids. J. Natl. Cancer Inst. 77, 1053-1062.

175. Hawkins, R.A., Sangster, K. and Arends, M.J. 1998. Apoptotic death of pancreatic cancer cells induced by polyunsaturated fatty acids varies with double bond number and involves an oxidative mechanism. J. Path. 185, 61-70.

176. Finstad, H.S., Drevon, C.A., Kulseth, M.A., Synstad, A.V., Knudsen, E. and Kolset, S.O. 1998. Cell proliferation, apoptosis and accumulation of lipid droplets in U937-1 cells incubated with eicosapentaenoic acid. Biochem. J. 336, 451-459.

177. Esterbauer, H., Schaur, R.J. and Zollner, H. 1991. Chemistry and biochemistry of 4-hydroxynonenals, malonaldehyde, and related aldehydes. Free Radical Biol. Med. 11, 81-128.

178. Dianzani, M.U. 1993. Lipid peroxidation and cancer. Crit. Rev. Oncol. Hematol. 15, 125-147.

179. Davidson, L.A., Lupton, J.R., Jiang, Y-H. And Chapkin, R.S. 1999. Carcinogen and dietary lipid regulate ras expression and localization in rat colon without affecting farnesylation kinetics. Carcinogenesis 20, 785-791.

180. Das, U.N. 1999. Essential fatty acids, lipid peroxidation and apoptosis. Prost. Leukot. Essential Fatty Acids 61, 157-163.

181. Joubert, A.M., Panzer, A., Joubert, F., Lottering, M.-L., Bianchi, P.C. and Seegers, J.C. 1999. Comparative study of the effects of polyunsaturated fatty acids and their metabolites on cell growth and tyrosine kinase activity in oesophageal carcinoma cells. Prost. Leukot. Essential Fatty Acids 61, 171-182.

182. Alexander, J.W. 1998. Immunonutrition: The role of ω-3 fatty acids. Nutrition 14, 627-633.

183. Slater, S.J., Kelly M.B., Taddeo, F.J., Ho, C., Rubin, E. and Stubbs, C.S. 1994. The modulation of protein kinase C activity by membrane lipid bilayer structure. J. Biol. Chem. 269, 4866-4871.

184. Nishizuka, Y. 1995. Protein kinase C and lipid signaling for sustained cellular responses. FASEB 9, 484-496.

185. Pajari, A. and Mutanen, M. 1999. Phospholipid fatty acid composition and protein kinase C activity in the large intestine of rats fed on butter and coconut-oil diets. Br. J. Nutr. 82, 411-418.

186. Shimabukkuro, M., Wang, M.Y., Zhou, Y.T., Newgard, C.B. and Unger, R.H. 1998. Protection against lipoapoptosis of beta cells through leptin dependent maintenance of BCL-2 expression. Proc. Natl. Acad. Sci. 95, 9558-9561.

187. Tyurina, Y.Y., Tyurina, Y.A., Certa, G., Quin, P.J., Schor, N.F. and Kagn, V.E. 1997. Direct evidence for antioxidant effect of BCL-2 in PC12 rat phacochromocytone cells. Arch. Biochem. Biophys. 344, 413-423.

188. Cao, Y., Pearman, A.T., Zimmerman, G.A., McIntyre, T.M. and Prescott, S.M. 2000. Intracellular unesterified arachidonic acid signals apoptosis. Proc. Natl. Acad. Sci. 97, 11280-11285.

189. Halder, S., Jena, N. and Croce, C.M. 1995. Inactivation of BCL-2 by phosphorylation. Proc. Natl. Acad. Sci. 92, 4507-4511.

190. Takahashi, M., Tsuboyama-Kasaoka, N., Nakatani, T., Ishii, M., Tsutsumi, S., Aburatani, H. and Ezaki, O. 2002. Fish oil feeding alters liver gene expressions to defend against PPAR-α activation and ROS production. Am. J. Physiol. Gastrointest. Liver Physiol. 282, G338-G348.

191. Fujimoto, Y, Uno, E. and Sakuma, S. 2004. Effects of reactive oxygen and nitrogen species on cyclooxygenase-1 and -2 activities. Prost. Leukot. Essent. Fatty Acids 71, 335-340.

192. Pervaiz, S. 2002. Anti-cancer drugs of today and tomorrow: are we close to making the turn from treating to curing cancer? Curr. Pharm. Des. 8, 1723-1734.

193. Buchwald, H. 1992. Cholesterol inhibition, cancer and chemotherapy. Hypothesis. Lancet 339, 1154-1156.

194. Abel, S., Smuts, C.M., De Villiers, C. and Gelderblom, W.C.A. 2001. Changes in essential fatty acid patterns associated with normal liver regeneration and the progression of hepatocyte nodules in rat hepatocarcinogenesis. Carcinogenesis 22, 795-804.

195. Garg, M.L., Wierzbicki, A.A., Thomson, A.B. and Clandinin, M.T. 1988. Fish,oil reduces cholesterol and arachidonic acid content more efficiently in rats fed diets containing low linolenic acid to saturated fatty acid ratios. Biochim. Biophys. Acta 962, 337-344.

196. Reddy, B.S. 1994. Chemoprevention of colon cancer by dietary fatty acids. Cancer Metastasis Rev. 13, 285-302.

197. Hong, M.Y., Chang, W.C.L., Chapkin, R.S. and Lupton, J.R. 1997. Relationship between colonocyte proliferation, differentiation and apoptosis as a function of diet and carcinogen. Nutr. Cancer 28, 20-29.

198. Colquhoun, A. and Schumacher, R.I. 2001. Gamma-linolenic acid and eicosapentaenoic acid induce modification in mitochondrial metabolism, reactive oxygen species generation, lipid peroxidation and apoptosis in Walker 256 rat carcinosarcoma cells. Biochim. Biophys. Acta 1533, 207-219.

199. Colquhoun, A. 2002. Gamma-linolenic acid alters the composition of mitochondrial membrane subfractions, decreases outer mitochondrial membrane binding of hexokinase and alters carnitine palmitoyltransferase I properties in the Walker 256 rat tumour. Biochim. Biophys. Acta 1583, 74-84.

200. Colquhoun, A. and Schumacher, R.I. 2001. Modifications in mitochondrial metabolism and ultrastructure and their relationship to tumour growth inhibition by gamma-linolenic acid. Mol. Cell Biochem. 218, 13-20.

201. Nwankwo, J.O. 2001. Repression of cellular anaplerosis as the hypothesized mechanism of gamma linolenic-induced cytotoxicity to tumour cells. Med. Hyotheses 56, 582-588.

202. Diaz, B.L. and Arm, J.P. 2003. Phospholipase A$_2$. Prost. Leukot. Essent. Fatty Acids 69, 87-97.

203. Balsinde, J., Winstead, M.V. and Dennis, E.A. 2002. Phospholipase A2 regulation of arachidonic acid mobilization. FEBS Letters 531, 2-6.

204. Laye, J.P. and Gill, J.H. 2003. Phospholipase A2 expression in tumours: a target for therapeutic intervention? DDT 8, 71-716.

205. Jayadev, S., Hayter, H.L., Andrieu, N., Gamard, C.J., Liu, B., Balu, R., Hayakawa, M., Ito, F. and Hannun, Y.A. 1997. Phospholipase A(2) is necessary for tumor-necrosis-factor alpha-induced ceramide generation in 1929 cells. J. Biol. Chem. 272, 17196-17203.

206. Mahdani, N-E. E., Ameyar, M., Cai, Z., Colard, O., Masliah, J. and Chouaib, S. 2000. Resistance to TNF-induced cytotoxicity correlates with an abnormal cleavage of cytosolic phospholipase A_2. J. Immun. 165, 6756-6761.

207. Cai, Z., Bettaieb, A., Mahdani, N.E., Legrès, L.G., Stancou, R., Masliah, J. and Chouaib, S. 1997. Alteration of the sphingomyelin/ceramide pathway is associated with resistance of human breast carcinoma MCF7 cells to TNFα mediated cytotoxicity. J. Biol. Chem. 272, 6918-6926.

208. Mahdani, N-E. E., Ameyar, M., Cai, Z., Colard, O., Masliah, J. and Chouaib, S. 2000. Resistance to TNF-induced cytotoxicity correlates with an abnormal cleavage of cytosolic phospholipase A_2. J. Immun. 165, 6756-6761.

209. Hayakawa, M., Ishida, N., Takeuchi, K., Shibamoto, S., Hori, T., Oku, N., Ito, F. and Tsujimoto, M. 1993. Arachidonic acid-selective cytosolic phospholipase A2 is crucial in the cytotoxic action of tumor necrosis factor. J. Biol. Chem. 268, 11290-11295.

210. Sapirstein, A., Spech, R.A., Witzgall, R. And Bonventre, J.V. 1996. Cytosolic phospholipase A_2 (PLA_2), but not secretory PLA_2, potentiates hydrogen peroxide cytotoxicity in kidney epithelial cells. J. Biol. Chem. 271, 21505-21513.

211. Reid, T., Ramesha, C.S. and Ringold, G.M. 1991. Resistance to killing by tumor necrosis factor in an adipocyte cell line caused by a defect in arachidonic acid biosynthesis. J. Biol. Chem. 266, 16580-16586.

212. Romano, M. and Clàra, J. 2003. Cyclooxygenase-2 and 5-lipoxygenase converging functions on cell proliferation and tumour angiogenesis: implications for cancer therapy. FASEB J. 17, 1986-1995.

213. Sano, H., Kawahito, Y. Wilder, R.L., Hashiramoto, A., Mukai, S., Asai, K., Kimura, S., Kato, H., Kondo, M. and Hla, T. 1995. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res. 55, 3785-3789.

214. Petrik, M., McEntee, M., Chiu, C. and Whelan, J. 2000. Antagonism of arachidonic acid is linked to the antitumorigenic effect of dietary eicosapentaenoic acid in Apc(Min/+) mice. J. Nutr. 130, 1153-1158.

215. Whelan, J., 1996. Antagonistic effects of dietary arachidonic acid and n-3 polyunsaturated fatty acids. J. Nutr. 126 (Suppl. 4), 1086S-1091S.

216.   Cao, Y. and Prescott, S.M. 2002. Many actions of cyclooxygenase-2 in cellular dynamics and in cancer. J. Cell Physiol. 190, 279-86.

217.   Fitzpatrick, F.A. and Soberman, R. 2001. Regulated formation of eicosanoids. J. Clin. Invest. 107, 1347-1351.

218.   Qiao, L., Hanif, R., Sphicas, E., Shiff, S.I. and Rigas, B. 1998. Effect of aspirin on induction of apoptosis in HT-29 human colon adenocarcinoma cells. Biochem. Pharmacol. 55, 53-64.

219.   Mohan, S. and Epstein, J.B. 2003. Carcinogenesis and Cyclooxygenase: the potential role of COX-2 inhibition in upper aerodigestive tract cancer. Oral Oncol. 39, 537-546.

220.   Sheng, H., Shao, J. and Dubois, R.N. 2001. K-Ras-mediated increase in Cyclooxygenase 2 mRNA stability involves activation of the protein kinase B1. Cancer Res. 61, 2670-2675.

221.   Miyata, Y., Koga, S., Kanda, S., Nishikido, M., Hayashi, T. and Kanetake, H. 2003. Expression of Cyclooxygenase-2 in renal cell carcinoma: correlation with tumour cell proliferation, apoptosis, angiogenesis, expression of matrix metalloproteinase-2, and survival. Clin. Cancer Res. 9, 1741-1749.

222.   Dannenberg, A.J., Altorki, N.K., Boyle, J.O., Dang, C., Howe, L.R., Weksler, B.B. and Subbaramaiah, K. 2001. Cyclo-oxygenase 2: a pharmacological target for the prevention of cancer. Lancet Oncol. 2, 544-551.

223.   Bakhle, Y.S. 2001. COX-2 and cancer: a new approach to an old problem. Br. J. Pharmacol. 134, 1137-1150.

224.   Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P. and Harkonen, M. 1997. Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res. 57, 1276-1280

225.   Zimmermann, K.C., Sarbia, M., Weber, A.A., Borchard, F., Gabbert, H.E. and Schrör, K. 1999. Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res. 59, 198-204.

226.   Koga, H., Sakisaka, S., Ohishi, M., Kawaguchi, T., Taniguchi, E., Sasatomi, K., Harada, M., Kusaba, T., Tanaka, M., Kimura, R., Nakashima, Y., Nakashima, O., Kojiro, M., Kurohiji, T. and Sata, M. 1999. Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumour dedifferentiation. Hepatology 29, 688-696

227.   Tucker, O.N., Dannenberg, A.J., Yang, E.K., Zhang, F., Teng, L., Daly, J.M., Soslow, R.A., Masferrer, J.L., Woerner, B.M., Koki, A.T. and Fahey, T.J.III. 1999. Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer. Cancer Res. 59, 987-990.

228.   Chan, G., Boyle, J.O., Yang, E.K., Zhang, F., Sacks, P.G., Shah, J.P., Edelstein, D., Soslow, R.A., Koki, A.T., Woerner, B.M., Masferrer, J.L. and Dannenberg, A.J. 1999. Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res. 59, 991-994.

229. Wolff, H., Saukkonen, K., Anttila, S., Karjalainen, A., Vainio, H. and Ristimaki, A. 1998. Expression of cyclooxygenase-2 in human lung carcinoma. Cancer Res. 58, 4997-5001.

230. Soslow, R.A., Dannenberg, A.J., Rush, D., Woerner, B.M., Khan, K.N., Masferrer, J. and Koki, A.T. 2000. COX-2 is expressed in human pulmonary, colonic, and mammary tumors. Cancer 89, 2637-2645.

231. Parrett, M.L., Harris, R.E., Joarder, F.S., Ross, M., Abou-Issa, H.M., Alshafie, G. and Harris, R.E. 1997. Cyclooxygenase-2 gene expression in human breast cancer. Int. J. Oncol. 10, 503-507.

232. Uotila, P., Valve, E., Martikainen, P., Nevalainen, M., Nurmi, M. and Harkonen, P. 2001. Increased expression of cyclooxygenase-2 and nitric oxide synthase-2 in human prostate cancer. Urol. Res. 29, 23-28.

233. Kulkarni, S., Rader, J.S., Zhang, F., Liapis, H., Koki, A.T., Masferrer, J.L., Subbaramaiah, K. and Dannenberg, A.J. 2001. Cyclooxygenase-2 is overexpressed in human cervical cancer. Clin. Cancer Res. 7, 429-434.

234. Patrono, C., Patrignani, P. and Rodríguez, L.A.G. 2001. Cyclooxygenase selective inhibition of prostanoid formation transducing biochemical selectivity into clinical readouts. J. Clin. Invest. 108, 7-13.

235. Prescott, S.M. and Fitzpatrick, F.A. 2000. Cyclooxygenase-2 and carcinogenesis. Biochim. Biophys. Acta 1470, M69-M78.

236. Smalley, W.E. and DuBois, R.N. 1997. Colorectal cancer and nonsteroidal anti-inflammatory drugs. Adv. Pharmacol. 39, 1-20.

237. Müller-Decker, K., Scholz, K., Marks, F. and Fürstenberger, G. 1995. Differential expression of prostaglandin H synthase isozymes during multistage carcinogenesis in mouse epidermis. Mol. Carcinog. 12, 31-41.

238. Tang, X., Sun, Y.J., Half, E., Kuo, M.T. and Sinicrope, F. Cyclooygenase-2 overexpression inhibits death receptor 5 expression and confers resistance to tumour necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human colon cancer cells. Cancer Res. 62, 4903-4908.

239. Surette, M.E., Fonteh, A.N., Bernatchez, C. and Chilton, F.H. 1999. Perturbations in the control of cellular arachidonic acid levels block cell growth and induce apoptosis in HL-60 cells. Carcinogenesis 20, 757-763.

240. Chan, T.A., Morin, P.J., Vogelstein, B. and Kinzler, K.W. 1998. Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. Proc. Natl. Acad. Sci. 95, 681-686.

241. Surette, M.E., Winkler, J.D., Fonteh, A.N. and Chilton, F.H. 1996. Relationship between arachidonic-phospholipid remodeling and apoptosis. Biochem. 35, 9187-9196.

242. Longo, W.E., Grossmann, E.M., Erickson, B., Panesar, N., Mazuski, J.E. and Kaminski, D.L. 1999. The effect of phospholipase A2 inhibitors on proliferation and apoptosis of murine intestinal cells. Surg. Res. 84, 51-56.

243. Jayadev, S., Linardic, C.M. and Hannun, Y.A. 1994. Identification of arachidonic acid as mediator of sphingomyelin hydrolysis in response to tumor necrosis factor alpha. J. Biol. Chem. 269, 5757-5763.

244. Sheng, H., Shao, J., Morrow, J.D., Beauchamp, R.D. and DuBois, R.N. 1998. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. Cancer Res. 58, 362-366.

245. Orlov, S.N., Thorin-Trescases, N., Dulin, N.O., Dam, T.V., Fortuno, M.A., Tremblay, J. and Hamet, P. 1999. Activation of cAMP signaling transiently inhibits apoptosis in vascular smooth muscle cells in a site upstream of caspase-3. Cell Death Differ. 6, 661-672.

246. Steele, V.E., Holmes, C.A., Hawk, E.T., Kopelovich, L., Lubet, R.A., Crowell, J.A., Sigman, C.C. and Kelloff, G.J. 1999. Lipoxygenase inhibitors as potential cancer chemopreventives. Cancer Epidemiol. Biomarkers Prev. 8, 467-483.

247. Ding, X.-Z., Hennig, R. and Adrian, T.E. 2003. Lipoxygenase and cyclooxygenase metabolism: new insights in treatment and chemoprevention of pancreatic cancer. Mol. Cancer 2, 10-21.

248. Ambs, S., Hussain, S.P. and Harris, C.C. 1997. Interactive effects of nitric oxide and the p53 tumor suppressor gene in carcinogenesis and tumor progression. FASEB J. 11, 443-448.

249. Whittle, B.J.R., Lopez-Belmonte, J. and Moncada, S. 1990. Regulation of gastric mucosal integrity by endogenous nitric oxide: interactions with prostanoids and sensory neuropeptides in the rat. Br. J. Pharmacol. 99, 607-611.

250. Nathan, C. and Xie, Q.W. 1994. Nitric oxide synthases: roles, tolls, and controls. Cell 78, 915-918.

251. Chang, A.D., Ramanujam, K.S. and Wilson, K.T. 1996. Co-expression of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2) and TGF-β in rat models of colitis. Gastroenterology 109, 1475-1478.

252. Singer, I.I., Kawka, D.W., Scott, S., Weidner, J.R., Mumford, R.A., Riehl, T.E. and Stenson, W.F. 1996. Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. Gastroenterology 111 871-885.

253. Ambs, S., Merriam, W.G., Bennett, W.P., Felley-Bosco, E., Ogunfusika, M.O., Oser, S.M., Klein, S., Shields, P.G., Billiar, T.R. and Harris, C.C. 1998. Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progression. Cancer Res. 58, 334-341.

254. Franco, L., Doria, D., Bertazzoni, E., Benini, A. and Bassi, C. 2004. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in pancreatic cancer. Prost. Lipid Mediat. 73, 51-58.

255. Nguyen, T., Brunson, D., Crespi, C.L., Penman, B.W., Wishnok J.S. and Tannenbaum, S.R. 1992. DNA damage and mutation in human cells exposed to nitric oxide in vitro. Proc. Natl. Acad. Sci. 89, 3030-3034.

256. Plummer, S.M., Hall, M. and Faux, S.P. 1995. Oxidation and genotoxicity of fecapentaene-12 are potentiated by prostaglandin H synthase. Carcinogenesis 16, 1023-1028.

257. Mordan, L.J., Burnett, T.S., Zhang, L.X., Tom, J. and Cooney, R.V. 1993. Inhibitors of endogenous nitrogen oxide formation block the promotion of neoplastic transformation in C3H 10T1/2 fibroblasts. Carcinogenesis 14, 1555-1559.

258. Tsujii, M. and DuBois, R.N. 1995. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 83, 493-501.

259. Cowing, B.E. and Saker, K.E. 2001. Polyunsaturated fatty acids and epidermal growth factor receptor/mitogen-activated protein kinase signaling in mammary cancer. J. Nutr. 131, 1125-1128.

260. Moller, P., Mechtersheimer, G., Kaufmann, M., Moldenhauer, G., Momburg, F., Mattfeldt, T. and Otto, H.F. 1989. Expression of epidermal growth factor receptor in benign and malignant primary tumours of the breast. Virch. Arch. A. Pathol. Anat. Histopathol. 414, 157-164.

261. Kinoshita, T., Yokota, T., Arai, K. and Miyajima, A. 1995. Suppression of apoptotic death in hematopoietic cells by signaling through the IL-3/GM-CSF receptors. EMBO J. 14, 266-275.

262. Lobenhofer, E.K., Huper, G., Iglehart, J.D. and Marks, J.R. 2000. Inhibition of mitogen-activated protein kinase and phophatidylinositol 3-kinase activity in MCF-7 cells prevents estrogen-induced mitogenesis. Cell Growth Differ. 11, 99-110.

263. Sakakura, C., Sweeney, E.A., Shirahama, T., Ruan, F., Solca, F., Kohno, M., Hakomori, S.I., Fischer, E.H. and Igarishi, Y. 1997. Inhibition of MAP kinase by sphingosine and its methylated derivative, N,N-dimethylsphingosine: a correlation with induction of apoptosis in solid tumor cells. Int. J. Oncol. 11, 31-39.

264. Tsai, M.-H.C.-L., Yu-Wei, F.-S. and Stacey, D.W. 1989. The effect of GTPase activating proteins upon Ras is inhibited by mitogenically responsive lipids. Science 243, 552-526.

265. Lester, D. 1990. In vitro linoleic acid activation of protein kinase c. Biochim. Biophys. Acta 1954, 297-303.

266. Fan, X., Huang, X., Da Salva, C. and Castagna, M. 1990. Arachidonic acid and related methyl ester mediate protein kinase C activation in intact platelets through the arachidonate metabolism pathways. Biochem. Biophys. Res. Commun. 169, 933-940.

267. Toker, A. 1998. Signaling through protein kinase C. Front. Biosci 3, 1134-1147.

268. Hardy, S., Langelier, Y. and Prentki, M. 2000. Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. Cancer Res. 60, 6353-6358.

269. Vacaresse, N., Lajoie-Mazenc, I., Auge, N., Suc, I., Frisach, M.F., Salvayre, R. and Negre-Salvayre, A. 1999. Activation of epithelial growth factor receptor pathway by unsaturated fatty acids. Circ. Res. 85, 892-899.

270. Burtrum, D., Zhu, Z., Lu, D., Anderson, D.M., Prewett, M., Pereira, D.S., Bassi, R., Abdullah, R., Hooper, A.T., Koo, H., Jimenez, X., Johnson, D., Apblett, R., Kussie, P., Bohlen, P., Witte, L., Hicklin, D.J. and Ludwig, D.L. 2003. A fully human monoclonal antibody to the insulin-like growth factor i receptor blocks ligand-dependent signaling and inhibits human tumor growth *in vivo*. Cancer Res. 63, 8912-8921.

271. Fan, Y.-Y. and Chapkin, R.S. 1998. Importance of dietary γ-linolenic acid in human health and nutrition. J. Nutr. 128, 1411-1414.

272. Pham, H., Banerjee, T. and Ziboh, V.A. 2004. Suppression of cyclooxygenase-2 overexpression by 15S-hydroxyeicosatrienoic acid in androgen-dependent prostatic adenocarcinoma cells. Int. J. Cancer 111, 192-197.

273. Jiang, W.G., Bryce, R.P. and Horrobin, D.F. 1998. Essential fatty acids: molecular and cellular basis of their anti-cancer action and clinical implications. Crit. Rev. Oncol. Hematol. 27, 179-209.

274. Menendez, J.A., Colomer, R. and Lupu, R. 2005. Inhibition of fatty acid synthase-dependent neoplastic lipogenesis as the mechanism of γ-linolenic acid-induced toxicity to tumour cells: an extension to Nwankwo's hypothesis. Med. Hypotheses 64, 337-341.

275. De Kock, M., Lottering, M.L., Grobler, C.J.S., Viljoen, T.C., le Roux, M. and Seegers, J.C. 1996. The induction of apoptosis in human cervical carcinoma (HeLa) cells by γ-linolenic acid. Prost. Leukot. Essent. Fatty Acids 55, 403-411.

276. Cai, J., Jiang, W.G. and Mansel, R.E. 1999. Inhibition of the expression of VE-cadherin/catenin complex by gamma linolenic acid in human vascular endothelial cells, and its impact on angiogenesis. Biochem. Biophys. Res. Commun.; 258, 113-118.

278. Jiang, W.G., Bryce, R.P., Horrobin, D.F. and Mansel, R.E. 1998. Regulation of tight junction permeability and occludin expression by polyunsaturated fatty acids. Biochem. Biophys. Res. Commun. 244, 414-420.

279. Kokura, S., Yoshikawa, T., Kaneko, T., Iinuma, S., Nishimura, S., Matsuyama, K., Naito, Y., Yoshida, N. and Kondo, M. 1997. Efficacy of hyperthermia and polyunsaturated fatty acids on experimental carcinoma. Cancer Res. 57, 2200-2202.

280. Johnson, M.M., Swan, D.D., Surette, M.E., Stegner, J., Chilton, T., Fontech, A.N. and Chilton, F.H. 1997. Dietary supplementation with γ-linolenic acid alters fatty acid content and eicosanoid production in healthy humans. J. Nutr. 127, 1435-1444.

281. Bolton-Smith, C., Woodward, M. and Tavendale, R. 1997. Evidence for age-related differences in the fatty acid composition of human adipose tissue, independent of diet. Eur. J. Clin. Nutr. 51: 619–624.

282. Grammatikos, S., Harvey, M.J., Subbaiah, P.V., Victor, T.A. and Miller, W.M. 1995. Loss of fatty-acid d-6 desaturating ability in human mammary epithelial cells that express an activated c-Ha-ras oncogene. Int. J. Oncol. 6, 1039-1046.

283. Kuhajda, F.P. 2000. Fatty acid synthase and human cancer: new perspectives on its role in tumour biology. Nutrition 16, 202-208.

284. Menendez, J.A., Mehmi, I., Atlas, E., Colomer, R. and Lupu, R. 2004. Novel signaling molecules implicated in tumour-associated fatty acid synthase-dependent breast cancer cell proliferation and survival: Role of exogenous dietary fatty acids, p53-p21WAF1/CIP1, ERK1/2 MAPK, p27KIP1, BRCA1, and NF-kappaB. Int. J. Oncol. 24, 591-608.

285. Menendez, J.A., Vellon, L., Mehmi, I., Oza, B.P., Ropero, S., Colomer, R. and Lupu, R. 2004. Inhibition of fatty acid synthase (FAS) suppresses *HER2/neu (erb*B-2) oncogenes overexpression in cancer cells. Proc. Natl. Acad. Sci. 101, 10715-10720.

286. von Schacky, C. 2000. n-3 fatty acids and the prevention of coronary atherosclerosis. Am. J. Clin. Nutr. 71, 224S-227S.

287. Daviglus, M.L., Stamler, J., Orencia, A.J., Dyer, A.R., Liu, K., Greenland, P., Walsh, M.K., Morris, D. and Shekelle, R.B. 1997. Fish consumption and the 30-year risk of fatal myocardial infarction. N. Engl. J. Med. 336, 1046-1053.

288. GISSI-Prevenzione Investigators (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico). 1999. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-prevenzione trial. Lancet 354, 447-455.

289. Marckmann, P. and Gronbaeck, M. 1999. Fish consumption and coronary heart disease mortality: a systematic review of prospective cohort studies. Eur. J. Clin. Nutr. 53, 585-590.

290. Albert, C.M., Hennekens, C.H., O'Donnell, C.J., Ajani, U.A., Carey, V.J., Willett, W.C., Ruskin, J.N. and Manson, J.E. 1998. Fish consumption and risk of sudden cardiac death. JAMA. 279, 23-28.

291. Hibbeln, J.R. 1998. Fish consumption and major depression and psychiatric disorders. Lancet 351, 1213.

292. Peet, M., Murphy, B., Shay, J. and Horrobin, D.F. 1998. Depletion of omega 3 fatty acid levels in red blood cell membranes of depressive patients. Biol. Psychiatry 43, 315-319.

293. Peet, M. 1999. New strategies for the treatment of schizophrenia: ω3 polyunsaturated fatty acids. In *Phospholipid spectrum disorder in psychiatry.* Peet, M., Glen, I. and Horrobin, D.F. eds. Marius Press, Lancashire. pp 189-192.

294. Gibson, R.A. and Makrides, M. 2000. n-3 polyunsaturated fatty acid requirements of term infants. Am. J. Clin. Nutr. 71, 251S-255S.

295. Hornstra, G. 2000. Essential fatty acids in mothers and their neonates. Am. J. Clin.Nutr. 71, 1262S-1269s.

296. Hibbeln, J.R. 2002. Seafood consumption, the DHA content of mothers' milk and prevalence rates of postpartum depression: A cross-national, ecological analysis. J. Affect. Disord. 69, 15-29.

297. Kato, I., Akhmedkhanov, A., Koenig, K., Toniolo, P.G., Shore, R.E., Riboli, E. 1997. Prospective study of diet and female colorectal cancer: The New York University Women's Health Study. Nutr Cancer 28, 276-281.

298. Okuyama, H., Kabayashi, T. and Watanabe, S. 1997. Dietary fatty acids - The n-6/n-3 balance and chronic elderly diseases: excess of linolenic acid and relative n-3 deficiencey syndrome seen in Japan. Prog. Lipid Res. 35, 409-457.

299. Shoda, R., Matsueda, K., Yamata, S. and Umeda, N. 1996. Epidemiological analysis of Crohn's disease in Japan: increased dietary intake of n-6 polyunsaturated fatty acids and animal protein relates to increased incidence of Crohn's disease in Japan. Am. J. Nutr. 63, 741-745.

300. Rose, D.P., Connolly, J.M., Rayburn, J. and Coleman, M. 1995. Influence of diets containing eicosapentaenoic or docosahexaenoic acid on growth and metastasis of breast cancer cells in nude mice. J. Natl. Cancer Inst. 87, 587-592.

301. Calviello, G., Palozza, P., Piccioni, E., Maggiano, N., Frattucci, A., Franceschelli, P. and Bartoli, G.M. 1998. Dietary supplementation with eicosapentaenoic and docosahexaenoic acid inhibits growth of Morris hepatocarcinoma 3924A in rats: effects on proliferation and apoptosis. Int. J. Cancer 75, 699-705.

302. Lai, P.B., Ross, J.A., Fearon, K.C., Anderson, J.D. and Carter, D.C. 1996. Cell cycle arrest and induction of apoptosis in pancreatic cancer cells exposed to eicosapentaenoic acid in vitro. Br. J. Cancer 74, 1375-1383.

303. Karmali, R.A. 1987. Fatty acids: inhibition. Am. J. Nutr. 45, 225-229.

304. Corey, R.J., Shih, C. and Cashman, J.R. 1980. Docosahexaenoic acid is a strong inhibitor of prostaglandin but not leukotrienes synthesis. Proc. Natl. Acad. Sci. 80, 3581-3584.

305. Kafrawy, O., Zerouga, M., Stillwell, W. and Jenski, L.J. 1998. Docosahexaenoic acid in phosphatidylcholine mediates cytotoxicity more effectively than other ω-3 and ω-6 fatty acids. Cancer Lett. 132, 23-29.

306. Pascale, A.W., Ehringer, W.D., Stillwell, W., Sturdevant, L.K. and Jenski, L.J. 1993. Omega-3 fatty acid modification of membrane structure and function. II. Alteration by docosahexaenoic acid of tumor cell sensitivity to immune cytolysis. Nutr. Cancer 19, 147-158.

307. Zerouga, M., Stillwell, W., Stone, J., Powner, A. and Jenski, L.J. 1996. Phospholipid class as a determinant in docosahexaenoic acid's effect on tumor cell viability. Anticancer Res. 16, 2863-2868.

308. Sessler, A.M. and Ntambi, J.M. 1998. Polyunsaturated fatty acid regulation of gene expression. J. Nutr. 128, 923-926.

309. Abel S., de Kock, M., Smuts, C.M., de Villiers, C. and Gelderblom, W.C.A. 2004. Dietary modulation of fatty acid profiles and oxidative status of rat hepatocyte nodules: effect of different n-6/n-3 fatty acid ratios. Lipids 39, 963-976.

310. Lai, P.B.S., Ross, J.A., Fearon, K.C.H., Anderson, J.D. and Carter, D.C. 1996. Cell cycle arrest and induction of apoptosis in pancreatic cancer cells exposed to eicosapentaenoic acid in vitro. Br. J. Cancer 74, 1375-1383.

311. Sauer, L.A., Dauchy, R.T. and Blask, D.E. 2000. Mechanism for the antitumor and anticachetic effects of n-3 fatty acids. Cancer Res. 60, 5289-5295.

312. Lands, W.E.M., Letellier, P.R., Rome, R.H. and Vanderhoek, J.Y. 1973. Inhibition of prostaglandin biosynthesis. Adv. Biosci. 9, 15-27.

313. Fay, M.P., Freedman, L.S., Clifford, C.K. and Midthune, D.N. 1997. Effect of different types and amounts of fat on the development of mammary tumors in rodents: a review. Canc. Res. 57, 3979-3988.

314. Li, B., Birdwell, C. and Whelan, J. 1994. Antithetic relationship of dietary arachidonic acid and eicosapentaenoic acid on eicosanoid production in vivo. J. Lipid Res. 35, 1869-77.

315. Price, P.T., Nelson, C.M. and Clarke, S.D. 2000. Omega-3 polyunsaturated fatty acid regulation of gene expression. Curr. Opin. Lipidol. 11, 3-7.

316. Novak, T.D., Babcock, T.A., Jho, D.H., Helton, W.S. and Espat, N.J. 2003. NF-κB inhibition by ω3 fatty acids modulates LPS-stimulated macrophage TNF-α transcription. Am. J. Physiol. Lung Cell Mol. Physiol. 284, L84-L89.

317. Arrigo, A-P. and Kretz-Remy, C. 1998. Regulation of mammalian gene expression by free radicals. In *Molecular Biology of free radicals in human diseases.* Aruoma, O.I. and Halliwell, B. eds. OICA International, Chelsea, London, UK. pp 183-223.

318. Iimuro, Y., Nishiura, T., Hellerbrand, C., Behrns, K., Shoonhoven, R., Grisham, J.W. and Brenner, D.A. 1998. NF-kB prevents apoptosis and liver failure during liver regeneration. J. Clin. Invest. 101, 902-811.

319. Mayo, M.W. and Baldwin, A.S. 2000. The transcription factor NF-κB: control of oncogenesis and cancer therapy resistance. Biochim. Biophys. Acta 1470, M55-M62.

320.   Wang, W., Abbruzzese, J., Evans, D., Larry, L., Cleary, K. and Chiao, P. 1999. The nuclear factor-κB RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. Clin. Cancer Res. 5, 119-127.

321.   Sovak, M., Bellas, R., Kim, D., Zanieski, G., Rogers, A., Traish, A. and Sonenshein, G. 1997. Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. J. Clin. Invest. 100, 2952-2960.

322.   Duffey, D., Chen, Z., Dong, G., Ondrey, F., Wolf, J., Brown, K., Siebenlist, U. and Van Waes, C. 1999. Expression of a dominant-negative mutant inhibitor-κBα of nuclear factor-κB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth *in vivo.* Cancer Res. 59, 3468-3474.

323.   Guttridge, D., Albanese, C., Reuther, J., Pestell, R. and Baldwin, A. 1999. NF-κB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol. Cell Biol. 19, 5785-5799.

324.   Ghosh, S., May, M. and Kopp, E. 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu. Rev. Immunol. 16, 225-260.

325.   Takeshita, H., Yoshizaki, T., Miller, W., Sato, H., Furukawa, M., Pagano, J. and Raab-Traub, J. 1999. Matrix metalloproteinase 9 expression is induced by Epstein-Barr virus latent membrane protein 1 C-terminal activation regions 1 and 2. J. Virol. 73, 5548-555.

326.   Collett, E.D., Davidson, L.A., Fan, Y-Y., Lupton, J.R. and Chapkin, R.S. 2001. N-6 and n-3 polyunsaturated fatty acids differentially modulate oncogenic Ras activation in colonocytes. Am. J. Physiol. Cell Physiol. 280, C1066-C1075.

327.   Schwartz, S.A., Hernandez, A. and Evers, B.M. 1999. The role of NF-κB/IκB proteins in cancer: Implications for novel treatment strategies. Surg. Oncol. 8, 143-153.

328.   Plümpe, J., Malek, N.P., Bock, C.T., Rakemann, T., Manns, M.P. and Trautwein, C. 2000. NF-κB determines between apoptosis and proliferation in hepatocytes during liver regeneration. Am. J. Physiol. Gastrointest. Liver Physiol. 278, G173-G183.

329.   Hardman, W.E. 2002. Omega-3 fatty acids to augment cancer therapy. J. Nutr. 132, 3508S-3512S.

330.   Mirnikjoo, B., Brow, S.E., Kim, H.F.S., Marangell, L.B., Sweatt, J.D. and Weeber, E.J. 2001. Protein kinase inhibition by ω-3 fatty acids. J. Biol. Chem. 276, 10888-10896.

331.   Lupulescu, A. 1996. Prostaglandins, their inhibitors and cancer. Prost. Leukot. Essent. Fatty Acids 54, 83-94.

332.   Rose, D.P. and Connolly, J.M. 2000. Regulation of tumor angiogenesis by dietary fatty acids and eicosanoids. Nutr. Cancer 37, 119-127.

333. Senzaki, H., Tsubura, A. and Takada, H. 2001. Effect of eicosapentaenoic acid on the suppression of growth and metastasis of human breast cancer cells in vivo and in vitro. World Rev. Nutr. Diet 88, 117-125.

334. Rose, D.P. 1997. Effects of dietary fatty acids on breast and prostate cancers: evidence from in vitro experiments and animal studies. Am. J. Clin. Nutr. 66, 1513S-1522S.

335. Klurfeld, D.M. and Bull, A.W. 1997. Fatty acids and colon cancer in experimental animals. Am. J. Clin. Nutr. 66, 1530S-1538S.

336. McCarthy, M.F. 1996. Fish oil may impede angiogenesis and invasiveness by down-regulating protien kinase C and modulating eicosanoid production. Med. Hypotheses 46, 107-115.

337. McEntee, M.F. and Whelan, J. 2002. Dietary polyunsaturated fatty acids and colorectal neoplasia. Biomed. Pharmacother. 56. 380-387.

338. Rao, C., Simi, B., Wynn, T., Garr, K. and Reddy, B. 1996. Modulating effect of amount and types of dietary fat on colonic mucosal phospholipase A2, phosphatidylinositol-specific phospholipase C activities, and cyclooxygenase metabolite formation during different stages of colon tumor promotion in male F344 rats. Cancer Res. 56, 532-537.

339. Grimm, H., Mayer, K., Mayser, P. and Eigenbrodt, E. 2002. Regulatory potential of n-3 fatty acids in immunological and inflammatory processes. Br. J. Nutr. 87, S59-S67.

340. von Schacky, C., Kiefl, R., Marcus, A.J., Broekman, M.J. and Kaminski, W.E. 1993. Dietary n-3 fatty acids accelerate catabolism of leukotriene B4 in human granulocytes. Biochim. Biophys. Acta 1166, 20-24.

341. Tsunomori, M., Fujimoto, Y., Muta, E., Nishida, H., Sakuma, S. and Fujita, T. 1996. 15-Hydroperoxyeicosapentaenoic acid inhibits arachidonic acid metabolism in rabbit platelets more potently than eicosapentaenoic acid. Biochim. Biophys. Acta 1300, 171-176.

342. Juan, H and Sametz, W. 1985. Dihomo-gamma-linolenic acid increases the metabolism of eicosapentaenoic acid in perfused vascular tissue. Prost. Leukot. Med. 19, 79-86.

343. Gil, Á. 2002. Polyunsaturated fatty acids and inflammatory diseases. Biomed. Pharmacother. 56, 388-396.

344. Solt, D. and Farber, E. 1976. New principles for the analysis of chemical carcinogenesis. Nature 263, 701-703.

345. Blumberg, B.S. and London, W.T. 1981. Hepatitis B virus and the prevention of primary hepatocellular carcinoma. New England J. Med. 304, 782-784.

346. Fay, M.P., Freedman, L.S., Clifford, C.K. and Midthune, D.N. 1997. Effect of different types and amounts of fat on the development of mammary tumors in rodents: a review. Cancer Res. 57, 3979-3988.

347.   Hardman, W.E. 2004. (n-3) Fatty acids and cancer therapy. J. Nutr. 134, 3427S-3430S.

348.   Black, H.S., Thornby, J.I., Gerguis, J. and Lenger, W. 1992. Influence of dietary omega-6, -3 fatty acid sources on the initiation and promotion stages of photocarcinogenesis. Photochem. Photobiol. 56, 195-199.

349.   Beck, S.A., Smith, K.L. and Tisdale, M.J. 1991. Anticachetic and antitumor effect of eicosapentaenoic acid and its effect on protein turnover. Cancer Res. 51, 6089-6093.

350.   Sakaguchi, M., Imray, C., Davis, A., Rowley, S., Jones, C., Lawson, N., Keighley, M.R., Baker, P.R. and Neoptolemos, J.P. 1990. Effects of dietary n-3 and saturated fats on growth rates of the human colonic cancer cell lines SW-620 and LS 1747 in vivo in relation to tissue and plasma lipids. Anticancer Res. 10, 1763-1768.

351.   Fritsche, K.L. and Johnston, P.V. 1990. Effect of dietary alpha-linolenic acid on growth, metastasis, fatty acid profile and prostaglandin production of two murine mammary adenocarcinomas. J. Nutr. 120, 1601-1609.

352.   Takata, T., Minoura, T., Takada, H., Sakaguchi, M., Yamamura, M., Hioki, K. and Yamamoto, M. 1990. Specific inhibitory effect of dietary eicosapentaenoic acid on N-nitroso-N-methylurea-induced mammary carcinogenesis in female Sprague-Dawley rats. Carcinogenesis 11, 2015-2019.

353.   Noguchi, M., Minami, M, Yagasaki, R., Kinoshita, K., Earashi, M., Kitagawa, H., Taniya, T. and Miyazaki, I. 1997. Chemoprevention of DMBA-induced mammary carcinogenesis in rats by low-dose EPA and DHA. Br. J. Cancer 75, 348-353.

354.   Paulsen, J.E., Stamm, T. and Alexander, J. 1998. A fish oil-derived concentrate enriched in eicosapentaenoic and docosahexaenoic acid as methyl esters inhibits the formation and growth of aberrant crypt foci in rat colon. Pharmacol. Toxicol. 82, 28-33.

355.   Chang, W.L., Chapkin, R.S. and Lupton, J.R. 1998. Fish oil blocks azoxymethane-induced rat colon tumorigenesis by increasing cell differentiation and apoptosis rather than decreasing cell proliferation. Nutrition 128, 491-497.

356.   Okuno, M., Tanak, T., Komaki, C., Nagase, S., Shiratori, Y., Muto, Y., Kajiwara, K., Maki, T. and Moriwaki, H. 1998. Suppressive effect of low amounts of safflower and perilla oils on diethylnitrosamine-induced hepatocarcinogenesis in male F344 rats. Nutr. Cancer 30, 186-193.

357.   Friborg, J., Koch, A., Wohlfarht, J., Storm, H.H. and Melbye, M. 2003. Cancer in Greenlandic Inuit 1973-1997: a cohort study. Int. J. Cancer 107, 1017-1022.

358.   Bingham, S.A. 1998. Epidemiology of colorectal cancer. In *Encyclopedia of human nutrition.* Strain, J., Sadler, M. and Caballero, B. eds. New York, Academic Press, Vol. 1. pp 230-235.

359. Simonsen, N., van't Veer, P., Strain, J.J., Martin-Moreno, J.M., Huttunen, J.K., Navajas, J.F.-C., Martin, B.C., Thamm, M., Kardinaal, A.F.M., Kok, F.J. and Kohlmeier, L. 1998. Adipose tissue omega-3 and omega-6 fatty acid content and breast cancer in the EURAMIC study. Am. J. Epidemiol. 147, 342-352.

360. Schloss, I., Kidd, M.S.G., Tichelaar, H.Y., Young, G.O. and O'Keefe, S.J.D. 1997. Dietary factors associated with a low risk of colon cancer in coloured West Coast fishermen. S. Afr. Med. J. 87, 152-158.

361. Gogos, C.A., Ginopouos, P., Salsa, B., Apostolidou, E., Zoumbos, N.C. and Kalfarentzos, F. 1998. Dietary omega-3 polyunsaturated fatty acids plus vitamin E restore immunodeficiency and prolong survival for severely ill patients with generalized malignancy: a randomized control trial. Cancer 82, 395-402.

362. Anti, M., Armelao, F., Marra, G., Percesepe, A., Bartoli, G.M., Palozza, P., Parrella, P., Canetta, C., Gentiloni, N. and De Vitis, I. 1994. Effects of different doses of fish oil on rectal cell proliferation in patients with sporadic colonic adenomas. Gastroenterology 107, 1709-1718.

363. Maillard, V., Bougnoux, P., Ferrari, P., Jourdan, M-L., Pinault, M., Lavillonnieree, F.F., Body, G., Le Floch, O. and Chajes, V. 2002. ω-3 and ω-6 fatty acids in breast adipose tissue and relative risk of breast cancer in a case-control study in Tours, France. Intl. J. Cancer 98, 78-83.

364. Terry, P.D., Terry, J.B. and Rohan, T.E. 2004. Long-chain (n-3) fatty acid intake and risk of cancers of the breast and the prostate: recent epidemiological studies, biological mechanisms, and directions for future research. J. Nutr. 134, 3412S-3420S.

365. Bartram, H. -P., Gostner, A., Reddy, B.S., Rao, C.V., Scheppach, W., Dusel, G., Richter, A., Richter, F. and Kasper, H. 1995. Missing anti-proliferative effect of fish oil on rectal epithelium in healthy volunteers consuming a high-fat diet: potential role of the n-3:n-6 fatty acid ratio. Eur. J. Cancer Prev. 4, 231-237.

366. Rose, D.P. 1997. Dietary fat, fatty acids and breast cancer. Breast Cancer (Japan) 4, 7-16.

THRESHOLDS AND KINETICS OF FATTY ACID REPLACEMENT IN DIFFERENT CELLULAR COMPARTMENTS IN RAT LIVER AS A FUNCTION OF DIETARY ω6/ω3 FATTY ACID CONTENT.

S Abel[a], WCA Gelderblom[a], CM Smuts[b] and M Kruger[b]

[a]Programme on Mycotoxins and Experimental Carcinogenesis and [b]National Research Programme for Nutritional Intervention, PO Box 19070, Tygerberg, 7505, South Africa.

ABSTRACT

The kinetics of fatty acid (FA) replacement in different membranal compartments in the rat liver was investigated using diets with varying ω6/ω3 FA ratios. Rats at different stages of growth i.e. after weaning and at 150 g of body weight, were either fed a modified AIN 76A diet containing sunflower oil as fat source or the same diet containing sunflower and fish oil to achieve ω6/ω3 FA ratios of 12:1 and 6:1 (diet A and B respectively). In the adult rats, fed diet A for 8 weeks, C18:2ω6 increased significantly at week 2 in the phosphatidylcholine (PC) fraction of the plasma membranes, microsomes and plasma but not in phosphatidylethanolamine (PE). C20:3ω6 increased significantly at week 2 in the plasma membranal and microsomal PC, but only increased in PE of both compartments by week 8. C20:4ω6 and the ω3 FAs significantly decreased and increased respectively at week 2 in PC and PE of both membranal compartments and plasma PC. The experimental diets led to a change in the plasma membranal fluidity but not in the microsomes. The FA changes in the weaned rats followed a similar pattern as in the adult rats although the changes were greater, depending on the phospholipid fraction and specific FA. The decrease in C20:4ω6 was significantly greater in the microsomal PC and PE and plasma PC but not in the plasma membranal PC and PE. The ω3 FAs increased significantly above the adult levels in the plasma membranal PC and PE respectively but not in the microsomal phospholipid fractions. A threshold for maximal ω3 and ω6 FA incorporation was achieved in the adult rats fed diet A in the microsomes after 2 weeks with no further alterations occurring with diet B. In the plasma PC and plasma membranes most of the ω3 FAs achieved a threshold incorporation after 2 weeks on diet A, except for C22:6ω3 in the plasma membranal PE and certain ω6 FAs in the plasma membrane PC and PE. The present data shows that differences exist in the kinetics of FA incorporation and replacement depending on the specific phospholipid fraction, membranal compartment, age and to a certain extent the dietary ω6/ω3 FA ratio.

INTRODUCTION

It has been suggested that dietary polyunsaturated fatty acids (FA) are important modulators of neoplastic development (1) since animal studies have indicated that they decrease the size and number of tumours as well as the lagtime of tumour appearance (2). The alteration in membrane phospholipid composition has been suggested to modify the physical and functional properties of membranal phospholipids which could alter the growth characteristics of the neoplastic cell or increase the sensitivity of the cell to therapy (1). It is known that the manipulation of the FA saturation and content of cell membranes induces changes in membrane fluidity which has important implications as it affects many membrane related responses such as membrane receptor-ligand interactions (1, 3). Dietary FA manipulation also has an important effect on membrane protein configuration and distribution and could therefore influence various membrane associated functions (4) such as the function of membrane proteins including ion channels and ATPases (5).

At present very little is known about the exact dietary ratios of the essential FAs (EFA) to be used in the dietary modulation of cancer development. It has been suggested that the human evolutionary diet contained a ω6/ω3 ratio of 1:1. At present it is estimated that this ratio has changed to 20-25:1 due to changes in modern dietary habits (2). Most dietary studies have utilised ω3 FAs at either a high or low dietary content with respect to the ω6 FAs or, in some cases, as the only source of dietary fat (6). It is known that the EFA share a common metabolic pathway including several desaturases and chain elongation steps (2). These desaturase enzymes preferentially metabolise the ω3 FAs over the ω6 FAs as substrates (5) while a recent study indicated that they also compete at the esterification step in phospholipid biosynthesis (7). These interactions between the different classes of FAs lead to the displacement of the ω6 FAs by the ω3 FAs in cellular membranes (5). However, excessive replacement of the dietary ω6 FAs could result in a decreased ω6 FA content of the cell and to a ω6 FA deficiency which could result in abnormal growth and development (2, 8). Therefore specific FA dietary ratios need to be selected which could effectively reduce neoplastic development while on the other hand not adversely affect the ω6 FA content of cellular membranes.

Partial replacement of dietary ω6 FAs with ω3 FAs has shown that maximal incorporation of ω3 FAs into membrane phospholipids occurs within a few weeks (9). A study on the phospholipid content of the rat nuclear envelope showed that maximal membrane incorporation of ω3 FAs occurred at a dietary level of 4-5% of the total dietary FAs (9).

There are indications that age also influences the membrane phospholipid FA content (10, 11). It has been observed that there is a difference in the polar head group profile of liver membrane phospholipids between weaned and older rats (10). Fluctuations in the activity of the desaturase enzymes have also been observed which could result in differing FA profiles in older rats compared to younger rats (11). The aim of the present study was to investigate different parameters such as the interaction of dietary ω3 and ω6 FAs and age on the kinetics and threshold of FA replacement in the different cellular compartments of rat liver.

MATERIALS AND METHODS

Preparation of Diets.

A diet (AIN 76A) with a total fat content of 5% (w/w) was used as control diet throughout the experiment with sunflower oil as fat source (12). In the experimental diets, the sunflower oil was partially replaced with fish oil (EPA 50, Callanish Ltd., Breasclete, Scotland). The dietary oil mixtures were analyzed on a gas chromatograph (Varian 4600) for the FA profiles from which the exact ω6 to ω3 FA ratios were calculated. The oil mixtures were added to the feed yielding two diets, diet A and B, with ω6/ω3 ratios of 12:1 and 6:1 respectively (Table 1). The diets were kept at -20EC under nitrogen until use.

Animals and Treatment.

Male Fischer rats of different ages, i.e. at 3 weeks (approximately 50 g body weight) and 7 weeks (approximately 150 g body weight), were used. The rats had free access to food and water and were housed separately in wire-bottomed cages under controlled lighting (12 hour cycles) and temperature (23-25EC). The rats were weighed three times a week.

In the first part of the experiment, two groups of rats (50 g body weight, 6 rats per group) received the AIN 76A control diet and the experimental diet (diet A) respectively immediately after being weaned. The rats were sacrificed after 4 weeks (150 g) and the livers removed, weighed and stored in saline at -80EC. Blood was collected for cholesterol analysis. In the second experiment, rats (body weight 150 g) were randomly divided into two groups of 20 rats each and fed the AIN 76A control diet and diet A respectively. Five rats from each group were killed at weeks 2, 4 and 8. The remainder of the rats that received diet A were fed diet B with a ω6/ω3 FA ratio of 6:1 for an additional 4 weeks after which they were terminated and samples were collected as described above.

Purification of Membrane Fractions. All procedures were performed at 4EC.

Plasma Membranes. Plasma membranes (PM) were isolated from the liver as described by Loten and Redshaw-Loten (13). The livers were homogenized in a 250 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 buffer and centrifuged at 1500 x *g* for 10 min. The resultant pellet was fractionated on a self-forming Percoll (Sigma Chemical Company, PO Box 14508, St Louis, MO 63178, USA) gradient from which the plasma membranes were isolated. The plasma membranes were washed by centrifugation and stored at -80EC in 10 mM Tris-HCl (pH 7.4) until analyzed.

Microsomes.   The supernatant from the low speed centrifugation of the plasma membranes was retained for the isolation of the microsomes (Mic) as described by Bartoli et al (14). The supernatant was centrifuged at 8 000 x *g* and 18 000 x *g* for 10 min to remove the mitochondrial and nuclear fractions and the microsomes were then pelleted at 105 000 x *g* for 60 min. The microsomal pellet was suspended in 10 mM Tris-HCl, centrifuged at 105 000 x *g* for 30 min and stored at -80EC in 10 mM Tris-HCl (pH 7.4) until used.   The protein concentration of the plasma membranes and microsomes was determined by the method of Kaushal and Barnes (15).
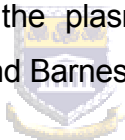
Lipid Analysis:

i)Extraction and Thin Layer Chromatography fractionation. One ml of the membranous fraction (approx. 1 mg protein/ml) was extracted with chloroform/methanol (CM; 2:1) containing butylated hydroxytoluene (0.01%) as antioxidant (16). All the samples were separated by thin layer chromatography (TLC) into phospholipid subfractions for concentration and FA determinations (17).

ii) Fatty Acid Determination. The phospholipid subfractions, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were transmethylated with 3 ml methanol/18 M sulphuric acid (95:5; v/v) at 70EC for 2 hours after which they were extracted with hexane and distilled water. The hexane was removed and evaporated under N_2 in a water bath (37EC) and the samples dissolved in CS_2 prior to analysis (18) by gas chromatography on a Varian 4600 Gas Chromatograph equipped with 30 m fused silica megabore DB-225 columns of 0.53 mm internal diameter (J&W Scientific, cat. no. 125-2232). The gas flow rates were: hydrogen (carrier gas), 5-8 ml/min.; medical air (21% oxygen in nitrogen), 250 ml/min.; and hydrogen, 25 ml/min. Temperature programming was linear at 3EC/min. with

initial temperature 165EC, final temperature 220EC, injection temperature 240EC and detector temperature 250EC. The results were expressed as a percentage of the total FA detected.

iii) <u>Cholesterol</u>. Total plasma cholesterol was measured enzymatically (Boehringer Mannheim, CHOD-PAP). Total cholesterol of the plasma membranes and microsomes was determined by an enzymatic iodide method on the chloroform/methanol extracts obtained from the above lipid extracts (19). The enzymes used were cholesterin-oxidase and -esterase (Boehringer Mannheim, South Africa).

iv) <u>Lipid Phosphorus Determination</u>. The lipid phosphorus content was quantified as described by Itaya and Ui (18), utilising a colorimetric method with malachite green.

Membrane Fluidity/Fluorescence Polarisation.

Fluorescence polarisation studies were performed on the isolated plasma membranes and microsomes with a fluorescence spectrofluorimeter (Perkin-Elmer MPF 44A). Samples were diluted to a concentration of 0.2-0.3 mg protein/ml (20) with 10 mM Tris-HCl, (pH 7.4) and sonicated for 10 seconds, whereafter 1.6-diphenyl-1.3.5-hexatrine (5 µl; 2 mM in tetrahydrofuran) was added. The suspension (2.5 ml) was equilibrated in a waterbath (37EC) for 30-60 minutes in the absence of light (20). Measurements were done manually with an emission polarizer at 0E (V component) and 90E (L component) with the excitation polarizer first at 0E (vertical component v) and then at 90E (horizontal component h). The excitation and emission slit widths were 14 nm and the excitation and emission wavelengths were 357 nm and 425 nm, respectively. The temperatures selected for screening ranged from 25EC to 41EC.

Statistical Analyses.

All the statistical analyses were carried using the analyses of variance. The Students-T Distribution Test was used to determine the significant differences between the means of the control and experimental groups. Values were considered significantly different if $p < 0.05$.

RESULTS

Sources of dietary FAs.

The fish oil and sunflower oil primarily consisted of 57% C20:5ω3 and 72% C18:2ω6, respectively (Table 1). The dietary fat (5%) in diet A (12:1 ω6/ω3) consisted of 62% C18:2ω6 and 4% C20:5ω3, whereas in diet B (6:1 ω6/ω3) the composition was 54% C18:2ω6 and 8% C20:5ω3.

Table 1:   Fatty acid composition of dietary oils.

Fatty acid	FO	SFO	Diet A SFO/FO	Diet B SFO/FO
16:0	0.76	5.47	5.68	5.37
16:1ω7	15.	0.0	1	2.21
18:0	0	4	6	3.50
1	7	17	19	25
1	3	71	62	53
1	2	<	0	0
1	1	<	0	0
2	0	<	0	0.
2	0	<	0	0.
2	5	<	4	7.
2	4	<	0	0.
2	8	<	0	1.
ω6	0	7	12	5.
Res	exp	d	p	tage	e
to	d			=Fi	il;

SFO=Sunflower oil.

Rat Growth Parameters.

Diet A had no significant effect on the % body weight gain and % relative liver weight during the treatment period for both the weaned and adult rats (Table 2). However, the increased ω3 FA content of diet B resulted in significant reductions in the % body weight gain ($p < 0.0001$), the % relative liver weight ($p < 0.001$) and the experimental group fed diet A for 8 weeks ($p < 0.001$) relative to that of the rats fed the control diet.

Membrane Phospholipid and Cholesterol Content.

There was no significant change in the cholesterol and phospholipid content (PC and PE) of the plasma membranes and microsomes in both the adult and weaned rats fed either diet A or B (data not shown, see Addendums 1 and 2). However, diet A and B resulted in a decrease of total plasma cholesterol (Table 2) of the adult rats from week 2 to week 12 ($p < 0.05$) and of the weaned rats ($p < 0.01$) as compared to the control group.

Table 2:   Effect of experimental diets on the relative liver weights, body weights and the plasma cholesterol.

Week	Relative liver weight (%)		Body weight gain (%)		Plasma cholestrol (mmol/l)	
	Cont	Experimental	Control	Experimental	Control	Experimental
Adult rats						
2[#]	4	4.03±0.27	20.9	26.95±1.90	2.10±0.	1.76±0.12[a]
4[#]	4	3.91±0.10	43	53.23±5.95	2.28±0.23	1
8[#]	3.30±0.13	3	7	83	2.6	2.2 [a]
1[##]	3.28±0.16	2 [c]	118	81 [d]	2.1	1.6 [a]
We d ra						
4	4.88±0.26	4.77±0.62	406.5±70.00	426.5±92.00	2.38±0.25	1.78±0.25

[#]Control rats received the AIN 76A diet and the experimental groups received diet A (ω6/ω3 ratio of 12:1). [##]Control rats received the AIN 76A diet and the experimental group received diet B (ω6/ω3 ratio of 6:1). Values are means ± standard deviation of 5 rats per group. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.0001$.

Membrane Fluidity.

Fluorescence polarisation at 37EC showed an increase in the fluidity of the plasma membranes (Fig. 1) but not in the microsomes of the adult and weaned rats fed diet A. This increase in fluidity was observed as early as 2 weeks after treatment with diet A in the adult rats and remained constant throughout the treatment period. This effect was sustained when diet B was fed for an additional 4 weeks.
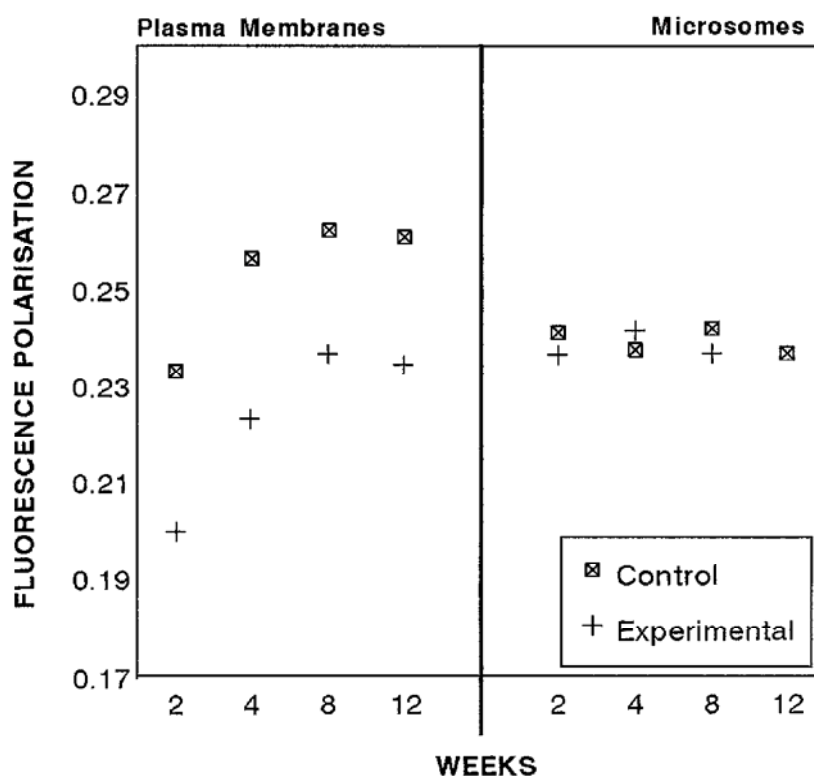
Fig. 1 Microviscosity of rat hepatocyte plasma membranes and microsomes as measured at 37°C.

Fatty acid profiles of plasma PC in weaned and adult rats.

The C18:2ω6 levels were significantly higher after 2 weeks (p<0.01) and 4 weeks (p<0.001), while no difference was observed after 8 weeks (Table 3). The C20:3ω6 level was significantly increased (p<0.0001) in the treated group at week 8 which was partly due to a concomitant decrease in the C20:3ω6 levels of the control rats. A significant (p<0.0001) reduction of C20:4ω6 was observed at week 2 as compared to the control rats. The levels of C20:5ω3, C22:5ω3 and C22:6ω3 were significantly higher (p<0.0001) in the treated adult rats from 2 weeks onwards. A similar pattern of FA changes was observed in the weaned rats (Table 3). However, the increase in C18:2ω6 (p<0.01), C20:5ω3 (p<0.01) and C22:6ω3 (p<0.05) and the decrease in C20:4ω6 (p<0.001) was significantly greater as compared to the adult rats fed diet A for 4 weeks.

Table 3:   Fatty acid composition of plasma phosphatidylcholine (PC) from adult and weaned rats fed diet A and B for different time intervals.

Fatty acid	Diet A (Adult rats)[1]						Diet B (Adult rats)[2]		Diet A (Weaned rats)[3]	
	Week 2		Week 4		Week 8					
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
18:2ω6	14.45±0.56	15.89±0.74[b]	13.99±0.18	15.94±0.56[c]	12.85±1.22	13.19±0.66	13.09±0.72	13.63±0.79	13.26±0.66	17.54±0.80[d]
18:3ω6	0.06±0.01	0.08±0.01	0.08±0.01	0.04±0.01	0.14±0.04	0.10±0.01	0.16±0.05	0.30±0.03a	0.05±0.02	0.05±0.02
20:3ω6	0.57±0.15	0.76±0.08	0.62±0.20	0.78±0.10	0.32±0.05	0.86±0.22[d]	0.29±0.03	0.67±0.09[d]	0.53±0.19	1.07±0.25[b]
20:4ω6	29.65±0.86	24.70±0.41[d]	29.17±0.98	24.23±0.86[d]	26.68±1.38	24.27±0.72[c]	25.68±1.31	24.24±1.10	27.69±1.04	19.94±1.54[d]
22:4ω6	0.81±0.06	0.26±0.04[d]	0.97±0.21	0.27±0.02[d]	0.98±0.14	0.20±0.01[d]	1.07±0.11	0.27±0.01[d]	0.76±0.11	0.23±0.02[d]
22:5ω6	4.13±0.32	0.43±0.06[d]	4.62±0.70	0.40±0.05[d]	4.97±0.18	0.33±0.04[d]	4.85±0.33	0.32±0.04[d]	5.25±0.50	0.27±0.04[d]
Total ω6	49.64±1.13	40.94±1.02[d]	49.44±1.07	41.46±0.42[d]	45.88±0.40	38.90±0.78[d]	45.19±0.94	38.96±0.93[d]	47.44±0.73	39.01±1.02[d]
18:3ω3	0.04±0.01	0.04±0.01	0.06±0.02	0.05±0.01	0.06±0.02	0.06±0.01	0.08±0.01	0.07±0.01	0.05±0.01	0.04±0.01
20:5ω3	<0.01	0.89±0.11[d]	<0.01	0.78±0.18[d]	<0.01	0.77±0.23[d]	<0.01	0.51±0.13[d]	<0.01	1.41±0.37[d]
22:5ω3	0.10±0.01	1.11±0.14[d]	0.09±0.01	0.96±0.17[d]	0.08±0.01	0.72±0.13[d]	0.13±0.01	0.97±0.28[d]	0.06±0.01	1.21±0.09[d]
22:6ω3	1.03±0.12	5.21±0.61[d]	0.96±0.09	5.53±0.69[d]	0.95±0.18	6.07±0.61[d]	0.94±0.10	6.29±0.64[d]	0.74±0.18	6.43±0.79[d]
Total ω3	1.08±0.15	7.24±0.85[d]	1.02±0.11	7.29±0.96[d]	0.97±0.21	7.59±0.92[d]	1.01±0.16	7.81±0.92[d]	0.79±0.21	9.06±0.95[d]
ω6/ω3	46.91±5.57	5.73±0.66[d]	49.18±5.77	5.79±0.81[d]	49.19±9.59	5.19±0.53[d]	45.65±7.15	5.05±0.54[d]	63.61±4.83	4.35±0.40[d]
p/s	1.24±0.07	1.09±0.07[c]	1.23±0.03	1.10±0.06[b]	1.01±0.02	0.99±0.05	1.00±0.04	1.03±0.05	1.08±0.03	1.06±0.07

Results are expressed as the percentage of the total identified fatty acids. Values are means ± standard deviation of 5 rats per group. [1]Adult rats fed diet A for 8 weeks. [2]Adult rats fed diet B for an additional 4 weeks after being fed diet A for 8 weeks. [3]Weaned rats fed diet A for 4 weeks. [a]$P < 0.05$, [b]$P < 0.01$ [c]$P < 0.001$ [d]$P < 0.0001$. ω6/ω3 = total ω6/ω3 ratio, p/s = polyunsaturate/saturate ratio.

Fatty acid profiles of membrane phospholipids.

ω6 FAs: Diet A resulted in a significant increase of C18:2ω6 from week 2 onwards in PC phospholipid fractions in the plasma ($p < 0.0001$) and microsomal ($p < 0.0001$) membranal compartments, while no significant change occurred in PE (Tables 4, 5). The level of C20:3ω6 was significantly increased in PC (Mic: $p < 0.0001$; PM: $p < 0.01$) from week 2 while in PE (Mic: $p < 0.01$; PM: $p < 0.0001$) only after week 8 (Tables 4, 5). The replacement of diet A with diet B for a further 4 weeks, did not result in any significant difference in the C20:3ω6 level in PE between the experimental groups (Table 7). A significant decrease in C20:4ω6 was noticed from week 2 in PC (Mic and PM: $p < 0.0001$) and PE (Mic: $p < 0.0001$; PM: $p < 0.01$) (Tables 4, 5).

ω3 FAs: With respect to the ω3 FAs, C20:5ω3, C22:5ω3 and C22:6ω3, a significant increase ($0.0001 < p < 0.001$) was noticed in the PC and PE fractions of both membranal compartments from week 2 onwards with diet A (Tables 4, 5). Although the level of C20:5ω3 was increased after 2 weeks, it tended to decrease as a function of time, more so in PC than PE in the plasma membranal compartment while to a similar extent in the phospholipid fractions of the microsomes. The C22:5ω3 and C22:6ω3 FAs were found to be incorporated to a greater extent in the PE fractions as compared to PC in both compartments.

In the weaned rats fed diet A for 4 weeks (Table 6), C18:2ω6 was significantly increased in PC (PM: $p < 0.0001$; Mic: $p < 0.01$) as described for the adult rats fed diet A for the same period (Tables 4 and 5). C20:3ω6 was significantly ($p < 0.0001$) increased in the PC phospholipid fraction of both compartments. The level of C20:4ω6 was significantly decreased in PC ($p < 0.0001$) and PE ($p < 0.001$) in both membranal compartments. With respect to the ω3 FAs a similar response was observed as described for the adult rats. However the increase for C18:2ω6 ($p < 0.01$) was significantly greater in the microsomal PC than in the adult rats fed diet A for 4 weeks (Tables 5, 6). The decrease of C20:4ω6 was significantly greater in the microsomal PC ($p < 0.01$) and PE ($p < 0.0001$) and plasma PC ($p < 0.001$), but not in the plasma membranes as compared to the adult rats at week 4. C20:5ω3 was significantly increased (PM PC: $p < 0.001$; PM PE: $p < 0.001$; Mic PC: $p < 0.001$; Mic PE: $p < 0.01$) above the levels of the adult rats in both the membranal compartments. In the plasma membranes (Table 4) the increase in the level of C22:5ω3 was significantly higher in PC ($p < 0.01$) and C22:6ω3 in PE ($p < 0.01$), whereas in the microsomes (Table 5) C22:5ω3 ($p < 0.01$) and C22:6ω3 ($p < 0.001$) were significantly increased only in the PC fraction.

Table 4: Fatty acid composition of liver plasma membrane phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from adult rats fed diet A.

	Phosphatidylcholine						Phosphatidylethanolamine					
	Week 2		Week 4		Week 8		Week 2		Week 4		Week 8	
Fatty acid	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
18:2ω6	8.15±0.60	10.60±0.72[d]	8.56±0.65	10.90±0.73[d]	7.57±0.34	8.99±0.40[b]	6.97±0.42	6.27±0.57	6.22±0.43	6.55±0.39	6.72±0.44	7.67±0.65
18:3ω6	0.08±0.02	0.06±0.02	0.20±0.04	0.14±0.03[b]	0.11±0.01	0.09±0.01	0.07±0.01	0.14±0.03[c]	0.08±0.01	0.15±0.03[c]	0.17±0.04	0.13±0.03[a]
20:3ω6	0.61±0.23	1.28±0.08[d]	0.72±0.24	0.95±0.08[a]	0.44±0.09	1.13±0.06[d]	0.52±0.10	0.52±0.09	0.50±0.13	0.53±0.07[d]	0.45±0.04	0.74±0.13[a]
20:4ω6	29.06±0.94	24.25±0.52[d]	30.69±1.01	25.40±0.38[d]	32.89±0.92	26.25±0.64[d]	31.66±0.61	30.42±0.59[b]	29.92±0.59	25.92±0.39[d]	29.64±0.52	26.09±0.89[d]
22:4ω6	0.67±0.07	0.23±0.01[d]	0.92±0.25	0.20±0.03[d]	0.51±0.11	0.23±0.02[d]	2.69±0.36	0.89±0.11[d]	2.72±0.28	1.00±0.14[d]	3.94±0.52	1.19±0.13[d]
22:5ω6	4.46±0.53	0.50±0.08[d]	5.53±0.50	0.45±0.10[d]	5.60±0.30	0.34±0.04[d]	7.54±0.64	0.63±0.09[d]	10.23±0.76	0.80±0.12[d]	10.86±0.20	0.85±0.13[d]
Total ω6	43.02±0.88	36.91±0.91[d]	46.61±1.37	38.05±0.98[d]	47.13±0.70	37.02±0.81[d]	49.59±0.48	38.63±1.10[d]	49.68±0.48	34.94±0.69	51.63±0.53	36.81±0.52[d]
18:3ω3	0.04±0.01	0.04±0.01	0.05±0.01	0.04±0.01	0.05±0.01	0.04±0.02	0.04±0.01	0.05±0.01	0.05±0.02	0.06±0.01	0.06±0.01	0.04±0.01
20:5ω3	<0.01	1.04±0.09[d]	<0.01	0.63±0.14[d]	<0.01	0.45±0.11[d]	<0.01	0.96±0.22[d]	<0.01	0.87±0.19[d]	<0.01	0.85±0.10[d]
22:5ω3	0.09±0.02	1.19±0.08[d]	0.12±0.02	1.02±0.12[d]	0.06±0.01	0.38±0.03[d]	0.22±0.01	2.69±0.27[d]	0.30±0.07	2.58±0.26[d]	0.17±0.03	2.81±0.35[d]
22:6ω3	1.22±0.29	6.77±0.70[d]	1.15±0.07	6.99±0.56[d]	0.85±0.05	7.49±0.59[d]	1.76±0.20	12.81±0.38[d]	2.49±0.30	13.95±0.31[d]	2.17±0.16	14.12±0.38[d]
Total ω3	1.31±0.34	9.03±0.83[d]	1.25±0.12	8.68±0.64[d]	0.89±0.06	8.29±0.63[d]	1.82±0.28	16.48±0.62[d]	2.72±0.26	17.45±0.20[d]	2.38±0.15	17.80±0.70[d]
ω6/ω3	35.09±9.14	4.13±0.49[d]	37.51±3.93	4.41±0.40[d]	52.96±4.12	4.50±0.42[d]	27.81±4.18	2.35±0.10[d]	18.42±1.94	2.00±0.03[d]	21.81±1.26	2.07±0.11[d]
p/s	0.98±0.03	1.04±0.03	1.14±0.05	1.07±0.03[a]	1.10±0.04	0.99±0.02[c]	1.30±0.06	1.42±0.11	1.36±0.02	1.29±0.04	1.39±0.04	1.44±0.03

Results are expressed as the percentage of the total identified fatty acids. Control rats were fed the AIN 76A diet and the experimental rats diet A for 8 weeks. Values are means ± standard deviation of 5 rats per group. [a]P < 0.05, [b]P < 0.01 [c]P < 0.001 [d]P < 0.0001. ω6/ω3 = total ω6/ω3 ratio, p/s = polyunsaturate/saturate ratio.

Table 5: Fatty acid composition of liver microsome phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from adult rats fed diet A.

	Phosphatidylcholine						Phosphatidylethanolamine					
	Week 2		Week 4		Week 8		Week 2		Week 4		Week 8	
Fatty acid	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
18:2ω6	8.27±0.78	11.31±0.92[d]	8.62±0.52	10.52±0.37[d]	8.09±0.58	10.31±0.42[d]	5.67±0.68	6.19±0.59	5.29±0.52	5.92±0.51	6.04±0.17	5.79±0.59
18:3ω6	0.07±0.02	0.05±0.02	0.09±0.03	0.08±0.03	0.11±0.03	0.08±0.03	0.25±0.18	0.08±0.03[b]	0.19±0.04	0.12±0.04	0.13±0.02	0.09±0.03
20:3ω6	0.53±0.10	0.96±0.03[b]	0.64±0.19	1.14±0.17[c]	0.42±0.06	1.22±0.34[d]	0.45±0.08	0.56±0.08	0.52±0.13	0.59±0.07	0.35±0.05	0.51±0.07[b]
20:4ω6	32.52±1.57	23.98±2.21[d]	30.57±0.51	27.57±0.64[c]	33.55±0.33	29.05±1.18[d]	29.87±0.79	24.31±0.47[d]	31.18±1.34	28.02±0.47[c]	29.71±0.36	25.67±0.73[d]
22:4ω6	0.62±0.15	0.18±0.02[d]	0.73±0.05	0.22±0.02[d]	0.93±0.11	0.20±0.02[d]	2.15±0.30	0.61 0.04[d]	3.37±0.63	1.02±0.18[d]	3.45±0.60	0.66±0.08[d]
22:5ω6	5.05±0.55	0.42±0.08[d]	5.35±0.70	0.45±0.07[d]	6.51±0.19	0.41±0.10[d]	9.01±1.06	0.84±0.28[d]	12.70±0.86	1.04±0.28[d]	13.47±0.79	0.63±0.14[d]
Total ω6	47.05±0.78	36.90±1.37[d]	46.01±0.94	39.97±0.53[d]	49.61±0.64	39.95±0.57[d]	47.40±1.66	32.59±1.13[d]	52.57±2.55	36.70±0.84[d]	53.15±1.60	33.35±0.87[d]
18:3ω3	0.05±0.01	0.05±0.01	0.04±0.01	0.05±0.01	0.05±0.01	0.05±0.01	0.06±0.01	0.04±0.01	0.07±0.02	0.07±0.01	0.06±0.02	0.05±0.01
20:5ω3	<0.01	0.84±0.10[d]	<0.01	0.62±0.18[d]	<0.01	0.56±0.20[d]	<0.01	1.18±0.22[d]	<0.01	0.98±0.25[d]	<0.01	0.83±0.24[d]
22:5ω3	0.10±0.01	1.25±0.13[d]	0.08±0.01	1.19±0.10[d]	0.11±0.01	0.90±0.30[d]	0.27±0.05	3.02±0.24[d]	0.48±0.21	3.42±0.52[d]	0.20±0.10	2.31±0.21[d]
22:6ω3	1.16±0.25	7.12±0.91[d]	1.21±0.11	7.65±0.60[d]	1.07±0.07	7.92±0.34[d]	2.56±0.60	15.38±0.52[d]	3.30±0.52	19.16±1.47[d]	2.69±0.37	16.28±0.79[d]
Total ω3	1.23±0.32	9.25±1.02[d]	1.33±0.09	9.27±0.35[d]	1.17±0.09	9.42±0.59[d]	2.57±0.62	16.84±0.59[d]	3.52±0.46	22.72±1.16[d]	3.08±0.45	20.07±1.69[d]
ω6/ω3	41.20±11.04	4.04±0.45[d]	34.82±2.73	4.32±0.17[d]	42.80±3.02	4.26±0.29[d]	19.43±4.31	1.94±0.12[d]	15.24±2.55	1.62±0.09[d]	17.59±2.19	1.67±0.16[d]
p/s	1.13±0.03	1.07±0.09	1.12±0.04	1.17±0.03	1.24±0.03	1.18±0.03	1.20±0.09	1.25±0.05	1.59±0.10	1.78±0.07	1.54±0.11	1.34±0.04

Results are expressed as the percentage of the total identified fatty acids. Control rats were fed the AIN 76A diet and the experimental rats diet A for 8 weeks. Values are means ± standard deviation of 5 rats per group. [a]$P < 0.05$, [b]$P < 0.01$ [c]$P < 0.001$ [d]$P < 0.0001$. ω6/ω3 = total ω6/ω3 ratio, p/s = polyunsaturate/saturate ratio.

Table 6: Fatty acid composition of liver plasma membrane and microsome phospholipids phophatidylcholine (PC) and phosphatidylethanolamine (PE) from weaned rats.

| | Plasma membranes | | | | Microsomes | | | |
| | Phosphatidylcholine | | Phosphatidylethanolamine | | Phosphatidylcholine | | Phosphatidylethanolamine | |
Fatty acid	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
18:2ω6	6.62±0.66	10.82±0.72[d]	4.40±0.53	4.77±0.73	7.47±0.59	12.16±0.92[b]	4.96±0.56	5.69±0.65
18:3ω6	0.07±0.02	0.18±0.04[d]	0.20±0.06	0.20±0.02	0.07±0.03	0.06±0.01	0.08±0.03	0.10±0.04
20:3ω6	0.67±0.11	1.32±0.11[d]	0.51±0.17	0.55±0.03	0.61±0.22	1.38±0.26[d]	0.42±0.07	0.49±0.08
20:4ω6	31.19±1.06	24.12±0.85[d]	28.90±1.83	24.85±0.89[c]	31.31±1.19	23.21±2.17[d]	30.59±2.59	23.48±0.39[c]
22:4ω6	0.87±0.12	0.29±0.09[d]	3.21±0.35	1.15±0.42[d]	0.64±0.24	0.22±0.05[d]	2.68±0.10	0.57±0.05[d]
22:5ω6	6.32±0.59	0.37±0.12[d]	14.74±0.59	0.93±0.43[d]	7.66±0.54	0.40±0.06[d]	12.99±5.90	0.58±0.11[d]
Total ω6	44.54±1.71	37.07±1.06[d]	51.97±0.98	32.46±0.76[d]	47.76±1.12	37.43±1.26[d]	51.70±3.03	30.92±0.25[d]
18:3ω3	0.07±0.01	0.05±0.01	0.10±0.04	0.09±0.04	0.04±0.01	0.06±0.01	0.04±0.01	0.05±0.01
20:5ω3	<0.01	1.13±0.15[d]	<0.01	1.64±0.23[d]	<0.01	2.17±0.53[d]	<0.01	1.60±0.32[d]
22:5ω3	0.14±0.01	1.43±0.22[d]	0.31±0.09	3.13±0.45[d]	0.08±0.02	1.65±0.18[d]	0.31±0.16	3.07±0.40[d]
22:6ω3	0.88±0.23	7.37±0.69[d]	1.47±0.18	15.99±0.82[d]	1.21±0.22	9.70±0.66[d]	2.57±0.65	16.97±0.97[d]
Total ω3	0.95±0.25	9.96±0.69[d]	1.77±0.18	20.86±1.02[d]	1.27±0.20	13.29±0.79[d]	4.51±0.88	18.79±0.66[d]
ω6/ω3	50.64±13.93	3.74±0.33[c]	29.62±3.26	1.56±0.09[d]	38.77±6.67	2.83±0.22[d]	11.99±2.87	1.65±0.07[c]
p/s	1.01±0.06	1.06±0.04	1.32±0.05	1.28±0.07	1.17±0.06	1.24±0.06	1.49±0.16	1.23±0.03

Results are expressed as the percentage of the total identified fatty acids. Control rats were fed the AIN 76A diet and the experimental rats diet A for 4 weeks. Values are means ± standard deviation of 5 rats per group. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0001$. ω6/ω3 = total ω6/ω3 ratio, p/s = polyunsaturate/saturate ratio.

Threshold effects of diet B.

To determine whether a threshold for a specific FA was obtained the data from the rats fed diet A for 8 weeks (Tables 4, 5) were compared with the rats fed diet B for an additional 4 weeks (Table 7).   In the plasma membranes C18:2ω6 ($p<0.0001$) and C22:6ω3 ($p<0.001$) were significantly increased in the PC and PE fractions respectively (Tables 4, 7).   C20:3ω6 was significantly lowered in the plasma membranal PC ($p<0.001$) and PE ($p<0.01$), whereas C20:4ω6 was significantly increased in PC ($p<0.001$) and significantly lowered ($p<0.01$) in PE.   No significant effects were observed in the microsomal PC and PE phospholipid fractions.

Table 7: Fatty acid composition of liver plasma membrane and microsome phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from adult rats fed diet B.

Fatty acid	Plasma membranes				Microsomes			
	Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylcholine		Phosphatidylethanolamine	
	Control	Experimental	Control	Experimental	Control	Experimental	Control	Experimental
18:2ω6	8.32±0.86	10.87±0.38[c]	6.71±0.31	6.41±0.48	7.91±0.68	10.02±0.67[d]	4.99±0.58	6.11±0.67
18:3ω6	0.17±0.03	0.23±0.02[a]	0.21±0.04	0.43±0.13[a]	0.12±0.02	0.19±0.04	0.19±0.02	0.25±0.01
20:3ω6	0.52±0.07	0.79±0.10[c]	0.44 0.03	0.48±0.08	0.41±0.06	0.90±0.14[c]	0.37±0.05	0.42±0.04
20:4ω6	31.26±2.67	29.15±0.51[c]	28.02±0.28	23.55±0.64[d]	32.58±0.73	28.78±1.62[d]	30.17±0.29	25.53±1.00[d]
22:4ω6	0.48±0.10	0.24±0.02[d]	3.65±0.17	0.56±0.12[d]	1.07±0.27	0.37±0.09[d]	3.46±0.26	0.61±0.13[d]
22:5ω6	6.13±0.66	0.33±0.08[d]	11.35±0.49	0.70±0.06[d]	5.67±0.35	0.44±0.15[d]	12.42±0.68	0.74±0.25[d]
Total ω6	46.87±2.44	41.61±0.84[d]	50.39±0.38	32.14±0.46[d]	46.42±2.03	40.70±1.14[d]	51.60±1.12	33.66±0.64[d]
18:3ω3	0.05±0.01	0.09±0.01[b]	0.06±0.01	0.08±0.05	0.07±0.02	0.08±0.01	0.07±0.01	0.08±0.01
20:5ω3	<0.01	0.38±0.07[d]	<0.01	0.77±0.13[d]	<0.01	0.34±0.14[d]	<0.01	0.72±0.23[d]
22:5ω3	0.07±0.01	0.52±0.09[d]	0.16±0.02	2.87±0.35[d]	0.14±0.03	0.88±0.15[d]	0.45±0.19	2.81±0.35[d]
22:6ω3	1.23±0.39	7.51±0.49[d]	2.32±0.24	15.88±0.63[d]	1.26±0.14	6.32±0.18[d]	2.59±0.52	16.72±1.00[d]
Total ω3	1.32±0.39	8.50±0.43[d]	2.55±0.25	19.60±0.78[d]	1.47±0.17	7.62±0.27[d]	2.78±0.53	19.42±0.97[d]
ω6/ω3	37.78±7.73	4.91±0.28[d]	19.99±2.13	1.64±0.07[d]	32.03± 4.54	5.35±0.09[d]	19.34±4.02	1.74±0.07[d]
p/s	1.13±0.13	1.21±0.04	1.34±0.03	1.23±0.03	1.10±0.09	1.12±0.06	1.42±0.04	1.35±0.08

Results are expressed as the percentage of the total identified fatty acids. Control rats were fed the AIN 76A diet for 12 weeks and the experimental rat group were fed diet B for an additional 4 weeks after being fed diet A for 8 weeks. Values are means ± standard deviation of 5 rats per group. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.0001$. Values are means ± standard deviation. ω6/ω3) = total ω6/ω3 ratio, p/s = polyunsaturate/saturate ratio.

Effect of age on FA profiles in adult rats.

The level of C20:3ω6 in the plasma PC of the control rats decreased significantly (p<0.01) at week 8 (Table 3). C20:4ω6 tended to increase (p<0.001) in the controls and C20:5ω3 tended to decrease (p<0.05) in the experimental groups over time in the plasma membrane PC (Table 4). The same pattern was observed in the microsomes for the two FAs but the change, as a function of time, was not significant (Tabel 5).

Polyunsaturate/saturate and ω6/ω3 ratios.

The polyunsaturate/saturate (p/s) ratio was significantly lower in the plasma PC of the experimental group at week 2 (p<0.001) and 4 (p<0.01; Table 3) and in the plasma membrane PC at week 4 (p<0.01) and 8 (p<0.0001; Table 4) compared to the control group. The ω6/ω3 ratio was significantly (p<0.0001) lower in the experimental group in all the compartments (Tables 3, 4, 5, 6).

DISCUSSION

Numerous studies have been conducted on the modulating role of dietary fat in the cancer initiating and promoting phases of cancer induction by chemicals in experimental animals (1, 2, 21). However, little information is available on the ω6/ω3 dietary ratios required and the mechanisms involved in the modulation of cancer induction by dietary FAs. Tumours are dependent on host circulation for the type and amount of PUFA available from the dietary FA intake (22). This suggests that it is possible to alter the FA composition of a tumour through dietary FA intake. The changes brought about by dietary FAs therefore, could alter the growth and membranal properties and/or characteristics of cancer cells and increase their sensitivity to cancer therapeutic treatment (1, 22).

The majority of studies investigating the role of various ω6/ω3 ratios in disease have used either a very high or low ratio of ω6 to ω3 FAs i.e. ranging from 40:1 to 3:1 in high fat diets (23, 24, 25, 26). The time of administering the diet also varied from either before, during or after the carcinogen application and/or cancer promoting regimens. Interpretation of these results is therefore very complex as it is difficult to separate the dietary induced changes in the relevant tissue compartments from the modulating effects on cancer induction. It is important to allow sufficient time for the diet-induced changes to occur prior to monitoring the effects related to cancer induction. The present study focused on the kinetics of tissue related changes induced by a low fat diet with different ω6/ω3 FA ratios as well as age related changes. The FA changes of specifically the liver plasma and

microsomal membranes were monitored since they are important compartments in the regulation of many cell functions such as growth and differentiation (9, 27, 28) and in the metabolism of cellular macromolecules and xenobiotics (29) respectively.

The present results indicate that differences exist in the kinetics of FA replacement of the plasma membrane and microsomal compartments as well as between the PC and PE fractions of the individual membranal compartments. With respect to the ω6 FAs, C18:2ω6 and C20:3ω6 increased in the plasma PC and plasma membrane and microsomal PC compartments within 2 weeks, while no changes were noticed in the plasma membrane and microsomal PE phospholipid fractions (Tables 4, 6). Therefore the accumulation of C18:2ω6 is not reflected in PE despite the fact that the level of C20:4ω6 was significantly lowered in this membranal compartment. The latter, however could be due to an increased replacement of C20:4ω6 by C20:5ω3, C22:5ω3 and C22:6ω3. This can be further substantiated from the fact that the levels of C22:5ω3 and C22:6ω3 were incorporated to a greater extent in the PE phospholipid fraction of both membranal compartments as compared to PC (Tables 4, 5). The increase in C18:2ω6 and C20:3ω6 is probably due to desaturase inhibition because C20:5ω3 has been shown to inhibit the delta-6 (30) and delta-5 (26) desaturation of the ω6 FAs. A similar effect was noticed in the weaned rats (Table 6). A final difference observed between the membranal compartments was that the level of C20:5ω3, which was increased in all compartments after two weeks, tended to decrease as a function of time only in the plasma membrane PC fraction (Table 4).

The difference in the kinetics of FA replacement between PC and PE could be due to the position of the phospholipids in the lipid bilayer as PC is mainly located in the outer and PE in the inner part of the lipid bilayer (28). This would imply that PC is more susceptible to dietary FA manipulation than PE while the mechanism and/or control of FA incorporation between PC and PE is probably different with regards to the rate of FA replacement. This can be deduced from: (i) the PE phospholipid fraction has a higher content of the long chain ω3 FAs i.e. C22:5 and C22:6 and (ii) the levels of C18:2ω6 and C20:3ω6 were affected less in PE than in PC as described above. As PC is located in the outer part of cellular membranes, the interaction between the plasma PC and plasma membrane and microsomal PC is therefore of interest. This study indicated that the FA profile of plasma PC does not necessarily reflect the profiles of the phospholipid fractions in the different membranous environments. The level of C18:2ω6 was significantly higher

(P<0.001) in plasma PC than in the plasma membrane and microsomal PC. The increase of C20:3ω6 was significant over that of the controls in the plasma PC only from week 8 on diet A as compared to the plasma membrane and microsomal PC fractions which was increased after 2 weeks.

The changes brought about with diet B were limited only to certain ω6 FAs in the plasma PC and plasma membrane compartments with no changes occurring in the microsomal compartments. Therefore, a threshold for the incorporation and replacement of both the ω6 and ω3 FAs was reached after 2 weeks in the microsomes with diet A. In the plasma PC and plasma membrane, most of the ω3 FAs achieved a threshold incorporation after 2 weeks on diet A, except for C22:6ω3 in the plasma membranal PE, which was further increased after introduction of diet B. However, diet B caused a further change in certain ω6 FAs. The level of C18:2ω6 increased in the plasma membranal PC, while C20:3ω6 decreased in the plasma membranal PC and PE. This subsequent decrease of C20:3ω6 could be related, as discussed above, to an increased replacement of the ω6 FAs by the ω3 FAs due to the increased ω3 FA content of diet B. C20:4ω6 decreased even further in the plasma membrane PE, but increased in the PC fraction as compared to week 8 of the adult experimental group. C20:4ω6 tended to increase steadily over time in the plasma membranal PC, but remained below the control levels in the rats fed diet A. It appears therefore that C20:4ω6 had reached a critical minimum level in PC by week 2 with diet A and that C20:4ω6 incorporation tended to increase despite the increased ω3 FA content of diet B. It is possible that other regulatory mechanisms exist preventing the decrease of C20:4ω6 below a critical level. It would appear that a balance exists with regards to inhibition of delta-6 and -5 desaturase by C20:5ω3 and the competition between C20:4ω6 and C20:5ω3 for incorporation into membranal phospholipids. Therefore further increases in C20:5ω3 will not affect C20:4ω6 levels in cellular membranes. The importance of this FA homeostasis in membranes could be related to the dual role of C20:4ω6 as a functional FA ie. precursor for prostaglandins (5) and structural FA ie. membrane fluidity and permeability (31). This inhibition effect was not reflected in the PE phospholipid fraction of the membranal compartments.

The ω6/ω3 ratio of the different membrane fractions were markedly decreased while the p/s ratios remained constant. The membrane fluidity increased markedly in the plasma membranes but not in the microsomes. Although changes in the degree of unsaturation of the FAs in membranes are known to affect membrane fluidity (1, 3), it has been shown

that a relationship between FA compositional changes in cellular membranes and changes in membrane fluidity does not exist in all cellular membranal compartments (3, 32). Microsomes appear to have a compensatory mechanism to resist changes in membrane fluidity as they are structurally less organized than plasma membranes and are therefore not readily influenced by changes in PUFA (1).

The FA exchange in the different cellular compartments for the weaned rats followed a similar pattern as in the adult rats fed the 12:1 diet for 4 weeks. However, the changes in the FA profiles of the phospholipids were greater, especially of the ω3 family, in the weaned rats. The decrease in C20:4ω6 content was greater in the microsomal compartment and plasma PC but not in the plasma membrane as compared to the adult group treated with the 12:1 diet for 4 weeks (Tables 3, 4, 6). This could be ascribed to a difference in the inhibitory pattern of the ω3 FAs over ω6 FA metabolism (7, 11) that is manifested differently in the various compartments as compared to adult rats. In addition, various studies have shown that the activity of the delta desaturases decrease with age and that this can influence the ω6 FA composition of different membranal fractions (2, 11).

The ω3 FA content of the 12:1 diet (diet A) constituted 8% of the total dietary fat content as compared to the 6:1 diet (diet B) with a ω3 FA content of 17% of total dietary fats. This study therefore suggests that a minimum level of ω3 FA constituting approximately 8% of the total dietary fat is, depending on the membranal compartment and structural component, adequate for a maximal effect on the incorporation of ω3 FAs. The present data indicated that a threshold for the maximal incorporation of the ω3 FAs had been reached after 2 weeks on the 12:1 ω6/ω3 PUFA diet. Regarding the ω6 FAs, a threshold was also reached after two weeks, but only in the microsomes and plasma membrane PC. With an increase in the ω3 dietary content further changes occurred in the ω6 FA profiles of the PC and PE components of the plasma membrane compartment. As the incorporation of the ω3 FAs into the different compartments depended directly on the diet, the membranal uptake stabilised more rapidly than in the case of the ω6 FAs. Therefore, the kinetics of replacement depends on the interaction of the different classes of FAs, the position of the structural component of the membrane and the age of the rats.

Studies where the role of dietary FA in the modulation of cancer is monitored need to be aware that differences exist in the kinetics of FAs replacement in the different cellular compartments. Even in the same compartment, FA replacement of the major membrane

structural components differ. Age also plays an important role in the interaction of ω6 and ω3 FAs as indicated in the present study by the difference in magnitude of FA incorporation between the weaned and adult rats in the various phospholipid fractions. A dietary FA ratio of between 12:1 to 6:1 appears to be optimal for the maximal replacement of FAs in rat cellular membranes. On the one end the 6:1 diet has a modulating effect in the rat regarding its body weight gain and relative liver weight while on the other the 12:1 diet does not induce a threshold effect within 8 weeks in the plasma membrane fraction with respect to the incorporation of ω6 FAs. The addition of dietary C18:3ω6, to counteract the lowering effects of the ω3 FAs on the C20:4ω6 levels, in combination with certain ω3 FAs could provide opportunities to establish specific ω3 FA levels in the major phospholipid fractions without interfering with the ω6 FA metabolic pathway.

REFERENCES

1. Spector A A, Burns C P. Biological and therapeutic potential of membrane lipid modification in tumors. Cancer Res 1987; 47: 4529-4537.

2. Simopoulos A P. Omega-3 fatty acids in health and disease and in growth and development. Am J Clin Nutr 1991; 54: 438-463.

3. Hagve T A. Effects of unsaturated fatty acids on cell membrane function. Scand J Clin Invest 1988; 48: 381-388.

4. Jakobsson-Borin A, Tollbom O, Dallner G. Effect of dietary fat on rat liver microsomal and mitochondrial/lysosomal Dolochol, phospholipid and cholesterol. Lipids 1991;26: 915-921.

5. Horrobin D F. Nutritional and medical importance of gamma-linolenic acid. Prog Lipid Res 1992; 31: 163-194.

6. Iritani N, Narita R. Changes of arachidonic acid and n-3 polyunsaturated fatty acids of phospholipid classes in liver, plasma and platelets during dietary fat manipulation. Biochim Biophys Acta 1984; 793: 441-447.

7. Boudreau M D, Chanmugam P S, Hart S B, Lee S H, Hwang D H. Lack of dose response by dietary n-3 fatty acids at a constant ratio of n-3 to n-6 fatty acids in suppressing eicosanois biosynthesis from arachidonic acid. Am J Clin Nutr 1991; 54: 111-117.

8. Singer P, Berger I, Moritz V, Förster D, Taube C. N-6 and N-3 PUFA in liver lipids, thromboxane formation and blood pressure from SHR during diets supplemented with evening primrose oil, sunflower or fish oil. Prostagland Leuk Essent Fatty Acids 1990; 39: 207-211.

9. Venkatraman T, Toohey T, Clandinin M T. Does a threshold for the effect of dietary omega-3 fatty acids on the fatty acid composition of nuclear envelope phospholipids exist? Lipids 1992; 27: 94-97.

10. Clandinin M T, Foot M, Robson L. Plasma membrane: Can its structure and function be modulated by dietary fat? Comp Biochem Physiol 1983; Vol 76B: 335-339.

11. Dinh T K L, Bourre J M, Durand G. Effect of age and alpha-linolenic acid deficiency on delta-6 desaturase activity and liver lipids in rats. Lipids 1993; 28: 517-523.

12. American Institute of Nutrition. Second report of the ad hoc committee on standards for nutritional studies. J Nutr 1980; 110: 1726.

13. Loten E G, Redshaw J C. Preparation of rat liver plasma membranes in a high yield. Analytical Biochem 1986; 154: 183-185.

14. Bartoli G M, Bartoli S, Galeotti T, Bertoli E. Superoxide dismutase content and microsomal lipid composition of tumours with different growth rates. Biochim Biophys Acta 1980; 620: 205-211.

15. Kaushal V, Barnes L D. Effect of zwitterionic buffers on measurement of small masses of protein with bicinchoninic acid. Analytical Biochem 1986; 157: 291-294.

16. Smuts C M, Weich H F H, Weight M J, Faber M, Kruger M, Lombard C J, Benadé A J S. Free cholesterol concentrations in the high-density lipoprotein subfraction-3 as a risk indicator in patients with angiographically documented coronary artery disease. Cor Art Dis 1994;5: 331-338.

17. Gilfillan A M, Chu A J, Smart D A, Rooney S A. Single plate separation of lung phospholipids including disaturated phosphatidylcholine. J Lipid Res 1983; 24: 1651-1656.

18. Itaya K, Ui M. A new micromethod for the colorimetric determination of inorganic phosphate. Clinica Chimica Acta 1966; 14: 361-366.

19. Richmond W. Preparation and properties of a cholesterol oxidase from Nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. Clin Chem 1973; 19: 1350-1356.

20. Victor T, Van der Merwe N, Benadé A J S, La Cock C, Lochner A. Mitochondrial phospholipid composition and microviscosity in myocardial ischaemia. Biochim Biophys Acta 1985; 834: 215-223.

21. Nassar B A, Das U N, Huang Y-S, Ells G, Horrobin D F. The effect of chemical hepatocarcinogenesis on liver phospholipid composition in rats fed n-6 and n-3 fatty acid-supplemented diets. Proc Soc Exp Biol Med 1992; 199: 365-368.

22. Burns C P, Spector A A. Biochemical effects of lipids on cancer therapy. J Nutr Biochem 1994; 5: 114-123.

23. Galli C, Butrum R. Dietary omega-3 fatty acids and cancer: An overview. In: Simopoulos A P, Kifer R R, Martin R E, Barlow S M, eds. Health effects of omega-3 polyunsaturated fatty acids in seafoods. Basel, Karger, 1991: 446-461,

24. Palombo J D, Bistrian B R, Fechner K D, Blackburn G L, Forse R A. Rapid incorporation of fish or olive oil fatty acids into rat hepatic sinusoidal cell phospholipids afetr continuous enteral feeding during endotoxemia. Am J Clin Nutr 1993; 57: 643-649.

25. Garg M L, Sbokova E, Wierzbicki A, Thomson A B R, Clandinin M T. Differential effects of dietary linoleic and alpha-linolenic acid on lipid metabolism in rat tissues. Lipids 1988; 23: 847-852.

26. Nassar B A, Huang Y S, Manku M S, Das U N, Morse N, Horrobin D F. The influence of dietary manipulation with n-3 and n-6 fatty acids on liver and plasma phospholipid fatty acids in rats. Lipids 1986; 21: 652-656.

27. Wahle K W J. Fatty acid modification and membrane lipids. Proc Nutr Soc 1983; 42: 273-287.

28. Berdanier C D. Role of membrane lipids in metabolic regulation. Nutr Rev 1988; 46: 145-149.

29. Balistreri W F, Shaw L M. Liver function. In: Tietz N W, eds. Fundamentals of clinical chemistry, third edition. W B Saunders Company, 1987: 729-761.

30. Garg M L, Thomson A B R, Clandinin M T. Interactions of saturated, n-6 and n-3 polyunsaturated fatty acids to modulate arachidonic acid metabolism. J Lipid Res 1990; 31:271-277.

31. Ballabriga A. Essential fatty acids and human tissue composition. An overview. Acta Paediatr 1994; Suppl 402: 63-68.

32. Stillwell W, Ehringer W, Jenski L J. Docosahexaenoic acid increases permeability of lipid vesicles and tumor cells. Lipids 1993; 28: 103-108.

CHAPTER 3

CHANGES IN ESSENTIAL FATTY ACID PATTERNS ASSOCIATED

WITH NORMAL LIVER REGENERATION AND THE PROGRESSION OF

HEPATOCYTE NODULES IN RAT HEPATOCARCINOGENESIS

CHANGES IN ESSENTIAL FATTY ACID PATTERNS ASSOCIATED WITH NORMAL LIVER REGENERATION AND THE PROGRESSION OF HEPATOCYTE NODULES IN RAT HEPATOCARCINOGENESIS.

S. Abel[a], C.M. Smuts[b], C. de Villiers[c] and W.C.A. Gelderblom[a]

[a]Programme on Mycotoxins and Experimental Carcinogenesis, [b]National Research Programme for Nutritional Intervention, [c]Experimental Biology Programme-Primate Unit, Medical Research Council (MRC), P.O. Box 19070, Tygerberg, 7505, South Africa.

ABSTRACT

Changes in lipid metabolism were monitored in rat hepatocyte nodules at certain time points over 9 months. Tissue obtained from partially hepatectomised rats collected over a period of 7 days, were included as a control for normal hepatocyte cell proliferation. Two important features regarding the lipid profiles of hepatocyte nodules and normal regenerating liver were the increased concentrations of phosphatidylethanolamine (PE) resulting in a decreased phosphatidylcholine/ phosphatidylethanolamine (PC/PE) ratio, and an increase in cholesterol. These changes coincided with increased membrane fluidity in the nodules and regenerating liver. With respect to the fatty acid (FA) profiles of the nodules, C18:1ᴛ9 and C18:2ᴛ6 increased in PE and PC whereas C20:4ᴛ6 decreased in PC and increased in PE. C22:5ᴛ6 and C22:6ᴛ3, the end products of the ᴛ6 and ᴛ3 metabolic pathways respectively, decreased in PC and remained unchanged in PE. The FA levels in PC reflected an impaired delta-6 desaturase enzyme, whereas this effect was masked in PE due to the increased concentration of this phospholipid fraction. In regenerating liver, the FA profiles of PC and PE showed the same pattern as described for the hepatocyte nodules, except for C18:1ᴛ9 which decreased in PC and increased non-significantly in PE. The increased C18:1ᴛ9 level, a FA with antioxidative properties, as well as the decreased levels of the long-chain polyunsaturated fatty acids (C20 and C22 carbon chains), have been associated with the decreased lipid peroxidation level in hepatocyte nodules. The resultant decrease in peroxidative metabolites, known to affect apoptosis, could be important in the progression of the nodules into neoplasia. The present results indicated that the altered lipid parameters associated with hepatocyte nodules closely mimics cellular proliferation in regenerating liver and could be responsible for the enhanced proliferation and/or altered growth pattern in these lesions. The altered FA profiles, especially the increased level of C20:4ᴛ6 in PE, suggest various pathways in which FA could play a role in transmembrane signaling related to the altered cell proliferative and apoptotic pathways. The persistent changes in the hepatocyte nodules suggest that the lipid metabolism escapes the regulatory mechanisms required for normal cellular homeostasis at different levels.

INTRODUCTION

The process of carcinogenesis is complex, resulting from alterations in the normal patterns of cellular growth (1). In the resistant hepatocyte model for liver carcinogenesis in the rat, a key event is the appearance of numerous altered or "resistant" cells during initiation which, upon promotion, results in the formation of hepatocyte nodules with a characteristic altered phenotype (2). Although the majority of these hepatocyte nodules disappear or re-differentiate to normal appearing liver, a few "persistent nodules" develop into malignant tumours (3). In the hepatocyte nodules, the balance between cell death and proliferation is disrupted resulting in a net increase in cell proliferation. This phenomenon changes, though, in the "persistent nodules" where cell death increases to counteract the increased cell proliferation, resulting in the retardation in growth of the persistent nodules. With the onset of cancer, this balance is again disturbed by an increased growth rate observed in the neoplastic tissue (4).

Studies have shown that the occurrence of potentially "neoplastic" hepatocyte lesions is associated with changes in the polyunsaturated fatty acid (PUFA) profile, especially the long-chain PUFA (C20 and C22 carbon chains, LCPUFA) and the lipid peroxidative status (5, 6). The differences in LCPUFA levels and extent of lipid peroxidation in preneoplastic lesions are possibly due to an abnormal essential FA metabolism involving delta-6 desaturase (7, 8, 9). Changes in the FA profiles have a wide range of effects regarding the integrity of cellular membranes. These changes are known to affect the membrane structure and fluidity, the activity of membranal enzymes and the affinity of growth factor receptors. Furthermore fatty acids act as signalling molecules involved in cell proliferation and/or apoptosis (5, 10).

Both LCPUFA, which form the main substrates for lipid peroxidation, and membrane lipid peroxidation have been found to be lower in hepatocyte nodules than in surrounding "normal" tissue (11, 12). The level of lipid peroxidation has also been found to influence tumour growth (11) and, together with changes in the membrane lipid status, is likely to play an important role in the abnormal cellular growth which prevails in preneoplastic lesions. The integrity of the cellular membrane is therefore important in the normal functioning of the cell and its responses to external growth stimulatory and/or inhibitory factors.

Studies in experimental animals have indicated that focal hepatocyte proliferations or hepatocyte nodules are the critical, relatively early lesions in the development of liver cancer in rats (2). The current study investigated the lipid profiles associated with the progression of hepatocyte nodules as well as in normal regenerating liver in order to delineate alterations in lipid metabolism with respect to cancer development in the liver of rats.

MATERIALS AND METHODS

Experimental Animals

Male Fischer rats (n=144) were fed the AIN 76A diet (13) *ad libitum* when weaned, and housed under controlled lighting (12 hour cycles) and temperature (23-25EC) with free access to water. Upon reaching a body weight of 150 g, they were housed separately in wire-bottomed cages and weighed three times a week.

The Resistant Hepatocyte model

Hepatocyte nodules were induced according to the method described by Solt and Farber (14). Briefly, the rats (body weight approximately 150 g) were injected intraperitoneally (i.p.) with diethylnitrosamine (DEN, 200 mg/kg body weight) to effect cancer initiation. Promotion was effected 3 weeks later by a daily intragastric dose of 2-acetylaminofluorene (2-AAF, 20 mg/kg body weight) on 3 consecutive days followed by partial hepatectomy on the fourth day. The rats (n=60) were terminated at intervals of one (n=15), three (n=15), six (n=15) and nine (n=15) months after cancer promotion and the hepatocyte nodules and surrounding tissue were collected. Control tissue was collected at similar time intervals from rats (n=60) that did not receive the initiating and promoting treatments. The tissue samples were immediately frozen on dry ice and stored at -80EC prior to analyses.

Regenerating Liver

Rat liver samples (n=18) were collected at intervals of 1, 2 and 7 days following partial hepatectomy in order to obtain tissue sections representing different stages of the regenerative response (15). Livers of untreated rats (n=6) were also collected at the time of partial hepatectomy. All the samples were stored at -80EC until analysed.

Lipid Analyses

Lipids were extracted from the control, nodule, surrounding and regenerating liver tissue with chloroform/methanol (CM; 2:1; v/v) (16) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant according to the method of Smuts *et al*. (17). In short, approximately 100 to 150 mg of the liver was ground to a fine powder in liquid nitrogen and weighed in glass-stoppered tubes. The tissue was suspended in 0.5 ml saline and the lipids were extracted with 24 ml CM. The CM mixture was filtered (sinterglass filters using Whatman glass microfibre filters, Cat No, 1820 866, Whatman International, Ltd, Maidstone, England) and the filtrate was evaporated to dryness *in vacuo* at 40℃, transferred to glass-stoppered tubes, washed with saline saturated with CM, and stored at 4℃ under nitrogen for 2 weeks until analysed. The lipid extracts were fractionated by thin layer chromatography (TLC) and the major phospholipid fractions, PC and PE, were collected for phospholipid and FA analyses (18). Phospholipid levels were determined colorimetrically using a malachite green dye after digestion with perchloric acid (16 N) at 170℃ for approximately 1 hr (19). For the FA analyses, the phospholipid fractions, PC and PE, were transmethylated with 2 ml methanol/18 M sulphuric acid (95:5; v/v) at 70℃ for 2 hrs. The FA methyl esters (FAME) were extracted in hexane and analysed by gas chromatography on a Varian 3400 Gas Chromatograph equipped with 30 m fused silica Megabore DB-225 columns of 0.53 mm internal diameter (J&W Scientific, cat. no. 125-2232). The individual FAME were identified by comparison of the retention times to those of a standard mixture of free FA, C14:0 to C24:1, and quantified using an internal standard (C17:0) and expressed as µg FA/100mg liver weight.

Total cholesterol of the lipid extracts was determined by an enzymatic iodide method using cholesterinoxidase and -esterase (20). The cholesterol/phospholipid molar ratio was calculated using the molar weights of 386.7, 787 and 744 for cholesterol, PC and PE, respectively.

Membrane Fluidity/Fluorescence Polarisation

Fluorescence polarisation studies were performed on homogenised control, surrounding, nodule and regenerating liver tissue with a fluorescence spectrofluorimeter (Perkin-Elmer MPF 44A). Samples were diluted to a concentration of 0.2 - 0.3 mg protein/ml with 10 mM Tris-HCl, (pH 7.4), sonicated for 10 seconds and

1.6-diphenyl-1.3.5-hexatrine (DPH; 5 µl; 2 mM in tetrahydrofuran) added (21, 22). The suspension (2.5 ml) was incubated in a water bath (37EC) for 30 - 60 minutes in the dark. Measurements were done manually with an emission polarizer at 0E (V component) and 90E (L component) with the excitation polarizer first at 0E (vertical component v) and then at 90E (horizontal component h). The excitation and emission slit widths were 14 nm and the excitation and emission wavelengths were 357 nm and 425 nm, respectively. The temperatures selected for screening ranged from 25EC to 41EC.

Protein determination

Powdered liver preparations (10-15 mg) were solubilised in 5% sodium dodecyl sulphate at 37EC and the protein content determined using a modified method of Lowry (23).

Statistical Analyses

The statistical analyses were performed using the analyses of variance (ANOVA). The Tukey Studentized Range Method was used to determine differences between the means. Lipid changes as a function of time were analysed with the Parametric Paired Difference T-Test. Values were considered significant if $P < 0.05$.

RESULTS

Cholesterol content   (Table 1)

The cholesterol content was significantly increased within the control, nodule and surrounding tissue, as a function of time. This increase was significant between months 3 and 6 ($P < 0.01$) in the control group, between months 6 and 9 ($P < 0.05$) in the nodules and between months 6 and 9 ($P < 0.05$) in the surrounding tissue. In the hepatocyte nodules, the cholesterol was significantly increased at months 1 ($P < 0.05$) and 9 ($P < 0.01$) compared to the respective controls. Compared to the surrounding tissue, the cholesterol in the nodules was significantly increased at months 6 ($P < 0.05$) and 9 ($P < 0.01$). No significant changes were observed between the controls and the surrounding tissue. The cholesterol concentration in the regenerating liver was significantly increased ($P < 0.01$) at days 2 and 7 when compared to the control.

Phospholipid content   (Table 1)

The PE level in the hepatocyte nodules, increased significantly at 1 (P<0.01), 3 (P<0.05), 6 (P<0.01) and 9 (P<0.01) months when compared to the respective controls and surrounding tissue (P<0.01). PE in the surrounding tissue was only significantly increased at months 1 (P<0.05) and 6 (P<0.05) compared to the respective controls. In contrast, PC in the nodules increased significantly only at month 1 compared to the control and surrounding tissue. The level of PE in the regenerating liver was significantly increased (P<0.01) at days 1, 2 and 7 compared to the control, whereas PC was significantly decreased (P<0.05) at day 2.

Table 1. Comparative lipid parameters in the Resistant Hepatocyte Model in control, nodule and surrounding tissue and in regenerating liver.

		Resistant Hepatocyte Model			Regenerating Liver	
Month		Control	Nodule	Surrounding		
Cholesterol (µg/100mg liver)						
1		15.80±3.26^a	21.77±3.98^b	17.60±0.96	Control	15.43±3.18^A
3		15.00±1.39	17.85±1.34	16.88±3.09	1 Day	15.08±3.17^A
6		22.15±0.38	25.30±2.36^a	19.68±3.74^b	2 Days	24.52±2.59^B
9		22.42±1.34^A	37.06±6.72^B	26.11±3.66^A	7 Days	29.88±4.96^B
Phospholipids (µg Pi/100mg liver)						
1	PC	133.84±23.29^A	183.65±23.28^B	129.71±11.68^A	Control	167.45±29.14^a
	PE	29.63±5.38^A	58.57±6.83^{bB}	40.72±1.97^{cA}		37.07±6.73^A
3	PC	160.66±16.73	161.26±28.75	132.29±20.22	1 Day	148.46±14.45
	PE	38.21±2.96^a	50.07±2.96^{bB}	36.35±5.92^{aA}		81.09±7.79^B
6	PC	153.82±45.71	162.13±18.64	129.29±12.15	2 Days	119.59±31.91^b
	PE	29.96±2.03^{aA}	72.82±5.66^{bB}	41.89±10.77^{cA}		65.93±18.78^B
9	PC	131.25±14.02	138.38±32.05	135.56±19.17	7 Days	146.42±18.90
	PE	39.30±3.08^A	62.63±7.99^B	45.59±4.94^A		63.93±7.53^B

Values are means ± SD. Significant differences are represented by upper caps (P<0.01) and lower caps (P<0.05). PC = phosphatidylcholine, PE = phosphatidylethanolamine.

Membrane Fluidity (Table 2)

The measurement of DPH-labelled membranes relates to membrane micro-viscosity which is inversely related to membrane fluidity i.e. an increase in micro-viscosity indicates a decrease in membrane fluidity (19). The membrane fluidity in the nodular tissue, decreased at months 1 (P<0.01), 3 (P<0.01) and 6 (P<0.05) as compared to the respective controls. However, at month 9 the membrane fluidity was significantly higher (P<0.01) than the respective control. The same pattern was observed when comparing the nodular tissue with the surrounding tissue. No significant differences were observed between the surrounding tissue and the respective controls. In the

regenerating liver, the membrane fluidity was significantly increased at days 1, 2 and 7 (P<0.01) when compared to the control.

A significant increase in the cholesterol/phospholipid molar ratio was observed in the surrounding tissue (P<0.05) at 3 months. At 9 months the ratio was significantly increased (P<0.05) in the nodules. The PC/PE ratio in the nodules was significantly decreased at months 1, 3, 6 and 9 (months 1, 6, 9; P<0.01, month 3; P<0.05) compared to the respective controls. The surrounding tissue PC/PE ratio was also significantly decreased from the controls at months 1 and 6 (P<0.01 and P<0.05, respectively). In the regenerating liver, the cholesterol/phospholipid molar ratio was significantly (P<0.01) increased at days 2 and 7 compared to the control and day 1. The PC/PE ratio was significantly (P<0.01) decreased from the control level at days 1, 2 and 7.

Table 2. Comparative membrane fluidity parameters of regenerating liver versus control, hepatocyte nodules and surrounding tissue generated in the Resistant Hepatocyte Model.

	Resistant Hepatocyte Model			Regenerating Liver	
Month	Control	Nodule	Surrounding		
Fluorescence polarisation					
1	0.249 ± 0.002^A	0.261 ± 0.004^B	0.245 ± 0.004^A	Control	0.238 ± 0.006^A
3	0.249 ± 0.003^A	0.267 ± 0.006^B	0.247 ± 0.002^A	1 Day	0.171 ± 0.007^B
6	0.243 ± 0.015^A	0.276 ± 0.013^B	0.240 ± 0.005^A	2 Days	0.130 ± 0.030^B
9	0.226 ± 0.004^A	0.175 ± 0.008^B	0.210 ± 0.002^A	7 Days	0.152 ± 0.030^B
Cholesterol/Phospholipid Molar Ratio*					
1	0.19 ± 0.01	0.18 ± 0.02^a	0.21 ± 0.01^b	Control	0.15 ± 0.01^A
3	0.15 ± 0.01^a	0.17 ± 0.02	0.20 ± 0.04^b	1 Day	0.13 ± 0.02^A
6	0.25 ± 0.05	0.22 ± 0.04	0.24 ± 0.07	2 Days	0.28 ± 0.05^B
9	0.27 ± 0.02^a	0.38 ± 0.02^b	0.29 ± 0.02^a	7 Days	0.28 ± 0.03^B
PC/PE Ratio					
1	4.54 ± 0.43^A	3.14 ± 0.16^B	3.20 ± 0.39^B	Control	4.54 ± 0.43^A
3	4.21 ± 0.43^a	3.21 ± 0.47^b	3.69 ± 0.73	1 Day	1.84 ± 0.17^B
6	5.14 ± 1.51^{aA}	2.22 ± 0.11^B	3.20 ± 0.54^b	2 Days	1.83 ± 0.26^B
9	3.34 ± 0.21^A	2.19 ± 0.24^B	2.97 ± 0.22^A	7 Days	2.31 ± 0.36^B

Values are means ± SD. Significant differences are represented by upper caps (P<0.01) and lower caps (P<0.05). PC = phosphatidylcholine, PE = phosphatidylethanolamine.

*The phospholipid molar ratio was calculated from the sum of PC and PE which constitute the major phospholipid fractions in rat liver.

Comparative FA profiles in PC and PE of hepatocyte nodules compared to surrounding and control tissue (Tables 3 & 4)

The individual FA were quantified against an internal standard (C17:0) and expressed as µg FA/mg protein (quantitative data).   The qualitative data, calculated as a percentage of total FA analysed, are given in the Addendum (Addendum 3, 4, 5).

The general trend of the quantitative FA profiles in the surrounding tissue tended to mimic that of the control tissue, especially after 3 months.   Before 3 months, some values fell in between the control and nodule values.

Saturated FA: (C16:0, C18:0)

In the nodules, the levels of C16:0 in PC increased significantly at months 1, 6 and 9 ($P<0.01$) compared to the controls, whereas C18:0 was significantly decreased at months 6 and 9 ($P<0.01$).   However, in PE C16:0 was significantly decreased at month 3 ($P<0.05$), whereas C18:0 was significantly increased at months 1, 6 and 9 ($P<0.01$). No significant changes were observed in the total saturated FA levels of PC, but in PE the levels increased significantly at months 1, 6 and 9 ($P<0.01$).

Monounsaturated FA: (C16:1, C18:1)

The values of C16:1 increased significantly in PC and PE in the nodules at months 1, 6 and 9 ($P<0.01$), while C18:1 increased significantly ($P<0.01$) in PC and PE at months 1, 3, 6 and 9 ($P<0.01$).   The total monounsaturated FA level was significantly increased in PC at 1, 6 and 9 months ($P<0.01$) in the nodules and in PE at 1, 3, 6 and 9 months ($P<0.01$).

Polyunsaturated FA

T6 PUFA: (C18:2, C20:4, C22:4, C22:5)

The level of C18:2 in the nodules increased ($P<0.01$) at months 1, 3, 6 and 9 in PC and PE.   In PC, C20:4 was significantly decreased only at month 3 ($P<0.05$), but tended to be slightly (not significant) lower at months 1, 6 and 9.   However, in PE C20:4 was significantly increased at 1, 6 and 9 months ($P<0.01$) with a non-significant increase at 3 months.   The level of C22:4 in PC was significantly decreased at month 3 ($P<0.05$) and 6 ($P<0.01$), but in PE the level was increased ($P<0.01$) at 1, 3, 6 and 9 months.   In PC, C22:5 was significantly decreased at 1 ($P<0.05$), 3, 6 and 9 months ($P<0.01$).   No significant changes were observed in PE.   The total T6 levels in PE were increased significantly at 1, 3, 6 ($P<0.01$) and 9 ($P<0.05$) months, while in PC it was significantly lower only at 3 months.

T3 PUFA: (C22:5, C22:6)

In PC, C22:5 was significantly decreased in the nodules at 3 and 6 months (P<0.01) but increased significantly in PE at 1, 6 (P<0.01) and 9 months (P<0.05). C22:6 was significantly decreased in PC at 1, 3, 6 and 9 months (P<0.01) compared to the controls, while no changes were observed in PE.   The total T3 level in PC was significantly decreased at 1, 3, 6 months (P<0.01) and 9 months (P<0.05). In PE, no significant changes were observed although the levels tended to increase (not significant) at months 1, 6 and 9.

Due to the decrease in T3 FA, the T6/T3 ratio was significantly increased in PC at 1, 3, 6 and 9 months (P<0.01), but only at months 1 and 6 (P<0.01) in PE. The PUFA level increased in PE at 1, 6 and 9 (P<0.01) months, and decreased significantly (P<0.01) at 3 months in PC. No significant changes in the P/S ratio was observed in the nodular tissue compared to the respective controls.

Table 3. Fatty acid analyses of the phosphatidylcholine (PC) fraction of control liver, nodule and surrounding liver in the Resistant Hepatocyte Model.

	1 Month			3 Months			6 Months			9 Months		
	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
Saturates												
C16:0	11.92±1.68^A	18.13±2.18^B	13.61±0.98^A	16.03±1.39^a	13.55±3.60	10.03±3.40^b	15.31±0.91^A	23.42±2.48^B	14.46±1.77^A	14.20±1.90^A	22.10±2.99^B	14.78±1.62^A
C18:0	13.13±1.80	11.34±1.96	10.90±0.51	15.50±0.84^a	10.64±3.18	9.22±2.38^b	16.71±2.08^A	10.99±1.85^B	15.89±2.24^A	21.40±1.05^A	12.98±3.76^B	19.13±1.87^A
Total	25.04±3.42	29.48±4.03	24.51±1.18	31.53±2.07^A	24.18±5.97	19.24±5.77^B	32.02±2.57	34.42±4.25	30.35±3.33	35.60±2.74	35.08±6.34	33.91±3.45
Monounsaturates												
C16:1	0.56±0.27^A	1.75±0.71^B	0.82±0.18^A	0.91±0.18	1.00±0.32	0.68±0.28	0.71±0.07^A	1.79±0.27^B	0.65±0.29^A	0.57±0.17^{aA}	1.71±0.34^{bB}	0.99±0.14^{cA}
C18:1	4.81±0.70^A	10.95±2.19^B	6.88±0.46^A	6.55±1.03	8.58±1.87^a	5.22±1.47^b	6.23±0.31^{aA}	12.73±0.91^{bB}	7.73±0.93^{cA}	5.77±0.70^A	16.70±4.51^B	7.23±0.78^A
Total	5.38±0.96^A	12.71±2.87^B	7.70±0.56^A	7.46±1.20	9.59±2.14	5.90±1.75	6.93±0.34^A	14.52±1.07^B	8.38±1.15^A	6.33±0.82^A	18.41±4.53^B	8.23±0.85^A
ω 6												
C18:2	6.13±1.33^A	11.92±2.09^B	7.80±1.10^A	7.22±0.70	9.04±2.61^a	5.45±1.60^b	7.80±0.89^A	13.57±2.25^B	8.97±1.47^A	8.37±1.56^A	15.97±5.17^B	8.58±1.12^A
C20:4	20.19±2.41	18.67±3.35	18.58±0.91	25.85±1.72^{aA}	18.53±5.39^{bAB}	15.40±4.45^{bB}	26.36±2.21	22.77±2.49	25.00±2.73	32.01±3.40	28.03±4.85	28.96±3.11
C22:4	0.56±0.06	0.54±0.11	0.71±0.14	0.74±0.07^a	0.50±0.15^b	0.51±0.11^b	0.69±0.07^{aAB}	0.57±0.06^{bB}	0.71±0.07^{aA}	0.97±0.19	1.26±0.82	0.78±0.09
C22:5	2.45±0.54^a	1.48±0.29^b	2.27±0.60	3.19±0.42^A	1.45±0.66^B	1.75±0.40^B	2.93±0.15^{aA}	0.92±0.19^{bB}	2.38±0.47^{cA}	4.49±0.85^A	1.66±0.78^B	4.19±0.55^A
Total	29.92±4.16	33.11±5.92	29.88±1.43	37.81±2.33^A	30.05±8.42	23.63±6.46^B	38.52±2.80	38.43±4.82	37.74±3.91	46.71±5.78	47.53±11.31	43.95±4.89
ω 3												
C22:5	0.18±0.03^a	0.18±0.04^a	0.25±0.03^b	0.23±0.01^{aA}	0.13±0.05^{bB}	0.15±0.06^{bAB}	0.23±0.02^A	0.16±0.02^B	0.24±0.03^A	0.11±0.03	0.08±0.03	0.07±0.01
C22:6	1.62±0.19^A	0.82±0.13^B	1.30±0.08^C	1.69±0.12^A	0.78±0.33^B	0.98±0.30^B	1.86±0.11^A	0.64±0.11^B	1.53±0.17^C	0.82±0.15^A	0.41±0.18^B	0.87±0.13^A
Total	1.88±0.21^A	1.13±0.18^B	1.64±0.08^A	2.02±0.12^A	1.08±0.42^B	1.22±0.35^A	2.24±0.09^A	0.94±0.14^B	1.88±0.19^C	1.04±0.20	0.58±0.23	1.01±0.14
Ω6/ω3	15.89±0.64^A	29.27±2.71^B	18.20±0.74^A	18.79±1.04^A	29.19±4.98^B	19.40±0.37^A	17.16±0.86^A	41.11±3.32^B	20.05±0.73^A	45.51±2.97^A	85.18±12.59^B	43.67±4.00^A
PUFA	31.80±4.36	34.24±6.08	31.52±1.48	39.83±2.41^A	31.13±8.79	24.85±6.81^B	40.76±2.08	39.37±4.94	39.63±4.09	47.74±5.97	48.12±11.53	44.97±5.00
P/S	1.27±0.05	1.16±0.09^a	1.29±0.08^b	1.26±0.03	1.28±0.09	1.30±0.05	1.27±0.04	1.15±0.08^a	1.31±0.10^b	1.34±0.12	1.37±0.16	1.33±0.06

Values are expressed as means ± SD µg FA/100mg liver weight. Significant differences are indicated by upper caps (P<0.01) and lower caps (P<0.05). Statistical comparisons (rows) of control, nodule and surrounding tissue was conducted within each time interval. PUFA = polyunsaturated fatty acids, P/S = polyunsaturated to saturated fatty acid ratio.

Table 4. Fatty acid analyses of the phosphatidylethanolamine (PE) fraction of control liver, nodule and surrounding liver in the Resistant Hepatocyte Model.

	1 Month			3 Months			6 Months			9 Months		
	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
Saturates												
C16:0	6.51 ± 1.44^a	8.08 ± 0.79	8.31 ± 0.75^b	$9.01\pm0.89a$	5.57 ± 0.95^b	6.01 ± 2.89	8.37 ± 0.74	10.02 ± 1.20	8.39 ± 1.05	4.32 ± 0.66	4.54 ± 0.64	5.34 ± 0.69
C18:0	8.55 ± 1.80^A	15.26 ± 1.26^B	10.41 ± 1.25^A	9.65 ± 0.49	12.81 ± 2.57^A	5.91 ± 2.38^B	10.24 ± 0.59^A	19.75 ± 1.90^B	11.70 ± 2.32^A	7.51 ± 0.62^A	11.15 ± 1.52^B	8.18 ± 0.98^A
Total	15.06 ± 3.12^{aA}	23.34 ± 1.97^{bB}	18.72 ± 1.48^a	18.66 ± 1.30	18.37 ± 3.40	11.92 ± 5.25	18.61 ± 0.47^A	29.77 ± 2.89^B	20.08 ± 3.19^A	11.83 ± 1.01^A	15.69 ± 1.55^B	13.51 ± 1.55
Monounsaturates												
C16:1	0.20 ± 0.08^A	0.61 ± 0.12^B	0.27 ± 0.05^A	0.33 ± 0.09	0.25 ± 0.08	0.23 ± 0.09	0.29 ± 0.03^A	0.65 ± 0.10^B	0.25 ± 0.11^A	0.12 ± 0.04^A	0.25 ± 0.07^B	0.26 ± 0.04^B
C18:1	2.50 ± 0.37^A	8.71 ± 1.39^B	4.47 ± 0.46^C	3.63 ± 0.45^a	5.79 ± 1.32^{bB}	2.46 ± 1.12^{aA}	3.58 ± 0.17^A	11.90 ± 1.44^B	4.96 ± 0.44^A	1.90 ± 0.31^A	7.03 ± 1.58^B	2.54 ± 0.32^A
Total	2.70 ± 0.38^A	9.32 ± 1.50^B	4.75 ± 0.42^C	3.96 ± 0.52^A	6.04 ± 1.40^B	2.69 ± 1.20^A	3.88 ± 0.17^A	12.55 ± 1.46^B	5.21 ± 0.46^A	2.02 ± 0.29^A	7.28 ± 1.63^B	2.79 ± 0.34^A
ω 6												
C18:2	2.38 ± 0.48^{aA}	5.70 ± 0.68^{bB}	3.56 ± 0.67^{cA}	3.12 ± 0.35	4.14 ± 1.38^a	2.35 ± 0.86^a	3.13 ± 0.32^A	6.94 ± 0.89^B	4.54 ± 1.45^A	2.15 ± 0.59^A	3.66 ± 0.51^B	2.48 ± 0.42^A
C20:4	11.27 ± 1.87^{aA}	18.19 ± 2.11^{bB}	14.84 ± 1.76^{cAB}	13.28 ± 1.10	16.27 ± 3.28^A	7.65 ± 3.99^B	13.69 ± 1.25^A	25.06 ± 2.29^B	16.20 ± 2.00^A	10.40 ± 1.27^{aA}	15.35 ± 2.86^{bB}	11.40 ± 1.36^a
C22:4	0.76 ± 0.12^A	1.52 ± 0.20^B	1.38 ± 0.22^B	1.09 ± 0.05	1.36 ± 0.36^A	0.64 ± 0.34^B	1.12 ± 0.22^A	2.46 ± 0.36^B	1.38 ± 0.13^A	1.05 ± 0.22^A	1.87 ± 0.37^B	1.01 ± 0.14^A
C22:5	3.31 ± 0.98	4.32 ± 0.69	4.21 ± 0.96	3.97 ± 0.26^A	3.17 ± 1.03	1.80 ± 0.94^B	4.09 ± 0.43	4.16 ± 0.80	4.01 ± 0.51	3.74 ± 0.49	3.04 ± 0.75	3.95 ± 0.58
Total	18.04 ± 3.34^A	30.12 ± 3.63^{bB}	24.38 ± 2.60^c	21.86 ± 1.36	25.33 ± 5.66^A	12.69 ± 6.11^B	22.38 ± 1.97^A	39.06 ± 3.60^B	26.57 ± 3.24^A	17.61 ± 2.30^a	24.15 ± 4.30^b	19.29 ± 2.47
ω 3												
C22:5	0.26 ± 0.04^A	0.50 ± 0.05^B	0.50 ± 0.11^B	0.34 ± 0.04	0.33 ± 0.10	0.19 ± 0.12	0.33 ± 0.05^A	0.62 ± 0.08^B	0.43 ± 0.06^A	0.09 ± 0.02^a	0.16 ± 0.06^b	0.10 ± 0.02
C22:6	2.54 ± 0.56	2.62 ± 0.14	2.72 ± 0.61	2.32 ± 0.24^a	2.16 ± 0.62^a	1.12 ± 0.68^b	2.81 ± 0.27	3.11 ± 0.44	2.82 ± 0.29	0.71 ± 0.10	0.89 ± 0.20	0.82 ± 0.12
Total	2.87 ± 0.58	3.28 ± 0.16	3.33 ± 0.73	2.75 ± 0.26^a	2.70 ± 0.76^a	1.42 ± 0.80^b	3.26 ± 0.33	3.89 ± 0.52	3.36 ± 0.35	0.86 ± 0.14	1.12 ± 0.27	0.96 ± 0.14
ω6/ω3	6.31 ± 0.36^A	9.18 ± 0.81^B	7.50 ± 1.03^A	7.98 ± 0.67	9.56 ± 1.16	8.94 ± 0.50	6.87 ± 0.17^A	10.08 ± 0.57^B	7.90 ± 0.47^C	20.68 ± 1.26	21.90 ± 1.40	20.23 ± 0.71
PUFA	20.91 ± 3.90^{aA}	33.40 ± 3.75^{bB}	27.72 ± 3.29^b	24.62 ± 1.51^a	28.03 ± 6.33^{aA}	14.12 ± 6.90^{bB}	25.64 ± 2.29^A	42.96 ± 4.08^B	29.93 ± 3.55^A	18.47 ± 2.43^A	25.27 ± 4.56^B	20.25 ± 2.60
P/S	1.39 ± 0.07	1.43 ± 0.05	1.48 ± 0.14	1.32 ± 0.03	1.52 ± 0.09^A	1.21 ± 0.08^B	1.38 ± 0.13	1.45 ± 0.12	1.50 ± 0.09	1.56 ± 0.12	1.60 ± 0.18	1.50 ± 0.07

Values are expressed as means ± SD µg FA/100mg liver weight. Significant differences are indicated by upper caps ($P<0.01$) and lower caps ($P<0.05$). Statistical comparisons (rows) of Control, Nodule and Surrounding tissue was conducted within each time interval. PUFA = polyunsaturated fatty acids, P/S = polyunsaturated to saturated fatty acid ratio.

FA profiles of PC and PE in regenerating liver   (Table 5)

<u>*Saturated FA*</u>*:   (C16:0, C18:0)*

The levels of both C16:0 (1, 2 and 7 days: P<0.05) and C18:0 (1 and 2 days: P<0.05, 7 days: P<0.01) decreased significantly in PC. No changes were observed with regards to C16:0 and C18:0 in PE, although a non-significant increase was noticed at days 1 and 2. As a result of these changes, the total saturate level in PC was significantly decreased at 1, 2 (P<0.05) and 7 days (P<0.01). In PE, no significant changes were observed, but there was a slight increase (not significant) in the level at days 1 and 2.

<u>*Monounsaturated FA*</u>*:   (C16:1, C18:1)*

No significant changes occurred with regards to C16:1 in PC and PE, although it decreased initially (not significant) in PC. The level of C18:1 was significantly decreased at 1 day (P<0.05) in PC, but increased thereafter at 2 and 7 days towards the control level. In PE, the level of C18:1 was significantly increased at 7 days after PH (P<0.05). The total monounsaturate level was significantly decreased in PC at day 1 (P<0.05) and thereafter the levels at days 2 and 7 tended to increase towards the control level. In PE, the total monounsaturate level was significantly increased at day 7 (P<0.05).

<u>*Polyunsaturated FA*</u>

ω6 PUFA: (C18:2, C20:3, C20:4, C22:4, C22:5)

At day 1 C18:2 increased in PC (P<0.05) and PE (P<0.01), after which the level tended to revert back to that in the controls. In PE, C20:3 was significantly increased 7 days (P<0.01) after partial hepatectomy (data not shown). The C20:4 level significantly decreased at 1, 2 (P<0.01) and 7 days (P<0.05) after partial hepatectomy in PC, but increased over time towards control levels. No significant changes were observed in PE, although there was a slight increase at day 1, but then tended to decrease back to the control level. C22:4 was significantly decreased in PC at day 1 only (P<0.05), but tended to increase thereafter. There was a significant increase in the level of C22:4 in PE at days 2 and 7 (P<0.05). The level of C22:5 decreased in PC at days 1 and 2 (P<0.01) and in PE at day 1 only (P<0.05). In both PC and PE, C22:5 was decreased initially at day 1 but tended to increase to the control levels at day 7. The total ω6 FA level was significantly decreased at days 1, 2 (P<0.01) and 7 (P<0.05) in PC. No significant changes were observed in PE, although the level tended to be higher than control levels.

ω3 PUFA: (C22:5, C22:6)

The level of C22:5 was significantly decreased at day 1 (P<0.05) only in PC, but increased significantly in PE at days 1 (P<0.05), 2 (P<0.01) and 7 (P<0.05). In PC, C22:6 was significantly decreased at days 1, 2 and 7 (P<0.01). No changes were observed in PE. The total ω3 FA level was significantly decreased in PC at 1, 2 and 7 days (P<0.01) compared to the control. Once again, no changes were observed in PE.

The ω6/ω3 ratios were significantly increased in PC (P<0.05) and PE (P<0.01) 1 day after partial hepatectomy compared to the control levels. The PUFA level was significantly decreased at 1, 2 (P<0.01) and 7 days (P<0.05) in PC, but no significant changes were observed in PE, although the level tended to be higher (not significant) over the experimental period. The P/S ratio in PC and PE was significantly increased from the respective controls at 7 days (P<0.01) after partial hepatectomy.

Table 5. Fatty acid analyses of the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions of regenerating liver.

	PC				PE			
	Control	1 Day	2 Days	7 Days	Control	1 Day	2 Days	7 Days
Saturates								
C16:0	22.17 ± 6.69^a	14.49 ± 2.03^b	15.14 ± 1.78^b	13.93 ± 1.55^b	10.94 ± 2.30	12.94 ± 0.81	12.88 ± 2.14	11.01 ± 1.64
C18:0	23.84 ± 5.31^{aA}	16.57 ± 2.89^b	17.30 ± 2.48^b	14.51 ± 2.24^{bB}	14.87 ± 3.80	20.27 ± 3.12	18.59 ± 2.85	15.03 ± 2.08
Total	46.01 ± 11.75^{aA}	31.05 ± 3.46^b	32.44 ± 4.16^b	28.44 ± 2.32^{bB}	25.82 ± 5.81	33.21 ± 3.63	31.48 ± 4.73	26.04 ± 2.96
Monounsaturates								
C16:1	1.16 ± 0.61	0.57 ± 0.24	0.53 ± 0.11	0.77 ± 0.27	0.33 ± 0.15	0.30 ± 0.09	0.21 ± 0.05	0.29 ± 0.05
C18:1	9.52 ± 3.56^a	5.59 ± 1.42^b	6.16 ± 0.94	8.10 ± 1.07	4.29 ± 0.67^a	5.24 ± 1.34	4.36 ± 0.74^a	6.31 ± 0.57^b
Total	10.68 ± 4.02^a	6.16 ± 1.62^b	6.69 ± 1.02	8.87 ± 1.33	4.62 ± 0.70^a	5.54 ± 1.41	4.57 ± 0.75^a	6.60 ± 0.57^b
ω6								
C18:2	10.90 ± 3.47	12.58 ± 3.36^a	8.59 ± 1.39	7.31 ± 0.79^b	4.25 ± 1.29^A	9.97 ± 2.09^B	5.31 ± 1.16^A	4.23 ± 0.61^A
C20:4	35.66 ± 6.74^{aA}	20.75 ± 2.58^{bB}	24.98 ± 2.83^{bB}	26.64 ± 1.72^b	18.93 ± 4.43	24.69 ± 3.25	23.59 ± 3.50	22.98 ± 3.23
C22:4	0.99 ± 0.24^a	0.66 ± 0.15^b	0.87 ± 0.12	0.85 ± 0.15	1.29 ± 0.32^a	1.44 ± 0.34	1.95 ± 0.37^b	2.02 ± 0.26^b
C22:5	4.32 ± 1.27^A	1.85 ± 0.67^B	2.35 ± 0.28^B	3.21 ± 0.57	5.68 ± 1.99^a	3.19 ± 0.75^{bB}	5.36 ± 0.58	7.35 ± 0.95^{aA}
Total	52.78 ± 11.22^{aA}	36.25 ± 4.80^{bB}	37.52 ± 4.39^{bB}	39.10 ± 1.50^b	30.64 ± 8.09	39.70 ± 5.73	36.74 ± 5.54	37.51 ± 3.26
ω3								
C22:5	0.31 ± 0.06^a	$0.20\pm0.04b^B$	0.35 ± 0.06^{aA}	0.28 ± 0.03	0.46 ± 0.11^{aA}	0.58 ± 0.08^{ab}	0.83 ± 0.18^{bB}	0.70 ± 0.10^b
C22:6	2.89 ± 0.59^A	1.59 ± 0.20^B	1.84 ± 0.20^B	1.83 ± 0.19^B	4.49 ± 1.13	4.59 ± 0.56	4.51 ± 0.58	5.00 ± 0.64
Total	3.34 ± 0.65^A	1.92 ± 0.23^B	2.34 ± 0.25^B	2.23 ± 0.17^B	5.09 ± 1.30	5.29 ± 0.58	5.48 ± 0.73	5.84 ± 0.71
ω6/ω3	15.82 ± 1.03^a	18.92 ± 2.21^b	16.07 ± 1.44^a	17.56 ± 0.95	6.01 ± 0.26^A	7.50 ± 0.66^{aB}	6.69 ± 0.23^b	6.46 ± 0.45^B
PUFA	56.12 ± 11.84^{aA}	38.17 ± 4.95^{bB}	39.86 ± 4.58^{bB}	41.33 ± 1.64^b	35.73 ± 9.37	44.99 ± 6.22	42.22 ± 6.26	43.35 ± 3.89
P/S	1.23 ± 0.09^A	1.23 ± 0.08^A	1.23 ± 0.06^A	1.46 ± 0.07^B	1.38 ± 0.10^A	1.35 ± 0.07^A	1.34 ± 0.07^A	1.67 ± 0.08^B

Values are expressed as means ± SD µg FA/100mg liver weight. Significant differences are indicated by upper caps (P<0.01) and lower caps (P<0.05). Statistical analyses (rows) of the phospholipid fractions of 1, 2 and 7 days after PH, were compared against the control. PUFA = polyunsaturated fatty acids, P/S = polyunsaturated to saturated fatty acid ratio.

DISCUSSION

The structure of plasma membranes in regenerating liver concerning the patterns of membrane proteins have shown no changes compared to that of control liver (5, 10). However, changes in the plasma membrane enzyme activity and receptor expression in regenerating liver have been described (24, 25). In hepatoma cells, alterations in membranal protein profiles have been reported resulting in changes in the activity of certain membrane enzymes, such as a decrease in 5-nucleotidase, an increase in (–glutamyltranspeptidase, as well as changes in the affinity of receptors (10, 26, 27). Changes in membrane protein turnover have also been shown to occur in HTC cells (Morris hepatoma 7288C) (28). Alterations in membrane fluidity can also influence the activity of certain enzymes and the affinity of receptors to their ligands (5, 10). Membrane fluidity has been shown to increase in the nuclear membrane following partial hepatectomy in rats (29). This increase is linked to nuclear membrane neutral-sphingomyelinase activity and the content of sphingomyelin. Alterations in lipid content have been closely linked with changes in membrane fluidity and which could play an important role in the control of signal transduction pathways and cellular regeneration in the altered growth pattern and progression of hepatocyte nodules to cancer development (5, 10).

Important indicators of membrane fluidity are the cholesterol/phospholipid molar ratio (Chol/PL), the PC/PE ratio and the degree of membrane unsaturation (P/S ratio) (5, 10). In the present study, fluorescence polarisation indicated that the nodule membranes where more rigid, i.e. less fluid, than the respective controls at 1, 3 and 6 months, in contrast to the increased fluidity in regenerating liver. However, at 9 months the nodule membrane fluidity increased above the control, mimicking regenerating liver. The PC/PE ratio in the nodules and the regenerating liver decreased early on due to the increased PE level. This increase in the PE concentration has also been observed in Morris hepatoma 7777 cells (5, 10). The Chol/PL molar ratio in the nodules increased only at 9 months due to a significant increase in the cholesterol level. This mimics regenerating liver where the cholesterol level also increased after 2 and 7 days. Cholesterol appears to play an important structural role in maintaining the fluidity of membranes making the outer part of the membrane less fluid, but causing the inner part of the lipid bilayer to be slightly more fluid by organizing the tails of the FA acyl chains (30). The non-polar membrane components, such as the FA acyl chains and cholesterol, seem to have a greater regulatory effect on the activity of membrane bound

proteins than the polar phospholipid head groups (31). In this way cholesterol can organize the movement of membrane bound proteins in regenerating liver and also in hepatocyte nodules. Finally, the unsaturation index (P/S ratio) in the hepatocyte nodules did not change, suggesting that this index is tightly controlled in the nodules and does not appear to have a significant effect on membrane fluidity in the hepatocyte nodule. The increased PUFA level in PE, due to the increased PE concentration, did not correlate with the resultant unchanged membrane fluidity, further indicating that unsaturation did not affect fluidity directly. A recent study showed that the FA unsaturation level in a membrane did not have such a large influence on membrane fluidity when compared to the effect of cholesterol (32). This increase in PE appears to be an early event in the nodular lipid profile compared to changes in the cholesterol and membrane fluidity which appear to be late events. Together, the increases in membrane cholesterol and PE are likely to be the major factors determining fluidity changes in the hepatocyte nodules. In regenerating liver, an increase in these two parameters was also associated with an increase in fluidity.

When comparing the lipid profiles of nodular liver with regenerating liver, some similarity in the pattern of lipid changes exists. In the regenerating liver, cholesterol increased significantly at days 2 and 7 while PE increased from day 1. The latter changes coincided with an increase in the fluidity as well as maximal liver regeneration, which occurs 2 to 3 days after partial hepatectomy (15). As seen in the hepatocyte nodules, the increased PE concentration in the regenerating liver is an early event with the changes in cholesterol occurring later. With regards to the fatty acids, C18:1ω9 decreased significantly in PC only at day 1, but was increased in PE at day 7, presumably due to the increase in the concentration of this phospholipid. It is not known whether the initial decrease in C18:1ω9, known to have antioxidant properties (6, 7), could be related to the decreased rate in lipid peroxidation noticed in regenerating liver prior to DNA synthesis (7). After an initial increase of C18:2ω6 in PC after day 1, the level was significantly reduced after 7 days, presumably due to an increased conversion of this FA to C20 and C22 fatty acids as a result of liver regeneration. In comparison with the nodules, it would therefore appear that the delta-6 desaturase enzyme is not impaired during normal regeneration following partial hepatectomy. The LCPUFA, C20:4ω6, C22:5ω6 and C22:6ω3, decreased significantly in the regenerating liver PC presumably due to a higher metabolism of these FA during

regeneration (7). It has been observed that proliferating cells have a higher utilisation of the delta-6 desaturated FA (11). The resultant decrease in these LCPUFA, supports the low lipid peroxidative status present in regenerating liver (33). In the nodules, the higher rate of cell proliferation as well as the impaired delta-6 desaturase enzyme, resulted in a similar decrease of LCPUFA in PC and which also appears to be an early event in the genesis of the nodules.

The lipid pattern associated with regeneration could be instrumental in the signal for cellular growth in regenerating liver under controlled conditions, while it prevails in hepatocyte nodules resulting in a steady increase in their size. However, in the majority of these nodules the lipid-associated stimulatory signals revert back to that prevailing in normal tissue, as observed in regenerating liver following partial hepatectomy, contributing to the remodeling process in the majority of the nodules. In the present study this was shown in the FA profiles of PC and PE of the 3 month nodules versus surrounding tissue with differences not being as prominent as that obtained at 6 and 9 months, presumably due to the large amount of nodules that are still remodelling at this stage. In a small subset of nodules, however, these changes "persist" supporting the increased rate of cell proliferation and facilitating their ultimate development into cancer. It has been reported that after 6 months post-initiation, certain hepatocyte nodules, termed "persistent" nodules, loose their ability to control cell proliferation (4) i.e. the normal "regulatory" processes of the cell cycle are impaired. After 9 months, the remaining subset of the original pre-neoplastic nodules develop into cancer (4). Therefore, the normal control processes that regulate cell proliferation, seen in regenerating liver, is not present in a small subset of these nodules. Up to a certain stage, 1 to 6 months, the hepatocyte nodules are still in a transitional phase i.e. at an early stage most of the nodules revert back/regress to normal hepatocytes and at a later stage, i.e. from 6 to 9 months, the persistent nodules lose their "pre-neoplastic" features/phase and advance to the neoplastic stage.

Critical events in the nodules are the persistent alterations in lipid parameters involving changes in FA metabolism (delta-6 desaturase), increased PE and cholesterol levels and changes in membrane fluidity (Fig. 1). Except for some changes in FA metabolism related to the impaired delta-6 desaturase enzyme, the other changes closely mimic that of regenerating liver but with the difference that in the latter, these changes revert back to normal liver. This implies that the normal regulatory mechanisms related to

lipid metabolism to ensure normal liver homeostasis, are disrupted in hepatocyte nodules. It is not known at present whether the increased level of PE is related to an increased synthesis or to a decrease in the conversion to PC involving phosphatidylethanolamine N-methyltransferase-2 (PEMT2). Recent studies imply the expression of this enzyme in the regulation of hepatocyte growth (34). PEMT2 expression is transiently inactivated after partial hepatectomy (35) and permanently disappears in hepatocellular carcinoma induced by the resistant hepatocyte model (36), while the transfection of the enzyme to a hepatoma cell line inhibits the cell growth rate (37). The higher PE concentration, a phospholipid normally situated on the inside of cellular membranes (38), together with the resultant increases in C20:4т6 appears to be an integral part of the growth stimulus in hepatocyte nodules and could play a role in sustaining cellular proliferation in these lesions (Fig. 1).
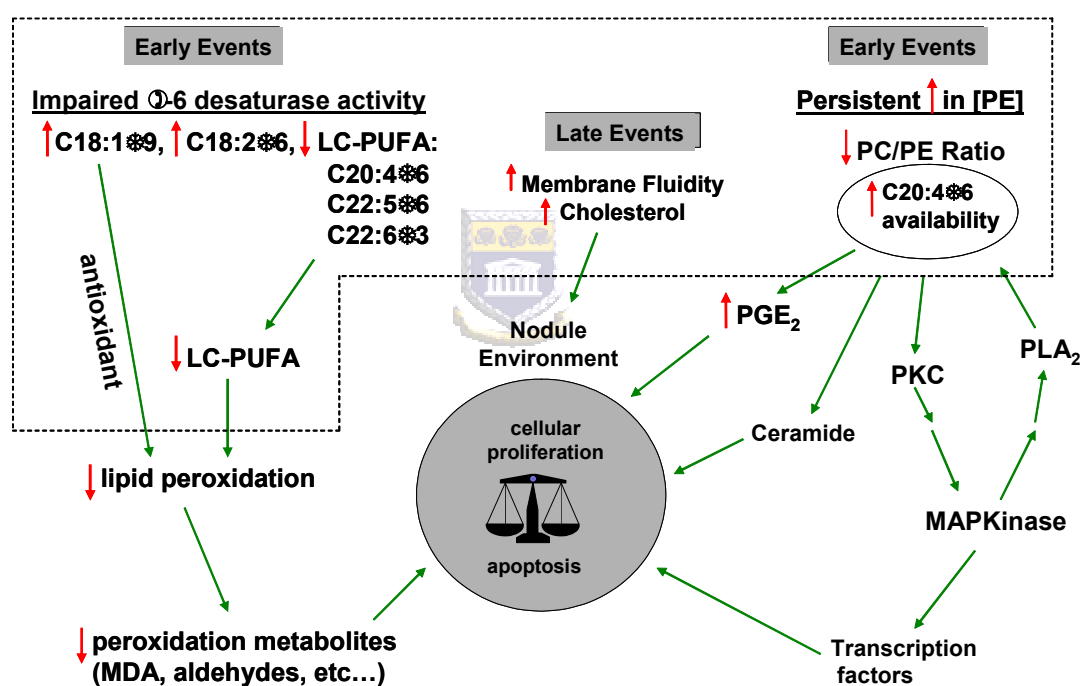
Figure1: Critical events associated with the altered growth pattern of hepatocyte nodules. The growth of preneoplastic nodules can be influenced by certain critical events with regards to lipid metabolism as summarised in the figure. This involves an impaired delta-6 desaturase, an increase in PE, cholesterol concentration and membrane fluidity (this study dotted block). The early events involving the delta-6 desaturase and increased PE level establish an environment critical for the continued proliferation of the nodules. This results in the later events such as the increased cholesterol and membrane fluidity affecting the functionality of the cellular membrane involving membrane enzymes and receptor affinity. The increased PE level is an important event leading to an increased membrane C20:4т6 availability affecting various systems such as prostaglandin synthesis and PKC, ceramide and MAP kinase activity. These factors in turn play a role in the regulation of cellular proliferation and apoptosis. The impaired delta-6 desaturase enzyme, as shown by increases in C18:1т9 and C18:2т6 and decreases in the LCPUFA, can result in a decreased lipid peroxidation status leading to an imbalance in the cell proliferation/apoptosis equilibrium in nodules, thereby favouring cell proliferation.

The dual role of C20:4T6, (i) structural as part of membrane phospholipids and (ii) functional as a precursor to the E_2-series eicosanoids and signal transduction pathways, are of particular interest with respect to its role in maintaining normal cellular homeostasis in the liver (39). The proteins, phospholipase A_2 (PLA_2), phospholipase C (PLC) and protein kinase C (PKC) play an important role in cell proliferation and have shown a tendency to be modulated by FA. Recent studies indicated that membranal FA, specifically C20:4T6, play an important role as second messengers in signal transduction pathways via the activation of protein kinase C (PKC), mitogen activated protein kinase (MAP kinase) and the generation of ceramide (40). C20:4T6 can also be involved in apoptosis via the release of ceramide by activating sphingomyelinase, which acts as a second messenger activating the apoptotic process (41). Increased levels of the E_2-series prostaglandins from C20:4T6 can also be involved in activating the apoptotic process (42). A recent study investigating the role of C20:4T6 in phenobarbital induced rat liver foci, indicated the involvement of this FA in the tumour promoting mechanisms of phenobarbital (43). It would appear that C20:4T6 plays a key role in controlling events which support the altered growth kinetics in heptocyte nodules (Fig. 1).

Apart from the role of FA in regulating prostaglandin production and signal transduction pathways, they are also key substrates for lipid peroxidation. LCPUFA can play a role in the control of cell proliferation by inhibiting cell growth and stimulating and/or enhancing apoptosis by the increase of cellular lipid peroxidation and the subsequent breakdown products such as malondialdehyde (44, 45). A key event with respect to the changes in FA metabolism in hepatocyte nodules, appears to be a change in the delta-6 desaturase enzyme and the implications this has on the LCPUFA levels (6, 30). The impairment of this enzyme has been observed in BL6 melanoma and Morris hepatoma 9618A cell lines and in various types of liver cancer with different origins (7, 8, 9). In the present study, impaired activity of this enzyme was observed in the nodule tissue indicated by the increased levels of the FA substrates C16:1T7, C18:1T9 and C18:2T6 in PC, while the LCPUFA products, C20:4T6, C22:5T6 and C22:6T3, were decreased. The decreased levels of C22:5T6 and C22:6T3 in PC can also be related to the impaired activity of delta-6 desaturase, as this enzyme has also been shown to be involved in the conversion of C22:4T6 and C22:5T3 to C22:5T6 and C22:6T3, repectively (46). The increased C18:1T9 level, a FA with antioxidative properties, as

well as the decrease in the LCPUFA have been associated with a decreased lipid peroxidation status in malignant lesions (6, 7). A loss of lipid peroxidation has also been observed in preneoplastic hepatocellular lesions in rats and a decrease in the cytostatic 4-hydroalkenals, other aldehydes and peroxides could be related to the increased growth patterns observed in these lesions (12). The subsequent decrease in peroxidative metabolites, which are known to induce apoptosis (47), is likely to negatively affect the apoptotic process in the nodular environment (Fig. 1). In normal regenerating liver, the level of C18:1T9 decreased, C18:2T6 increased and the LCPUFA in PC decreased after day 1, implying a possible controlled involvement of the delta-6 desaturase enzyme to reduce the lipid peroxidation level. It has been shown that the rate of lipid peroxidation is reduced in regenerating liver following partial hepatectomy which fits into the general hypothesis that increased cell proliferation is associated with a decreased rate of lipid peroxidation (7, 48, 49). This would suggest that the decrease in lipid peroxidation is another important event in sustaining hepatocyte regeneration both under normal and abnormal conditions (Fig. 1).

The present study indicated that the persistent alterations in lipid metabolism in the hepatocyte nodules (dotted block in Fig. 1) are likely to play an important role in the development of the malignant phenotype. The dynamic state of the lipid bilayer of cellular membranes would allow the manipulation of the lipid content of the membrane by dietary means, thereby modulating the activity of membrane proteins, the availability of FA for signal transduction pathways and prostaglandin synthesis and thereby determining cell survival (31). The altered cholesterol, phospholipid and FA profiles in the hepatocyte nodules could provide unique targets for developing strategies in chemoprevention with the inclusion of dietary manipulation in order to counteract the increased cellular proliferation and, therefore the progression and subsequent development of these lesions into neoplasia.

ACKNOWLEDGEMENTS

REFERENCES

1.   Pitot, H.C. Fundamentals of Oncology, 3rd ed. New York: Marcel Dekker, p532, 1986.

2.   Farber, E. (1996) The step-by-step development of epithelial cancer: From phenotype to genotype. *Adv. Cancer Res.,* **70**, 21-48.

3.   Farber, E. (1991) Hepatocyte proliferation in stepwise development of experimental liver cell cancer. *Digestive Diseases Sciences,* **36**, 973-978.

4.   Farber, E. and Sarma, D.S.R. (1987) Hepatocarcinogenesis: A dynamic cellular perspective. *Lab. Invest*., **56**, 4-22.

5.   Mahler, S.M., Wilce, P.A. and Shanley, B.C. (1988) Studies on regenerating liver and hepatoma plasma membranes - I. Lipid and protein composition. *Int. J. Biochem.,* **20**, 605-611.

6.   Horrobin, DF. (1990) Essential fatty acids, lipid peroxidation, and cancer. In: *Omega-6 essential fatty acids: Pathphysiology and Roles in Clinical Medicine.* (Horrobin, DF, ed.), pp. 351-377, Alan R. Liss, Inc., New York, USA.

7.   Horrobin, DF. (1994) Unsaturated lipids and cancer. In: *New approaches to cancer treatment: Unsaturated lipids and photodynamic therapy*. (Horrobin, D.F., ed.), pp. 3-64, Churchill Communications Europe.

8.   Gardiner, N.S. and Duncan, J.R. (1991) Possible involvement of delta-6-desaturase in control of melanoma growth by gamma-linolenic acid. *Prostaglandins Leukot. Essent. Fatty Acids*, **42**, 149-153.

9.   Hrelia, S., Bordoni, A., Biagi, P.L., Galeotti, T., Palombini, G. and Masotti, L. (1994) The role of delta-6- and delta-9-desaturase in the fatty acid metabolism of hepatomas with different growth rate. *Biochem. Mol. Biol. Int*., **34**, 449-455.

10.   Mahler, S.M., Wilce, P.A. and Shanley, B.C. (1988) Studies on regenerating liver and hepatoma plasma membranes - II. Membrane fluidity and enzyme activity. *Int. J. Biochem.,* **20**, 613-619.

11.   Horrobin D F. (1992) Nutritional and medical importance of gamma-linolenic acid. *Prog. Lipid Res.,* **31**, 163-194.

12.   Benedetti, A. Malvaldi, G., Fulceri, R. and Comporti, M. (1984) Loss of lipid peroxidation as a histochemical marker for preneoplastic hepatocellular foci of rats. *Cancer Res.,* **44**, 5712-5717.

13.   American Institute of Nutrition. (1980) Second report of the ad hoc committee on standards for nutritional studies. *J. Nutr.,* **110**, 1726.

14.   Solt, D. and Farber, E. (1976) New principles for the analysis of chemical carcinogenesis. *Nature,* **263**, 701-703.

15.   Michalopoulos, G.K. and DeFrances, M.C. (1997) Liver regeneration. *Science,* **276**, 16-66.

16.   Folch, J., Lees, M. and Stanley, G.H.S. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*., **226**, 497-509.

17.   Smuts, C.M., Weich, H.F.H., Weight, M.J., Faber, M., Kruger, M., Lombard, C.J. and Benadé, A.J.S. (1994) Free cholesterol concentrations in the high-density lipoprotein subfraction-3 as a risk indicator in patients with angiographically documented coronary artery disease. *Cor. Art. Dis.,* **5**, 331-338.

18.   Gilfillan, A.M., Chu, A.J., Smart, D.A. and Rooney, S.A. (1983) Single plate separation of lung phospholipids including disaturated phosphatidylcholine. *J. Lipid Res.,* **24**, 1651-1656.

19.   Itaya, K. and Ui, M. (1966) A new micromethod for the colorimetric determination of inorganic phosphate. *Clinica Chimica Acta,* **14**, 361-366.

20.   Richmond, W. (1973) Preparation and properties of a cholesterol oxidase from Nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. *Clin. Chem.,* **19**, 1350-1356.

21.   Victor, T., Van der Merwe, N., Benadé, A.J.S., La Cock, C. and Lochner, A. (1985) Mitochondrial phospholipid composition and microviscosity in myocardial ischaemia. *Biochim. Biophys. Acta,* **834**, 215-223.

22.   Zubenko, G.S., Cohen, B.M., Reynolds, C.F., Boller, F., Malinakova, M and Keefe, N. (1987) Platelet membrane fluidity in Alzheimer's Disease and major depression. *Am. J. Psychiatry,* **144**, 860-868.

23.   Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.,* **87**, 206-210.

24.   Wright, G.H. (1977) Changes in plasma membrane enzyme activities during liver regeneration in the rat. *Biochim. Biophys. Acta,* **470**, 368-381.

25.   Sandnes, D., Tor-Erik, S., Sager, G., Brønstad, G.O., Refsnes, M.R., Gladhaug, I.P., Jocobsen, S. and Christofferson, T. (1986) Elevated level of *?*-adrenergic receptors in hepatocytes from regenerating rat liver. *Expl. Cell Res.,* **165**, 117-126.

26.   Lopez-Saura, P., Trouet, A. and Tulkens, P. (1978) Analytical fractionation of cultured hepatoma cells (HTC cells). *Biochim. Biophys. Acta,* **543**, 430-449.

27.   Barouki, R., Finidori, J., Chobert, M., Aggerbeck, M., Laperche, Y. and Hanoune, J. (1984) Biosynthesis and processing of gamma-glutamyl transpeptidase in hepatoma tissue culture cells. *J. Biol. Chem.,* **259**, 7970-7974.

28. Chu, F.F. and Doyle, D. (1985) Turnover of plasma membrane proteins in rat hepatoma cells and primary cultures of rat hepatocytes. *J. Biol. Chem.,* **260**, 3097-3107.

29. Tomassoni, M.L., Albi, E. and Magni, M.V. (1999) Changes of nuclear membrane fluidity during rat liver regeneration. *Biochem. Mol. Biol. International.,* **47**, 1049-1059.

30. Darnell, J., Lodisch, H. and Baltimore, D. (Ed.) (1986) Molecular Cell Biology, Scientific American Inc. Pp 576-577.

31. Spector, A.A. and Yorek, M.A. (1985) Membrane lipid composition and cellular function. *J. Lipid Res.,* **26**, 1015-1035.

32. Galli, C. and Butrum, R. (1991) Dietary T3 fatty acids and cancer: An overview. In: Health effects of T3 polyunsaturated fatty acids in seafoods, *World Rev. Nutr. Diet.* (Simopoulos, A.P., Kifer, R.R., Martin, R.E., and Barlow, S.M., eds.), pp. 446-461, Karger, Basel.

33. Cheeseman, K.H., Collins, M., Maddix, S., Milia, A., Proudfoot, K., Slater, T.F., Burton, G.W., Webb, A. and Ingold, K.U. (1986) Lipid peroxidation in regenerating rat liver. *F.E.B.S.,* **209**, 191-196.

34. Tessitore, L., Sesca, E., Bosco, M. and Vance, D.E. (1999) Expression of phosphatidylethanolamine *N*-methyltransferase in Yoshida ascites hepatoma cells and the liver of host rats. *Carcinogenesis,* **20**, 561-567.

35. Houweling, M., Cui, Z., Tessitore, L. and Vance, D.E. (1997) Induction of hepatocyte proliferation after partial hepatectomy is accompanied by a markedly reduced expression of phosphatidylethanolamine *N*-methyltransferase-2. *Biochem. Biophys. Acta,* **1346**, 1-9.

36. Tessitore, L., Cui, Z. and Vance, D.E. (1996) Phosphatidylethanolamine *N*-methyltransferase-2 (PEMT-2): a potential oncosuppressor specific for the liver. *Proc. Am. Assoc. Cancer Res*., **37**, 127.

37. Cui, Z., Houweling, M. and Vance, D.E. (1994) Suppression of rat hepatoma cell growth by expression of phosphatidylethanolamine *N*-methyltransferase-2. *J. Biol. Chem*., **269**, 24531-24533.

38. Berdanier , C.D. (1988) Role of membrane lipids in metabolic regulation. *Nutr. Rev*., **46**, 145-149.

39. Khan, W.A., Blobe, G.C. and Hannun, Y.A. (1995) Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C. *Cell Signaling,* **7**, 171-184.

40. Lin, L-L., Wartmann, M., Lin, A.Y., Knopf, J.L., Seth, A. and Davis, R. (1993) cPLA$_2$ is phosphorylated and activated by MAP Kinase. *Cell,* **72**, 269-278.

41. Obeid, L.M., Hannun, Y.A. (1995) Ceramide: a stress signal and mediator of growth suppression and apoptosis. *J. Cell. Biochem.,* **58**, 191-198.

42.   De Kock , M., Lottering, M.L., Grobler, C.J.S., Viljoen, T.C., Le Roux, M., Seegers, J.C. (1996) The induction of apoptosis in human cervical carcinoma (HELA) cells by gamma-linoleic acid. *Prostaglandins Leukot. Essent. Fatty Acids,* **55***,* 403-411.

43.   Tang, Q., Denda, A., Tsujiuchi, T., Tsutsumi, M., Amanuma, T., Maruyama, H. and Konishi, Y. (1993) Inhibitory effects of inhibitors of arachidonic acid metabolism on the evolution of rat liver preneoplastic foci into nodules and hepatocellular carcinomas with or without phenobarbital exposure. *Jpn. J. Cancer Res.,* **84**, 120-127.

44.   Bartsch, H., Nair, J. and Owen, R.W. (1999) Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. *Carcinogenesis,* **20**, 2209-2218.

45.   Ji, C., Rouzer, C.A., Marnett, L.J. and Pietenpol, J.A. (1998) Induction of cell cycle arrest by the endogenous product of lipid peroxidation, malondialdehyde. *Carcinogenesis,* **19**, 1275-1283.

46.   Marzo, I., Alava, M.A., Piñeiro, A. and Naval, J. (1996) Biosynthesis of docosahexaenoic acid in human cells: evidence that two different delta 6-desaturase activities may exist. *Biochim. Biophys. Acta,* **1301**, 263-272.

47.   Palmer, H.J., Paulson, K.E. (1997) Reactive oxygen species and antioxidants in signal transduction and gene expression. *Nutr. Rev.,* **55***,* 353-361.

48.   Cheeseman, K.H., Collins, M., Proudfoot, K., Slater, T.F., Burton, G.W., Webb, A.C. and Ingold, K.U. (1986) Studies on lipid peroxidation in normal and tumour tissues. *Biochem. J.,* **235**, 507-514.

49.   Diplock, A.T., Rice-Evans, C.A. and Burdon, R.H. (1994) Is there a significant role for lipid peroxidation in the causation of malignancy and for antioxidants in cancer prevention? *Cancer Research,* **54**, 1952s-1956s.

CHAPTER 4

DIETARY MODULATION OF FATTY ACID PROFILES AND OXIDATIVE

STATUS OF RAT HEPATOCYTE NODULES: EFFECT OF DIFFERENT

ω6/ω3 FATTY ACID RATIOS

DIETARY MODULATION OF FATTY ACID PROFILES AND OXIDATIVE STATUS OF RAT HEPATOCYTE NODULES: EFFECT OF DIFFERENT ω6/ω3 FATTY ACID RATIOS.

S. Abel[a*], M. De Kock[b], C.M.Smuts[c], C. de Villiers[d], S. Swanevelder[e] and W.C.A. Gelderblom[a]

[a]Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC Unit), [c]Nutritional Intervention Research Unit, [d]Diabetes Research Group/Primate Unit, [e]Biostatistics Unit, Medical Research Council (MRC), P.O. Box 19070, Tygerberg, 7505, South Africa. [b]Department of Physiology, University of the Western Cape, Private Bag X17, Bellville, 7530.

ABSTRACT

Male Fischer rats were fed the AIN 76A diet containing varying ω6/ω3 fatty acid ratios using sunflower oil (SFO), soybean oil (SOY) and SFO supplemented with EPA-50 and GLA-80 (GLA) as fat sources. Hepatocyte nodules, induced using diethylnitrosamine followed by 2-acetylaminofluorene/partial hepatectomy promotion, were harvested, with surrounding and respective dietary control tissues, 3 months after partial hepatectomy. The altered growth pattern of hepatocyte nodules in rats fed SFO, is associated with a distinct lipid pattern entailing an increased concentration of phosphatidylethanolamine (PE) resulting in increased levels of C20:4ω6. In addition, there is an accumulation of C18:1ω9 and C18:2ω6 and a decrease in the end products of the ω3 metabolic pathway in phosphatidylcholine (PC) suggesting a dysfunctional delta-6-desaturase enzyme. The hepatocyte nodules of the SFO-fed rats exhibited a significantly reduced lipid peroxidation level which was associated with an increase in the glutathione (GSH) concentration. The low ω6/ω3 fatty acid ratio diets significantly decreased C20:4ω6 in PC and PE phospholipid fractions with a concomitant increase in C20:5ω3, C22:5ω3 and C22:6ω3. The resultant changes in the C20:4/C20:5 FA ratio and the C20:3 FA level in the case of the GLA diet suggest a reduction of prostaglandin synthesis of the 2-series. The GLA diet also counteracted the increased level of C20:4ω6 in PE by equalizing the nodule/surrounding ratio. The low ω6/ω3 ratio diets significantly increased lipid peroxidation levels in hepatocyte nodules, mimicking the level in the surrounding and control tissue while glutathione (GSH) was decreased. An increase in ω3 fatty acid levels and oxidative status resulted in a reduction in the number of glutathione-S-transferase positive foci in the liver of the GLA-fed rats. Modulation of cancer development with low ω6/ω3 ratio diets containing specific dietary FA could be a promising tool in cancer intervention in the liver.

INTRODUCTION

Investigations into the lipid content of tumour tissue indicate that the process of carcinogenesis is associated with an altered lipid profile which appears to play an important role in cell survival and the subsequent development into neoplasia. Fatty acids (FA), as integral components of cell membranes, may influence neoplastic development by altering cellular integrity, the activation state of pre-carcinogens, and the capacity of the cell to respond to growth regulatory signals (1). Studies by Dyerberg and Bang (1979) indicated that dysfunctions in eicosanoid metabolism (2) can lead to certain illnesses and disorders such as cardiovascular and gastrointestinal diseases as well as an increased incidence of cancer (3). These disorders have been linked to an imbalanced polyunsaturated fatty acid (PUFA) intake, related to diets with a high $\omega6$ and/or low $\omega3$ FA content (3). Diets high in $\omega6$ FA content have been shown to promote colon and breast cancer which is associated with the up-regulation of cyclo-oxygenase-2 (COX-2) and p21*ras* expression (4, 5). In the colon, phospholipase A_2 (PLA_2) and COX-2 (5) are overexpressed in neoplastic cells which may lead to the release of $C20:4\omega6$ from membrane phospholipids with an increased production of prostaglandins such as PGE_2 (6, 7, 8). Overproduction of PGE_2 has been implicated in tumour initiation and promotion, cell proliferation and differentiation and shown to modulate cellular and humoral immune responses by inhibiting the production of lymphocytes, interleukin and antibodies. It has also been shown to inhibit the macrophage mediated cytotoxicity to cancer cells (6). However, $C20:4\omega6$ 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 (9). As $C20:4\omega6$ appears to be one of the major players in the progression of hepatocyte nodules into neoplasia, the modulation of the $C20:4\omega6$ level is therefore of importance (10). The addition of $\omega3$ PUFA in the diet is known to displace and therefore decrease the $\omega6$ FA content of cellular membranes (3, 6, 11). This displacement and decrease is of particular importance with respect to $C20:4\omega6$. To prevent excessive $\omega6$ FA replacement, it is suggested that a dietary combination of $C18:3\omega6$ and $C20:5\omega3$ should be used to maintain a critical level of $C20:4\omega6$ in cell membranes (11, 12). It has been shown, in vitro and in vivo, that supplementation with $C18:3\omega6$ or evening primrose oil can

maintain a steady state in the C20:4ω6 level (13, 14). Dietary supplementation with C18:3ω6 and C20:5ω3 is also important with regard to supplying FA downstream of the delta-6-desaturase enzyme, which is known to be impaired in cancer tissue (15). In addition, C18:3ω6 is rapidly converted to C20:3ω6, a substrate for the 1-series prostaglandins that counteract the activities of the 2-series prostaglandins (11).

An important property of cancer cells is the low level of lipid peroxidation, partly due to unusually high levels of anti-oxidants such as vitamin E and C18:1ω9 (15, 16). Another key molecule in determining the redox status in cells is the antioxidant glutathione which appears to be altered in cancer tissue (17, 18). One of the main reasons, however, appears to be the low PUFA levels, especially ω3 PUFA, due to the impairment of the delta-6-desaturase enzyme (11, 15). A previous study utilising a liver cancer model in rats, indicated that a distinct pattern with regard to FA metabolism exists in hepatocyte nodules (10). This entails low levels of the long-chain PUFA (LCPUFA) C22:5ω6 and C22:6ω3, and high levels of C18:1ω9 and C18:2ω6 typical of a delta-6-desaturase impairment. The decrease in the LCPUFA is also likely to be associated with a low oxidative status in hepatocyte nodules. Other important properties associated with hepatocyte nodule development include: (i) an increased concentration of the phosphatidylethanolamine (PE) phospholipid fraction, resulting in a decrease in the PC/PE ratio and an increase in the level of 20:4ω6, (ii) elevated cholesterol and (iii) an increase in membrane fluidity (10). Wood *et al.* (1986) found similar elevations of PE in the plasma membrane and endoplasmic reticulum from Hepatoma (7288CTC) cells grown in the hind legs of rats (19). With respect to FA levels, C18:1ω9 was increased in the PC and PE phospholipid fractions, while the level of C22:6ω3 was very low.

As tumours are dependent on host circulation for the type and amount of PUFA available, it should be possible to alter the FA composition by dietary means (20). Several studies have shown that the FA composition of cancer cells can be altered *in vitro* and *in vivo* by supplementation with dietary FA (21, 22, 23). The resultant changes in the FA content of the tumour may alter prostaglandin synthesis and relevant signaling pathways, thereby modulating the apoptotic/proliferative imbalance (24). The present study was conducted to monitor the effect of low ω6/ω3 FA ratio diets on the PUFA and oxidative status, and the modulating role on hepatocyte nodule development.

MATERIALS AND METHODS

Chemicals

Eicosapentaenoic acid (EPA-50) and gamma-linolenic acid (GLA-80) were obtained from Callanish Ltd., Breasclete, Scotland.

Animals and Diets

The use of laboratory animals in this study was approved by the Ethics Committee of the Medical Research Council of South Africa. At weaning (body weight 50 g), male Fischer-344 rats were divided into three treatment groups (n=20 rats per group) and their respective control groups (n=5 rats per group) and fed the AIN 76A diet (25) containing fat (5% of diet) with varying ω6/ω3 FA ratios for the duration of the experiment. The different fat sources consisted of:

i)   sunflower oil with a ω6/ω3 FA ratio of 250:1 (SFO, high ω6/ω3 ratio diet),

ii)   SFO supplemented with EPA-50 and GLA-80 yielding a ω6/ω3 ratio of 12:1 (GLA, low ω6/ω3 ratio diet),

iii)   soybean oil yielding a ω6/ω3 ratio of 5:1 (SOY, low ω6/ω3 ratio diet).

To examine the influence of GLA on the nodule FA profile, a separate treatment group (n=20) was fed the AIN 76A diet containing SFO and EPA-50 (without GLA) with a ω6/ω3 FA ratio of 12:1 (EPA diet). A schematic outline of the experimental design is illustrated in Figure 1.

Experimental Design
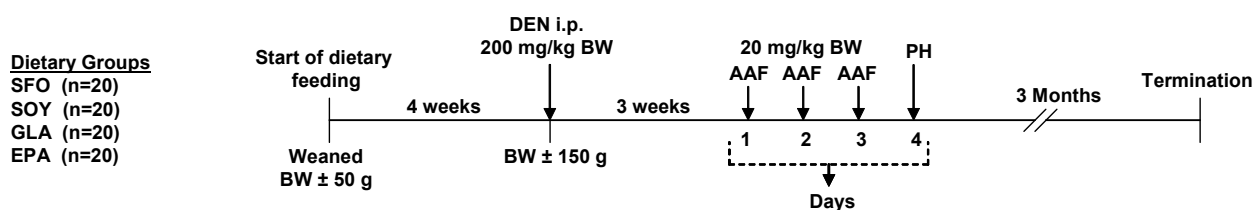
Figure 1: Weaned male Fischer-344 rats (body weight, BW, 50 g), were divided into four experimental treatment groups (n=20 rats per group) and fed the AIN 76A diet containing fat (5% of diet) with varying ω6/ω3 fatty acid (FA) ratios consisting of sunflower oil (SFO), soybean oil (SOY), sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 (GLA) and sunflower/eicosapentaenoic acid-50 (EPA) for the duration of the experiment. Control groups included rats fed SFO, SOY and GLA diets without the carcinogen treatment. Hepatocyte nodules were induced in the experimental treatment groups as described in Materials and Methods. Rats were terminated 3 months after the cancer promotion treatment and hepatocyte nodules and surrounding tissue were collected. BW, body weight; DEN, diethylnitrosamine; AAF, 2-acetylaminofluorene; PH, partial hepatectomy.

The ω6/ω3 FA ratios of the different dietary oils used (Table 1) were determined by gas chromatography (Varian 3300). The diets were prepared and stored at 4°C under nitrogen for the duration of the experiment. The rats were housed separately in wire-bottomed cages under controlled lighting (12 hour cycles), humidity and temperature (23-25°C) with free access to water. They were fed *ad libitum* and weighed three times weekly.

Induction and harvesting of hepatocyte nodules

Hepatocyte nodules were induced in the treatment groups according to the method described by Solt and Farber (26). Briefly, the rats (body weight approximately 150 g) were injected intraperitoneally (i.p.) with a single dose (200 mg/kg body weight) of diethylnitrosamine (DEN) to effect cancer initiation. Promotion was effected 3 weeks later by a daily intragastric dose (20 mg/kg body weight) of 2-acetylaminofluorene (2-AAF) on 3 consecutive days followed by partial hepatectomy on the fourth day. The rats were terminated 3 months following cancer promotion and hepatocyte nodules and surrounding tissue were collected. Control tissue was collected from rats only fed the SFO, SOY and GLA diets without the carcinogen treatment. Tissue sections were immediately frozen on dry ice and stored at -80°C prior to analyses.

Lipid extraction

Lipids were extracted with chloroform/methanol (CM; 2:1; v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant (27, 28). Approximately 100 to 150 mg of the liver tissue was ground to a fine powder in liquid nitrogen and weighed in glass-stoppered tubes. The tissue was suspended in 0.5 ml saline (0.9 % NaCl in distilled water) and the lipids were extracted with 24 ml CM. The CM mixture was filtered (sinterglass filters using Whatman glass microfibre filters, Cat No, 1820 866, Whatman International, Ltd, Maidstone, England) and evaporated to dryness *in vacuo* at 40°C. The extract was transferred to glass-stoppered tubes, washed with saline saturated with CMS (chloroform/methanol/saline; 86:14:1; v/v/v) containing 0.01% BHT, and stored at 4°C until analysed.

Fatty acid analyses

The lipid extracts were fractionated by thin layer chromatography (TLC) and the major phospholipid fractions, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were collected for phospholipid and FA analyses (29). For FA analyses, the

phospholipid fractions were transmethylated with 2 ml methanol/18 M sulphuric acid (95:5; v/v) at 70°C for 2 hrs. The FA methyl esters (FAME) were extracted in hexane and analysed by gas chromatography on a Varian 3300 Gas Chromatograph equipped with 30 m fused silica Megabore DB-225 columns with a 0.53 mm internal diameter (cat. no. 125-2232; J&W Scientific, Agilent Technologies. Palo Alto, CA). The individual FAME were identified by comparison of the retention times to those of a standard mixture of free FA, C14:0 to C24:1 (Nu-Chek-Prep Inc., Elysian, Minnesota, USA), and quantified using an internal standard (C17:0, Sigma-Aldrich) and expressed as μg FA/mg protein.

Phospholipid and cholesterol analyses

The phospholipid concentrations of PC and PE (ug/mg protein) were determined colourimetrically using malachite green after digestion with perchloric acid (16 N) at 170°C for approximately 1 hr (30). Total cholesterol (ug/mg protein) from the lipid extracts was determined by an enzymatic iodide method (31) using cholesterin-oxidase and -esterase (cat. no.125512; Preciset Cholesterol kit; Roche/Boehringer Mannheim, Indianapolis, IN). The cholesterol/phospholipid molar ratio (Chol/PL) was calculated by adding PC and PE together representing the major membrane phospholipids and using the molar weights of 386.7, 787 and 744 for cholesterol, PC and PE, respectively.

Lipid peroxidation

Liver homogenates were prepared (1:19 m/v) in a 1.15% KCl/0.01 M phosphate buffer (pH 7.4) on ice and a 0.5 ml aliquot (2 mg protein/ml) incubated with 2.5 mM ferrous sulphate for 1 hour at 37°C (32). Malondialdehyde (MDA) was measured by determining the thiobarbituric reactive substances (TBARS) level according to the method of Hu *et al*. (33) and the results were expressed as TBARS representing the μmole MDA equivalents/mg protein, using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} at 532 nm for MDA (34). Non-specific lipid peroxidation was prevented by the incorporation of EDTA in the buffers and BHT in the reaction solutions for the TBARS assay.

Glutathione (GSH and GSSG) analysis

The glutathione, reduced (GSH) and oxidized forms (GSSG), was determined according to the method of Tietze (35). Tissue samples were homogenized (1:10 ratio) in a 15% trichloroacetic acid (TCA, w/v) and 1 mM EDTA solution for GSH and 6%

perchloric acid (PCA, v/v), 3 mM M2VP (1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate) and 1 mM EDTA solution for GSSG, on ice. The homogenates were centrifuged at 10 000 *g* for 10 minutes and 50 µl of the supernatant was added to glutathione reductase (5 Units) and 75 µM DTNB [5,5' dithiobis-(2-nitrobenzoic acid)], in a microtiter plate. The reaction was initiated with the addition of 0.25 mM NADPH (50 µl) to a final reaction volume of 200 µl, the absorbance monitored at 410 nm for 5 minutes and the levels of GSH and GSSG determined from standard GSH and GSSG curves, respectively. The results were expressed as mM GSH or GSSG/g wet liver weight.

Immunohistochemistry

Tissue sections of the major liver lobes were taken from all treatment and control groups at termination of the rats and preserved in buffered formalin for GSTP staining according to the method of Ogawa *et al.* (36). Dewaxed tissue sections (5 µm) were immunostained with a strepavidin-biotin-peroxidase complex and an affinity-purified biotin-labeled goat anti-rabbit IgG serum (Vector Laboratories, Burlingame, CA). Negative controls, without the antibody, were also included to test the specificity of the anti-GSTP antibody binding. The number and size (internal diameter using the largest transverse of longitudinal measurement) of the GSTP$^+$ foci were quantified microscopically (4x objective) and categorized according to the internal diameter of the foci (10 to 20, 21 to 50, 51 to 100, >100 μm and total i.e. >20 μm). The results were expressed as number per cm^2.

Protein determination

Powdered liver preparations (10-15 mg) from the liquid nitrogen homogenisation step were first solubilised in 5% sodium dodecyl sulphate at 37°C and the protein content determined using a modified method of Lowry (37). The protein content in the liver homogenate prepared for the lipid peroxidation determination was determined as described by Kaushal and Barnes (38).

Statistical analyses

Descriptive statistics performed on the data indicated that all groups were normally distributed (Kolmogorov-Smirnof Test) with homogeneity among the variances (Levene's Test). Initial statistical analyses included 2-way ANOVA's testing for interaction effects between diet and tissue type, which was followed by 1-way ANOVA's

testing for diet effects across all tissue types and also testing for tissue effects across all dietary groups. One-way ANOVA's were also used to test for dietary group differences within each tissue type separately, as well as for tissue type differences within each dietary group. When dietary group differences or tissue type differences were present, Tukey's Studentized Range Test was used, testing for multiple pairwise comparisons between the means of the different groups. As the data were unbalanced, the Tukey-Cramér adjustment was made automatically. When only 2 groups were present, group differences were tested using Student's T-test. Statistical significance was considered at $P<0.05$.

RESULTS

Fatty acid content of dietary oils (Table 1)

Fatty acid analyses of the dietary oils showed that with regards to PUFA, the SFO oil mainly contained C18:2ω6 (62.25%) with a ω6/ω3 FA ratio of approximately 250:1 consisting of total ω6 and ω3 FA levels of 62.31% and 0.25%, respectively. The SOY oil primarily consisted of C18:2ω6 (48.53%) and C18:3ω3 (9.2%). The total ω6 and ω3 FA levels were 48.57% and 9.2%, respectively, with a ω6/ω3 ratio of approximately 5:1. The GLA dietary oil contained C18:2ω6 (53.42%) and C18:3ω6 (6.2%) compared to 60.15% and 0.12% in the EPA dietary oil. Both the GLA and EPA dietary oils contained total ω6 and ω3 FA levels of 60% and 5%, respectively, with a ω6/ω3 ratio of approximately 12:1. Both dietary oils also contained relatively high levels of C20:5ω3 (3.5%).

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

	Sunflower	Soybean	Low ω6/ω3 fatty acid ratio oils	
			Sunflower EPA-50 GLA-80	Sunflower EPA-50
	(SFO)	(SOY)	(GLA)	(EPA)
C14:0	0.07	0.10	0.14	0.11
C16:0	6.68	11.26	5.77	5.59
C17:0	0.04	ND	0.24	0.22
C18:0	4.45	4.20	4.23	4.15
C20:0	0.29	0.36	0.29	0.32
C22:0	0.75	0.43	0.79	0.77
C24:0	0.25	ND	ND	ND
Total	*12.53*	*16.35*	*11.46*	*11.16*
C16:1	0.06	0.06	1.17	1.02
C18:1	25.17	25.60	24.35	22.08
C20:1	0.21	0.24	0.28	0.24
C22:1	0.01	0.01	0.04	0.02
C24:1	0.05	ND	ND	ND
Total	*25.50*	*25.91*	*25.84*	*23.36*
ω6				
C18:2	62.25	48.53	53.42	60.15
C18:3	0.01	ND	6.2	0.12
C20:2	0.02	ND	ND	ND
C20:3	ND	ND	0.08	0.02
C20:4	ND	ND	0.12	0.07
C22:4	0.02	0.04	0.03	0.03
C22:5	0.01	ND	0.01	0.02
Total	*62.31*	*48.57*	*59.90*	*60.41*
ω3				
C18:3	0.25	9.20	0.31	0.40
C18:4	ND	ND	0.35	0.30
C20:3	ND	ND	0.05	0.13
C20:5	ND	ND	3.40	3.58
C22:5	ND	ND	0.27	0.23
C22:6	ND	ND	0.56	0.44
Total	*0.25*	*9.20*	*4.94*	*5.08*
ω6/ω3	249.24	5.28	12.12	11.89
PUFA	62.56	57.77	64.84	65.49
P/S Ratio	4.99	3.53	5.66	5.87

ω6/ω3= ω6 fatty acid to ω3 fatty acid ratio, PUFA=Polyunsaturated fatty acid, ND=not detected, P/S ratio=Polyunsaturated fatty acid to Saturated fatty acid ratio.

Tissue Lipid Analyses

Due to the complexity and amount of data, all data for the lipid parameters and FA were first analysed by 2-way ANOVA for any diet and tissue interactions. Where a significant (P<0.05) interaction occurred the data was further analysed by 1-way ANOVA for diet effects across all tissue types and tissue effects across all dietary groups. Where no diet-tissue interactions were observed, the data are described as a diet and/or tissue effect, independent of each other. Because only overall interactions and effects are observed with this type of data analyses, the data were also separately analysed by 1-way ANOVA in which the different diet and tissue types were not grouped together as for the 2-way ANOVA. The data in Tables 2, 3 and 4 and Figures 2 and 3 depict the results from these 1-way ANOVA analyses.

Lipid parameters: Phospholipid and Cholesterol content (Table 2)

Phospholipids: No diet-tissue interaction was observed. Two-way ANOVA indicated significant (P<0.05) effects due to the diet and tissue for PC and PE. Overall, the PC and PE levels were significantly (P<0.05) higher in the nodule tissue. Overall the tissues the EPA diet decreased (P<0.05) the PC and PE level in the nodules when compared to the SFO diet. The separate 1-way ANOVA revealed a significantly (P<0.05) higher PE level in the nodule tissue compared to the respective surrounding and control in all the diet groups (Table 2).

Cholesterol: No diet-tissue interaction was observed. Two-way ANOVA revealed significant (P<0.05) effects due to the diet and tissue. The cholesterol level was significantly (P<0.05) higher in the nodule tissue while the low ω6/ω3 ratio diets significantly (P<0.05) decreased the cholesterol level when compared to the SFO dietary group. A similar effect was noticed when the data were analysed by the separate 1-way ANOVA. The GLA diet also significantly (P<0.05) decreased the cholesterol level in the surrounding compared to the SFO surrounding tissue.

Lipid parameter ratios (PC/PE and Chol/PL): No diet-tissue interaction was observed for the PC/PE ratio. Two-way ANOVA revealed a significant (P<0.05) tissue effect with a lower PC/PE ratio in the nodule tissue. A significant (P<0.05) diet and tissue interaction was observed for the Chol/PL ratio. Analyses by 1-way ANOVA showed a significant (P<0.05) diet effect reflected by a decrease in the Chol/PL ratio with the GLA diet when compared to the EPA diet. Separate 1-way ANOVA revealed that the Chol/PL ratio was significantly (P<0.05) decreased in the nodules by the GLA

and EPA diets compared to the SFO diet (Table 2).

Table 2: Comparative Phospholipid and Cholesterol content and parameters in the nodule, surrounding and control liver of rats fed a diet with varying $\omega6/\omega3$ fatty acid ratios.

DIET		Phospholipid Concentration (µg/mg protein)			PC:PE Phospholipid Ratio		
		Nodule	Surrounding	Control	Nodule	Surrounding	Control
SFO	PC	113.63 ± 31.56^{A}	102.32 ± 11.08^{A}	107.87 ± 14.55^{A}	1.99 ± 0.53^{a}	3.07 ± 0.46^{b}	2.83 ± 0.30^{b}
	PE	57.20 ± 5.08^{A}	35.58 ± 7.05^{B}	39.71 ± 5.68^{B}			
SOY	PC	104.76 ± 11.33^{A}	95.40 ± 13.13^{A}	91.94 ± 6.89^{A}	2.19 ± 0.30^{a}	3.09 ± 0.39^{b}	2.90 ± 0.32^{b}
	PE	51.77 ± 6.71^{A}	30.80 ± 5.46^{B}	32.05 ± 4.64^{B}			
GLA	PC	106.90 ± 20.70^{A}	87.82 ± 10.55^{A}	99.09 ± 9.30^{A}	2.17 ± 0.19^{a}	2.60 ± 0.17^{b}	2.91 ± 0.45^{b}
	PE	49.09 ± 8.45^{A}	35.29 ± 4.72^{B}	31.95 ± 8.60^{B}			
EPA	PC	101.95 ± 21.65^{A}	82.10 ± 12.70^{A}	---------	2.19 ± 0.36^{a}	2.83 ± 0.49^{b}	---------
	PE	48.73 ± 5.24^{A}	29.71 ± 8.23^{B}	---------			

DIET	Cholesterol Concentration (µg/mg protein)			Cholesterol/Phospholipid Molar Ratio		
	Nodule	Surrounding	Control	Nodule	Surrounding	Control
SFO	$19.73\pm3.30a$	$15.88\pm3.12a$	14.82 ± 2.45	$0.24\pm0.05a$	$0.22\pm0.05ab$	0.20 ± 0.04
SOY	$15.17\pm2.02^{A}b$	$13.97\pm2.14^{AB}ab$	12.32 ± 1.98^{B}	$0.19\pm0.02ab$	$0.22\pm0.04ab$	0.20 ± 0.02
GLA	$13.99\pm2.22b$	$11.93\pm1.66b$	11.94 ± 2.98	$0.18\pm0.01b$	$0.19\pm0.02a$	0.19 ± 0.04
EPA	$13.84\pm1.67b$	$13.05\pm1.06ab$	---------	$0.18\pm0.02^{A}b$	$0.25\pm0.04^{B}b$	---------

Values are means SD of 5-6 replications. Initial statistical analyses included 2-way ANOVA testing for interaction effects between diet and tissue type, followed by 1-way ANOVA testing for overall diet effects across all tissue types and testing for overall tissue effects across all dietary groups. Separate 1-way ANOVA was performed to test for significance between different tissue types separately within the same dietary group, as indicated by superscript uppercase letters within a row ($P<0.05$). One-way ANOVA was also used to test separately for differences within the same tissue type, but compared between the different dietary groups, as indicated by lowercase letters ($P<0.05$) and identical colour in a column. PC=phosphatidylcholine, PE=phosphatidylethanolamine, SFO=sunflower oil diet, SOY=soybean oil diet, GLA=sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 oil diet, EPA=sunflower/eicosapentaenoic acid-50 oil diet. The Cholesterol/Phospholipid molar ratio was calculated using the sum of PC and PE which constitute the two major phospholipid fractions in rat liver.

Comparative FA parameters: Effect of SFO, SOY, GLA and EPA diets on the FA content of the PC and PE phospholipid fraction of hepatocyte nodule, surrounding and control tissues. (Tables 3 and 4)

The individual FA were quantified against an internal standard (C17:0) and expressed as µg FA/mg protein (quantitative data). The qualitative data, calculated as a percentage of total FA analysed, are given in the Addendum (Addendum 6, 7).

Saturated FA: (C16:0, C18:0)

PC fraction: No significant diet-tissue interactions were observed.   Two-way ANOVA showed a significant ($P < 0.05$) tissue effect for C16:0, C18:0 and the total saturated FA (SATS), as well as a diet effect ($P < 0.05$) for C18:0. Overall, the levels of C16:0 and the total SATS were higher in the nodule tissue than in the surrounding and control, except for C18:0 which was higher in the control tissue. Separate 1-way ANOVA showed that the level of C18:0 was also significantly ($P < 0.05$) decreased in the nodules by the SOY diet when compared to the SFO diet (Table 3).

PE fraction: There were no diet-tissue interactions. Two way ANOVA revealed a significant ($P < 0.05$) tissue effect for C16:0, C18:0 and total SATS. Overall, the levels of C16:0, C18:0 and total SATS were higher in the nodules than in surrounding and control tissues. Separate 1-way ANOVA revealed that C16:0 was significantly ($P < 0.05$) increased in the nodules by the GLA diet when compared to the SFO diet (Table 4).

Monounsaturated FA: (C16:1, C18:1)

PC fraction: A significant ($P < 0.05$) diet-tissue interaction was observed for C16:1, C18:1 and the total monounsaturated FA (MUFA). Analyses by 1-way ANOVA showed a significant ($P < 0.05$) tissue effect for these FA and the total MUFA. Overall these FA levels were highest in the nodules compared to surrounding and control tissue. Separate 1-way ANOVA indicated that the SOY and GLA diets significantly ($P < 0.05$) decreased the levels of these FA in the control tissue when compared to the SFO diet (Table 3).

PE fraction: A significant ($P < 0.05$) diet-tissue interaction was observed for C18:1 and the total MUFA only. One-way analyses revealed a significant ($P < 0.05$) tissue effect with the highest levels observed in the nodule tissue compared to the surrounding and control. Two way ANOVA for C16:1 indicated a significant ($P < 0.05$)

tissue effect with the highest level observed in the nodules. Separate 1-way ANOVA revealed that C18:1 and total MUFA were significantly (P<0.05) decreased in the nodule and control tissues by the SOY and GLA diets when compared to the SFO diet (Table 4).

ω6 PUFA: (C18:2, C18:3, C20:3, C20:4, C22:4, C22:5)

   PC fraction: Significant (P<0.05) diet-tissue interactions were observed for C18:3, C20:3 and C22:5. One-way ANOVA showed a significant (P<0.05) diet effect for C18:3 and C22:5, whereas a diet and tissue effect (P<0.05) was observed for C20:3. Overall, the SOY and EPA diets decreased C18:3 when compared to the SFO and GLA fed groups. The GLA diet also increased C20:3 when compared to the other diets while C22:5 was decreased by the low ω6/ω3 ratio diets. The tissue effect of C20:3 was reflected by a lower level in the nodule tissue. Separate 1-way ANOVA indicated that the SOY diet significantly (P<0.05) decreased C18:3 in the nodule tissue while C22:5 was decreased by the low ω6/ω3 ratio diets (Table 3).

Two-way ANOVA revealed significant (P<0.05) tissue effects for C18:2, a diet effect for the total ω6 PUFA level and both diet and tissue effects for C20:4 and C22:4. Overall, the C18:2 level was the highest in the nodule tissue, whereas C20:4 and C22:4 were decreased. With regards to the diet, C20:4 was decreased by the SOY and EPA diets compared to the SFO and GLA dietary groups, while C22:4 and the total ω6 PUFA levels were decreased by the low ω6/ω3 ratio diets. A similar effect was noticed when conducting the separate 1-way ANOVA on the levels of C20:4 and C22:4 in the nodules, surrounding and control tissues (Table 3).

   PE fraction: Significant (P<0.05) diet-tissue interactions were observed for C18:3, C20:3, C20:4 and C22:4. One-way ANOVA showed a significant (P<0.05) diet effect for C20:3 and C22:4, whereas a diet and tissue effect was observed for C18:3 and C20:4. C20:3 was decreased by the SOY diet while C22:4 was decreased by the low ω6/ω3 ratio diets when compared to the SFO diet. Overall, C18:3 was decreased by the SOY and EPA diets, while the level was lower in the nodule tissue when compared to the control. The SOY diet decreased the C20:4 level when compared to the SFO diet but, overall, this FA remained higher in the nodule tissue compared to surrounding and control. C22:4 was decreased by the low ω6/ω3 ratio diets when compared to the SFO fed group.

Two-way ANOVA revealed a significant (P<0.05) tissue effect for C18:2, a diet effect for C22:5 and a diet-tissue effect for the total ω6 PUFA. Overall, C18:2 was the highest

in the nodule tissue. With regards to diet, C22:5 was decreased by the low ω6/ω3 ratio diets compared to the SFO fed group. Although the total ω6 PUFA level was decreased by the low ω6/ω3 ratio diets, overall the level remained the highest in the nodules. When considering the separate 1-way ANOVA, C20:4 was decreased in the nodules while C22:4 and C22:5 were decreased in all the tissues by the low ω6/ω3 ratio diets. This resulted in a significant decrease in the total ω6 FA in the nodules (Table 4).

ω3 PUFA: (C18:3, C20:5, C22:5, C22:6)

PC fraction: Significant (P<0.05) diet-tissue interactions were observed for C18:3, C20:5, C22:5, C22:6 as well as for the total ω3 PUFA. One-way ANOVA showed a significant (P<0.05) diet and tissue effect for C18:3, C20:5 and C22:6, but only a diet effect (P<0.05) for C22:5 and the total ω3 PUFA. The C18:3, C20:5, C22:6 levels were increased by the low ω6/ω3 ratio diets compared to the SFO fed group. Overall, C18:3 and C20:5 were higher in the nodule tissue, whereas C22:6 was lower when compared to surrounding and control tissue. The overall levels of C22:5 and total ω3 PUFA were increased by the low ω6/ω3 ratio diets.

Separate 1-way ANOVA indicated that C18:3 was increased by the low ω6/ω3 ratio diets in the nodule with the highest level obtained with the SOY diet. C20:5 was increased significantly (P<0.05) by the GLA and EPA diets when compared to the SOY diet. C22:5, C22:6 and total ω3 PUFA were significantly increased by the low ω6/ω3 ratio diets in the nodules with the highest levels obtained with the GLA and EPA diets (Table 3).

PE fraction: Two-way ANOVA showed a significant (P<0.05) diet and tissue effect for C18:3. The low ω6/ω3 ratio diets increased the C18:3 level when compared to the SFO fed group, with the highest level achieved by the SOY diet. Overall, C18:3 was the highest in the nodule tissue. Significant (P<0.05) diet-tissue interactions were observed for C20:5, C22:5, C22:6 and the total ω3 PUFA. One-way ANOVA showed significant (P<0.05) effects due to the diet and tissue for C20:5 and C22:5, but only a diet effect for C22:6 and the total ω3 PUFA. The low ω6/ω3 ratio diets increased the levels of C20:5, C22:5, C22:6 and the total ω3 PUFA. However, of the three diets the level of C22:5 was lowest in the SOY fed group whereas C22:6 and the total ω3 PUFA were the highest in the GLA fed group. Overall the tissue, C20:5 and C22:5 were the highest in the nodules when compared to the control tissue. Separate 1-way ANOVA showed a significant (P<0.05) increase of the ω3 FA in all the tissues when compared

to the SFO diet. The highest incorporation was obtained with the GLA and EPA diets (Table 4).

PUFA and LCPUFA

PC fraction: No significant diet-tissue interactions were observed. Two-way ANOVA only revealed significant (P<0.05) effects due to the diet and tissue for LCPUFA. Overall the tissue the LCPUFA level was decreased by the SOY and EPA diets when compared to the SFO diet in contrast to the GLA diet where the tissue level of LCPUFA was not affected. The level of the LCPUFA was significantly (P<0.05) lower in the nodule and surrounding tissue when compared to the control. Separate 1-way ANOVA indicated that the LCPUFA was decreased in the nodules only by the SOY diet when compared to the SFO diet (Table 3).

PE fraction: Two-way ANOVA for PUFA revealed a significant (P<0.05) diet and tissue effect. The PUFA level was lowered by the SOY diet when compared to the SFO and GLA diets. Overall, the PUFA was the highest in the nodule tissue compared to surrounding and control. A significant (P<0.05) diet-tissue interaction was observed for the LCPUFA while the 1-way ANOVA indicated a significant diet and tissue effect. Overall the tissue, the GLA diet effected a higher level of LCPUFA when compared to the SOY diet, while the level was higher in the nodule tissue compared to the surrounding and control. Separate 1-way ANOVA indicated that the SOY diet significantly (P<0.05) decreased the levels of LCPUFA and PUFA in the nodules (Table 4).

Table 3: Comparison of the fatty acid profiles (μg fatty acid/mg protein) in the phosphatidylcholine (PC) phospholipid fraction of the nodule, surrounding and control liver of rats fed a diet with varying ω6/ω3 fatty acid ratios.

DIET	∀	SFO			SOY			GLA			EPA	
		Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
SATS												
C16:0	N	13.96±1.41	10.30±0.91	9.13±2.87	14.78±1.49	11.68±1.36	10.38±0.72	14.59±2.54	9.45±1.51	9.10±2.94	13.62±2.09	9.04±3.02
C18:0	N	10.35±2.31a	10.41±1.47	13.25±1.41	7.10±0.81b	9.66±1.02	12.10±0.87	8.68±1.51ab	9.65±1.71	11.91±3.04	8.24±1.01ab	7.71±2.49
Total	N	24.31±3.23	20.71±1.66	22.37±3.88	21.88±2.28	21.34±2.21	22.47±1.48	23.28±3.76	19.10±3.05	21.02±5.45	21.87±3.06	16.75±5.50
MUFA												
C16:1	Y	0.80±0.13^A	0.49±0.17^B	0.40±0.11^{B}a	0.73±0.09^A	0.43±0.11^B	0.27±0.07^{C}b	0.98±0.20^A	0.45±0.10^B	0.17±0.05^{C}b	0.95±0.18^A	0.44±0.15^B
C18:1	Y	7.53±0.99^A	4.69±0.88^B	4.78±0.60^{B}a	6.04±0.56^A	4.81±0.50^B	3.73±0.37^{C}b	7.14±1.24^A	4.76±0.70^B	3.37±0.61^{C}b	7.08±0.90^A	3.72±1.34^B
Total	Y	8.33±1.03^A	5.18±0.91^B	5.18±0.72^{B}a	6.78±0.64^A	5.25±0.60^B	4.01±0.39^{C}b	8.12±1.35^A	5.21±0.73^B	3.54±0.60^{C}b	8.03±1.05^A	4.16±1.49^B
ω6												
C18:2	N	8.89±1.38	5.50±1.07	5.30±1.36	8.99±1.22	6.30±0.45	5.02±0.58	8.24±1.46	5.91±0.87	5.03±1.11	10.28±1.32	5.77±2.62
C18:3	Y	0.23±0.05a	0.24±0.05ab	0.32±0.05a	0.14±0.02^{A}b	0.22±0.02^{B}ab	0.17±0.04^{A}b	0.26±0.05a	0.28±0.05a	0.20±0.03b	0.19±0.02a	0.18±0.07b
C20:3	Y	0.23±0.05^A	0.33±0.10^A^{B}a	0.41±0.10^B	0.24±0.04^A	0.32±0.02^{B}a	0.36±0.08^B	0.32±0.06^A	0.57±0.09^{B}b	0.36±0.09^A	0.30±0.06	0.41±0.16ab
C20:4	N	18.32±2.85a	15.57±2.38a	19.10±1.75a	11.47±1.21b	14.56±1.37ab	16.10±1.15b	14.12±2.48ab	15.84±2.65a	15.83±1.80b	13.36±1.68b	11.97±2.38b
C22:4	N	0.41±0.1a	0.56±0.14a	0.44±0.11a	0.08±0.01b	0.19±0.11b	0.11±0.03b	0.12±0.02b	0.13±0.03b	0.12±0.04b	0.09±0.01b	0.07±0.03b
C22:5	Y	1.24±0.32^{A}a	2.43±0.32^{B}a	2.33±0.18^{B}a	0.05±0.02^{A}b	0.16±0.05^{B}b	0.19±0.02^{B}b	0.08±0.02b	0.12±0.03b	0.16±0.07b	0.05±0.02^{A}b	0.09±0.02^{B}b
Total	N	28.42±5.39a	25.21±4.47a	27.90±3.07a	20.97±2.30b	22.08±2.16ab	21.94±1.79b	23.14±4.04ab	22.85±3.67ab	21.69±4.88b	24.26±2.94ab	17.54±6.12b
ω3												
C18:3	Y	0.04±0.01a	0.05±0.01a	0.05±0.02a	0.13±0.02b	0.10±0.02b	0.08±0.01b	0.08±0.01c	0.08±0.02bc	0.05±0.01a	0.08±0.04bc	0.06±0.02ac
C20:5	Y	ND	ND	ND	0.16±0.03a	0.11±0.02a	0.15±0.05	0.25±0.05^{A}b	0.27±0.11^{A}b	0.09±0.05^B	0.30±0.04b	0.27±0.12b
C22:5	Y	0.02±0.01a	0.03±0.01a	0.03±0.01a	0.34±0.05b	0.45±0.12b	0.47±0.10b	0.60±0.15c	0.65±0.15b	0.44±0.12b	0.52±0.06c	0.46±0.24b
C22:6	Y	0.17±0.04^{A}a	0.24±0.06^A^{B}a	0.30±0.06^{B}a	1.03±0.20^{A}b	2.61±1.02^{B}b	3.45±0.47^{B}b	1.96±0.44^{A}c	3.04±0.54^{B}b	3.28±1.10^{B}b	1.78±0.37^{A}c	2.49±0.65^{B}b
Total	Y	0.23±0.06^{A}a	0.31±0.08^A^{B}a	0.37±0.07^{B}a	1.66±0.25^{A}b	3.27±1.16^{B}b	4.15±0.55^{B}b	2.90±0.58^{A}c	4.04±0.78^{B}b	3.86±1.23^{B}b	2.69±0.46c	3.28±0.97b
PUFA	N	28.66±5.45	25.53±4.54	28.27±3.13	22.6±32.47	25.36±1.72	26.10±2.24	26.04±4.51	26.89±4.43	25.55±6.01	26.95±3.31	20.82±7.07
LCPUFA	N	19.49±0.43a	19.74±3.30	22.61±1.21	13.36±1.33b	18.74±1.25	20.83±1.50	17.47±2.80ab	20.62±3.22	20.27±4.46	16.40±1.96ab	14.81±4.09
FA Ratios												
C20:4 N/S		1.18±0.09a		----------	0.79±0.12b		----------	0.87±0.14b		----------	1.12±0.15a	
20:4/20:5	Y	3.23±0.08a	3.20±0.08a	3.28±0.04a	1.87±0.06^{A}b	2.12±0.11^{B}b	2.04±0.11^A^{B}b	1.75±0.04^{A}bc	1.80±0.15^{A}c	2.27±0.24^{B}b	1.65±0.02c	1.63±0.06c
Δ6 S/P	Y	5.86±0.73^{A}a	2.11±0.31^B	1.96±0.34^B	8.11±1.77^{A}b	2.18±0.36^B	1.50±0.15^B	4.01±0.73^{A}c	1.97±0.04^B	1.60±0.18^B	5.52±0.79^{A}ac	2.24±0.56^B
ω6/ω3	Y	123.21±10.67^{A}a	81.42±6.70^{B}a	76.85±11.30^{B}a	12.79±1.64^{A}b	7.94±2.13^{B}b	5.32±0.50^{B}b	8.07±1.02^{A}c	5.69±0.36^{B}b	5.80±1.08^{B}b	9.12±0.92^{A}c	5.28±0.41^{B}b
P/S	Y	1.17±0.10a	1.23±0.17ab	1.29±0.21	1.03±0.06^{A}b	1.19±0.06^{B}a	1.16±0.03^B	1.12±0.09^{A}ab	1.42±0.15^{B}b	1.23±0.15^A^{B}	1.24±0.05a	1.24±0.03ab

Values are means SD of 5-6 replications. ∀, A dietary group-tissue type interaction, analysed by 2-way ANOVA, Y=a significant ($P<0.05$) diet-tissue interaction and N=no interaction. Initial statistical analyses included 2-way ANOVA testing for interaction effects between diet and tissue type, followed by 1-way ANOVA testing for overall diet effects across all tissue types and testing for overall tissue effects across all dietary groups. Separate 1-way ANOVA was performed to test for significance between different tissue types separately within the same dietary group, as indicated by superscript uppercase letters within a row ($P<0.05$). One-way ANOVA was also used to test separately for differences within the same tissue type across the different dietary groups, as indicated by lowercase letters ($P<0.05$) and identical colour in a row. SFO=sunflower dietary oil, SOY=soybean dietary oil, GLA=sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 dietary oil, EPA=sunflower/eicosapentaenoic acid-50 dietary oil, SATS=saturated fatty acids, MUFA=monounsaturated fatty acids, ND=not detected, 20:4/20:5=log ratio of C20:4ω6 to C20:5ω3, ω6/ω3=ω6 to ω3 fatty acid ratio, PUFA=polyunsaturated fatty acids, LCPUFA=long-chain polyunsaturated fatty acids, P/S=polyunsaturated to saturated fatty acid ratio, Δ6 S/P=ratio of the substrates to products of the delta-6-desaturase enzyme, C20:4 N/S= C20:4ω6 nodule to surrounding tissue ratio.

Table 4: Comparison of the fatty acid profiles (µg fatty acid/mg protein) in the phosphatidylethanolamine (PE) phospholipid fraction of the nodule, surrounding and control liver of rats fed a diet with varying ω6/ω3 fatty acid ratios.

DIET	∀	SFO			SOY			GLA			EPA	
		Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
SATS												
C16:0	N	2.98±0.63a	3.06±0.60	2.43±0.72	3.77±0.44ab	3.14±0.55	2.72±0.45	3.92±0.63b	3.01±0.50	2.46±0.72	3.73±0.48ab	2.65±1.27
C18:0	N	8.18±1.20	4.17±0.85	5.67±1.69	6.40±0.85	3.84±0.30	4.73±0.44	6.84±1.92	4.97±1.04	4.89±1.96	7.13±0.98	3.43±1.43
Total	N	11.16±1.44	7.24±1.05	8.10±1.31	10.17±1.24	6.98±0.66	7.45±0.88	10.77±2.08	7.98±1.36	7.34±2.48	10.86±1.31	6.08±2.66
MUFA												
C16:1	N	0.13±0.03	0.09±0.04	0.07±0.04	0.14±0.03	0.08±0.04	0.05±0.02	0.15±0.03	0.07±0.01	0.03±0.01	0.16±0.03	0.05±0.02
C18:1	Y	$3.97±0.50^A$a	$1.56±0.36^B$	$1.76±0.36^B$a	$2.93±0.24^A$b	$1.62±0.30^B$	$1.05±0.15^C$b	$3.00±0.58^A$b	$1.79±0.25^B$	$1.02±0.27^C$b	$3.36±0.46^A$ab	$1.22±0.55^B$
Total	Y	$4.10±0.50^A$a	$1.66±0.34^B$	$1.83±0.33^B$a	$3.07±0.25^A$b	$1.70±0.33^B$	$1.09±0.16^C$b	$3.15±0.58^A$b	$1.86±0.26^B$	$1.05±0.27^C$b	$3.52±0.46^A$ab	$1.27±0.57^B$
ω6												
C18:2	N	2.61±0.34	1.27±0.35	1.36±0.43	2.35±0.17	1.46±0.67	0.98±0.16	2.06±0.26	1.51±0.18	0.99±0.21	2.55±0.34	1.25±0.68
C18:3	Y	$0.02±0.01^A$	$0.02±0.01^A$	$0.04±0.01^B$	0.01±0.01	0.01±0.01	0.02±0.01	$0.01±0.01^A$	$0.03±0.01^B$	$0.02±0.01^{AB}$	0.01±0.01	0.01±0.01
C20:3	Y	$0.10±0.02^{AB}$	$0.09±0.04^A$a	$0.17±0.07^B$a	0.08±0.04	$0.05±0.03$a	$0.08±0.02$b	$0.09±0.02^A$	$0.15±0.03^B$b	$0.08±0.03^A$b	0.08±0.01	0.09±0.05ab
C20:4	Y^D	$10.25±1.54^A$a	$5.66±1.49^B$ab	$7.78±2.92^{AB}$	$6.56±0.72^A$b	$4.60±0.75^B$ab	$5.20±0.59^B$	$7.93±1.41^A$b	$6.87±1.34^{AB}$a	$5.36±2.05^B$	$7.93±1.07^A$b	$4.23±1.83^B$b
C22:4	Y^D	$1.04±0.20^A$a	$0.63±0.20^B$a	$0.69±0.35^{AB}$a	$0.22±0.02$b	$0.21±0.08$b	$0.15±0.02$b	$0.21±0.08^A$b	$0.24±0.06^{AB}$b	$0.15±0.05^B$b	$0.21±0.04^A$b	$0.12±0.05^B$b
C22:5	N	2.38±0.45a	2.13±0.45a	2.74±1.09a	0.10±0.02b	0.16±0.04b	0.14±0.01b	0.14±0.02b	0.14±0.04b	0.12±0.06b	0.09±0.02b	0.07±0.02b
Total	N	$16.40±2.49^A$a	$9.80±2.40^B$a	$12.78±4.83^{AB}$a	9.32±0.82b	6.68±1.44ab	6.55±0.79b	10.43±1.85b	8.92±1.62ab	6.73±2.39b	10.87±1.46b	5.78±2.63b
ω3												
C18:3	N	0.03±0.01a	0.02±0.01a	0.02±0.01a	0.08±0.01b	0.06±0.01b	0.04±0.01b	0.05±0.02c	0.04±0.01ab	0.02±0.01a	0.05±0.01c	0.03±0.02ab
C20:5	Y	ND	ND	ND	$0.09±0.01^A$a	$0.05±0.02^B$a	$0.08±0.04^{AB}$	$0.14±0.03^A$b	$0.15±0.03^A$b	$0.05±0.02^B$	0.19±0.03b	0.13±0.07b
C22:5	Y	$0.05±0.01^A$a	$0.03±0.01^B$a	$0.04±0.02^{AB}$a	$0.65±0.09^A$b	$0.34±0.12^B$b	$0.39±0.08^B$b	$0.99±0.30^A$c	$0.83±0.19^A$c	$0.40±0.17^B$b	$0.94±0.14^A$c	$0.47±0.29^B$b
C22:6	Y^D	$0.35±0.06^A$a	$0.23±0.08^B$a	$0.37±0.16^{AB}$a	$2.32±0.23^A$b	$2.10±0.76^A$b	$3.07±0.39^B$b	3.63±0.56c	3.91±0.75c	3.26±1.49c	$3.45±0.52^A$c	$2.31±0.76^B$b
Total	Y^D	$0.42±0.07^A$a	$0.28±0.09^B$a	$0.43±0.19^{AB}$a	$3.14±0.31^{AB}$b	$2.55±0.93^A$b	$3.58±0.48^B$b	4.80±0.88c	4.94±0.98c	3.74±1.66b	$4.62±0.69^A$c	$2.94±1.11^B$b
PUFA	NY	16.83±2.56a	10.08±2.49ab	13.21±5.00	12.46±1.05b	9.23±1.87a	10.13±1.25	15.23±2.71ab	13.86±2.57b	10.47±4.04	15.49±2.06ab	8.72±3.73a
LCPUFA		$14.17±1.05^A$a	$8.77±1.99^B$ab	$11.80±1.10^B$	$10.01±0.90^A$b	$7.70±1.15^B$a	$9.10±0.98^{AB}$	13.23±2.17a	12.28±2.19b	9.44±3.41	$12.88±1.59^A$a	$7.42±2.77^B$a
FA Ratios												
C20:4 N/S		1.81±0.31a		----------	1.43±0.27ab		----------	1.15±0.25b		----------	1.87±0.24a	
20:4/20:5	Y	$3.01±0.06^A$a	$2.74±0.10^B$a	$2.87±0.13^{AB}$a	$1.89±0.02$b	$1.99±0.08^A$b	$1.83±0.12^B$b	$1.76±0.02^A$c	$1.65±0.05^B$c	$2.00±0.10^C$b	$1.63±0.02^A$d	$1.53±0.03^B$d
Δ6 S/P	Y	$1.37±0.11^A$a	$0.82±0.13^B$	$0.68±0.10^B$a	$1.37±0.13^A$a	$0.83±0.18^B$	$0.48±0.03^C$b	$0.86±0.08^A$b	$0.65±0.03^B$	$0.49±0.08^C$ab	$1.06±0.09^A$b	$0.75±0.21^B$
ω6/ω3	N	39.03±2.49a	36.11±5.01a	31.67±7.64a	2.98±0.24b	2.61±1.47b	1.84±0.09b	2.17±0.09b	1.82±0.11b	1.86±0.17b	2.36±0.20b	1.92±0.21b
P/S	Y	1.51±0.12a	1.39±0.26	1.61±0.38	1.23±0.10b	1.32±0.20	1.36±0.04	$1.43±0.17^A$a	$1.74±0.20^B$	$1.43±0.26^{AB}$	1.43±0.05a	1.45±0.12

Values are means SD of 5-6 replications. ∀, A dietary group-tissue type interaction, analysed by 2-way ANOVA, Y=a significant (P<0.05) diet-tissue interaction and N=no interaction. Initial statistical analyses included 2-way ANOVA testing for interaction effects between diet and tissue type, followed by 1-way ANOVA testing for overall diet effects across all tissue types and testing for overall tissue effects across all dietary groups. Separate 1-way ANOVA was performed to test for significance between different tissue types separately within the same dietary group, as indicated by superscript uppercase letters within a row (P<0.05). One-way ANOVA was also used to test separately for differences within the same tissue type across the different dietary groups, as indicated by lowercase letters (P<0.05) and identical colour in a row. SFO=sunflower dietary oil, SOY=soybean dietary oil, GLA=sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 dietary oil, EPA=sunflower/eicosapentaenoic acid-50 dietary oil, SATS=saturated fatty acids, MUFA=monounsaturated fatty acids, ND=not detected, 20:4/20:5=log ratio of C20:4ω6 to C20:5ω3, ω6/ω3=ω6 to ω3 fatty acid ratio, PUFA=polyunsaturated fatty acids, LCPUFA=long-chain polyunsaturated fatty acids, P/S=polyunsaturated to saturated fatty acd ratio, Δ6 S/P=ratio of the substrates to products of the delta-6-desaturase enzyme, C20:4 N/S= C20:4ω6 nodule to surrounding tissue ratio.

Membrane FA Ratios (Tables 3 and 4):

C20:4/C20:5, Δ6 S/P, ω6/ω3, P/S ratios

    PC fraction: A significant ($P<0.05$) diet-tissue interaction was observed for the C20:4ω6 to C20:5ω3 FA ratio (C20:4/C20:5), delta-6-desaturase FA substrate to product ratio (Δ6 S/P), total ω6 to total ω3 PUFA ratio (ω6/ω3) and the polyunsaturated to saturated FA ratio (P/S). One-way ANOVA indicated a significant ($P<0.05$) diet effect for the C20:4/C20:5 and ω6/ω3 ratios, a tissue effect for the Δ6 S/P ratio and a diet and tissue effect for the P/S ratio. Overall the tissues, the C20:4/C20:5 and ω6/ω3 ratios were decreased by the low ω6/ω3 ratio diets, with the lowest C20:4/C20:5 ratio observed in the EPA dietary group. The Δ6 S/P ratio was overall the highest in the nodule tissue. The SOY diet significantly decreased the P/S ratio when compared to the GLA diet. Overall, the ratio was lower in the nodule tissue.

Separate 1-way ANOVA indicated that the C20:4/C20:5 and ω6/ω3 ratios were decreased in the nodules by the low ω6/ω3 ratio diets with the highest decrease obtained with the GLA and EPA diets. The Δ6 S/P ratio was increased by the SOY diet but decreased by the GLA diet when compared to the SFO diet. The P/S ratio was decreased only by the SOY diet (Table 3).

    PE fraction: A significant ($P<0.05$) diet-tissue interaction was observed for the C20:4/C20:5, Δ6 S/P and the P/S ratios. One-way ANOVA indicated a significant ($P<0.05$) diet effect for the C20:4/C20:5 and P/S ratios and a diet and tissue effect for the Δ6 S/P ratio. Overall the tissues, the C20:4/C20:5 ratio was decreased by the low ω6/ω3 ratio diets with the EPA diet exhibiting the lowest ratio. The P/S ratio was decreased by the SOY diet when compared to the SFO and GLA diets. The Δ6 S/P ratio was decreased by the GLA diet compared to the SFO diet, while overall the ratio was the higher in the nodule tissue. Separate 1-way ANOVA indicated that the C20:4/C20:5 ratio was decreased in the nodules with the low ω6/ω3 ratio diets with the lowest ratio obtained with the EPA diet. The Δ6 S/P ratio was decreased in the nodules by the GLA and EPA diets. The P/S ratio was reduced in the nodules by the SOY diet (Table 4).

The C20:4ω6 nodule to surrounding ratio (C20:4ω6 N/S) was significantly ($P<0.05$) decreased by both the SOY and GLA diets in PC and the GLA diet ($P<0.05$) in PE.

Lipid peroxidation (Figure 2)

Significant ($P < 0.05$) diet and tissue interactions were revealed for the TBARS level. For the diet, one-way ANOVA showed that the overall TBARS level was significantly ($P < 0.05$) increased by the low $\omega 6/\omega 3$ ratio diets compared to the SFO diet (Figure 2-A). Overall the diets, the lowest TBARS level ($P < 0.05$) was observed in the nodules compared to the control tissue.

Separate 1-way ANOVA showed that the TBARS level in the nodules of the low $\omega 6/\omega 3$ ratio diets was significantly ($P < 0.05$) increased to a similar level as the respective surrounding tissue (Figure 2-A). This resulted in a significant ($P < 0.05$) increase in the TBARS nodule/surrounding ratio in the low $\omega 6/\omega 3$ ratio diets with the highest ($P < 0.05$) ratio observed in the GLA diet (Figure 2-B). The low $\omega 6/\omega 3$ ratio diets exhibited a shift in the ratio towards an equilibrium of 1 between the nodule and surrounding tissue (Figure 2-B). The TBARS level in the nodules of the EPA and GLA diets was significantly higher than the SOY diet (Figure 2-A).
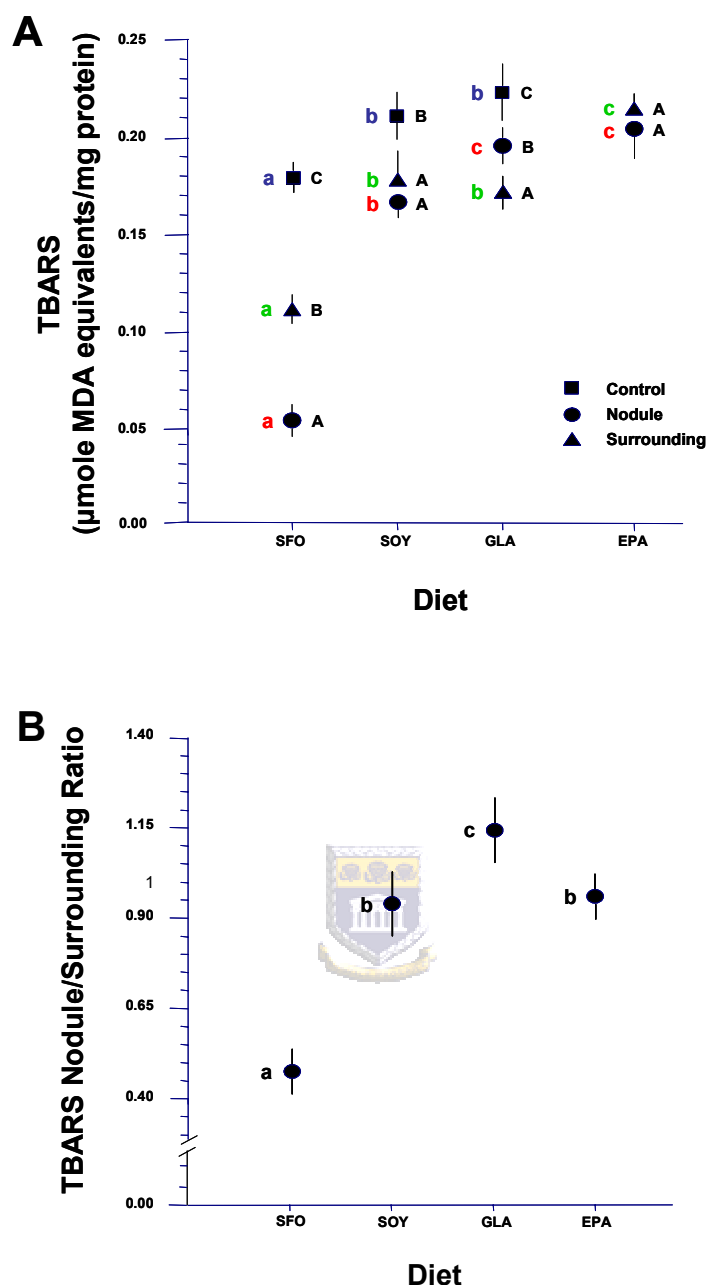
Figure 2: The TBARS level (μmole MDA equivalents/mg protein) was determined in hepatocyte nodules, surrounding and control tissue samples from rat livers modulated with diets containing sunflower oil (SFO), soybean oil (SOY), sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 (GLA) and sunflower/eicosapentaenoic acid-50 (EPA) as fat sources (Figure 2-A). Two-way ANOVA indicated a significant diet-tissue interaction. One-way ANOVA showed that the overall TBARS level was significantly (P<0.05) increased by the low ω6/ω3 ratio diets compared to the SFO fed group.   Overall the diets, the lowest TBARS level (P<0.05) was observed in the nodules compared to the control tissue. Statistical analyses by one-way ANOVA of the separate tissue types and dietary groups were also done. This was performed to test for significance between different tissue types within the same dietary group, as indicated by uppercase letters within a column, and for differences within the same tissue type, but compared between the different dietary groups, as indicated by lowercase letters (P<0.05) and identical colour from left to right across the groups. Figure 2-B represents the change in ratio between nodule and surrounding tissue (nodule/surrounding ratio) compared to a theoretically value of 1 (i.e. no difference between nodule and surrounding tissue). Statistical analyses by one-way ANOVA was performed to test for significance (P<0.05) between different dietary groups, as indicated by lowercase letters.

Glutathione (GSH and GSSG; Figure 3)

Both the GSH and GSSG levels showed a significant ($P<0.05$) diet-tissue interaction, although this was related to a significant ($P<0.05$) tissue effect in the case of GSH only. Overall the diets, the GSH level was significantly ($P<0.05$) higher in the nodule tissue.

Separate 1-way ANOVA showed that the GSH level was significantly higher ($P<0.05$) in the nodules of the SFO dietary group compared to the respective surrounding and control tissue (Figure 3-A). A similar pattern was also observed with the SOY diet, although the level was significantly increased ($P<0.05$) in the surrounding tissue. The EPA and GLA diets significantly reduced ($P<0.05$) the GSH levels in the nodules (Figure 3-A), while the GLA diet significantly ($P<0.05$) increased the level in control tissue. These changes resulted in a significant ($P<0.05$) reduction in the GSH nodule to surrounding ratio (Figure 3-B) with the low $\omega6/\omega3$ ratio diets. The lowest GSH nodule to surrounding ratio was observed with the EPA diet, while the SOY and GLA diets exhibited similar ratios. With respect to GSSG, only the SOY diet significantly ($P<0.05$) reduced the level in the nodules (Figure 3-C). The GSSG nodule to surrounding ratio (Figure 3-D) was significantly ($P<0.05$) lowered by the SOY and GLA diets and significantly ($P<0.05$) increased with the EPA diet compared to the SFO diet.
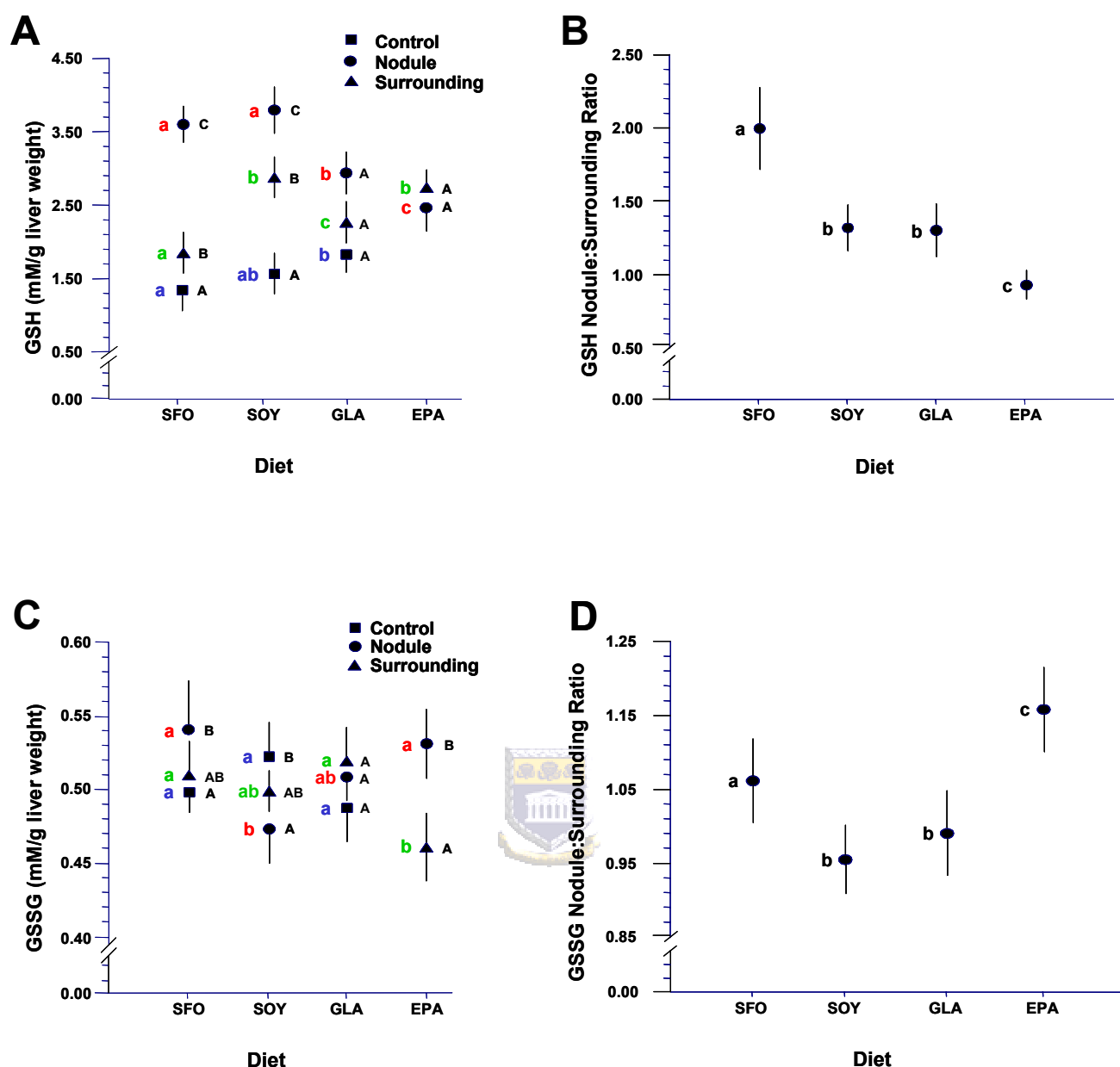
Figure 3: The GSH (Figure 3-A) and GSSG (Figure 3-C) levels were determined in hepatocyte nodules, surrounding and control tissue from rats fed diets containing sunflower oil (SFO), soybean oil (SOY), sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 (GLA) and sunflower/eicosapentaenoic acid-50 (EPA) as fat sources. Two-way ANOVA indicated a significant (P<0.05) diet-tissue interaction for GSH and GSSG, although only a significant (P<0.05) effect due to tissue was observed for GSH. Overall the diets, the GSH level was significantly (P<0.05) higher in the nodule tissue. Statistical analyses by one-way ANOVA of the separate tissue types and dietary groups were also done. This was performed to test for significance between different tissue types within the same dietary group, as indicated by uppercase letters within a column, and for differences within the same tissue type, but compared between the different dietary groups, as indicated by lowercase letters (P<0.05) and identical colour from left to right across the groups. Figures 3-B and 3-D indicate the change in ratio in GSH and GSSG between nodule and surrounding tissue, respectively. Statistical analyses by one-way ANOVA was performed to test for significance (P<0.05) between different dietary groups, indicated by lowercase letters.

Induction of GSTP[+] foci (Figure 4)

No significant effect on the induction of GSTP[+] foci was observed with the SOY and EPA diets as compared to the SFO diet (Figure 4). The GLA diet markedly lowered foci in all the size categories when compared to the SFO and SOY diets, except for the size category >100 μm/cm^2. The GLA diet marginally (P=0.052) reduced the 21 to 50 μm/cm^2 focal size category when compared to the SFO, SOY and EPA diets (Figure 4 insert). This size category comprised 70 to 76% of the total focal count in these diets compared to the 64% obtained with the GLA diet.
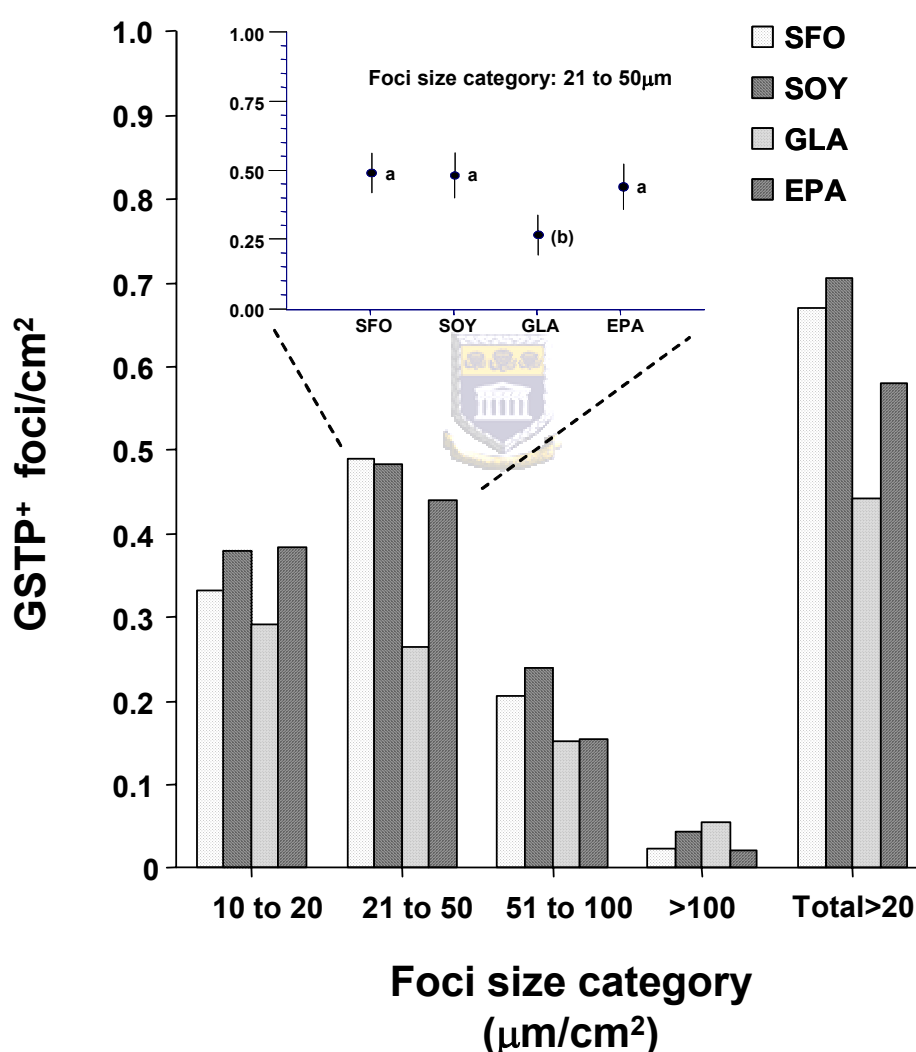
Figure 4: Effect of diets containing sunflower oil (SFO), soybean oil (SOY), sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 (GLA) and sunflower/eicosapentaenoic acid-50 (EPA) as fat sources on number of GSTP[+] foci in liver sections. The number and size of the GSTP[+] foci were quantified by microscope (4x objective) and categorized according to the internal diameter of the foci as follows; 10 to 20, 21 to 50, 51 to 100, > 100 μm/cm^2 and total>20. Statistical analyses by one-way ANOVA was performed to test for significance between different dietary groups within a foci size category. The graph insert shows the number GSTP+ foci in the 21 to 50 μm/cm^2 foci size category of the different dietary groups. The letter in parentheses (b) indicates a marginally significant (P<0.1) effect.

DISCUSSION

Alterations in lipid metabolism are associated with cancer development affecting the function and growth of neoplastic cells (6, 10). It appears that the regulatory mechanisms related to normal lipid metabolism are disrupted thereby altering the growth and survival of preneoplastic cells (10, 39). The interaction between cholesterol, phospholipids and FA is of importance in maintaining the integrity and functioning of cell membranes (40, 41). A study by Blom *et al.* (42) reported that, in response to cholesterol loading, there was an increase in the phospholipid species containing PUFA resulting in an increased membrane unsaturation. In the present study increased levels of cholesterol and PE in hepatocytes nodules was associated with changes in the SATS, MUFA and PUFA. The low $\omega 6/\omega 3$ FA ratio diets mainly altered the LCPUFA content of the major phospholipid fractions as well as the cholesterol level and Chol/PL ratio in the nodule tissue, while the PC and PE phospholipid concentrations were decreased. These changes were shown to affect the membrane fluidity of hepatocyte nodules in a previous study (10). Alterations in these parameters could be of importance when considering the potential role of lipid rafts in controlling certain cell signaling events by modulating the activity of raft proteins (43, 44).

PUFA are known to affect various cellular processes including proliferation and/or apoptosis through the formation of oxidation products and prostaglandins (11, 45). In the present study, the low $\omega 6/\omega 3$ ratio diets significantly increased the $\omega 3$ FA content, especially C20:5ω3 and C22:6ω3, with a concomitant decrease in the $\omega 6$ PUFA levels. The regulation of C20:4ω6 in cancer cells by dietary FA intervention utilising C20:5ω3, a precursor of the 3-series prostaglandins, has been suggested as it competes for the cyclo-oxygenase enzyme. The C20:4ω6 to C20:5ω3 FA ratio is, therefore, of importance indicating a shift in the type of prostaglandin synthesized (46). The low $\omega 6/\omega 3$ ratio diets significantly decreased C20:4ω6 and increased C20:5ω3 in PC and PE resulting in a decreased C20:4/C20:5 ratio. The kinetics of changes in these parameters differed between the diets with the EPA diet inducing the largest effect. However, as C20:4ω6 has been implicated in the induction of apoptosis via the stimulation of ceramide release (47, 48), excessive reduction of C20:4ω6 could impact negatively on the apoptotic rate in the nodules in counteracting the enhanced cell proliferation in this tissue type. Therefore, control over the level of C20:4ω6 by dietary intake of C20:5ω3 and C22:6ω3 can be exercised, due to their feedback inhibition on the activity of the delta-5- and delta-6-desaturases (8, 12). Dietary GLA also modulates

the levels of C20:3ω6 and C20:4ω6, thereby influencing prostaglandin synthesis (49, 50). In the present study, the GLA diet modulated the C20:4/C20:5 ratio by stabilizing the replacement/decrease of C18:3ω6, C20:3ω6 and C20:4ω6 by the SOY diet and C18:3ω6 and C20:4ω6 by the EPA diet. The characteristic difference in the C20:4ω6, PUFA and LCPUFA pattern between the nodule and surrounding tissue was equalized by the GLA diet in PE. GLA decreased the C20:4ω6 nodule/surrounding ratio to approximately 1 by bypassing the impaired delta-6-desaturase. This could compensate for the increased level of C20:4ω6 due to the persistent high concentration of PE in the nodule tissue. In contrast, the EPA diet had a comparable nodule to surrounding ratio to the SFO diet as the replacement of C20:4ω6 by the ω3 PUFA was similar in the nodule and surrounding tissue. Therefore, changes in the levels of both C18:3ω6 and C20:3ω6, together with a shift in the C20:4 nodule/surrounding and C20:4/C20:5 ratios in hepatocyte nodules, is likely to direct prostaglandin synthesis away from C20:4ω6. In addition, the ω3 PUFA inhibit the phospholipase-induced release of C20:4ω6 (51, 52), while C20:5ω3 has a potentiating effect on the growth regulatory effects of C20:3ω6 presumably via the production of the 1-series prostaglandins and 15-OH-dihomo-gamma-linolenic acid which inhibits cell proliferation (11). This potentiating effect is manifested by the lack of interference of ω3 PUFA on the elongation of C18:3ω6 to C20:3ω6 which will favor the formation of PGE_1, thereby further modulating PGE_2 formation (11).

High LCPUFA and lipid peroxidation levels have been shown to be important mechanisms in the inhibition of cancer cell proliferation (15, 53). Impairment of the delta-6-desaturase enzyme could therefore play an important role in maintaining low LCPUFA levels, contributing to a low oxidative status in cancer tissue, with respect to hepatocyte nodules (10, 12). Changes in cholesterol, phospholipids and FA unsaturation level of PC have been shown to be important for the optimal functioning of the delta-6-desaturase enzyme (54, 55). In the present study the dysfunctional desaturase enzyme was not affected by the low ω6/ω3 ratio diets, indicated by the persistent elevated levels of C18:1ω9 and C18:2ω6 in the nodule PC and PE fractions and persistent lower level of C22:6ω3 in the nodule tissue. The increase in lipid peroxidation can therefore be attributed to the increase in the ω3 LCPUFA, specifically C22:6ω3 which is known to be a good substrate for lipid peroxidation (56). Although C22:6ω3 was increased by the low ω6/ω3 ratio diets, it remained lower in the nodules in comparison to the surrounding and control tissue. This is probably due to the

impaired delta-6-desaturase enzyme which also catalyzes the conversion of C22:5ω3 to C22:6ω3. Of interest is that the level of C18:1ω9, reported to be an effective anti-oxidant (16), was decreased in PE by the SOY and GLA diets which could contribute to the increased lipid peroxidation level in the nodules. C18:1ω9 also promotes cell proliferation and is a negative regulator of apoptosis (57).

The regulation of the oxidative status by GSH is of importance with regards to tumour growth (58). A higher GSH level in breast cancer tumours is associated with an increased level of cell proliferation (59). GSH is also elevated in a number of drug-resistant tumour cell lines and tumour cells isolated from patients resistant to drug therapy (60). In the present study, the GSH level was significantly increased in the nodules of the SFO and SOY-fed dietary rats, but was decreased in the nodules of the GLA and EPA dietary groups, which coincided with the increased TBARS formation. A study by Kokura et. al. (2002) showed a similar effect with respect to an increased lipid peroxidation and decreased GSH levels in tumour cells of rats dosed with C20:5ω3 (61). The decreased GSH, associated with the EPA and GLA diets, can be attributed to the high level of dietary C20:5ω3 which lies downstream of the delta-6-desaturase. In contrast, the SOY diet did not decrease the GSH level in the nodules, presumably as stated above, due to the source of dietary ω3 FA, i.e. C18:3ω3, which lies upstream of the delta-6-desaturase. As mentioned earlier the nodules of the SOY dietary group had lower ω3 FA levels in contrast to the GLA and EPA groups (Tables 3 and 4). The increased lipid peroxidation mainly targeted the hepatocyte nodules with a far less significant impact on the oxidative status in the surrounding and control tissue. In the case of the GLA diet, the increased oxidative status in the nodule tissue contributed to the reduced number of GSTP$^+$ foci and/or nodules in the foci category comprising the largest number of foci. This decrease can be ascribed to the so-called "GLA effect" which seems to stabilize the level of C20:4ω6, when considering the C20:4ω6 nodule/surrounding ratio. In addition to the potential modulating effect of C18:3ω6, via 20:3ω6, and C20:5ω3 on prostaglandin synthesis in tumour cells, these PUFA, including C20:4ω6, also elicit responses by directly influencing intracellular signaling pathways and transcription factor activity (6, 8, 12, 23). Other FA such as C18:1ω9 have also been shown to exhibit anti-apoptotic effects and stimulate cell proliferation in MDA-MB-231 breast cancer cells. This effect was found to be linked to C18:1ω9 stimulation of phosphatidylinositol 3-kinase (PI3-K) activity, a key signal transduction enzyme involved in the control of cell growth (57). Of interest is that both the SOY and

GLA diets significantly reduced the level of C18:1ω9 in the PE phospholipid fraction of the nodules.

The decrease in LCPUFA levels, due to impairment of the delta-6-desaturase in hepatocyte nodules, is likely to play an important role in the subsequent modulatory effect on nodule development. In this regard, the role of dietary FA in controlling cellular homeostasis seems to be important in balancing events related to apoptosis and cell proliferation in order to sustain normal growth. Certain dietary ω6 and ω3 FA, therefore, could create a multiple control mechanism for regulating cancerous growth depending on the dietary ω6/ω3 FA ratio as well as the type of FA constituting this ratio.

REFERENCES

1. Kolonel,L.N., Nomura,A.M.Y., and Cooney,R.V. (1999) Dietary fat and prostate cancer: Current status, *J. Nat. Cancer Institute 91*, 414-428.

2. Dyerberg,J., and Bang,H.O. (1979) Haemostatic function and platelet polyunsaturated fatty acids in Eskimos, *Lancet 2*, 433-435.

3. Gill,I., and Valivety,R. (1997) Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications, *TIBTECH 15*, 401-409.

4. Singh,J., Hamid,R., and Reddy,B.S. (1997) Dietary fat and colon cancer: modulation of cyclooxygenase-2 by types and amount of dietary fat during the postinitiation stage of colon carcinogenesis, *Cancer Res. 57* 3465-3470.

5. Badawi,A.F., El-Sohemy,A., Stephen,L.L., Ghoshal,A.K., and Arcger,M.C. (1998) The effect of dietary n-3 and n-6 polyunsaturated fatty acids on the expression of cyclooxygenase 1 and 2 and levels of $p21^{ras}$ in rat mammary glands, *Carcinogenesis 19*, 903-910.

6. Bartsch,H., Nair,J., and Owen,R.W. (1999) Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers, *Carcinogenesis 20*, 2209-2218.

7. Marks,F., Müller-Decker,K., and Fürstenberger,G. (2000) A causal relationship between unscheduled eicosanoid signaling and tumor development: cancer chemoprevention by inhibitors of arachidonic acid metabolism, *Toxicology 153*, 11-26.

8. Rose,D.P., and Connolly,J.M. (1999) Omega-3 fatty acids as cancer chemopreventive agents, *Pharm. Therap. 83*, 217-244.

9. Khan,W.A., Blobe,G.C., and Hannun,Y.A. (1995) Arachidonic acid and free fatty acids as second messengers and the role of protein kinase C, *Cell Signaling 7*, 171-184.

10. Abel,S., Smuts,C.M., de Villiers,C., and Gelderblom,W.C.A. (2001) Changes in essential fatty acid patterns associated with normal liver regeneration and the progression of hepatocyte nodules in rat hepatocarcinogenesis, *Carcinogenesis 22*, 795-804.

11. Horrobin,D.F. (1992) Nutritional and medical importance of gamma-linolenic acid, *Prog. Lipid Res. 31*, 163-194.

12. Tapiero,H., Ba,G.N., Couvreur,P., and Tew,K.D. (2002) Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies, *Biomed. Pharmacother. 56*, 215-222.

13. Fujiyama-Fujiwara,Y., Ohmori,C., and Igarashi,O. (1989) Metabolism of ⍵-linolenic acid in primary cultures of rat hepatocytes and in Hep G2 cells, *J. Nutr. Sci. Vitaminol. 35*, 597-611.

14.   Ishikawa,T., Fujiyama,Y., Igarashi,O., Morino,M., Tada,N., Kagami,A., Sakamoto,T., Nagano,M., and Nakamura,H. (1989) Effect of gammalinolenic acid on plasma lipoproteins and apolipoproteins, *Atherosclerosis 75*, 95-104.

15.   Horrobin,D.F. (1994) Unsaturated lipids and cancer, in *New approaches to cancer treatment: Unsaturated lipids and photodynamic therapy* (Horrobin,D.F., ed.), pp. 3-64, Churchill Communications, Nordic House, London, UK.

16.   Diplock,A.T., Balasubramanian,K.A., Manohar,M., Mathan,V.I., and Ashton,D. (1988) Purification and chemical characterization of the inhibitor of lipid peroxidation from intestinal mucosa, *Biochim. Biophys. Acta 962*, 42-50.

17.   Klein,C.B., Snow,E.T., and Frenkel,K. (1998) Molecular mechanisms in metal carcinogenesis: role of oxidative stress, in *Molecular Biology of Free Radicals in Human Diseases* (Aruoma,O.I., and Halliwell,B., eds.), pp. 79-138, OICA International, London, UK.

18.   Pastore,A., Federici,G., Bertini,E., and Piemonte,F. (2003) Analysis of glutathione: implication in redox and detoxification, *Clin. Chim. Acta 333*, 19-39.

19.   Wood,R., Upreti,G.C., and de Antueno,R.J. (1986) A comparison of lipids from liver and hepatoma subcellular membranes, *Lipids 21*, 292-300.

20.   Burns,C.P., and Spector,A.A. (1994) Biochemical effects of lipids on cancer therapy, *J. Nutr. Biochem. 5*, 114-123.

21.   Conklin,K.A. (2002) Dietary polyunsaturated fatty acids: impact on cancer chemotherapy and radiation, *Altern. Med. Rev. 7*, 4-21.

22.   Kokura,S., Nakagawa,S., Hara,T., Boku,Y., Naito,Y., Yoshida,N., and Yoshikawa,T. (2002) Enhancement of lipid peroxidation and of the antitumor effect of hyperthermia upon combination with oral eicosapentaenoic acid, *Cancer Lett. 185*, 139-144.

23.   Diggle,C.P. (2002) In vitro studies on the relationship between polyunsaturated fatty acids and cancer: tumour or tissue specific effects? *Prog. Lipid Res. 41*, 240-253.

24.   Baronzio,G.F., Solbiati,L., Ierace,T., Barzaghi,F., Suter,F., Airold,M., Belloni,G., Ravagnani,F., Notti,P., Gramalia,A., Galante,F., and Livraghi,T. (1995) Adjuvant therapy with essential fatty acids (EFA) for primary liver tumours: Some hypotheses, *Medical Hypotheses 44*, 149-154.

25.   American Institute of Nutrition (AIN). (1980) Second report of the ad hoc committee on standards for nutritional studies, *J. Nutr. 110*, 1726.

26.   Solt,D., and Farber,E. (1976) New principles for the analysis of chemical carcinogenesis, *Nature 263*, 701-703.

27.   Folch,J., Lees,M., and Stanley,G.H.S. (1957) A simple method for the isolation and purification of total lipids from animal tissues, *J. Biol. Chem. 226*, 497-509.

28.   Smuts,C.M., Weich,H.F.H., Weight,M.J., Faber,M., Kruger,M., Lombard,C.J., and Benadé,A.J.S. (1994) Free cholesterol concentrations in the high-density lipoprotein subfraction-3 as a risk indicator in patients with angiographically documented coronary artery disease, *Cor. Art. Dis. 5*, 331-338.

29.   Gilfillan,A.M., Chu,A.J., Smart,D.A., and Rooney,S.A. (1983) Single plate separation of lung phospholipids including disaturated phosphatidylcholine, *J. Lipid Res. 24*, 1651-1656.

30.   Itaya,K., and Ui,M. (1966) A new micromethod for the colorimetric determination of inorganic phosphate, *Clinica Chimica Acta 14*, 361-366.

31.   Richmond,W. (1973) Preparation and properties of a cholesterol oxidase from Nocardia sp. and its application to the enzymatic assay of total cholesterol in serum, *Clin. Chem. 19*, 1350-1356.

32.   Yen,G.C., and Hsieh,C.L. (1998) Antioxidant activity of extracts from Du-zhong (*Eucommia ulmoides*) toward various lipid peroxidation models in vitro, *J. Agric. Food Chem. 46*, 3952-3957.

33.   Hu,M.L, Frankel,E.N., Leibovitz,B.E., and Tappel,A.L. (1989) Effect of dietary lipids and vitamin E on in vitro lipid peroxidation in rat liver and kidney homogenates, *J. Nutr. 119*, 1574-1582.

34.   Esterbauer,H., and Cheeseman,K.H. (1990) Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal, *Methods Enzymol. 186*, 407-421.

35.   Tietze,F. (1969) Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues, *Anal. Biochem. 27*, 502-522.

36.   Ogawa,K. Solt,D., and Farber,E. (1980) Phenotypic diversity as an early property of putative preneoplastic cells in liver carcinogenesis, *Cancer Res. 40*, 725-733.

37.   Markwell,M.A.K., Haas,S.M., Bieber,L.L., and Tolbert,N.E. (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, *Anal. Biochem. 87*, 206-210.

38.   Kaushal,V., and Barnes, L.D. (1986) Effect of zwitterionic buffers on measurement of small masses of protein with bicinchoninic acid, *Anal. Biochem. 157*, 291-294.

39.   Mahler,S.M., Wilce,P.A., and Shanley,B.C. (1988) Studies on regenerating liver and hepatoma plasma membranes - II. Membrane fluidity and enzyme activity, *Int. J. Biochem. 20*, 613-619.

40.   Tabas,I. (2001) Consequences of cellular cholesterol accumulation: basic concepts and physiological implications, *J. Clin. Invest. 110*, 905-911.

41.   Darnell,J., Lodisch,H., and Baltimore,D. (1986) The plasma membrane, in *Molecular Cell Biology* (Darnell,J., Lodisch,H., and Baltimore,D., eds.), pp. 576-577, Scientific American Books, NY, USA.

42.   Blom,T.S., Koivusalo,M., Kuismanen,E., Kostiainen,R., Somerharju,P., and Ikonen,E. (2001) Mass spectrometric analysis reveals an increase in plasma membrane polyunsaturated phospholipid species upon cellular cholesterol loading, *Biochemistry 40*, 14635-14644.

43.   Stulnig,T.M., Huber,J., Leitinger,N., Imre,E-M., Angelisová,P., Nowotny,P., and Waldhäusl,W. (2001) Polyunsaturated eicosapentaenoic acid displaces proteins from membrane rafts by altering raft lipid composition, *J. Biol. Chem. 276*, 37335-37340.

44.   Zajchowski,L.D., and Robbins,S.M. (2002) Lipid rafts and little caves: Compartmentalized signaling in microdomains, *Eur. J. Biochem. 269*, 737-752.

45.   Kohlmeier,L. (1997) Biomarkers of fatty acid exposure and breast cancer risk, *Am. J. Clin. Nutr. 66*, 1548S-1556S.

46.   Sears,B. (2002) *The omega Rx zone*, 1st edn., pp. 149, 184, HarperCollins Publishers Inc., New York, USA.

47.   Jayadev,S., Linardic, C.M., and Hannun, Y.A. (1994) Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor-α, *J. Biol. Chem. 269*, 5757-5763.

48.   Surette,M.E., Fonteh,A.N., Bernatchez,C., and Chilton,F.H. (1999) Perturbations in the control of cellular arachidonic acid levels block cell growth and induce apoptosis in HL-60 cells, *Carcinogenesis 20*, 757-763.

49.   Tocher,D.R., Bell,J.G., Farndale,B.M., and Sargent,J.R. (1997) Effects of dietary γ-linolenic acid rich borage oil combined with marine fish oils on tissue phospholipid fatty acid composition and production of prostaglandins E and F of the 1-, 2- and 3-series in a marine fish deficient in $\Delta5$ fatty acyl desaturase, *Prost. Leukot. Essent. Fatty Acids 57*, 125-134.

50.   Barham,J.B., Edens,M.B., Fonteh,A.N., Johnson,M.M., Easter,L., and Chilton,F.H. (2000) Addition of eicosapentaenoic acid to γ-linolenic acid-supplemented diets prevents serum arachidonic acid accumulation in humans, *J. Nutr. 130*, 1925-1931.

51.   Calder,P.C. (2001) Polyunsaturated fatty acids, inflammation and immunity, *Lipids 36*, 1007-1024.

52.   Lands,W.E.M., Le Tillier,P.R., Rome,L.H., and Vanderhoek,J.Y. (1973) Inhibition of prostaglandin biosynthesis, *Adv. Biosci. 9*, 15-17.

53.   Bougnoux,P. (1999) N-3 Polyunsaturated fatty acids and cancer, *Curr. Opin. Clin. Nutr. Metab. Care 2*, 121-126.

54. Leikin,A., and Shinitzky,M. (1995) Characterization of the lipid surrounding the Δ-6-desaturase of rat liver microsomes, *Biochim. Biophys. Acta 1256*, 13-17.

55. Shimada,Y., Morita,T., and Sugiyama,K. (2003) Dietary eritadenine and ethanolamine depress fatty acid desaturase activities by increasing liver microsomal phosphatidylethanolamine in rats, *J. Nutr. 133*, 758-765.

56. Song,J.H., Fujimoto, K., and Miyazawa,T. (2000) Polyunsaturated (n-3) fatty acids susceptible to peroxidation are increased in plasma and tissue lipids of rats fed docosahexaenoic acid-containing oils, *J. Nutr. 130*, 3028-3033.

57. Hardy,S., Langelier,Y., and Prentki,M. (2000) Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects, *Cancer Res. 60*, 6353-6358.

58. Navarro,J., Obrador,E., Carretero,J., Petschen,I., Perez,P., and Estrela,J.M. (1999) Changes in glutathione status and the antioxidant system in blood and in cancer cells associate with tumour growth in vivo, *Free Rad. Biol. Med. 26*, 410-418.

59. Perquin,M., Oster,T., Maul,A., Froment,N., Untereiner,M., and Bagrel,D. (2001) The glutathione-related detoxification system is increased in human breast cancer in correlation with clinical and histopathological features, *J. Cancer Res. Clin. Oncol. 127*, 368–374.

60. Huang,Z.Z., Chen,C., Zeng,Z., Yang,H., Oh,J., Chen,L., and Lu,S.C. (2001) Mechanism and significance of increased glutathione level in human hepatocellular carcinoma and liver level in human hepatocellular carcinoma and liver regeneration, *FASEB 15*, 19-21.

61. Kokura,S., Nakagawa,S., Hara,T., Boku,Y., Naito,Y., Yoshida,N., and Yoshikawa,T. (2002) Enhancement of lipid peroxidation and of the antitumor effect of hyperthermia upon combination with oral eicosapentaenoic acid, *Cancer Lett. 185*, 139-144.

CHAPTER 5

ALTERED LIPID PROFILE AND OXIDATIVE STATUS IN HUMAN

HEPATOCELLULAR CARCINOMA

ALTERED LIPID PROFILE AND OXIDATIVE STATUS IN HUMAN HEPATOCELLULAR CARCINOMA.

S. Abel[a], M. De Kock[c], C.M.Smuts[d], D.J. van Schalkwyk[e], S. Swanevelder[f], M.C. Kew[b] and W.C.A. Gelderblom[a]

[a]PROMEC Unit, [b]Molecular Hepatology Research Unit, [d]Nutritional Intervention Research Unit, [f]Biostatistics Unit, Medical Research Council, P.O. Box 19070, Tygerberg, 7505, South Africa. [c]Department of Physiology, University of the Western Cape, South Africa. [e]The UK Renal Registry, Southmead Hospital, Bristol, United Kingdom.

ABSTRACT

Carcinogenesis is associated with an altered lipid metabolism affecting cellular membrane structure and function. Detailed lipid analyses of hepatocellular carcinoma and surrounding non-tumorous liver tissue obtained from cancer patients, revealed a decreased phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratio due to a decreased PC phospholipid concentration, while cholesterol was increased resulting in an increased cholesterol/phospholipid ratio. The decreased PC level was associated with a decrease ($P<0.05$) in the quantitative levels of the total saturated (SATS; C16:0, C18:0), $\omega6$ (C18:2, C20:4) and $\omega3$ (C22:5, C22:6) fatty acids (FA), the total polyunsaturated fatty acids (PUFA), long-chain (LC) PUFA and $\omega3$ LCPUFA levels. In PE, SATS and the $\omega3$ (C22:5, C22:6) FA decreased significantly ($P<0.05$) whereas the total $\omega6$ FA level was not affected. The decreased $\omega3$ FA level resulted in an increased $\omega6/\omega3$ FA ratio in PC and PE. Increased levels of the total monounsaturated FA (MUFA), especially the qualitative level of C18:1$\omega9$, the quantitative level of C20:2$\omega6$ and reduction in the level of 22:6$\omega3$ in PC and PE suggest a dysfunctional delta-6 desaturase enzyme. Of importance was the decreased level of C20:4$\omega6$ in PC as compared to PE, implying an increased level in the latter membrane phospholipid compartment relative to PC. A lower TBARS level was observed in the hepatitis B virus (HBV)$^-$ carcinoma tissue, while no significant difference was observed between the HBV$^+$ carcinoma and surrounding tissue. Reduced glutathione (GSH) was decreased in the carcinoma tissue, while HBV$^+$ significantly ($P<0.05$) enhanced the level in the surrounding tissue. Alterations in membrane cholesterol, phospholipids and FA parameters as well as the low level of lipid peroxidation, are likely to provide a selective growth environment for the cancerous cells.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most prevalent human cancers, ranking the fifth most common cancer worldwide (1). The geographical distribution of the disease varies greatly i.e. in parts of Asia and Africa the prevalence is more than 100 per 100 000 population and in Europe and North America it is estimated to be 2 to 4 per 100 000 population (2, 3). The main risk factors associated with HCC are hepatitis B (HBV) and C (HCV) viral infection which account for more than 80% of reported cases worldwide (4, 5). Other factors that play a role in HCC development, either alone or in conjunction with viral infection, include aflatoxin B_1 (AFB_1) exposure, cigarette smoking, heavy alcohol consumption, p53 gene mutations, repeated cycles of necrosis and regeneration and chronic inflammation (6, 7, 8). Experimental and clinical observations suggest that persistent proliferation of liver cells plays a key role in the progression of a chronic hepatitis into cancer (9). Surgical resection and liver transplantation are at present the only treatment options that offer potential for long-term survival or cure in limited-stage hepatocellular carcinoma (10). In general the prognosis is poor, and 1-year survival is rare.
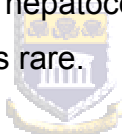
Carcinogenesis is the breakdown or dysfunction of normally closely regulated processes regarding cell differentiation, proliferation and apoptosis. The control of cell proliferation and apoptosis has been recognised as key events in cancer development depending on multiple extracellular and intracellular signals (11, 12, 13). The role of dietary constituents such as fatty acids (FA), known to regulate cell proliferation and apoptosis, has been recognised to be an important tool in cancer therapy (14, 15). Studies showed that the antitumour effects of ω3 FA such as C20:5ω3 and C22:6ω3 are mainly attributed to the suppression and induction of cell proliferation and apoptosis, respectively (16, 17). In contrast, C20:4ω6 from the ω6 FA family is known to stimulate cell proliferation via the formation of PGE_2 due to an over-expression of COX-2 (18, 19). However, C20:4ω6 is also an important signaling molecule for apoptosis via the generation of ceramide (20, 21). Increased ω3 PUFA dietary intake can modulate the ω6 FA content of cellular membranes with a resultant decrease in C20:4ω6 (22). In addition, C20:5ω3 may function as an alternate substrate for COX-2 yielding prostaglandin of the 3-series thereby counteracting the cell proliferative effects of the prostaglandins of the 2-series (18, 19, 22).

Cancer development is known to be associated with alterations in lipid metabolism affecting cellular function and growth. Changes in membrane lipid composition affect membrane function altering the responsiveness of cells to growth related processes and activity of membrane-bound enzymes and receptors (23, 24). Studies in rats indicated that the occurrence of potentially "neoplastic" hepatocyte lesions is associated with an increase in cholesterol and PE resulting in a decreased cholesterol/phospholipid and PC/PE ratios, a decrease in the polyunsaturated fatty acid (PUFA), especially the long-chain PUFA (LCPUFA) which was associated with a low oxidative status (25, 26). It would appear that the regulatory mechanisms related to lipid metabolism ensuring normal liver homeostasis, are disrupted in hepatocyte nodules and these changes play a role in the survival and growth of malignant cells (22). Cancer cells exhibit low levels of lipid peroxidation, partly due to unusually high levels of anti-oxidants such as vitamin E and the monounsaturated FA (MUFA), C18:1ω9 (27). However, the main reason for the lower lipid peroxidation levels appears to be due to low PUFA levels resulting from either an increased requirement during cell proliferation or an impaired functioning of the delta-6 desaturase enzyme, a rate-limiting enzyme in the PUFA biosynthetic pathway (28, 29). The impairment of this enzyme has been observed in BL6 melanoma and Morris hepatoma 9618A cell lines and in various types of liver cancer from different origins (28, 29, 30). LCPUFA may play a role in the control of cell proliferation by inhibiting cell growth and stimulating and/or enhancing apoptosis either directly (22) or via increased lipid peroxidation (31). Due to dependence on host circulation for the type and amount of PUFA available (32), opportunities exist to alter the FA composition of tumours by modulating dietary FA intake to alter the membranal composition thereby altering the growth characteristics of cancer cells.

The present study investigated the lipid composition and oxidative status of human hepatocellular carcinoma as compared to the matching surrounding tissue. Information regarding changes in the lipid profile could be important in the modulation of the altered growth pattern associated with liver cancer development.

MATERIALS AND METHODS

Ethical considerations

Ethical approval was granted by the Ethics Committee of the Medical Research Council of South Africa. Consent for necropsy and the removal of cancerous and normal liver issue was obtained from close relatives of the patient and consent for resection of the tumour from the patient prior to the surgical procedure.

Hepatocellular carcinoma and surrounding non-tumorous liver tissue

Hepatocellular carcinoma and surrounding non-tumorous liver tissue were obtained from 13 patients either at necropsy or at the time of surgical resection of the tumour at the Baragwanath and Rand Mutual Hospitals, Johannesburg, South Africa. The tissues obtained were immediately 'snap frozen' in liquid nitrogen and stored at -80ºC until analysed. Except for the 3 tumours that were resected, the HCC's were all at an advanced stage. Nine of the tumours were well or moderately well differentiated trabecular HCC's, whereas the remainder were poorly differentiated. The surrounding non-tumorous tissue was normal in the 3 patients in whom the tumour was resected and in 4 patients in whom a necropsy was performed. Cirrhosis was present in the remaining livers and was of a macronodular variety. Metastases (most often in the lungs and the regional lymph nodes) were present in 63% of the patients at necropsy. The biopsies were screened for HBV and HIV infection and five of the carcinoma and corresponding surrounding tissue were found to be HBV positive (HBV^{+}).

Lipid extraction

Lipids were extracted with chloroform/methanol (CM; 2:1; v/v) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant (33, 34). In short, approximately 100 to 150 mg of the liver tissue was ground to a fine powder in liquid nitrogen and weighed in glass-stoppered tubes. The tissue was suspended in 0.5 ml saline and the lipids were extracted with 24 ml CM. The CM mixture was filtered (sinterglass filters using Whatman glass microfibre filters, Cat No, 1820 866, Whatman International, Ltd, Maidstone, England) and evaporated to dryness *in vacuo* at 40°C. The extract was transferred to glass-stoppered tubes, washed with saline saturated with CMS (chloroform/methanol/saline; 86:14:1; v/v/v) containing 0.01% BHT, and stored at under nitrogen at 4°C until analysed.

Lipid analyses

The lipid extracts were fractionated by thin layer chromatography (TLC) and the major phospholipid fractions, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were collected for phospholipid and FA analyses (35). An aliquot of the lipid extract was used for cholesterol determination.

Phospholipid and cholesterol analyses*:* The phospholipid concentrations of PC and PE (ug/mg protein) were determined colourimetrically using malachite green after digestion with perchloric acid (16 N) at 170°C for approximately 1 hr (36). Total cholesterol content (ug/mg protein) was determined by an enzymatic iodide method (37) using cholesterin-oxidase and -esterase (Roche/Boehringer Mannheim kit, Preciset Cholesterol, cat. no.125512). The cholesterol/phospholipid molar ratio (Chol/PL) was calculated by using the combined PC and PE concentrations representing the main membrane phospholipids.

Fatty acid analyses*:* For FA analyses, the PC and PE phospholipid fractions were transmethylated with 2 ml methanol/18 M sulphuric acid (95:5; v/v) at 70°C for 2 hrs. The FA methyl esters (FAME) were extracted in hexane and analysed by gas chromatography on a Varian 3300 Gas Chromatograph equipped with 30m BPX-70 capillary columns with a 0.32 mm internal diameter (cat. no. 054616, SGE Inc., Austin, Texas, USA). The individual FAME were identified by comparison of the retention times to those of a standard mixture of free FA, C14:0 to C24:1, and quantified using an internal standard (C17:0) and expressed as µg FA/mg protein. The qualitative values were calculated as a percentage of the total FA content.

Oxidative Parameters

Lipid peroxidation*:* The thiobarbituric reactive substances (TBARS) level, representing the formation of malondialdehyde (MDA), was measured according to the method of Hu *et al*. (38). Briefly, liver homogenates were prepared (1:19 m/v) in a 1.15% KCl/0.01 M phosphate buffer (pH 7.4) on ice and a 0.5 ml aliquot (2 mg protein/ml) incubated with 2.5 mM ferrous sulphate for 1 hour at 37°C (39). Thereafter, 2 ml cold trichloro-acetic acid (10% TCA) containing 0.01% butylated hydroxytoluene (BHT) was added to the incubated homogenate and centrifuged (3000 rpm) for 10 min. Subsequently, 2 ml of the supernatant was mixed with 0.67% thiobarbituric acid (TBA, 2 ml) and incubated at 90°C for 20 minutes. The TBARS results were expressed as µmole MDA equivalents/mg protein using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} at 532

nm for MDA (40). Non-specific lipid peroxidation was prevented by the incorporation of EDTA in the buffers and BHT in the reaction solutions for the TBARS assay (40).

<u>Glutathione (GSH and GSSG) analyses</u>*:*   The glutathione status, including both reduced (GSH) and oxidized forms (GSSG), was determined according to the method of Tietze (41). Tissue samples were homogenized (1:10, w/v) in a 15% TCA and 1 mM EDTA solution for GSH and 6% perchloric acid (v/v), 3 mM 1-methyl-2-vinyl-pyridinium trifluoromethane sulfonate and 1 mM EDTA solution for GSSG, on ice.   The homogenates were centrifuged at 10 000 g for 10 minutes and 50 µl of the supernatant was added to glutathione reductase (5 Units) and 75 µM 5,5= dithiobis-(2-nitrobenzoic acid), in a microtiter plate. The reaction was initiated with the addition of 0.25 mM NADPH (50 µl) to a final reaction volume of 200 µl. The absorbance was monitored several times at 410 nm over 5 minutes and the sample levels were determined utilizing pure GSH and GSSG in standard curves, respectively. The results were expressed as mM GSH or GSSG/g liver.

Protein determination

Powdered liver preparations (10-15 mg) from the liquid nitrogen homogenisation step were first solubilised in 5% sodium dodecyl sulphate at 37°C and the protein content determined using a modified method of Lowry (42). The protein content in the liver homogenate prepared for the lipid peroxidation determination was determined as described by Kaushal and Barnes (43).

Statistical Analyses

The data were first analyzed by testing for each variable whether HBV group differences (HBV$^-$; HBV$^+$) were present, using the Two-sample T-test.   For those variables that did have significant group differences, separate Two-sample T-tests were performed testing for tissue group differences (carcinoma vs surrounding tissue) within each HBV group (HBV$^-$ and HBV$^+$, respectively). However, when no significant HBV$^-$ or HBV$^+$ group differences were present, the HBV group variables were combined and only tested for tissue group differences (carcinoma vs surrounding tissue), again using the Two-sample T-test. Depending on whether the variances were equal or unequal, the Pooled or Satterthwaite method was used for the Two-sample Test. A statistical significance of 5% was used (p<0.05).

RESULTS

Phospholipid and cholesterol parameters (Table I)

No significant difference in the phospholipid and cholesterol levels was observed between the HBV$^-$ and HBV$^+$ tissue samples. The PC concentration was significantly ($P<0.05$) decreased in the carcinoma compared to surrounding tissue, while no significant effect was observed in the PE concentration.   The decreased PC concentration resulted in a significant ($P<0.05$) decrease in the PC/PE ratio in the carcinoma tissue.   The cholesterol concentration was marginally ($0.05<P<0.1$) increased in the carcinoma compared to surrounding tissue.   The cholesterol/phospholipid molar ratio (Chol/PL ratio) was significantly ($P<0.05$) increased in the carcinoma tissue, due to the increase in cholesterol and decreased PC concentration.

Table I: Comparative phospholipid and cholesterol content and lipid parameters in hepatocellular carcinoma and surrounding non-tumour tissue from human patients.

Lipid Parameter		Carcinoma	Surrounding
Phospholipid	PC	62.32±12.57*	75.74±14.78$^{\#}$
(μg/mg protein)	PE	46.34±14.72*	49.37±10.74*
PC/PE Ratio		1.51±0.14*	1.90±0.23$^{\#}$
Cholesterol (µg/mg protein)		0.76±0.55$^{(*)}$	0.41±0.24$^{(\#)}$
Chol/PL Molar Ratio		0.016±0.008*	0.006±0.004$^{\#}$

Values are expressed as means ± SD, (carcinoma, n=13; surrounding, n=13). Significant differences between the carcinoma and surrounding tissue are indicated by differing superscript symbols (* and $^{\#}$) in a row. Statistical indicators in parenthesis indicate a marginal difference ($0.1>P>0.05$). Differences were considered significant if $P<0.05$. PC = phosphatidylcholine, PE = phosphatidylethanolamine.   The Cholesterol/Phospholipid molar ratio (Chol/PL Molar Ratio) was calculated using the sum of PC and PE which constitute the two major phospholipid fractions in the liver.

Fatty acid composition (Table II)

The individual FA were calculated as a percentage of total FA analysed (qualitative data) and quantified against an internal standard (C17:0) and expressed as μg FA/mg protein (quantitative data). No significant difference in the different FA parameters levels was observed between the HBV⁻ and HBV⁺ tissue samples.

Saturated FA (SATS):

The total saturated FA (SATS) level was significantly ($P<0.05$) decreased quantitatively in the phospholipid PC and PE fractions in the carcinoma tissue. This was due to decreases in the main saturated FA, C16:0 and C18:0, both of which decreased significantly ($P<0.05$) in PC. In PE only C16:0 decreased significantly ($P<0.05$), while there was a marked decrease (not-significant) in C18:0. Qualitatively, no significant effect was observed in the PC fraction. In PE, C16:0 was also decreased significantly ($P<0.05$) resulting in a significant ($P<0.05$) decrease in the total SATS level.

Monounsaturated FA (MUFA):

No significant quantitative change in the total monounsaturated fatty acid (MUFA) level was observed, although the individual FA levels tended to be higher in the carcinoma in PC and PE. Qualitatively, the total MUFA level was significantly ($P<0.05$) increased in the carcinoma in PC and PE. This resulted from a significant ($P<0.05$) increase mainly in C18:1, C20:1 and C24:1.

ω6 Polyunsaturated fatty acids (PUFA)

The total ω6 PUFA level was significantly ($P<0.05$) decreased quantitatively in the PC phospholipid fraction only, mainly due to significant ($P<0.05$) decreases in C18:2 and C20:4. In PE, C18:2 was marginally ($0.05<P<0.1$) decreased in the carcinoma. Only C20:2 was significantly increased ($P<0.05$) in PC and PE of the carcinoma tissue. Qualitatively, the ω6 PUFA level was significantly ($P<0.05$) decreased in PC and PE due to a decrease in C18:2 ($P<0.05$) in the carcinoma. However, C20:2 increased ($P<0.05$) in PC and PE and C20:3 ($P<0.05$) in PE only.

ω3 PUFA

The total ω3 PUFA level was significantly ($P<0.05$) decreased quantitatively in PC and PE in the carcinoma. This effect was due to significant ($P<0.05$) decreases in C22:5 and C22:6. A similar effect was noticed in the qualitative levels.

PUFA and LCPUFA

Quantitatively, the total PUFA and LCPUFA were significantly ($P<0.05$) decreased only in PC. No significant effect on the total ω6 LCPUFA was observed, although it tended to be lower in PC, while the total ω3 LCPUFA level was significantly ($P<0.05$)

decreased in PC and PE. Qualitatively, the total PUFA was significantly ($P<0.05$) decreased in PC of the carcinoma. The total ω6 LCPUFA was only significantly ($P<0.05$) increased in PE of the carcinoma tissue, while the total ω3 LCPUFA was significantly decreased in PC and PE.

Membrane FA Ratios

The ω6/ω3 FA ratio was significantly ($P<0.05$) increased in the carcinoma PC and PE tissue. The delta-6 desaturase substrate to product (delta-6 Sub/Prod) ratio was significantly ($P<0.05$) higher in the carcinoma PE and to some extent in PC. No significant changes in the P/S or C20:4/C20:5 ratios were observed.

Oxidative Parameters and Effect of HBV Status (Table III)

Within the HBV$^-$ group, both the TBARS and GSH levels were significantly ($P<0.05$) decreased in the carcinoma tissue compared to surrounding, whereas the GSSG level was marginally ($0.05<P<0.1$) increased. This resulted in a significantly ($P<0.05$) lower GSH/GSSG ratio in the carcinoma tissue. With regards to the HBV$^+$ group, no significant effect on the TBARS level was observed between the carcinoma and surrounding tissue. A significant decrease in the TBARS level was noticed in the surrounding tissue with HBV$^+$ infection. The GSH and GSSG levels were significantly ($P<0.05$) decreased and increased, respectively, in the carcinoma compared to the corresponding surrounding tissue. The GSH/GSSG ratio was significantly ($P<0.05$) lower in the carcinoma tissue, while it was markedly and significantly ($P<0.05$) increased in HBV$^+$ carcinoma and surrounding tissues, respectively.

Table II: Comparative fatty acid profiles (µg fatty acid/mg protein and percentage of total fatty acids analysed) of the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) phospholipid fractions in the hepatocellular carcinoma and surrounding non-tumorous liver tissue from human patients with hepatocellular carcinoma.

FA parameters	PC (µg fatty acid/mg protein)		PC (Percentage of total fatty acids)		PE (µg fatty acid/mg protein)		PE (Percentage of total fatty acids)	
	Carcinoma	Surrounding	Carcinoma	Surrounding	Carcinoma	Surrounding	Carcinoma	Surrounding
SATS								
C14:0	0.21±0.11	0.22±0.08	0.41±0.18	0.38±0.16	0.11±0.06a	0.16±0.07b	0.33±0.19	0.48±0.20
C16:0	11.54±5.46a	17.72±3.90b	32.51±3.89	31.78±3.69	3.13±2.03a	5.74±1.84b	13.47±4.36a	16.60±2.59b
C18:0	4.55±2.02a	7.91±1.90b	13.55±4.12	14.28±2.64	6.35±3.74	8.18±2.27	24.76±4.22	24.06±1.70
C20:0	0.09±0.04	0.13±0.04	0.24±0.06	0.23±0.08	0.15±0.05(a)	0.10±0.04(b)	0.54±0.22a	0.31±0.10b
C22:0	0.07±0.03	0.08±0.04	0.23±0.10(a)	0.15±0.05(b)	0.03±0.02	0.02±0.01	0.22±0.13a	0.07±0.02b
C24:0	0.12±0.06	0.11±0.06	0.35±0.23	0.20±0.09	0.03±0.02	0.02±0.01	0.06±0.04	0.07±0.04
Total	17.03±6.11a	26.06±5.00b	46.89±4.50	46.84±3.44	10.01±5.22a	14.12±3.85b		41.28±1.97b
MUFA								
C16:1	0.52±0.45	0.70±0.27	1.09±0.52	1.33±0.57	0.16±0.15	0.15±0.09	1.02±0.67a	0.45±0.26b
C18:1	10.50±2.34	9.06±1.67	23.75±3.50a	16.73±3.89b	4.20±2.41	3.28±0.73	16.99±6.02a	10.30±2.56b
C20:1	0.16±0.13	0.08±0.03	0.39±0.21a	0.15±0.07b	0.14±0.11	0.07±0.02	0.50±0.27a	0.24±0.11b
C22:1	ND	0.02±0.01		0.04±0.01	ND	0.01±0.01	ND	0.02±0.01
C24:1	0.11±0.06	0.08±0.05	0.37±0.17a	0.15±0.06b	0.04±0.03	0.02±0.01	0.31±0.23a	0.07±0.03b
Total	11.27±2.38	9.89±1.81	25.13±3.97a	18.28±4.34b	4.46±2.54	3.49±0.77	18.12±6.76a	10.91±2.76b
ω6								
C18:2	4.63±2.76a	9.30±3.76b	10.19±4.25a		2.07±1.33(a)	3.12±1.25(b)	6.20±1.08a	9.05±1.97b
C18:3	0.22±0.10	0.15±0.06	0.39±0.17	0.34±0.18	0.13±0.06	0.07±0.04	0.34±0.12	0.22±0.09
C20:2	0.98±0.96a	0.23±0.11b	2.10±1.52a	0.35±0.11b	1.16±1.07a	0.16±0.13b	1.89±1.21a	0.51±0.46b
C20:3	1.17±0.84	1.28±0.44		2.50±0.78	0.73±0.44	0.49±0.20	2.26±0.74a	1.46±0.49b
C20:4	3.04±0.93a	5.13±1.48b	7.74±2.92	9.20±2.00	5.37±2.36	6.31±1.43	21.72±4.44	19.37±2.14
C22:2	0.06±0.03	0.08±0.05	0.15±0.07	0.14±0.09	0.08±0.05	0.05±0.04	0.35±0.28	0.15±0.10
C22:4	0.20±0.24	0.18±0.06	0.52±0.39	0.34±0.13	0.38±0.15	0.39±0.12	2.07±1.71	1.19±0.41
C22:5	0.11±0.07	0.14±0.07	0.32±0.19	0.27±0.11	0.30±0.19	0.30±0.16	0.93±0.45	1.00±0.50
Total	10.09±5.23a	16.30±5.12b	22.42±6.31a	30.94±4.63b	10.46±4.94	10.74±2.58	34.31±6.94	32.68±3.51
ω3								
C18:3	0.03±0.02	0.06±0.03	0.06±0.04	0.09±0.03	0.01±0.01	0.03±0.03	0.05±0.01	0.07±0.02
C20:5	0.22±0.16	0.32±0.20	0.44±0.25	0.49±0.13	0.28±0.21	0.24±0.10	0.82±0.50	0.65±0.20
C22:5	0.12±0.07a	0.36±0.13b	0.29±0.11a	0.65±0.17b	0.28±0.19a	0.52±0.10b	1.16±0.76(a)	1.59±0.32(b)
C22:6	0.67±0.51a	1.97±0.92b	1.50±0.79a	3.47±1.33b	1.67±0.96a	4.62±1.94b	5.89±1.84a	12.11±3.66b
Total	1.00±0.70a	2.57±1.17b	2.12±1.03a	4.35±1.58b	2.29±1.14a	5.40±2.06b	7.78±2.30a	14.33±3.62b
Total PUFA	12.05±5.23a	18.77±5.93b	24.96±6.94a	35.46±5.22b	13.33±5.41	16.14±3.68	41.64±7.55	46.22±5.59
Total LCPUFA	6.82±3.67a	9.67±2.44b	14.44±5.62	16.81±3.52	11.51±4.57	13.13±2.89	36.60±6.33	37.04±5.39
Total ω6 LCPUFA	5.83±3.12	7.06±1.68	12.41±4.85	12.51±2.79	8.23±4.45	7.60±1.60	29.33±5.52a	23.52±2.03b
Total ω3 LCPUFA	0.99±0.69a	2.61±1.15b	2.11±1.03a	4.30±1.58b	2.28±1.14a	5.39±2.06b	7.77±2.29a	14.31±3.61b

FA Ratios	Carcinoma (PC)	Surrounding (PC)	Carcinoma (PE)	Surrounding (PE)
ω6/ω3	12.42±3.75a	6.52±1.64b	5.06±1.75a	2.26±0.86b
P/S	0.66±0.25	0.72±0.16	1.27±0.26	1.16±0.11
delta-6 Sub/Prod	5.44±1.81	4.46±1.21	1.54±0.65a	0.94±0.36b
C20:4/C20:5	16.91±7.98	18.13±7.21	23.50±6.09	28.79±9.48

Values are expressed as means ± SD from 13 carcinoma and 13 matching surrounding samples. Significant differences within the same phospholipid group are indicated by differing lowercase letters within a row ($P < 0.05$). Marginal differences ($0.05 < P < 0.1$) are indicated by differing lowercase letters in parentheses. Unshaded columns represent the quantitative data (µg fatty acid/mg protein) and the shaded columns represent the qualitative data (Percentage of total fatty acids). ND = not detected, C20:4/C20:5 = ratio of C20:4ω6 to C20:5ω3, ω6/ω3 = ω6 to ω3 fatty acid ratio, PUFA = polyunsaturated fatty acids, LCPUFA = long-chain polyunsaturated fatty acids, P/S = polyunsaturate:saturate ratio, delta-6 Sub/Prod = ratio of the substrates to products of the delta-6-desaturase enzyme.

Table III: Comparative oxidative parameters and the effect of hepatitis B virus (HBV) infection in hepatocellular carcinoma and surrounding non-tumour tissue.

Biochemical parameter	HBV Tissue Status	Carcinoma	Surrounding
TBARS	HBV$^-$	0.155±0.071*A	1.306±0.101#A
(µmole MDA eq./mg protein)	HBV$^+$	0.652±0.319*B	0.442±0.216*B
GSH	HBV$^-$	0.387±0.287*A	1.120±0.182#A
(mM/g liver)	HBV$^+$	0.498±0.291*A	1.408±0.251#B
GSSG	HBV$^-$	0.147±0.091$^{(*)}$A	0.078±0.012$^{(\#)}$A
(mM/g liver)	HBV$^+$	0.099±0.016*A	0.053±0.009#B
GSH/GSSG	HBV$^-$	3.961±2.037*A	17.152±2.647#A
	HBV$^+$	5.900±1.373*A	25.126±3.481#B

Values are expressed as means ± SD, (HBV$^-$, n=8 carcinoma and matching surrounding; HBV$^+$, n=5 carcinoma and matching surrounding). Significant difference between the carcinoma and surrounding tissue within each respective HBV group is indicated by differing superscript symbols (* and #) in a row. Significant differences between the same tissue type, but differing HBV status are indicated by uppercase letters, within a column. Statistical indicators in parentheses indicate a marginal difference (0.1>P>0.05). Differences were considered significant if P<0.05.

DISCUSSION

Alterations in the lipid profile in malignant tissue is of importance due to their effect in maintaining membrane integrity, fluidity and regulation of cellular processes related to the growth and survival of cancer cells (22, 25, 44, 45). The dietary modulation of these carcinoma-associated lipid changes is gaining rapid prominence in the prevention and treatment of cancer (46). In this regard, the effects of LCPUFA, especially the ω3 PUFA known to reduce neoplastic growth in various experimental studies, are of importance. The main mechanisms whereby these FA affect cancer growth are (i) alteration of the cell membrane structure and fluidity, (ii) inhibition of C20:4ω6-derived eicosanoid synthesis affecting the immune response, inflammation, cell proliferation, apoptosis, metastasis and angiogenesis, (iii) modulating lipid peroxidation status and (iv) altering signal transduction pathways, influencing transcription factor activity and gene expression which may lead to changes in cell growth and differentiation.

Regarding membrane structure, the present study indicated a decrease in the PC phospholipid fraction, resulting in a decreased PC/PE ratio. The decreased ratio indicates a shift in the phospholipid type and content of the membrane resulting in a higher membranal PE content. A recent study (26) reported a similar decrease in the PC/PE ratio in rat hepatocyte nodules and normal regenerating hepatocytes due to an increase in PE. The alteration of the PC/PE ratio could be an important indicator of cell proliferation and therefore, survival of a cancer cell. Studies indicated that the phospholipid distribution in the cell membrane is not symmetrical, with PC mostly situated in the outer bilayer and PE in the inner cytoplasmic leaflet (47). Therefore, changes in the level of membrane phospholipids may affect membrane functionality via alterations in membrane phospholipid distribution (48). Disruption of the asymmetric phospholipid membrane distribution is also associated with cell survival signals. Translocation of the phospholipids phosphatidylserine (PS) and PE from the inner to the outer membrane leaflet of red blood cells signals for apoptosis and the removal of injured or damaged cells (49, 50). Therefore, disruption of the membrane lipid distribution, especially with regards to phospholipids, can change the responsiveness of the cell to normal physiological control mechanisms. The present study indicated an increase in the cholesterol level in the carcinoma tissue, possibly due to a membrane compensatory mechanism (48) for maintaining the integrity and membrane functionality in response to the decreased PC and the LCPUFA levels. A similar increase in cholesterol was noticed in hepatocyte nodules after 9 months, implying that it was a late event during cancer development in rat liver (26). In the cell membrane, cholesterol is usually situated in the curve of the PUFA with the 3-hydroxyl group next to the phospholipid phosphate group (51) and plays an important role in maintaining the structural integrity of membranes by arranging the configuration of the FA acyl chains (52). The increase in cholesterol and Chol/PL ratio in carcinoma tissue could also result in a more rigid or less fluid membrane structure (53). Similar changes were observed in preneoplastic hepatocyte nodules, whereby an increase in these parameters corresponded to a decrease in membrane fluidity (26).

FA metabolism in tumour cells is altered due to the impaired functioning or decrease in the activity of the delta-6 desaturase enzyme (29, 45). Alterations in the concentration of membrane PC and cholesterol as well as the FA unsaturation index were shown to regulate the function of the delta-6 desaturase enzyme (54). The present study

revealed changes in the total PUFA level, PC and cholesterol concentrations altering the Chol/PL and PC/PE ratios in the carcinoma tissue. Impairment of the activity of the enzyme was demonstrated by an increased substrate/product ratio, indicative of an increase in the enzyme substrate levels. This was largely due to an increase in qualitative levels of C18:1ω9, which forms the endogenous FA synthesis pathway and is usually increased when the ω6 and ω3 FA levels are low or decreased (28, 55). It is not known at present whether the significantly increased level of C20:2ω6 in HCC is related to an impaired delta-6 desaturase. A study by Kew *et al.* (56) conducted in mice also suggested that inhibition of the delta-6 desaturase resulted in the elongation of C18:2ω6 to C20:2ω6. The accumulation of C20:2ω6, therefore, further corroborates a disrupted FA metabolic pathway in cancerous tissue. The impairment of the desaturase enzyme and resultant decrease in LCPUFA, such as C22:6ω3, may also be a major factor for the characteristically low lipid peroxidation level observed in carcinoma tissue (26, 27, 29, 48, 57, 58, 59). Lipid peroxidation is also reduced in regenerating liver following partial hepatectomy which is in agreement with the concept that increased cell proliferation is associated with decreased lipid peroxidation (60, 61). This would suggest that the decrease in lipid peroxidation is an important event in sustaining hepatocyte proliferation, both under normal and abnormal conditions such as cancer. In the present study, a lower TBARS level was observed in the HBV$^-$ carcinoma tissue compared to the corresponding surrounding tissue. In contrast, no significant difference was observed between the HBV$^+$ infected tissues, although the TBARS level in both HBV$^+$ tissues was lower compared to the HBV$^-$ surrounding tissue. The latter could be related to an increase in GSH synthesis in response to HBV infection. It would appear that HBV infection negates the characteristic low TBARS level in carcinoma tissue compared to the surrounding. In addition to the low levels of PUFA, the low lipid peroxidation can also be attributed to increased levels of anti-oxidants, such as vitamin E, and C18:1ω9 which may have anti-oxidant properties, in the carcinoma tissue (27, 29, 62). As discussed above, the total MUFA, specifically C18:1ω9, was significantly increased qualitatively in PC and PE of the carcinoma tissue.

GSH (reduced glutathione form) is an important cellular constituent that protects against oxidative damage and is suggested to play a role in the survival of cancer cells (63). In this regard, increased levels of GSH have been observed in rat preneoplastic nodules as well as in some human cancers (26, 63). However, decreased GSH levels

have been observed in foetal rat liver and in the Novikoff hepatoma (64, 65), while viral hepatitis also depletes GSH levels (63, 66). Decreased GSH levels may be due to increased utilization in the carcinoma tissue, possibly to maintain a low peroxidation level characteristic of carcinoma tissue (48). GSH synthesis is dependent on ATP (67) and due to faster GSH metabolism in cancer cells (64), energy depletion leading to mitochondrial deterioration could occur. A decrease in the number of mitochondria as well as ATP concentration has been demonstrated in neoplastic cells (64, 68). Therefore, a decreased GSH level may be due to mitochondrial and ATP depletion. Furthermore, alterations in the GSH level have been observed at different stages of tumour growth, whereby a decrease in GSH correlates with a decline in tumour growth rate (56, 69). In addition, liver cancer cells such as the HepG2 and HuH7 cells have a block in the methionine pathway in the synthesis of GSH (70). Patients with liver cirrhosis have an impaired methionine clearance resulting in hypermethioninemia which is postulated to be a factor in decreased GSH levels (71). The present study indicated a lower GSH and higher GSSG level in the carcinoma tissue compared to the respective surrounding tissue, presumably due to the reasons discussed above. When considering the GSH/GSSG ratio, a significant ($P<0.05$) decrease was noticed in the carcinoma tissue due to the decreased level of GSH and increase in GSSG. The increased GSH/GSSG ratio in the HBV$^+$ tissue was more evident in the surrounding tissue due to an increase in GSH and decrease in GSSG implying an enhanced redox cycling of GSSG which could explain the decreased level of lipid peroxidation. This implies that the carcinoma tissue is more resistant towards changes to the GSH/GSSG redox cycle, presumably due to impairment of GSH metabolism, as discussed above.

Changes in the phospholipid content have important implications not only on the structural integrity of the membrane, but also on the content and type of FA. The present study showed a decreased PC phospholipid in the carcinoma tissue associated with a decrease in the total SATS (C16:0, C18:0), ω6 (C18:2, C20:4) and ω3 (C22:5, C22:6) FA, total PUFA, total LCPUFA and ω3 LCPUFA levels. In PE, only a decrease in the SATS and ω3 FA was observed. These changes resulted in an increased ω6/ω3 FA ratio in PC and PE, mainly due to the lower ω3 FA levels in the carcinoma tissue. A similar decrease in ω3 FA was found in the gastric mucosa of patients with stomach cancer as well as in rat hepatocyte nodules resulting in an increased ω6/ω3 FA ratio (26, 72). Qualitatively, the total MUFA level increased in both PC and PE carcinoma tissue possibly due to an impairment of the delta-6 desaturase or increase in the

endogenous synthesis of the ω9 FA, resulting in accumulation of C18:1ω9 in response to the decrease in the ω6 and ω3 PUFA (28, 55). The increased qualitative levels of C18:1ω9 and decrease in SATS, known to be negative and positive regulators of apoptosis, respectively, is of interest with respect to increased cell survival (73, 74).

In rat hepatocyte nodules an increased PE level resulted in a significant increase in the concentration of C20:4ω6 (26). As PE occurs mostly on the inside of the cell membrane, it has been suggested that the enhanced level of C20:4ω6 in PE and availability is important in the growth of hepatocyte nodules (22). Inhibition of the remodeling or movement of C20:4ω6 from the outer cell membrane leaflet phospholipids, such as PC, to inner membrane phospholipids, such as PE, has been implicated in decreasing cell proliferation and increasing apoptosis (75, 76). These studies illustrate important aspects regarding the association of C20:4ω6 with certain membrane phospholipid fractions and cell survival. The decrease in ω3 and increased ω6 FA in PE, especially C20:4ω6, appear to be important determinants in the survival and growth of malignant tissue (26, 46, 77, 78). In the present study, the decreased PC concentration was associated with a quantitative decrease in C20:4ω6, while no significant change was observed in PE. The level of C20:4ω6 is normally higher in PE and replacement by ω3 FA was shown to be more prominent in PE than in PC (79). In the carcinoma tissue, the decreased PC level and stabilisation of C20:4ω6 in PE further shifted the concentration gradient of this FA towards PE. This shift in C20:ω4 concentration towards PE increases the availability of the FA affecting various systems such as prostaglandin metabolism (26). Increased levels of PGE$_2$ have been reported in many tumours and the regulation of COX-2 by non-steroidal antiinflammatory drugs (NSAIDs) has been utilised in chemopreventative trials in colon cancer patients (80, 81, 82). In rat intestinal cultured cells, increased COX-2 expression together with an increase in PGE$_2$ was associated with a decrease in apoptosis (70). In human HCC, COX-2 expression has also been found to be up-regulated, depending on the histological differentiation of the tumour (83).

Modulation of the C20:4ω6 level in the cell membrane can be achieved by the ω3 FA, C20:5ω3 and C22:6ω3, via competitive incorporation into the membrane as well as by feedback inhibition on the activity of delta-5 and delta-6 desaturase (45, 46). Prostaglandin synthesis from C20:4ω6 can also be modulated by dietary intake of C18:3ω6 which is metabolised into C20:3ω6, a precursor for PGE$_1$ (27), while

C20:5ω3, a precursor for PGE$_3$, competes for COX-2 in prostaglandin synthesis (46). Modulation of the ω6 FA, especially the C20:4ω6 status in heptatocyte nodules utilising a specific low ω6 to ω3 FA ratio diet in combination with C18:3ω6, resulted in reduced nodule growth associated with an increased oxidative status (77). This implies a shift in the type of prostaglandin synthesis, effected by C20:5ω3 and C18:3ω6, and was associated with a reduction in the development of preneoplastic lesions in the liver. C18:3ω6, in the form of evening primrose oil, was observed to retard tumour growth in patients with HCC and improve their survival time (84, 85).

The present study showed that changes in the lipid profiles and oxidative parameters of human hepatocellular carcinoma closely mimics that observed in rats. Modulation of these parameters by specific dietary FA retarded the growth of hepatocyte nodules in rats provides evidence that similar intervention initiatives could be conducted in humans.

REFERENCES

1.   Wang, X.W., Hussain, S.P., Huo, T.-I., Wu, C.-G., Forgues, M., Hofseth, L.J., Brechot, C. and Harris, C.C. 2002. Molecular pathogenesis of human hepatocellular carcinoma. *Toxicology* **181-182:** 43-47.

2.   Bain, I. and McMaster, P. 1997. Benign and malignant liver tumours. *Surgery* **15:** 169-174.

3.   Duvoux, C. 1998. Epidemiology and diagnosis of HCC in cirrhosis. *Ann. Chir.* **52:** 511-517.

4.   Kew, M.C. 2002. Epidemiology of hepatocellular carcinoma. *Toxicol.* **181-182:** 35-38.

5.   Wallner, I., Hartmann, H. and Ramdori, G. 1994. Current therapeutic strategies in HCC, Part 1. *Leber Magen Darm.* **24:** 150154.

6.   Chen, C.J., Yu, M.W. and Liaw, Y.F. 1997. Epidemiological characteristics and risk factors of hepatocellular carcinoma. *J. Gastroenterol. Hepatol.* **12:** S294-S308.

7.   Sherman, M. 1995. Hepatocellular carcinoma. *Hepatogastroenterology* **3:** 55-66.

8.   Cotran, R.S., Kumar, V. and Robbins, S.L. 1994. Robbins Pathologic basis of disease. 5th ed. Saunders Publications, Philadelphia, USA, 879-882.

9.   Romeo, R. and Colombo, M. 2002. The natural history of hepatocellular carcinoma. *Toxicol.* **181-182:** 39-42.

10.   Badvie, S. 2000. Hepatocellular carcinoma. *Postgrad. Med. J.* **76:** 4-11.

11.   Chang, W.C.L., Chapkin, R.S. and Lupton, J.R. 1997. Predictive value of proliferation, differentiation and apoptosis as intermediate markers for colon tumorigenesis. *Carcinogenesis* **18:** 721-730.

12.   Evan, G., Harrington, E., Fanidi, A., Land, H., Amati, B. and Bennett, M.. 1994. Integrated control of cell proliferation and cell death by the c-myc oncogenes. *Phil. Trans. R. Soc. Lond.* **345:** 269-275.

13.   Evan, G.I. and Vousden, K.H.. 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature* **411:** 342-348.

14   McDonnel, S.J., Meyn, R.E. and Robertson, L.E. 1995. Implications of apoptotic cell death regulation in cancer therapy. *Cancer Biol.* **6:** 53-60.

15   Thompson, C.B. 1995. Apoptosis in the pathogenesis and treatment of disease. *Science* **267:** 1456-62.

16   Calviello, G., Palozza, P., Piccioni, E., Maggiano, N., Frattucci, A., Franceschelli, P. and Bartoli, G.M. 1998. Dietary supplementation with eicosapentaenoic and docosahexaenoic acid inhibits growth of Morris hepatocarcinoma 3924A in rats: effects on proliferation and apoptosis. *Int. J. Cancer* **75:** 699-705.

17   Lai, P.B., Ross, J.A., Fearon, K.C., Anderson, J.D. and Carter, D.C. 1996. Cell cycle arrest and induction of apoptosis in pancreatic cancer cells exposed to eicosapentaenoic acid in vitro. *Br. J. Cancer* **74:** 1375-1383.

18.   Petrik, M., McEntee, M., Chiu, C. and Whelan, J. 2000. Antagonism of arachidonic acid is linked to the antitumorigenic effect of dietary eicosapentaenoic acid in Apc(Min/+) mice. *J. Nutr.* **130:** 1153-1158.

19.   Whelan, J. 1996. Antagonistic effects of dietary arachidonic acid and n-3 polyunsaturated fatty acids. *J. Nutr.* **126 (Suppl. 4):** 1086S-1091S.

20.   Jayadev, S., Linardic, C.M. and Hannun, Y.A. 1994. Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor-α. *J. Biol. Chem.* **269:** 5757-5763.

21.   Surette, M.E., Fonteh, A.N., Bernatchez, C. and Chilton, F.H. 1999. Perturbations in the control of cellular arachidonic acid levels block cell growth and induce apoptosis in HL-60 cells. *Carcinogenesis* **20:** 757-763.

22.   Bartsch, H., Nair, J. and Owen, R.W. 1999. Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. *Carcinogenesis* **20:** 2209-2218.

23.   Das, U.N., Madhavi, N., Sravan Kumar, G., Padma, M. and Sangeetha, P. 1998. Can tumour cell drug resistance be reversed by essential fatty acids and their metabolites? *Prostaglandins Leukot. Essent. Fatty Acids* **58:** 39-54.

24.   Bougnoux, P. 1999. n-3 Polyunsaturated fatty acids and cancer. *Curr. Opin. Clin. Metab. Care* **2:** 121-126.

25.   Mahler, S.M., Wilce, P.A. and Shanley, B.C. 1988. Studies on regenerating liver and hepatoma plasma membranes - II. Membrane fluidity and enzyme activity. *Int. J. Biochem.* **20:** 613-619.

26.   Abel, S., Smuts, C.M., de Villiers, C. and Gelderblom, W.C.A. 2001. Changes in essential fatty acid patterns associated with normal liver regeneration and the progression of hepatocyte nodules in rat hepatocarcinogenesis. *Carcinogenesis* **22:** 795-804.

27.   Horrobin, D.F. 1994. Unsaturated lipids and cancer. In *New approaches to cancer treatment: Unsaturated lipids and photodynamic therapy*. Horrobin, D.F., ed. Churchill Communications Europe, London, UK, 3-29.

28.   Horrobin, D.F. 1992. Nutritional and medical importance of gamma-linolenic acid. *Prog. Lipid Res.* **31:** 163-194.

29. Hrelia, S., Bordoni, A., Biagi, P.L., Galeotti, T., Palombini, G. and Masotti, L. 1994. The role of delta-6- and delta-9-desaturase in the fatty acid metabolism of hepatomas with different growth rate. *Biochem. Mol. Biol. Int.* **34:** 449-455.

30. Gardiner, N.S. and Duncan, J.R. 1991. Possible involvement of delta-6-desaturase in control of melanoma growth by gamma-linolenic acid. *Prostaglandins, Leukot. Essent. Fatty Acids* **42:** 149-153.

31. Ji, C., Rouzer, C.A., Marnett, L.J. and Pietenpol, J.A. 1998. Induction of cell cycle arrest by the endogenous product of lipid peroxidation, malondialdehyde. *Carcinogenesis* **19:** 1275-1283.

32. Burns, C.P. and Spector, A.A. 1994. Biochemical effects of lipids on cancer therapy. *J. Nutr. Biochem.* **5:** 114-123.

33. Folch, J., Lees, M. and Stanley, G.H.S. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497-509.

34. Smuts, C.M., Weich, H.F.H., Weight, M.J., Faber, M., Kruger, M., Lombard, C.J. and Benadé, A.J.S. 1994. Free cholesterol concentrations in the high-density lipoprotein subfraction-3 as a risk indicator in patients with angiographically documented coronary artery disease. *Cor. Art. Dis.* **5:** 331-338.

35. Gilfillan, A.M., Chu, A.J., Smart, D.A. and Rooney, S.A. 1983. Single plate separation of lung phospholipids including disaturated phosphatidylcholine. *J. Lipid Res.* **24:** 1651-1656.

36. Itaya, K. and Ui, M. 1966. A new micromethod for the colorimetric determination of inorganic phosphate. *Clinica Chimica Acta* **14:** 361-366.

37. Richmond, W. 1973. Preparation and properties of a cholesterol oxidase from Nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. *Clin. Chem.* **19:** 1350-1356.

38. Hu, M.L, Frankel, E.N., Leibovitz, B.E. and Tappel, A.L. 1989. Effect of dietary lipids and vitamin E on in vitro lipid peroxidation in rat liver and kidney homogenates. *J. Nutr.* **119:** 1574-1582.

39. Yen, G.C. and Hsieh, C.L. 1998. Antioxidant activity of extracts from Du-zhong (*Eucommia ulmoides*) toward various lipid peroxidation models in vitro. *J. Agric. Food Chem.* **46:** 3952-3957.

40. Esterbauer, H. and Cheeseman, K.H. 1990. Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. *Methods Enzymol.* **186:** 407-421.

41. Tietze, F. 1969. Enzymatic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* **27:** 502-522.

42.   Markwell, M.A.K., Haas, S.M., Bieber, L.L. and Tolbert, N.E. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **87:** 206-210.

43.   Kaushal, V. and Barnes, L.D. 1986. Effect of zwitterionic buffers on measurement of small masses of protein with bicinchoninic acid. *Anal. Biochem.* **157:** 291-294.

44.   Larsson, S.C., Kumlin, M., Ingelman-Sundberg, M. and Wolk, A. 2004. Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. *Am. J. Clin. Nutr.* **79:** 935-945.

45.   Tapiero, H., Nguyen Ba, G., Couvreur, P. and Tew, K.D. 2002. Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. *Biomed. Pharmacother.* **56:** 215-222.

46.   Rose, D.P. and Connolly, J.M. 1999. Omega-3 fatty acids as cancer chemopreventive agents. *Pharmacol. Ther.* **83:** 217-244.

47   Daleke, D.L. 2003. Regulation of transbilayer plasma membrane phospholipid asymmetry. *J. Lipid Res.* **44:** 233-242

48.   Kolanjiappan, K., Ramachandran, C.R. and Manohara, S. 2003. Biochemical changes in tumor tissues of oral cancer patients. *Clin. Biochem.* **36:** 61-65.

49.   Pomorski, T., Hrafnsdóttir, S., Devaux, P.F. and van Meer, G. 2001. Lipid distribution and transport across cellular membranes. *Sem. Cell Dev. Biol.* **12:** 139-148.

50.   Rudolph, I.L., Kelley, D.S., Klasing, K.C. and Erickson, K.L. 2001. Regulation of cellular differentiation and apoptosis by fatty acids and their metabolites. *Nutr. Res.* **21:** 381-393.

51.   Sinclair, H.M. 1984. Essential fatty acids in perspective. *Hum. Nutr.: Clin. Nutr.* **38C:** 245-260.

52.   Darnell, J., Lodisch, H. and Baltimore, D. 1986. *Molecular Cell Biology*. Scientific American, New York, USA. 576-577.

53.   Mahler, S.M., Wilce, P.A. and Shanley, B.C. 1988 Studies on regenerating liver and hepatoma plasma membranes - II. Membrane fluidity and enzyme activity. *Int. J. Biochem.* **20:** 613-619.

54.   Leikin, A. and Shinitsky, M. 1995. Characterization of the lipid surrounding the delta-6-desaturase of rat liver microsomes. *Biochim. Biophys. Acta* **1256:** 13-17.

55.   Holman, RT. 1960. The ratio of trienoic:tetraenoic acids in tissue lipids as a measure of essential fatty acid requirement. *J Nutr*. **60:** 405-410.

56. Kew, S., Gibbons, E.S., Thies, F., McNeill, G.P., Quinlan, P.T. and Calder, P.C. 2003. The effect of feeding structured triacylglycerols enriched in eicosapentaenoic or docosahexaenoic acids on murine splenocyte fatty acid composition and leucocyte phagocytosis. *Br. J. Nutr.* **90:** 1071-1080.

57. Saintot, M., Cecile, A., Pujol, H. and Gerber, M.. 1996. Tumor progression and oxidant-antioxidant status. *Carcinogenesis* **17:** 1267-1271.

58. Kokura, S., Nakagawa, S., Hara, T., Boku, Y., Naito, Y., Yoshida, N., and Yoshikawa, T. 2002. Enhancement of lipid peroxidation and of the antitumor effect of hyperthermia upon combination with oral eicosapentaenoic acid. *Cancer Lett.* **185:** 139-144.

59. Cheeseman, K.H., Emery, S., Maddix, S.P., Slater, T.F., Burton, G.W. and Ingold, K.U. 1988. Studies on lipid peroxidation in normal and tumor tissues. *Biochem. J.* **250:** 247-252.

60. Cheeseman, K.H., Collins, M., Proudfoot, K., Slater, T.F., Burton, G.W., Webb, A.C. and Ingold, K.U. 1986. Studies on lipid peroxidation in normal and tumour tissues. *Biochem. J.* **235:** 507-514.

61. Diplock, A.T., Rice-Evans, C.A. and Burdon, R.H. 1994. Is there a significant role for lipid peroxidation in the causation of malignancy and for antioxidants in cancer prevention? *Cancer Research* **54:** 1952s-1956s.

62. Diplock, A.T., Balasubramanian, K.A., Manohar, M., Mathan, V.I. and Ashton, D. 1988. Purification and chemical characterization of the inhibitor of lipid peroxidation from intestinal mucosa. *Biochim. Biophys. Acta* **962:** 42-50.

63. Pastore, A., Federici, G., Bertini, E. and Piemonte, F. 2003. Analysis of glutathione: implication in redox and detoxificatiom. *Clinica Chimica Acta* **333:** 19-39.

64. Marinho, H.S., Baptista, M. and Pinto, R.E. 1997. Glutathione metabolism in hepatomous liver of rats treated with diethylnitrosamine. *Biochim. Biophys. Acta* **1360:** 157-168.

65. Wirth, P.J. and Thorgeirsson, S.S. 1978. Glutathione synthesis and degradation in fetal and adult rat liver and Novikoff hepatoma. *Cancer Res.* **38:** 2861-2865.

66. Altomare, E., Vendemiale, G. and Alano, O. 1988. Hepatic glutathione content in patients with alcoholic and non alcoholic liver diseases. *Life Sci.* **43:** 991-998.

67. Dalton, T.P., Chen, Y.C., Schneider, S.N., Nebert, D.W. and Shertzer, H.G. 2004. Genetically altered mice to evaluate glutathione homeostasis in health and disease. *Free Rad. Biol. Med.* **37:** 1511-1526.

68. Estrela, J.M., Hernandez, R., Terradez, P., Asensi, M., Puertes, I.R. and Viňa, J. 1992. Regulation of glutathione metabolism in Ehrlich ascites tumour cells. *Biochem. J.* **286:** 257-262.

69.   Navarro, J., Obrador, E., Carretero, J., Petschen, I., Aviňo, J., Perez, P. and Estrela, J.M. 1999. Changes in glutathione status and the antioxidant system in blood and in cancer cells associate with tumour growth in vivo. *Free Rad. Biol. Med.* **26:** 410-418.

70.   Lu, S.C. and Huang, H.Y. 1994. Comparison of sulfur amino acid utilization for GSH synthesis between HepG2 cells and cultured rat hepatocytes. *Biochem. Pharmacol.* **47:** 859-869.

71.   Mato, J.M., Alvarez, L., Corrales, F.J. and Pajares, M.A. 1994. S-adenosylmethionne and the liver. In *The Liver: Biology and Pathobiology.* Arias, I.M., Boyer, J.L., Fausto, N., Jakoby, W.B., Schachter, D.A. and Shafritz, D.A., eds. Third Edition. Raven Press, New York, USA. pp 461-470.

72.   Ahn, J., Park, I.S., Lee, K.S., Kim, S.Y., Chung, E.J., Kim, J., Kim, D.J., Yoon, S. and Lee-Kim, Y.C. 2001. Fatty acid patterns in gastric mucosa of stomach cancer patients. *Yonsei. Med. J.* **42:** 220-226.

73.   Hardy, S., Langelier, Y. and Prentki, M. 2000. Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. *Cancer Res.* **60:** 6353-6358.

74.   Iturralde, M., Gamen, S., Pardo, J., Bosque, A., Piňeiro, A., Alava, M.A., Naval, J. and Anel, A. 2003. Saturated free fatty acid release and intracellular ceramide generation during apoptosis induction are closely related processes. *Biochim. Biophys. Acta* **1634:** 40-51.

75.   Chan, T.A., Morin, P.J., Vogelstein, B. and Kinzler, K.W. 1998. Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *PNAS* **95:** 681-686.

76.   Surette, M.E., Fonteh, A.N., Bernatchez, C. and Chilton, F.H. 1999. Perturbations in the control of cellular arachidonic acid levels block cell growth and induce apoptosis in HL-60 cells. *Carcinogenesis* **20:** 757-763.

77.   Abel, S., de Kock, M., Smuts, C.M., de Villiers, C., Swanevelder, S., and Gelderblom, W.C.A. 2004. Dietary modulation of fatty acid profiles and oxidative status of rat hepatocyte nodules: effect of different n-6/n-3 fatty acid ratios, *Lipids* **39:** 963-976.

78.   McCarthy, M.F. 1996. Fish oil may impede angiogenesis and invasiveness by down-regulating protien kinase C and modulating eicosanoid production. *Med. Hypotheses* **46:** 107-115.

79.   Abel, S., Gelderblom, W.C.A., Smuts, C.M. and Kruger, M. 1997. Thresholds and kinetics of fatty acid replacement in different cellular compartments in rat liver as a function of dietary n-6/n.3 fatty acid content. *Prost. Leukot. Essent. Fatty Acids* **56:** 29-39.

80.   Conklin, KA. 2002. Dietary polyunsaturated fatty acids: impact on cancer chemotherapy and radiation. *Alt. Med. Rev.* **7:** 4-21.

81. McEntee, M.F. and Whelan, J. 2002. Dietary polyunsaturated fatty acids and colorectal neoplasia. _Biomed. Pharmacother._ **56:** 380-387.

82. Dommels, Y.E.M., Alink, G.M., van Bladeren, P.J. and van Ommen, B. 2002. Dietary n-6 and n-3 polyunsaturated fatty acids and colorectal carcinogenesis: results from cultured colon cells, animal models and human studies. _Environ. Toxicol. Pharmacol._ **12:** 233-244.

83. Bae, S.H., Jung, E.S., Park, Y.M., Kim, B.S., Kim, B.K., Kim, D.G. and Ryu, W.S. 2001. Expression of cyclooxygenase-2 (COX-2) in hepatocellular carcinoma and growth inhibition of hepatoma cell lines by a COX-2 inhibitor, NS-398. _Clin. Cancer Res._ **7:** 1410-1418.

84. Van der Merwe, C.F., Booyens, J. and Katzeff, I.E. 1987. Oral γ-linolenic acid in 21 patients with untreatable malignancy - an ongoing pilot open clinical-trial. _Br. J. Clin. Pract._ **41:** 907-915.

85. Van der Merwe, C.F., Booyens, J., Joubert H.F. and van der Merwe, C.A. 1990. The effect of γ-linolenic acid, an in vitro cytostatic substance contained in evening primrose oil, on primary liver cancer - a double-blind placebo controlled trial. _Prost. Leukot. Essent. Fatty Acids_ **40:** 199-202.

CHAPTER 6

SUMMARY and CONCLUSIONS

SUMMARY AND DISCUSSION

As research has progressed over the last few decades, evidence has grown providing a strong link between changes in lifestyle associated dietary habits and a reduction in cancer risk. Attention shifted towards the role of nutrition as a modifiable factor in reducing cancer incidence rates leading to investigations regarding the role of dietary substances or compounds in the prevention of cancer. This process, defined as chemoprevention by Sporn *et al.,* (1), is complex but the understanding of the mechanisms involved in combating cancer can increase our knowledge in the treatment of this disease.

Molecular studies demonstrate that cancer cells contain defects in the regulatory mechanisms controlling cell proliferation and apoptosis. There are more than 100 distinct types of cancer, and subtypes of tumors can be found within specific organs. This complexity provokes a number of questions (2). How many distinct regulatory systems within each type of target cell must be disrupted before a cell becomes cancerous? Are the same sets of regulatory systems disrupted in the appearance of different neoplasms? Which of these systems are coupled to the signals that cells receive from their surrounding microenvironment within a tissue? Can the large and diverse collection of cancer associated genes be linked to a certain group involved in regulatory systems? Research showed that from a vast catalog of cancer cell phenotypes, the manifestation of six essential alterations in cell physiology dictate malignant growth, i.e., independence/self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential (dependent on telomere length or shortening), sustained angiogenesis and tissue invasion and metastasis. Each of these physiological changes acquired during tumour development represents the successful breakdown of an anti-cancer defense mechanism in cells and tissues. These six characteristic acquirements of cancer cells are shared by most types of human tumours (2). Therefore, in the struggle against cancer, an ideal strategy would be to regain control of these deregulated processes. A major challenge in the development of effective anti-cancer agents is the search for tumour-specific substances which do not detrimentally affect normal cells. In this regard, experimental evidence has indicated the potential of dietary compounds/elements such as the ω3 FA in cancer prevention.

In 1956, Hugh Sinclair (3) claimed that most of the "diseases of civilization" such as coronary heart disease, thrombosis, cancer, diabetes, inflammation and skin diseases are caused by a disturbance in fat metabolism. The main reason for the occurrence of these diseases was associated with the increased intake of processed foods rich in SATS and *trans* FA and a subsequent alteration in EFA intake, such as a shift towards a higher ω6 FA intake. Evidence from animal models and epidemiologic human studies suggest that a high intake of ω6 FA together with a very low ω3 FA intake increases the risk for the development of cancer. During the 1970s the beneficial health effects of ω3 FA gained recognition and since then a growing body of evidence from *in vitro* studies in cell cultures, animal cancer models, epidemiological and clinical studies in humans has provided evidence to support their use in the prevention of cancers such as in the colon, breast and prostrate. The proposed chemopreventive mechanisms of ω3 FA in cancer are multiple and include the suppression of C20:4ω6-derived eicosanoid production, enhancement of apoptosis, inhibition of tumor cell proliferation and an increase in anti-angiogenic activity (4). The incorporation and association of ω6 and ω3 FA with membrane dynamics involving cholesterol, phospholipids and membrane proteins, significantly impact on cellular processes such as signal transduction pathways and cell to cell communication in the regulation of cell growth. Although research suggests that ω3 FA can protect against cancer development, it appears that the ω6/ω3 dietary FA ratio, rather than the quantity of ω3 FA, is the major determinant responsible for the chemopreventive effect (5, 6, 7).

The current research presented in this thesis was conducted to characterise specific lipid changes associated with cancer development in the liver of the rat and in humans. The modulating role of dietary fat with varying ω6/ω3 FA ratios on cancer development was subsequently investigated in rat liver to provide information on their possible use as a chemopreventive tool in humans. In an initial study, the kinetics of FA replacement in blood plasma, as well as in the microsomes and plasma membrane phospholipids of rat liver were investigated. Changes in the lipid composition may affect the structural and functional properties of membranes, thereby altering the growth characteristics of neoplastic cells. The source of fat used in the diets was either SFO (control diet) or a SFO/EPA-50 oil mixture with a ω6/ω3 FA ratio of 12:1 or 6:1. The effects of the 12:1 ω6/ω3 FA ratio diet were monitored in adult rats for 2, 4 and 8 weeks and in weaned rats for 4 weeks. The adult rats were fed the 6:1 ω6/ω3 FA ratio diet for an additional 4 weeks to determine a threshold effect on FA replacement and

incorporation by qualitative analyses. Results indicated that the kinetics of membrane FA incorporation and replacement were dependent on (i) the specific phospholipid fraction, (ii) the membrane tissue compartment, (iii) age and to some extent, (iv) the dietary ω6/ω3 FA ratio. A threshold for the maximal incorporation of the ω3 FA was reached after 2 weeks on the 12:1 ω6/ω3 PUFA diet, except for C22:6ω3 which was further increased by the 6:1 diet in the plasma membrane PE. Regarding the ω6 FA, a threshold was reached after two weeks in the microsomes and to some extent in the plasma membrane PC. Feeding with the 6:1 diet resulted in further changes for certain ω6 FA in the plasma membrane. The level of C20:4ω6 was maximally decreased after two weeks on the 12:1 diet. However, C20:4ω6 tended to increase over time in the microsomes and plasma membrane PC, although it still remained below control diet levels. With the 6:1 diet, C20:4ω6 was not affected in the microsomes, but decreased further in the plasma membrane PE, while it increased in PC despite the increased ω3 FA content of the 6:1 ratio diet. Possibly, C20:4ω6 is regulated to prevent a decrease below a critical level which could be related to the dual role of C20:4ω6 as an important functional and structural PUFA. The FA exchange in the cellular compartments of the weaned rats fed the 12:1 diet generally followed the same pattern as in the adult rats fed the 12:1 diet for 4 weeks. However, certain FA were affected to a greater extent, such as a higher incorporation of the ω3 PUFA and a greater decrease in C20:4ω6 content in the microsomes and plasma PC, indicating that age affected dietary FA incorporation and displacement. This could also be a reflection of age differences in delta-6 desaturase activity as well as differences in incorporation into the different membrane fractions (8, 9, 10).

The FA replacement differed between the two phospholipid fractions as the ω3 FA were incorporated to a higher extent into the PE fraction and certain ω6 FA were decreased more in PC than PE. This could also be a reflection of PC being more susceptible to dietary FA alterations. Tissue compartments also reacted differently to FA replacement with the blood plasma compartment not reflecting the same changes as in the membrane tissue. Further tissue differences were reflected in the membrane fluidity which increased in the plasma membranes with both diets and stabilised after 8 weeks with the 12:1 diet. No effect was observed in the microsome membranes. The results demonstrated the importance of considering the kinetics affecting FA replacement in different tissue compartments when conducting dietary intervention studies, specifically regarding carcinogenesis. It is important to allow sufficient time for diet-induced changes to occur

prior to monitoring the effects related to cancer induction. This study showed that the kinetics of FA replacement depends on the interaction of different classes of FA, the membrane phospholipid fractions and the age of the rats.

Subsequently, alterations in lipid metabolism during the development of hepatocyte nodules were monitored over a period of 9 months utilising the resistant hepatocyte cancer model in rat liver. The results showed that the altered lipid parameters associated with hepatocyte nodule development mimicked normal cellular proliferation in regenerating liver. However, a major difference between the two proliferating tissue compartments was the persistence of the lipid changes in the hepatocyte nodules suggesting that nodule lipid metabolism escapes the regulatory mechanisms required for normal cellular homeostasis during proliferation. This indicates that the impaired regulation of lipid metabolism may be responsible for the enhanced proliferation and altered growth pattern in hepatocyte nodules. Similarities regarding altered lipid parameters in the nodules and normal regenerating liver were, (i) an increased PE concentration with a resultant decreased PC/PE ratio, and (ii) an increase in cholesterol and membrane fluidity as late events. With regards to specific FA in the PC phospholipid fraction, C18:1ω9 and C18:2ω6 increased in the nodules, whereas in the regenerating tissue C18:1ω9 and C18:2ω6 were initially decreased and increased, respectively. In the nodule PE tissue, C18:1ω9 and the ω6 PUFA, C18:2ω6 and C20:4ω6, were increased. In the regenerating liver, C18:1ω9 only increased after 7 days as a late event, while C18:2ω6 was initially increased but decreased to control levels over time in PE. An important difference between the nodule and regenerating tissue was the apparent impairment of the desaturase enzyme and the increased C20:4ω6 in PE in the nodules. These aspects have implications for signaling pathways regarding the control of C20:4ω6 metabolites and their involvement in stimulating cellular proliferation. Furthermore, differences in membrane structure were indicated by alterations in the nodule membrane fluidity which initially decreased and then increased as a late event. In contrast, the fluidity in regenerating tissue increased as an early event. This study demonstrated that alterations in lipid content can be linked with changes in membrane fluidity which could play an important role in the control of signal transduction pathways and cellular regeneration in an altered growth pattern and progression of hepatocyte nodules in the development of cancer.

Results obtained from these studies were integrated to examine the effect of different dietary fats with varying low ω6/ω3 FA ratios on rat hepatocyte nodule development. Different fats consisting of SFO (250:1 ω6/ω3 FA ratio) or SFO in combination with GLA-80 and/or EPA-50 to obtain the GLA and EPA diets, respectively (12:1 ω6/ω3 FA ratio), or SOY (5:1 ω6/ω3 FA ratio) were used to obtain the various low ω6/ω3 FA dietary ratios. Feeding with SFO was associated with the typical hepatocyte nodule growth and distinct lipid pattern observed in the aforementioned study. The level of lipid peroxidation in the nodules of the SFO-fed rats was decreased compared to the surrounding tissue which was associated with an increased GSH concentration. The low ω6/ω3 FA ratio diets (EPA, GLA and SOY diets) mainly affected the phospholipid LCPUFA composition in the nodules by increasing the ω3 FA content to varying degrees, especially C20:5ω3 and C22:6ω3, with a concomitant decrease in the ω6 LCPUFA. Together with the changes in the FA content, these diets also decreased the cholesterol and Chol/PL ratio in the nodules, which is likely to alter the structure and function of the membranes. However, changes in these parameters differed amongst the three diets as the SOY diet resulted in a lower ω3 PUFA content in the nodules compared to the GLA and EPA fed groups. Of importance were the different effects exerted on the nodule C20:4ω6 level by the three diets. The SOY and EPA diets decreased C20:4ω6 in PC and PE, whereas the GLA diet did not decrease C20:4ω6 to the same extent. Only the GLA diet decreased the C20:4ω6 nodule/surrounding ratio in both PC and PE fractions, while the EPA diet induced a comparable nodule/surrounding ratio to the SFO diet. Alteration in the C20:4ω6/C20:5ω3 ratio and C20:4ω6 level by the GLA diet is also suggestive of a decreased PGE_2 synthesis from C20:4ω6. With regards to the nodule oxidative status, the GLA and EPA diets increased lipid peroxidation while decreasing the GSH level more effectively than the SOY diet. However, only the GLA diet was associated with a reduction in the development of the nodules which could be related to the interaction between C20:5ω3 and C20:3ω6 which appear to synergistically control the metabolism of C20:4ω6 and also serve as substrates for less potent prostanoid metabolites, such as PGE_3 and PGE_1. This control over C20:4ω6 metabolism by the C20:5ω3/C18:3ω6 dietary combination has important implications for the regulation of transcription factors and genes involved in signaling pathways affecting cell proliferation and apoptosis which can influence the development of hepatocyte nodules (11, 12, 13).

The final study describes the alterations in membrane lipid parameters and oxidative status associated with human HCC. Results obtained were compared with lipid alterations observed in rat hepatocyte nodules. In the carcinoma tissue, PC was decreased and cholesterol increased resulting in a decreased PC/PE and increased Chol/PL ratio. With regards to alterations in specific FA, C18:2ω6, C20:4ω6, C22:5ω3 and C22:6ω3 levels decreased in PC, probably due to the lower PC level. In PE, a similar decrease in C22:5ω3 and C22:6ω3 was noticed, while the total ω6 PUFA was not altered. This resulted in an increased ω6/ω3 ratio in PC and PE. The decreased PC concentration was associated with a decreased C20:4ω6, while no change was observed in PE, implying a higher membrane C20:4ω6 content in PE relative to PC. The decrease in ω3 PUFA in PC and PE and the stabilisation of C20:4ω6 in PE has implications for stimulating cellular signalling involved in cell proliferation and the inhibition of apoptosis, contributing to a growth stimulus in the HCC tissue. In this regard, the decreased lipid peroxidation indicates a decreased oxidative status in the carcinoma, contributing to an environment for enhancing cell growth and survival. In contrast to the nodular tissue in rat liver, GSH levels were decreased in the HCC tissue. The decreased GSH could be due to various factors such as an increased utilization in the carcinoma tissue (14) or due to an impaired methionine clearance (15). In the present study, HBV infection did not affect the GSH concentration in the carcinoma tissue whereas it was increased in the surrounding tissue, presumably due to an impairment of the GSH/GSSG redox cycle. Alterations in membrane cholesterol and phospholipids, perturbations in C20:4ω6 phospholipid content as well as the low lipid peroxidation and ω3 PUFA levels, create an environment for a selective growth advantage promoting the survival of cancer cells. As demonstrated in the *in vivo* dietary modulation study in rat liver, this environment creates the opportunity for the modulation of the lipid profiles of cancerous tissue by dietary PUFA, especially the ω3 FA, which may alter the growth characteristics of cancer cells.

The rat liver and human HCC studies showed similarities in the altered lipid profile, associated with liver cancer development. These similarities are as follows:

i)   Increased cholesterol and Chol/PL ratio

ii)   Low PC/PE ratio, due to alterations in phospholipid concentration

iii)   Low ω3 PUFA levels resulting in a high ω6/ω3 PUFA ratio

iv)   Low level of lipid peroxidation

v)   Increased level and/or stabilisation of C20:4ω6 in PE relative to PC.

Increased cholesterol in the carcinoma tissue is possibly a compensatory mechanism in the membrane to counter the decreased PUFA level to maintain the integrity and functionality of the cell membrane. Cholesterol is also involved in arranging the configuration of the membrane FA acyl chains in maintaining the membrane structural integrity (16). Alterations in the membrane phospholipid and cholesterol levels, affecting the membrane Chol/PL and PC/PE ratios, influence membrane structure, fluidity and functionality. The various phospholipid classes are not evenly distributed and arranged in the cell membrane creating a characteristic asymmetric phospholipid membrane distribution. Disruption of the asymmetrical arrangement is associated with cellular signalling resulting in events such as apoptosis (17, 18). Changes in these parameters are also known to regulate the function of delta-6-desaturase enzyme, a key rate limiting enzyme in FA metabolism (19). Various studies have demonstrated an impairment of the delta-6 desaturase in cancer tissue affecting PUFA synthesis (20, 21, 22).

Impairment of the delta-6 desaturase enzyme and resultant low PUFA levels, especially ω3 PUFA, have been associated with the reduced oxidative status of cancer cells (23). From these observations the question arises of whether the low lipid peroxidation status in cancer tissue can be directly linked to a low ω3 PUFA status. Modulation of hepatocyte nodules with the SOY, GLA and EPA diets in the rat, demonstrated an increased lipid peroxidation level in the nodules. This increase in lipid peroxidation was attributed to the increased ω3 PUFA in the nodule tissue, specifically C22:6ω3 which is known to be a good substrate for lipid peroxidation (24). Lipid peroxidation was also decreased in regenerating liver indicating that increased cell proliferation is associated with decreased lipid peroxidation (23, 25, 26). This suggests that decreased lipid peroxidation plays a role in hepatocyte proliferation, both under normal and disease

states, such as cancer. In this regard, the effects of an increased FA and antioxidant intake is gaining prominence, and is of relevance when considering diseases such as cancer. In cell cultures, the addition of an antioxidant such as vitamin E has been shown to negate the cytotoxic effect of the ω3 FA, indicating that lipid peroxidation is one of the mechanisms whereby these FA regulate cancer cell growth (27, 28). It appears that ω3 FA, such as C20:5ω3 and C22:6ω3, also increase the susceptibility of cancer cells to lipid peroxidation, and when used in conjunction with certain chemotherapeutic agents, may increase the efficacy of the drug in inhibiting cancer growth (27, 28). These chemotherapeutic drugs, as well as the ω3 FA, increase the generation of free radicals which target the cancer cells and antioxidant supplements can negate this desired effect. Fish oil has been shown to upregulate certain antioxidant genes, such as glutathione transferase, UCP-2 and Mn-SOD, in the liver (29). Presumably, this upregulation counteracts excessive ROS production in normal cells and could explain the selective toxicity of PUFA to cancer cells (30). Studies in cell culture have also demonstrated that cancer cells produce more peroxidation products than normal cells when supplemented with PUFA (31, 32). The level of lipid peroxidation has been shown to regulate the balance between cell proliferation and apoptosis during liver cell regeneration (33). The addition of antioxidants, such as vitamin C and E, enhanced hepatocyte proliferation and was associated with increased DNA synthesis, suggesting an inverse relationship between lipid peroxidation and mitosis (34). These findings caution the unnecessary use of antioxidant supplements, especially regarding disease treatment.

Apart from altering the oxidative status in the nodules, the low ω6/ω3 FA ratio diets have important implications for the regulation of C20:4ω6 metabolism by altering its role as, (i) a substrate for prostaglandin synthesis, involved in the promotion of cell proliferation, or (ii) in stimulating apoptosis by enhancing ceramide production. In this regard, the modulation of C20:4ω6 in the PE phospholipid fraction is important. The higher incorporation of ω3 PUFA in the PE phospholipid, compared to PC (35), may be a possible mechanism to regulate the membrane C20:4ω6 content. However, excessive reduction of C20:4ω6 by the ω3 FA could impact negatively on the multiple role of C20:4ω6 in maintaining cellular homeostasis, such as the regulation of apoptosis. A dietary FA combination consisting of C20:5ω3 and C18:3ω6 appeared to counteract undue displacement of the membrane C20:4ω6, modulated rat hepatocyte nodule growth and normalised the C20:4ω6 nodule/surrounding ratio by bypassing the

impaired delta-6-desaturase. Furthermore, a synergistic effect between C18:3ω6 and C20:5ω3 seems to exist in normalising the C20:4ω6 level in PE and directing prostaglandin synthesis towards the bioactively less potent PGE$_1$ and PGE$_3$. It is not known whether the diet will also modulate the growth of HCC in humans. Comparison of the lipid profiles of cancerous lesions from the rat and human liver demonstrated changes to the membrane C20:4ω6 content associated with a decreased PC/PE ratio, although this manifested differently depending on the specific phospholipid type. In human HCC, PC decreased which was associated with a decrease in C20:4ω6 while it was stabilised in PE. Recent studies indicated that COX-2 is overexpressed in human HCC (36), while supplementation with C18:3ω6 was shown to retard the tumour growth (37, 38). Modulation of the C20:4ω6 content of human HCC, therefore, could be a feasible objective in regulating the adverse effects of this FA.

Various *in vitro* and *in vivo* studies have demonstrated the beneficial potential of ω3 FA in the prevention and inhibition of cancer growth, proposing several mechanisms for their protective effects (39, 40, 41, 42). Results from this thesis support these findings and expands on the importance of using specific dietary FA to alter the ω6/ω3 FA ratio in the regulation the growth of hepatocytes nodules. Dietary modulation of the characteristic altered lipid profile in rat hepatocyte nodules demonstrated changes to the following parameters:

i)   membrane cholesterol

ii)   ω3 PUFA level and ω6/ω3 FA ratio

iii)   C20:4ω6/C20:5ω3 ratio

iv)   oxidative status

Apart from the ω6 and ω3 FA, some SATS and MUFA have also demonstrated an ability to alter signal transduction pathways related to the regulation of cell proliferation and apoptosis. *In vitro*, C18:1ω9 (MUFA) and C16:0 (SATS) may stimulate cell proliferation and apoptosis, respectively (43, 44). Signaling pathways affected involve PI3-K, a second messenger for pathways relating to cell proliferation and apoptosis. C18:1ω9 can activate PI3-K as well as the membrane bound EGFR, thereby acting as a growth stimulator (45). In contrast, C16:0 can inhibit PI3-K activation and induce apoptosis. In this regard, C18:1ω9 was increased in the rat hepatocyte nodule PC and PE phospholipid fractions, while C16:0 was increased in PC and decreased in PE.

Dietary modulation with the SOY and GLA diets decreased C18:1ω9 and increased C16:0 in the PE phospholipid fraction in the nodules which could attenuate or stimulate the signaling events regulating cell proliferation and apoptosis, respectively. Changes in membrane lipid parameters such as cholesterol, phospholipids, SATS and PUFA, especially ω3 in conjunction with C18:3ω6, may affect membrane functionality and cellular survival.

Dietary modulation of the altered lipid profile in cancerous tissue by dietary ω3 FA demonstrates the importance of changes to the membrane structure and function as well as the effect on cellular oxidative status in the regulation of cell growth. These FA can modulate C20:4ω6-derived eicosanoid biosynthesis, influence transcription factor activity, gene expression and signal transduction pathways, affect the level of free radical and reactive oxygen species and membrane fluidity. Further studies are needed to evaluate and verify these mechanisms in humans to gain more understanding of the effects of ω3 FA intake on cancer prevention. Epidemiologic studies with more detailed information about ω3 and ω6 FA intake and the biological interaction between other nutritional factors in cancer development should also be considered. The emergence of a "multifocal signal modulation therapy" strategy in combating cancer as proposed by McCarty (46) whereby a combination of substances which have been individually shown to counteract alterations in signal transduction in cancer tissue, provides an important motivation for FA research in cancer prevention. As FA affect cell function in various ways, their role as a single unit may encompass the whole aspect of controlling certain signal transduction pathways instead of a mixture of substances as proposed by McCarty.

CONCLUSIONS

The mechanisms related to cancer induction, progression and metastasis are complex. Cancer may be caused by a wide array of events, including the interaction between chemical, environmental, dietary, lifestyle and hereditary factors. Although research related to the efficacy of nutritional substances appears to offer significant promise to cancer prevention and treatment, these findings do not at present offer any guaranteed certainty as chemopreventive substances in the battle against cancer. Although there has been significant progress in identifying FA with cytotoxic effects and/or growth inhibitory activity towards cancer cells, much remains to be discovered regarding the underlying mechanisms involved. This complexity can be attributed to the vast and integrated involvement of FA in many body functions. However, the vast numbers of published studies do indicate a positive effect on cancer prevention as well as the treatment of cancer in conjunction with regular cancer therapy strategies (27, 47). Taking into account the well-established effects of ω3 FA in preventing cardiovascular disease and the therapeutical effect on other diseases such as schizophrenia, Alzheimer's, certain skin disorders, agencies such as the American Food and Drug Administration (FDA), American Heart Association (AHA) and International Society for the Study of Fatty Acids and Lipids (ISSFAL) recommend an increased intake of dietary ω3 FA (48, 49, 50). In a recent publication, the US Department of Health and Human Services together with the Department of Agriculture recommend that most dietary fats should be from sources containing PUFA and MUFA, such as fish, nuts, and vegetable oils (51).

With more official recommendations being published concerning the health benefits of an increased ω3 FA intake, such as C20:5ω3 and C22:6ω3 in reducing cardiovascular disease, it may not be long before official recognition is also given to their benefits in preventing cancer. These recommendations for altering dietary habits to improve the health or to counter the predisposition for certain diseases could be an affordable and maintainable proposition. Liver cancer is one of the most frequent malignant tumours in the world and in sub-Saharan Africa HCC is the leading cause of cancer-related deaths in adults (52, 53). The incidence rates amongst men in sub-Saharan Africa and Asia may be 20 times higher than in the USA (49). The liver is a common site of metastases from a variety of organs such as lung, breast, colon and rectum and is

frequently involved due to its rich blood supply and contact with the abdominal organs via the portal vein. Various studies in colon, breast and prostate have shown that FA dietary intervention can be applied in the prevention of carcinogenesis, especially when considering the fish or marine long-chain FA, C20:5ω3 and C22:6ω3 (11, 27, 30, 40, 42, 47, 54). At present very little information is available with regards to FA dietary intervention of HCC in humans, although similar carcinogenic causative mechanisms, such as COX-2 overexpression in colon cancer, also appear to play a role in HCC development (36). A study in patients with HCC indicated that evening primrose oil, containing C18:3ω6, retarded tumour growth (37, 38). These investigations, as well as results from the present study, demonstrate the potential effect of modulating lipid parameters associated with liver cancer by dietary FA.

FUTURE RESEARCH

As information concerning the health properties of the ω3 FA increases, food manufacturers have explored the opportunity of fortifying everyday foods with these FA (55). ω3-enriched eggs are already available as well as fortified bread in New Zealand and Australia. Malaysia has a cracker fortified with vitamins, minerals and ω3 and ω6 FA as well as a fortified margarine. In Spain fruit juices fortified with milk, fibre, vitamins and ω3 are available as well as soups and milkshakes with ω3 FA. In Finland a blackcurrant juice with added ω6 and ω3 FA is being marketed. Other foodstuffs fortified with ω3 FA include infant milks as supplements.

Future studies need to address the effect of dietary FA modulation of cancer by:

i)   Developing dietary fats containing certain FA within a specific ω6/ω3 FA ratio for modulating carcinogenesis in humans.

ii)   Elucidating the interaction between antioxidants and FA in modulating the oxidative status of cancer cells.

iii)   Investigating the extent of delta-6 desaturase impairment in human cancers.

iv)   Developing biomarkers, including the C20:4ω6/C20:5ω3 tissue ratio, COX-2 expression and certain markers for cell proliferation and apoptosis, to monitor the effectiveness of cancer modulation by dietary FA.

REFERENCES

1. Sporn, M.B., Dunlop, N.M., Newton, D.L. and Smith, J.M. 1976. Prevention of chemical carcinogenesis by vitamin A and its synthetic analogs (retinoids). Fed. Proc. 35, 1332-1338.

2. Hanahan, D. and Weinberg, R.A. 2000. The hallmarks of cancer. Cell 100, 57-70.

3. Sinclair, H.M.. 1956. Deficiency of essential fatty acids and atherosclerosis, etcetera. Lancet i:381-383.

4. Rose, D.P. and Connolly, J.M. 2000. Regulation of tumor angiogenesis by dietary fatty acids and eicosanoids. Nutr. Cancer 37, 119-127.

5. Simopolous, A.P. 2002. The importance of the ratio of ω6/ω3 essential fatty acids. Biomed. Pharmacother. 56, 365-379.

6. Simonsen, N., van't Veer, P., Strain, J.J., Martin-Moreno, J.M., Huttunen, J.K., Navajas, J.F.-C., Martin, B.C., Thamm, M., Kardinaal, A.F.M., Kok, F.J. and Kohlmeier, L. 1998. Adipose tissue omega-3 and omega-6 fatty acid content and breast cancer in the EURAMIC study. Am. J. Epidemiol. 147, 342-352.

7. Bartram, H.-P., Gostner, A., Reddy, B.S., Rao, C.V., Scheppach, W., Dusel, G., Richter, A., Richter, F. and Kasper, H. 1995. Missing anti-proliferative effect of fish oil on rectal epithelium in healthy volunteers consuming a high-fat diet: potential role of the n-3:n-6 fatty acid ratio. Eur. J. Cancer Prev. 4, 231-237.

8. Prentice, R.L., Pepe, M., Self, S.G. 1989. Dietary fat and breast cancer: a quantitative assessment of the epidemiologic literature and a discussion of methodologic issues. Cancer Res. 49, 3147-3156.

9. Farber, E. 1996. The step-by-step development of epithelial cancer: from phenotype to genotype. Adv. Cancer Res. 70, 21-48.

10. inh T K L, Bourre J M, Durand G. Effect of age and alpha-linolenic acid deficiency on delta-6 desaturase activity and liver lipids in rats. Lipids 1993; 28: 517-523.

11. Anti, M., Armelao, F., Marra, G., Percesepe, A., Bartoli, G.M., Palozza, P., Parrella, P., Canetta, C., Gentiloni, N. and De Vitis, I. 1994. Effects of different doses of fish oil on rectal cell proliferation in patients with sporadic colonic adenomas. Gastroenterology 107, 1709-1718.

12. Cao, Y., Pearman, A.T., Zimmerman, G.A., McIntyre, T.M. and Prescott, S.M. 2000. Intracellular unesterified arachidonic acid signals apoptosis. Proc. Natl. Acad. Sci. 97, 11280-11285.

13. Jayadev, S., Linardic, C.M. and Hannun, Y.A. 1994. Identification of arachidonic acid as mediator of sphingomyelin hydrolysis in response to tumor necrosis factor alpha. J. Biol. Chem. 269, 5757-5763.

14.   Marinho, H.S., Baptista, M. and Pinto, R.E. 1997. Glutathione metabolism in hepatomous liver of rats treated with diethylnitrosamine. Biochim. Biophys. Acta 1360, 157-168.

15.   Mato, J.M., Alvarez, L., Corrales, F.J. and Pajares, M.A.. 1994. S-adenosylmethionne and the liver. In *The Liver: Biology and Pathobiology.* Arias, I.M., Boyer, J.L., Fausto, N., Jakoby, W.B., Schachter, D.A. and Shafritz, D.A. eds. Third Edition. Raven Press, New York, USA. pp 461-470.

16.   Darnell, J., Lodisch, H. and Baltimore, D. 1986. *Molecular Cell Biology*. Scientific American, New York, USA. 576-577.

17.   Pomorski, T., Hrafnsdóttir, S., Devaux, P.F. and van Meer, G. 2001. Lipid distribution and transport across cellular membranes. Sem. Cell Dev. Biol. 12, 139-148.

18.   Rudolph, I.L., Kelley, D.S., Klasing, K.C. and Erickson, K.L. 2001. Regulation of cellular differentiation and apoptosis by fatty acids and their metabolites. Nutr. Res. 21, 381-393.

19.   Leikin, A. and Shinitzky, M. 1995. Characterization of the lipid surrounding the Δ^6-desaturase of rat liver microsomes. Biochim. Biophys. Acta 1256, 13-17.

20.   Gardiner, N.S. and Duncan, J.R. 1991. Possible involvement of delta-6-desaturase in control of melanoma growth by gamma-linolenic acid. Prostaglandins, Leukot. Essent. Fatty Acids 42, 149-153.

21.   Hrelia, S., Bordoni, A., Biagi, P.L., Galeotti, T., Palombini, G. and Masotti, L. 1994. The role of delta-6- and delta-9-desaturase in the fatty acid metabolism of hepatomas with different growth rate. Biochem. Mol. Biol. Int. 34, 449-455.

22.   Das, U.N. 2004. From bench to the clinic: -linolenic acid therapy of human gliomas. Prost. Leukot. Essent. Fatty Acids 70, 539-552.

23.   Horrobin, D.F. 1994. Unsaturated lipids and cancer. In *New approaches to cancer treatment: unsatsed lipids and photodynamic therapy*. Horrobin, D.F. ed. Churchill Communications, Nordic House, London. pp 3-64.

24.   Song, J.H., Fujimoto, K. and Miyazawa, T. 2000. Polyunsaturated (n-3) fatty acids susceptible to peroxidation are increased in plasma and tissue lipids of rats fed docosahexaenoic acid-containing oils. J. Nutr. 130, 3028-3033.

25.   Cheeseman, K.H., Collins, M., Proudfoot, K., Slater, T.F., Burton, G.W., Webb, A.C. and Ingold, K.U. 1986. Studies on lipid peroxidation in normal and tumour tissues. Biochem. J. 235, 507-514.

26.   Diplock, A.T., Rice-Evans, C.A. and Burdon, R.H. 1994. Is there a significant role for lipid peroxidation in the causation of malignancy and for antioxidants in cancer prevention? Cancer Res. 54, 1952s-1956s.

27.   Conklin, K.A. 2002. Dietary polyunsaturated fatty acids: impact on cancer chemotherapy and radiation. Alt. Med. Rev. 7, 4-21.

28.   Cognault, S., Jourdan, M.L., Germain, E., Pitavy, R., Morel, E., Durand, G., Bougnoux, P. and Lhuillery, C. 2000. Effect of an alpha-linolenic acid-rich diet on rat mammary tumour growth depends on the dietary oxidative status. Nutr. Cancer 36, 33-41.

29.   Takahashi, M., Tsuboyama-Kasaoka, N., Nakatani, T., Ishii, M., Tsutsumi, S., Aburatani, H. and Ezaki, O. 2002. Fish oil feeding alters liver gene expressions to defend against PPAR-α activation and ROS production. Am. J. Physiol. Gastrointest. Liver Physiol. 282, G338-G348.

30.   Jiang, W.G., Bryce, R.P. and Horrobin, D.F. 1998. Essential fatty acids: molecular and cellular basis of their anti-cancer action and clinical implications. Crit. Rev. Oncol. Hematol. 27, 179-209.

31.   *Das, U.N., Begin, M.E., Ells, G., Huang, Y.S. and Horrobin, D.F. 1987. Polyunsaturated fatty acids augment free radical generation in tumor cells in vitro. Biochem. Biophys. Res. Commun. 145, 15-24.*

32.   *Begin, M.E., Ells, G. and Horrobin, D.F. 1988. Polyunsaturated fatty acid-induced cytotoxicity against tumour cells and its relationship to lipid peroxidation. J. Natl. Cancer Inst. 80, 188-194.*

33.   Ronco, M.T., Alvarez, M.L., Monti, J., Carrillo, M.C., Pisani, G., Lugano, M.C. and Carnovale, C.E. 2002. Modulation of balance between apoptosis and proliferation by lipid peroxidation (LPO) during rat liver regeneration. Mol. Med. 8, 808-817.

34.   Aguilar-Delfin, I., Lopez-Barrera, F. and Hernández-Muñoz, R. 1996. Selective enhancement of lipid peroxidation in plasma membrane in two experimental models for liver regeneration: partial hepatectomy and acute CCl4 administration. Hepatology 24, 657-662.

35.   Abel, S., Gelderblom, W. C. A., Smuts, C. M. and Kruger, M. 1997. Thresholds and kinetics of fatty acid replacement in different cellular compartments in rat liver as a function of dietary n-6/n.3 fatty acid content. Prost. Leukot. Essent. Fatty Acids 56, 29-39.

36.   Bae, S.H., Jung, E.S., Park, Y.M., Kim, B.S., Kim, B.K., Kim, D.G. and Ryu, W.S. 2001. Expression of cyclooxygenase-2 (COX-2) in hepatocellular carcinoma and growth inhibition of hepatoma cell lines by a COX-2 inhibitor, NS-398. Clin. Cancer Res. 7, 1410-1418.

37.   Van der Merwe, C.F., Booyens, J. and Katzeff, I.E. 1987. Oral γ-linolenic acid in 21 patients with untreatable malignancy - an ongoing pilot open clinical-trial. Br. J. Clin. Pract. 41, 907-915.

38.   Van der Merwe, C.F., Booyens, J., Joubert, H.F. and Van der Merwe, C.A. 1990. The effect of γ-linolenic acid, an in vitro cytostatic substance contained in evening primrose oil, on primary liver cancer - a double-blind placebo controlled trial. Prost. Leukot. Essent. Fatty Acids 40, 199-202.

39. Bartsch, H., Nair, J. and Owen, R.W. 1999. Dietary polyunsaturated fatty acids and cancers of the breast and colorectum: emerging evidence for their role as risk modifiers. Carcinogenesis 20, 2209-2218.

40. Terry, P.D., Rohan, T.E. and Wolk, A. 2003. Intakes of fish and marine fatty acids and the risks of cancers of the breast and prostate and of other hormone-related cancers: a review of the epidemiologic evidence. Am. J. Clin. Nutr. 77, 532-543.

41. Simopoulos, A.P. 2003. Omega-3 fatty acids and cancer. Indoor Built. Environ. 12, 405-412.

42. Terry, P.D., Terry, J.B. and Rohan, T.E. 2004. Long-chain (n-3) fatty acid intake and risk of cancers of the breast and the prostate: recent epidemiological studies, biological mechanisms, and directions for future research. J. Nutr. 134, 3412S-3420S.

43. Hardy, S., Langelier, Y. and Prentki, M. 2000. Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. Cancer Res. 60, 6353-6358.

44. Iturralde, M., Gamen, S., Pardo, J., Bosque, A., Piňeiro, A., Alava, M.A., Naval, J. and Anel, A. 2003. Saturated free fatty acid release and intracellular ceramide generation during apoptosis induction are closely related processes. Biochim. Biophys. Acta. 1634, 40-51.

45. Vacaresse, N., Lajoie-Mazenc, I., Auge, N., Suc, I., Frisach, M.F., Salvayre, R. and Negre-Salvayre, A. 1999. Activation of epithelial growth factor receptor pathway by unsaturated fatty acids. Circ. Res. 85, 892-899.

46. McCarty, M.F. 2004. Targeting multiple signaling pathways as a strategy for managing prostate cancer: multifocal signal modulation therapy. Integr. Cancer Therap. 3, 349-380.

47. Larsson, S.C., Kumlin, M., Ingleman-Sundberg, M. and Wolk, A. 2004. Dietary long-chain n-3 fatty acids for the prevention of cancer: a review of potential mechanisms. Am. J. Clin. Nutr. 79, 935-945.

48. American Heart Association (AHA) Scientific Statement: AHA Dietary Guidelines: Revision 2000, #71-0193. Circulation 102, 2284-2299.

49. Office of Nutritional Products, Labeling, and Dietary Supplements, Center for Food Safety and Applied Nutrition, US Food and Drug Administration. Letter responding to a request to reconsider the qualified claim for a dietary supplement health claim for omega-3 fatty acids and coronary heart disease. Docket No. 91N-0103. February 8, 2002.
http://www.cfsan.fda.gov/~dms/ds-ltr28.html

50. ISSFAL Policy statements. http://www.issfal.org.uk/policystatements.htm.

51.   U.S. Department of Health and Human Services and U.S. Department of Agriculture. *Dietary Guidelines for Americans,* 2005. 6th Edition, Washington, DC: U.S. Government Printing Office, January 2005.

52.   Wang, X.W., Hussain, S.P., Huo, T.-I., Wu, C.-G., Forgues, M., Hofseth, L.J., Brechot, C. and Harris, C.C. 2002. Molecular pathogenesis of human hepatocellular carcinoma. Toxicol. 181-182, 43-47.

53.   Kew, M.C. 2002. Epidemiology of hepatocellular carcinoma. Toxicol. 181-182, 35-38.

54.   Sherlock, S. 1989. Hepatic tumours. In *Diseases of the liver and biliary system*. 8th Edition. Blackwell Scientific, London, UK. pp 585.

55.   Omega-3 Information Service website. The fats of life.
        http://www.omega-3info.com/wheredoifind.htm

SUPPLEMENT

A.   **Ethical Approval**

B.   **Public Awareness and Understanding of**

Science and Technology (PAUSET)

ETHICAL APPROVAL

All studies performed in this dissertation conformed to the ethical guidelines as stipulated by the MRC Ethics Committee.

MEDIESE NAVORSINGSRAAD
MEDICAL RESEARCH COUNCIL

Kantoor van die President/Office of the President

✉ 19070 Tygerberg 7505 • Suid-Afrika/South Africa • ☎ (021) 938-0211/938-0911 • Fax (021) 938-0201

30 September 1993

Dr S Wolfe-Coote
Experimental Biology
MRC
PO Box 19070
TYGERBERG

Dear Dr Wolfe-Coote
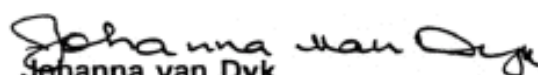
APPLICATION FOR ETHICAL APPROVAL - MR S ABEL

Thank you for your note and addendum in connection with above application.

I have pleasure in advising that Mr Abel's application "Modulation of the cancer initiating and promotional stages in chemical carnogenesis by dietary essential fatty acids" has been ethically approved.

I wish him everything of the best with this study.

Yours sincerely

Johanna van Dyk
for **CHAIRMAN: ETHICS COMMITTEE**

ETHICAL APPROVAL

MRC
SOUTH AFRICA
The
**Medical
Research
Council**

Ethics Committee

P.O. Box 19070, Tygerberg 7505, South Africa.
Francie van Zijl Drive, Parow Valley 7500, Cape Town.
Tel: +27 (0)21 938 0341, Fax: +27 (0)21 938 0201
E-mail: adri.labuschagne@mrc.ac.za
http://www.sahealthinfo.org/Modules/ethics/ethics.htm

14 November 2001

Mr S Abel
PROMEC Unit
MRC

Dear Mr Abel

**RE: Investigating the regulation of fatty acid metabolism in human
carcinogenesis**

Thank you for your response to the Ethics Committee, dated 1 October 2001.
The Committee has approved the redone patient information sheet. I am
pleased to inform you that ethics approval is now granted for the study.

Wishing you well with your research.

Yours sincerely

**PROF PE CLEATON-JONES
CHAIRPERSON: MRC ETHICS COMMITTEE**

ETHICAL APPROVAL

PAUSET

The dissemination of information regarding science and technology amongst the general public, termed as "Public Awareness and Understanding of Science and Technology" (PAUSET), is an important aspect for empowering people through knowledge information as well as demonstrating the importance and relevance of research with regards to improving health. This is important for the involvement of public participation in health information and for creating interest for recruiting people into jobs in the various science disciplines. The success of such a campaign lies in transferring the knowledge in ways that are easily understood and appreciated by the general public. Furthermore, institutions should also provide opportunities for pre- and post-graduate students to increase their knowledge within a research environment for the benefit of science. In this regard, this study contributed towards PAUSET in the following aspects:

(i)   Student training

Since January 1997, students from abroad have been trained in various aspects of FA analyses in furthering their pre- or post-graduate studies (Honours, Master and PhD level). Techniques learned include:

- the isolation of primary hepatocytes and use in certain *in vitro* assays,
- fatty acid extraction, analyses and interpretation,
- analyses and interpretation of data regarding certain lipid parameters, such as phospholipids, cholesterol and lipid peroxidation.

Participation in Student Day visits to the MRC, demonstrating various aspects of fatty acid research to pre-graduate students from UWC, US and UWC.

(ii)   Lay publications

- Abel, S. 2002. The case of the good fats. MRC News, vol. 33, No. 6 (December). pp 16-19.
- Smuts, C.M. and Abel, S. 2005. The relevance of essential fatty acids to health and disease. Specialist Medicine 5, 38-48.

PAUSET

(iii)   Radio interviews

▪   Mr S. Abel, together with Prof V. Sewram and Dr J. Marnewick, were invited by the Cancer Association of South Africa (CANSA) to attend a press conference as part of the World Cancer Day programme held at Delft Community Centre, Western Cape, South Africa, 5 February 2005. All three PROMEC Unit staff members were interviewed by Bush Radio (89.5 FM), where they discussed aspects of their research and how it impacts on South African communities.

▪   Mr S. Abel and Dr J. Marnewick were invited for a live radio interview by Bush Radio at their Salt River studios, where they discussed aspects of their research and in relation to promoting and improving the health of South African communities (28 Feb 2005, Salt River, Western Cape, South Africa).

PAUSET

PAUSET

ADDENDUM

Supplementary Data Tables

PAUSET

ADDENDUM 1

Chapter 2: *Membrane Phospholipid Content.*

Effect of experimental diets on the liver membrane phospholipid content in a

| | | Phospholipid (µg Pi/mg protein) | | | |
| | | Plasma Membranes | | Microsomes | |
Week	Dietary Group	PC	PE	PC	PE
Adult rats					
2*	Control	316.93±21.31	56.24±4.18	172.03±15.56	56.17±4.61
	Experimental	258.25±63.77	66.25±8.22	271.54±17.88	71.74±17.92
4*	Control	191.32±11.07	46.23±9.14	323.01±83.04	
	Experimental	237.59±41.89	63.13±14.25	302.01±34.41	69.00±16.28
8*	Control	298.55±36.16	49.00±9.95	216.87±47.37	
	Experimental	278.88±22.45	46.52±8.98	266.08±42.76	61.17±8.05
12#	Control	294.18±13.61	89.20±14.03	359.89±19.42	74.08±19.71
	Experimental	276.79±92.05	95.26±15.95	360.03±24.79	84.60±9.14
Weaned rats					
4*	Control	203.14±13.27	55.89±4.69	236.00±23.35	44.50±6.84
	Experimental	254.46±15.14	63.74±7.04	213.06±16.51	45.31±8.19

*Control rats received the AIN 76A diet and the experimental groups received diet A ($\omega6/\omega3$ ratio of 12:1). #Control rats received the AIN 76A diet and the experimental group received diet B ($\omega6/\omega3$ ratio of 6:1). Values are means ± standard deviation of 5 rats per group. No significant differences were observed. PC, Phosphatidylcholine; PE, Phosphatidylethanolamine.

ADDENDUM 2

Chapter 2: *Membrane Cholesterol Content.*

Effect of experimental diets on the liver membrane cholesterol content in adult and weaned rats fed diets A and B over different time periods.

	Cholesterol (µg/mg protein)				
	Plasma Membranes			Microsomes	
Week	Control	Experimental		Control	Experimental
Adult rats					
2*	63.91±15.28	51.23±5.85		40.57±6.29	41.70±2.28
4*	60.76±5.28	52.58±6.20		31.69±2.92	33.27±4.19
8*	61.35±10.20	50.42±4.66		31.43±2.49	27.65±5.46
12#	60.08±11.89	58.99±12.92		30.95±2.16	28.92±3.15
Weaned rats					
4*	71.74±13.14	70.58±6.69		32.24±4.81	27.02±5.54

*Control rats received the AIN 76A diet and the experimental groups received diet A (ω6/ω3 ratio of 12:1). #Control rats received the AIN 76A diet and the experimental group received diet B (ω6/ω3 ratio of 6:1). Values are means ± standard deviation of 5 rats per group. No significant differences were observed.

ADDENDUM 3

Chapter 3:   *Comparative FA profiles in PC and PE of hepatocyte nodules compared to surrounding and control tissue (addendum to Table 3).*

Table 3. Fatty acid analyses (percentage of total fatty acids) of the phosphatidylcholine (PC) fraction of control liver, nodule and surrounding liver in the Resistant Hepatocyte Model.

	1 Month			3 Months			6 Months			9 Months		
	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
Saturates												
C16:0	19.14±0.29a	23.93±1.76b	21.34±1.14c	20.32±0.34	21.07±2.42	19.83±1.19	19.22±0.73a	26.56±1.27b	18.47±1.32a	15.84±1.29a	22.10±2.71b	16.97±0.64a
C18:0	21.11±0.97a	14.84±0.58b	17.11±0.44c	19.70±0.82	16.29±1.84	18.55±0.57	20.93±1.39a	12.39±0.73b	20.26±1.57a	23.95±1.12a	12.66±1.56b	21.98±0.33a
Total	40.25±1.10	38.77±1.80	38.45±0.92	40.02±0.67	37.36±1.04	38.39±0.70	40.15±0.69	38.95±1.47	38.73±1.53	39.79±1.86	34.76±2.78	38.95±0.91
Monounsaturates												
C16:1	0.89±0.33a	2.24±0.53b	1.28±0.26a	1.14±0.13	1.57±0.33	1.33±0.17	0.89±0.06a	2.02±0.11b	0.81±0.28a	0.63±0.16a	1.74±0.48b	1.14±0.13c
C18:1	7.75±0.65a	14.28±0.62b	10.80±0.38c	8.27±0.66a	13.40±1.26b	10.47±0.40a	7.82±0.25a	14.50±1.12b	9.86±0.34c	6.44±0.58a	16.42±2.88b	8.31±0.14c
Total	8.64±0.93a	16.52±1.14b	12.08±0.56c	9.42±0.78a	14.98±1.54b	11.80±0.42	8.71±0.24a	16.52±1.08b	10.67±0.59c	7.07±0.68a	18.16±2.95b	9.45±0.03c
ω6												
C18:2	9.83±1.22a	15.57±0.54b	12.27±1.65c	9.18±0.83a	13.95±1.26b	10.95±0.72a	9.80±0.95a	15.30±0.90b	11.43±1.11a	9.29±0.98a	15.45±1.80b	9.83±0.28a
C20:4	32.50±0.84a	24.38±0.85b	29.15±0.59c	32.81±0.46a	28.33±2.02b	30.79±1.00	33.06±0.69a	25.79±0.75b	31.94±1.89a	35.69±1.17a	27.73±1.71b	33.24±0.38a
C22:4	0.90±0.09	0.70±0.05a	1.10±0.17b	0.94±0.09	0.77±0.10a	1.04±0.07b	0.87±0.10a	0.66±0.09b	0.91±0.07a	1.07±0.12	1.17±0.52	0.89±0.06
C22:5	3.91±0.46a	1.93±0.08b	3.55±0.76a	4.05±0.37a	2.16±0.60b	3.55±0.42a	3.69±0.19a	1.04±0.16b	3.04±0.46c	4.98±0.45a	1.58±0.36b	4.80±0.35a
Total	48.08±0.78a	43.23±1.45b	46.89±1.36a	48.00±0.58	46.03±1.69	47.37±0.94	48.32±0.59a	43.46±1.12b	48.19±1.67a	51.99±1.89a	46.53±2.06b	50.43±0.79
ω3												
C22:5	0.29±0.04a	0.24±0.02a	0.39±0.03b	0.29±0.01a	0.20±0.03b	0.29±0.02a	0.29±0.03a	0.19±0.02b	0.30±0.03a	0.12±0.02	0.08±0.01	0.08±0.01
C22:6	2.62±0.10a	1.07±0.07b	2.03±0.14c	2.14±0.13a	1.16±0.27b	1.95±0.10a	2.33±0.11a	0.72±0.06b	1.95±0.10c	0.91±0.07a	0.39±0.08b	1.00±0.11a
Total	3.03±0.12a	1.49±0.10b	2.58±0.12c	2.56±0.12a	1.63±0.33b	2.44±0.07a	2.82±0.14a	1.06±0.10b	2.41±0.14c	1.15±0.09	0.56±0.10	1.16±0.11
ω6/ω3	15.89±0.57a	29.27±2.42b	18.20±0.67a	18.79±0.93a	29.19±4.45b	19.40±0.33a	17.16±0.77a	41.11±2.97b	20.05±0.65a	45.51±2.66a	85.18±11.26b	43.67±3.58a
PUFA	51.11±0.82a	44.71±1.43b	49.47±1.44a	50.56±0.59	47.66±2.01	49.81±0.99	51.14±0.66a	44.53±1.20b	50.60±1.78a	53.14±1.96a	47.08±2.15b	51.60±0.89a
P/S	1.27±0.05	1.16±0.08a	1.29±0.07b	1.26±0.03	1.28±0.08	1.30±0.05	1.27±0.04	1.15±0.07a	1.31±0.09b	1.34±0.11	1.36±0.14	1.33±0.05

Values are expressed as means ± SD of the percentage of the total fatty acids analysed. Significant differences are indicated by lower caps (P<0.05). Statistical comparisons (rows) of Control, Nodule and Surrounding tissue was conducted within each time interval. ω6/ω3 = ratio of total ω6 fatty acids to total ω3 fatty acids, PUFA = polyunsaturated fatty acids, P/S = polyunsaturated to saturated fatty acid ratio.

ADDENDUM 4

Chapter 3:   *Comparative FA profiles in PC and PE of hepatocyte nodules compared to surrounding and control tissue (addendum to Table 4).*

Table 4.   Fatty acid analyses (percentage of total fatty acids) of the phosphatidylethanolamine (PE) fraction of control liver, nodule and surrounding liver in the Resistant Hepatocyte Model.

	1 Month			3 Months			6 Months			9 Months		
	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
Saturates												
C16:0	16.82±1.22a	12.25±0.51b	16.37±1.96a	19.06±0.67a	10.75±1.02b	21.36±1.31a	17.46±1.89a	11.75±1.01b	15.22±1.01a	13.40±1.55a	9.62±2.06b	14.63±1.07a
C18:0	22.05±0.82a	23.15±0.56a	20.29±0.56b	20.45±0.64a	24.45±0.48b	19.79±0.44a	21.28±0.83	23.16±1.22	21.04±1.37	23.32±0.82	23.15±1.03	22.37±0.44
Total	38.87±1.10a	35.40±0.76b	36.66±1.76	39.51±0.49a	35.20±0.95b	41.14±1.53	38.74±1.80a	34.91±1.93b	36.26±1.21	36.71±1.59	32.77±2.62a	37.00±0.90b
Monounsaturates												
C16:1	0.51±0.19a	0.91±0.07b	0.54±0.13a	0.69±0.12	0.47±0.08	0.78±0.09	0.61±0.05	0.76±0.10a	0.46±0.19b	0.38±0.12a	0.52±0.11	0.70±0.10b
C18:1	6.52±0.43a	13.14±0.68b	8.74±0.37c	7.66±0.49a	11.05±0.71b	8.81±0.39a	7.45±0.11a	13.99±1.42b	9.01±0.38a	5.87±0.55a	14.53±2.07b	6.94±0.19a
Total	7.03±0.39a	14.05±0.74b	9.28±0.37c	8.35±0.57a	11.52±0.78b	9.59±0.46	8.06±0.12a	14.74±1.43b	9.47±0.52a	6.24±0.53a	15.05±2.14b	7.65±0.21c
ω6												
C18:2	6.18±0.66a	8.64±0.52b	6.92±0.69a	6.59±0.49a	7.82±1.13	8.75±1.25b	6.49±0.44	8.13±0.66	8.10±1.60	6.57±1.15	7.62±0.75	6.75±0.41
C20:4	29.25±1.02a	27.53±0.50b	28.95±1.14	28.11±0.71a	31.07±0.75b	26.50±1.41a	28.40±1.17	29.38±0.60	29.35±0.42	32.15±0.84	31.69±1.49	31.21±0.79
C22:4	1.97±0.15a	2.30±0.07	2.71±0.35b	2.30±0.10	2.58±0.26	2.16±0.30	2.32±0.42	2.89±0.40	2.51±0.23	3.21±0.33	3.86±0.21a	2.75±0.08b
C22:5	8.42±1.05	6.51±0.40	8.28±1.83	8.45±0.79a	5.97±1.05b	6.15±1.06b	8.48±0.48a	4.87±0.61b	7.36±1.20a	11.61±1.10a	6.26±0.72b	10.78±0.54a
Total	46.68±0.51	45.56±0.63a	47.62±1.52b	46.31±0.52	48.17±0.91a	44.47±1.29b	46.44±1.59	45.78±0.95a	48.15±0.65b	54.41±1.37a	49.89±1.42b	52.74±0.83a
ω3												
C22:5	0.69±0.09a	0.76±0.03a	0.97±0.12b	0.71±0.04	0.62±0.06	0.63±0.15	0.68±0.06	0.72±0.06	0.77±0.06	0.27±0.04	0.32±0.07	0.26±0.01
C22:6	6.55±0.45a	3.99±0.29b	5.27±0.65c	4.92±0.44	4.09±0.55	3.70±0.74	5.84±0.27a	3.64±0.25b	5.13±0.32c	2.20v0.12	1.83±0.13	2.23±0.11
Total	7.42±0.42a	4.99±0.36b	6.44±0.78a	5.83±0.42	5.11±0.67	4.80±0.69	6.76±0.32a	4.56±0.32b	6.11±0.36a	2.64±0.15	2.29±0.19	2.61±0.11
ω6/ω3	6.31±0.32a	9.18±0.73b	7.50±0.92a	7.98±0.60	9.56±1.03	9.47±1.43	6.87±0.15a	10.08±0.51b	7.90±0.42c	20.68±1.13	21.90±1.25	20.23±0.64
PUFA	54.10±0.82a	50.55±0.61b	54.06±1.87a	52.14±0.63	53.28±1.46a	49.27±1.91b	53.20±1.89a	50.34±1.25b	54.27±0.93a	57.05±1.43a	52.18±1.60b	55.35±0.92a
P/S	1.39±0.06	1.43±0.04	1.48±0.12	1.32±0.03	1.52±0.08a	1.20±0.09b	1.38±0.12	1.45±0.11	1.50±0.08	1.56±0.11	1.60±0.16	1.50±0.06

Values are expressed as means ± SD of the percentage of the total fatty acids analysed. Significant differences are indicated by lower caps (P<0.05). Statistical comparisons (rows) of Control, Nodule and Surrounding tissue was conducted within each time interval. ω6/ω3 = ratio of total ω6 fatty acids to total ω3 fatty acids, PUFA = polyunsaturated fatty acids, P/S = polyunsaturated to saturated fatty acid ratio.

ADDENDUM 5

Chapter 3:   *FA profiles of PC and PE in regenerating liver   (addendum to Table 5)*

Table 5.   Fatty acid analyses (percentage of total fatty acids) of the phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions of regenerating liver.

	PC				PE			
	Control	1 Day	2 Days	7 Days	Control	1 Day	2 Days	7 Days
Saturates								
C16:0	19.45±1.17	19.28±2.18	19.18±0.78	17.69±1.27	16.64±1.79	15.58±1.22	16.46±1.14	14.48±1.37
C18:0	21.22±1.52	21.96±2.16	21.86±0.86	18.44±2.27	22.43±1.14a	24.18±1.38a	23.74±0.97a	19.74±1.41b
Total	40.67±1.48a	41.25±1.33a	41.04±1.12a	36.13±1.26b	39.07±1.31a	39.76±1.41a	40.20±1.33a	34.22±1.11b
Monounsaturates								
C16:1	1.00±0.38	0.75±0.27	0.68±0.14	0.98±0.27	0.51±0.22	0.35±0.08	0.28±0.07	0.39±0.07
C18:1	8.29±1.03	7.37±1.35a	7.80±0.62a	10.30±1.10b	6.58±0.52a	6.23±1.16a	5.58±0.48a	8.34±0.71b
Total	9.28±1.25	8.12±1.59a	8.48±0.73a	11.28±1.34b	7.09±0.55	6.58±1.23a	5.85±0.52a	8.73±0.74b
ω6								
C18:2	9.64±1.34a	16.49±2.66b	10.85±0.93a	9.31±0.94a	6.38±0.61a	11.82±1.39b	6.74±0.56a	5.57±0.45a
C20:4	31.91±1.45a	27.61±2.29b	31.65±0.44a	33.89±1.57a	28.65±0.86	29.47±0.35	30.14±0.48	30.18±2.23
C22:4	0.88±0.09	0.89±0.21	1.11±0.06	1.09±0.18	1.96±0.19ab	1.75±0.44b	2.48±0.11ac	2.66±0.32c
C22:5	3.79±0.45	2.53±1.00a	2.99±0.31	4.07±0.53b	8.41±0.89ac	3.78±0.52b	6.89±0.57c	9.74±1.35a
Total	47.06±1.48a	48.06±1.55	47.52±1.08	49.75±0.83b	46.14±1.37a	47.31±1.06	46.93±1.01a	49.38±0.84b
ω3								
C22:5	0.28±0.04a	0.27±0.07a	0.44±0.03b	0.36±0.04	0.70±0.09a	0.70±0.15a	1.06±0.08b	0.92±0.09b
C22:6	2.58±0.19a	2.13±0.24b	2.33±0.15	2.32±0.15	6.78±0.29ac	5.49±0.31b	5.77±0.19b	6.57±0.43c
Total	2.99±0.21	2.57±0.29	2.97±0.17	2.84±0.13	7.69±0.33a	6.34±0.42b	7.01±0.13	7.67±0.43a
ω6/ω3	15.82±0.92a	18.92±1.98b	16.07±1.28a	17.56±0.85	6.01±0.23a	7.50±0.59b	6.69±0.20a	6.46±0.41a
PUFA	50.05±1.60	50.63±1.71	50.49±0.97	52.59±0.85	53.84±1.56a	53.65±0.94a	53.94±1.01a	57.05±0.93b
P/S	1.23±0.08a	1.23±0.07a	1.23±0.05a	1.46±0.06b	1.38±0.09a	1.35±0.06a	1.34±0.07a	1.67±0.08b

Values are expressed as means ± SD of the percentage of the total fatty acids analysed. Significant differences are indicated by lower caps ($P<0.05$). Statistical analyses (rows) of the phospholipid fractions of 1, 2 and 7 days after PH, were compared against the control. ω6/ω3 = ratio of total ω6 fatty acids to total ω3 fatty acids, PUFA = polyunsaturated fatty acids, P/S = polyunsaturated to saturated fatty acid ratio.

ADDENDUM 6

Chapter 4: *Comparative FA parameters: Effect of SFO, SOY, GLA and EPA diets on the FA content (percentage of total fatty acids analysed) of the PC phospholipid fraction of hepatocyte nodule, surrounding and control tissues. (addendum to Table 3).*

Table 3: Comparison of the fatty acid profiles (percentage of total fatty acids) in the phosphatidylcholine (PE) phospholipid fraction of the nodule, surrounding and control liver of rats fed a diet with varying ω6/ω3 fatty acid ratios.

DIET	SFO			SOY			GLA			EPA	
	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
SATS											
C16:0	23.01±2.56^{A}a	20.30±3.12	16.16±4.22^B	28.83±0.68^{A}b	22.45±1.35^{B}a	19.76±0.66^C	25.45±2.31^{A}a	18.48±1.67^{B}b	18.04±3.38^B	23.91±0.94^{A}a	21.68±0.35^{B}a
C18:0	16.74±1.62^{A}a	20.20±0.60^{B}a	23.75±0.53^C	13.83±0.43^{A}b	18.58±0.65^{B}b	23.02±0.58^C	15.10±0.44^{A}b	18.82±1.40^B	23.65±1.78^C	14.51±0.47^{A}b	18.55±0.65^{B}b
Total	39.75±1.40ac	40.51±2.83	39.91±3.83	42.67±0.98b	41.02±1.23a	42.78±0.59	40.56±1.87c	37.30±2.43^{A}b	41.69±2.91^B	38.42±0.71^{A}a	40.23±0.68^B
C16:1	1.35±0.38	0.98±0.42	0.72±0.37	1.43±0.08^A	0.83±0.17^B	0.52±0.12^C	1.71±0.25^A	0.89±0.17^B	0.36±0.12^C	1.67±0.18^A	1.05±0.04^B
C18:1	12.39±1.62^A	9.11±1.12^B	8.61±1.06^{B}a	11.79±0.35^A	9.27±0.68^B	7.09±0.23^{C}b	12.42±0.55^A	9.32±0.62^B	6.85±0.89^{C}b	12.45±0.08^A	8.86±0.27^B
Total	13.74±1.97^A	10.09±1.39^B	9.33±1.26^{B}a	13.23±0.39^A	10.10±0.82^B	7.61±0.31^{C}b	14.14±0.36^A	10.20±0.59^B	7.21±0.92^{C}b	14.12±0.18^A	9.92±0.28^B
ω6											
C18:2	14.49±0.25^{A}a	10.63±0.95^{B}a	9.39±1.45^B	17.49±1.22^{A}b	12.14±0.38^{B}a	9.52±0.49^C	14.32±0.88^{A}a	11.57±0.70^B	10.11±1.01^C	18.11±0.81^{A}b	13.42±1.93^{B}b
C18:3	0.38±0.04^{A}a	0.47±0.04a	0.58±0.12^B	0.28±0.04^{A}b	0.42±0.04^{B}a	0.32±0.05^A	0.45±0.04^{A}a	0.54±0.04^{B}b	0.41±0.07^A	0.34±0.02^{A}b	0.42±0.03^{B}a
C20:3	0.38±0.04^{A}a	0.63±0.15^{B}a	0.75±0.22^B	0.47±0.06^{A}bd	0.61±0.04^{B}a	0.68±0.12^B	0.56±0.04^{A}cd	1.12±0.09^{B}b	0.76±0.25^A	0.52±0.06^{A}d	0.96±0.07^{B}c
C20:4	28.23±2.22^{A}a	31.23±3.03a	34.40±3.33^B	22.39±0.97^{A}b	28.05±1.38^{B}a	30.64±0.34^C	24.57±0.84^{A}b	30.92±0.95^{B}a	31.65±2.97^B	23.52±0.71^{A}b	26.72±1.26^{B}b
C22:4	0.66±0.09^{A}a	1.07±0.22^{B}a	0.77±0.12^{A}a	0.15±0.02^{A}b	0.37±0.19^{B}b	0.21±0.04b	0.20±0.02b	0.24±0.03b	0.23±0.04b	0.16±0.04b	0.17±0.01b
C22:5	1.99±0.31^{A}a	4.76±0.54^{B}a	4.19±0.22^{B}a	0.09±0.03^{A}b	0.94±0.08^{B}b	0.36±0.03^{C}b	0.15±0.03^{A}b	0.23±0.02^{B}b	0.30±0.07^{B}b	0.09±0.02^{A}b	0.22±0.05^{B}b
Total	46.14±2.56a	48.79±3.45a	50.09±3.70a	40.87±1.16b	42.52±2.37b	41.72±0.30b	40.24±1.49^{A}b	44.62±1.48^{B}b	43.46±2.72	42.73±0.70b	41.89±0.80b
ω3											
C18:3	0.07±0.01a	0.09±0.01a	0.08±0.02a	0.26±0.02^{A}b	0.20±0.04^{B}b	0.15±0.02^{B}b	0.13±0.01c	0.16±0.03^{A}bc	0.10±0.02^B	0.14±0.01c	0.14±0.01c
C20:5	N/D	N/D	N/D	0.30±0.04a	0.22±0.05a	0.29±0.09	0.44±0.06^{A}b	0.52±0.17^{A}b	0.20±0.03^B	0.52±0.03^{A}c	0.62±0.07^{B}b
C22:5	0.04±0.01a	0.04±0.01a	0.05±0.01a	0.67±0.06b	0.88±0.24b	0.89±0.14b	1.05±0.15c	1.27±0.12^{A}c	0.89±0.15^{B}b	0.92±0.03c	1.06±0.22bc
C22:6	0.27±0.03^{A}a	0.46±0.06^{B}a	0.53±0.12^{B}a	2.01±0.39^{A}b	5.06±0.16^{B}b	6.56±0.61^{B}b	3.43±0.61^{A}c	5.94±0.41^{B}b	6.45±0.29^{B}b	3.14±0.47^{A}c	6.13±0.75^{B}b
Total	0.38±0.05^{A}a	0.61±0.08^{B}a	0.67±0.13^{B}a	3.24±0.41^{A}b	6.35±0.33^{B}b	7.89±0.67^{B}b	5.05±0.62^{A}c	7.88±0.68^{B}b	7.64±1.11^{B}b	4.73±0.49^{A}c	7.96±0.48^{B}b
T6/T3	123.21±10.67^{A}a	81.42±6.70^{B}a	76.85±11.30^{B}a	12.79±1.64^{A}b	7.94±2.13^{B}b	5.32±0.50^{B}b	8.07±1.02^{A}b	5.69±0.36^{B}b	5.80±1.08^{B}b	9.12±0.92^{A}b	5.28±0.41^{B}b
PUFA	46.52±2.62	49.40±3.54	50.76±3.83	44.11±1.35^{A}a	48.88±1.20^{B}a	49.61±0.67^B	45.29±1.59^A	52.49±2.12^{B}b	51.10±2.91^B	47.46±0.85^{A}b	49.86±0.47^B
LCPUFA	31.58±0.52^{A}a	38.21±1.02^{B}a	40.71±0.87^B	26.08±0.63^{A}b	36.12±1.12^{B}a	39.62±2.33^B	30.39±1.21^{A}a	40.22±0.49^{B}b	40.48±0.65^B	28.87±0.32^{A}a	35.88±0.57^{B}a
P/S	1.17±0.10a	1.23±0.17	1.29±0.21	1.03±0.06^{A}b	1.19±0.06^{B}a	1.16±0.03^B	1.12±0.09^A	1.42±0.15^{B}b	1.23±0.15	1.24±0.05a	1.24±0.03

Values are means ̠ SD of 5-6 replications and expressed as percentage of total fatty acids analysed. Statistical analyses consisted of separate 1-way ANOVA to test for significance between different tissue types separately within the same dietary group, as indicated by superscript uppercase letters within a row ($P<0.05$). One-way ANOVA was also used to test separately for differences within the same tissue type across different dietary groups, as indicated by lowercase letters ($P<0.05$) and identical colour in a row. SFO=sunflower dietary oil, SOY=soybean dietary oil, GLA=sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 dietary oil, EPA=sunflower/eicosapentaenoic acid-50 dietary oil, SATS=saturated fatty acids, MUFA=monounsaturated fatty acids, ND=not detected, ω6/ω3=ω6 to ω3 fatty acid ratio, PUFA=polyunsaturated fatty acids, LCPUFA=long-chain polyunsaturated fatty acids, P/S=polyunsaturated to saturated fatty acid ratio.

ADDENDUM 7

Chapter 4:   *Comparative FA parameters: Effect of SFO, SOY, GLA and EPA diets on the FA content (percentage of total fatty acids analysed) of the PE phospholipid fraction of hepatocyte nodule, surrounding and control tissues. (addendum to Table 4).*

Table 4:   Comparison of the fatty acid profiles (percentage of total fatty acids) in the phosphatidylcholine (PE) phospholipid fraction of the nodule, surrounding and control liver of rats fed a diet with varying $\omega6/\omega3$ fatty acid ratios.

DIET	SFO			SOY			GLA			EPA	
	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding	Control	Nodule	Surrounding
SATS											
C16:0	$9.37\pm2.32^{A}a$	16.56 ± 4.19^{B}	11.48 ± 5.19	$14.66\pm0.86^{A}b$	17.66 ± 3.06^{B}	14.52 ± 0.89^{A}	$13.73\pm2.61b$	12.88 ± 2.30	13.55 ± 4.12	$12.53\pm1.15^{A}b$	16.26 ± 2.09^{B}
C18:0	25.47 ± 0.72^{A}	21.96 ± 0.51^{B}	24.42 ± 0.72^{C}	24.83 ± 1.53^{A}	21.62 ± 1.50^{B}	25.39 ± 1.02^{A}	23.17 ± 3.52	20.84 ± 1.16^{A}	25.54 ± 1.64^{B}	23.84 ± 0.60^{A}	21.45 ± 0.47^{B}
Total	$34.84\pm1.74a$	38.52 ± 3.84	35.89 ± 5.05	$39.49 1.87b$	39.28 ± 3.43	39.91 ± 0.80	36.89 ± 2.67	33.72 ± 2.43^{A}	39.09 ± 3.96^{B}	$36.37\pm0.65a$	37.71 ± 1.73
MUFA											
C16:1	0.42 ± 0.12	0.53 ± 0.29	0.35 ± 0.27	0.56 ± 0.13^{A}	0.43 ± 0.16	0.25 ± 0.07^{B}	0.53 ± 0.12^{A}	0.31 ± 0.07^{B}	0.18 ± 0.08^{B}	0.54 ± 0.12^{A}	0.35 ± 0.06^{B}
C18:1	$12.39\pm0.45^{A}a$	8.23 ± 0.66^{B}	$7.69\pm0.54^{B}a$	$11.41\pm0.71^{A}b$	$9.05\pm1.01^{B}a$	$5.60\pm0.24^{C}b$	$10.30\pm0.77^{A}b$	$7.63\pm0.66^{B}b$	$5.61\pm0.95^{C}b$	$11.25\pm0.59^{A}b$	$7.53\pm0.25^{B}b$
Total	$12.81\pm0.54^{A}a$	8.75 ± 0.84^{B}	$8.04\pm0.77^{B}a$	$11.97\pm0.83^{A}a$	$9.48\pm1.09^{B}a$	$5.84\pm0.31^{C}b$	$10.83\pm0.74^{A}b$	$7.93\pm0.70^{B}b$	$5.79\pm1.02^{C}b$	11.79 ± 0.56^{A}	$7.88\pm0.24^{B}b$
ω6											
C18:2	$8.14\pm0.39^{A}a$	6.61 ± 0.74^{B}	5.83 ± 0.63^{B}	$9.20\pm0.97^{A}b$	7.89 ± 2.19^{A}	5.23 ± 0.34^{B}	$6.67\pm0.52^{A}c$	6.42 ± 0.62^{A}	5.44 ± 0.67^{B}	$8.54\pm0.39^{A}ab$	7.48 ± 0.87^{B}
C18:3	0.06 ± 0.01^{A}	0.11 ± 0.03^{B}	$0.18\pm0.03^{C}a$	$0.060\pm.02$	0.08 ± 0.06	$0.08\pm0.02b$	0.05 ± 0.03^{A}	0.12 ± 0.01^{B}	$0.11\pm0.02^{B}b$	$0.030\pm.02^{A}$	0.09 ± 0.01^{B}
C20:3	0.33 ± 0.04^{A}	$0.45\pm0.12^{A}a$	$0.71\pm0.14^{B}a$	0.29 ± 0.12	$0.29\pm0.14a$	$0.41\pm0.08b$	0.31 ± 0.04^{A}	$0.62\pm0.02^{B}b$	$0.45\pm0.16^{A}b$	0.28 ± 0.05^{A}	$0.57\pm0.05^{B}b$
C20:4	$31.89\pm1.57a$	$29.5\pm02.60ac$	$33.06\pm3.12a$	$25.50\pm0.91^{A}b$	$25.68\pm2.20b$	$27.86\pm0.59^{B}b$	$27.22\pm1.12b$	$28.85\pm1.04c$	28.26 ± 1.86	$26.55\pm0.85b$	$26.35\pm0.64b$
C22:4	$3.24\pm0.24a$	$3.30\pm0.71a$	$2.88\pm0.57a$	$0.86\pm0.07bc$	$1.17\pm0.40b$	$0.80\pm0.08b$	$1.06\pm0.12^{A}b$	$0.99\pm0.12b$	$0.81\pm0.15^{B}b$	$0.69\pm0.05c$	$0.73\pm0.03b$
C22:5	$7.38\pm0.57^{A}a$	$11.30\pm1.55^{B}a$	$11.61\pm1.40^{B}a$	$0.38\pm0.11^{A}b$	$0.98\pm0.12^{B}b$	$0.73\pm0.04^{B}b$	$0.49\pm0.03^{A}b$	$0.56\pm0.06b$	$0.64\pm0.13^{B}b$	$0.30\pm0.05^{A}b$	$0.48\pm0.12^{B}b$
Total	$51.04\pm1.89a$	$51.27\pm4.25a$	$54.26\pm5.13a$	$36.29\pm0.82b$	$37.09\pm4.36b$	$35.11\pm0.74b$	$35.79\pm1.58b$	$37.57\pm1.10b$	$35.71\pm2.14b$	$36.39\pm1.18b$	$35.69\pm0.95b$
ω3											
C18:3	$0.08\pm0.02a$	0.10 ± 0.01	$0.08\pm0.02a$	$0.32\pm0.03b$	$0.33\pm0.19b$	$0.21\pm0.03b$	$0.17\pm0.04^{A}c$	0.19 ± 0.02^{A}	$0.11\pm0.02^{B}a$	$0.16\pm0.02^{A}c$	0.20 ± 0.02^{B}
C20:5	N/D	N/D	N/D	$0.33\pm0.02a$	$0.27\pm0.05^{A}a$	0.44 ± 0.17^{B}	$0.47\pm0.03^{A}b$	$0.65\pm0.09^{B}b$	0.29 ± 0.09^{C}	$0.63\pm0.04^{A}c$	$0.78\pm0.06^{B}b$
C22:5	$0.16\pm0.03a$	$0.17\pm0.05a$	$0.17\pm0.05a$	$2.54\pm0.21^{A}b$	$1.90\pm0.55^{B}b$	$2.05\pm0.23b$	$3.34\pm0.55^{A}c$	$3.49\pm0.42^{A}c$	$2.17\pm0.87^{B}b$	$3.15\pm0.13c$	$2.77\pm0.51d$
C22:6	$1.07\pm0.08^{A}a$	$1.19\pm0.24a$	$1.56\pm0.45^{B}a$	$9.05\pm1.03^{A}b$	$11.65\pm3.71^{A}b$	$16.45\pm0.65^{B}b$	$12.50\pm0.45^{A}c$	$16.45\pm1.31^{B}c$	$16.85\pm1.90^{B}b$	$11.53\pm0.81^{A}c$	$14.97\pm2.29^{B}bc$
Total	$1.31\pm0.11a$	$1.46\pm0.30a$	$1.81\pm0.50a$	$12.24\pm1.10^{A}b$	$14.15\pm4.36^{A}b$	$19.14\pm0.67^{B}b$	$16.48\pm0.66^{A}c$	$20.78\pm1.81^{B}c$	$19.42\pm2.50^{B}b$	$15.45\pm0.93^{A}c$	$18.72\pm1.84^{B}c$
T6/T3	$39.03\pm2.49a$	$36.11\pm5.01a$	$31.67\pm7.63a$	$2.98\pm0.23b$	$2.61\pm1.46b$	1.84 ± 0.09	$2.17\pm0.09^{A}b$	$1.82\pm0.11^{B}b$	$1.86\pm0.16^{B}b$	$2.36\pm0.19^{A}b$	$1.92\pm0.21^{B}b$
PUFA	$52.35\pm1.98a$	52.73 ± 4.56	56.07 ± 5.54	$48.54\pm1.78^{A}b$	$51.24\pm3.53a$	54.25 ± 0.62^{B}	$52.27\pm2.01^{A}a$	$58.35\pm2.86^{B}b$	55.13 ± 4.43	$51.84\pm0.96^{A}a$	54.42 ± 1.91^{B}
LCPUFA	$44.07\pm0.17^{A}a$	$45.91\pm2.57a$	49.98 ± 2.23^{B}	$38.96\pm1.75^{A}b$	$42.94\pm2.89a$	48.73 ± 3.12^{B}	$45.38\pm2.68a$	$51.62\pm3.41b$	49.47 ± 2.92	$43.11\pm2.11a$	$46.65\pm1.29a$
P/S	$1.51\pm0.12a$	1.39 ± 0.25	1.61 ± 0.38	$1.23\pm0.10b$	$1.32\pm0.21a$	1.36 ± 0.04	$1.43\pm0.16a$	1.74 ± 0.20	1.43 ± 0.26	$1.43\pm0.05a$	1.45 ± 0.12

Values are means SD of 5-6 replications and expressed as percentage of total fatty acids analysed. Statistical analyses consisted of separate 1-way ANOVA to test for significance between different tissue types separately within the same dietary group, as indicated by superscript uppercase letters within a row ($P<0.05$). One-way ANOVA was also used to test separately for differences within the same tissue type across different dietary groups, as indicated by lowercase letters ($P<0.05$) and identical colour in a row. SFO=sunflower dietary oil, SOY=soybean dietary oil, GLA=sunflower/eicosapentaenoic acid-50/gamma-linolenic acid-80 dietary oil, EPA=sunflower/eicosapentaenoic acid-50 dietary oil, SATS=saturated fatty acids, MUFA=monounsaturated fatty acids, ND=not detected, $\omega6/\omega3=\omega6$ to $\omega3$ fatty acid ratio, PUFA=polyunsaturated fatty acids, LCPUFA=long-chain polyunsaturated fatty acids, P/S=polyunsaturated to saturated fatty acid ratio
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